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UNIVERSITY OF CALIFORNIA

Ernest O. Lawrence Radiation Laboratory

BIO-ORGANIC CHEMISTRY QUARTERLY REPORT

March through May 1961

DISCLAIMER

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Contents

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*Preceding Quarterly Reports: UCRL-9652, UCRL-9519

BIO-ORGANIC CHEMISTRY QUARTERLY REPORT

March through May 1961

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

June 29, 1961

1. INVESTIGATION OF ORGANIC MATTER IN THE METEORITES ORGUEIL AND MURRAY

Susan V. Himes

The study of meteorite Murray has been reported in previous Quarterly Reports.[1,2,3] This report gives further results with Murray, and information on another meteorite, Orgueil.

A sample of Orgueil was sent to us from the Muséum National d'Histoire Naturelle, Paris. It fell in several pieces over an area of 2 square miles near Orgueil, France, in 1864. The elemental analysis of this meteorite is shown in Table I. We extracted a 10.07-g sample of this meteorite with water, using the same procedure as that for Murray.[1] The water extracted 1.32 g, which is at least twice as much material as was water-extracted from Murray. The elemental analysis of the water extract is given in Table II[4] and its uv spectrum is shown in Fig. 1. From an x-ray diffraction pattern it was determined that the water extract contained mostly $MgSO_4 \cdot 6H_2O$ with some calcium sulfate.[5] The ir spectrum (Fig. 2) shows a strong $SO_4^=$ band at 1100 cm^{-1}, = strong H_2O bands at 1650 cm^{-1} and 3200-3600 cm^{-1}, and some unidentified peaks at 2300, 1400, and 980 cm^{-1}. The approximately 8 g of Orgueil left after the water extraction was then extracted with purified chloroform. Approximately 50 mg of yellow material was extracted. Its uv spectrum is shown in Fig. 3 and is identical to the spectrum of elemental sulfur. Whatever else may be extracted from the meteorites by organic solvents, the uv spectra show only sulfur.

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1. Susan K. Vaughn, in Bio-Organic Chemistry Quarterly Report UCRL-8961, Sept. 1959, p. 44.
2. Susan K. Vaughn and M. Calvin, in Bio-Organic Chemistry Quarterly Report UCRL-9041, Dec. 1959, p. 24.
3. Susan V. Himes, in Bio-Organic Chemistry Quarterly Report, UCRL-9208, March, 1960, p. 7.
4. We are grateful to Dr. John Conway and Mr. George Shalimoff for the spectrochemical analysis of the metals.
5. We are grateful to Mrs. Helena Ruben for the x-ray diffraction pattern.

Table I. Elemental analysis of meteorite Orgueil.

Element	Percent
C	3.06
H	1.95
N	0.08
P	<0.02
S, combustible	1.68
S, noncombustible, from residue	2.31
Residue	64.3

Table II. Elemental analysis of the water extract of Orgueil

Element	Percent
C	0.79
H	4.27
N	0.20
P	0.0
S, combustible	0.53
S, noncombustible, from residue	15.81
Residue	59.0
Mg	10[a]
Ca	3[a]
Al	0.2[a]
Fe	0.1[a]
Mn	0.1[a]
Si	0.01[a]
B	<0.01[a]
Ni	<0.05[a]

[a]This value is accurate only within a factor of two.

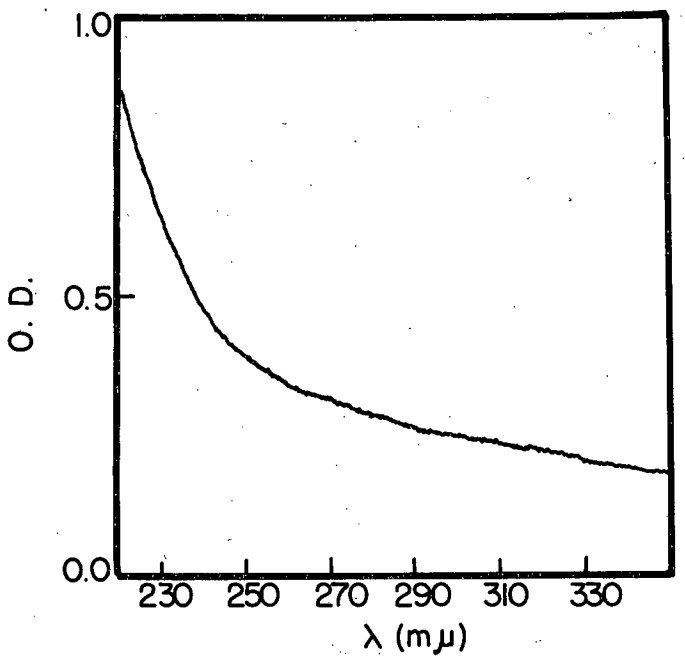

Fig. 1. The ultraviolet spectrum of the water extract of Orgueil.

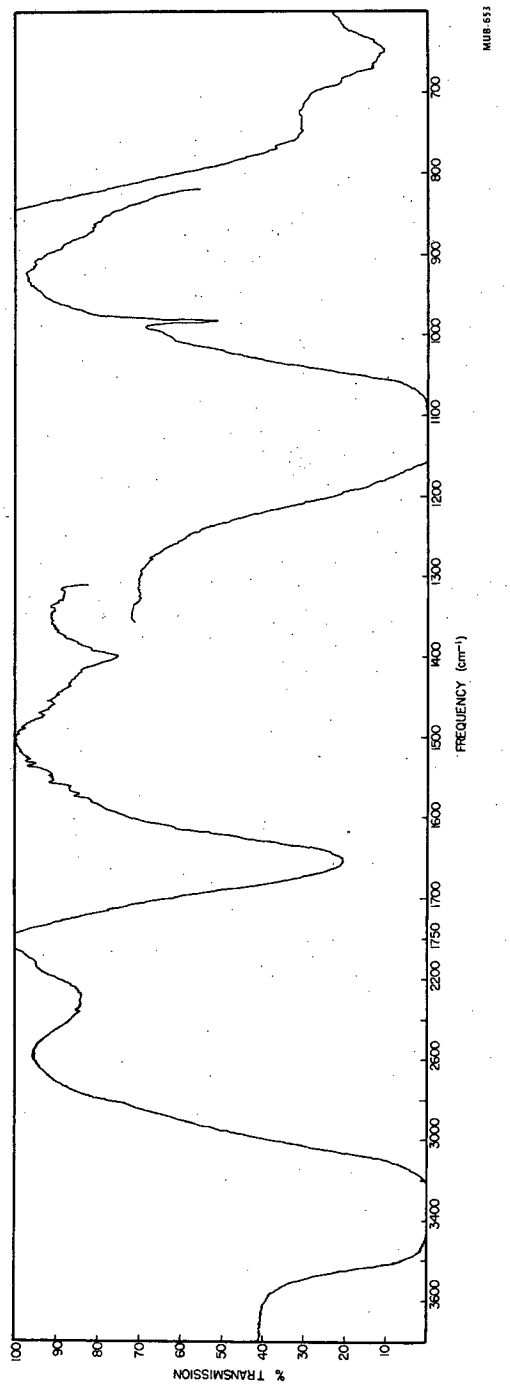

Fig. 2. Infrared spectrum of water extract from Orgueil.

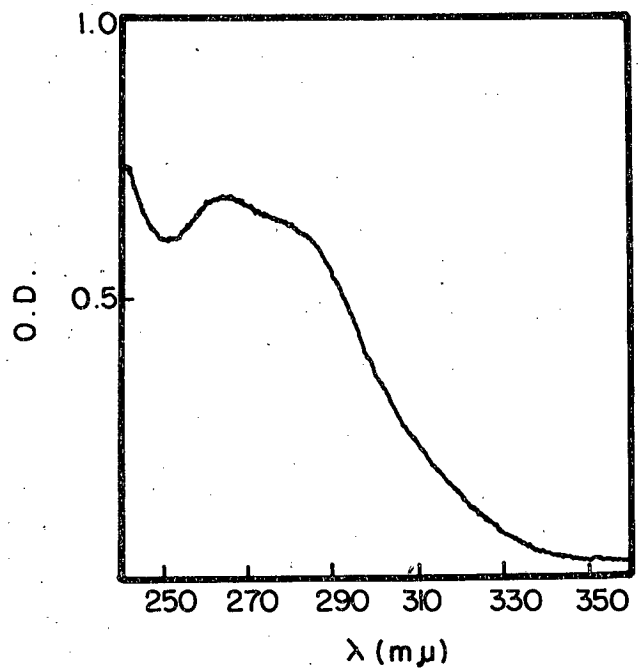

Fig. 3. The ultraviolet spectrum of the chloroform extract of Orgueil.

A portion of the water extract (400 mg) was applied to a Dowex-1-Cl column (22×0.9 cm) which had been treated according to the procedure reported previously.[3] The column was first washed with water then with, successively, 0.001 N, 0.005 N, 0.01 N, and 0.05 N HCl. All of the weighable and uv-absorbing material came through with the water wash. The specific absorption at 245 mμ of the water-wash fractions is shown in Fig. 4. The uv spectrum of Fraction 1 (Fig. 5) has a maximum near 245 mμ and a minimum near 220 mμ. This peak persists through the first nine fractions, but gradually fades out. A satisfactory material balance was obtained, since an amount of $MgCl_2 \cdot 6H_2O$ came off the column equivalent to the amount of $MgSO_4 \cdot 6H_2O$ applied. A portion of Fraction 1 was subjected to a pH change. The spectrum did not change in acid, and the effect of base was obscured by the precipitation of $Mg(OH)_2$.

In order to rid the uv-absorbing material of salt, the first seven fractions from the water wash (105 mg) were applied to an Amberlite IRC-50 column in the ammonium form (18×0.7 cm). The resin had first been thoroughly washed. Then $MgCl_2 \cdot 6H_2O$ was applied to ascertain that Mg^{++} would exchange for NH_4^+, and that Mg^{++} would not displace uv-absorbing material from the column. After the Orgueil sample was applied, the column was first washed through with water. These water-wash fractions were concentrated from 150 ml to 1 ml and the uv spectrum taken. No uv-absorbing material had come off the column. Approximately 150 ml of 0.005 N NH_4OH was then run through the column in order to elute the uv-absorbing material. The NH_4OH had already been checked for contamination on a stock column of Amberlite IRC-50. The eluted fractions were combined and concentrated to 1 ml and the spectrum taken. The uv-absorbing material still was not present. The same volume of higher-concentration NH_4OH (0.01 N) was then used for elution. After concentration, the eluent had uv absorption only slightly above the blank. The sample was made acid to see if maxima and minima would appear, but the spectrum did not change (Fig. 6). Still higher concentration of NH_4OH (0.05 N) was used for elution. Upon concentration, there appeared some white powder in the evaporating flask. This may mean that the excess NH_4^+ was displacing Mg^{++}. The spectrum of the 0.05 N NH_4OH elution is shown in Fig. 7. The absorption at 320 mμ is an enigma.

This method of getting rid of salt was also used on a sample of the water extract of Murray. The sample used was the first few fractions (containing 90 mg of material) off a Dowex-1-Cl^- column which had been washed with water.[3] Since the water extract of Murray was mostly $CaSO_4$, this material was mostly $CaCl_2 \cdot xH_2O$. An Amberlite-IRC-50-NH_4^+ column (15×0.7 cm) identical to the one used for the Orgueil sample, was prepared and washed. Calcium chloride was applied to the column to ascertain that Ca^{++} would exchange with NH_4^+ and that Ca^{++} did not displace any uv-absorbing material. The uv spectrum of the Murray sample before it was applied to the column is shown in Fig. 8A. After application, the column was washed well with water. The absorbing material did not stay on the column but came right through with the water. Two-ml fractions were collected and the uv absorption persisted through about 12 fractions before fading out. Figure 8B shows the spectrum of Fraction 8; this spectrum exhibits the same shape as that of all the fractions. Thus, although the Ca^{++} was removed, the spectrum remained the same. The NH_4Cl that was present in Fraction 6 was removed by sublimation in vacuo

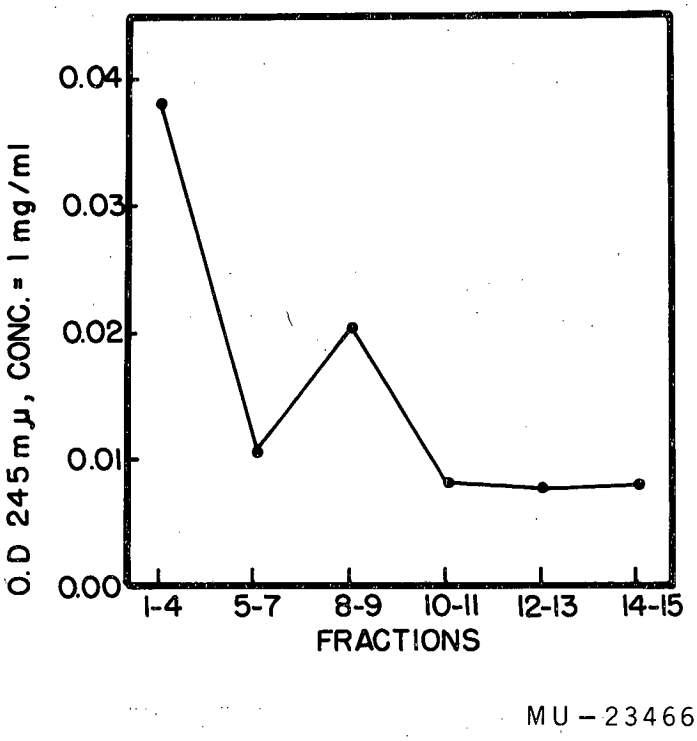

Fig. 4. The specific absorption at 245 mμ of successive water-wash fractions of meteorite Orgueil material (water extract) from a Dowex-1-Cl-column.

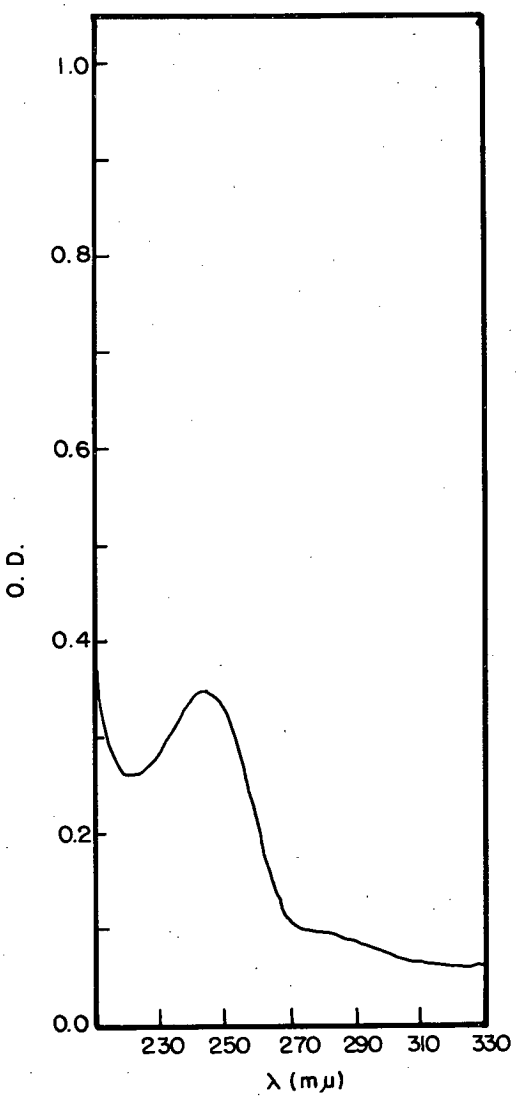

Fig. 5. The ultraviolet spectrum of water-wash fraction 1 (see Fig. 4).

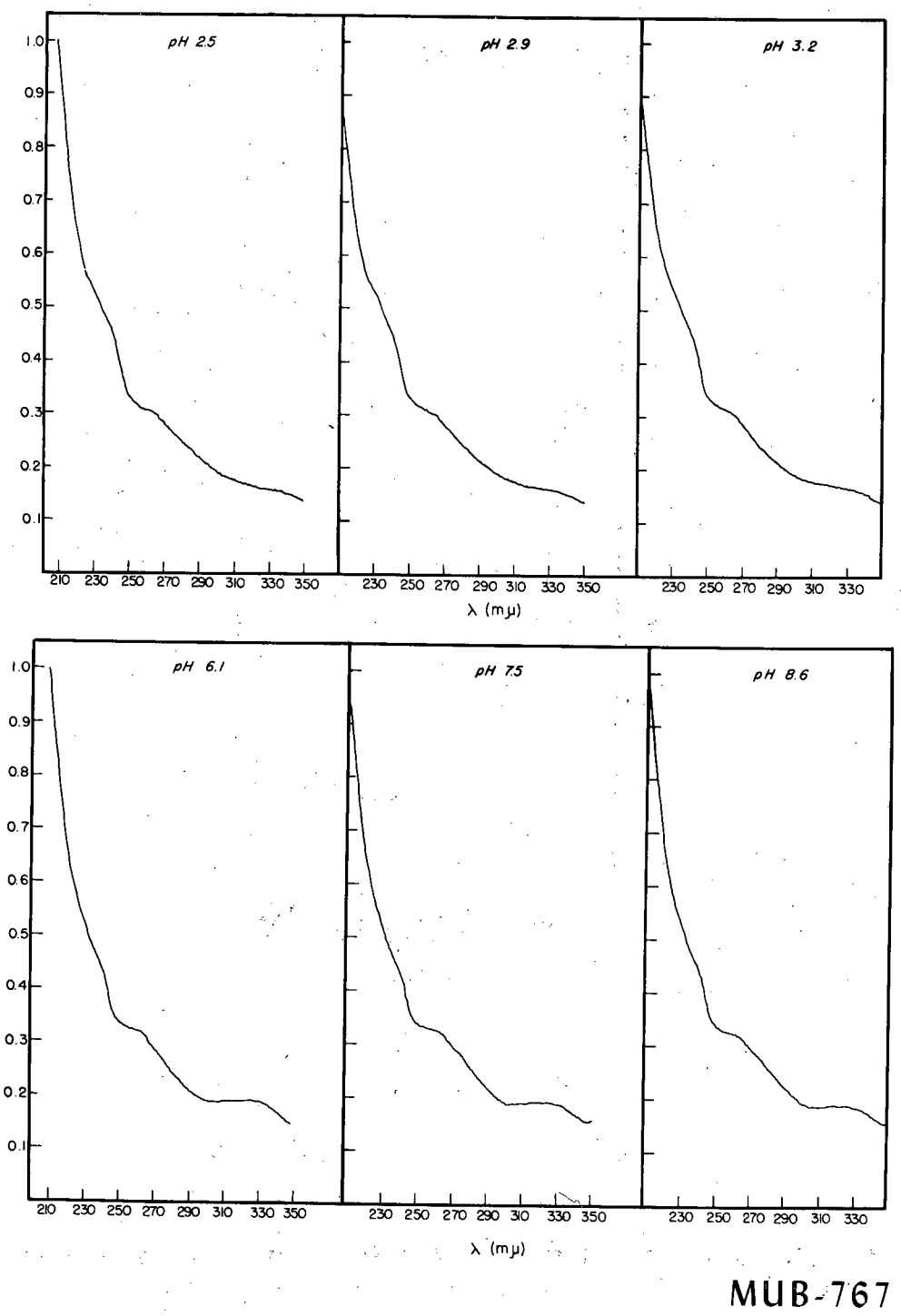

Fig. 6. Effect of acid and base on the ultraviolet spectrum of Orgueil material (water extract) removed from Amberlite IRC-50 by 0.01 N NH_4OH.

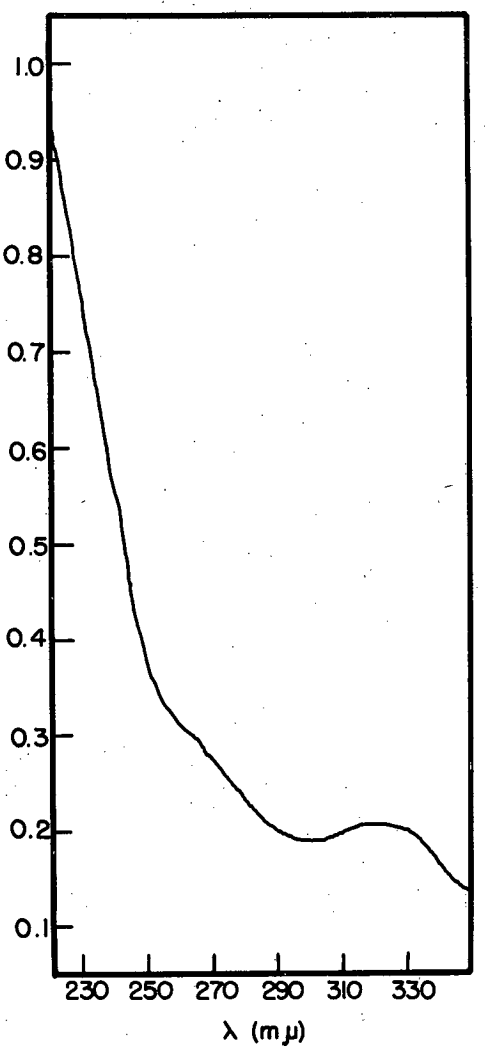

Fig. 7. Ultraviolet spectrum of Orgueil material (water extract) removed from Amberlite IRC-50 by 0.05 N NH_4OH.

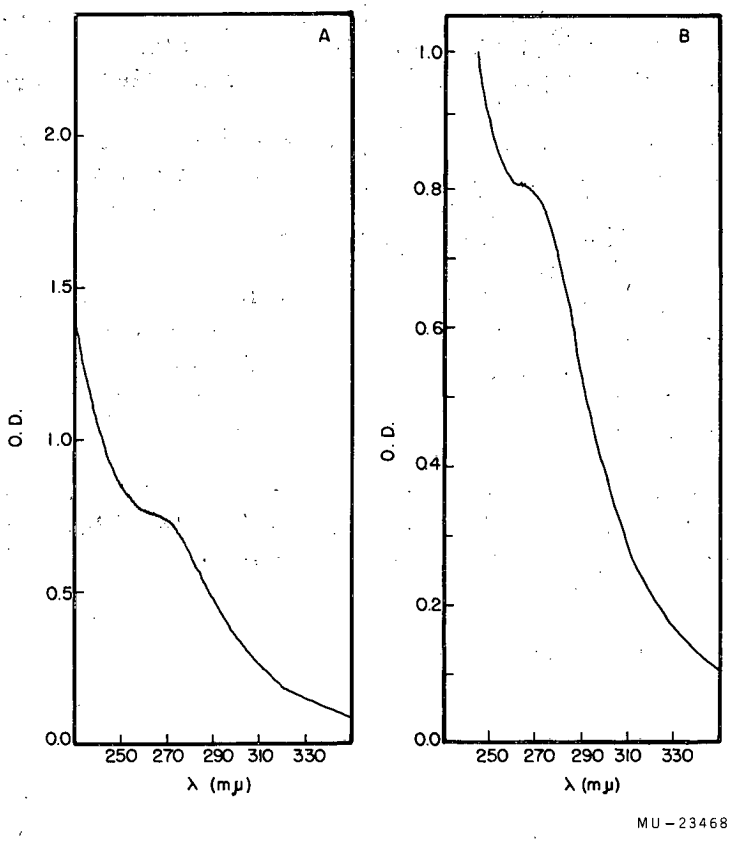

Fig. 8. The ultraviolet spectra of meteorite Murray material (water extract)
A. before chromatography on Amberlite IRC-50,
B. after removal by water from Amberlite IRC-50.

at 100°. The spectrum of the residue, not readily visible, was the same as in Fig. 8B. Fraction 6 without the NH_4Cl and Fraction 9, which still contained NH_4Cl, were subjected separately to a pH change. Neither of the spectra changed in either acid or base. Thus, in this case, the removal of salt did not leave behind a definite organic substance with a uv spectrum having characteristic maxima and minima.

The spectral study in acid and base of various substituted pyrimidines was continued in order to gain knowledge of their behavior for possible comparison with unknown from the meteorites. The effect of acid and base on 6-methyl-2-thiouracil is shown in Fig. 9. The pK lies between 5 and 7.

6-Methyl-4-pyrimidone was synthesized from 6-methyl-2-thiouracil with Raney nickel catalyst.[6] Data reported and found are compared in Table III. The effect of acid and base on the uv spectrum of 6-methyl-4-pyrimidone is shown in Fig. 10. The pK lies between 5 and 8.

N, 6-Dimethyl-4-pyrimidone was synthesized according to the method of Marshall and Walker from 6-methyl-4-pyrimidone and diazomethane.[7] Table IV gives a comparison of data reported and found for this compound. The effect of acid and base on its uv spectrum is shown in Fig. 11. The pK lies between 2 and 5.

In conclusion, according to uv spectral studies, there appear to be some substances of biological interest in meteorites Murray and Orgueil. What they are, however, has not been satisfactorily determined.

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6. T. L. Cairns, ed., Organic Syntheses Vol. 35, (John Wiley and Sons, Inc., New York, N. Y., 1955), p. 81.

7. J. R. Marshall and James Walker, J. Chem. Soc., 1951, 1004.

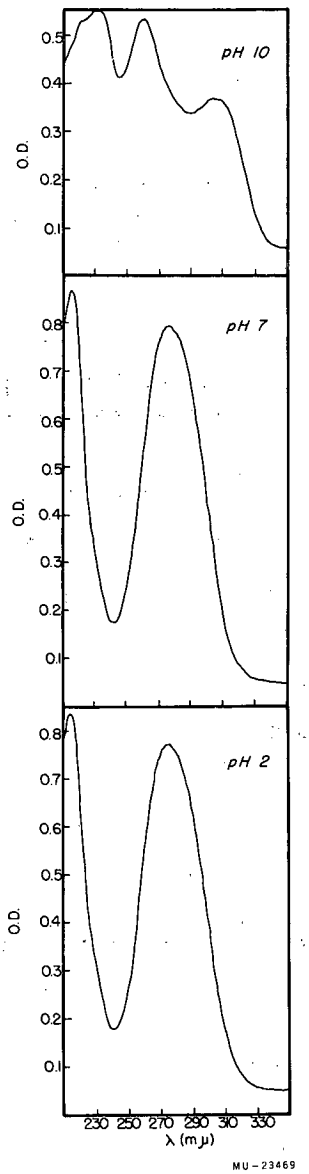

Fig. 9. The effect of acid and base on the ultraviolet spectrum of 6-methyl-2-thiouracil.

Table III. Data on 6-Methyl-4-pyrimidone.

	Reported	Found
mp	148 – 149°	148 – 151°
C	54.5%	54.66%
H	5.45%	5.34%
N	25.0%	25.09%
log ϵ_{263}	3.5	3.61
log ϵ_{228}	3.86	3.79
Yield	93%	99%

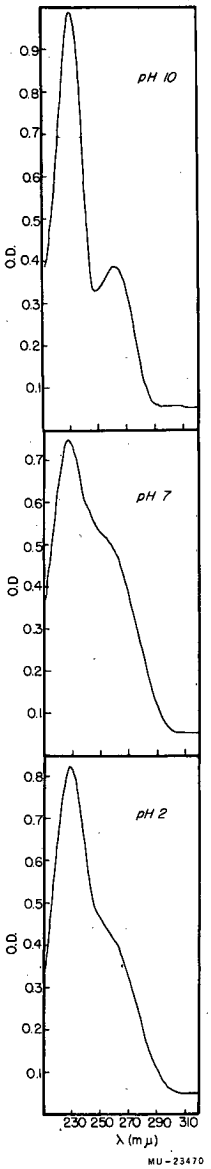

Fig. 10. The effect of acid and base on the ultraviolet spectrum of 6-methyl-4-pyrimidone.

Table IV. Data on N, 6-Dimethyl-4-pyrimidone.

	Reported	Found
mp	80 - 82°	79 - 81°
C	58.1%	57.88%
H	6.45%	6.51%
N	22.6%	22.42%
log ϵ_{268}	3.56	3.53
log ϵ_{225}	3.75	3.71
Yield	48%	1.5%

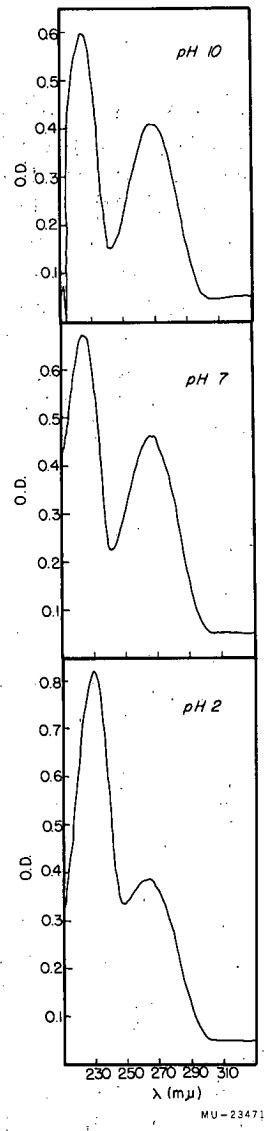

Fig. 11. The effect of acid and base on the ultraviolet spectrum of N, 6-dimethyl-4-pyrimidone.

2. CHANGES OF CONDUCTIVITY AND ESR OF A COMPLEX ORGANIC SOLID AS A RESULT OF HEATING

David Ilten, John Eastman, and M. Calvin

It has been found that a one-to-one solid complex of o-chloranil and perylene can be prepared by dissolving equimolar quantities of these two substances in boiling ethylene dichloride and allowing the resulting solution to cool.[1] Electron spin resonance (ESR) data showed the room-temperature concentration of unpaired electrons in the freshly prepared solid complex to be extremely small. However, when the complex was heated at moderate temperatures and then cooled to room temperature there was a decided, irreversible increase in the concentration of unpaired electrons. This concentration continued to increase as a function of the length of time the complex was heated--until a maximum level was reached. From this point, there was a gradual decrease in the concentration of unpaired electrons with further heating (Fig. 12).

The purpose of this work was to determine if there is a corresponding change in the conductivity of the complex as a function of heating time. Dark conductivity measurements were made on commercial samples of o-chloranil and perylene, and on the complex. Samples of the complex for both conductivity and ESR measurements were prepared and heated simultaneously for various periods of time so that changes in these two properties as a result of heating could be correlated. Table V and Fig. 12 summarize the results.

Dark Conductivity

Dark conductivity was determined on samples of the materials that had been pulverized and then pressed into pellets approximately 1.5 cm in diameter and 0.1 cm thick. These were prepared by placing the powdered sample into a die, evacuating to remove moisture, and then applying 25,000 lb force for at least 2 minutes. This was equivalent to a pelleting pressure of 125,000 psi. The conductivity apparatus used was a slightly modified version of that described by David Kearns.[2] The sample was placed between two steel cylinders and surrounded by a glass cylinder. Platinum discs in contact with the ends of the steel cylinders served as electrodes for connecting the sample into the circuit for measuring resistance. The resistance was determined by using a vibrating-reed electrometer to measure the current flowing through a standard 10^8-ohm resistor which was placed in series with the sample and a dc source of 17.5 volts. The sample, cylinders, and electrodes were enclosed in a brass chamber which could be evacuated. The temperature of the system was controlled by circulating a methanol-water mixture from a thermostat bath through coils surrounding the chamber. A copper-constantin thermocouple, fitted into the chamber through a Radiation Laboratory Seal and an epoxy plug, was used for temperature determinations.

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1. John W. Eastman and Melvin Calvin, in Bio-Organic Chemistry Quarterly Report, UCRL-9652, April 1961, p. 36.
2. David R. Kearns, Electrical Properties of Organic Solids (Thesis), UCRL-9120, March 25, 1960, p. 24.

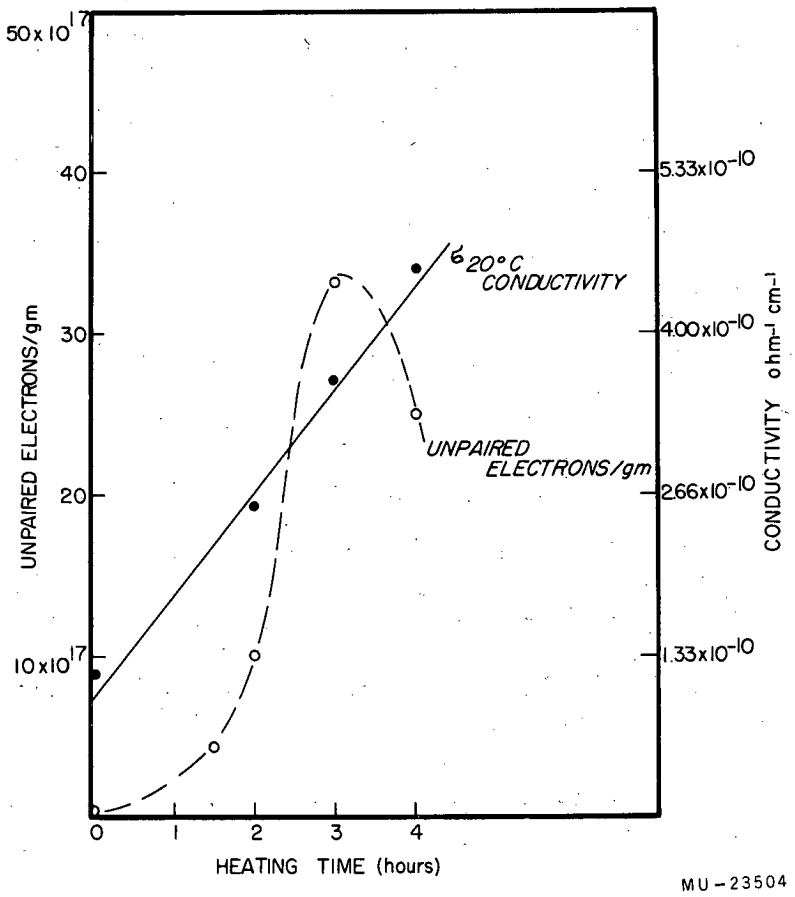

Fig. 12. Comparison of changes in conductivity and number of unpaired electrons per g (for o-chloranil-perylene) as function of heating time.

Table V. Activation energies and ESR measurements.

Substance	Activation energy of conduction, ΔE, (ev)		Concentration per gram of unpaired electrons (at room temperature)
o-chloranil	2.42 ± 5%		
perylene	0.55	(0.98[a])	
complex (unheated)	0.49		$< 1 \times 10^{16}$
complex (heated, 1 hr)			4.8×10^{17}
complex (heated, 2 hr)			1.0×10^{18}
complex (heated, 3 hr)			3.3×10^{18}
complex (heated, 4 hr)	0.25	(0.05 ± 15%[b])	

[a] Literature value for sublimed film: D. C. Northrop and O. Simpson, Proc. Roy. Soc. (London) A234, 124 (1956). The impurity in our sample may explain the difference between these two values.

[b] ESR value which may be the ΔE for a phenomenon unrelated to the conductivity.

A pressure of approximately 100 kg/cm^2 was applied to the sample by a lever arm. As the conductivity was greatly changed if air were in the system, all measurements were carried out at 10^{-4} mm pressure. The high volatility of the materials made it more difficult to obtain low pressures under conditions of even moderate temperatures (60°).

Dark-conductivity measurements were made on o-chloranil, perylene, and the complex. The resistivity of the sample, ρ, in ohm-centimeters was computed as

$$\rho = AR/d,$$

where

R = measured resistance (in ohms),
A = area of the sample (in cm^2),
d = sample thickness (in cm).

The activation energies of conduction for these substances were calculated by plotting resistivity, r, vs $(\text{temperature})^{-1}$ and then applying the formula

$$\Delta E = 2.303\ mk \qquad \text{or} \qquad \Delta E = 1.99\times10^{-4}\ m\cdot ev,$$

where

ΔE = activation energy for conduction (in electron volts),
k = Boltzmann constant = 8.63×10^{-5} ev,
m = slope of the semilog plot.

This calculation was based on the assumption that the activation energy of conductivity followed the Boltzmann relation,

$$\rho = \rho_0 e^{(\Delta E/kt)}.$$

Pellets of the complex were pressed as described previously and then placed into sample tubes. These tubes were evacuated to 10^{-4} mm pressure and then filled with nitrogen gas. One pellet was powdered and 0.025 g of the resulting powder was placed into each of five ESR tubes. These, also, were evacuated and then filled with nitrogen. Four ESR and four conductivity tubes were placed into an oil bath that was maintained at $85\pm4^{\circ}$. Samples were removed after 1-hr intervals and cooled in an ice bath to prevent further reaction. The ESR and conductivities of these samples were then measured. The temperature dependence of these properties was determined for the sample that had been heated for 4 hr.

Results

The unheated materials

As illustrated in Figs. 13 and 14, the Arrhenius-type plot of log ρ vs $(\text{temperature})^{-1}$ yielded the expected linear relationship over the temperature range investigated for o-chloranil and perylene. This was true, also, for the unheated complex up to about 60°. However, at this point there was a marked positive deviation of the curve from a straight line. The resistivity was actually

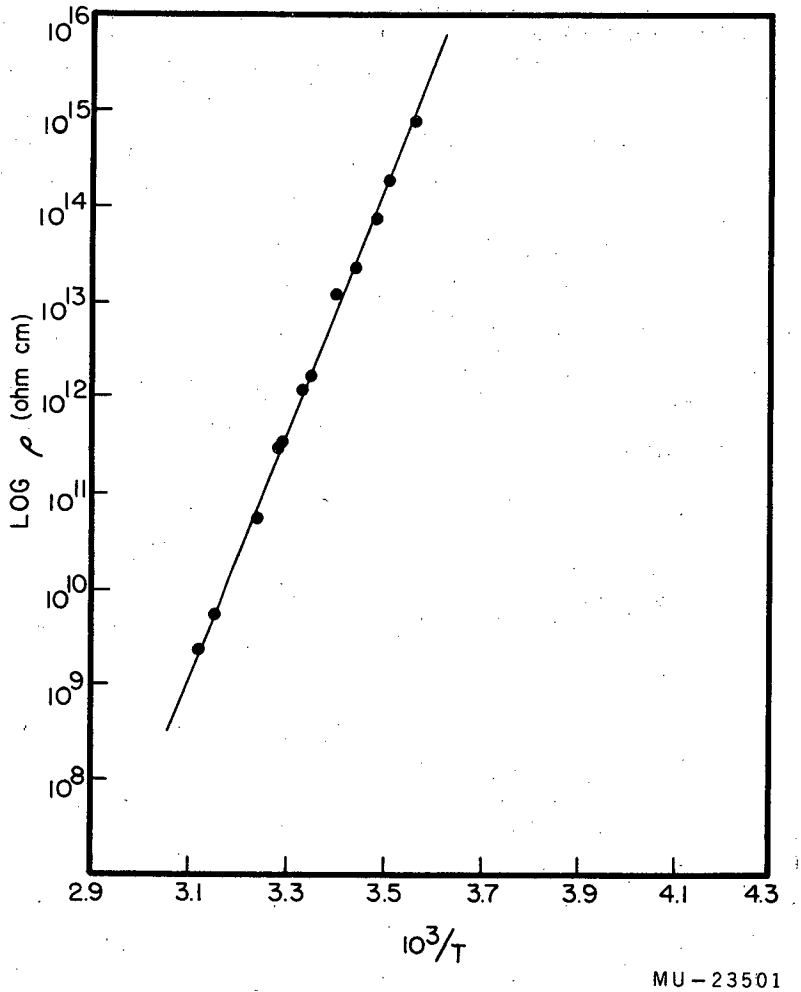

Fig. 13. Log resistivity vs (temperature)$^{-1}$ for a pellet of perylene.

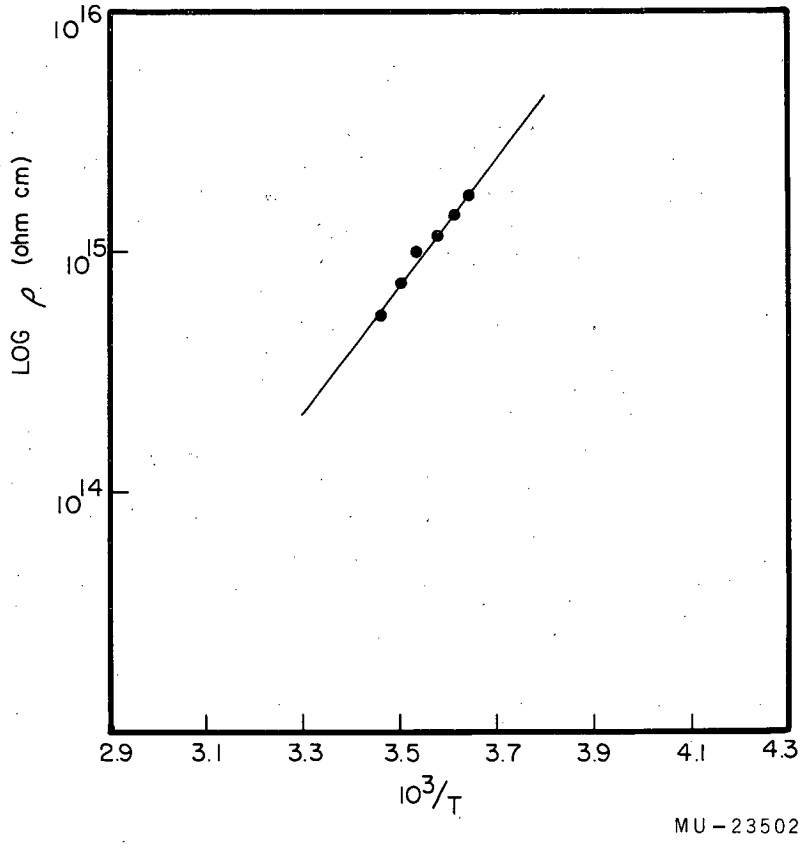

Fig. 14. Log resistivity vs (temperature)$^{-1}$ for pellet of o-chloranil, 0.08 cm thick, 1.29 cm in diameter.

higher than would be expected if the linear relationship were obeyed. This deviation was demonstrated to be reversible by cooling the sample to 40°, at which a point was obtained that fell on the original line. The pellet was once more heated to 66° and the original deviation was observed. This may indicate a temporary thermal disruption of the crystalline lattice which tends to decrease conductivity.

The heated complex

The experimental results for the complex that had been heated in the oil bath, given in Fig. 15, show that conductivity at 20° does indeed increase as a function of the length of time the sample was heated. However, under the conditions of this experiment, the conductivity at 20° did not pass through a maximum, nor did it follow the ESR curve. Rather, it increased linearly with time. This would seem to indicate that the concentration of unpaired electrons cannot be the only factor determining conductivity at 20°. It may serve to substantiate the point of view that crystalline imperfections are being introduced irreversibly as a result of heating, and that conductivity increases as the concentration of these imperfections increases.[1] If this were the case, the linear increase of conductivity with time would suggest that the introduction of imperfections also proceeds linearly with time at a given temperature, or that zero-order kinetics are followed.

The activation energy of conduction, ΔE, for the complex that had been heated 4 hours was found to be lower than that for the unheated complex (Table V). The variation of the log of ESR signal with $(\text{temperature})^{-1}$ for the 4-hr sample is plotted in Fig. 16, and provides a rough estimate of an activation energy for this process.

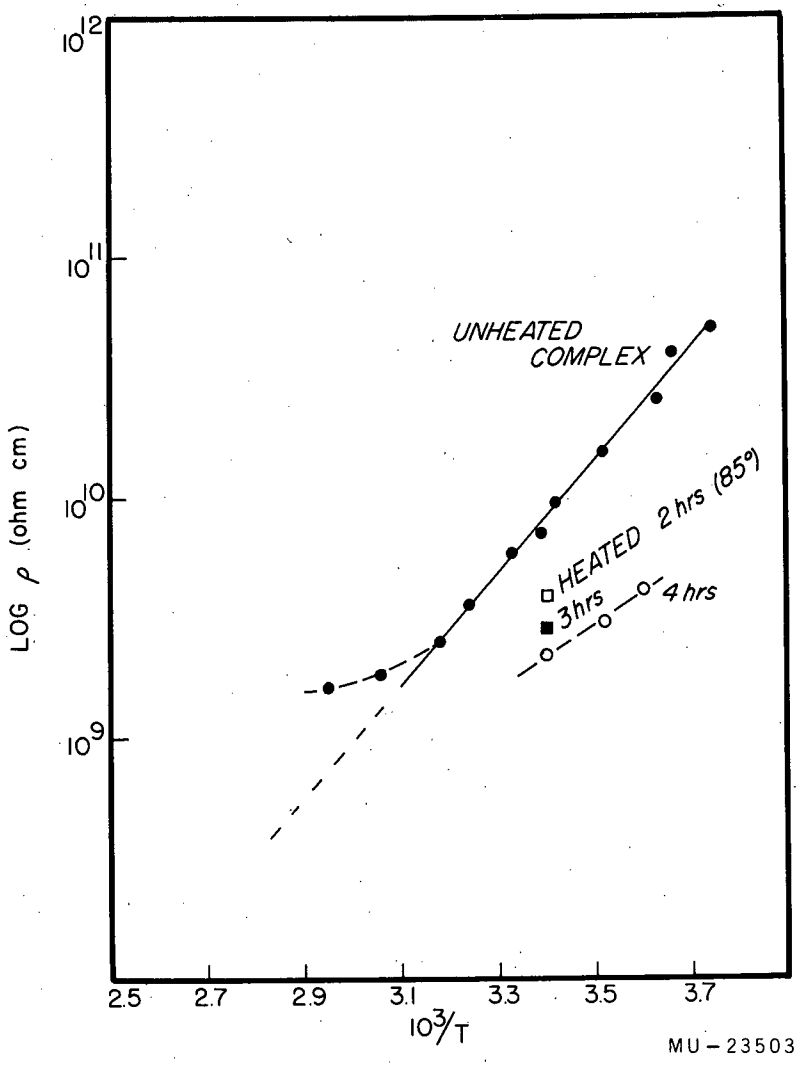

Fig. 15. Log resistivity as a function of temperature for heated and unheated complexes of o-chloranil and perylene.

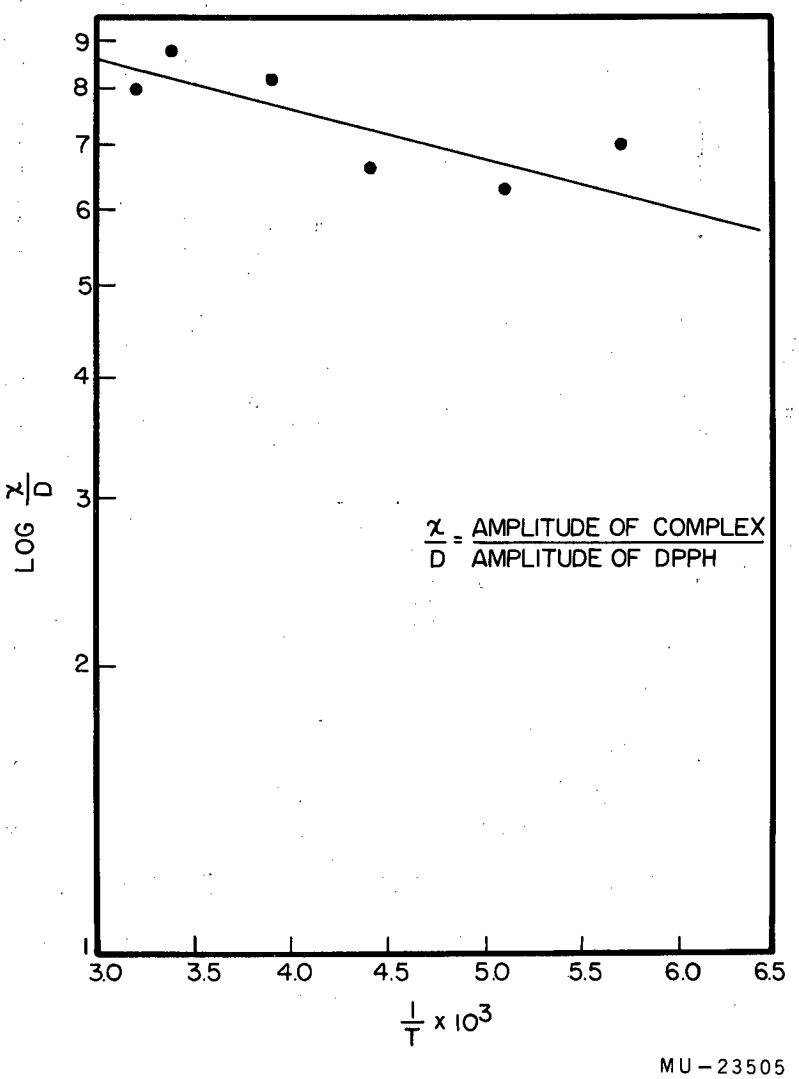

Fig. 16. Temperature dependence of ESR for solid complex of o-chloranil and perylene. (DPPH = diphenylpicrylhydrazyl.)

3. STUDIES ON THE ESTERS OF INORGANIC OXYACIDS

P. R. Hammond

Knowledge of the π bonding between the oxygen and the hetero atom in the inorganic oxyacids and their anions may be expected to be of value in interpreting the acid strength and alkylating properties of these compounds.[1] Bond energy and bond-distance measurements have been given differing interpretations.[2] A study is therefore being made of the physical properties of the esters, assuming the bonding in these parallels the bonding in the undissociated acids. This study is an attempt to gain an independent method of measurement. A previous report[3] recorded the extinction coefficients and positions of maximum absorption in the ultraviolet spectra of a limited number of phenyl esters and showed that variations in these occurred which exhibited a rough correlation with anticipated variations in π bonding.

The proton magnetic resonance spectra of some methyl esters[4] at infinite dilution in carbon tetrachloride are reported here. Among the oxyacids of the first and second rows of the periodic table, the proton spectra of the methyl esters of boric, carbonic, silicic, and sulfuric acid have been measured.[5] The position of resonance of the methyl protons in a given external magnetic field depends on the perturbation caused by the magnetic shielding due to bonding electrons, and on the net bulk magnetic susceptibility of the sample. The last effect may be eliminated[5,6] by comparing infinitely dilute solutions in inert, magnetically isotropic (spherical) solvents. The methyl esters of nitric, phosphoric, and perchloric acids are reported here.

Experimental Procedure

Measurements were made in spinning cylindrical tubes, 5 mm o.d., by using a Varian spectrometer operating at 60 megacycles. A fine capillary, containing water, was supported symmetrically in the sample by a plasticene seal at the top of the tube, and the positions of resonance from water were determined by measuring with respect to a 60-cycle side band imposed on the spectra by an audio-oscillator.

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1. M. Anbar, I. Dostrovky, D. Samuel, and A. D. Yoffe, J. Chem. Soc. 1954, 3603.
2. G. M. Phillips, J. S. Hunter, and L. E. Sutton, J. Chem. Soc. 1945, 146; A. F. Wells, J. Chem. Soc. 1949, 55.
3. P. R. Hammond, in Bio-Organic Chemistry Quarterly Report UCRL-9519, Jan. 1961.
4. In these compounds, steric interactions between the ester groups are considerably less than in the phenyl esters.
5. A. L. Allred and E. G. Rochow, J. Am. Chem. Soc. 79, 5361 (1957).
6. A. A. Bothner-By and C. Naar-Colin, J. Am. Chem. Soc. 80, 1728 (1958).

Carbon tetrachloride was commercial reagent-grade material. Commercial trimethyl phosphate was purified by fractional distillation (bp 90° at 30 mm). Methyl nitrate was prepared by the method of Black and Babers[7] and was used without further purification.[8]

Methyl perchlorate solutions were prepared by the reaction of silver perchlorate[9] and a small, known excess of methyl iodide in carbon tetrachloride (10 ml). The mixtures were sealed and shaken overnight, and then filtered from the yellow silver iodide through fine sintered glass funnels, and were used directly in the spectrometer tubes. The spectra of these mixtures showed two peaks, about 2.4 and 0.28 chemical shifts from water. The ratio of these areas was approximately in the proportion of excess methyl iodide added to silver perchlorate used. The extrapolation to infinite dilution for methyl perchlorate was made by assuming complete conversion of the silver perchlorate to the ester. This was not seriously in error, as the position of resonance varied only slightly with concentration. All values of chemical shifts quoted come from at least five measurements.

Results

In Table VI are the concentrations of the three esters, studied the methyl protium molarity (M), and the chemical shifts (σ) observed relative to water. The graph (Fig. 17) shows the variation in σ with M; for the extrapolation to infinite dilution, a linear relationship was assumed. All measurements were reproducible to ± 0.01 chemical shift (room temperature was 26°).

Conclusions

The spectra of the purified trimethyl phosphate always showed two sharp peaks and the frequency separation between them did not vary on dilution. This is attributed to coupling between the P^{31} and the proton nuclei (j = 11 cycles). The positions of resonance of methyl nitrate (σ = 0.40) and methyl perchlorate (σ = 0.27) are the lowest yet recorded for methyl-containing compounds.

The chemical shifts obtained here (for the phosphate, the average of the two) and those quoted by Allred and Rochow are shown below, together with values for other methyl compounds under similar conditions.

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7. A. P. Black and F. H. Babers, Organic Syntheses, Coll. Vol. II, 412.
8. No lines were seen in the NMR spectrum that could be attributed to protium-containing impurities.
9. Dried overnight in a vacuum desiccator over phosphorus pentoxide and sodium hydroxide and handled only in a dry box.

Table VI. Positions of chemical shifts for methyl esters in carbon tetrachloride.

Sample	Concentration (M)	Methyl protium molarity	1	2	M = 0 (inf. dilution)
$(CH_3)_3PO_4$	0.100 M	0.90	0.805	0.622	$\sigma_1 = 0.96$
	0.050 M	0.45	0.885	0.694	$\sigma_2 = 0.77$
	0.033 M	0.30	0.907	0.722	$\sigma_{av} = 0.87$
	0.025 M	0.23	0.915	0.726	j = 11 cycles
CH_3NO_3	0.39 M	1.17	0.450		
	0.23 M	0.69	0.426		$\sigma = 0.40$
	0.16 M	0.48	0.423		
CH_3ClO_4	$(AgClO_4)$ (CH_3I)				
	0.64 M 0.73 M	1.92	0.289		
	0.29 M 0.39 M	0.87	0.284		$\sigma = 0.27$
	0.12 M 0.22 M	0.36	0.263		

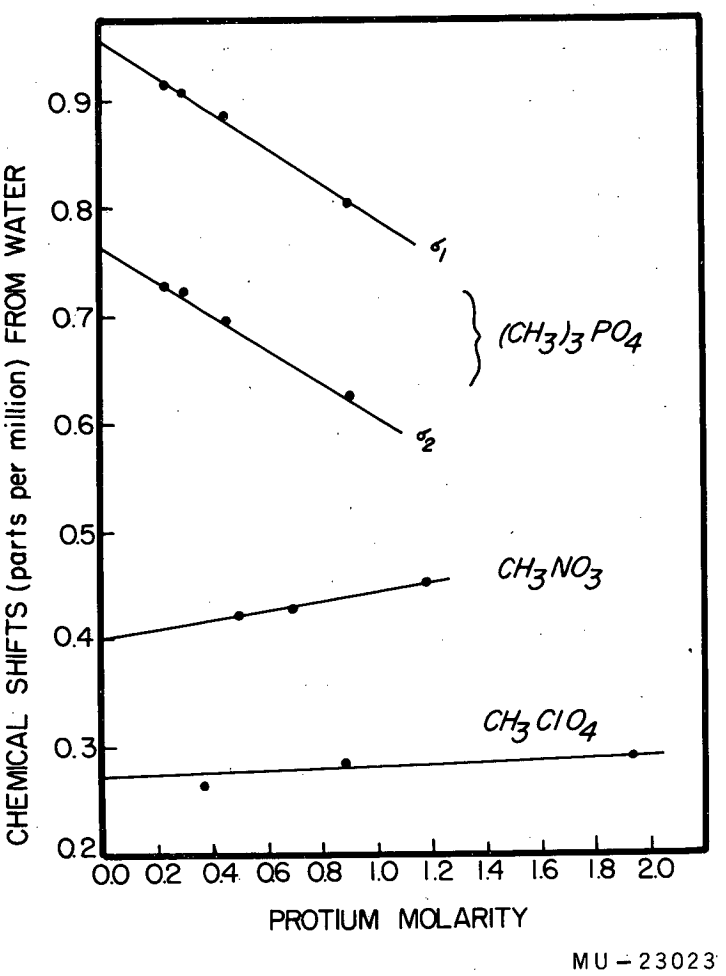

Fig. 17. Concentration dependence in chemical shift for methyl esters in carbon tetrachloride.

$(CH_3)_3BO_3$	$(CH_3)_2CO_3$		CH_3NO_3			
1.26	1.02		0.40			
	$(CH_3)_4SiO_4$		$(CH_3)_3PO_4$	$(CH_3)_2SO_4$		CH_3ClO_4
	1.23		0.87	0.85		0.27
CH_3OH		$CH_3OC_6H_5$			CH_3F	
1.39		1.10			0.53	

For increasing electronegativity of the hetero atom, or for increasing acid strength, a drift of the methyl resonance to decreasing values of the applied field occurs. From the figures quoted, however, no simple relationship applies.

A number of publications[5; 10, 11], have drawn attention to linear relationships between element electronegativity and the position of protium resonance[12] of similar methyl compounds, although the gradients of the lines are different. For the acids of the esters above, the variations in chemical shifts can be considered to arise from changes in a parameter that closely resembles classical group electronegativity. Thus, assuming a slope of -0.7 unit per chemical shift, taking 4.0 as the electronegativity value for fluorine,[13] and comparing with methyl fluoride, one has for the acids the electro-negativity values shown below.

Boric	Carbonic	Nitric		
3.5	3.7	4.1		
	Silicic	Phosphoric	Sulfuric	Perchloric
	3.5	3.8	3.8	4.2

Probably the most important influences on the positions of resonance are those affecting electron density on the methyl hydrogens. These include

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10. A. A. Bothner-By and C. Naar-Colin, Ann. N. Y. Acad. Sci. 70, 833 (1958).
11. A. L. Allred and E. G. Rochow, J. Inorg. Nuclear Chem. 5, 269 (1958).
12. For the methyl halides -0.76 electronegativity units per chemical shift (p.p.m.), for the first row elements -0.38, and for the group IVB element, -0.78.
13. L. Pauling, Nature of the Chemical Bond, 2nd Edition (Cornell University Press, 1944), p. 60.

contributions from the structure $C - \overset{+}{O} = X$ and electronegativity differences between oxygen and the hetero atom. Long-range interactions between the hydrogens and the hetero atom are also likely to be significant.[14] Thus the coupling with the phosphorus in the phosphate ester ($j = 11$ cycles) is too large to be attributed completely to bond interaction,[10] and the effect this has on the position is not known. Magnetic anisotropy, particularly of the O-X bond systems, may also influence the shieldings. Further calculation, as yet, is therefore not justified.

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14. H. M. McConnell, J. Chem. Phys. 24, 460 (1956); G. A. Williams and H. S. Gutowsky, J. Chem. Phys. 30, 717 (1959); L. Petrakis and C. H. Sederholm, NMR Flourine-Flourine Coupling Constants in Saturated Organic Compounds UCRL-9565, Feb. 1961.

4. A SPECTRAL STUDY OF SOME COMPLEXES OF THE ESTERS OF PHOSPHOROUS ACID

P. R. Hammond

Triethyl phosphite that has been purified by fractional distillation dissolves trinitrotoluene (TNT), s-trinitrobenzene (TNB), p-chloranil (C) and p-bromanil (B) forming pink, red, dark-red, and dark-red solutions respectively. The first part of this article is concerned with a spectral study of these solutions in the visible region using a Beckman DK-2 spectrophotometer, an instrument suitable for observing transient peaks.

None of the solutions is stable. With TNT the pink color rapidly changes to yellow. The TNB solution changes to an orange-red, and both quinones fade to a pale yellow. The triethyl phosphite–TNB color was also studied in ethanol solution.

Experimental Procedure

Commercial samples of TNT, TNB, and C were purified by crystallizing twice from ethanol, whereas B was crystallized twice from a large volume of carbon tetrachloride. The materials had melting points in agreement with literature values.

Triethyl phosphite and diethyl phosphonate had been purified by fractional distillation 3 months before, stored at 0°, and samples removed during the period. The triester showed no impurities in the infrared spectrum. Nevertheless, this and a commercial sample of trimethyl phosphite were purified before use by fractional distillation in an atmosphere of nitrogen. Fractions boiling within a 1 degree range were collected. The infrared spectrum of the diethyl phosphonate showed weak OH frequencies, and the solution in water was acid. After fractional distillation, the aqueous solutions were neutral, both before and after the experiment.

Dimethyl aniline was purified by refluxing for an hour with acetic anhydride. The liquid was washed five times with water and the base was dried with sodium sulfate followed by sodium hydroxide pellets. Finally, it was fractionally distilled.

Triethylamine and pyridine were fractionally distilled, dried over potassium hydroxide, redistilled, and stored in nitrogen atmospheres. ACS-grade carbon tetrachloride and 95% ethanol were fractionally distilled and the first and last fractions were discarded. The anthracene was recrystallized twice from ethanol. The spectra were observed by using a 0.25-cm optical path and were compared with the solvent in the compensating beam.

For the rapidly changing complexes, in which half lives of less than a minute were found, the finely powdered materials were weighed directly into the absorption cell, the solvent added and stirred with a glass rod, and the

spectra obtained immediately. By using the fast recording speed of the instrument, a complete visible spectrum could be obtained within 45 seconds of the time of mixing.

Three recording speeds were used: fast, medium, and slow. The slow speed was found to record spectra in agreement with quoted literature values; for example, the position and fine structure of the benzene in cyclohexane transition centered at 256 mμ. For the faster speeds, loss of resolving power and peak displacement were found. Thus, corrections of 15 and mμ had to be added to the fast and medium speeds respectively. The correction also depended on the shape of the absorption peak, and for this reason the final corrected values estimated for the fast runs are accurate to ± 5 mμ. All measurements were taken at room temperature (24-25°).

Results

a. p-Chloranil and p-bromanil solutions (0.1 M) in triethyl phosphite produced rapidly decaying absorption maxima at 530 mμ and 538 mμ respectively (corrected values of 545 and 553 mμ). Figure 18 shows the spectra obtained, their time dependence, and a comparison with the absorption for 0.02 M solutions of the quinones in benzene[1] with a 1-cm optical path. The rate of decay could be measured by setting the pen in the region of the absorption maximum, inserting fresh sample, and timing the motion of the pen. Plots of log $(D-D_\infty)$ and $(D-D_\infty)$ against t are shown in the graph (Fig. 19) for both quinones.[2] The decays are of first order, with rate constants of 3.0×10^{-2} sec^{-1} for C and 1.6×10^{-2} sec^{-1} for B.

b. For TNB the results obtained in ethanol solution overshadow other measurements, and these are therefore recorded first. One-cm cells of the magenta solution, 1 M in triethyl phosphite and 10^{-2} M in trinitrobenzene, showed absorption spectra of two peaks at 456 and 556 mμ (corrected 461 and 561 mμ) together with a large absorption below 400 mμ that is attributed to the trinitrobenzene (Fig. 22). Triethyl phosphite had negligible absorption. The peaks steadily increased, maintaining a constant ratio $D_{456} : D_{556}$ of about 2:1. A plot of the increase of the absorption at 556 mμ, where the trinitrobenzene spectrum may be neglected, is shown in Fig. 20. An initial rapid increase settles down to a linear rise with time (0.016 optical density unit per min).

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1. The charge transfer band of the benzene chloranil complex has been identified in the 360 mμ region, (R. Foster, Nature 187, 337 (1960) and studies on bromanil (R. Foster, D. L. Hammick and J. P. Placito, J. Chem. Soc. 1956, 38881) suggest its band with benzene will be near this and will certainly not appear in the region studied. Thus the intense absorption below 450 mμ may be assigned to the quinones.

2. D is the optical density at time t and D_∞ are the densities (0.03 and 0.04) after long periods (10 minutes).

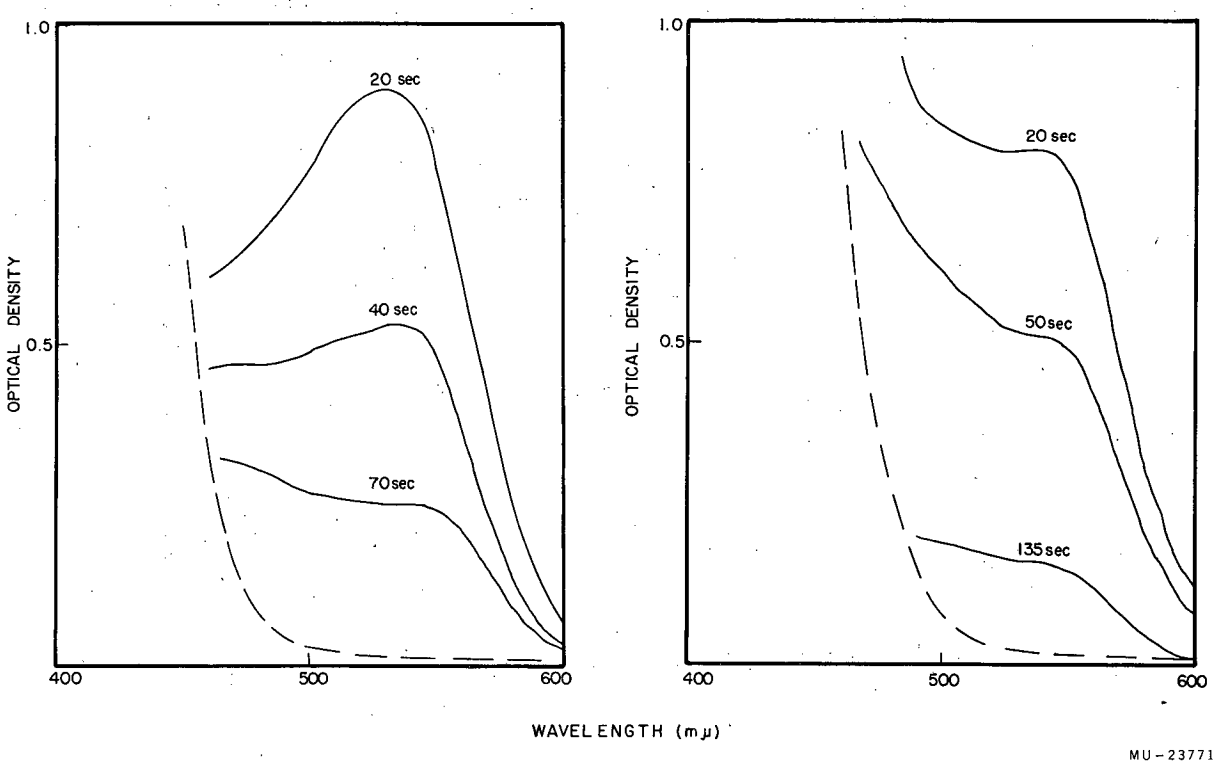

Fig. 18. Absorption of quinones in triethyl phosphite.
_____ complex
- - - - - - quinone in benzene

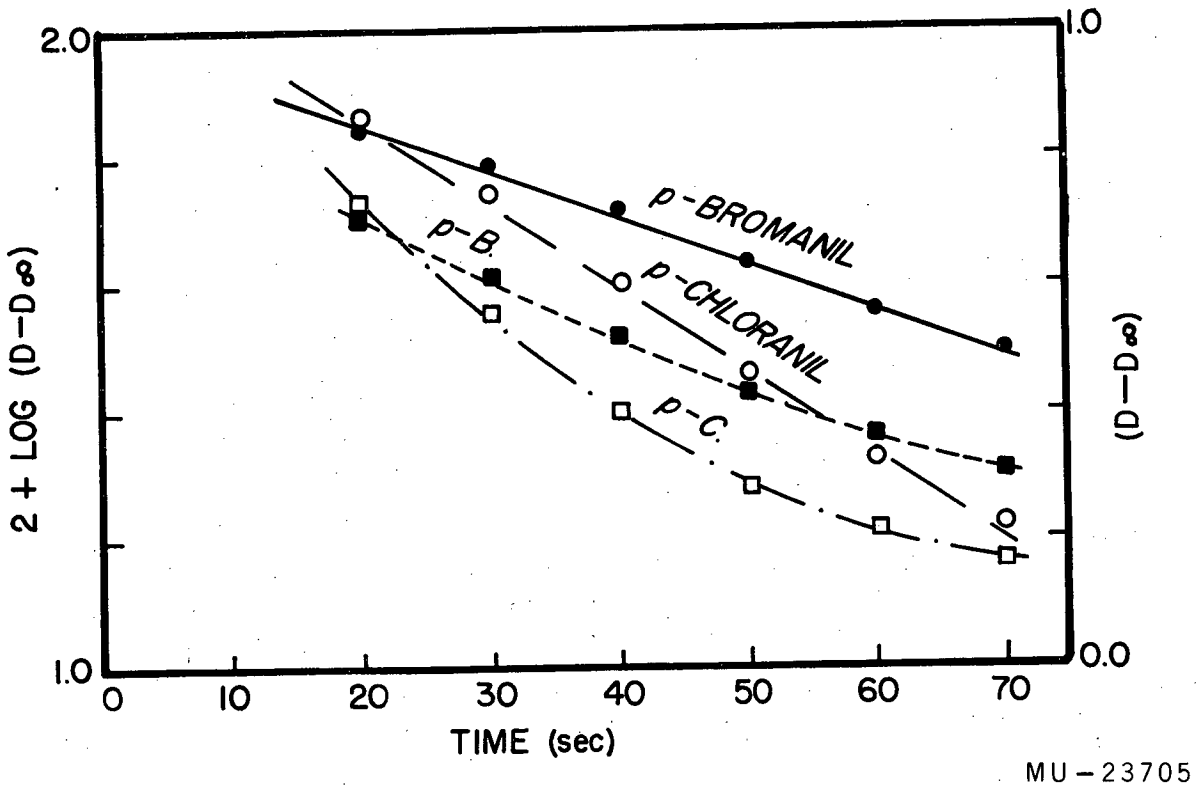

Fig. 19. Decay of absorption maxima of triethyl phosphite–quinone complexes

● and o $\log (D - D_\infty)$ against t

■ and □ $(D - D_\infty)$ against t.

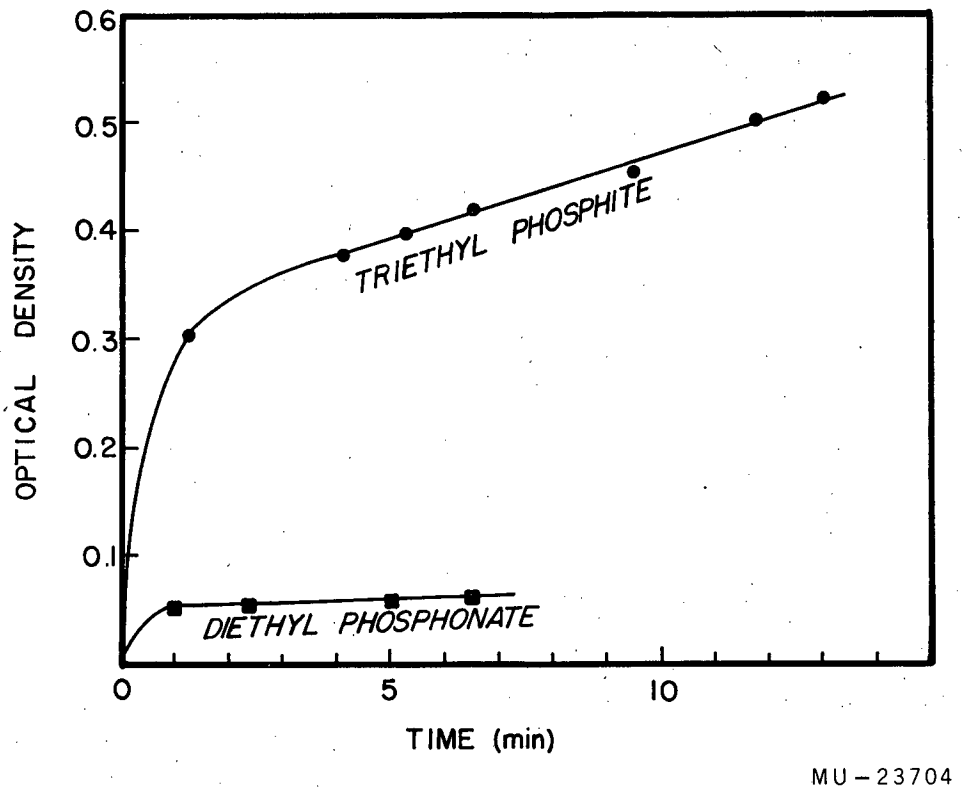

Fig. 20. Change with time of absorption of the 556-mμ peak obtained from molar solutions of triethyl phosphite or diethyl phosphonate and s-trinitrobenzene (10^{-2} M) in alcohol.

This is in fact identical to the results obtained with diethyl phosphonate and TNB in alcohol in the presence of a base, reported below. The same spectrum (maxima at 456 and 556 mμ, ratio about 2:1) was very sensitive to traces of the phosphonate, and it must be concluded that traces of this material were present in the alcohol solution.

The infrared spectrum of the solution showed a small peak in the 2,400-cm^{-1} region where the P-H vibrational frequency of diethyl phosphonate occurs, whereas the triethyl phosphite sample did not show this absorption.

Of particular interest, a solution of diethyl phosphonate (1 M) in alcohol in the presence of TNB developed the same absorption peaks only very slowly (Fig. 20).

Trinitrobenzene (0.3 M) dissolved in triethyl phosphite produced the spectrum of Fig. 21, where the peaks in the 560-mμ region gradually increased with time and where the intense absorption below 450 mμ steadily grew until it swamped the 560-mμ band (after 60 minutes).

c. Trinitrotoluene (0.5 M) dissolved in triethyl phosphite produced a band, centered at 530 mμ (corrected 545 mμ), that was weak (A = 0.12 after 45 seconds) and transient (half-life about 15 seconds).

d. Trimethyl phosphite containing dissolved trinitrobenzene produced peaks at 560 and 440 mμ (corrected 575 and 455 mμ). Chloranil and bromanil colors were associated with weak transient peaks at 540 and 550 mμ (corrected 545 and 555 mμ). The decays were more rapid and a precipitate developed in the chloranil solution.

Diethyl phosphonate complexes are relevant to the above problem and are examined here. Some of these have been described in an interesting note by Saunders and Stark[3]--a number of nitroaromatic compounds, in particular dinitrobenzoic acid, developed intense colorations with dialkyl phosphonates in basic solution. Trinitrobenzene is used here to avoid complications of proton transfer.

Results

Studies were made on ethanol solutions of trinitrobenzene at 10^{-2} M and 10^{-3} M containing triethylamine (10^{-2} M) or pyridine (10^{-2} M) and varying concentrations of diethyl phosphonate.

Addition of the phosphonate to the TNB-triethylamine solution produced a gradually deepening magenta color, and this was associated with two absorption bands centered at 456 and 556 mμ (corrected 461 and 561 mμ), whose maxima grew together and maintained a constant ratio of about 2:1 (Fig. 22).

The peak at 556 mμ was away from the trinitrobenzene absorption and its growth was therefore studied. Rapid initial rises were found which were

3. B. C. Saunders and B. P. Stark, Tetrahedron 4, 197 (1958).

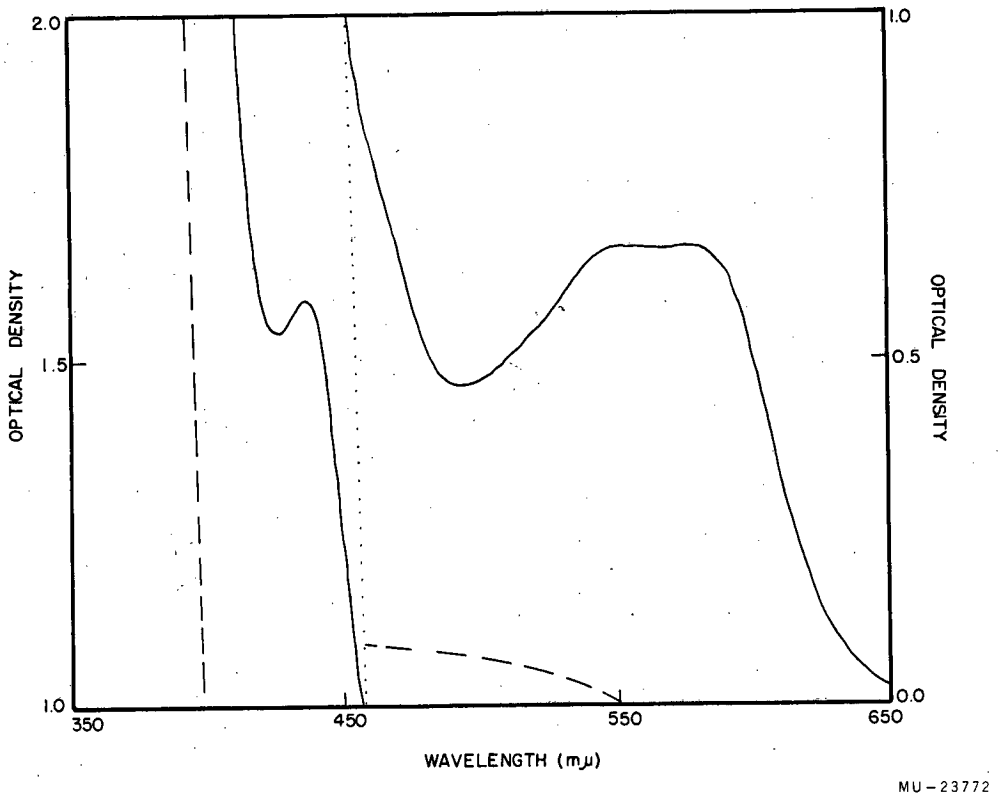

Fig. 21. Spectrum of s-trinitrobenzene in triethyl phosphite, 6 minutes after dissolving.

———— Complex

- - - - Trinitrobenzene in alcohol (corrected for concentration and path length)

Right hand side - scale 0 to 1.
Left hand side - scale 1 to 2.

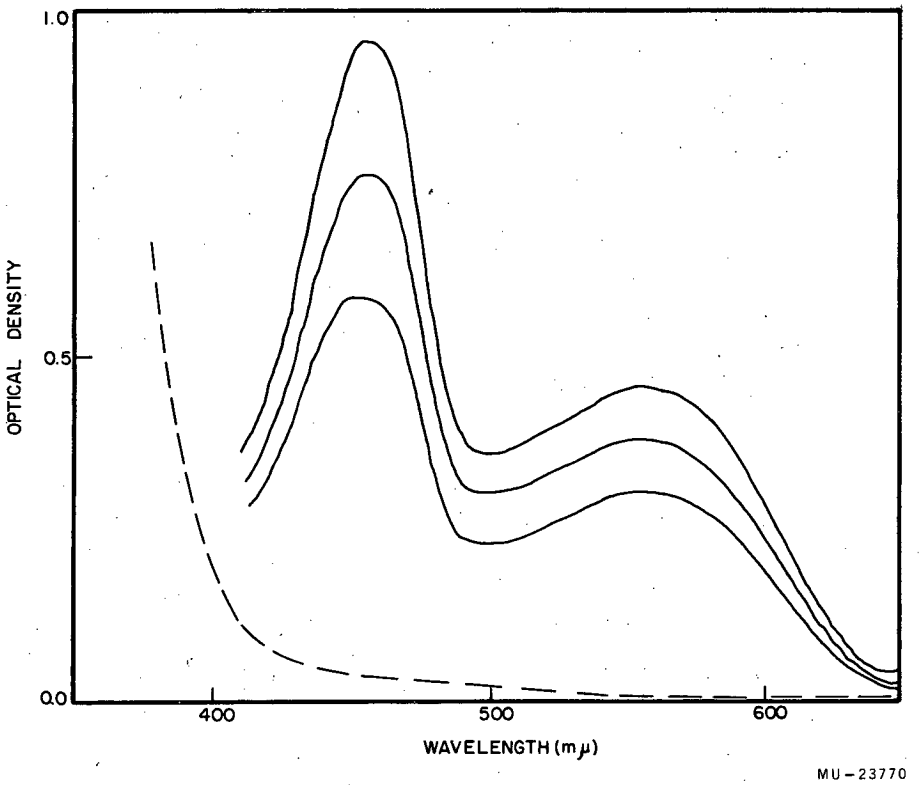

Fig. 22. Absorption of the complex formed in an alcohol solution containing diethyl phosphonate, trinitrobenzene, and triethylamine.

followed by linear increases with time (Fig. 23). For fixed concentrations of TNB (10^{-2} M) and triethylamine (10^{-2} M), the slope of the linear portion followed the concentration of phosphonate added.

	I	II	III	IV	V
Phosphonate concentration (M) $\times 10^4$	100	25	10	5	3
Slope: optical density units per minute $\times 10^4$	310	155	90	8.4	1

At lower TNB concentration (Fig. 24 shows curves for diethyl phosphonate concentrations of 10^{-2} M VI, 5×10^{-3} M VIII, 3.3×10^{-3} in VIII) the curves are less critically dependent on the ester molarity.

After a considerable period (24 hours) the spectra had passed through a maximum absorption and were starting to fade. The absorption at 556 mμ had shifted slightly to shorter wave lengths.

The color development without base was not dependent on ester concentration. Thus, after 30 minutes, the absorption of solutions 1 M and 10^{-2} M in ester at the second band had reached 0.07. Pyridine (10^{-2} M) did not catalyze the color formation, and after 20 minutes, a solution of TNB (10^{-2} M) and ester (10^{-2} M) reached the same absorption.

Discussion

I. The transient bands of the quinone (Q) solutions in triethyl phosphite (P) are not present in the original compounds, are formed as soon as the solutions are prepared, and occur close together (545 mμ and 553 mμ). They indicate the formation of complexes. An interpretation is that they are the charge-transfer bands[4] arising from the complex of the triethyl phosphite donor and the quinone acceptors. On the other hand, they do not exhibit the broad absorption frequently associated with this transition.[5] Thus, approximate half-widths for the dimethyl aniline-TNB, the anthracene-C, and anthracene-B complexes were 160, 150, 160 mμ, whereas the triethyl phosphite–quinone complexes were 80 mμ.

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4. The formation of red solutions with chloranil has also been interpreted as charge-transfer complex formation by F. Ramirez and S. Dershowitz, J. Org. Chem. 22, 856 (1957).

5. L. E. Orgel and R. S. Mulliken, J. Am. Chem. Soc. 79, 4839 (1957).

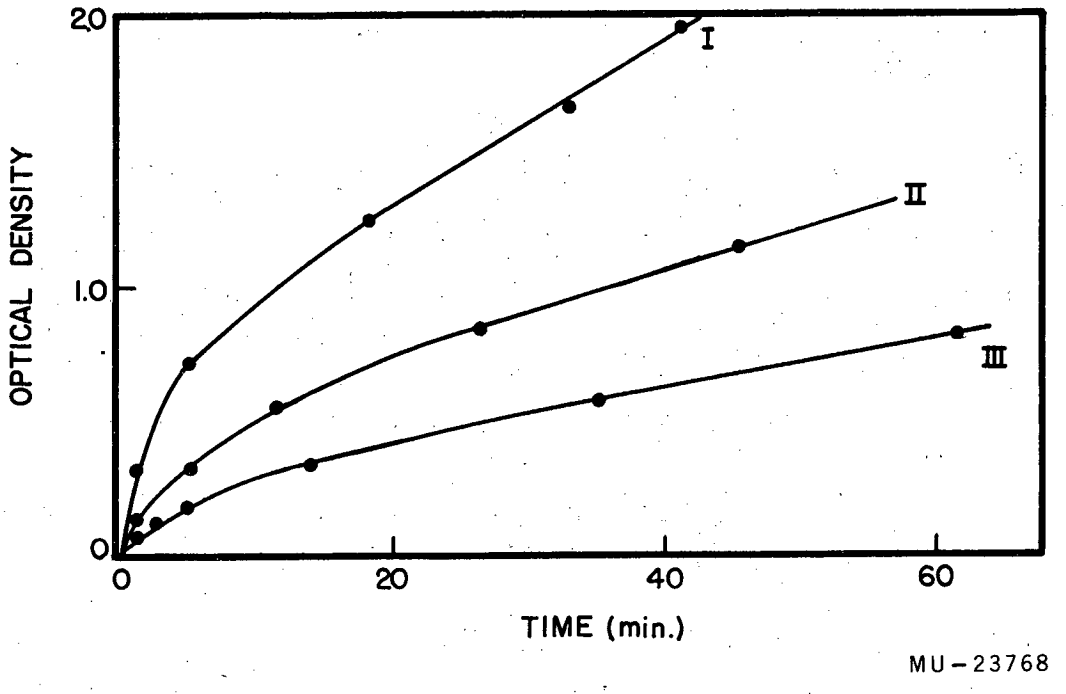

Fig. 23. Change of absorption of the 556-mμ peak obtained from diethyl phoshonate with trinitrobenzene (10^{-2} M) and triethylamine (10^{-2} M) in alcohol.

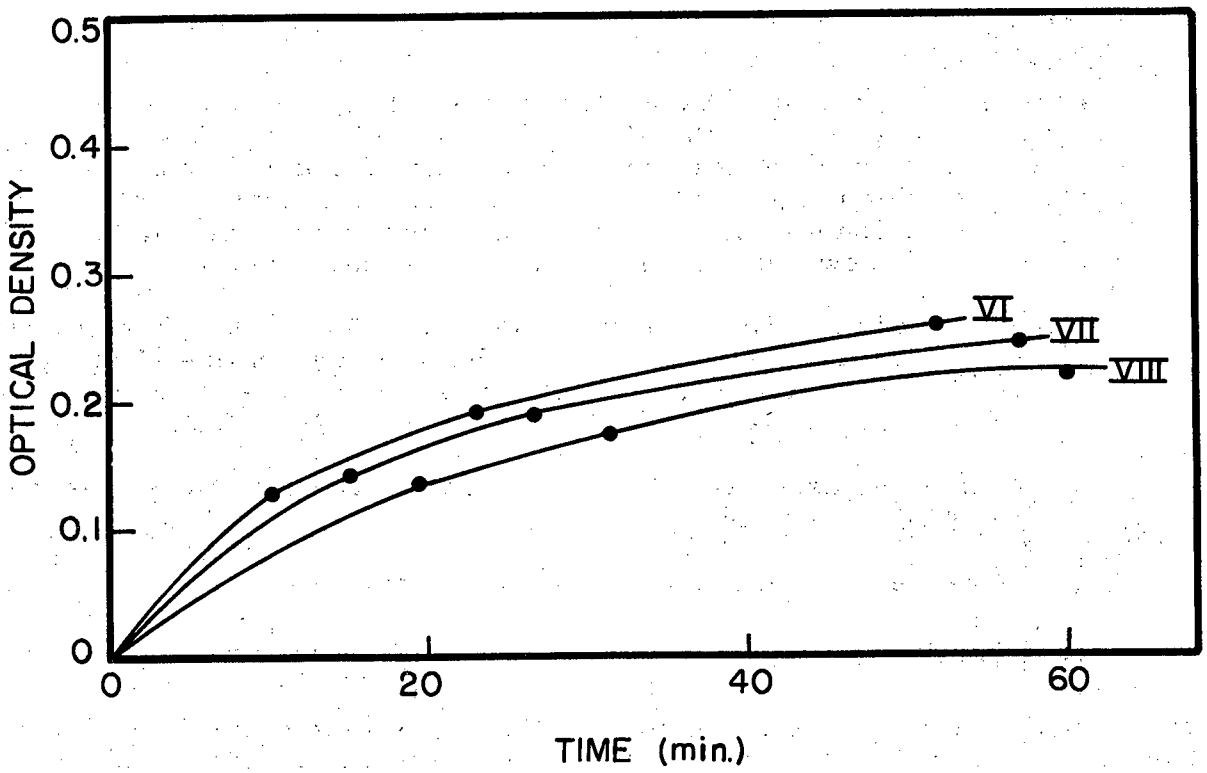

Fig. 24. Change of absorption of the 556-mμ peak obtained from diethyl phosphonate with trinitrobenzene (10^{-3} M) and triethylamine (10^{-2} M) in alcohol.

Assuming they are charge-transfer bands, one may estimate the ionization potential of the triethyl phosphite molecule to be 7.7 ev, using the relation[6]

$$h\nu = I_D - E_A - \Delta.$$

6(a) ν is the frequency of the charge-transfer band, I_D and E_A the ionization potential of the donor and the electron affinity of the acceptor, respectively; Δ is a constant dependent on the acceptor and donor molecules and also to a small extent on the solvent. An approximation is that Δ is a constant for a series of donors with one acceptor, hence by comparing the bands with donors of known ionization potential, the estimate above is made.

(b) A band of dimethyl aniline with trinitrobenzene in triethyl phosphite was centered at 457 mμ. The maximum of the dimethyl aniline-TNB band in a variety of solvents has been reported to range between 465 and 492 mμ--R. Foster and D. L. Hammick, J. Chem. Soc. 1954, 2685.

(c) A 1:1 complex is assumed. The transient nature of the peaks made concentration studies impossible.

(d) Estimations were made from the following information:

Donor	I_D	Acceptor	Solvent	ν_{CT}
Hexamethyl benzene	7.9	Bromanil	Carbon tetrachloride	527[a]
Hexamethyl benzene	7.9	Chloranil	Carbon tetrachloride	518[a]
Anthracene	7.4	Bromanil	Carbon tetrachloride	638[b]
Anthracene	7.4	Chloranil	Carbon tetrachloride	629[b]

The I_D values are from G. Briegleb and J. Czekalla, Z. Elek. 63, 6 (1959).

[a]R. Foster, Tetrahedron 10, 96 (1960).

[b]Solvent was added to a mixture of the donor and acceptor directly, the flask shaken for 5 minutes, and samples withdrawn after excess material had settled.

(e) General references: H. McConnell, J. S. Ham, and J. R. Platt, J. Chem. Phys. 21, 66 (1953); D. Booth, F. S. Dainton, and K. J. Ivin, Trans. Farad. Soc. 55, 1293 (1959); S. H. Hastings, J. L. Franklin, J. C. Schiller, and F. A. Matsen, J. Am. Chem. Soc. 75, 2900 (1953).

Also, the appearance of the bands is in accord with the two reaction schemes

$$P + Q \underset{\text{fast equilibrium}}{\rightleftharpoons} \text{Complex} \xrightarrow[\text{slow}]{} \text{Products}, \qquad (1)$$

$$\begin{array}{l} P + Q \longrightarrow \text{Products}. \qquad (2) \\ \downarrow\uparrow \text{ fast equilibrium} \\ \text{Complex} \end{array}$$

Again, assuming the existence of a charge-transfer complex, it is probable that Scheme (2) may be ignored, for an intermediate in which a partial electron transfer has occurred is most likely to be further along the reaction path than the reactions.

Charge-transfer complexes are well established with, for example, the unreactive aromatic hydrocarbons, which make ideal examples of donor molecules. Their existence as intermediates in a wide number of chemical reactions has been postulated.[7] The experimental difficulties encountered with these esters are typical of what is to be expected if attempts are made to extend the concept to other fields, and rapid-flow techniques[8] will be more profitable means of investigation.

The difference in reaction rates for C and B can probably be attributed to the bulkier groups on the bromanil molecule. For comparison, benzoquinone was found to react immediately (and strongly exothermally) with the triethyl ester.

Further studies can profitably be made on esters with bulkier alkyl groups and in suitable solutions (benzene or dioxan).

The appearance of the bands for the triethyl phosphite in ethanol with TNB can be attributed simply to the slow hydrolysis,[9] for it has been shown that the color formation is very sensitive to the diester. The color formation with the diester depends on the presence of a strong base in solution such as

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7. [a]W. Brackman, Rec. trav. chim. 68, 147 (1949); [b]R. S. Mulliken, J. Am. Chem. Soc. 74, 811 (1952).
8. J. B. Ainscough and E. F. Caldin, J. Chem. Soc. 1956, 2546.
9. A. E. Arbuzov and M. G. Imaev, Dokl. Akad. Nauk SSSR. 112, 856 (1957).

triethylamine or hydroxide ion. Pyridine was found not to work. The only possibly basic molecule in the solution was the triester itself, and it must be concluded that this is a strong base.[10]

In view of the results in ethanol, the triethyl phosphite-TNB spectrum (Fig. 21) must be attributed to a small amount of hydrolysis,[11] and the TNT spectrum may require similar qualification.

II. Saunders and Stark proposed a number of chemical products to account for the color from diethyl phosphonate, base, and nitrobenzenes.

An interesting possibility is that this may be an outer complex between the Lewis acid and the strongly basic phosphonate anion, and that the color arises from the charge-transfer transition of the complex. This may be quickly eliminated:

(a) the spectrum does not resemble a charge-transfer spectrum;

(b) a complex of this nature, particularly one involving the anion and in which very little steric interaction is to be expected, would be formed rapidly[12] from its components, probably as fast, in fact, as the anion was generated in solution. The half life of the P-H bond of the phosphonate[13] in an aqueous solution 10^{-2} M in triethylamine is about 10^{-3} second if OH^- catalysis alone is considered. This will not be different by many orders of magnitude in ethanol solution. Thus the spectrum could not have shown the kinetics found.

However, it is likely that this outer complex formation would facilitate the production of an inner complex or chemical product, and it is also likely that this would be colored because of the nature of the organic electron acceptor. Relevant to this, the rapid initial rise followed by a steady increase of absorption (Fig. 23) is in accordance with a rapid establishment of an equilibrium concentration of a nonabsorbing species, followed by a steady removal of this species to the complex. But a number of equilibria are possible in this solution. Also, the stable quality of the spectra obtained would favor just one product.

Other electron acceptors were tried in the color reaction with diethyl phosphonate and base and were compared with acceptors and base in neighboring tubes. Thus it was found that p-chloranil in carbon tetrachloride,

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10. The author has a considerable body of information to support this, as well as an interpretation. This will be reported later.

11. From a comparison with other spectra, an estimate of 10^{-2}% is sufficient to account for the results.

12. Compare reference 7b and G. N. Lewis and G. T. Seaborg, J. Am. Chem. Soc. 61, 1886 (1939).

13. Estimated from kinetic studies, P. R. Hammond, Ionization of the P-H Bond. Part I. Diethyl Phosphonate, J. Chem. Soc. in press.

Conclusions

1. p-Chloranil and p-bromanil dissolve in triethyl phosphite, producing transient absorption bands centered at 545 and 553 mμ. Their disappearance is first-order, with rate constants 3.0×10^{-2} and 1.6×10^{-2} sec^{-1}. Assuming these are charge-transfer bands arising from a complex between the triethyl phosphite donor and the quinone acceptors, the ionization potential of the ester is 7.7 ev.

2. The color produced in an alcoholic solution containing diethyl phosphonate, trinitrobenzene, and triethylamine is a chemical product and not a loose complex.

5. COMPLEXES OF SOME WEAK ACIDS

P. R. Hammond

It has been suggested in the preceding article that interaction of a strong base with the organic Lewis acids is a likely method for producing colored complexes, for even if the outer or charge-transfer complex is not stable enough to be observed, electron rearrangement to chemical products, or inner complexes, is likely to give colored materials because of the nature[1] of the organic acceptors used.

This concept has been applied to account for the color reaction between nitrobenzenes, bases, and diethyl phosphonate or other phosphorous acids.[2]

Similar color reactions have been known for some time in the carbon series,[3] and for this reason, the theory outlined above was investigated by using electron acceptors whose complex bands with donors frequently appear in the visible region.[4] In particular, p-chloranil, p-bromanil, s-trinitrobenzene, tetranitromethane, and tetracyanoethylene were used in the presence of the bases triethylamine or pyridine, and in a number of solvents.

As the field covered is broad, no attempt at spectral investigation was made and merely the colors of the solutions were recorded.

It may be seen that a large number of color reactions is found and hence the theory is a sensible interpretation.

Experimental Procedure

Acceptors were purified by crystallizing twice [chloranil (ethanol), trinitrobenzene (ethanol), and bromanil (carbon tetrachloride)]; by sublimation

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1. The electronegative groups responsible for enhancing the acceptor properties of the molecule are also likely to make the products derived from the acceptor colored.

2. B. C. Saunders and B. P. Stark, Tetrahedron 4, 197 (1958).

3. J. V. Janovsky and L. Erb, Ber. deut. chem. Ges. 19, 2155 (1886); W. J. Hickinbottom, Reactions of Organic Compounds, 2nd Edition (Longmans, Green and Company, London, 1948, p. 353; F. G. Mann and B. C. Saunders, Practical Organic Chemistry, 3rd Edition (Longmans, Green and Co., London 1952, pp. 216, 261, 293; M. J. Newlands and F. Wild, J. Chem. Soc. 1956, 3686.

4. L. J. Andrews, Chem. Revs. 54, 713 (1953); R. Foster, Tetrahedron 10, 96 (1960).

(tetracyanoethylene); and by fractional distillation under reduced pressure (tetranitromethane).

Bases were purified by double fractional distillation (triethylamine, pyridine, and dimethylaniline). (In addition, the last was refluxed with acetic anhydride before distillation).

Commercial solvents were distilled and dried over sodium (benzene, cyclohexane, dioxan, and ether); or fractionally distilled and the first and last fractions neglected ethanol, carbon tetrachloride).

Good commercial samples of carbon and nitrogen acids were used without further purification. Indene was fractionally distilled.

For investigating colors, to 1 ml of approximately 0.01 M acceptor solution was added 0.05 ml of base and 0.05 ml of the material to be tested. For the solid compounds, approximately 0.1 M solutions in ethanol were used. The colors with some solid materials were weak, (4, 4'-dinitrodiphenylmethane, phthalimide, ethyl carbamate), and these were discovered by saturating the acceptor solution with the compound and adding base.

Comparisons were made in every test with (1) acceptor plus base (2) acceptor plus compound, (3) compound plus base. Case (1) frequently gave colored solutions, but these were weak and did not interfere with the tests. The following general interactions for Case (1) were obtained (the abbreviations used are given below) although small variations occurred for different solvents.

	Acceptor Solutions				
Bases	TNB	C	B	TNM	TCE
TEA	O	B-G	G	X	X
Py	-	-	O	L-Y	L-Y

Case (2) produced colors only with the materials fluoradene, indene, and pyrrole whereas for (3) the nitro compounds gave very pale yellow solutions. These did not obscure the tests.

The following abbreviations are used:

B, blue; Bl, black; Br, brown; D, dark; G, green, Gr, gradual; L, light or pale; M, magenta; O, orange; P, purple; R, red; T, turbid solution; V, violet; Y, yellow;

* intense;
- nothing detected;
X, not possible to study because of vigorous reaction, precipitate or intensely colored solutions.

TEA, triethylamine; Py, pyridine; TNB, s-trinitrobenzene; C, p-chloranil; B, p-bromanil; TNM, tetranitromethane; TCE, tetracyanoethylene.

Results

Series I Carbon Acids

(a) p-Bromanil (acceptor)

Carbon acids	Benzene		Dioxan	
	TEA	Py(T)	TEA	Py(T)
1. 1-Chloro-1-nitropropane	Br	O	D-G	O
2. Nitroethane	Br-G	O	D-B-G	O
3. Ethyl cyanoacetate	D-G	B-Bl	B*	-
4. Nitromethane	R-Br	O	V	O
5. Ethyl acetoacetate	D-G	R	D-G	-
6. Diethyl malonate	D-G	-	L-G	L-O
7. Phenylacetonitrile	D-G	-	-	-
8. Phenylacetone	B	-	L-B	-
9. Acetone	-	-	-	
10. Acetonitrile	-	-	-	
11. Ethyl acetate	-	-	-	

In general, colors are unstable

(b) p-Chloranil

Acid	Benzene		Carbon Tetrachloride		Dioxan		Ethanol	
	TEA	Py(G_1)	TEA	Py	TEA	Py	TEA	Py
1.	Br	O	Br	-	Br	O	O	Y
2.	D-G	O	D-B-G	L-O	D-G	Y	O	P
3.	D-B-G	G	D-B-G	L-G	B*	Y	D-B-G	B
4.	V	O	V	L-O	V*	L-Y	O	P
5.	D-G	R-O	G	L-R	D-G	L-R	B-G	R
6.	L-G	L-O	G	-	-	-	G	R
7.	L-G	-			-	L-O	L-G	-
8.	B	-			B	O	-	-
9.	-	-	L-G	-	-	L-O	-	-
10.	L-G	L-O			-	O	-	-
11.	-	-			-	-	-	-

In general, colors are unstable.

(c) s-Trinitrobenzene

Acid	Benzene		Carbon Tetrachloride		Dioxan		Ethanol	
	TEA	Py(Gr)	TEA	Py	TEA	Py	TEA	Py
1.	L-O	L-O			L-O	-	D-R	-
2.	D-R	L-O			D-R	-	D-R	L-R
3.	D-R*	-			R	L-O	D-R*	L-R
4.	D-R*	L-O	D-R*	R	D-R	-	D-R*	R
5.	L-Y	-			-	-	D-R	L-R
6.	L-O	-	L-R	-	L-R	-	D-R	-
7.	L-O	-			-	-	R*	-
8.	-	-			O	-	D-M*	L-O
9.	-	-	-	-	-	-	R	-
10.	-	-			-	-	-	L-Y
11.	L-O	-			-	-	-	-

Colors are stable.

(d) Tetranitromethane

Acid	Carbon Tetrachloride	Ethanol	Ether
	Py	Py	Py
1.	L-Y	L-Y	-
2.	L-Y	L-Y	-
3.	L-Y	Y	-
4.	L-Y	L-Y	L-Y
5.	L-Y	L-Y	-
6.	-	-	-
7.	-	-	-
8.	L-Y	L-Y	-
9.	-	-	-
10.	-	-	-
11.	-	-	-

Colors are stable.

With pyridine as a base no color was observed with tetracyanoethylene. On the other hand, when one started with the intensely colored solutions containing dimethylaniline as a base, color removal occurred with the above carbon acids.

Propyl vinyl ether in ethanol with trinitrobenzene, chloranil, bromanil, and tetranitromethane gave pink, colorless, colorless, and yellow solutions respectively, whilst dihydropyran gave pale yellow, pale orange, pale orange, and orange solutions.

Addition of excess acetic acid to TNM, Py, and ethyl cyanoacetate in ethanol or to TNB, carbon acid, TEA solutions in ethanol caused slow color removals

Series II Carbon Acids

Trinitrotoluene, 4,4'-dinitrodiphenylmethane, indene, and fluoradene[5] produced red colors with TNB and TEA in ethanol, whereas fluorene and triphenylmethane did not.

The indene color was intense, and it was found that this compound with benzene or dioxan solutions of chloranil or bromanil in the presence of triethylamine gave dark-green, practically black colors.

Similarly, TNT in dioxan in the presence of C and B with TEA gave dark blue solutions which rapidly changed to purple precipitates. In comparison, C and B with TEA were light turquoise and TNT with TEA was light yellow.

5. H. Rapoport and G. Smolinsky, J. Am. Chem. Soc. 80, 2910 (1958). The color with fluoradene was found to fade within a minute.

Nitrogen Acids

Succinimide, phthalimide, and ethyl carbamate gave red colors in alcohol solution with trinitrobenzene and triethylamine. Cyanamide, diphenylurea, and acetanilide did not work, whereas a red color with pyrrole did not require the presence of a base. Addition of acetic acid removed the colors, and base caused them to return.

Discussion

The following discussion contains some interesting hypotheses on the interpretations of the results. They are merely tentative and will serve to make further predictions that can be investigated.

The base is necessary for the complex formation. This causes ionization of the H-X system and the strong base X^- is liberated in small concentrations in solution.

A. Return of the proton to X^- can produce a tautomeric form of many of the acids, and this form can interact with electron acceptors to form a colored complex.[6] Although this may occur with many of the systems described it is unlikely to occur with the Series II carbon acids, and the colors produced with vinyl propyl ether and dihydropyran are by no means as intense as those found for the Series I.

B. The strong base X^- interacts with the Lewis-acid acceptors to give a complex which is probably of Mulliken outer or charge-transfer type.[7]

Three cases apply:

(i) The complex is stable to further electron rearrangement. Reacidification should remove the anion and hence the color, and color formation should be reversible, depending on the acidity of the solution. Of the number tried, only one carbon acid, trinitrotoluene in the presence of TNB and TEA, and the nitrogen acids behaved in this way.

If the relation (preceding report, Reference 6)

$$h\nu = I_D = E_A - \Delta$$

may be applied to bases as strong as those under consideration, then the ionization potential of the anions may be estimated, a quantity which is not accessible by other measurements.

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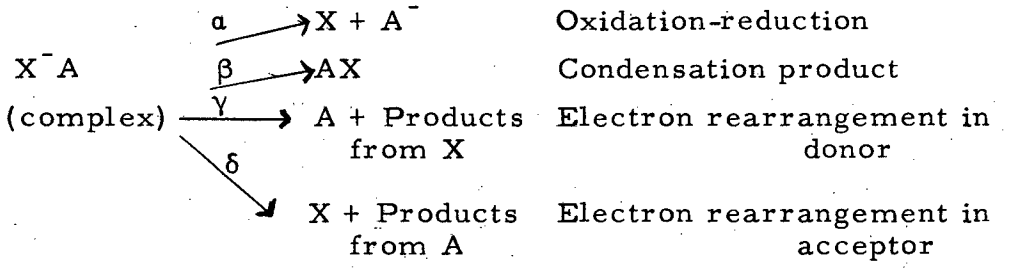
6. Compare complexes of dihydropyran, J. E. Collin, Z. Elektrochem. 64, 936 (1960).

7. R. S. Mulliken, J. Am. Chem. Soc. 74, 811 (1952).

(ii) The complex is not stable to further electron rearrangement and chemical products appear. This is likely to be a more general case than (i), thus the trinitrobenzene–diethyl phosphonate case may be considered as an example, and it may well apply to the acids of Series I, in which reacidification does not result in immediate color removal. It happens in the examples described that the products are colored. In the case of tetracyanoethylene they are not.[8]

Four cases apply for product formation



(iii) For cases in which the complex has intermediate stability, light, which promotes the transition to an $XA^{\pm}$ excited state, quickens chemical reaction if this state is unstable. Decomposition to products does not require a separation of charge.

C. Both Cases A and B imply that base-catalyzed reactions of the acids with electron acceptors may be sensitive to traces of other acceptor or donor molecules. Thus the halogenations of acetone and diethyl phosphonate are to be investigated. The effect of cupric ion on the bromination of ethyl acetoacetate is well known.[9]

D. The question of inner or outer complex formation should be resolved by examination of the visible spectrum and by the simple kinetic studies outlined in the preceding article.

E. For cases in which product formation is suspected, free-radical intermediates may appear and may be observable by electron spin resonance spectroscopy. Thus, the strong signals obtained with basic solutions of TNB in acetone are of interest.[10] The phosphonate case particularly should be worth examining.

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8. Compare J. K. Williams, D. W. Wiley, and B. C. McKusick, Abstracts of 139th Meeting of the American Chemical Society, Organic Chemistry Section, Abstract 9-0.

9. K. J. Pedersen, Acta Chem. Scand. 2, 252 (1948).

10. R. E. Miller and W. F. K. Wynne-Jones, Nature 186, 149 (1960).

F. The conclusions from spectral studies performed with acceptors, particularly TNB, base, and any of the above acids as solvent, e.g., acetone and acetonitrile, may need re-examination.[11]

G. Some further points that require investigation are as follows:

(i) The intensity of color formation, particularly for the Series I carbon acids and TNB, decreases with increasing pK of the acids.[12] Thus 4, 4'-dinitrodiphenylmethane is weakly acidic.[13]

(ii) The interaction of hydroxide ion with trinitrobenzene,[14] triethylamine with chloranil,[15] and the intense brown and red-brown solutions obtained by adding aqueous sodium hydroxide to dioxan solutions of p-chloranil and p-bromanil may be considered to be specific examples of the theory, particularly as the first is light-sensitive.

(iii) The deep red color from TNB, TEA, and TNT in ethanol was removed by acids, was returned by fresh base, and was completely reversible. Similarly, the purple coloration obtained by adding base to TNT solutions was completely reversible, depending on the acidity of the solution.

It is of interest that kinetic studies by Caldin and workers[16] on the TNT-ethoxide ion reaction in ethanol indicated a very fast reaction which had been ascribed to formation of either a charge-transfer complex or an addition compound. This was followed by a second fast reation which was ascribed to the formation of the colored TNT anion.

Another possibility to add to this is that the anion interacted with the Lewis-acid TNT in solution and the color observed was the charge-transfer band of the complex. Trinitroaniline, a nitrogen acid, was also studied by these authors and similar arguments apply.

This idea will require detailed comparison with the kinetic information reported. The color of TNT in basic solutions should depend on the ratio of HX/X^- being a maximum when the ratio is the same as the ratio for the complex.

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11. G. Briegleb, W. Liptay, and M. Cantner, Zeit. Physik. Chem. (Frankfurt) 26, 55(1960); R. E. Miller and W. F. K. Wynne-Jones, J. Chem. Soc. 1959, 2377 and Reference 9.

12. R. G. Pearson and R. L. Dillon, J. Am. Chem. Soc. 75, 2439 (1953).

13. Compare N. C. Deno, J. Am. Chem. Soc. 74, 2039 (1952).

14. V. Gold and C. H. Rochester, Proc. Chem. Soc. 1960, 403.

15. D. Buckley, S. Dunstan, and H. B. Henbest, J. Chem. Soc. 1957, 4880.

16. E. F. Caldin and G. Long, Proc. Royal Soc. A228, 263(1955); J. B. Ainscough and E. F. Caldin, J. Chem. Soc. 1956, 2546.

The disappearance of color in strongly basic solutions is perhaps relevant to this. On the other hand, inner complex formation, as has been ascribed to TNB complexes, is undoubtedly of importance.[17] The same argument cannot be applied to TNB, as it contains no dissociable hydrogens.[18]

(iv) The color estimation of creatinine, with dinitrobenzoic acid in basic solutions, has been known for some time.[19] The color was ascribed to a Janowsky-type[3] complex formation in which the compound functioned as a carbon acid. Materials necessary to make a detailed comparison were not available, but in view of the experiments with nitrogen acids it is likely that creatinine may be included with these.

17. D. Booth, Science Progress 48, 435 (1956).
18. J. A. A. Ketelaar, A. Bier, and H. T. Vlaar, Rec. trav. chim. 73, 37 (1954).
19. J. J. Carr, Anal. Chem. 25, 1859 (1953).

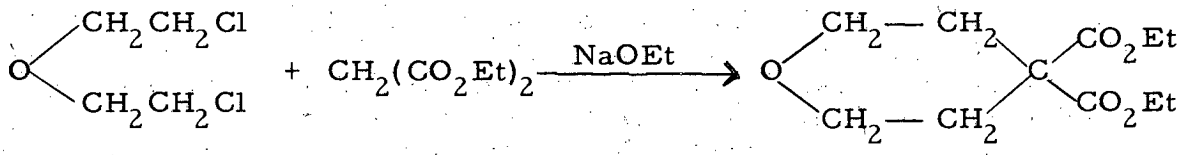
6. CYCLIC DISULFIDES

George C. Palmer

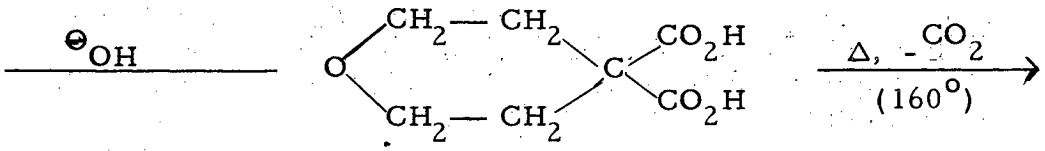
The original purpose of this particular work on cyclic disulfides was to determine the energy barrier to rotation about the sulfur-sulfur bond.

1,2-Dithiacycloheptane-5-carboxylic acid (Formula VI below) was synthesized with the expectation that it could be resolved and the rotational barrier could then be calculated from the temperature coefficient of the racemization. This acid, however, showed an odd instability, and studies are now underway to determine the nature of this instability. It was suggested that it was due to the presence of the carboxyl group adjacent to the dithiacycloheptane ring; therefore, it was felt that some other 7-ring disulfides should be studied. 1,2-Dithiacycloheptane was synthesized and does not seem to show this type of instability; 1,2-Dithiacycloheptane-5-acetic acid is being synthesized to test its stability; 1,2-Dithiacyclohexane-3-acetic acid was also synthesized, and attempts to purify and resolve it are not in progress.

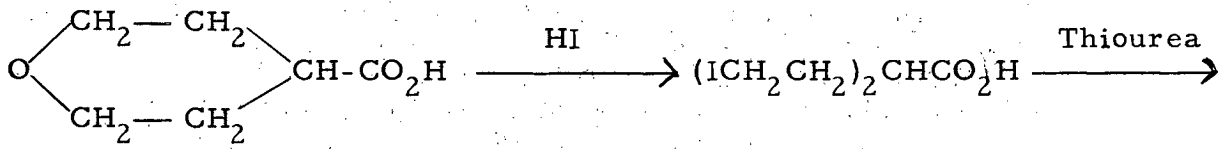
1,2-Dithiacycloheptane-5-carboxylic acid was synthesized according to the scheme shown below:[1]



(I)

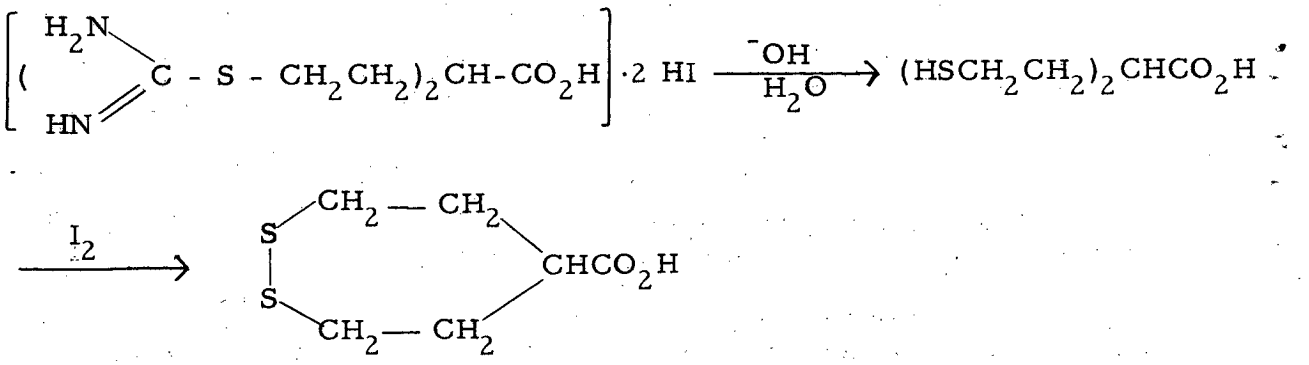


(II)



(III) (IV)

1. G. E. Utzinger, unpublished work from this Laboratory.



(VI)

Diethyl-tetrahydropyran-4,4-dicarboxylate (I). 715 g (5.0 moles) of β,β'-dichloroethyl ether, 801 g (5.0 moles) diethyl malonate, and 230 g (10.0 moles) of sodium dissolved in 3200 ml absolute alcohol were reacted as follows: (1) The malonic ester, plus half of the sodium ethoxide solution, were added with stirring to the reaction flask. (2) The dichloroethyl ether and the second half of the ethoxide solution were added simultaneously at the rate of about 1 mole per hour; stirring and refluxing were carried on during the addition. (3) After the addition was completed, the refluxing was continued for 4 to 6 hours. (4) The reaction mixture was then made acidic with acetic acid. (5) About 80 to 90% of the alcohol was removed by distillation. (6) Sufficient water to dissolve the NaCl was added, and the phases were separated. The remaining organic material was extracted from the aqueous phasw with ether, the organic phase was dried, the solvents removed, and the product distilled.

Yield: 675 g (57.5%); bp 104.5–105.5° at 2.3 mm.

Tetrahydropyran-4,4-dicarboxylic acid (II). 310 g diester (from above) was added to a solution of 260 g of KOH in 1.5 liters of water, and then refluxed for 2 hr. The solution was acidified with HCl, and the products extracted with ether. Yield: 210 g (90%); mp 173–174°.

Tetrahydropyran-4-carboxylic acid (III). 351 g of the diacid was decarboxylated by heating to 160° in vacuo, then the product was distilled; the vacuum fluctuated considerably during the distillation. Yield: 233 g (87%); bp about 112° at 1.7 mm; mp 90°.

1,5-Diiodo-3-pentanoic acid (IV). 32.5 g of the tetrahydropyran-4-carboxylic acid dissolved in 450 cc HI (d = 1.7) was reacted in the dark for 2 days, then the diluted HI was distilled off at 50 to 70° at about 70 mm pressure. An additional 100 ml of HI was added, the diluted acid distilled off, and this process continued twice more until the diiodo acid "crystallized" or oiled out of the solution. Yield: 48 g (52%); mp 91.0–91.2°.

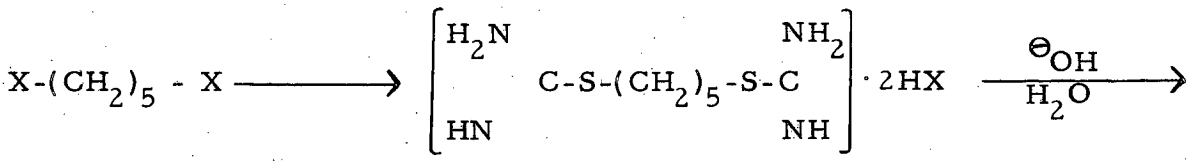
1,5-Diisothiuronium salt of 3-pentanoic acid (V). 18.4 g (0.050 moles) of diiodide was reacted with 7.5 g (0.10 moles) of thiourea in 140 ml absolute methanol, and the solvent then evaporated. Then, in succession two 110-ml portions of $CHCl_3$ were added and evaporated. The product was an oil, insoluble in $CHCl_3$, but was used as such in the following reaction.

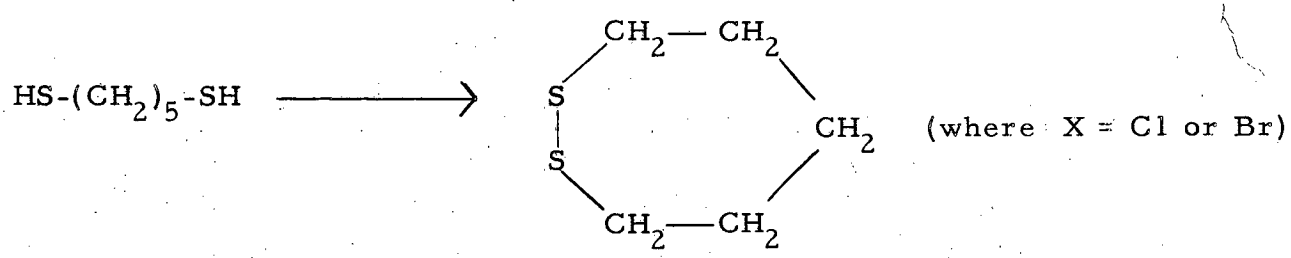
1,2-Dithiacycloheptane-5-carboxylic acid (VI). The "diisothiuronium salt" from the above reaction was dissolved in water, 250 ml 1 N NaOH added, the solution brought to a volume of 2 liters and allowed to stand 2 to 3 hr, titrated with 900 ml (theoretical 100 ml) of 0.1 N KI_3 solution. The solution was acidified with HCl, and extracted with ether. Yield: 3.4 g (38%).

The over-all yield was about 7%. The product had a λ_{max} = 256 mμ, ϵ = 480 (Fig. 25), mp 72–75°.

Upon standing in dilute alcohol solution in the refrigerator for 20 days, the product showed an altered spectrum--the original maximum at 256 mμ shifted to 254 mμ, and a new maximum appeared at 248 mμ (Fig. 26). After standing much longer, the original maximum disappeared, with only the 248-mμ maximum showing (Fig. 27). (This could be the result of polymerization, with the new maximum due to a nonstrained disulfide linkage.)

1,2-Dithiacycloheptane was synthesized as follows.[2,3,4]





(where X = Cl or Br)

2. Grogan, Rice, and Sullivan, J. Org. Chem. 18, 728 (1953).
3. Grogan, Rice, and Reid, J. Org. Chem. 20, 50 (1955).
4. Schoberl and Grafje, Ann. 614, 66 (1958).

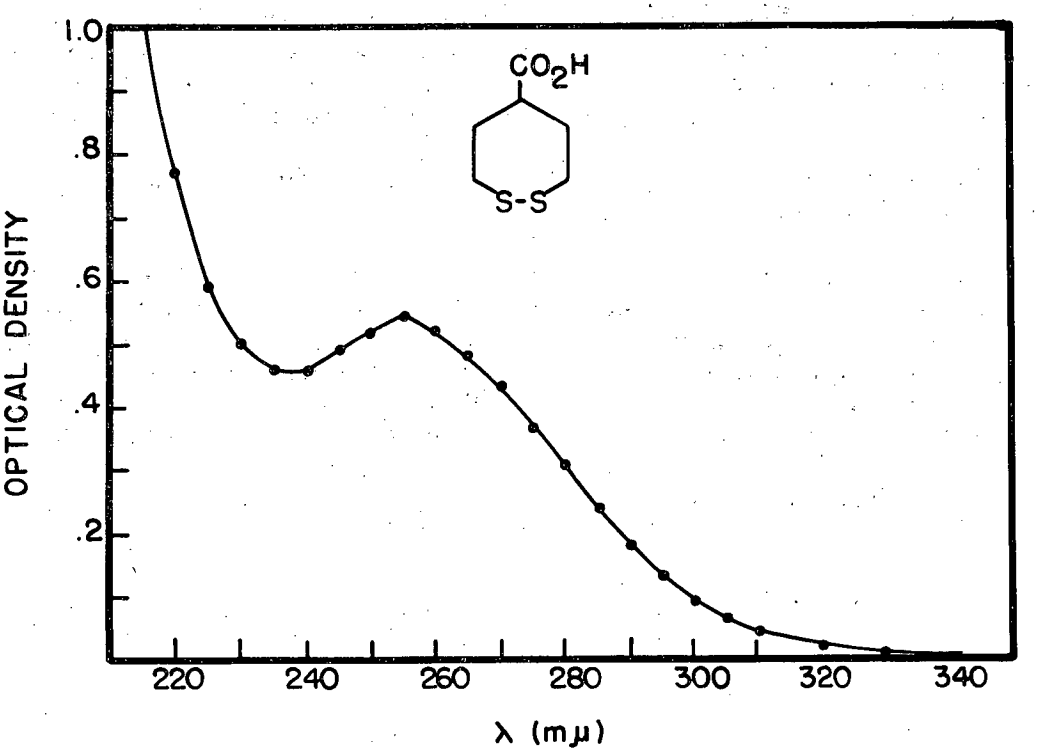


Fig. 25. 1,2-Dithiacycloheptane-5-carboxylic acid (VI). Fresh solution. 0.02% in 95% EtOH.

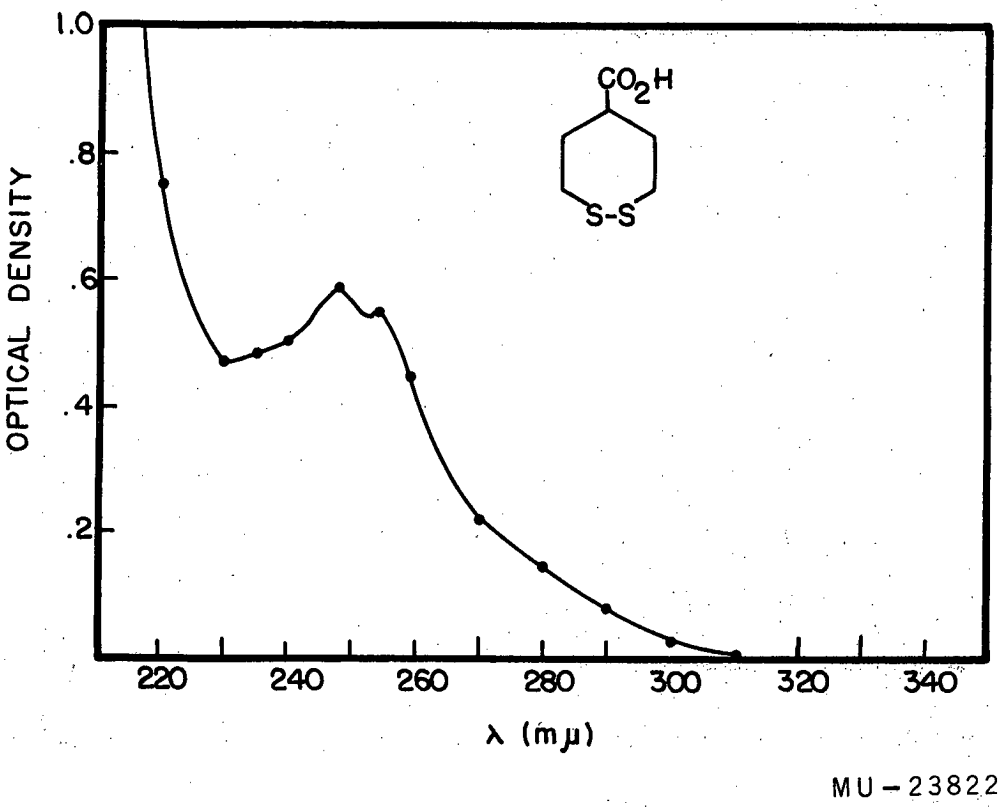


Fig. 26. 1,2-Dithiacycloheptane-5-carboxylic acid (VI) stored 20 days in the refrigerator as a 0.02% solution in 95% EtOH.

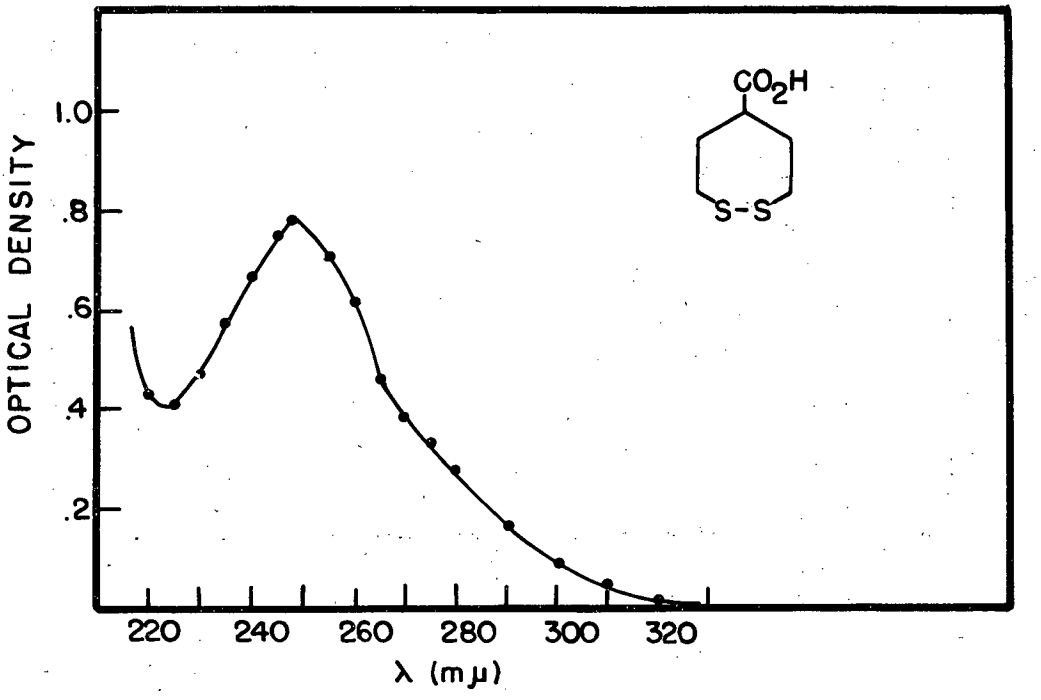


Fig. 27. 1,2-Dithiacycloheptane-5-carboxylic acid (VI) stored 18 months in the refrigerator as a 0.02% solution in 95% EtOH.

Pentane-1, 5-diisothiuronium salt.[2] 155 g (1/2 mole) of 1, 5-dibromopentane and 76.1 g (1 mole) of thiourea were dissolved in 700 ml absolute alcohol (commercial) and the mixture refluxed for 9 hr. After the solution was cooled, 500 ml of ether was added slowly to the solution, precipitating the product. Yield: 184.2 g (96%); mp 164–165°.

Pentane-1, 5-dithiol.[3] 184 g (0.482 mole) of the above diisothiuronium salt was dissolved in 450 ml of water, a solution of 160 g (2.84 moles) of KOH in 170 ml wqter was added, and the mixture refluxed for 24 hr. On cooling, the solution was acidified with 400 ml 6 N HCl, then extracted four times with ether. The extracts were dried and the solvent evaporated. Yield: 62.3 g (95%), bp 135–138° at 72 mm; main fraction: 56.7 g (86.5%), bp 136.5–138.0° at 72 mm.

1, 2-Dithiacycloheptane.[4] 30 g (0.22 mole) of pentane-1, 5-dithiol (in 500 ml of 95% ethanol containing 40 g $NaHCO_3$) was oxidized with 0.35 equivalent of 0.2 N KI_3 solution, with a small amount of $FeCl_3$ as catalyst and indicator. The reaction mixture was extracted three times with ether, the ether extracts washed four times with saturated aqueous NaCl solution, then dried, and the solvent removed. Yield: 8.5 g (35%); bp 79° at 10 mm.

The over-all yields were 30 to 35%. The product has a λ_{max} 258 mμ, ϵ = 400. After it had at room temperature for a month in dilute alcoholic solution, there appeared to be only small changes in the spectrum, either qualitatively or quantitatively (Fig. 28). The significance of these differences is questionable.

1, 2-Dithiacycloheptane-5-acetic acid is being synthesized according to the following route.[5]

5. Burger, Turnbull, and Dinwiddie, J. Am. Chem. Soc. 72, 5512 (1950).

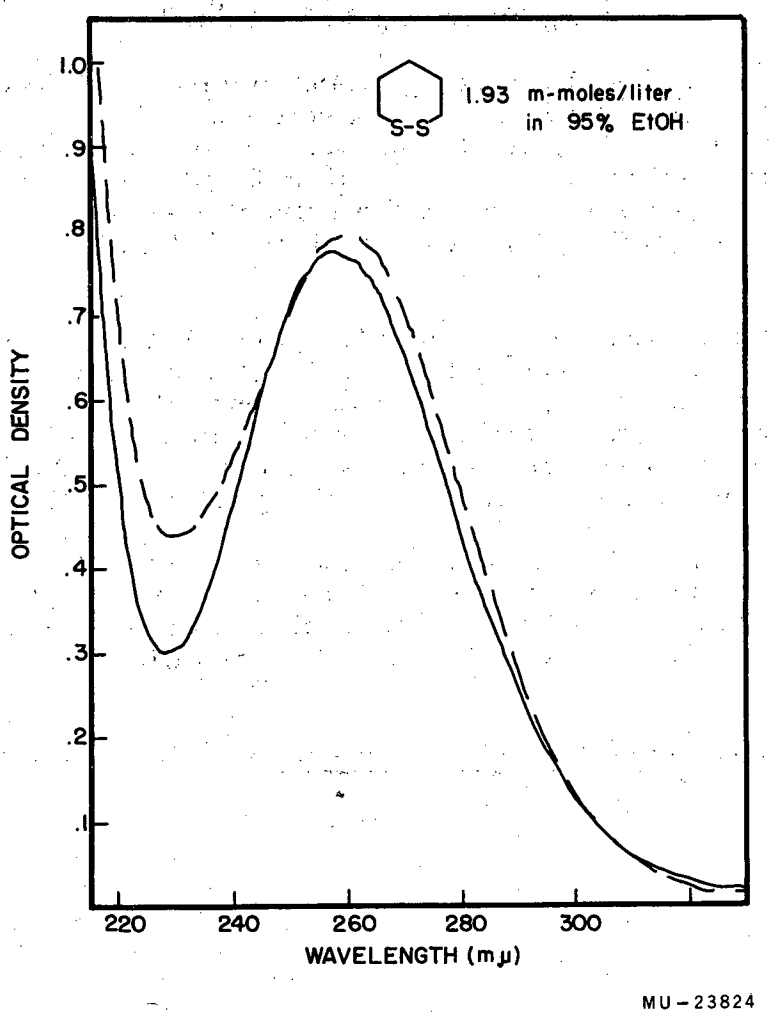
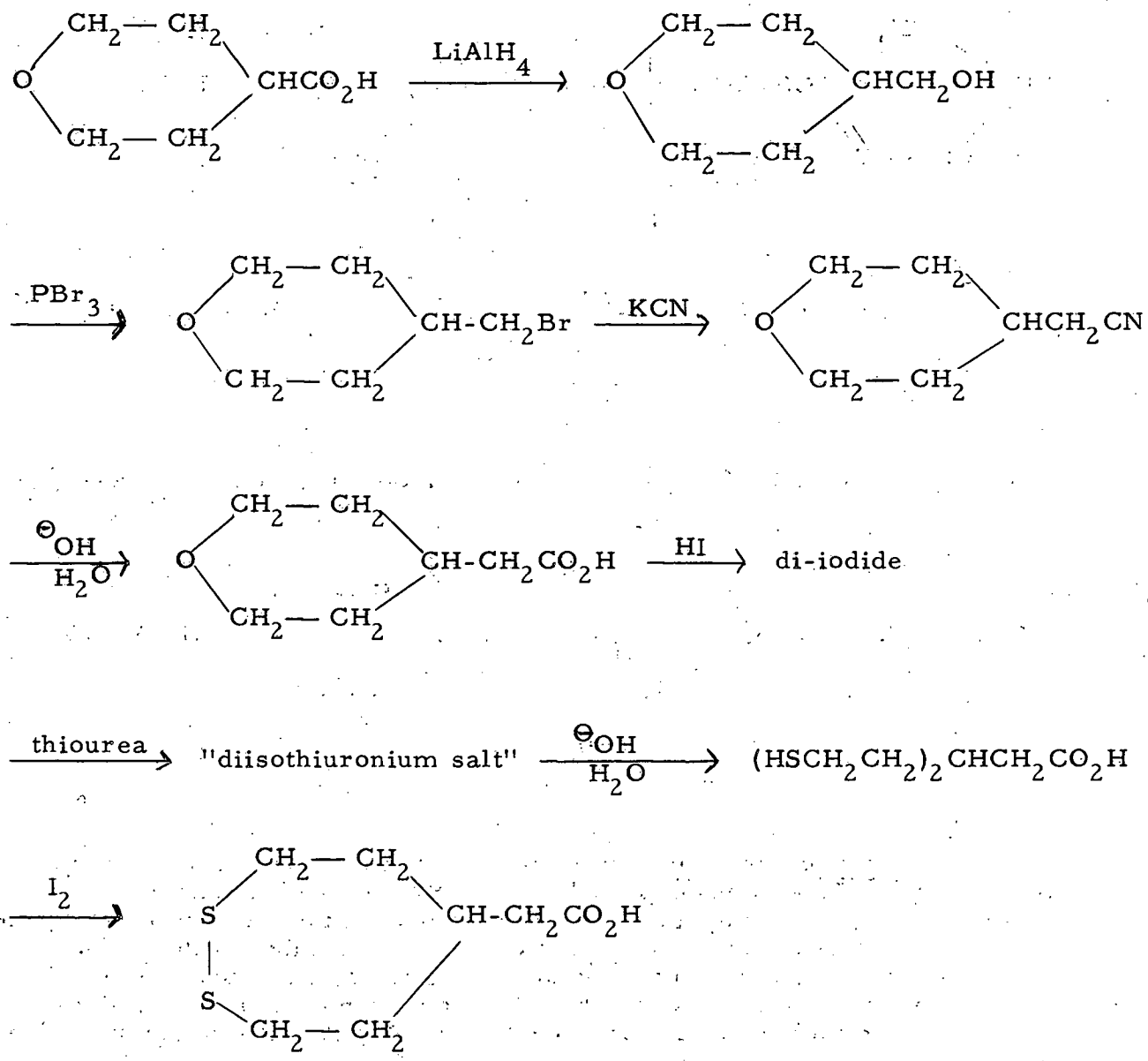
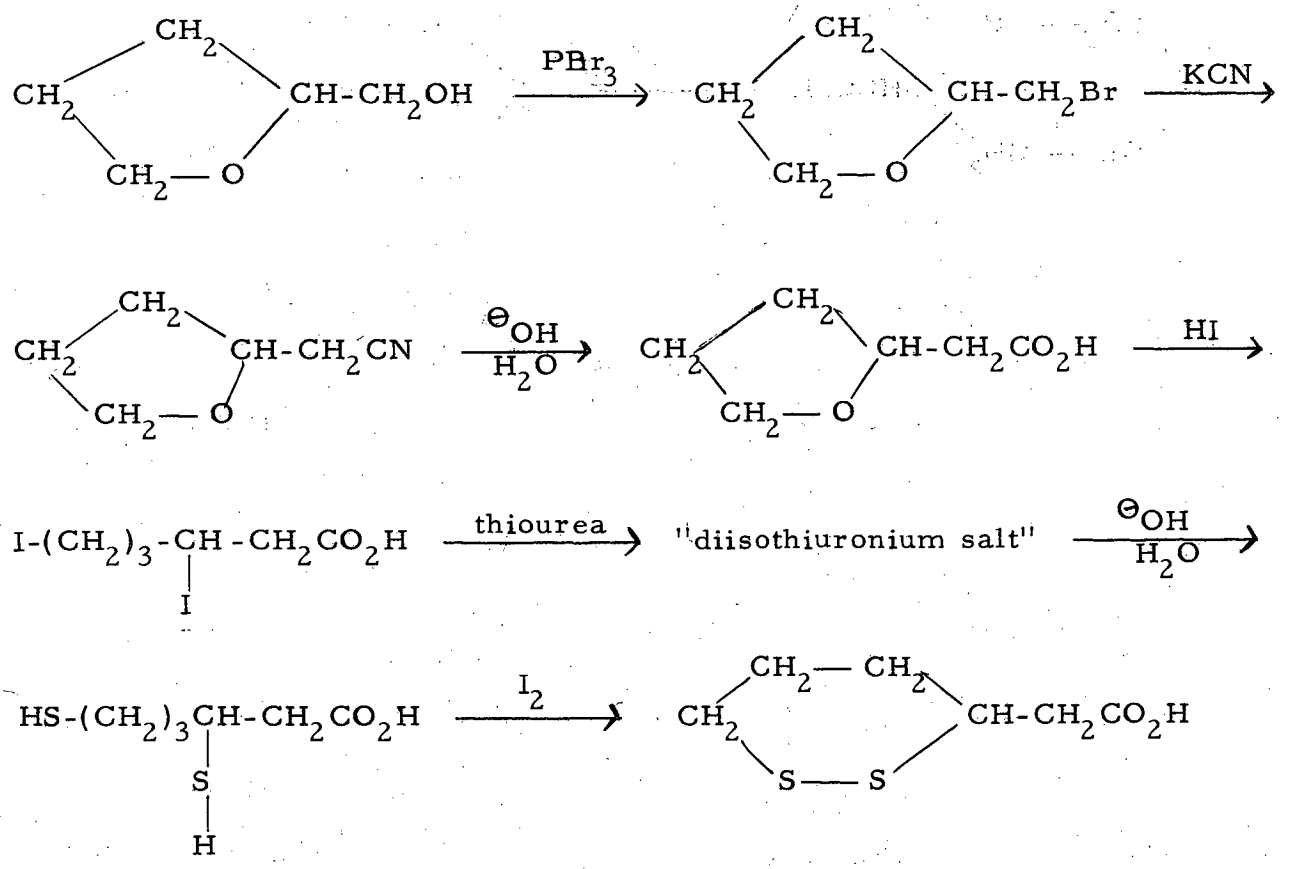


Fig. 28. 1,2-Dithiacycloheptane, 1.93 mmoles/liter in 95% EtOH.

——— Fresh solution
----- Solution stored for one month at room temperature



4-Hydroxymethyl tetrahydropyran.[5] 77.5 g (0.597 mole) tetrahydropyran-4-carboxylic acid in 1250 ml dry ether was added with stirring over a 2-hr period to 59 g (1.55 mole) of $LiAlH_4$ in 1500 ml of dry ether. The mixture was refluxed for 10 hr, the excess $LiAlH_4$ was destroyed with dilute (1:5) H_2SO_4, and the solution was made alkaline with $NaHCO_3$ before extraction. After the extracts were dried and the solvent was evaporated, 55 g of crude carbinol was obtained. The product was distilled; the yield was 50.1 g (72%), bp 115-118° at 25 mm. (Literature value: 105-110° at 20 mm.) Acidification of the mother liquors yielded 12.5 g of starting material, giving a yield of 86%, considering this recovery.



Tetrahydrofurfuryl bromide was prepared according to Organic Syntheses.[6] To a solution of 103.1 g of PBr_3 in dry benzene was added (with stirring) 15 g of pyridine. To this was added a solution of 6 g pyridine in 109 g of tetrahydrofurfryl alcohol over a 4-hr period, with the temperature of the reaction mixture kept at -3 to -5°. Stirring was continued for 1 hr, then the mixture was allowed to stand at room temperature for 2 days. Yield: 89.8 g (50%), bp 34–35° at 2.5 mm. (Literature value: 49–50° at 4mm.)

Tetrahydrofurfuryl nitrile.[7] 300 g of tetrahydrofurfuryl bromide, 200 g KCN, 10 g NaI, 350 ml of 95% EtOH, and 200 ml water were refluxed for 35 hr. The ethanol was distilled off and the nitrile isolated with ether. Yield: 52.8 g (26%), bp 92–93° at 15 mm. (Literature value: bp 92.4° at 13 mm.)

Tetrahydrofurfuryl carboxylic acid.[7] 52.8 g of tetrahydrofurfuryl nitrile, 65 g KOH, 500 ml of 95% EtOH, and 130 ml water were refluxed overnight (about 15 hr). Upon cooling, the reaction mixture was acidified with 100 ml of 12 N HCl, then extracted with ether. Yield: 45.8 g (74%), bp 117–118° at 2.3 mm. (Literature value: pb 144–146° at 16 mm.)

3,6-Diiodocaproic acid. 32 g of tetrahydrofurfuryl carboxylic acid reacted in the dark with 450 ml of HI (d = 1.7). After standing 24 hr, the mixture was heated to boiling at 80 mm pressure and the diluted HI plus an oil were distilled (total about 150 ml). 100 ml of HI (d = 1.7) was added to the reaction mixture and an additional 50 to 75 ml was distilled over, and an oil was formed in the reaction flask. This oil was dissolved in $CHCl_3$, washed eight times with aqueous KI solution, then dried, and the solvent removed. The remaining oil (45 g, 49%) was used to make the diisothiuronium salt.

3,6-Diisothiuronium salt of caproic acid. The diiodide (an oil) from the preparation above (45 g = 0.122 mole) was reacted for about 1 hr with 19 g thiourea in refluxing methanol. The resulting oil was insoluble in chloroform, and was used, unpurified, for the next reaction.

1,2-Dithiacyclohexane-3-acetic acid. The diisothiuronium salt was dissolved in about 500 ml water, and 425 ml of 1 N NAOH was added. After standing for 2 to 3 hr, the solution was titrated with 0.1 N KI_3 solution (1650 ml required--theory: 2400 ml). The solution was acidified with 6 N HCl and extracted with ether. Ten grams of an oil was obtained (about 50% yield and about 5% over-all yield).

The crude product has a λ_{max} at 285 mμ, with an $\epsilon = \sim 250$, and with a small maximum at 260 mμ (Fig. 29) which is evidently due to the presence of a 7-ring isomer caused by a rearrangement during the HI treatment. After two "sublimations" the maximum due to the 7-ring was gone (Fig. 30), but the product could not be obtained crystalline.

4-Bromomethyl tetrahydropyran. To a solution of 45 g (16 ml) of PBr_3 in 25 ml of dry benzene was added, with stirring, 7 ml of pyridine. A solution of 3 ml of pyridine in 50 g 4-hydroxymethyl tetrahydropyran was then added to the mixture over a 4.5-hr period; during the addition, the reaction temperature was kept at -6 to -3°. The mixture was allowed to stand at room temperature for a week. The mixture was then distilled, and the fraction boiling between 85 and 91° at 30 mm was collected. Redistillation of this yielded about 50 g of product boiling at 82.5–84.0° at 17 mm. Upon standing overnight, this turned yellow. The product was therefore dissolved in ether, washed with 5% H_2SO_4 (twice), followed by two washes with distilled water, then dried and solvent removed. Distillation yielded 45 g, bp 84.0–85.0° at 18 mm. This,[5] too, developed a yellow discoloration in a few hours. (Literature value,[5] bp 84–86° at 20 mm.)

Thus, the largest problem in this synthesis has been the purification of the bromide. However, the impure bromide may be adequate for the next step--the nitrile synthesis. This work is now in progress.

1,2-Dithiacyclohexane-3-acetic acid has been prepared according to the following scheme.[6,7]

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6. L. H. Smith, Tetrahydrofurfuryl Bromide, in Organic Syntheses, Vol. 23, 88-89 (1943).

7. Barger, Robinson, and Smith, J. Chem. Soc. 1937, 718.

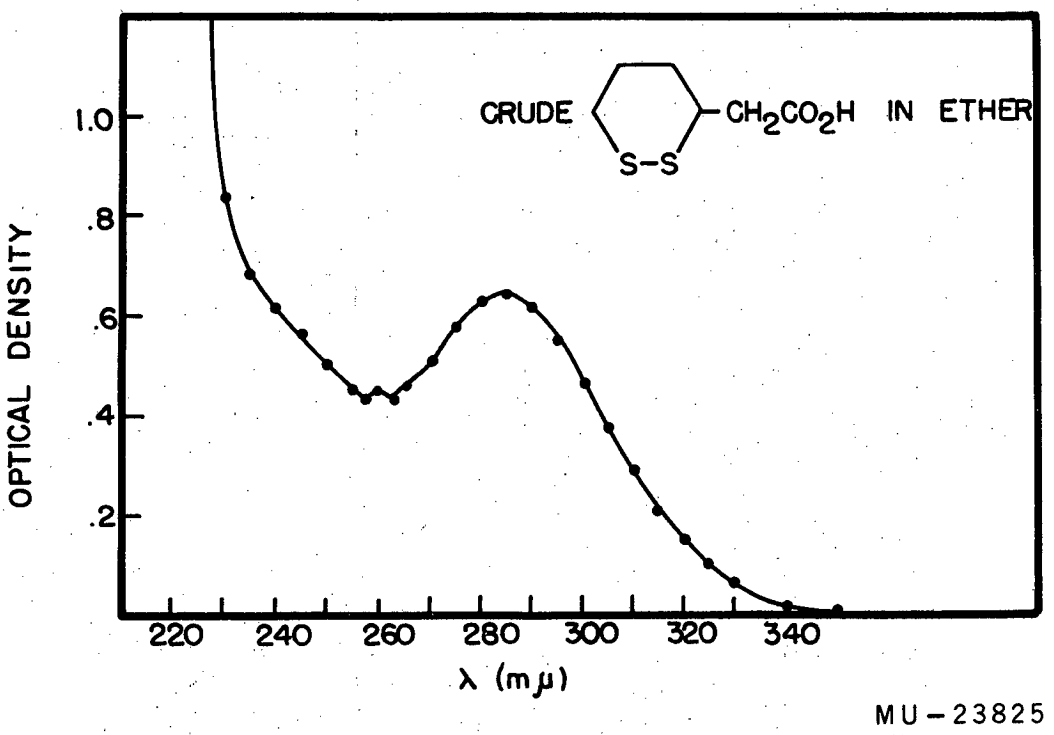

Fig. 29. Crude 1,2-dithiacyclohexane-3-acetic acid in ether.

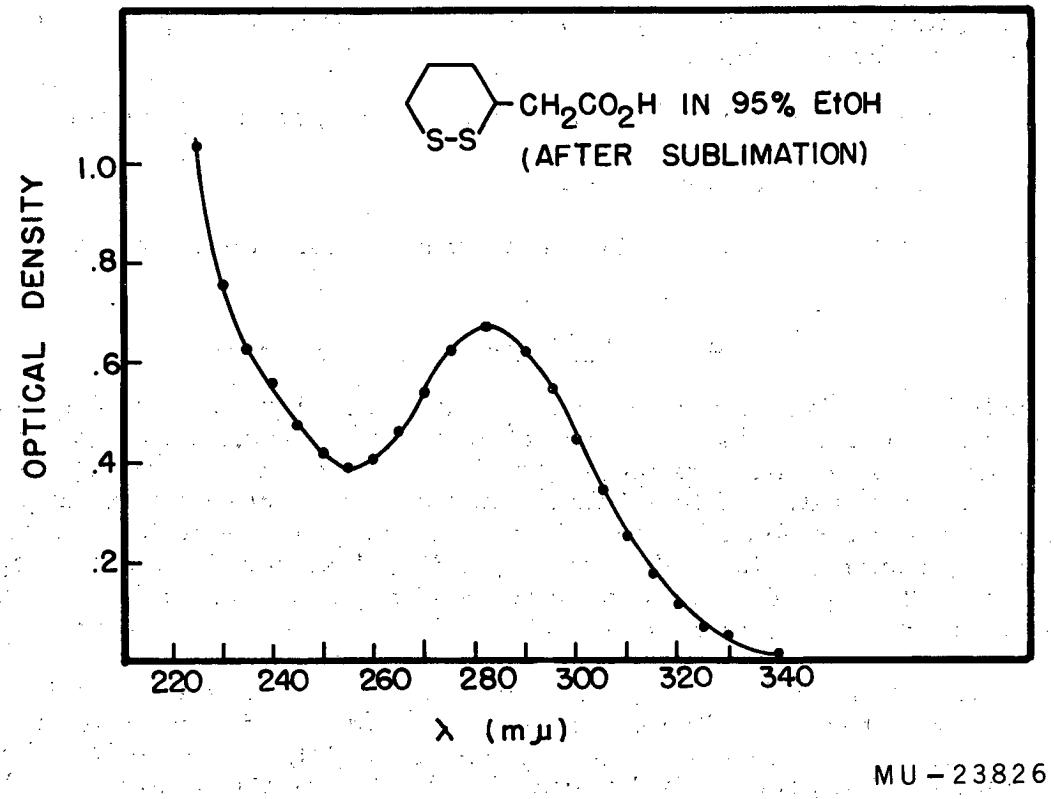

Fig. 30. 1,2-Dithiacyclohexane-3-acetic acid in 95% EtOH (after sublimation).

7. THE CHEMICAL EFFECTS OF IONIZING RADIATION ON ADENINE AND GUANINE

Cyril Ponnamperuma and Richard M. Lemmon

In a further study of the radiation decomposition of aqueous solutions of purines,[1] we have investigated

I. The production of formamidopyrimidines from adenine.

II. The deamination of guanine.

III. The breakdown of the purine ring in guanine and adenine.

I. Formamidopyrimidines from Adenine

Scholes and Weiss have shown that in the x-irradiation of aqueous solutions of purine nucleosides and nucleotides the purine ring system is the most sensitive to radiation damage.[2,3] Hems examined the attack on the base in greater detail, and found that when aqueous solutions of guanosine and guanylic acid are irradiated in the absence of oxygen, the imidazole ring of the purine system opens to form the corresponding 4-amino-5-formamidopyrimidine riboside.[4,5] This product was found to be similar in ultraviolet spectral characteristics to the products obtained by the alkylation of guanylic acid.[6]

In our study of the γ-radiation decomposition of aqueous solutions of adenine (I), we have identified the 4-amino-6-hydroxy-5-formamidopyrimidine (II) and the 4,6-diamino-5-formamidopyrimidine (III).[1] There is some evidence to show that a third product is 4,5-diamino-6-formamidopyrimidine (IV).

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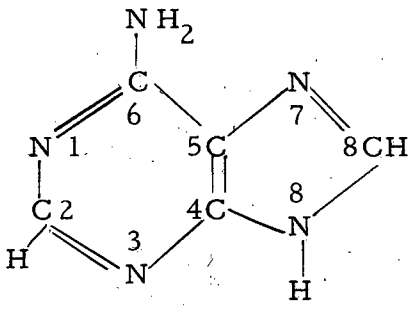
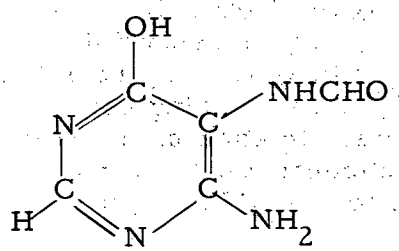
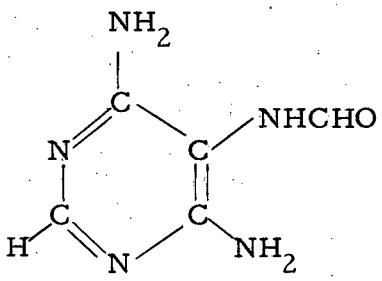
1. Cyril Ponnamperuma and Richard M. Lemmon, in Bio-Organic Chemistry Quarterly Report, UCRL-9652, April 1961, p. 50.
2. G. Scholes and J. Weiss, Biochem. J. 56, 65 (1954).
3. M. Daniels, G. Scholes, and J. Weiss, Experientia 11, 219 (1955).
4. G. Hems, Nature 181, 1721, (1958).
5. G. Hems, Radiation Research 13, 777 (1960).
6. P. D. Lawley and C. A. Wallik, Chem. & Ind. (London) 633 (1957).



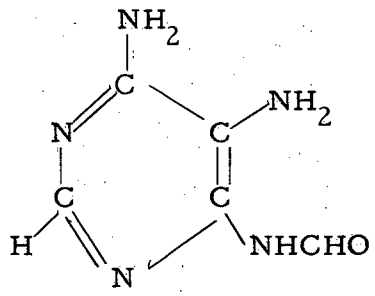
I



II



III



IV

Experimental Procedure

Adenine-2-C^{14} of specific activity 1.3 mC/mmole was obtained from Isotope Specialties Co., Burbank, Calif. The method of irradiation was the same as described in our earlier reports.[7] Two hundred and fifty µl of a 0.1% solution of adenine in water was sealed in vacuo. Dissolved oxygen was previously expelled by bubbling nitrogen through the solution. The 1.5-kilocurie cobalt-60 source was used for the irradiation. The intensity of radiation, calculated by Fricke ferrous sulfate dosimetry, was 3.45×10^5 rad per hour.

The irradiated products were separated by paper chromatography by using Whatman No. 4 paper with propanol:ammonia:water and butanol:propionic acid:water as solvents.[8] Twenty-five µl of the solution of adenine-2-C^{14} irradiated at 2×10^6 rads was chromatographed with 10 µl of 0.1% solution each of the inactive 4,6-diamino-5-formamidopyrimidine and 4-amino-6-hydroxy-5-formamidopyrimidine.[9] The ultraviolet-absorbing areas were carefully marked

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7. C. Ponnamperuma, R. M. Lemmon, and E. L. Bennett, in Bio-Organic Chemistry Quarterly Report, UCRL-9408, Sept. 1960, p. 32.
8. E. L. Bennett, Biochim. et Biophys. Acta, 11, 487 (1953).
9. These compounds were kindly supplied to us by Dr. George B. Brown of the Sloan-Kettering Cancer Research Insitute.

out. X-ray film autoradiographs showed darkening of the film corresponding to the areas representing the two formamido compounds. These spots were eluted with water and rechromatographed with fresh carrier formamido-pyrimidines by using butanol:water[10] and isobutyric acid:ammonia:EDTA[11] as solvents. In each case (that is, in all four solvent systems), the radioactivity was concentrated on the pyrimidine spot, confirming the formation of radioactive formamido compounds.

A third as yet unidentified spot is possibly the 5,6-diamino-4-formamido compound (IV), which is the isomer of (III). Its R_f value is very close to that of (III) and, possibly, this compound arises from the fission of the N_7-C_8 bond of the imidazole ring,[1] instead of the N_9-C_8 bond.

The formation of these formamido compounds may be of some biological importance, especially in light of the work of Scholes and Weiss[2,3] and Hems[4,5] with the nucleosides and nucleotides. The imidazole ring opens before the loss of the sugar moiety. It is possible that under the influence of radiation, a ring opening of this type could take place while the nucleotide is still attached to the double helical DNA. A similar effect has been observed with the mutagenic nitrogen mustards when they react with the nucleic acids.[12] An opening of the imidazole ring while the monomeric nucleotide is still part of the long-chain acid would interfere with the orderly process of replication of the DNA molecule.

II. The Deamination of Guanine

When adenine is irradiated in solution, one of the effects observed is the replacement of the amino group in the 6-position by a hydroxyl group, giving rise to hypoxanthine.[1,7] Guanine could be expected to react in the same way, giving rise to xanthine. Similarly, cytosine would be converted into uracil.

Preliminary experiments with guanine indicate that this conversion takes place.

Experimental Procedure

Guanine-8-C^{14} sulfate $(C_5H_5ON_5)_2 \cdot H_2SO_4$ of specific activity 2.18 mC/mmole was obtained from the Oak Ridge National Laboratory. On account of the very low solubility of guanine sulfate, a 0.04% solution was used for the irradiation.

The method of irradiation, and of chromatographic analysis, was the same as that described for the adenine. Two hundred µl of a 0.5% solution of inactive xanthine was chromatographed (propanol:ammonia and butanol:propionic acid) together with 250 µl of guanine sulfate solution irradiated at 2×10^6 rads.

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10. J. Smith and R. Markham, Biochem. J. 45, 294 (1949).
11. H. A. Krebs and R. Hems, Biochim. et Biophys. Acta, 12, 173 (1953).
12. Professor E. Boyland, Chester Beatty Reseach Institute, London, personal communication.

Autoradiographs with x-ray film showed darkening of the film corresponding to the xanthine. The activity in this area was measured with an end-window Geiger-Mueller tube. About 3% conversion had taken place.

These findings have to be confirmed by further two-dimensional chromatography and liquid scintillation counting. The present results, however, would appear to show that the deamination of guanine takes place more readily than that of adenine. The maximum yield of hypoxanthine from adenine was 1.9%.[13]

III. The Breakdown of the Purine Ring in Guanine and Adenine

To estimate the amount of radioactivity lost during the irradiation, 25 μl of each of the irradiated solutions and of the control were counted with a liquid scintillation counter. An internal C^{14} standard was used. The loss of activity may be due to several causes: the release of C^{14}-labeled volatile material, the adsorption of C^{14}-labeled compounds on the glass container, or the chelation of radioactive products by trace impurities. But, in every case, the loss of activity is a measure of the breakdown of the molecule. Figure 31 presents graphically the results obtained for guanine-8-C^{14} sulfate and guanine-2-C^{14} sulfate irradiated from 10^6 to 20×10^6 rads. The results for the breakdown of adenine, which were reported earlier,[1] are included in the same figure. The results for guanine-8-C^{14} sulfate and guanine-2-C^{14} sulfate cannot as yet be extrapolated to zero. Further measurements at dose levels below 10^6 rads must be made.

The guanine was labeled in the 8-position, i.e., in the imidazole ring, while the adenine was labeled in the 2-position, which is in the pyrimidine ring. The greater degradation of guanine seems to show that the imidazole ring is more labile to radiation than the pyrimidine ring. This fact is confirmed by a preliminary experiment with guanine-2-C^{14} sulfate in which the loss of radioactivity was less than with guanine-8-C^{14}. The formation of the formamido compounds described in the first part of this report also points to the ease of disruption of the imidazole ring.

In view of the resonance stability of the pyrimidine ring, it is not surprising that the imidazole ring is attacked more easily than the pyrimidine ring.

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13. C. Ponnamperuma, R. M. Lemmon, and E. L. Bennett, in Bio-Organic Chemistry Quarterly Report, UCRL-9519, Jan. 1961, p. 26.

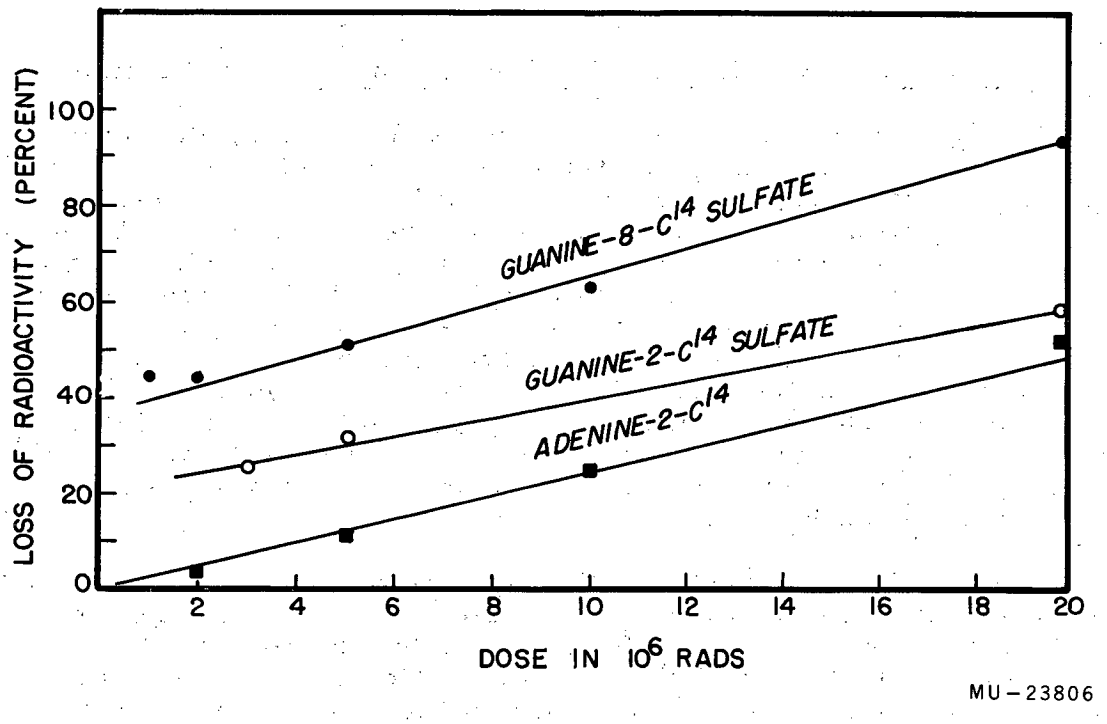

Fig. 31. Dose-vs-decomposition data for aqueous solutions of guanine-8-C^{14} sulfate, guanine-2-C^{14} sulfate, and adenine-2-C^{14}.

8. DETERMINATION OF HEXOKINASE ACTIVITY IN BRAIN

Edward L. Bennett and J. B. Drori*

(In collaboration with David Krech and Mark R. Rosenzweig, Department of Psychology, and M. Diamond, Department of Anatomy, University of California, Berkeley.)

Studies by our group have shown a correlation of brain cholinesterase activity with behavior in rats.[1] Variations in environment can alter the amount and distribution of cholinesterase activity in the brain.[2] Glucose is the principal energy source of cerebral tissue. The hexokinase (Hk) activity may be of prime importance in determining the maximal metabolic rate of brain and may be correlated with an animal's learning ability or behavior.

The activity of a number of ensymes, including Hk, has been investigated, principally in the tracts of the central nervous system and the cortex.[3,4] However, no detailed study of the distribution of Hk in the rat brain is available.

Two methods for the determination of Hk activity have been studied. The method and results obtained using 2-deoxyglucose as a substrate have been summarized.[5]

However, the determination of hexokinase activity by this method was not without its disadvantages for our purposes--it required relatively large samples (at least 40 to 50 mg in order to do duplicate assays), and so precluded the analyses of small functional areas of a single rat brain. The method was laboroius, and, more important, since it is a "difference" method, we felt that variations of 5% or less in hexokinase activity of pairs of animals with different behavior scores or treatment could not be reliably detected.

We have accordingly investigated the coupled reaction

$$\text{glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-phosphate} + \text{ADP},$$

$$\text{glucose-6-phosphate} + \text{TPN}^{+} \xrightarrow{\text{glucose-6-phosphate dehydrogenase}} \text{6-phosphogluconic acid} + \text{TPNH} + \text{H}^{+}.$$

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*Kaiser Foundation Hospital, Oakland, California

1. Mark R. Rosenzweig, David Krech, and Edward L. Bennett, Psychol. Bull. 57, 476 (1960).

2. David Krech, Mark R. Rosenzweig, and Edward L. Bennett, J. Comp. and Physiol. Psychol. 53, 509 (1960).

3. Eli Robins, J. Histochem. and Cytochem. 8, 431 (1960).

4. D. B. McDougal, Jr., D. W. Schultz, J. V. Passoneau, J. R. Clark, M. A. Reynolds, and O. H. Lowry, J. Gen. Physiol. 44, 487 (1961).

5. E. L. Bennett, J. B. Drori, H. Morimoto, and A. Orme, in Bio-Organic Chemistry Quarterly Report, UCRL-9408, Sept. 1960, p. 19.

The rate of the second reaction can be readily followed by the increase in optical density of the system at 340 mμ. Determination of the change of optical density has been greatly facilitated by the use of an automatic cuvette positioner and optical density converter whereby the optical density of four cells can be recorded sequentially in automatic rotation.

A very large excess of dehydrogenase must be added, since it is necessary to maintain an extremely low glucose-6-phosphate concentration in order to avoid inhibition of the hexokinase reaction. Crane and Sols found that animal hexokinases were inhibited 50% by 4×10^{-4} M glucose-6-phosphate.[6] Although we have not yet directly determined the steady-state glucose-6-phosphate concentration obtained during our assays, we estimate that it is between 10^{-5} and 10^{-6} M.

Two sources of glucose-6-phosphate dehydrogenase were investigated--a partially purified enzyme from rat mammary gland, and a commercial source (Boehringer glucose-6-phophate dehydrogenase prepared from yeast). Although either preparation is satisfactory, we chose the commercial enzyme for these studies inasmuch as it is essentially free of contaminating enzymes, particularly hexokinase and 6-phosphogluconate dehydrogenase. Under our conditions of assay, the commercial enzyme in the absence of brain homogenate gave a blank equivalent to less than 1% of the optical density change obtained with the complete system including brain homogenate.

Six buffers used by previous investigators for hexokinase studies were evaluated. We finally chose 0.1 M potassium phosphate buffer, pH 8.0, as the homogenizing medium, and 0.15 M glycylglycine buffer, pH 8.0, as the incubation buffer. The complete incubation mixture contained the 0.15 M glycylglycine buffer (pH 8.0), 0.02 M $MgCl_2$, 0.009 M ATP, 0.001 M TPN, and 0.025 M glucose. One-half or double the concentration of any of the above components produced less than 5 to 10% change in the hexokinase activity of a brain homogenate. One ml of this incubation mixture was prewarmed to 37°, and to it was added the appropriate aliquot of brain homogenate containing approximately 0.25 mg of brain, and 20 μl of the glucose-6-phosphate dehydrogenase (containing 0.8 μg of protein). The resulting changes in optical density at 340 mμ were determined over a 10- to 15-min period. A typical record obtained is shown in Fig. 32. Cuvette 1 contained 3 μl dehydrogenase and glucose-6-phosphate. The optical density change was 0.18 density units per minute, which is equivalent to the oxidation of 3×10^{-8} mole of glucose-6-phosphate per minute. In cuvettes 2, 3, and 4, 20μl (0.8 μg) of dehydrogenase was used with 0.12, 0.24, and 0.36 mg of dorsal cortex respectively (but with no glucose-6-phosphate). The optical density change in cuvette 4 was approximately 0.03 density unit per minute, with approximately seven times as much dehydrogenase as in cell 1. If the difference in substrate concentrations is ignored, the dehydrogenase was present in 40-fold excess. Under these conditions, the reaction rate was closely proportional to the amount of tissue used, and linear over the 10 to 15 minutes used for the enzyme determination.

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6. A. Sols and R. K. Crane, J. Biol. Chem. 210, 581 (1954); R. K. Crane and A. Sols, J. Biol. Chem. 210, 597 (1954).

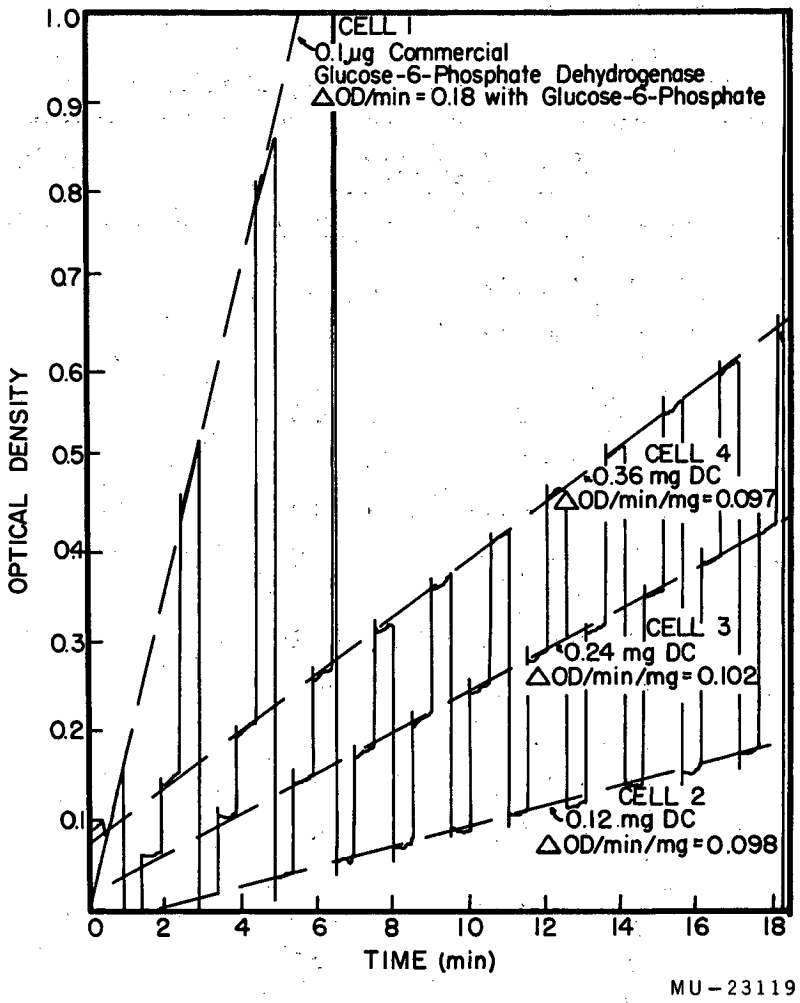


Fig. 32. Typical reaction rate curves of (1) glucose-6-phosphate and glucose-6-phosphate dehydrogenase, and (2, 3, 4) 0.12, 0.24, 0.36 mg of dorsal cortex with complete incubation mixture including glucose and dehydrogenase.

For comparing the activities of different areas of the brain, the homogenate concentration and aliquots were so chosen as to obtain closely similar hexokinase activity in each assay and a rate equivalent to approximately 0.3 optical density unit per 10 minutes. Results from analyses of 15 areas of 8 rat brains are shown in Table VII. These results are

Table VII. Comparison of hexokinase activity of fifteen areas of rat brain.

	Glucose-6-phosphate dehydrogenase assay		2-deoxyglucose assay	Cholinesterase activity
Area	Δ OD/min/mg	(% Cerebellum)	(% Cerebellum)	(% Cerebellum)
Visual cortex	0.147±.018[a]	118±5%	106	128
Somesthetic cortex	0.140±.018	113±4		150
Remainder of dorsal cortex	0.133±.018	107±8	93	155
Hypothalamus	0.125±.015	101±10	79	220
Cerebellum	0.124±.014	100±0	100	100
Superior colliculus	0.120±.014	97±8		650
Medial nuclei of thalamus	0.120±.022	96±10		280
Olfactory tubercules	0.120±.011	96±4		1850
Ventral cortex	0.116±.012	94±5	86	
Caudate	0.108±.010	87±3	73	1260
Inferior colliculus	0.099±.013	80±10		190
Sample of the reticular formation	0.096±.007	78±6		410
Olfactory bulbs	0.096±.013	77±7	70	190
Remainder of subcortex	0.092±.010	75±4	67	320
Medulla and pons	0.080±.006	65±6	57	290

[a] Standard deviation $= \sqrt{\frac{\Sigma X^2}{N-1} - \frac{(\Sigma X)^2}{(N)(N-1)}}$

expressed in terms of a change of optical density per mg wet weight tissue per minute, and as a percent of cerebellum activity. These latter figures can be compared to the results obtained with 2-deoxyglucose. A change of optical density

of 0.105 density units/min/mg is equivalent to 1000 μM of glucose phosphorylated per g per hr. Previous values for hexokinase activity for whole rat brain have been given as 400 μM/hr/g.[7] Thus, the method using a coupled reaction with glucose-6-phosphate dehydrogenase has given values 2-1/2 to 3 times those previously reported. The relative activities are very similar by this method to those obtained using 2-deoxyglucose for the brain areas compared. The highest activity was generally found in the cortical areas, and the lowest in the subcortical areas. Essentially the same hexokinase activity has been found in homogenates of fresh and frozen brain. Only slight loss of activity is observed during 6 to 8 hours in homogenates prepared in the phosphate buffer kept at 0°. However, the addition of either 2-mercaptoethanol (0.1%) or cysteine (0.01 to 0.05 M) to the homogenates causes an increase in hexokinase activity (Table VIII).

Table VIII. Hexokinase activity of homogenates as a function of time after homogenizing. Percent of initial activity in absence of mercaptoethanol (ME).

	Time after homogenizing (hr)			
Area	0 to 1	3 to 4	24	144
Cerebellum	100	97	95	66
Cerebellum + 0.1% ME	128	145	172	167
Visual cortex	100	99	90	45
Visual + 0.1% ME	147	173	189	183
Dorsal cortex	100	93	86	63
Dorsal + 0.1% ME	140	150	183	195
Hypothalamus	100	92	88	64
Hypothalamus +0.1% ME	148	160	198	186
Reticular formation	100	92	64	45
Reticular + 0.1% ME	141	157	175	160

The stability of hexokinase activity in homogenates varied from area to area, and it was generally less in the small areas such as reticular formation, olfactory tubercle, etc. The increase in activity produced by mercaptoethanol seemed to vary from area to area, but in many cases hexokinase activity approached twice the value obtained in a homogenate in its absence. This effect is being studied further.

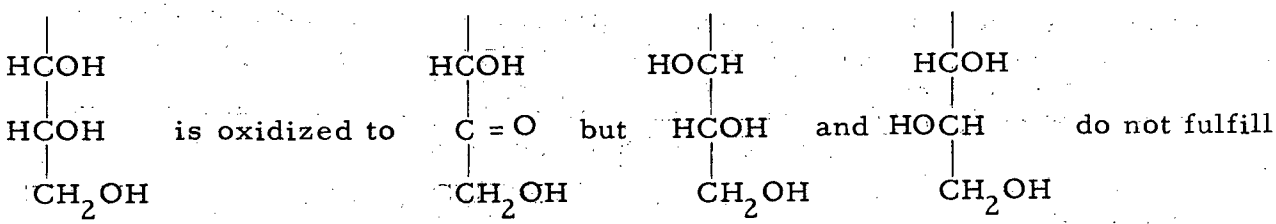
Comparisons of hexokinase activity in strains of rats which have large differences in cholinesterase activity or maze-solving ability are planned. The effect of training and experience in mazes on hexokinase activity will also be investigated.

7. C. Long, Biochem. J. 57, 407 (1952).

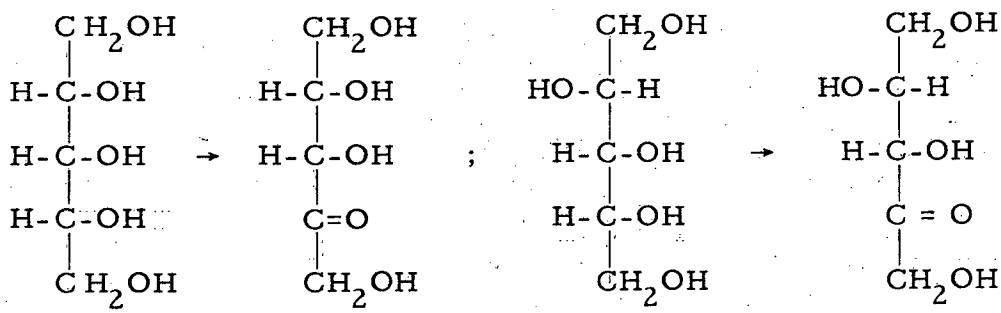
9. THE OXIDATION OF POLYOLS BY ACETOBACTER SUBOXYDANS

V. Moses and R. J. Ferrier

A requirement in this Laboratory for multigram quantities of the ketopentoses ribulose and xylulose prompted the use of the bacterium Acetobacter suboxydans for their preparation. This organism has been known for nearly 40 years to be capable of the oxidation of a particular secondary alcoholic group in polyols to the corresponding ketone when certain steric requirements are met: Two contiguous D-secondary alcohol groups must be adjacent to a primary alcohol group. Thus,



the steric requirements and are not oxidized. In particular, it is known that the bacterium will oxidize ribitol and D-arabitol to L-ribulose and D-xylulose respectively:



The organism was grown in Roux bottles on a solid medium containing yeast extract, KH_2PO_4, $MgSO_4$, glucose, $CaCO_3$, and agar. After growth for 2 to 4 days at 28°, the cells were scraped off the agar, washed twice with water by decantation after centrifugation, and resuspended in distilled water. Adonitol (ribitol) or D-arabitol in aqueous solution was supplied to the cells and the mixtures were shaken at 28°. The production of L-ribulose and D-xylulose, respectively, was followed polarimetrically until completion. The yields were >95%, and apart from small amounts of xylose and possibly L-arabitol mixed with the D-xylulose, products were pure chromatographically and polarimetrically. The pentuloses were isolated simply by removing the bacteria by centrifugation and evaporating the supernatants to dryness.

From previous work with this organism, it was known that cyclohexane-1,2,3-cis-cis-triol is oxidized at the 1-position to give the corresponding

ketone. Among compounds in which we were interested were two glycosides, methyl D-ribopyranoside and methyl-β-L-arabopyranoside. The methyl-D-ribopyranosides, having a 1,2,3-cis-cis-triol structure similar to cyclohexane-1,2,3-cis-cis-triol, might be expected to undergo oxidation by A. suboxydans, while the methyl-β-L-arabopyranoside, not having this steric configuration, would not be expected to be attacked. However, it was found experimentally that little oxidation of either compound was observed as indicated by changes in the optical rotations of the solutions in a period sufficient for the complete oxidation of adonitol or D-arabitol. It was therefore decided to investigate this situation in greater detail by using Warburg respirometry to study the bacterial oxidations kinetically, and paper chromatography to follow the production of carbonyl compounds. Cells for these experiments were resuspended in 0.067 M potassium phosphate buffer, pH 6.67.

It was found that the five substrates investigated could be divided into three different groups from the viewpoint of their bacterial metabolism: sorbitol and D-arabitol; adonitol (ribitol); and methyl riboside and methyl arabinoside.

Sorbitol and D-arabitol

Under the experimental conditions used, both these substances were rapidly oxidized by washed bacterial cells; one atom of oxygen was consumed per molecule of substrate within 30 min. Thereafter the rate of oxygen uptake was somewhat reduced, but continued without a pause until about 80% of the oxygen required for complete oxidation had been consumed, this taking about 2 days. Carbon dioxide was released as soon as the substrate was added to the cells, though the rate of release was relatively slow during the period in which the first atom of oxygen per molecule of substrate was being consumed. The rate of carbon dioxide production gradually increased during this period until the respiratory quotient was about unity during the second stage of oxidation. Chromatographic analysis in two solvent systems of the supernatant medium from the time one atom of oxygen per molecule of substrate had been consumed until the end of the experiment showed only sorbose and xylulose, respectively, as products of the reactions. The amounts of these sugars gradually decreased with time, and they had disappeared entirely when respiratory activity ceased. At no time were any other products observed chromatographically, the reduction of silver nitrate being used for location of reducing substances on the chromatograms. Polarimetry showed that the D isomer of xylulose was formed. No rotation measurements were performed with the sorbose, but this is known from previous work to be the L isomer.

Adonitol (Ribitol)

This substance was also oxidized rapidly until by about 1 hour after its addition to the cells, 1 atom of oxygen had been consumed per molecule of added substrate. There was no release of carbon dioxide at this stage, and chromatographic analysis of the supernatant medium revealed the presence of ribulose as the sole product. This was shown polarimetrically to be the L isomer.

Respiratory activity (except for a low rate of endogenous respiration then ceased, and was eventually resumed after a lag period of several hours.

The resumed respiration had a respiratory quotient of about unity and proceeded for at least 5 days, by which time about 60% of the oxygen required for total oxidation had been consumed. Chromatography of the supernatant medium during this period showed the presence only of gradually decreasing quantities of ribulose. These findings with adonitol, sorbitol, and arabitol suggested the following metabolic situation:

(a) the cells as grown possessed a constitutive enzyme system capable of oxidizing adonitol to L-ribulose;

(b) the cells could subsequently produce one or more adaptive enzymes which permitted the further metabolism of L-ribulose;

(c) the cells possessed constitutive enzymes capable of oxidizing sorbitol and D-arabitol to L-sorbose and D-xylulose, respectively, as well as for the subsequent oxidation of these free sugars to carbon dioxide;

(d) however, the formation of L-sorbose and D-xylulose was much more rapid than their subsequent utilization, and this accounted for release of large amounts of these substances from the cells into the medium.

The possibility of enzyme adaptations being involved in the later stages of adonitol metabolism was investigated by several methods. Chloramphenicol (chloromycetin) is well known as a specific inhibitor of protein synthesis. It does not affect the activity of preformed enzymes, but prevents processes such as enzyme adaptation, which involve the manufacture of new enzyme molecules. The antibiotic had almost no effect on any stage of the oxidation of sorbitol or D-arabitol by A. suboxydans, nor did it affect the first stage of adonitol oxidation with regard to the uptake of one atom of oxygen per molecule of substrate and the quantitative formation of L-ribulose. Even preincubation of the cells with chloroamphenicol for 24 hours prior to the addition of adonitol had no action on the subsequent first-stage oxidation of the polyol. However, during the second stage of adonitol metabolism, chloramphenicol inhibited both the oxygen uptake and CO_2 liberation by nearly 80%, and increased the lag period between the end of the first stage and the beginning of the second stage from 6 hr to nearly 48 hr.

The oxidation of L-ribulose itself by the bacteria closely paralled the second stage of adonitol oxidation, both as regards oxygen uptake and CO_2 release; i.e., there was a lag period of several hours before oxidation commenced. Chromatography at various times during the oxidation of L-ribulose showed only decreasing amounts of this substance in the medium.

A further batch of cells was incubated with adonitol for 5 days to promote enzyme adaptation, then washed several times and incubated with adonitol and with L-ribulose. The cells demonstrated no lag period between the first and second stages of oxidation when supplied with a fresh quantity of adonitol; in the second stage the rate of oxygen uptake remained lower than during the first stage, a situation resembling those with sorbitol and D-arabitol. There was no lag period before the oxidation of L-ribulose, which again closely paralleled the second stage of adonitol oxidation. Chloramphenicol did not inhibit the second stage of adonitol oxidation, indicating that adaptation had already taken place during the preincubation period. Whereas unadapted cells

produce carbon dioxide from adonitol only after a lag period, adapted cells began to release this substance as soon as they were supplied with the sugar alcohol, demonstrating again their capability of metabolizing L-ribulose with no lag period.

Some confirmation of the specific requirement of the cells for adaptation to metabolize L-ribulose before the second stage of adonitol oxidation was obtained by supplying cells with D-ribulose. Only the D-isomer has the necessary stereochemical configuration for the characteristic oxidation of a secondary alcohol group to a ketone. D-Ribulose showed a kinetic pattern of oxidation similar to those of sorbitol and D-arabitol: a rapid first-stage oxidation corresponding to one atom of oxygen per molecule of sugar, followed by a slower second stage (which proceeded without a lag period) during which CO_2 was produced and O_2 absorbed with a respiratory quotient greater than 1. Carbon dioxide was formed as soon as the cells were supplied with the sugar. Chromatography of the supernatant medium showed, in contrast to that of L-ribulose, the formation of a reaction product which would be expected to be 1, 3, 5-trihydroxypentan-2, 4-dione. Some confirmatory evidence for the structure of this product has been obtained from its ultraviolet absorption spectra under varying conditions of pH.

Methyl D-ribopyranoside

Very slow oxidation of this substance took place. In the course of 4 days one atom of oxygen was absorbed per molecule of glycoside, with no production of carbon dioxide. This was followed by a period in which some CO_2 was produced, together with oxygen absorption, until at 9 days, when the experiment was terminated, about 14% of the oxygen required for complete oxidation had been absorbed. As the rate of oxygen uptake was so low it was difficult to determine whether any increase in the rate took place as a result of enzyme adaptation. However, in the presence of chloramphenicol the total oxygen absorbed in the first 4 days was about 20% less than the corresponding value in the absence of the antibiotic, suggesting that adaptation was less pronounced than in the adonitol. Chromatographic analysis of the supernatant medium showed the presence of a product formed in the course of the first 4 days; this gradually decreased in quantity during the subsequent period of incubation, and, simultaneously, a second product made its appearance. There is some evidence from ultraviolet spectroscopy that the first product is the expected methyl-2-oxo-riboside; the second product appeared from chromatographic data to be acidic.

Methyl-β-L-arabopyranoside

This glycoside was oxidized even more slowly than methyl riboside. After 9 days no CO_2 had been produced, while the oxygen uptake corresponded to about one atom per molecule of substrate. During the first 4 days of incubation the total oxygen uptake was some 25% lower in the presence of chloramphenicol than with the corresponding control sample, a finding similar to that with methyl riboside. Two products were formed from methyl arabinoside which were chromatographically similar to those from methyl riboside, but no further investigations were made of these substances.

The Use of A. suboxydans in Sugar Chemistry

As a preparative tool for the oxidation of an appropriate secondary alcohol to a ketone, the study presented here was shown that four types of situation may be encountered with Acetobacter suboxydans. The first and experimentally most satisfactory of these is exemplified by adonitol. With this substance there is a quantitative oxidation of the secondary alcohol to the corresponding carbonyl, compound followed by a long lap period prior to the subsequent complete oxidation of the ketonic product. Thus, provided sufficient time is allowed for the initial reaction to be completed, there is little danger (for a considerable time) of loss of product as a result of further metabolism. The second situation is illustrated by sorbitol, D-arabitol, and D-ribulose. Here, although the first-stage oxidation is quantitative, the second stage starts before the first is finished and there is no lag period between the two. It therefore becomes desirable to stop the reaction as soon as the first stage has been completed, because continued incubation will result in a loss of the desired product, although the product will remain pure.

The methylglycosides are examples of the third possibility. Here, oxidation proceeds very slowly and may possibly not be complete before the cells die unless a relatively high cell to substrate ratio is used. Although with methyl riboside there appeared to be no lag period between the two stages of oxidation, the oxidation of these substances to specific products is complicated by the eventual appearance of a second product. It is therefore necessary to stop the reaction at an appropriate point in order to obtain the required product in maximum purity and yield.

Fourthly, there are substances which undergo oxidation but from which the bacterium produces no chromatographically detectable product. Such substances are D-xylulose, L-ribulose, and L-sorbose. It appears in this fourth case that, once these substances enter the metabolic pathways of the organism, no product is released from the cells until the substrates have been completely oxidized to carbon dioxide and water. Obviously, such substances cannot be used as starting materials for organic preparations.

10. STUDIES ON A MECHANISM FOR THE PHOTOSYNTHETIC PHOSPHORYLATION

Gerrit Engelsma

The initial stage of the photosynthetic cycle involves the conversion of light energy into chemical energy.[1] It is now commonly accepted that the light-induced reactions by which the light energy is stored are the synthesis of ATP from ADP and orthophosphate and the reduction of pyridine nucleotides with simultaneous oxygen evolution.[2] How these reactions are linked together is still unknown. Recent studies have provided evidence that manganese is involved in this initial stage of the photosynthetic cycle.[3-7] Markham[4] showed that manganese is present in the photosynthetic centers of plants. Kessler et al. assume that the role of manganese in the oxygen-evolving system of photosynthesis is connected with the formation of a "photoperoxyd."[7] With solutions of manganese phthalocyanine in pyridine, Markham[4] and Yamamoto[8] have found evidence for a light-dependent reaction as follows.

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

2. D. I. Arnon, Nature 184, 10 (1959).

3. C. Eyster, T. E. Brown, H. A. Tanner, and S. L. Hood, Plant Physiol. 33, 235 (1958).

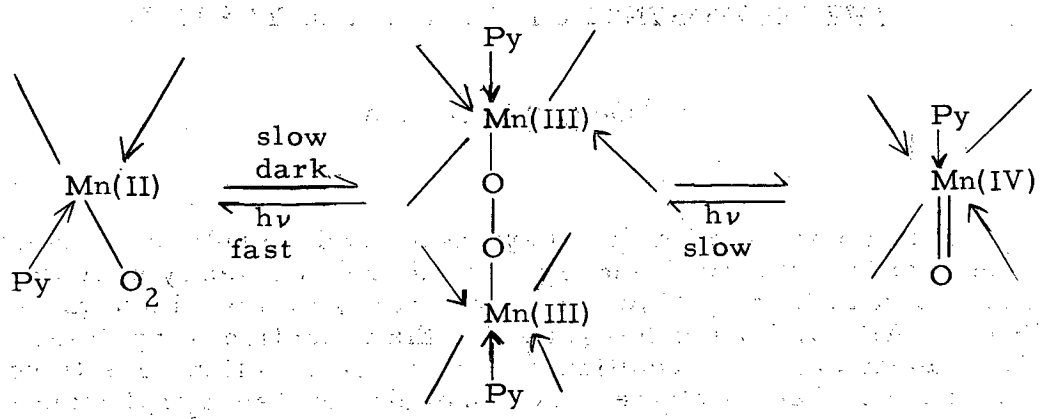
4. Edward Markham, in Bio-Organic Chemistry Quarterly Report UCRL-9208, June 1960, p. 40.

5. E. Kessler, R. Moraw, B. Runberg, and H. T. Witt, Biochim. Biophys. Acta 43, 134 (1960).

6. E. Kessler, Planta 49, 435 (1957).

7. E. Kessler, W. Arthur, and J. E. Brugger, Arch. Biochem. Biophys. 71, 326 (1957).

8. Akio Yamamoto, in Bio-Organic Chemistry Quarterly Report UCRL-9652, April 1961, p. 55.



A reversible dissociation of a peroxide complex, as shown here for the bis-manganese complex, has so far not been found with any other metal. The hypothesis that the function of manganese in photosynthesis depends on this special character has led to the suggestion that the photochemical reactions of photophosphorylation and oxygen evolution are linked together as shown in Fig. 33. Divalent manganese is reoxydized in the system to the quadrivalent state whereas DPN^+ is reduced to DPNA. This scheme implies that at least part of the oxygen evolved during illumination comes from the orthophosphate. On hydrolysis of the ATP, oxygen from the water will be incorporated into the phosphate. This oxygen will be given off on resynthesis of ATP under the influence of light. This makes our scheme in agreement with the findings of Ruben, Kamen, et al. that oxygen liberated during photosynthesis has the same isotope distribution as the water in the plant.[9]

If it could be proved that this hypothesis is in agreement with the actual processes in vivo, it would form an attactive basis for model studies. In order to check if oxygen evolved during illumination comes (at least partly) from the orthophosphate, we have carried out photosynthetic experiments with algae (Chlorella pyrenoidosa) in a medium which contained oxygen-18-labeled inorganic phosphate.

In isolated chloroplasts the evolution of oxygen under the influence of light can be realized in the presence of a nonphysiological electron acceptor such as ferricyanide (Hill reaction). Phosphorylation and oxygen evolution are then linked:

$$2Fe^{+++}Cy + ADP + P\ (+H_2O) \rightarrow 2\ Fe^{++}\ Cy + ATP + 1/2\ O_2\ (+H_2O).$$

We also carried out experiments with spinach chloroplasts in a medium which contained oxygen-18-labeled inorganic phosphate, ADP, and ferricyanide in order to see if the oxygen given off on illumination would show any isotope enrichment.

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9. S. Ruben, M. Rendall, M. Kamen, and J. L. Hyde, J. Am. Chem. Soc. 63, 877 (1941).

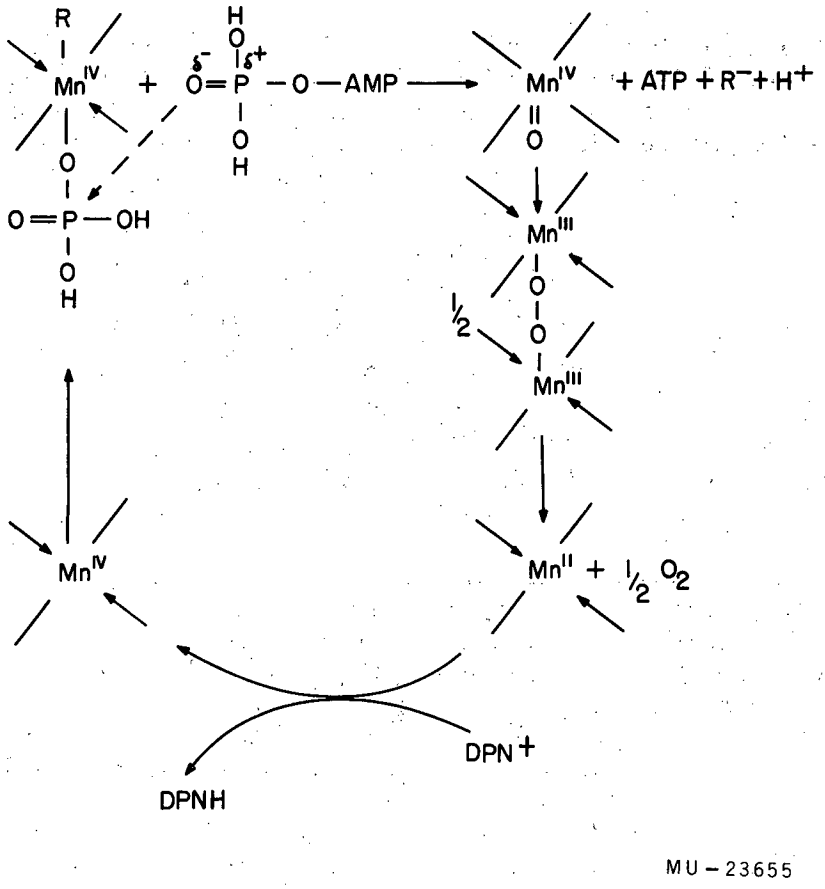

Fig. 33. Hypothesis for photophosphorylation linked with oxygen evolution.

Algae Experiments

Phosphate labeled with oxygen-18 was prepared from P_2O_5 and H_2O^{18} (30% O^{18}) according to Cohn and Drysdale.[10] The oxygen-18 content was determined by heating the phosphate together with mercuricyanide in an evacuated sealed tube followed by mass spectrometric analysis (CEC, Model 21-130) of the CO_2 thus formed (Williams and Hager[11]).

The alga used in this work was the Emerson strain of Chlorella pyrenoidosa. The algae were centrifuged from the culture medium (120 to 140 mg dry weight). To remove the phosphate of the original medium the algae were washed twice with distilled water. After this, the algae pellet was suspended in 100 ml of a medium with the following composition:

$(NH_4)_2CO_3$	1.5 mM	Modified Arnon A4	1/10 ml
$MgSO_4$	0.15 mM	NH_4VO_3 (0.023 g/l)	1/10 ml
$MgCl_2$	0.15 mM	KCl	0.04 mM
$Ca(NO_3)_2$	0.005 mM		
KNO_3	0.005 mM	$FeCl_3$	0.01 mM

The reaction vessel, shown in Fig. 34, was filled with this suspension. Under continuous stirring the algae were illuminated with two lamps (GE Photospot No. RSP 2). After 25 min of illumination the oxygen that had accumulated was discarded. We assume that during this period part of the soluble phosphates originally present had been converted to insoluble phosphates. There was now added 0.15 mM of $KH_2PO_4^{18}$ (10.3% O^{18}); the final pH, 7.3. Every 10 min the oxygen that was given off during the preceding interval was collected in a gas receiver.

A typical result of an analysis of four such 10-min samples, with a mass spectrometer, was as follows:

	O_2 (%)	$O_2^{34}/O_2^{32} \times 100$
Sample 1	56.4	0.393 ± .01
Sample 2	87.3	0.401 ± .01
Sample 3	70.3	0.395 ± .01
Sample 4	52.7	0.390 ± .01
Linde oxygen	100.0	0.400 ± .01
Air	20.8	0.380 ± .01

10. M. Cohn and G. R. Drysdale, J. Biol. Chem. 180, 771 (1949).

11. F. R. Williams and L. P. Hager, Science 128, 1434 (1958).

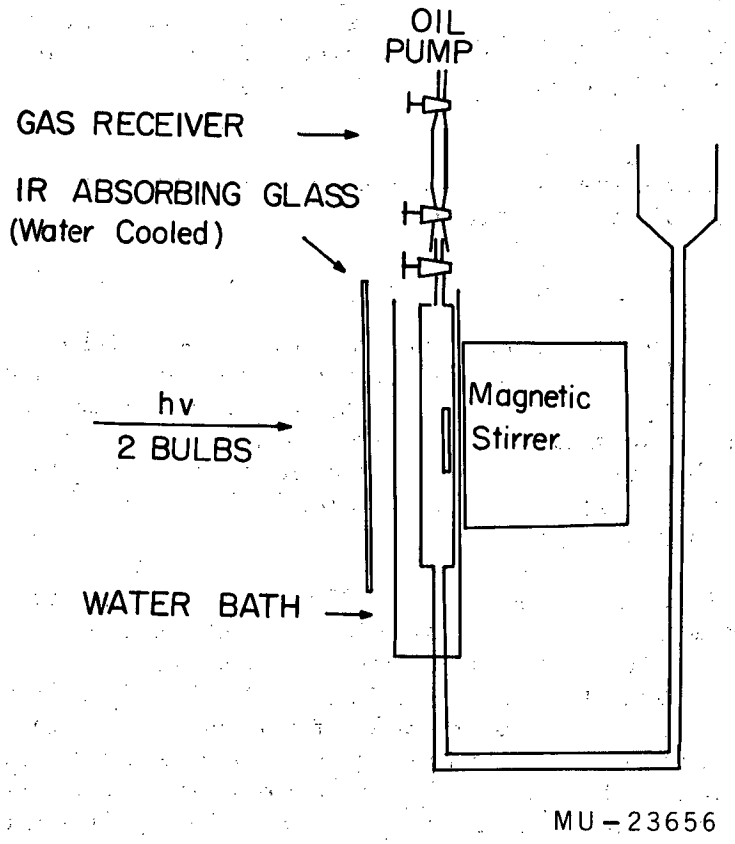

Fig. 34. Apparatus for the algae experiments.

Because the medium was saturated with air, all the samples contained a certain percentage of Ar, N_2, and CO_2. In none of the experiments was the ratio for $O_2^{34} / O_2^{32} \times 100$ found to be higher than 0.40, which it would have to be to support the proposed mechanism. Conclusions, however, are limited by two facts:

(a) Analysis showed that the algae contain 1.65% phosphorus. If we make the assumption that 30% of the phosphorus is present in the proposed cycle and the phosphate taken up from the medium gives off during the experiment 50% of its oxygen in the form of molecular oxygen, then at least 5 to 10% of the phosphate in the cycle has to be renewed to give a measurable result.

(b) No data are available about a possible exchange of oxygen between water and phosphate within the algae.

Hill Reaction with Chloroplasts

The experiments were performed with spinach chloroplasts. The progress of the Hill reaction was estimated from the amount of oxygen which evolved on illumination, whereas the amount of radioactive phosphate incorporated into organic phosphates was taken as a measure of the rate of photophosphorylation. Phosphate that had not been used during the reaction, as well as the inorganic phosphate liberated on hydrolysis of the organic phosphate had exchanged its oxygen.

Chloroplasts were preapred as follows: Spinach leaves whose midribs had been removed were cut into small pieces. About 125 g of these leaf parts were placed in the cup of a Waring Blendor and 250 ml of chilled homogenizing medium (0.4 M sucrose, 0.05 M Tris, 0.01 M NaCl, pH 7.8)was added. Grinding was carried out for 30 sec with the homogenizer at full speed. The slurry was then squeezed through eight layers of cheesecloth and centrifuged at 0 to 2° for 4 min at 900 rpm. The sediment was discarded and the supernatant centrifuged now for 12 min at 1700 rpm. The pellet from this centrifugation was resuspended in 50 ml of homogenizing medium and recentrifuged for 12 min at 1800 rpm. After this, the sediment was suspended in a Tris buffer solution (0.03 M Tris, 0.01 M NaCl, 0.004 M $MgCl_2$, pH 7.8) and centrifuged again for 12 min at 1800 rpm.

The Hill reaction was carried out in an apparatus as shown in Fig. 35. Twice-washed chloroplasts prepared form 250 g spinach leaves were suspended in 95 ml of a Tris buffer of pH 7.8 containing, in moles: Tris, 3; NaCl, 2; $MgCl_2$, 0.4; ADP, 0.25. This suspension was placed in the flask at the left side of the apparatus. Under cooling with ice and exclusion of light, a stream of helium was passed through to sweep out the air. The reaction vessel, which contained a solution of 1.5 mM $K_3Fe(CN)_6$, 0.75 mM $KH_2PO_4^{18}$, and 1.0 nM Tris in 5 ml water was swept with helium at the same time. After 15 min under the pressure of a reservoir filled with helium, the chloroplast suspension was conveyed to the reaction vessel. The pH of the combined solutions was 8.1. Under continuous stirring the reaction mixture was illuminated with four lamps (GE Photospot No. RSP 2) for 80 min. The temperature was kept between 12 and 15°. Every 20 min the evolved gas was collected. The O_2^{34} / O_2^{32} ratio of the four gas samples was measured with the mass spectrometer.

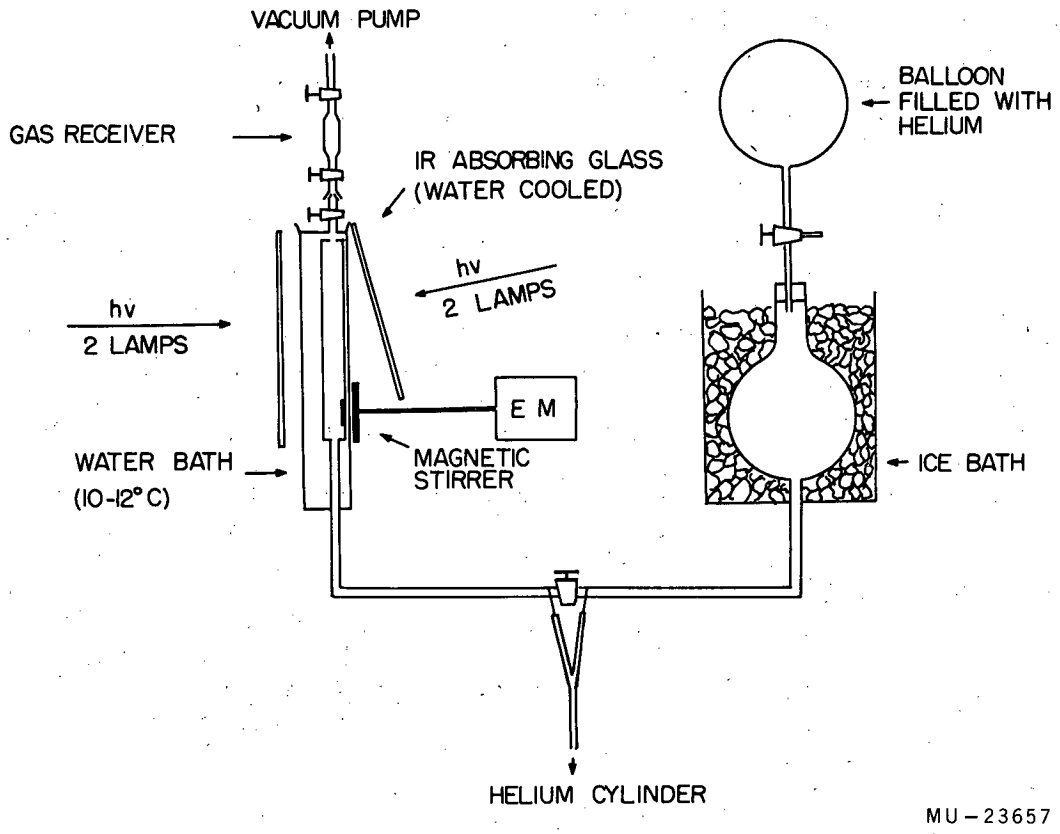

Fig. 35. Apparatus for the Hill reaction.

To isolate phosphate from the ATP formed during the reaction and to recover phosphate which has not been used, we followed the same procedure as Avron and Sharon.[12] Four and a half ml $HClO_4$ (70%) was added to the ice-cooled reaction mixture and the precipitate was removed by centrifugation. The solution was stirred twice with portions of 4 g of acid-washed charcoal to adsorb the ATP (and the ADP that had not reacted). The charcoal was washed four times with distilled water and then placed in N HCl for 10 min to liberate the inorganic phosphate (β phosphate of ADP and β + γ phosphate of ATP). This phosphate was then precipitated as $MgNH_4PO_4$ (Umbreit et al.[13]). The phosphate from the medium was precipitated the same way. After they were intensively washed, both samples of $MgNH_4PO_4$ were dissolved in 0.1 N $HClO_4$, and then precipitated as the silver salt (Anbar et.al.[14]). The Ag_3PO_4 was washed several times and dried in vacuo over P_2O_5 at 60°. Afterwards, it was heated at 1000° for 15 min in an evacuated quartz tube. The oxygen that was given off was also analyzed with the mass spectrometer.

Since the results were irreproducible in later experiments, the Ag_3PO_4 was converted into KH_2PO_4 in order to determine the oxygen-18 content according to the earlier-described method of Williams and Hager. Forty mg Ag_3PO_4 was powdered together with 30 mg KCl. Then, 2 ml water was added and enough 1 N HCl to adjust the pH of the supernatant to 4.4. The solids were filtered off and two volumes of ethyl alcohol were added to the filtrate to precipitate KH_2PO_4. This was filtered off, washed twice with ethyl alcohol and twice with ether. The product was dried at 100° in vacuo for 1 hour.

To determine the amount of phosphate incorporated into the organic phosphate fraction, we followed a procedure described by Avron.[15] The Hill reaction was carried out with P^{32}-labeled phosphate (40μC). After illumination for 80 min the reaction mixture was cooled to 0° and 4.5 ml $HClO_4$ (70%) was added. The precipitate was centrifuged down and 1 ml of the supernatant was placed in a small test tube; 1.2 ml acetone was added and the contents mixed and allowed to stand for 10 min. The water content of the tube was made up to 2.5 ml with water saturated with a 1:1 mixture of isobutanol and benzene, 7.0 ml of a 1:1 mixture of isobutanol and benzene saturated with water was added, and the tube contents mixed. After phase separation 0.8 ml of molybdate reagent (made by dissolving 5 g ammonium molybdate in 40 ml of 10 N H_2SO_4 and making it up to 100 ml with water) was added to the side of the tube and the water layer gently mixed, and the tube was allowed to stand for 5 min. Each tube was now vigorously shaken for 30 sec. After phase separation, the

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12. M. Avron and N. Sharon, Biochem. Biophys. Research Comms. 2, 336 (1960).
13. W. W. Umbreit, R. H. Burris, and J. F. Stauffer, Manometric Techniques, (Burgess Publishing Co., Minneapolis, 1957), p. 276.
14. M. Anbar, Anal. Chem. 32, 841 (1960).
15. M. Avron, Biochim. Biophys. Acta 40, 257 (1960).

upper layer was removed and 0.02 ml of 0.02 N KH_2PO_4 was added to the aqueous layer, followed by 7.0 ml of the isobutanol-benzene, and the tube contents vigorously mixed for 30 sec. After phase separation, the upper layer was again removed and 50 λ of the water phase was mixed with 10 ml of a liquid scintillation solution. Of the supernatant from the $HClO_4$-treated reaction mixture, 50 λ was also mixed with 10 ml of the scintillation solution. Both solutions were counted with an automatic "Tri-carb" liquid scintillation spectrometer. The amount of inorganic phosphate that has been used can be calculated from the equation

$$\text{mmoles of inorganic phosphate used} = (\text{counts/min in organic phosphate fraction}) \times 3.3 \times \frac{(\text{volume in flask after } HClO_4 \text{ addition})}{(\text{original volume})} \times \frac{(\text{mmoles } KH_2PO_4 \text{ in flask})}{(\text{total counts/min in flask})}$$

Results: A typical result of two experiments was as follows:

	$O_2^{34}/O_2^{32} \times 100$		$O_2^{34}/O_2^{32+34} \times 100$	
Sample	Expt. 1	Expt. 2	Expt. 1	Expt. 2
1	0.389	0.384[a]		
2	0.390	0.392		
3	0.393	0.387		
4	0.385	0.387		
air	0.388	0.382		
Original $KH_2PO_4^{18}$			16.8	16.8[b]
Recovered $KH_2PO_4^{18}$			16.6	16.7
$KH_2PO_4^{18}$ from org. phosphate			4.76	4.58

[a] Results accurate to within ± 0.01

[b] Results accurate to within ± 0.1

The experiments thus give no evidence for the proposed mechanism. In two experiments with $KH_2P^{32}O_4$ we found that respectively 220 and 232 μM of inorganic phosphate had been used. If the phosphorylation of ADP were the only reaction, this would mean that, in the first case, 88% and, in the second, 93% of the 250 μM of ADP used had been converted into ATP.

The isotope distribution of the inorganic phosphate recovered from the ATP fraction would then indicate that the phosphate which had reacted with ADP had exchanged 40 to 50% of its oxygen. This makes our results roughly in agreement with the findings of Avron and Sharon,[12] who performed photophosphorylation experiments with Swiss-chard chloroplasts and found a light-dependent loss of two atoms of oxygen from inorganic phosphate during its conversion to ATP.

11. FURTHER STUDIES ON THE NEW PHOSPHATE DONOR FORMED BY CHLOROPLASTS

Johannes Ullrich

Some previous reports[1] dealt with the formation of monomethylphosphate by killing $P^{32}O_4$-fed chloroplasts with four volumes of methanol. These reports show that the ester is formed in a nonenzymatic reaction from a still unknown probably high-energy phosphorylating agent arising only in an enzymatic reaction during the incubation of the chloroplasts. This agent is surprisingly stable to water, even at high temperature.

Some of the most important experiments of Tyszkiewicz[1] were reproduced. They were also repeated with some variations designed to establish the reactivity and stability of the unknown phosphorylating agent and to increase its yield with the hope of later isolation. All the experiments were performed at room temperature in the light and under aerobic conditions. They gave the following new results:

1. Extending the incubation time from the original 5 minutes up to some hours increases the yield of the methylphosphate formed by the usual killing procedure.

2. n-Propanol as killing agent also forms an ester with the phosphorylating agent, probably n-propylphosphate which, in the solvent systems used (I: isobutyric acid - aqueous 1 N ammonia - EDTA; and II: propionic acid-n-butanol-water) runs very near the solvent front and therefore was probably not detected in the previous experiments. The R_F values of ethylphosphate are between those of methyl and n-propylphosphates.

3. The phosphate donor giving monoalkylphosphates by killing the incubation mixture with primary low-molecular-weight alcohols is also formed when all the cofactors and metal ions added in the previous experiments[1] are omitted. Sometimes the yield of methylphosphate was even higher than in simultaneous experiments with addition of metal ions and cofactors.

4. To prove the stability of the phosphate donor in an aqueous solution containing much organic solvent, the incubation mixture was killed with two volumes of acetone, and after different times, samples of the resulting mixture were treated with methanol. The donor appeared to be quite stable under these conditions. Considerable amounts of methylphosphate were still formed when the methanol was added 24 hours after the acetone. This shows that its alcoholysis is much faster than hydrolysis.

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1. Edwige Tyszkiewicz, in Bio-Organic Chemistry Quarterly Report, UCRL-9519, Dec. 1960, and UCRL-9652, April 1961. E. Tyszkiewicz, G. Gingras, M. Calvin, preliminary communication, Biochim. et Biophys. Acta, in press.

5. In order to get the unknown phosphate donor as a spot on the paper chromatograms, acetone-killed mixtures were chromatographed with a modified solvent system I (trimethylamine used in the same molecular quantity instead of ammonia) and with the unchanged solvent II. The trimethylamine is used because it is expected that ammonia might cause aminolysis of the phosphorylating agent, and because, in general, aminolysis of acylating agents is much faster than hydrolysis or alcoholysis. These experiments have not been successful yet, but the separation of the organic phosphates by the new solvent combination is even better than by the previous one.

6. Treatment of the sonicated chloroplast suspension with four volumes of methanol different times before the addition of the $P^{32}O_4$ substrate prevents the formation of all organic phosphates except the monomethylphosphate, which is still formed when the $P^{32}O_4$ is added 30 minutes after the methanol. The enzyme responsible for the formation of the phosphate donor seems, under these conditions, to be one of the longest-surviving ones in the complex enzyme system of the chloroplasts.

In one case (the experiment has not yet been reproduced), when the sonicated chloroplasts suspension was treated with methanol a few minutes before the addition of the $P^{32}O_4$ substrate, methylphosphate was formed in 20% yield from the P^{32}-orthophosphate, without formation of any other organic phosphate.

12. IMPROVEMENT OF STUDY-STATE APPARATUS AND EXPERIMENTAL CONDITIONS TO PERMIT STUDIES OF AMINO ACID SYNTHESIS FROM CARBON-14 AND NITROGEN-15 SIMULTANEOUSLY

Martha Kirk and J. A. Bassham

In previous reports[1,2] we described experiments in which we approached conditions of steady-state photosynthesis. These studies permitted measurements of the rates of uptake of carbon dioxide and ammonium ion from the medium. At the same time, we could calculate the rate of flow of carbon through certain actively turning-over intermediates in the metabolic sequence. We found that some amino acids, particularly alanine, aspartic acid, and serine, were synthesized directly from intermediates derived from the carbon reduction cycle. The rates of synthesis of these amino acids accounted for an appreciable fraction of the total carbon fixed. Alanine and aspartic acid were labeled more rapidly with carbon-14 than was glutamic acid, despite the fact that glutamic acid is commonly considered to be the primary entry point of nitrogen in the form of ammonia in biosynthesis. The carbon-labeling experiments could not tell us what the points of entry of ammonia are in the synthesis of amino acids during photosynthesis. Therefore we are undertaking experiments in which we will employ simultaneously C^{14} and N^{15} as isotopic tracers to study the biosynthesis of these and other amino acids.

In order to make such studies, we needed not only to develop the necessary techniques for analyzing the N^{15}-labeled compounds by mass spectrometry, but also to improve upon the steady-state conditions which we had previously achieved. It is particularly important that the levels of amino acids should remain entirely constant during the course of the experiments and that the rates of flow of carbon and ammonia through these pools should not change. To accomplish this we have added another type of control, in this case a density control, to our apparatus.

We altered the medium in which the algae were grown in such a way that two different nutrieint solutions could be added to the algae by means of the automatic controls. One solution, which would be added to maintain pH control, contains only 0.1 N ammonium hydroxide. As the algae photosynthesize and take up ammonium ion from the medium, their pH tends to become more acidic. This change in pH is registered by the pH meter, which sends a signal to a control relay, which in turn activates a solenoid admitting the ammonium hydroxide to the algae suspensions.

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1. J. A. Bassham and Martha Kirk, Biochim. et Biophys. Acta 43, 447 (1960).

2. David C. Smith, J. A. Bassham and Martha Kirk, Biochim. et Biophys. Acta 48, 299 (1961).

The density control is achieved by mounting a photoresistor on the face of the algae vessel so that the amount of light entering the photoresistor is dependent upon the algae density (see Fig. 36). The light source is the same one used to illuminate the algae for photosynthesis. Any change in the amount of light impinging on the photoresistor (densitometer) results in a change in its current. This current controls a solenoid which admits a medium containing all of the ions (except a nitrogen source-ammonium ion) to the algae. Thus, the algae density is automatically controlled.

The medium without ammonia contains phosphate and a number of other mineral requirements of the algae, as shown in Table IX. This medium is about pH 4. At the beginning of the experiment, some of the medium is brought to pH 6 with 0.1 N NaOH, and the algae are suspended in it. The amount of NaOH required to bring the medium with the algae to pH 6 is such that the steady-state concentration of NH_4^+ ion at pH 6 is about 1 mM. As a result, the algae have a constant adequate supply of NH_4^+ ion for amino acid and protein synthesis.

Table IX. Composition of medium.

KH_2PO_4	2.00 mM
$MgSO_4$	2.00 mM
$MgCl_2$	0.20 mM
$Ca(NO_3)_2$	0.02 mM
KNO_3	0.05 mM
Modified Arnon's A-4 trace elements containing 0.05 M $MnCl_2$	2 ml/l
NH_4VO_3	.001 nM
Saturate with 2% CO_2 in air	
$FeCl_3$	0.1 mM

Another control has been installed to maintain a constant volume of algae suspension in the cell during times when we are not removing samples manually (see Fig. 36). This control is mounted on a glass standpipe in back of the algae cell into which the algae suspension can circulate. As the level of algae rises in the standpipe it cuts off a beam of light which comes from a small light bulb and passes into another photoresistor. The current in the photoresistor thus changes, activating a solenoid which causes a sample of about 1 ml volume of algae suspension to be taken out of the algae cell. The samples taken out are collected. The rate of growth of the algae can be

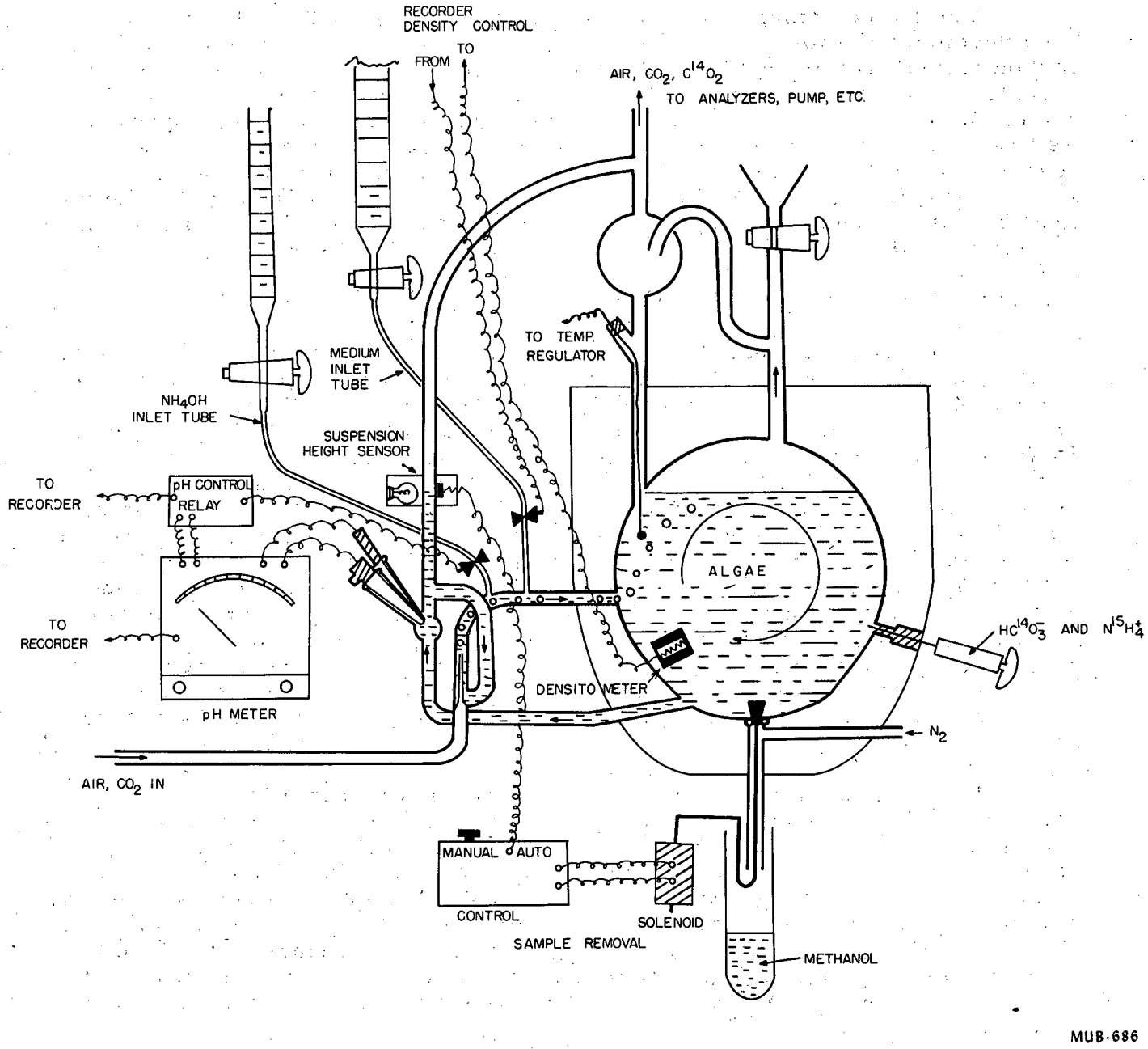

Fig. 36. Algae steady-state apparatus.

determined over a period of time by measuring the volume of algae suspension automatically removed. We have found under our steady-state conditions that the algae doubling time is about 10 to 12 hours.

For the beginning of the actual exposure of the algae to the tracers, we have developed the following technique. C^{14}-labeled bicarbonate and C^{14}-carbon dioxide are added to the algae simultaneously, as described in previous reports, so as to bring the specific activity of C^{14} very quickly to it final value. In order to bring the specific activity of N^{15} to something approaching its final value, it is necessary to deplete the supply of ammonia in the algae suspension briefly. This is, admittedly, not ideal from the standpoint of steady-state photosynthesis, but we have found that for short periods of NH_4^+ depletion the rate of photosynthesis is not affected. We believe that the supply of ammonia within the algae cells probably remains sufficient to maintain a healthy condition of growth approaching steady state provided the depletion does not extend for more than about 5 minutes.

To reduce the steady-state ammonium concentration we turn off the pH control, allowing the algae to pick up the ordinary ammonium ion and the pH to become more acidic. From previously determined pH curves for the medium, and with the algae present, we know that when the pH drops to about 4.6 most of the ammonium ion is depleted. At that point we inject, by means of a hypodermic needle inserted through a rubber plug in the algae cell, a solution of N^{15}-labeled ammonium hydroxide. This is timed to coincide with the admission of C^{14}. Additional N^{15}-labeled ammonium hydroxide is added later from time to time as necessary to maintain pH control. We then proceed to take samples as frequently as we wish by means of our manual push-button sampler, and these will be analyzed subsequently by paper chromatography, by radioautography to determine the location of C^{14}, by ninhydrin estimation to determine the total amino acid, and by mass spectroscopic analysis to determine the N^{15} content of each individual amino acid. The techniques used in the amino acid determination are described in a separate report.[3]

By using the new steady-state apparatus and the conditions we have described here, it has been possible to maintain algae in a condition of steady-state growth for as long as 48 hours, at which time we initiated our first pilot experiment with C^{14} and N^{15}. The first experiment lasted 2 hours, and the samples are now being analyzed by paper chromatography and radioautography. The rates of uptake of carbon dioxide and evolution of oxygen during the course of this experiment are shown in Table X. In this preliminary experiment, the density control did not operate perfectly and there is some variation in the rates as recorded. However, we believe this trouble can be corrected in future experiments.

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3. G. J. Crowley and J. A. Bassham, this Bio-Organic Chemistry Quarterly Report, p. 102.

Table X. Steady-state experiment No. 38.

Time (hr) O = "lights on"	PS rate (μM/min/g O_2	PS rate (μM/min/g CO_2	PS rate (μM/min/g N	0.1 M NH_4OH Total ml	0.1 M NH_4OH ml/hr	Medium Total ml	Medium ml/hr	Volume of suspension (ml)	% Wet packed algae
1	9.89	8.44	3.46	2.7	2.7	11	11	70	2
2-1/4	9.43	9.38	2.26	2.2	1.76	6	4.8	75	
6-1/2	9.00	8.09							
21	5.64	5.54	1.74	29.6	2.04	46	3.17	75	2.6
23-1/3			2.13	4.1	1.76	6.0	2.57	69	2.6
26	6.60	6.41		4.0	1.5	14.0	5.25	75	2
41-1/4			2.39	24.6	1.61	72.5	4.75	75	1.5
43-1/4	10.40	10.19						75	1.5
43-1/2			2.37	3.8	1.60	12.5	5.56	75	1.5
45	10.40	9.69							
47	10.40	9.89						75	1.5
47-1/3			2.25	6.6	1.52				
50		9.84	2.08	4.0	1.50	40	6.15	80	1.5

13. METHODS OF ANALYZING N^{15}-LABELED AMINO ACIDS OBTAINED FROM ANALYSIS OF STEADY STATE

Gerald J. Crowley and J. A. Bassham

We are investigating methods for determining the fate of $N^{15}H_4OH$ introduced into a culture of algae photosynthesizing under steady-state conditions.[1] Since N^{15} is a nonradioactive isotope, the task of locating and identifying N^{15}-labeled products on chromatograms is greatly simplified by the decision to use C^{14} and N^{15} concurrently. The problem reduces to an analysis of the spots identified by radioautography, since most of the compounds in which we are interested will become labeled with both C^{14} and N^{15}.

Amino acids are the primary nitrogen-containing compounds found in our experiments to be labeled with C^{14},[2] and are therefore the first compounds we will study using both tracer elements. The two principal analytical problems to be solved for the amino acid studies are (a) estimation of total amount of each amino acid, and (b) measurement of the N^{15} isotopic content of each amino acid.

Attempts to use previously employed methods[2] for ninhydrin estimation of amino acids indicated that modifications would be required in order to achieve the sensitivity and reliability the proposed experiments demanded. After a number of studies of the variables, intensity of color development and of background, the following method has been developed.

Reagents

Reagents are according to Yemm and Cocking.[3]

1. KCN reagent: 0.01 M KCN in deionized water.

2. KCN-methylcellusolve reagent: 10 ml 0.01 M KCN diluted to 300 ml with methylcellusolve.

3. 5% ninhydrin reagent: 5% (w/v) triketohydrindens hydrate in methylcellusolve.

4. Ninhydrin-KCN reagent: 1 volume 5% ninhydrin to 3 volumes KCN-methylcellusolve reagent. Prior to use, this should be stored overnight in dark. Examination of the absorption spectrum for several days indicates that this reagent retains its composition for a least 3 days.

5. Citric acid reagent: 0.8 N citric acid, preserved with 10^{-4} (w/w) thymol solution in water.

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1. Martha Kirk and J. A. Bassham, preceding report, p. 97.
2. David Smith, J. A. Bassham, and M. Kirk, Biochim. Biophys. Acta 48, 299 (1961).
3. Yemm and Cocking, Analyst 80, 209 (1955).

6. NaOH reagent: 0.5 N NaOH. When mixed in equal volumes (5) and (6) buffer at pH 5.0 ± 0.1.

7. 60% ethanol.

Nitrogen gas is bubbled through all reagents to dispel air, and the reagents are stored under N_2 in the refrigerator until used.

Procedure

The amino acid spots to be determined are cut from chromatograms and eluted with 2.0 ml deionized water into 12-ml centrifuge tubes. To each tube is then added 100 µl 0.5 N NAOH, and the tubes are evaporated to dryness in a desiccator, in vacuo, and over conc. H_2SO_4 to drive off any ammonia.[4] When dry, the residue is reconstituted in 100 µl of 0.8 N citric acid solution (to buffer sample to pH 5.0) and 0.5 ml N_2-bubbled-deionized water, bringing the total volume to 0.6 ml. To each sample is next added 0.4 ml ninhydrin-KCN reagent; all the tubes are then stoppered with glass marbles and boiled for 20 minutes in a boiling water bath to develop the color. After boiling, they are cooled for 5 minutes in an ice water bath and diluted to the 2-ml mark with 60% ethanol. The absorption is read immediately with a Beckman DU Spectrophotometer at 570 mµ. This method has been found reproducible, and 1 µg glutamic acid diluted to 2 ml gives an optical density reading of about 0.075. When eluates are analyzed, it is imperative to take paper blanks from as near the spot as possible to ascertain the background value. It is further essential that amino acid standards be analyzed along with eluates and paper blanks.

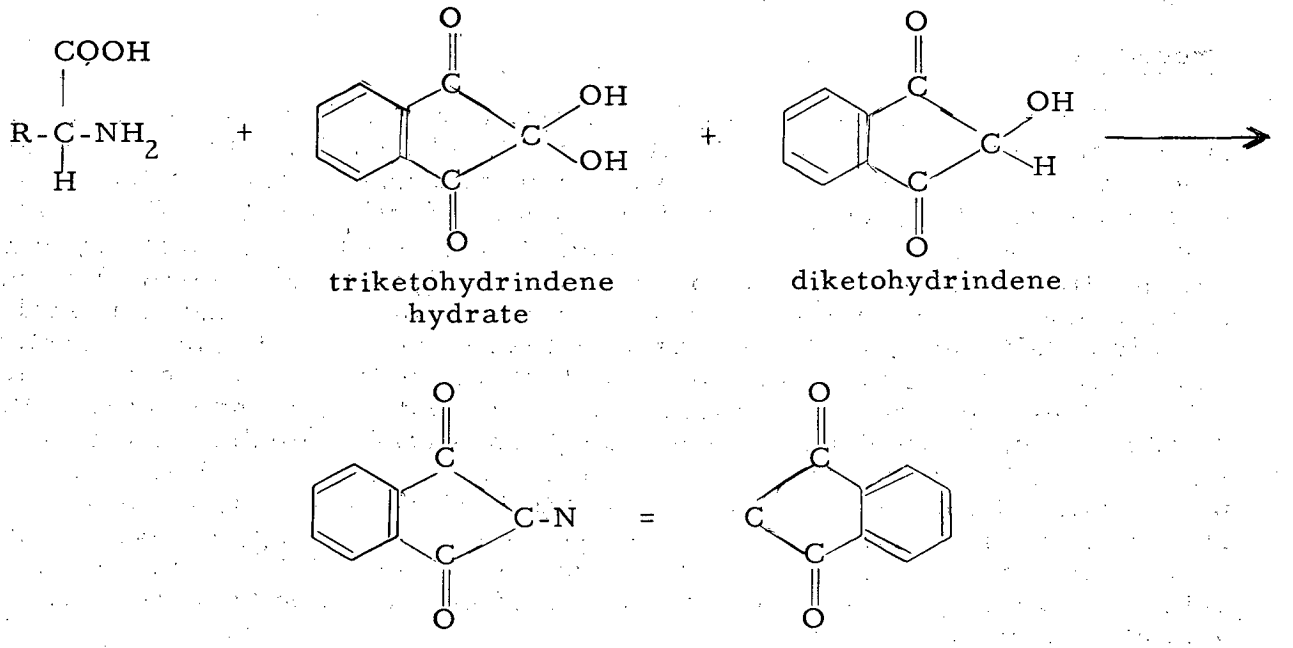
At present a study is under way to identify the source of a nonammonia ninhydrin-positive background which has been found to occur occasionally despite all above-mentioned precautions. The paper chromatographic solvents and amino acid breakdown products are being examined.[5,6]

The isotopic N^{15} abundance must be determined by measurements with a mass spectrometer. This requires the conversion of the N^{15}-labeled amino acid to a gaseous form of nitrogen. Because of reported disadvantages in using ammonia gas in the mass spectrometer, we felt that the amino nitrogen should be converted to gaseous nitrogen for analysis. Two methods of accomplishing this conversion have been reported in the literature. One involves the formation of the reaction product between the amino acid and ninhydrin,

4. Method of Leslie Fowden (University College, London); personal communication to Dr. V. Moses.

5. DeVay, Weinhold, and Zweig, Anat. Chem. 31:5, 821 (1959).

6. Thompson, Morris, and Gering, Anat. Chem. 31:6, 1031 (1959).



and the subsequent decomposition of DYDA by HCl at pH = 3.0 to give a nitrogen product which is then converted to ammonium chloride.[7] The ammonium chloride is oxidized with sodium hypobromite to yield N_2.[7] The other method, and the one we are currently investigating, involves first a Kjehldahl digestion of the amino acid with conc. H_2SO_4, crystalline selenium, and potassium sulfate to yield ammonium sulfate. The ammonium sulfate we then convert in the same reaction flask with sodium hypobromite to gaseous nitrogen.[8] To date a 50-μg sample of unlabeled amino acid has been successfully taken through this process, but in the future it is hoped that the N^{15}-labeled amino acid analysis will be in the 20-to-200-μg range. The method of nitrogen gas liberation via hypobromite oxidation has been studied with N^{15}-labeled ammonium nitrate. It was found that the apparatus best suited to this purpose is a modified Toepler pump[9] and reaction vessel, as shown in Fig. 37.

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7. Folkes and Yemm, New Phytol. 57, 106 (1958).
8. Glascock, Isotopic Gas Analyses (Edwards, Ann Arbor, Michigan, 1954).
9. Rittenberg, Preparation and Measurement of Isotopic Tracers, (Edwards, Ann Arbor, Mich., 1956).

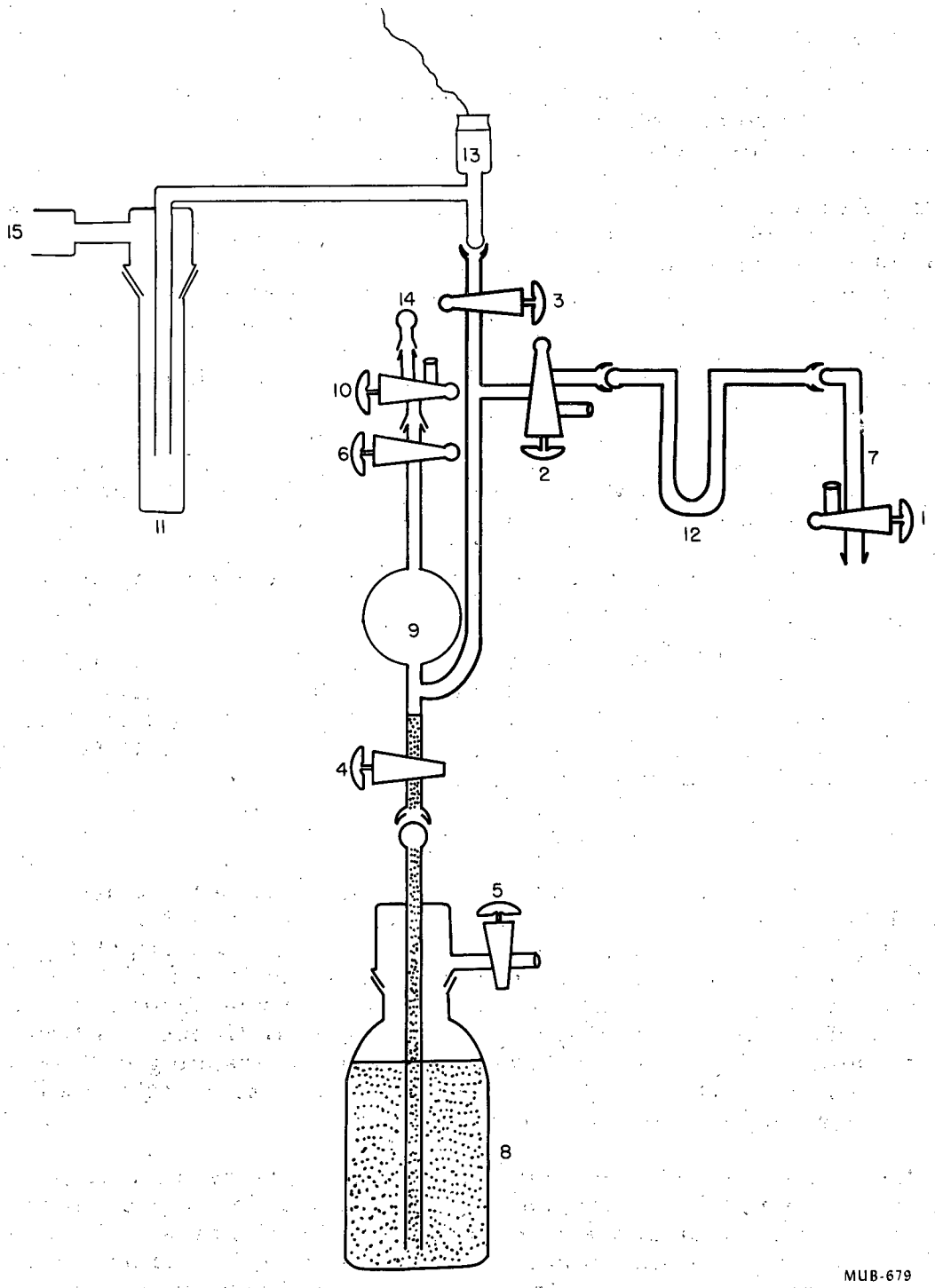


Fig. 37. Apparatus for preparation of N_2 for mass spectrometer.

The procedure is as follows:

1. Prior to use, connect gas collecting trap (10, 14) and turn three-way stopcock (10) so that the bulb (14) may be evacuated. Open entire system to vacuum except Stopcocks 1 and 4. These must be closed off.

2. Evacuate entire system. When pressure on gauge registers about 100 μ, as measured by vacuum thermocouple at 13, open Stopcock 5 to 3, let air into mercury reservoir (8). Then temporarily close Stopcock 3, open Stopcock 4, and permit mercury to rise to level indicated by shading. Then close Stopcock 4, and open Stopcock 3 to resume evacuation.

3. Let entire system evacuate for about 1 hour without using liquid nitrogen on trap (11).

4. Flush system with helium by attaching tube from helium tank to standard taper joint at the point to which the reaction flask is attached (below Stopcock 1). Pass a little helium into system while pump is working.

5. Close Stopcock 1 and allow system to evacuate completely. Now place liquid nitrogen Dewar flask on trap 11.

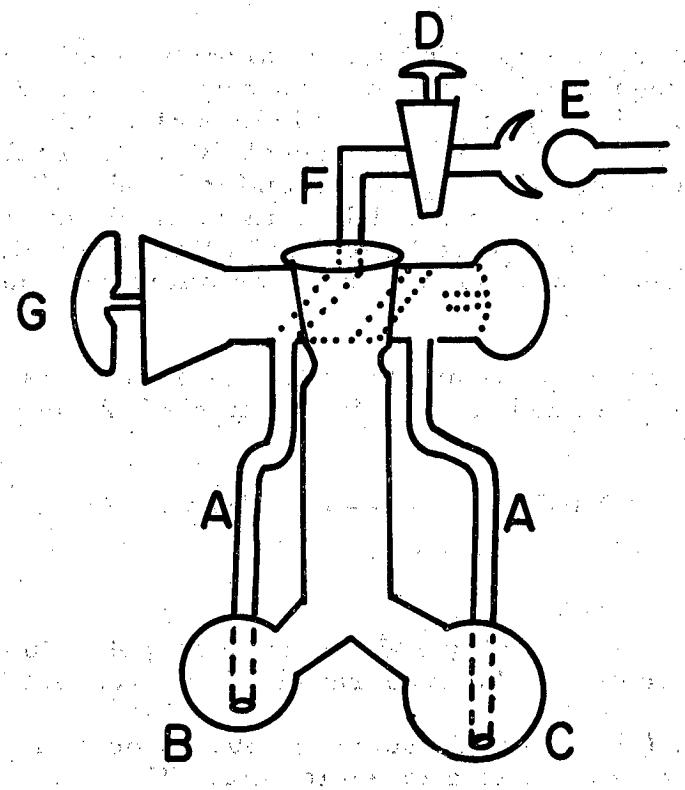
6. Now, very carefully, introduce digested sample for analysis into the smaller bulb (B) of reaction flask (see Fig. 38). Next introduce 3 ml of NaOBr solution (see below), mixed with saturated NaOH solution, into the larger flask (C); this is sufficient to neutralize the acid as well as to react completely with the ammonium salt.

Note: 1 ml NaOBr ≈ 10 mg NH_3.

7. Place the reaction flask on apparatus (sealing the standard taper joints with high-vacuum silicone grease) and turn two-way Stopcock 1 so that gas will be permitted to escape from reaction flask. Then attach line from helium tank via ball joint (E) to reaction flask. Open Stopcock D and G to permit helium to flow via tubes (a) into chambers of reaction flask. Allow helium to bubble through solution in each chamber (B, C) one at a time, for about 5 minutes. Then close off Stopcocks G and D, trapping in Tube F exactly 1 ml of gas. Open Stopcock 1 so as to evacuate reaction flask. Turn this stopcock slowly to avoid bumping.

When pressure on gauge reads about 50 μ, freeze the solutions in the reaction vessel with liquid nitrogen and allow evacuation to proceed to gauge reaction = 0 μ. Close Stopcock 1 and allow solutions to thaw. Then, detach helium line at E and tilt reaction flask on swivel arm (7), to permit NaOBr to mix with ammonium salt solution. When violent bubbling subsides, open Stopcock (G) only and allow trapped helium in Tube (F) to bubble into reaction mixture as a carrier gas. Then, freeze the reaction flask with liquid nitrogen to remove NO_2, CO_2, NO, etc.

9. Close off Stopcocks 2 and 3. Place liquid nitrogen Dewar flask under trap (12) and open Stopcock 1. Allow N_2 in helium carrier gas to expand into volume between Stopcocks 1 and 2 for about 2 minutes to permit thorough trapping of water vapor, etc. Then open Stopcock 2 and allow N_2 in



MU-23681

Fig. 38 . Reaction flask.

carrier gas to expand into expansion volume of Toepler pump.

NOTE: Ratio $\frac{\text{expansion volume (9)}}{\text{reaction vessel}} = 30.$

Close off Stopcock 2, open Stopcock 5 (if not already open), and slowly open Stopcock 4 so as to regulate ascent of mercury up column. Allow Hg to climb to Stopcock 10. Then close Stopcock 10 and close Stopcock 4. Open Stopcock 2 to external atmosphere, followed by Stopcock 10 to external atmosphere. Slowly open Stopcock 4 and allow Hg to descend to level. Close 4, remove trap from pump, close Stopcocks 6 and 1, remove liquid nitrogen Dewar flask from trap (12), and open Stopcocks 2 and 3 to initiate evacuation of system. Do not remove nitrogen Dewar flask from 11 or water will enter diffusion pump at 15.

NOTE: Run mass spectrometric analysis as soon as possible. The above procedure should exclude all air from sample. A source of O_2 was found in the NaOBr:[10]

$$2\ NaOBr \xrightarrow[\text{trace } Cu^{++}]{} 2\ NaBr + O_2.$$

This reaction can be eliminated by addition of 0.1% potassium iodide to NaOBr reagent when it is made up. The preparation of this reagent is as follows:

NaOH pellets (200 g) are dissolved in 300 ml deionized water. After 0.2 g KI is added, the mixture is allowed to cool. Then add 60 ml bromine while the solution is stirred over a 10-minute period. It is helpful to divide the NaOH solution into two portions. Into one portion pour the bromine; then add the other portion to the mixture. Let mixture stand in refrigerator for 3 days to precipitate NaBr. Centrifuge at 2000 rpm for 30 minutes and pour the supernate into container for storage in refrigerator. This solution retains its potency for several months if stored in the dark in the refrigerator.

The procedure for mass spectrometric analysis of the gas sample is dictated by a number of factors peculiar to the mass spectrometer. There are, first of all, several variables which profoundly affect the use of this machine as an analytical instrument:

(a) Temperature and volume. In our mass spectrometer (Consolidated Electrodynamics Corp. Model 21-130), both the temperature (range = 25° to 150°) and volume (expansion volume = 1000 x injection volume) are fixed. Thus, only the pressure may vary, depending upon the amount of the gas injected.

(b) Pressure. Peak heights as recorded by the mass spectrometer are a function of the molecular concentrations of the several gases in the sample in the analyzer tube and of their ionization potentials. The molecular

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10. Simms and Cocking, Nature 181, 474 (1958).

concentration of a given gas in the analyzer tube depends both upon its partial pressure in expansion volume and upon its diffusion coefficient which determines its rate of flow through the leak between the expansion volume and the analyzer tube. For these reasons another parameter (time) is important in the analysis.

(c) Time. The time elapsing between (i) the opening of the leak valve between the expansion volume and the cycloid analyzer and (ii) the scanning of the sample determines the actual concentrations of the various gases in the analyzer. Therefore, it is necessary to choose a fixed time interval between these operations. In the present series of experiments, as shown in Table XI, the time interval of 2 minutes was established as the arbitrary constant between the opening of the leak-through valve and the scanning. In order to keep this time interval constant, it is also necessary to start the scan at the same voltage and to use the same scan rate each time. In addition, the emission current must be kept constant.

Table XI. Ratio of mass peaks under varying conditions of operating mass spectrometer.[a]

Sample	Air	Air	Air	Air	Air	Air	Air	Air	Air	Air	Air
Duodial reading	10	70	90	110	115	120	144	170	195	203	288
Sample pressure ($\times 10^{-7}$ mm)	0.8	1.2	1.75	2.75	2.85	3.80	5.95	8.60	15.3	16.55	30.85
Ratio 28/32	12	5.32	5.5	6.02	7.28	5.76	5.04	5.77	5.0	5.25	5.04
Ratio 28/29	--	132.07	115.79	116.33	91	108.63	116.0	125.91	126.47	126	157.5
Ratio 28/14	8	9.12	9.17	8.73	9.1	9.66	9.51	9.23	9.11	9.0	9.0

[a] Emission Current = 50 μamp; field plate voltage range = 250 to 50 v; time interval between opening valve expansion volume and analyzer = 2 min.

As indicated in Table XI, the theoretically constant mass ratio has yet to be established. However, indications are that this constant value may yet be established. Table XII contains the values obtained for N^{15}-enriched samples of NH_4NO_3 under anaerobic conditions.

Table XII. Mass spectrometric analyses of ammonium nitrate mixtures.[a]

Sample	1 mg $N^{15}H_4NO_3$[b]	1 mg $N^{15}H_4NO_3$[b] to 5 mg $N^{14}H_4NO_3$	0.5 mg $N^{15}H_4NO_3$[b]	0.3 mg $N^{15}H_4NO_3$[b]
Duodial reading	143	180	86	12
Sample pressure ($\times 10^{-7}$ mm)	2.30	10.8	1.63	0.7
Atom % N^{15} (by analyses of peaks 14, 14.5, 15)	56.55	12.17	55.88	56.04
Atom % N^{15} (by analyses of peaks 28, 29, 30)	56.90	10.13	56.18	56.63

[a] Emission current = 50 μamp; field plate voltage range = 250 to 50 v; time interval between opening valve between expansion volume and analyzer = 2 min.

[b] Chemical determination: 1.0 g N^{15}/8.39 g $N^{14}H_4NO_3$. Isotopic ratio reported = 62.6 atom % N^{15}.

As indicated in Table XII, there is a slight variation in individual samples even though conditions are maintained as constant as possible. Further experiments are necessary to achieve truly standardized criteria of analysis.

14. A SEMIAUTOMATIC DEVICE FOR MEASURING RADIOACTIVITY ON TWO-DIMENSIONAL PAPER CHROMATOGRAMS

V. Moses and Karl K. Lonberg-Holm

Much of the biochemical research in this Laboratory is based on the technique of incubating the tissue under consideration with a labeled substrate for a known period of time under particular experimental conditions. At the end of the incubation period the tissue is killed and the soluble metabolic products extracted from the cells with alcohol. The constituents of the alcoholic extract are analyzed by two-dimensional paper chromatography, and the locations of the radioactive materials on the chromatograms are found by x-ray film radioautography. For quantitative studies the amount of radioisotope in each separate spot on the chromatogram must be measured and this is done in the following manner.

A large-diameter (2.25-in.) end-window Geiger-Muller tube, fitted with a thin replaceable Mylar window (density about 1 mg/cm^2) and flushed with Q gas, is placed on the chromatogram at each position known from radioautography to be the location of a radioactive substance. The number of counts from each location is recorded for a 1-minute period. Many spots on the chromatograms are too large to be counted with the G-M tube in a single counting operation, and part of the spot must then be shielded in a suitable manner (index cards are used for C^{14}) while another part is counted. The position of the shield is then moved so that the area previously shielded may be counted and the area previously counted is shielded. Because of the sizes of many spots, it is frequently necessary to count them in several, rather than in two operations. Spots on the chromatogram lying close to the one being assayed must also be shielded lest they interfere with the one being counted. Finally, a particular spot usually gives different counts on the two sides of the paper, probably owing to differences in the rates of evaporation of the solvent used for development of the chromatogram. It is therefore necessary, in all investigations requiring a reasonably quantitative treatment, for each spot to be counted on both sides of the paper.

A typical chromatogram obtained in an investigation of the pathways of intermediary metabolism may have as many as twenty spots. Perhaps half of these spots will be too large to be counted in one positioning of the G-M tube, and will need to be counted in sections. Thus, on both sides of the paper there may be a total of some sixty different positions to be counted, each for 1 minute; in practice, 30 seconds must be added to each counting period for aligning the shields and recording the results, making some 90 minutes in all for counting each chromatogram. It is difficult to keep more than one counter in operation for 1 minute counts, as the operator rapidly becomes confused and over-all efficiency and accuracy of placing the masking shields deteriorates considerably. Equally important is the general tedium of the whole operation, and although it might theoretically be possible to count five or six chromatograms in one working day, this places an undue mental strain on the operator, again with a fall in efficiency and an increase of carelessness. In addition, a 1 minute counting period is insufficient to achieve a high degree of statistical accuracy,

whereas increasing the counting period to, say, 5 minutes would slow the whole operation down to a quite unacceptable degree if this were to be done manually.

There have been a few attempts to make the process automatic. Strip counters, usually working in conjunction with a ratemeter and pen recorder, have been in use for many years for one-dimensional chromatograms, and these are available commercially. However, it is not possible satisfactorily to cut a two-dimensional chromatogram into a number of strips unless the spots are very widely separated; in this case there are usually few spots present on each sheet of paper and counting these by hand becomes less of a problem. With a chromatogram having twenty spots it is probable that there will be some overlap of adjacent spots, resulting in incomplete resolution no matter how the chromatogram is cut into strips. Furthermore, other spots would be cut into more than one piece and time would be spent collecting counts pertaining to one spot from different places on the record.

Some efforts have been made to construct automatic two-dimensional scanners, and at least one model is available commercially (Packard Instrument Co., La Grange, Ill.). These are subject to the same objections as cutting the chromatograms into strips and counting the latter with a one-dimensional scanner: poor resolution unless the spots are widely spaced, and time wasted because for most of its operating period the counter is scanning blank areas of the paper.

It continues to be our experience that x-ray film radioautography of the chromatogram is the only method for obtaining the location of labeled compounds with sufficient accuracy and resolution. However, a device is being developed to render obsolete the counting of chromatograms by hand, with the dual advantages of greatly increased statistical accuracy and a considerable reduction in the demands on the time of the operator. The principle of the apparatus is to "convert" a two-dimensional chromatogram into a one-dimensional strip by cutting out the areas of paper bearing radioactive substances after these have been located by radioautography, and mounting them between two continuous strips of thin plastic. The strips are then passed automatically between two G-M tubes which detect the emitted radiation on both sides of the paper simultaneously, and feed the pulses into one scaler. After a predetermined counting period, the counts and the time are printed onto paper tape together with a sample number, and the plastic strips advance automatically to the position of the next piece of chromatogram paper, when counting recommences.

Two pieces of equipment are required: a "loader: and a "counter." The principle of the loader is shown in Fig. 39. A strip of brown paper, 5 in. wide, is unwound from a spool along a table. Simultaneously, a strip of Mylar (0.00025 in. thick and 5 in. wide) is also unwound from a spool and lies on top of the brown paper. The Mylar and paper together pass over a window in the table, 3 in. in diameter, illuminated by an electric light bulb from below. With the Mylar and paper stationary, the excised chromatogram spot is placed on the Mylar over the illuminated window, with a small spot of glue on the leading edge of the chromatogram paper to ensure adequate adhesion to the Mylar. A felt-tipped pen is operated to inscribe a black mark on the Mylar at some definite position in relation to the center of the illuminated window. The plastic-plus-brown-paper strip is then moved on and is covered by a

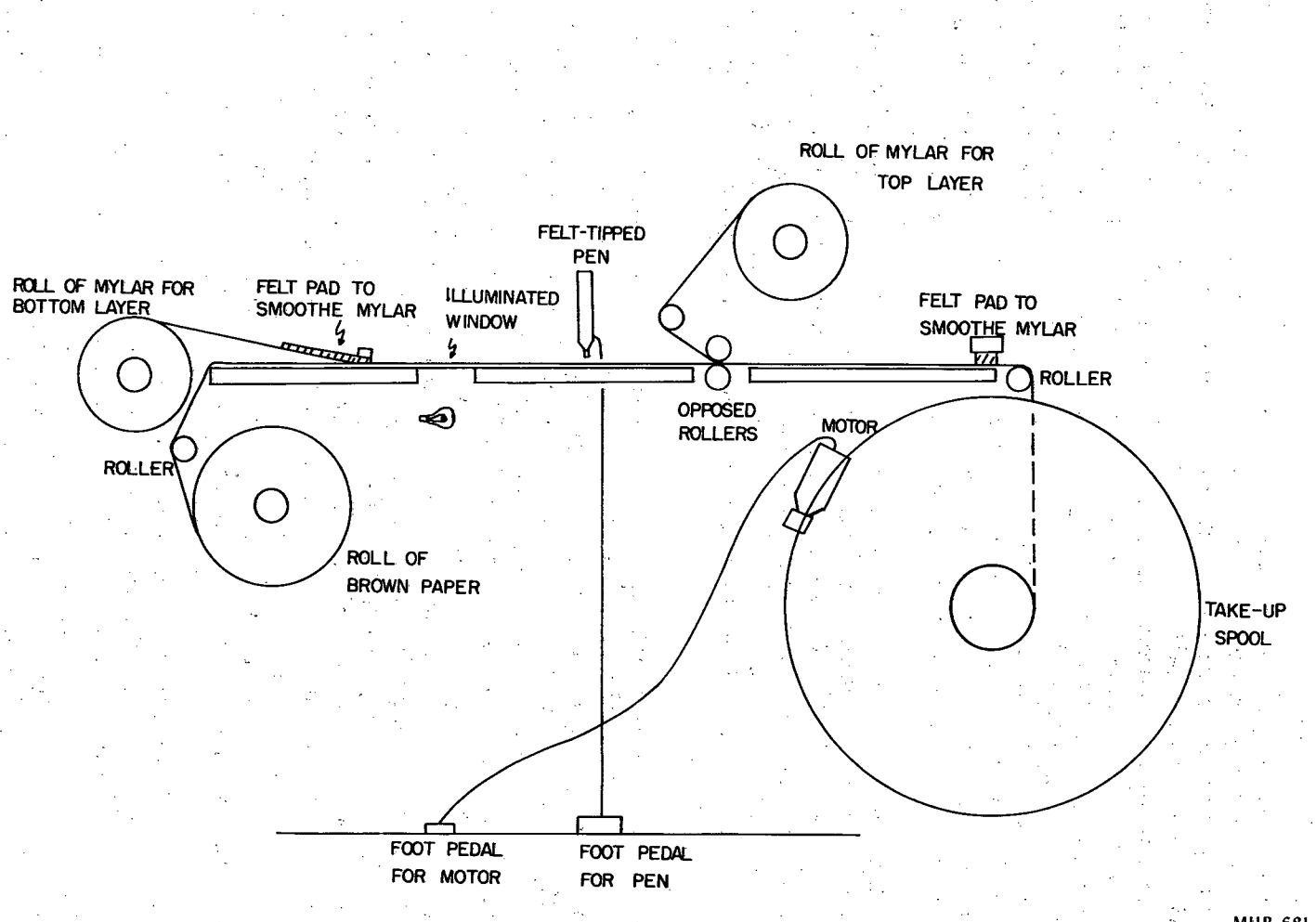

Fig. 39. Mylar strip loader.

second layer of Mylar wound off another spool. The brown paper plus two layers of Mylar, the latter bearing between them the piece of chromatogram paper, is passed between a pair of opposed rubber-covered rollers, the purpose of which is to press the layers of Mylar into firm contact with the piece of chromatogram paper and to make certain that the glue holds the chromatogram paper firmly to both layers of plastic. The combined strip is wound up onto a large take-up spool.

As the Mylar is so thin, it has very little resistance to bending and folding. It is, therefore, important that the axles of all the spools and rollers be accurately aligned, else the combined strip will not wind uniformly onto the take-up spool. A considerable measure of support is provided by the brown paper. This has a twofold purpose: to support the bottom layer of Mylar, which tends to adhere to the brown paper, and to provide a continuously smooth surface on which to roll up the combined Mylar-paper strip on the take-up spool. As the chromatogram paper is about fifteen times as thick as the double layer of Mylar, and the chromatogram spots are confined to the center of the strip, a bulge develops in the accumulated strip on the take-up spool unless the backing of brown paper is provided.

The operation of loading the take-up spool, once the brown paper and the two layers of Mylar have been threaded over the rollers and attached with Scotch tape to the core of the spool, consists of placing the chromatogram paper, together with a dab of glue, on the bottom layer of Mylar at the correct position as shown by the illuminated window. Then, the marking pen is operated by a foot pedal. Next, a second foot pedal is pressed which operates an electric motor driving the rim of the take-up spool, thus drawing the combined strip through the whole apparatus at a minimum speed of 3.5 ft/min. While the strip is advancing, glue is applied to the next chromatogram spot, and the motor is stopped by the foot switch when the previous spot has moved 4 or 5 inches from the center of the illuminated window. The next spot is placed in position on the Mylar and the process repeated; the interval between loading successive spots is less than 10 seconds. When all the spots have been loaded, the motor is run for sufficient time to wind a few feet of empty strip onto the take-up spool, the Mylar and brown paper are cut off, and the spool removed to the counter. The over-all diameter of the spool is 2 feet and the core diameter 6 inches; about 2700 feet of combined strip, or about 6500 spots, may be loaded onto the take-up spool in one continuous strip.

In the counter the strip is unwound from the spool, the brown paper separated from the double layer of Mylar, and the latter passed between two end-window G-M tubes. The brown paper is then brought into contact again with the Mylar strip and the two taken up together on another motor-driven spool as in the loader (Fig. 40). A photoelectric cell is mounted so that it bears the same relation to the G-M tubes that the felt-tipped pen bears to the illuminated window on the loader. At the start of the counting sequence, the driving motor continues to run and advance the tape until the photoelectric cell observes the first black ink mark on the Mylar strip. The photocell stops the motor (which is braked electrically) and at this point the first chromatogram spot is lying exactly between the two counters. The radioactivity on the paper is counted for a preset time, or until a preset number of counts has been recorded; at that time the counter operates the printer and the driving motor is switched on until the next black ink mark is located by the photocell and the

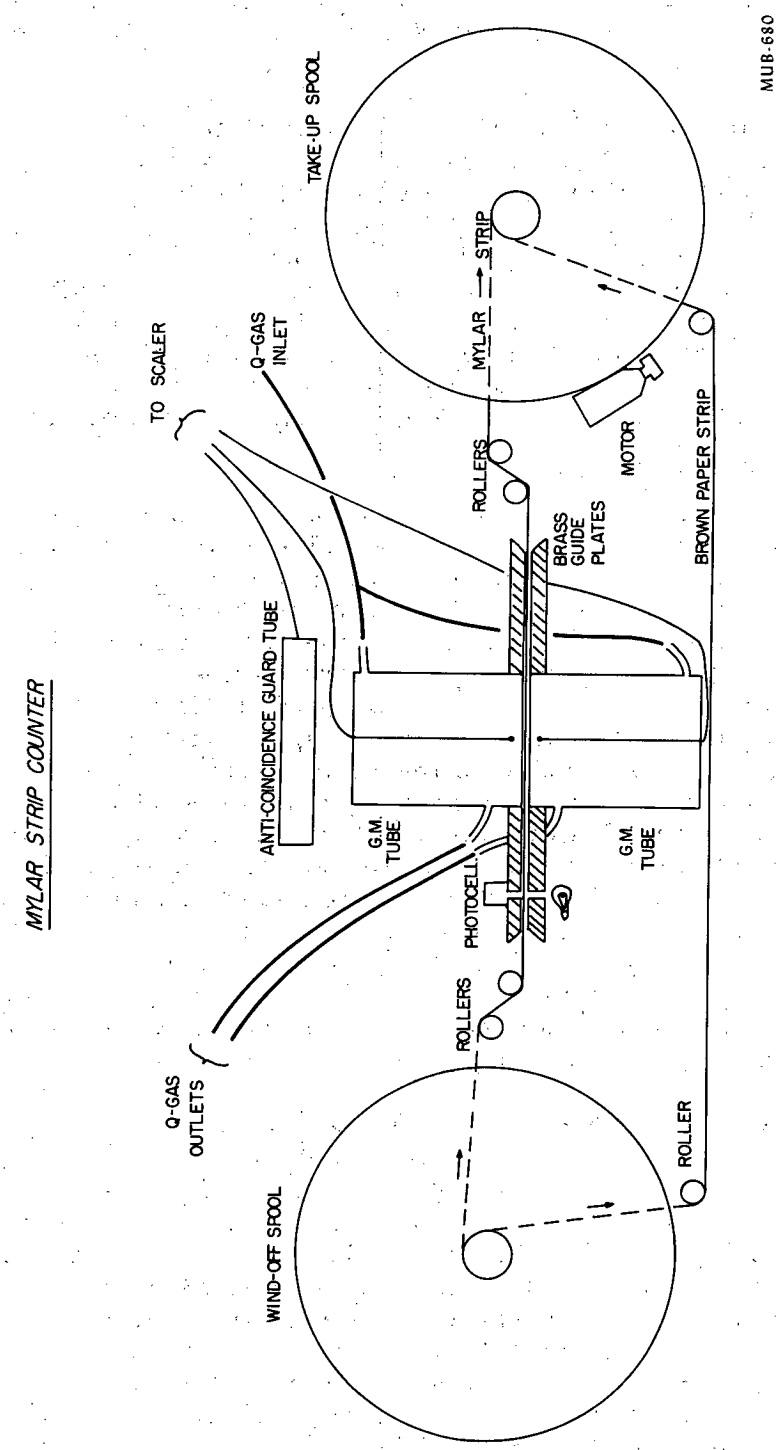

Fig. 40. Mylar strip counter.

next chromatogram spot is between the counters. The cycle is then repeated until all the spots have been counted.

The counting tubes are standard Scott-type G-M tubes, about 3.75 in. internal diameter. The tubes are flushed with Q gas (99.05% He, 0.95% isobutane) and have a plateau about 300 volts long between 1550 and 1850 volts. Gold-sputtered Mylar windows (0.00025 in. thick) are used and the complete tube has a very uniform sensitivity over a radius of 1.625 in. from the center of the window. Preliminary trials of the equipment have demonstrated that the counting array has a satisfactory statistical reproducibility. The "dead time" of the tubes is about 265 μsec, and the two tubes are virtually identical as regards over-all counting efficiency. The positive pressure of Q-gas inside the tubes is maintained at about 6 mm. This pressure is sufficient to ensure that the flexible windows of the two G-M tubes are in contact with the Mylar strip bearing the chromatogram paper, and serves also to flatten out any ripples in the Mylar of the strip. The movement of many feet of Mylar strip past the G-M tube Mylar windows has not resulted in appreciable damage to the latter; it is, however, necessary to prevent dust and grit from falling on the Mylar strip and being carried past the windows, where damage might well result.

The over-all efficiency of each counter tube after coincidence corrections is 6% of the total disintegrations using Whatman No. 4 filter paper; i.e., with two counters operating simultaneously, 12% of the total disintegrations of C^{14} are recorded with this type of chromatography paper.