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Ernest O. Lawrence

Radiation Laboratory

## BIO-ORGANIC CHEMISTRY QUARTERLY REPORT
September through November 1960

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# BIO-ORGANIC CHEMISTRY QUARTERLY REPORT

September through November 1960

M. Calvin, Director

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

January 31, 1961

## I. COACERVATION OF SALTS OF POLYVINYLSULFONIC ACID INDUCED BY HEAVY-METAL IONS

Frank Millich

### Introduction

The importance of heavy-metal ions in the chemical mechanisms of biological reactions is readily acknowledged. The catalytic activities of at least eight metals-- i.e., Fe, Co, Mg, Ca, Zn, Mn, Cu, and K--have been identified with enzyme systems.[1] The need exists, however, for detailed knowledge of metal-ion interaction with polyelectrolytes. Detailed "proposed mechanisms" for enzyme-coenzyme substrate interplay are plentiful in the literature,[2] yet quantitative treatment of electrolyte-polyelectrolyte interactions are contrastingly meager, especially in condensed systems. It is reasonable that such systems of high concentration of interacting groups are capable of high kinetic rates without the necessity of having reactive sites positioned by primary bonds. Such systems profit from the advantages of numerous associative forces, entropy factors, and high encounter frequencies.

Current studies pertinent to the subject of enzyme action are those covering the physics of colloid systems; studies of ion binding of polyelectrolytes and their properties; and "super-rate" kinetics of intramolecularly reacting chemical groups, usually located vicinally to one another in a molecule, or separated by four carbon bond lengths. It remains to be demonstrated in exactly what way condensed media provide highly beneficial conditions for interaction of diffusible reactants. This, however, is precluded by the present lack of knowledge and difficulties of precise definition of physical factors. Most theories, such as that of ionic strength, quantitatively describe only relatively dilute solutions, even in the absence of polymeric species. A discussion of unsuccessful attempts to apply various theoretical approaches to quantitative description of bolaform electrolytes is given by Rice and Harris.[3]

---

[1] V.A. Najjar in symposium on Phosphorus Metabolism, Vol. I, ed. by W.D. McElroy and B. Glass (Johns Hopkins University Press, Baltimore, Md., 1951), p. 500.

[2] M. Dixon and E.C. Webb, in The Enzymes (Academic Press, Inc., New York, N.Y., 1958), Chap. VII.

[3] S.A. Rice and F.E. Harris, J. Phys. Chem. 58, 725, 733 (1954).

The present study seeks to give a preliminary profile of experimental conditions affecting the interaction of heavy-metal ions with salts of polyvinylsulfonic acid (PVSA), a polymer which is known to yield aqueous complex-coacervate solutions.[4]

## Experimental Procedures

### Materials

Three polymer samples of polyvinulsulfonic acid were used. One polymer, sample M, was polymerized by irradiation of a 70% aqueous solution at room temperature for 12 hours with ultraviolet light from a 1000-watt G.E. AH-6 lamp at a distance of 6 inches. The monomer (b.p. 128° at 2 mm, $n_D^{25}$ 1.4500) and the polymerization mixture were made up in a quartz reaction vessel by distilling, in turn, monomer (under reduced pressure) and water directly into a quartz reaction vessel before sealing, to exclude oxygen. The two other samples, H and D, were obtained through the courtesy of Dr. D.S. Breslow of the Hercules Powder Company, Wilmington, Delaware. Samples H and D originated from spontaneous polymerization of concentrated aqueous solutions of sodium ethylenesulfonate.

All three polymer samples were filtered as aqueous solutions of their sodium salts through fine-porosity sintered glass filters. Polymers M and D, after treatment with Nuchar decolorizing charcoal, were precipitated twice by adding 20 ml of saturated sodium chloride to an otherwise stable milky suspension which is produced when 700 ml of methanol is added, with stirring, to a solution of 20 g of polymer in 300 ml of water. In this way 95% of the original material is recovered, and only unreacted monomer and low-molecular-weight oligomers are separated out. Dialysis through cellulose tubing (Visking Co.) of 24-A pore size removed contaminants capable of reducing neutral or acidic permanganate solution, as evaluated by iodimetry.

### Methods

Sedimentation velocity analyses of sodium salts of the polymer samples at 1.5% concentration in 0.5 M NaCl were conducted in a Spinco Model E ultracentrifuge at 52,640 rpm for 1 to 2 hr at 26°. The sedimentation constant for sample D was also determined in 0.5 M NaCl at three different concentrations: 0.94, 1.56, and 2.60 weight %. One determination of the sedimentation constant in 0.1 M $Na_2SO_4$ was made on a 1.5% solution of sample D.

Viscosities of samples of sodium polyvinylsulfonic acid (Na-PVSA) in solution of two different salts were determined in an Ostwald viscometer at 24.82° ± .01°. Refractive indices were determined in a Bausch and Lomb six-decimal-place refractometer at 26.0 ± .1°, with monochromatic light. Ultraviolet spectra were studied with a Beckman DU spectrophotometer.

[4] H. Eisenberg and G. Ram Mohan, J. Phys. Chem. 63, 671 (1959).

The qualitative screening of distribution of colored ionic species between phases of a coacervate solution was performed by visual observation at $0^{o}$ of the color resulting from one or more drops of a concentrated solution of each ion, e.g., approx 2 M with respect to metal ion, millimolar with dye ion added, to 1 ml of Na-PVSA sample M (0.3 eq. in 1.5 M NaCl solution). The latter solution is monophasic at room temperature, but yields two phases at $0^{o}$.

Phase diagrams at $24.82 \pm .01^{o}$ were obtained by titration of decimilliliter volumes of polymer solution, with solutions of metal ion. In one series (Fig. 1) a variety of concentrations of a simple electrolyte was also included in the polymer solution. Polymer solutions were metered with micropipettes, the titrant with a microburet (L.S. Starrett Co., Athol, Mass.) which was graduated to $0.1\lambda$, though only droplets of $2\ \lambda$ or larger were transferred. Points shown in the phase diagrams indicate the very first and last indication of opacity of solutions.

## Results and Discussion

### A. Molecular Weights of Samples

Sedimentation velocity analysis showed dc/dx distribution patterns that cannot be distinguished from Gaussian distribution within the precision of the determination. There seems to be little or no dependence of the sedimentation constant or concentration in the range investigated. Table I summarizes some of the properties that have been determined for the three samples. Sedimentation velocity data were obtained for our samples in 0.5 M NaCl solution, as well as viscosity data in the same solvent, to check the correspondence of physical properties reported by Dialer and Kerber.[5] However, calculated molecular weights were lower than values from viscosity-molecular weight correlation made by Breslow and Kutner[6] in a different salt solution using data from osmometry and light scattering.

Referring viscosity measurements in 0.1 M $Na_2SO_4$ for samples H, M, and D to the data of the latter authors, we obtain indication of both number-and weight-average molecular weights which are numerically larger (the latter by 300%) than indicated by the ultracentrifuge average molecular weights. Viscosity data would undoubtedly show a limited dependence on rate of shear, but the magnitude of the discrepancy found above points to the probable need of closer scrutiny of the influence of solvent on polymers that are capable of coacervation. The large dependence of intrinsic viscosity on sodium sulfate concentration shown by Breslow and Kutner[6] accentuates this point. It has been stated that the comparison of intrinsic viscosity with ultracentrifuge molecular weights and perhaps with light scattering is valid only if the exponent in the Staudinger equation is close to unity; this is not the case with Na-PVSA.[5] For present purposes, it is necessary to establish only the relative order of molecular weights for the three samples in order to demonstrate if molecular weight differences are significant in coacervation. For the present the samples H, M, and D,

[5] K. Dialer and R. Kerber, Makromol. Chem. 17, 56 (1956).

[6] D.S. Breslow and A. Kutner, J. Polymer Sci. 27, 295 (1958).

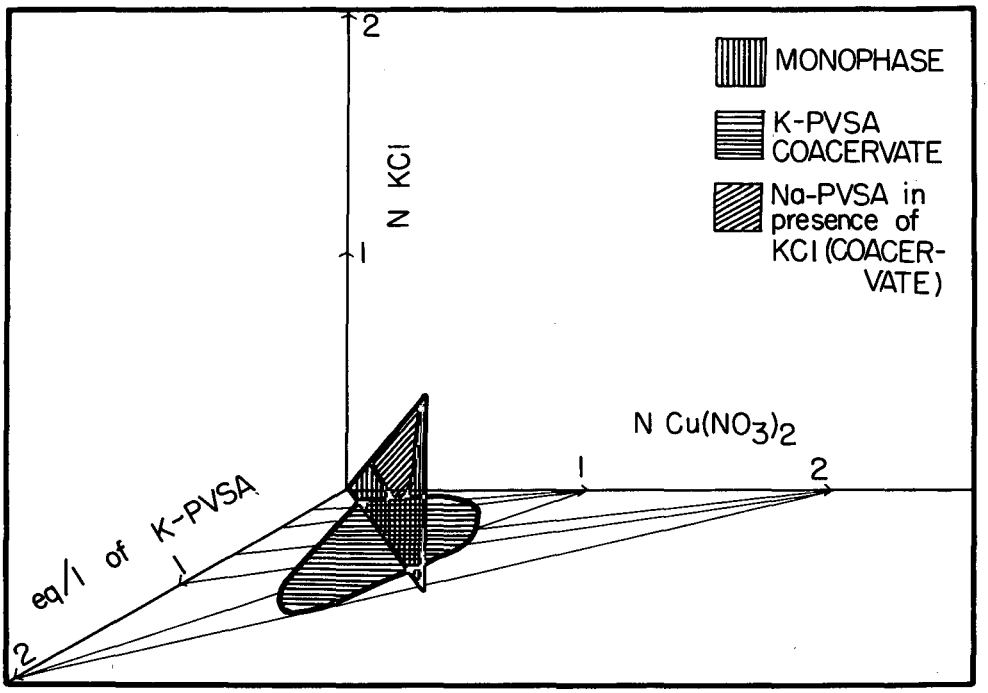


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Fig. 1. Phase diagram of coacervation of salts of polyvinylsulfonic acid in the presence of simple electrolytes at 24.82°C. Equivalence of PVSA = g equivalent of segmer unit, $-CH_2CH(SO_3^-)-$.

Table I. Physical properties of polymers

| Sample | $[\eta]$ (a) (dl/g) | $\overline{M}_n$ (b) | $\overline{M}_W$ (b) | $[\eta]$ (c) (dl/g) | $\overline{M}_W$ (c) | $dn^{26.0}_D/dc$ (dl/g) | 1% (e) $\eta$ | $S_{20,w}$ (x $10^{13}$ $sec^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| Na-PVSA, H | 0.290<br>0.27 (e) | 40,000 | 85,000 | 0.183 | 30,000 | 0.125 | 1.25 | 4.01 (d) |
| Na-PVSA, M | 0.214 | 27,000 | 60,000 | 0.150 | 21,900 | 0.130 | -- | 3.67 (d) |
| Na-PVSA, D | 0.144 | 18,000 | 40,000 | 0.094 | 11,000 | -- | 0.44 | 2.06 (d)<br>2.50 (a) |

(a) Solvent: 0.1 M $Na_2SO_4$

(b) Molecular weights were evaluated from the graphs by Breslow and Kutner[6] which correlate viscosity in 0.1 M $Na_2SO_4$ with light scattering.

(c) Molecular weights were evaluated from the data of Dialer and Kerber[5] which correlate viscosity in 0.5 M NaCl with ultracentrifuge sedimentation velocity.

(d) Solvent: 0.5 M NaCl

(e) Data of Breslow in 0.1 N $Na_2SO_4$ (private communication).

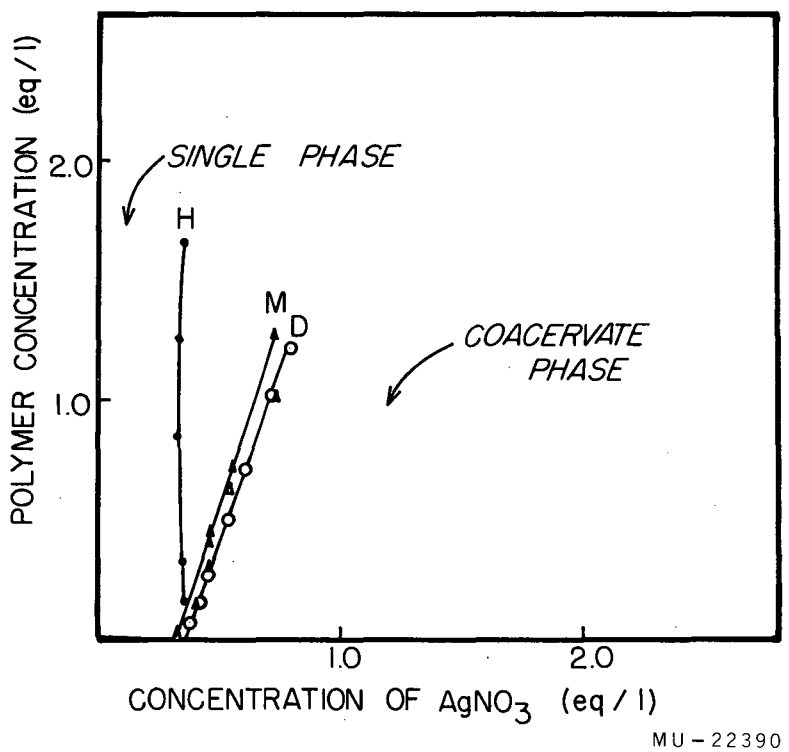



Fig. 2. Phase diagram of the coacervation of potassium polyvinylsulfonate by silver nitrate, as a function of samples of different average molecular weight (see Table I).

listed in order of decreasing molecular weight, remain characterized by viscosity data in two different solvents and sedimentation constants.

B. Distribution of Ions Between Coacervate and Equilibrium Phases

The results of a qualitative screening of the distribution of colored ions between the heavier, coacervate phase and the more polymer-dilute equilibrium phase are as follows: the anions--i.e., dichromate, fluorescein, Rose Bengal, and ferricyanide--became localized in the equilibrium phase, as did Nile Blue monocation; the cations--i.e. cerium (IV), iron (III), copper (II), nickel (II), manganese (II), silver (I), and the monoprotonated Proflavin--became localized in the coacervate phase; while ionic forms of acridine orange, thionine, Safranin, and Congo Red were insoluble and salted out of solution. Of the cations, those of the heavy metals induced coacervation at room temperature without the need for cooling.

The generalization arises that the distribution is largely determined by the sign of the charge of each ion--cations of added simple electrolyte being localized in the polyanionic coacervate phase and anions being repelled from this phase. Only one exception to this rule was found: Nile Blue hydrochloride became a solute of the equilibrium phase and was repelled completely by the high ionic strength of the medium of the coacervate phase. Since several dyes salt out of this medium it is not surprising that ionic strength effect on aromatic dyes may predominate in certain cases. A wider spectrum of the influence of ionic strength on distribution of proteins--for instance between two neutral coacervate phases--may be appreciated by reference to the data of Albertsson and Nyns.[7]

Quantitatively, in contrast with the anionic dyes, which showed strong differentiation between the two phases of the system, the chromate ion phase preference was poor and could be noticeably demonstrated only at low chromate concentration. This observation also reflects upon the sensitivity to ionic strength imparted to the character of ionic dyes by their partial lyophillic nature.

C. Coacervation Induced by Heavy Metals

Following the observation that when heavy-metal ions were employed in the above experiments they induced formation of coacervate phases without the need for cooling, quantitative studies were designed to reveal the extent of these effects. The three samples of PVSA, differing in molecular weight, were employed in the form of their sodium and potassium salts. The results are summarized in Figs. 1 to 4. Several conclusions about the factors affecting the degree of phase separation may be drawn from inspection of these diagrams.

1. Molecular weight of the polyanion

In each case--i.e., with silver ion, Fig. 2; with cupric ion, Fig. 3; and with ferric ion, Fig. 4--the tendency to develop a second phase is greatest with the PVSA sample of highest molecular weight, and vice versa, when equivalent concentrations in gram ionic weight per liter of metal and polyion are compared.

[7] P. Albertsson and E. J. Nyns, Nature 184, 1466 (1959).

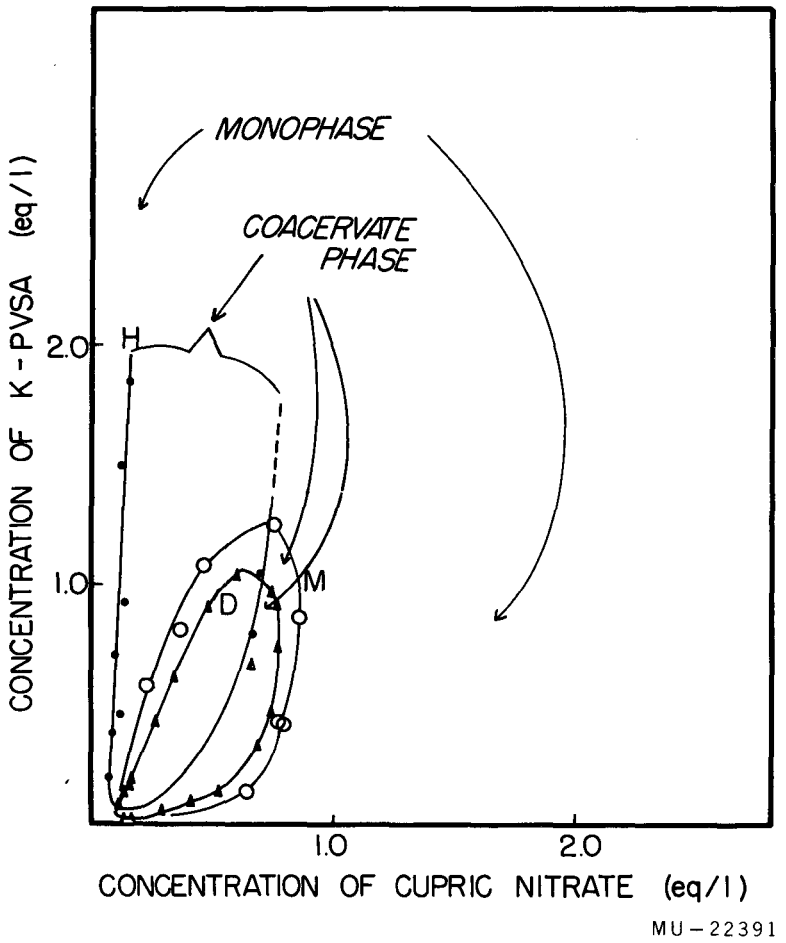



Fig. 3. Phase diagram of the coacervation of potassium polyvinylsulfonate by cupric nitrate, as a function of samples of different average molecular weight (see Table I).

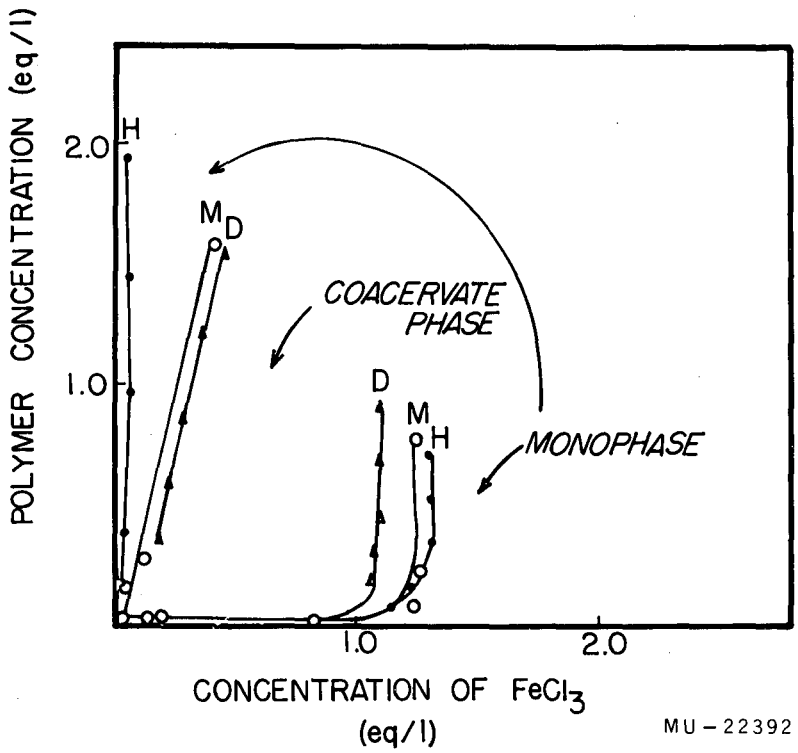



Fig. 4. Phase diagram of the coacervation of potassium polyvinylsulfonate by ferric chloride, as a function of samples of different average molecular weight (see Table I).

2. Sensitivity of coacervate to ionic strength of the medium

From the slopes of the curves in Fig. 2, depicting an inverse dependence of coacervate formation on the concentration of interacting constituents, and thus on ionic strength, it is apparent that with the samples of higher molecular weight the dependency is less, being almost nil for sample H, which shows a slope of 90 deg. The abscissa intercept, however, is apparently the same for all three samples. Calculation of the ionic strength of the metal titrant for the extrapolated abscissa value of zero concentration of polymer (sample H, Figs. 2-4) along the slope of incipient phase separation gives the following values: silver nitrate, 0.34; cupric nitrate, 0.24; ferric chloride, 0.06.

3. Resolubilization by high concentration of metal ion

The phase diagrams of the interaction of cupric and of ferric ion with K-PVSA contrast with those of silver (Figs. 3 and 4 vs. Fig. 2) in showing an area of high concentration of metal ion at which the coacervate redissolves. One factor which undoubtedly plays a role is the acidity developed in solution by solvolysis of cupric and ferric ions, as distinct from the properties of silver ion in aqueous solution. The solubilizing effect of adding HCl to the above systems can be demonstrated. Alternatively, polyvalency of metals can afford ampholyte character to the polymer-metal complex. The phenomenon of redissolution is not new, having been reported as occurring with polyphosphates.[8] The phenomenon has an apparent analogy with the solubility properties of antibody-antigen complexes.

4. Temperature dependence of coacervate phase separation

The amount of coacervate separating out increases with decrease in temperature. Gross difference in temperature dependence was visibly apparent upon dilution of coacervate mixture. It is possible to induce phase separation for some one concentrated solution of metal and polymer simply by titrating with water. The copious coacervate solutions initially formed can be redissolved merely by raising the temperature of the mixture by 5 or 10°. However, as addition of water progresses, this temperature dependence diminishes until, for instance, a coacervate mixture produced by equivalent volumes of 0.1 N ferric chloride and 0.1 N K-PVSA can be boiled without dissolution. The apparent color of this latter presumably ionically tightly cross-linked material is of appreciably more intense red color. An x-ray crystallographic analysis failed to show any pattern of regularity for this latter material. Van Wazer distinguishes types of association between metal ions and polyphosphates that can be differentiated experimentally.[9]

5. Specific ion association

As previously reported, there appears to be a selective association between PVSA and potassium ion in preference to ammonium, hydrogen, lithium, cesium, rubidium and sodium ions, as shown by the ease of coacervate formation (i.e., the concentration and temperature ranges over which formation occurs), although sodium and rubidium show the coacervation phenomenon to a lesser degree in the temperature range that is

[8] Persoz, Ann. Chem. Liebigs 65, 163 (1848).
[9] J.R. Van Wazer, discussion, Reference 3.

practically attainable with aqueous solutions. This is somewhat curious, since Dowex-50 (sulfonate polystyrene) associates ions by order of valency and ratio of hydrated ion.[10,11]

The difference for the contrasting property of PVSA probably does not lie in the density of charge difference between an aliphatic and aromatic sulfonate group, since that would not explain a maximum effect for potassium. To say that aqueous KCl solution is a poorer solvent than, for example, aqueous NaCl is begging the question, though some solvent effect is probable, as seen in the differential effect among KCl, KBr, and KI solutions upon the coacervation of K-PVSA.[3] The nature of the specificity seems to lie rather in the steric pliability of PVSA when contrasted with the rigid ion-exchange resin, which enables it to group anionic sites about a cation. Consideration of the dominant role which flexibility of polymer chains plays in ability to complex are indicated in studies such as those of Bradley[12] and Rice and Harris.[3]

Figure 1 supports the assumption that the ease of coacervation which is induced by a heavy metal, as in the case of cupric ion, is strongly dependent upon whether the counter ion of PVSA is $K^+$ or $Na^+$. As seen, the copper coacervate could not be formed from the sodium salt of PVSA at room temperature without the addition of monovalent electrolyte (KCl). This coacervate formation was possible with the K-PVSA.

In an attempt to determine a constant of ionization for the interaction of ferric ion with PVSA, the ultraviolet spectrum of ferric chloride was studied at a concentration of $8 \times 10^{-4}$ equivalent per liter in the presence of the various concentrations of Na-PVSA (sample H), over the range of $10^{-4}$ to $10^{-2}$ eq./liter of the polymer. A stock solution of ferric chloride at concentrations higher than $10^{-3}$ eq./liter is distinctly acid and thus is stable. On hundredfold dilution, however, the pH of the solution rises and hydroxide formation of the ferric ion takes place over a conveniently observable time span of 1/2 to 1 hour. Concomitantly, the absorbtivity index increases. Over the range of spectrum studied (Fig. 5) the absorption spectrum appears as a straight sloping line. The hyperchromic change gives a parallel line. With standing, ferric hydroxide precipitate appears after several days.

When a stock solution of ferric chloride was freshly diluted one-hundredfold to a concentration of $8 \times 10^{-4}$ in the presence of sodium 2-propanesulfonate, a monomolecular analog of Na-PVSA, the hyperchromic

---

[10] Dowex Ion Exchange (Dow Chemical Co., Midland, Michigan, 1959), p. 9. The series given for monovalent ions is, in the order of decreasing associative ability, Ag, Tl, Cs, Rb, $NH_4$, K, Na, H, Li.

[11] J. Kielland, J. Am. Chem. Soc. 59, 1675 (1937) gives values for hydrated ionic radii. Neither ionic nor covalent radii offer a uniform correlation.

[12] D.F. Bradley and M.K. Wolf, Proc. Natl. Acad. Sci. U.S. 45, 944 (1959).

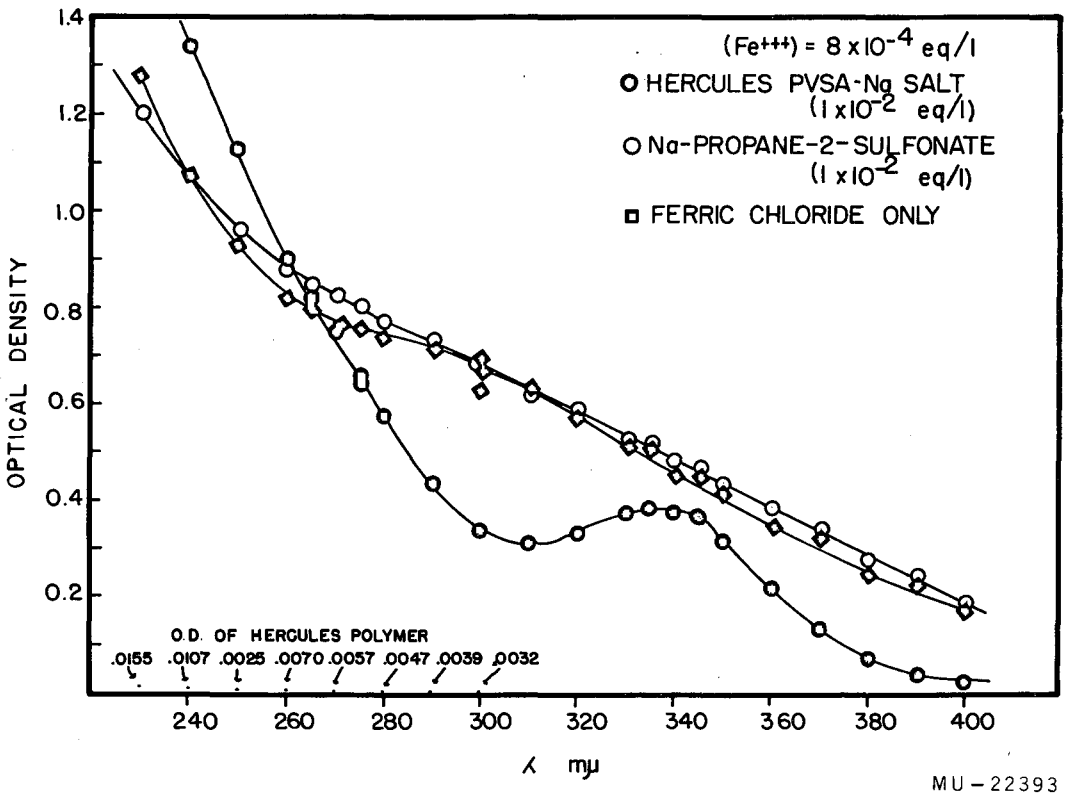



Fig. 5. Spectra of ferric ion in the presence of various ligands.

change takes place instantly, giving the same resulting spectrum. When the dilution is made in the presence of Na-PVSA, a specific band develops with a maximum at 330mμ, with a molar extinction coefficient of about $1.4 \times 10^3$. Since, intrinsically, the associative property of a sulfonic acid group would not be expected to differ between the monomeric and polymeric compound, and, in any case, is known to be extremely weak (PVSA titrates as a monofunctional strong acid of the strength of sulfuric acid[13]), the development of a specific band reflects upon the pliability of the polymeric chain in its ability to group several groups around the cation, an effect promoted by gain in entropy considerations for solvent molecules of hydration of the polymer. Figure 6a shows the dependence of the spectra of freshly diluted solutions of ferric ion upon the concentration of Na-PVSA. The spectra of these solutions are stable for 24 hours, as shown in Fig. 6b. When a ferric solution is first diluted and later mixed with Na-PVSA (after hydroxide formation had already been allowed to occur), the action of developing a specific band is successful at room temperature only at considerably higher polymer concentration, as is shown in Fig. 6c and 6d. It can be seen in Fig. 7, in which there are plotted as a function of concentration of Na-PVSA the optical densities of the maxima and minima of the specific band, that the ferric ion exists in a seemingly metastable association at polymer-to-ion equivalence ratio of 10:8.

## Summary

In summary, the following observations have been made to further describe the interaction of metal ions in coacervation of salts of PVSA.

(a) The polyelectrolyte concentrates ions of opposite charge;
(b) Coacervation occurs with heavy-metal ions over a wider concentration range with alkali metals.

In contrast with the latter, Ag-PVSA could not be brought back into solution at high silver concentrations. Also in contrast with the alkali metals is the loss of sensitivity of coacervate phase to temperature under certain conditions. Increase in molecular weight of the polyelectrolyte promotes coacervate formation, and causes the solubility of the coacervate to be less sensitive to high ionic strength.

[13] W. Kern, W. Herold, and B. Scherhag, Makromol. Chem. 17, 231 (1956).

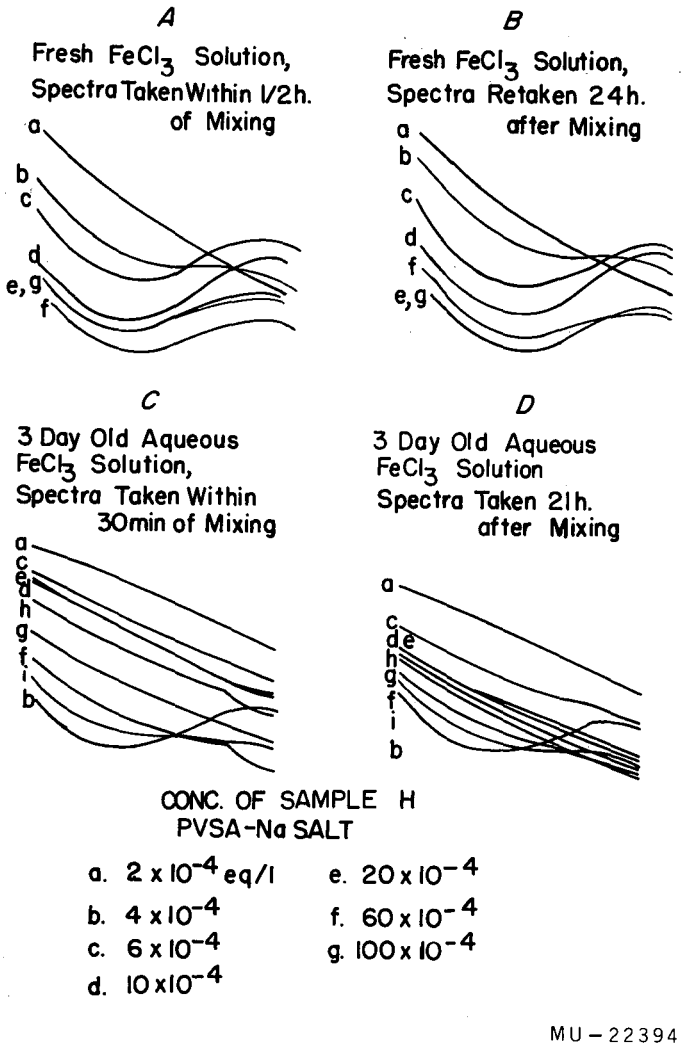



Fig. 6. Dependence of the ultraviolet spectra of ferric ion upon the concentration of Na-PVSA: 6a and 6b = dilutions made in the presence of polymer in solution; 6c and 6d = dilutions in water, to which polymer was later added. Concentration of ferric chloride = $8 \times 10^{-4}$ eq/l.

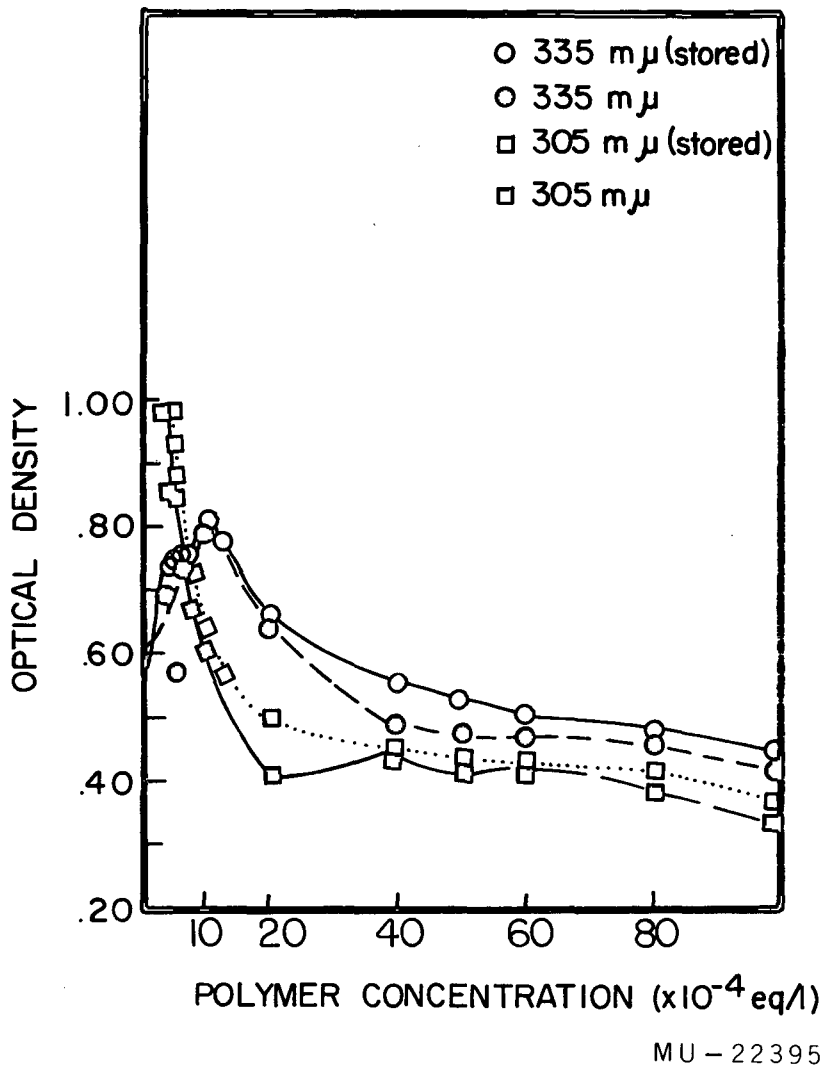
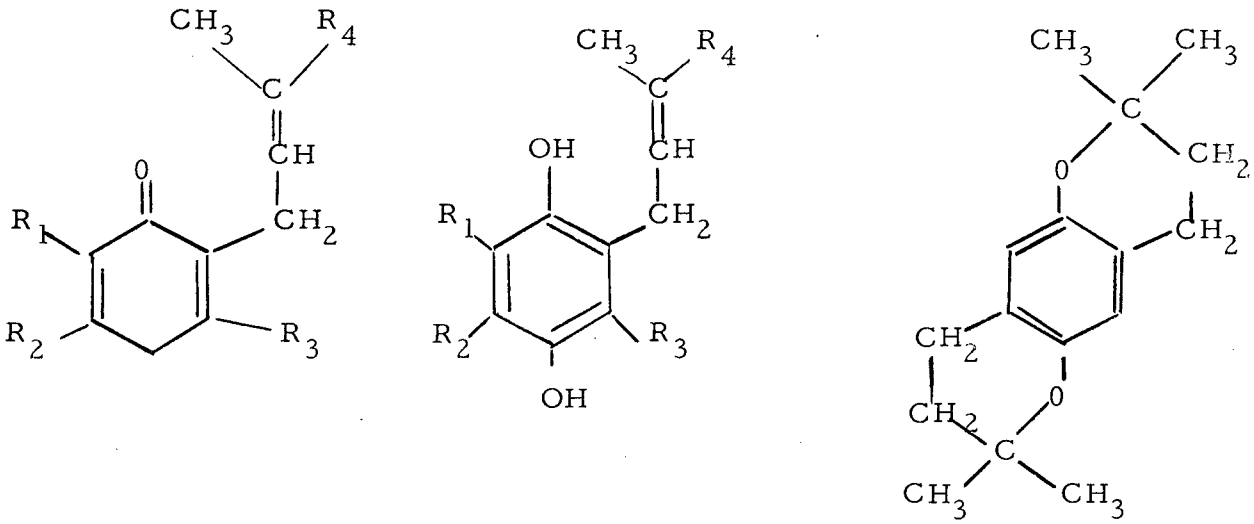



Fig. 7. Dependence of the optical density of ferric ion absorption at two wave lengths as a function of the concentration of Na-PVSA. Concentration of ferric ion = $8 \times 10^{-4}$ eq/l.

## II. THE CONDENSATION OF HYDROQUINONE WITH ISOPRENE

P. R. Hammond

Condensation of isoprene in the presence of phosphoric acid with phenols[1] was investigated as a synthetic route to analogs of the Q coenzymes (I).



I II III

The readily available hydroquinone would be expected to give II ($R_1$, $R_2$, $R_3$ = H; $R_4$ = $CH_3$ ), which on oxidation would be converted to 3-methylcrotyl benzoquinone I($R_1$, $R_2$, $R_3$ = H; $R_4$ = $CH_3$ ), the parent quinone of the Q coenzymes.

Reaction in toluene at room temperature overnight gave a product A, soluble in toluene, which set to a waxlike solid. This crude material showed strong O-H infrared vibration frequencies, and was readily oxidized by o-chloranil, potassium dichromate in glacial acetic, or silver oxide in dry ether. The latter gave a copper-colored powder whose nuclear magnetic resonance (NMR) spectrum showed that the required quinone was only a minor product.[2]

The NMR spectrum of A, crystallized twice from aqueous ethanol, showed a trace of the hydroquinone, whereas the major product had no O-H frequencies. When A was refluxed with glacial acetic acid in the presence of a catalytic amount of sulfuric acid, it was converted to a compound

[1] Bader and Bean, J. Am. Chem. Soc. 80, 3073 (1958).

[2] The NMR spectra were determined in a Varian Model V-4311 spectrometer operating at 60 megacycles.

(m.p. 172° C, λ max at 302 mμ) whose reactions and spectra are in accord with the benzodipyran III. (The 2, 3 isomer is possible as well as the 2,5.) It was not oxidized by o-chloranil in dioxan at 90° for thirty minutes, and the infrared spectrum showed no O-H frequency. The NMR spectrum, shown in Fig. 8 has peaks at $\tau$ = 3.8 (aromatic), $\tau$ = 8.7 (methyl), triplets centered at $\tau$ = 7.3 (7.2, 7.3, 7.4) ($CH_2$ next to a benzene ring), and $\tau$ = 8.3 (8.2, 8.3, 8.4) ($CH_2$ isolated from electronegative substituents). The area ratios under the peaks are 1:2:2:6, in ascending order.

These experiments show that

(a) the condensation results in a product with the atoms arranged as required;

(b) dialkylation is preferred under the reaction conditions whereby the starting hydroquinone is practically insoluble in toluene (less than 0.1 g per 100 ml);

(c) the quantity of acid (0.8 mole of 85%) and the time and temperature (16 hr at room temperature) favor formation of chroman (structure III) over the hydroquinone.

Further experiments on this and a similar condensation using boron trifluoride, together with hydrogen-exchange reactions of the products, will be reported later.

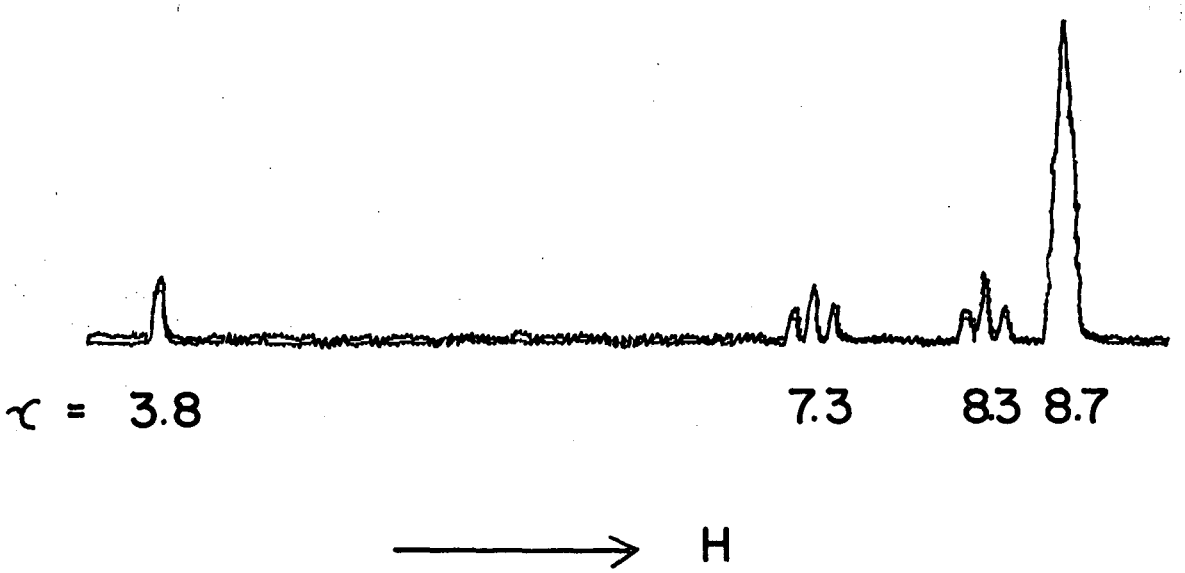



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Fig. 8. Nuclear-magnetic-resonances spectrum of the benzodipyran.

## III. π BONDING IN INORGANIC OXYACIDS

P. R. Hammond

Method of estimating the π bonding of the oxygen atoms of the inorganic oxyacids with the hetero-atom are based on measurements of bond distances[1] and on heats of formation.[2] In this way a number of phosphorus compounds have been studied.[3] Knowledge of π bonding in these compounds is of interest in interpreting their acid strength and alkylating properties,[4] and as the above procedures are not precise, an independent method of measurement is of value.

The ultraviolet absorption of benzene nuclei attached to phosphorus and other elements[5] has been used to detect conjugation in these systems. Hydroquinone groups in natural products have been identified by acetylation where reduction of the ultraviolet absorption to that of the parent hydrocarbon occurs.[6] The spectra of some phenyl esters were measured here to investigate the relationship between bonding and the absorption.

In all the esters studied the absorption band in the 265-mμ region was clearly visible, and although small variations could be seen, the vibrational fine structure was present in every case.

The band observed can be considered to arise from the electronic transitions in benzene and a series of its derivatives. The increase in intensity and wave length of this benzenoid absorption in the compounds phenol and anisole, which is attributed mainly to participation of the oxygen unshared electrons in the resonance hybrid, suggests that the spectrum is sensitive to this bonding. Hence, as a working hypothesis, it was assumed that any π bonding of the oxygen-to-hetero atom, tending to reduce this effect, would be seen in the uv spectrum.

### Results

The esters were purified by crystallization or fractional distillation. Spectra were obtained in 95% ethanol and were corrected (approx 3%) against runs of solvent alone. Absorption of the oxyacids in water was not detected at the concentrations used for the phenyl esters.

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[1] L. Pauling, J. Phys. Chem. 56, 361 (1952).

[2] L. Pauling, J. Am. Chem. Soc. 54, 3570 (1932).

[3] J. Van Wazer, J. Am. Chem. Soc. 78, 5709 (1956).

[4] M. Anbar, I. Dostrovsky, D. Samuel, and A. D. Yoffe, J. Chem. Soc. 3603 (1954).

[5] H. H. Jaffe, J. Chem. Phys. 22, 1430 (1954).

[6] A. W. Johnson, Sci. Progr. 45, 86 (1957).

The results are given in Table II, which shows the position of maximum absorption and the logarithm of the molar extinction coefficient per phenyl group, and in Fig. 9.

Table II. Ultraviolet absorption of some phenyl esters

| Compound | $\lambda_{max}$ (mμ) | $\text{Log}_{10} \frac{\epsilon}{N}$ |
|---|---|---|
| Phenol | 273 | 3.31 |
| Anisole | 272 | 3.30 |
| Triphenyl borate | 273 | 3.21 |
| Triphenyl phosphite | 268 | 2.79 |
| Triphenyl phosphate | 262 | 2.50 |
| Triphenyl thiophosphate | 263 | 2.48 |
| Phenyl stearate | 259 | 2.40 |
| Diphenyl succinate | 260 | 2.37 |

It may be seen that a hypsochromic shift and a hypochromic effect occur together. Arranging the compounds in decreasing absorption, or in decreasing $\lambda_{max}$, does give an approximate order in which increasing π bonding is to be expected.[3,7,8]

The influence of other factors (for example, the electronegativity difference between the oxygen and the hetero-atom) on the spectra together with a quantitative relationship, if one exists, will come from further study of these compounds.

[7] S.H. Bauer and J.Y. Beach, J. Am. Chem. Soc. 63, 1394 (1941).

[8] J.M. O'Gorman, W. Shand, and V. Schomaker, J. Am. Chem. Soc. 72, 4222, (1950).

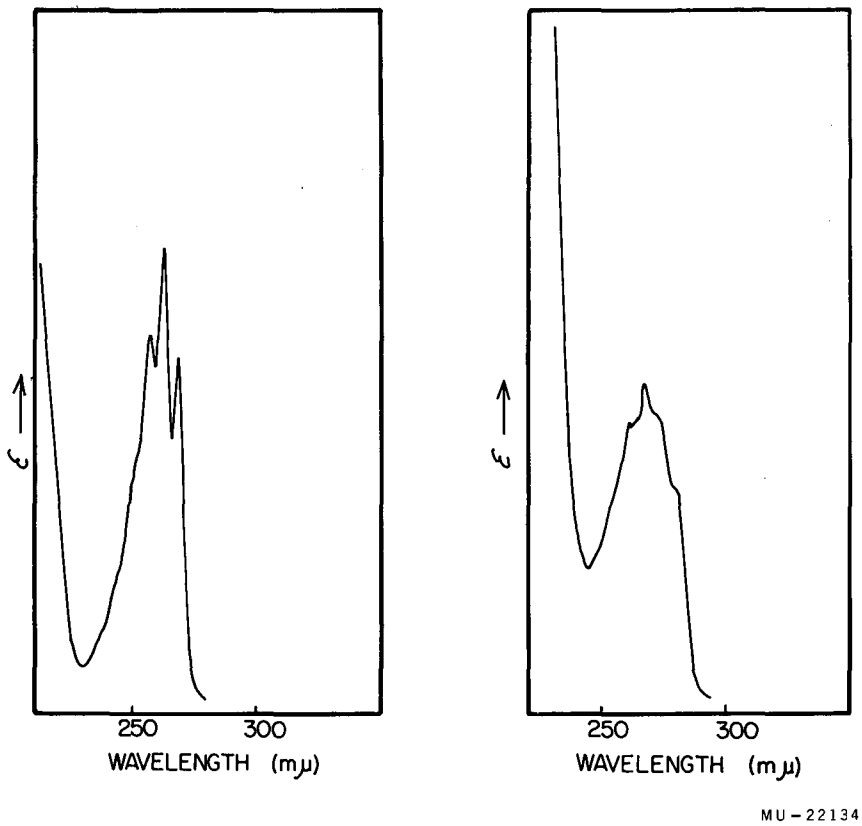


Fig. 9. Ultraviolet absorption of phenyl esters.
Left-hand side: triphenyl phosphate
Right-hand side: diphenyl succinate

# IV. THE DEAMINATION OF ADENINE, ADENOSINE, AND 3'-ADENYLIC ACID BY THE ACTION OF IONIZING RADIATION

Cyril Ponnamperuma, Richard M. Lemmon, and Edward L. Bennett

In continuing our study of the chemical effects of ionizing radiation on nucleotides,[1,2] we have found that adenine is deaminated to hypoxanthine. The adenine moiety of adenosine and 3'-adenylic acid appears to undergo a similar change.

Deamination of this type takes place quite readily when an aminopurine or an aminopyrimidine is hydrolyzed with acid.[3] Ten percent hydrochloric acid converts cytosine into uracil,[4] adenine into hypoxanthine, and guanine into xanthine.[5] Nitrous acid deaminates adenosine to inosine,[6] and guanosine to xanthosine.[7] Certain enzymes also bring about the same change. Intestinal deaminases, for example, convert adenine to hypoxanthine, adenosine to inosine, and adenylic acid to inosinic acid.[8]

Previous workers had concluded that conversion of aqueous solutions of aminopurines and pyrimidines to the hydroxy compounds did not take place under the influence of ionizing radiation.[9] Our studies, however, have shown a small but significant yield of hypoxanthine from adenine. With adenosine and adenylic acid, preliminary experiments show a similar effect. Further work is in progress to confirm these tentative results.

## Experimental Procedure

Adenine-2-$C^{14}$ (sp. act. 1.3 mC/mmole) was obtained from Isotope Specialities, Inc., Burbank, California. Adenosine-8-$C^{14}$ (sp. act. 5μC/mg) and adenylic acid, uniformly labeled with $C^{14}$ (sp. act. 0.5 μC/mg) were supplied by Schwarz Laboratories, Mount Vernon, New York.

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[1] Cyril Ponnamperuma, R.M. Lemmon, and E.L. Bennett, in Bio-Organic Chemistry Quarterly Report, UCRL-9135, March 1960, p. 27.

[2] C. Ponnamperuma, R.M. Lemmon, and F.H. Woeller, Bio-Organic Chemistry Quarterly Report, UCRL-9408, Sept. 1960, p. 9.

[3] H.S. Loring, J.L. Fairley, H.W. Bortner, and H.L. Seagram, J. Biol. Chem. 197, 809 (1952).

[4] E. Vischer and E. Chargaff, J. Biol. Chem. 176, 703 (1948).

[5] E. Vischer and E. Chargaff, J. Biol. Chem. 176, 715 (1948).

[6] J. M. Gulland and E.R. Holliday, J. Chem. Soc. 765 (1936).

[7] J.M. Gulland and T.F. Macrae, ibid, 662 (1933).

[8] M. Laskowski, in The Enzymes, James Sumner and Karl Myrback, Editors, Vol. I (Academic Press, Inc., New York, 1951), P. II, p. 956.

[9] G. Scholes and J. Weiss, J. Exptl. Cell Res. Suppl. 2, 219 (1952).

For the irradiations, 200 λ of a 0.1% solution of each compound was sealed in glass tubes under vacuum. To eliminate dissolved $O_2$, a steady stream of $N_2$ was bubbled through the solution for about 10 minutes. The 1500-curie $Co^{60}$ source was used for the irradiations. The calculation of dosage and the method of irradiation were the same as described in a previous report.[2] The dose range was from $10^6$ to $10^7$ rads at an intensity of $5 \times 10^6$ rads/hour.

The irradiated products were separated by paper chromatography using Whatman No. 4 paper with propanol-ammonia-water and butanol-propionic acid—water as solvents.[10] Twenty-five to 50 λ of each irradiated solution, together with 10 λ of a 0.1% solution of inactive hypoxanthine as carrier, were chromatographed together. The ultraviolet-light-absorbing areas were carefully marked out. Autoradiographs on x-ray film showed darkening of the film corresponding to the hypoxanthine area.

Liquid scintillation counting was used to measure the amount of hypoxanthine formed. The hypoxanthine was eluted with 0.1% formic acid solution. Almost all the activity came off the paper with the first drop of solvent. The second and third drops were also collected as a precautionary measure. The liquid scintillator solution was made up of toluene, 2000 ml; dioxane, 2000 ml; ethanol, 1200 ml; napthalene, 260 g; PPO, 26 g; POPOP, 0.5 g. The internal-standard method of counting was used; 100 λ of toluene-$C^{14}$, equivalent to 12,300 dpm, was used as the standard.

## Results

The appearance of hypoxanthine on the irradiations of 0.1% aqueous solutions of adenine, adenosine, and adenylic acid is shown in Table III.

The hypoxanthine spots from the chromatograms of adenine irradiated at $10^6$ rads were eluted and rechromatographed with different solvents: butanol saturated with water in one direction, and isobutyric acid—$NH_3$—EDTA[12] in the other. Almost all the radioactivity was concentrated on the hypoxanthine spot, confirming the formation of radioactive hypoxanthine. When the adenine was irradiated at $10^6$ rads in the presence of air, the amount of hypoxanthine formed decreased by about 50%. It is thus apparent that the $HO_2$ radical, formed in the presence of $O_2$ but not in its absence, interferes with the processes leading to the replacement of $-NH_2$ by -OH.

The biological importance of deamination of this type is perhaps far reaching. If it seems reasonable to extrapolate from the results at a high dose level to those at a low level, we may have a possible clue to the origin of a radiation mutation.

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[10] Edward L. Bennett, Biochim. Biophys. Acta 11, 487 (1953).

[11] J. Smith and R. Markham, Biochem. J. 45, 294 (1949).

[12] H. Krebs and R. Hems, Biochim. Biophys. Acta 12, 173 (1953).

Table III. Formation of hypoxanthine by irradiation of adenine-2-$C^{14}$, adenosine-8-$C^{14}$, and adenylic acid-$C^{14}$

| Compound irradiated | Initial activity (dpm) | Dose (rads) | Activity of hypoxanthine (dpm) | Yield of hypoxanthine (%) |
|---|---|---|---|---|
| Adenine | $4.1 \times 10^5$ | 0 | 330 | 0.08 |
| | | $10^6$ | 3602 | 0.88 |
| | | $2 \times 10^6$ | 3807 | 0.93 |
| | | $5 \times 10^6$ | 7840 | 1.91 |
| | | $10 \times 10^6$ | 3897 | 0.95 |
| Adenosine-8-$C^{14}$ | $2.5 \times 10^5$ | 0 | 340 | 0.13 |
| | | $10^6$ | 1349 | 0.53 |
| 3'-adenylic acid | $5.0 \times 10^4$ | 0 | 104 | 0.22 |
| | | $10^6$ | 422 | 0.84 |
| | | $5 \times 10^6$ | 280 | 0.56 |
| | | $10 \times 10^6$ | 385 | 0.77 |

A comparison may be made with deamination by nitrous acid. When the RNA of tobacco mosaic virus is heated with $HNO_2$, the amino bases are converted to the hydroxy bases without splitting of the nucleotide chain. Deamination of a large number of nucleotides may result in the inactivation of the molecule.[13] More gentle treatment by controlled action of nitrous acid gave a maximum of mutations when an average of one deamination per molecule resulted.[14,15] Similarly, whereas a large dose of radiation may destroy the entire molecule, a small dose may give rise to the change that brings about a mutation.

Hypoxanthine, xanthine, and uracil normally exist in the keto form.[16] This has been demonstrated experimentally for hypoxanthine.[17] In the Watson and Crick structure of DNA,[18] adenine (A) is H-bonded to thymine (T), while guanine (G) is linked to cytosine (C). Deamination of adenine to hypoxanthine, and of cytosine to uracil, would not permit the H bonding between A and T, and G and C. From theoretical considerations Lavalle[19] has pointed out that deamination would result in the replacement of an A-T pair of bases by a G-C pair. The net result of deamination may thus be a change in the molecular code of heredity.

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[13] H. Schuster and G. Schramm, Z. Naturforsch. 13 b, 697 (1958).

[14] A. Gierer and K. W. Mundry, Nature 182, 1457 (1958).

[15] K. W. Mundry and A. Gierer, Z. Vererbungsforsch. 89, 614 (1958).

[16] D. O. Jordon, in The Nucleic Acids, E. Chargaff and J. N. Davidson, Editors, Vol. I, p. 447 (Academic Press, Inc., New York, 1955).

[17] S. F. Mason in Ciba Foundation Sympossium on Chemistry and Biology of Purines (Little Brown and Co., Boston, 1957), p. 60.

[18] J. D. Watson and F. H. C. Crick, Nature 171, 737 (1953).

[19] R. Lavalle, Compt. rend. 250 1134 (1960).

## V. IRRADIATION OF METHANE, AMMONIA, HYDROGEN, AND WATER

Christof Palm and Melvin Calvin

Chemical evolution has been suggested as the process preceding biological evolution.[1] It has been of interest, therefore, to investigate the effect of different forms of energy on mixtures of simple gases containing carbon, hydrogen, nitrogen, and oxygen. Although little is known about the conditions on the primitive earth, speculation on possible pathways of chemical evolution from the simplest carbonaceous compound (methane) to the highest ones of organized life is an incentive for investigations of the effects of radiation on mixtures of simple organic and inorganic compounds.

In 1951 Calvin et al.[2] showed the formation of organic acids and formaldehyde as a result of α irradiation of $CO_2$ in water. Miller and others[3] have shown that amino acids and carboxylic acid are produced by the action of an electric discharge on mixtures of $CH_4$, $NH_3$, $H_2$, and $H_2O$. The number of identified compounds formed by irradiation of simple gases is still very small. They account for only a small fraction of the total material formed.

Since they are essential constituents of nucleic acids, heterocyclic bases are assumed to be necessary for evolution. Until now, no heterocyclic base has been detected as a result of irradiation of simple gases. However, Kimball and Oro[4] have reported that the two bases, 5-aminoimidazole carboxylic acid and adenine, are formed on heating an aqueous solution of ammonium cyanide. Since cyanide ions are produced by the action of electric discharge on simple gas mixtures, and since they are also produced by the action of high-energy irradiation (see below), it should be possible to find heterocyclic bases after irradiation of mixtures of simple gases.

We are investigating the influence of radiation of mixtures of simple hydrocarbons (primarily $CH_4$, but also $C_2H_4$ and $C_2H_6$), $NH_3$, $H_2$, and $H_2O$. Irradiations with γ rays from a $Co^{60}$ source were restricted to small volumes and doses. Therefore we used 5-Mev electrons from a linear accelerator as the energy source. The dose rate can be made much higher within a short time (about $10^9$ rep/2 hr), and larger volumes can be irradiated. It is assumed that the kinds of reactions caused by electrons are essentially the same as those induced by γ rays.

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[1] M. Calvin, Am. Scientist 44, 248 (1956).

[2] W.M. Garrison, D.C. Morrison, J.G. Hamilton, A.A. Benson, and M. Calvin, Science 114, 416 (1951).

[3] S.L. Miller, J. Am. Chem. Soc. 77, 2351 (1955); S.L. Miller and H.C. Urey, Science 130, 245 (1959).

[4] J. Oro and A.P. Kimball, Abstr. 138th Meeting Am. Chem. Soc. 1960, p. 25c.

When a mixture of gases containing $CH_4$ is irradiated with the linear accelerator, approximately 10 mg of nonvolatile compounds is obtained. Since many compounds are present in this mixture, the amount of material obtained is not sufficient for general chemical identification methods.

The sensitivity of isolation and identification procedures can be increased manyfold by the use of radioactive techniques; we have used $C^{14}H_4$ in these experiments.

In some experiments, possible catalysts for formation of higher-molecular-weight organic compounds were added. As a basis for selection of possible catalysts, we chose compounds of types that are abundant in our solar system or which are similar to compounds of known importance in life today. Olivine, a magnesium-iron silicate, $(Mg, Fe)_2SiO_4$, is abundant not only on earth, but also in many meteorites. It may resemble possible surfaces on primitive worlds. Phosphorus is found in all known living systems. Polyphosphates, as the kurrol salt $(NaPO_3)_x$, have a linear structure and may be evolutionary precursors of organophosphorus polymers as polynucleotides. To avoid $Na^+$, the exchanged ammonium salt, $(NH_4PO_3)_x$, was used. In one experiment phosphines were added as the phosphorus-containing compound in accordance with the assumed reducing conditions in primitive worlds.

The gaseous mixtures were irradiated in a sealed glass tube or flask with a thin window through which the electrons entered the vessel (Fig. 10a). A modification (Fig. 10b) was used in a later experiment (M22). The bottom well can be heated to reflux the water and thus increase the partial pressure of the water. At the same time, the condensing water washes down and out of actual radiation the nonvolatile compounds formed during the experiment.

Precautions were taken to avoid contaminations of chemical or biological origin. The reaction tube was cleaned with sulfuric acid—dichromate, and then water was boiled in it for 30 minutes. After the tube was emptied, it was attached to a vacuum line, heated with a Bunsen burner, and evacuated to less than 100 μ. The catalysts for the reactions were added at this time.

The evacuated flask was then cooled with dry ice, and 20 ml of previously boiled water condensed into it. If phosphines were used, they were evolved from $Ca_3P_2$ and added together with the water at this time. The flask was briefly re-evacuated, and $C^{14}H_4$ was introduced from a storage flask. The hydrocarbon ($CH_4$, $C_2H_4$, or $C_2H_6$), $H_2$, and $NH_3$ were then added. All gases were analytical grade and obtained from cylinders. Before addition of each gas, the gas-transfer system and the manifold were thoroughly swept and evacuated to avoid contamination by $O_2$. The $NH_3$ was added rapidly after the dry ice bath was removed.

The reaction tube was then sealed and irradiated. The radiations were usually done at about 30°; recently, the temperature has been raised to 80 to 90° in order to reflux the water at the reduced pressure of the system. The radiation was carried out with the 5-Mev electrons for 2 hr.

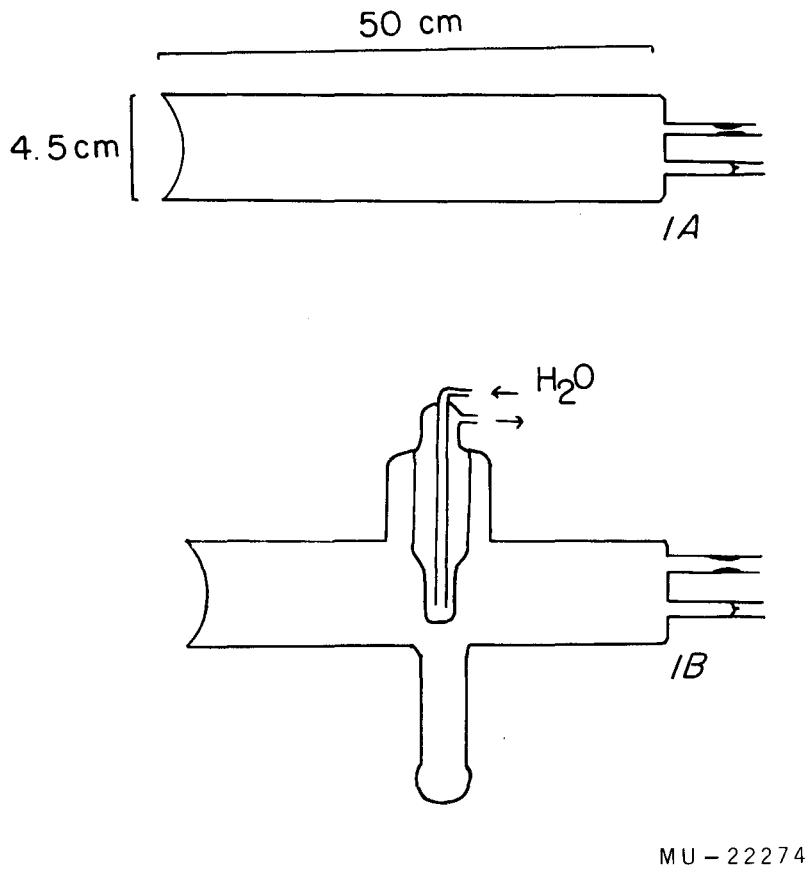



Fig. 10. Flasks for electron irradiations.

The accelerator delivered 30 pulses/sec, each pulse lasting 6 μsec. The integrated dose rate was 9 μamp. Assuming a Feather relationship for the energy loss per electron, it can be calculated that the total dose was approximately $10^9$ rads. No attempt was made to evaluate the exact dose with a chemical dosimeter.

After irradiation, the reaction flask is reattached to the vacuum line and opened, and the gases are pumped to a storage flask. The aqueous phase was analyzed. The colorless or occasionally slightly yellow solution had a putrefactive odor which was particularly unpleasant when $C_2$ hydrocarbons were used. The pH, depending upon the $NH_3$ content, was higher than 11. An aliquot portion of the aqueous phase was removed and evaporated to dryness in vacuo. This was done in an inverted U-shaped tube with one side cooled in liquid $N_2$ in order not to lose volatile material. The $C^{14}$ content of the original solution and the condensate was determined. The total activity in the original solution was assumed to represent conversion of $C^{14}H_4$ to higher-molecular-weight molecules. Any solubility of $C^{14}H_4$ in the aqueous phase was disregarded.

The difference between the total activity and that found in the condensate was assumed to represent the nonvolatile compounds.

Experimental conditions and a summary of the fixation and distribution of $C^{14}$ in the several fractions are presented in Table IV. The compounds added in addition to $CH_4$, $H_2$, $NH_3$, and $H_2O$ are also listed. Not all the experiments are strictly comparable, inasmuch as conditions have been modified during the course of these experiments in an effort to achieve optimum conditions for the conversion of $C^{14}H_4$ to other organic compounds.

After the distribution of $C^{14}$ between volatile and nonvolatile compounds had been determined, the residue was redissolved in water and aliquot portions were chromatographed on Whatman No. 1 or No. 4 filter paper. Two-dimensional chromatograms were generally made, using phenol-water and then butanol—propionic acid—water. The distribution of the $C^{14}$-labeled compounds was determined by radioautography.

As only a small fraction of $C^{14}H_4$ had been converted to nonvolatile organic compounds in the first experiments, little was learned from the chromatograms made from them. In later experiments, larger amounts of $C^{14}H_4$ were used and it was possible to put about $5 \times 10^5$ dpm on the origin of the chromatogram.

About 20 spots were detected on the first chromatogram; an example is shown in Fig. 11. This substantiates the opinion that numerous compounds can result from the irradiation of mixtures of simple gases. However, the problem of identifying such a large number of compounds present in such extremely small amounts is a difficult one. It should be emphasized that in addition to the well-defined separated spots, there is a background of radioactivity over most of the chromatogram. This activity increases with $R_f$. Thus, besides polymerized material, many more compounds present in even smaller amounts are indicated.

Table IV. Distribution of $C^{14}$ after the irradiation of $C^{14}H_4$, $NH_3$, $H_2$, and $H_2O$ under various conditions

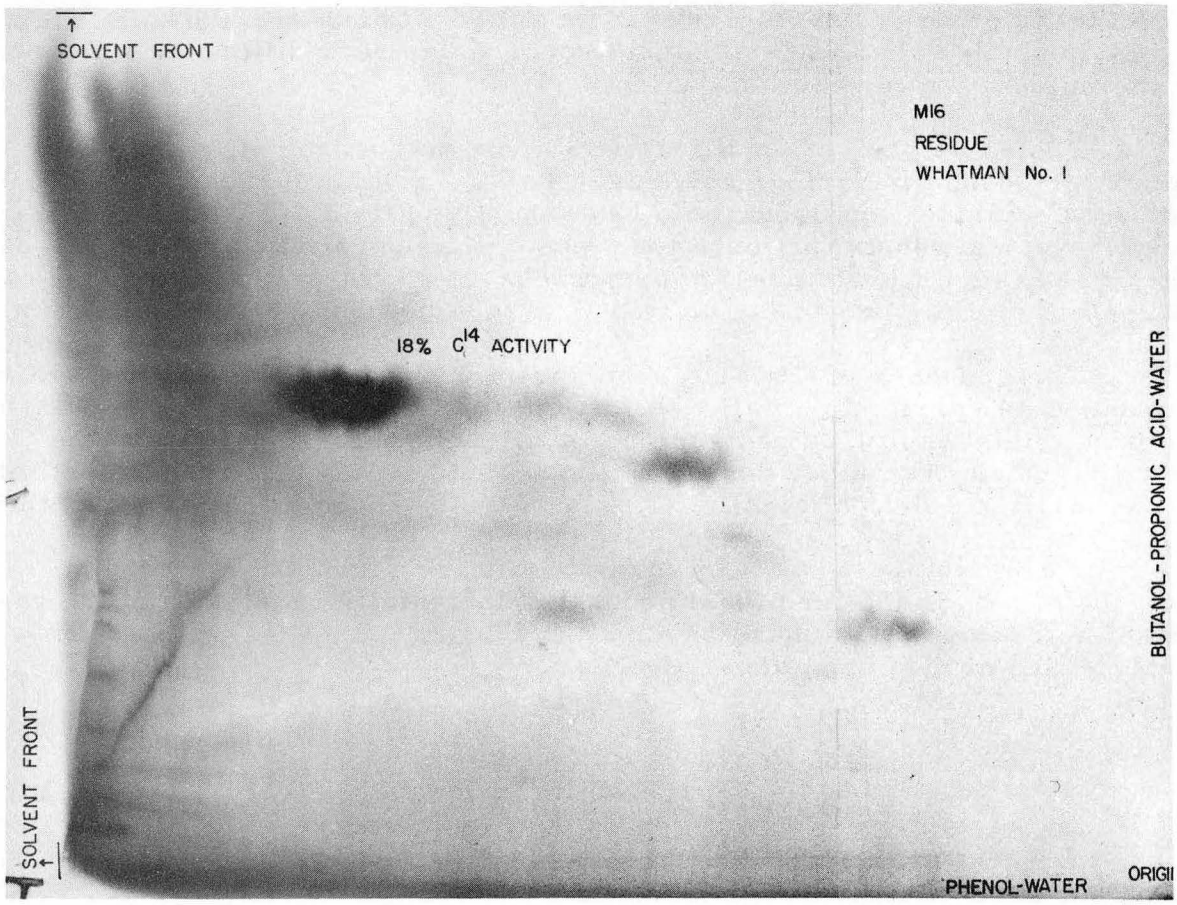
| Expt. | $P_{CH_4}$ [a] (cm) | $CH_4$ (mg) | $C^{14}$ in $CH_4$ inc (mC) | $P_{H_2}$ (cm) | $P_{NH_3}$ (cm) | Time of irradiation (hr) | $C^{14}$ in liq. phase μC | $C^{14}$ in condensate, μC | Non-volatile $C^{14}$, μC (by diff.) | % Conversion of $C^{14}$ activity [b] | Residue mg. [c] | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M11 | 45.65 | 256 | 0.104 | 2.5 | 27 | 2 | 2.26 | 0.67 | 1.59 | 2.16 | 12.5 | 20 ml $H_2O$, 2.28 g olivine |
| M12 | 35.1 | 216 | 0.132 | 9.0 | 30 | 6 | 8.88 | 1.89 | 6.99 | 6.72 | 24 | 20 ml $H_2O$, 3.1 g olivine |
| M14 | 35.7 | 220 | 0.181 | 6.0 | 30 | 2 | 2.74 | 1.6 | 1.14 | 1.51 | 23.2 | 20 ml $H_2O$, 3.1 g olivine |
| M16 | 27 | 166 | 1.4 | 5.0 | 20 | 2 | 78 | 27.2 | 50.8 | 5.55 | 17.7 | 17 ml $H_2O$, 3 g olivine, 20 cm Hg pressure of $N_2$, due to air infiltration in $^{14}CH_4$, but $O_2$ was removed. |
| M18 | 36.8 | 226 | 0.345 | 5.5 | 30 | 2 | 6.85 | 1.4 | 5.45 | 1.98 | | 20 ml $H_2O$, 1 g $(NH_4PO_3)_x$ |
| M19 | 36.8 | 226 | 0.445 | 5 | 30 | 2 | 7.82 | 4.85 | 2.97 | 1.75 | 25.6 | 20 ml $H_2O$, no additives |
| M20 | 42 | 270 | 0.93 | 5 | 30 | 2 | 15.3 | | | 1.65 | | 20 ml $H_2O$, 1 g $(NH_4PO_3)_x$ |
| M21 | 24 | | 0.93 | | 20 | 2 | 46.8 | 23.4 | 23.4 | 5.04 | 16.4 | 20 ml $H_2O$ |
| M22 | 30.6 [d] | 326 | 1.16 | 0.20 | 20 | 2 | 33 | 4.5 | 28.5 | 2.84 | | 13 ml $H_2O$, 100 mg $(NH_4PO_3)_x$ $PH_3$ evolved from 0.6 g $Ca_3P_2$ |

[a] Total volume of flask was 657 ml.

[b] % of activity in $C^{14}H_4$ converted in activity in liquid phase at end of irradiation.

[c] The divergence in the conversion rate as calculated from $C^{14}$ specific activity in the starting methane and found in the nonvolatile residue must be due to dissolved impurities, either from the glass or from the olivine.

[d] 810 ml flask.




ZN-2757

Fig. 11. Chromatogram of crude residue

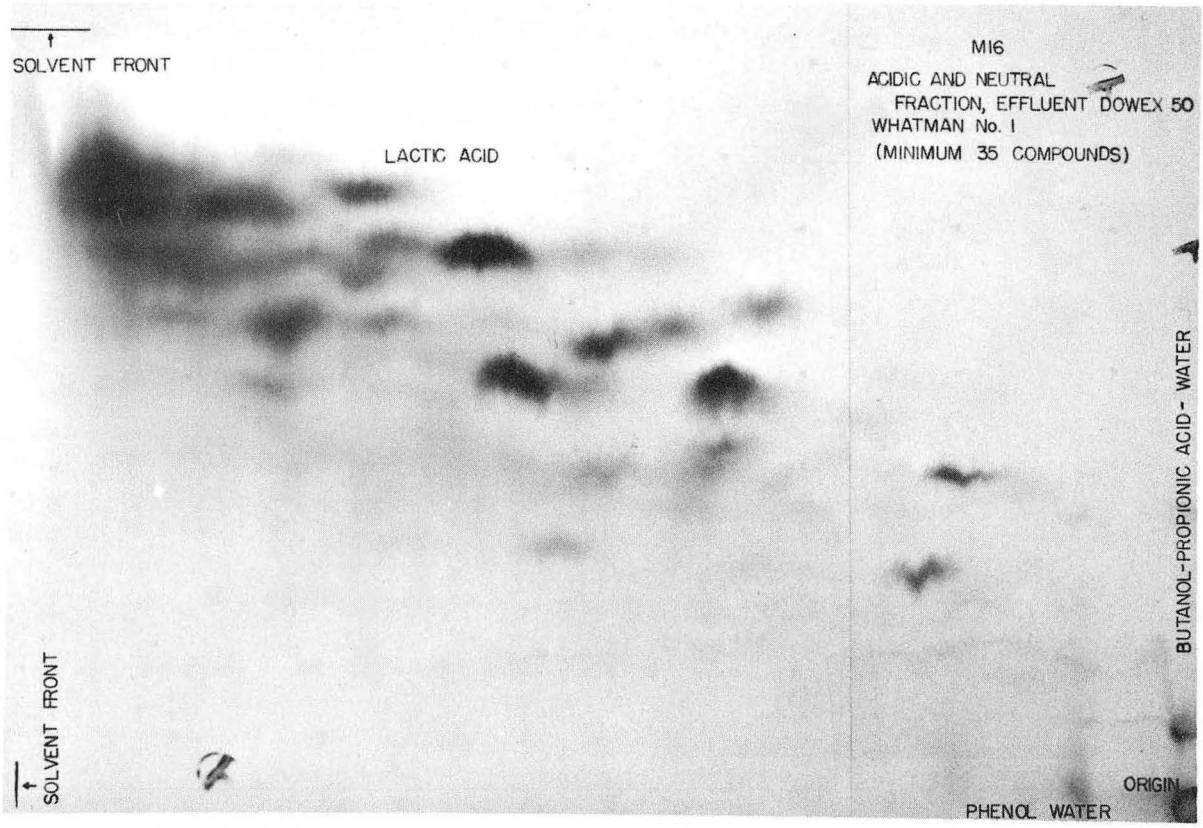
The numerous nonvolatile compounds obtained after irradiation may be separated into three fractions by means of ion-exchange columns. Passage through a Dowex-50 $H^+$ column and elution with 2 N HCl yields bases and amino acids.

Organic acids in effluent from the above column can be retained on a Dowex-1 $CO_3^=$ column, and neutral compounds will be in the effluent from this column. The radioautographs from such fractions are shown in Figs. 12, 13, and 14. The yields of each compound are very different, as shown by the intensity of the spot on the film.

The spots can be identified by elution and cochromatography with known compounds in various solvents. To date glycine (present in small amounts), alanine, and lactic acid have been identified. The major compound, which accounts for more than 18% of the nonvolatile compounds, has not yet been identified. The compound behaves like a base, but attempts to estimate the pK by two-phase distribution have not been successful so far.

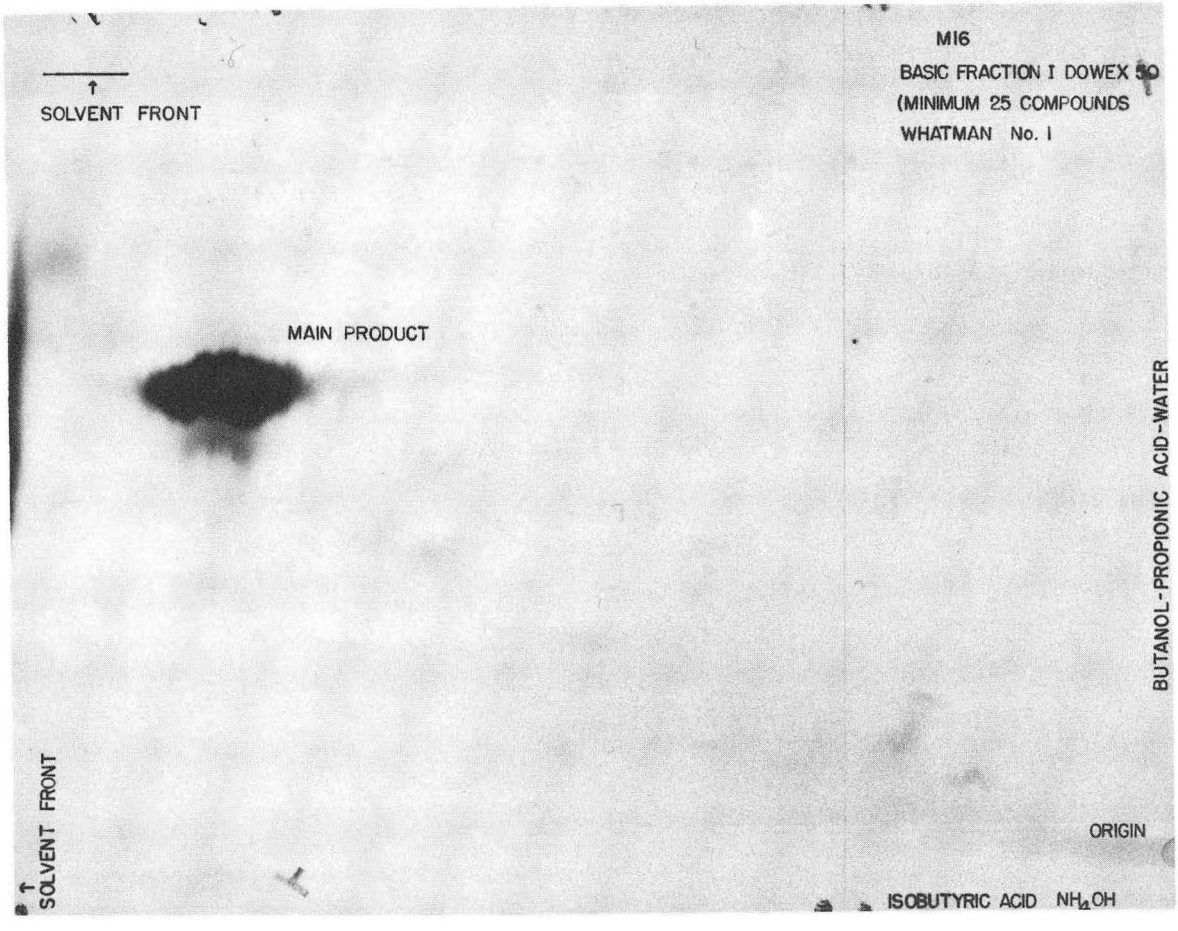
The presence of $CN^-$ has been shown in the reaction mixture. KCN was added as carrier to an aliquot portion, and precipitated as AgCN. After the precipitate was twice redissolved in $NH_4OH$ and reprecipitated, the specific activity remained constant. It amounts to 0.45% of the $C^{14}$ activity in the reaction solution (M22). No urea could be found by the same method.

As our interest has been primarily in the nonvolatile or higher-molecular-weight and functional products, informative analyses on the gas phase, either by vapor-phase chromatography or with a mass spectrometer, have not yet been done.



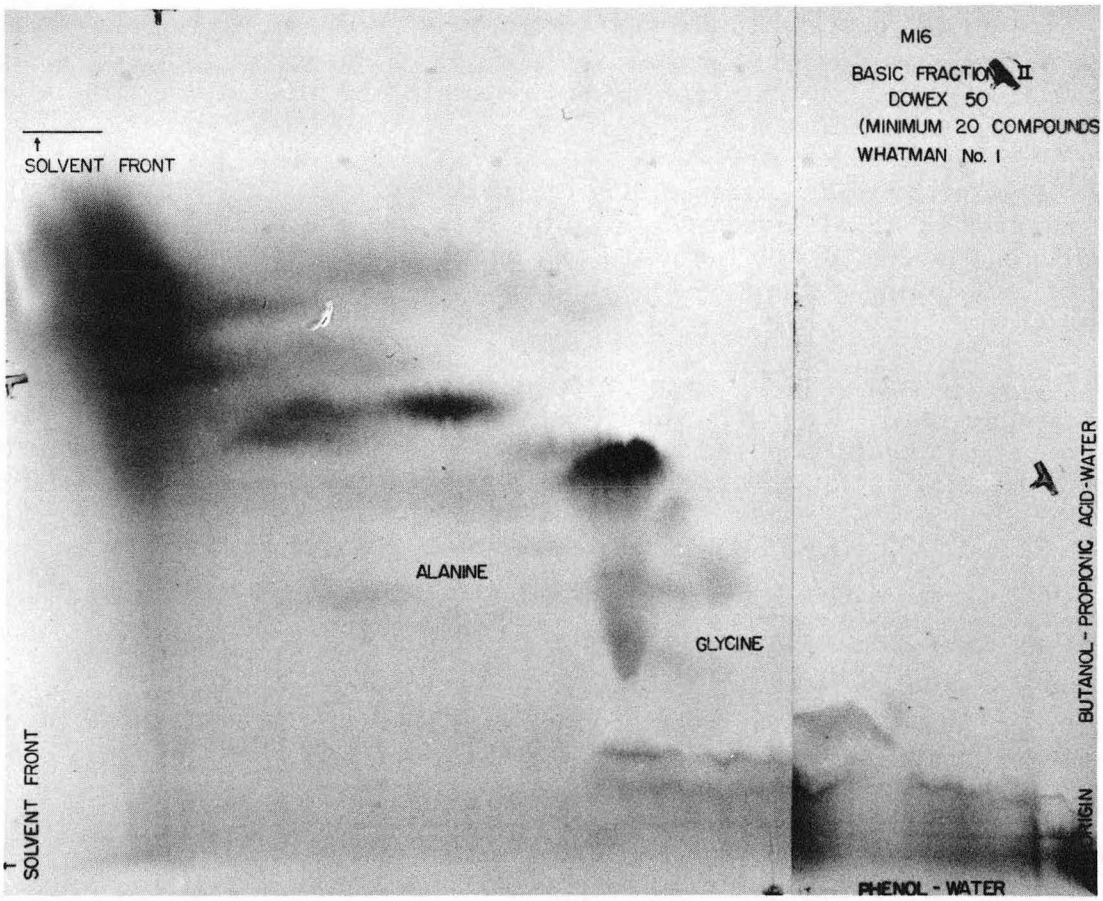

ZN-2758

Fig. 12. Chromatogram of acid and neutral fractions.




ZN-2759

Fig. 13. Chromatogram of Basic Fraction I.
a = 621; b = 619




ZN-2760

Fig. 14. Chromatogram of Basic Fraction II.

# VI. CRYSTAL VIOLET AS A REACTION PRODUCT OF THE CHARGE-TRANSFER COMPLEX OF DIMETHYL ANILINE AND o-CHLORANIL

Gerrit Engelsma and John W. Eastman

It was previously shown that on solution of o-chloranil in dimethyl aniline a charge-transfer complex is formed.[1] This complex decays slowly. The reaction mixture turns from blue to violet, and after some time a violet compound precipitates.

The infrared and visible spectra of this product are identical with these spectra of crystal violet. Simultaneous chromatography on paper, both with a mixture of ethyl acetate and butyl alcohol and with chloroform alone as developing solvent, has confirmed that our reaction product is crystal violet.

The crystal violet precipitates mainly in the form of a chloranil hydroquinone salt. When the reaction takes place in the presence of air the product is crystalline and is nearly 100% crystal violet salt. The ratio of crystal violet cation to hydroquinone that best fits the analysis is 1 to 1.

Found: C, 63.52%; H, 5.87; N, 8.58; Cl, 17.98

Calc. for $\left[(CH_3)_2NC_6H_4\right]_3C \cdot C_6Cl_4O_2H$ : C, 60.10; H, 5.08; N, 6,79; Cl, 22.8.

Carried out under nitrogen, the reaction leads to a product with a glassy appearance. It consists of about 30% crystal violet, the remainder being one or more colorless compounds.

In one experiment, 7 millimoles of o-chloranil was dissolved in 50 ml of dimethylaniline and kept at 55° for 24 hours in an open bottle. The change of absorption at 780 mμ and 610 mμ as a function of time was determined. The disappearance of the dimethyl aniline—o-chloranil complex (determined from the change in absorption at 780 mμ) is shown by the solid line in Fig. 15. The formation of the crystal violet is shown by the dotted line. This was determined by the difference between the measured absorption at 610 mμ and calculated absorption of the dimethyl aniline—chloranil complex at this wave length.

After about 8 hr the crystal violet started to precipitate from the solution. In 24 hr 200 mg crystal violet had been formed. This corresponds to 14% of the o-chloranil. After the reaction mixture had been kept at room temperature for 6 days more, the yield had increased to 30%.

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[1] J. W. Eastman and M. Calvin, in Bio-Organic Chemistry Quarterly Report, UCRL-9041, Dec. 1959, p. 40.

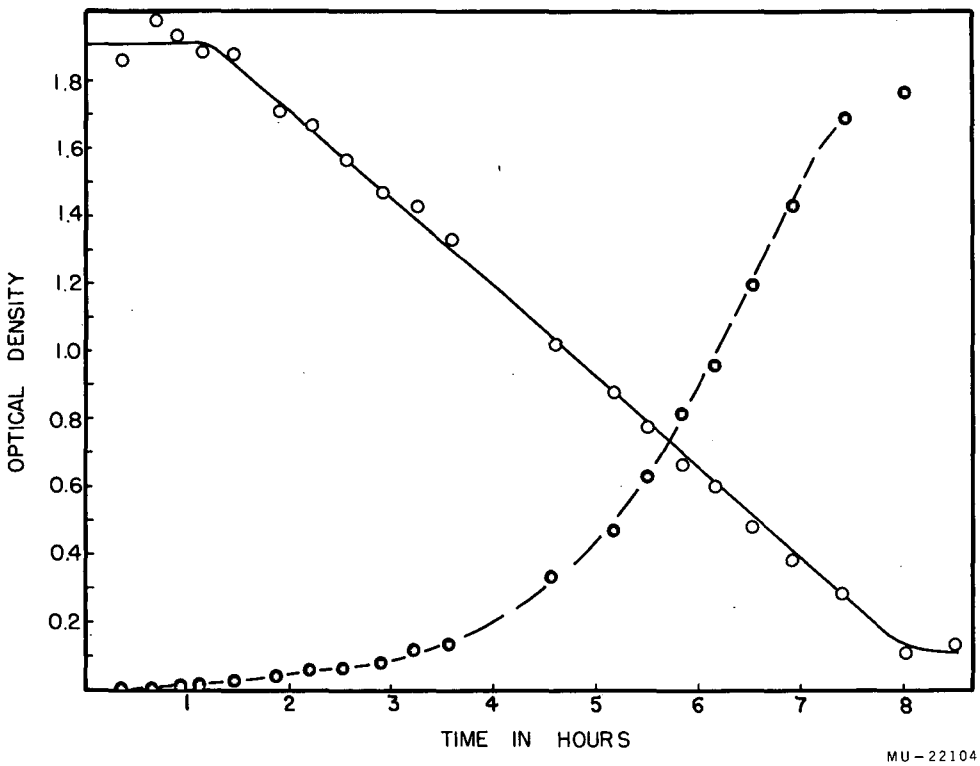


Fig. 15. Decay of the dimethylaniline-o-chloranil complex and formation of crystal violet as functions of time.

# VII. ON THE CORRELATION OF THE PHOTOINDUCED ESR SIGNAL IN SPINACH CHLOROPLASTS WITH THE PRESENCE OF PIGMENTS IMPORTANT TO HILL REACTIVITY

Gaylord Androes and Mary C. Singleton

Electron spin resonance (ESR) techniques have been used to observe a light-induced free radical in photosynthetic systems. Thus far the radical's position and function in the photosynthetic cycle have not been established. This report concerns another approach in trying to characterize this radical.

Recently Bishop has shown that a benzoquinone, $Q_{255}$, is important in the Hill reaction.[1] An extraction procedure which removes $Q_{255}$ (as well as other pigments) from spinach chloroplasts also reduces the Hill reactivity to approximately 35% of its original value. Although several substances, when added again to the extracted chloroplast residue, are effective in partially restoring Hill reactivity, $Q_{255}$ restores it completely.

Model systems proposed for chloroplasts have linked chlorophyll and quinone-type molecules.[2] This suggested that any connection the quinone or Hill reactivity has with the light-induced free radical could be discovered by following the ESR signal through Bishop's extraction and recombination procedure.

The experiment is shown diagrammatically in Fig. 16. Spinach chloroplasts were extracted three or four times under Bishop's conditions. In recombining the extract with the residue, about half the residue was combined with all the extracts from a given extraction. The residue was placed in a funnel with a sintered glass filter. The concentrated extract solution was added dropwise while nitrogen gas was passed through the filter from the bottom, thus providing a nitrogen atmosphere and evaporating the excess petroleum ether from the solution. This is essentially Bishop's procedure, except that his extract solution was first chromatographed. He then added again, in excess, the individual components of the extract as well as other interesting compounds.

The ESR experiments were run with the sample material plus water painted on a rod, in air and at room temperature. The variation in ESR line widths and positions was negligible compared with other experimental uncertainties. The photosignals were induced with 710-mμ light of about $10^{15}$ quanta/sec intensity. Several types of experiments were carried out. The equilibrium dark- and photosignals were determined at the points in Fig. 16 designated as ESR #1, #2, #3, #4. The decay of the photosignal as a function of time after turning off the light was determined at the same points. In addition, in two instances a Hill-reaction experiment was carried out in parallel with the ESR experiment. This was done to determine if we were actually repeating Bishop's results in our experiments. For dye substance in the Hill reaction we used the sodium salt of 2, 3, 6-trichlorophenol-indolphenol.

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[1] N.I. Bishop, Proc. Natl. Acad. Sci. U.S. 45, 1696 (1959).

[2] G. Tollin, Radiation Research, Suppl. 2, 387 (1960).

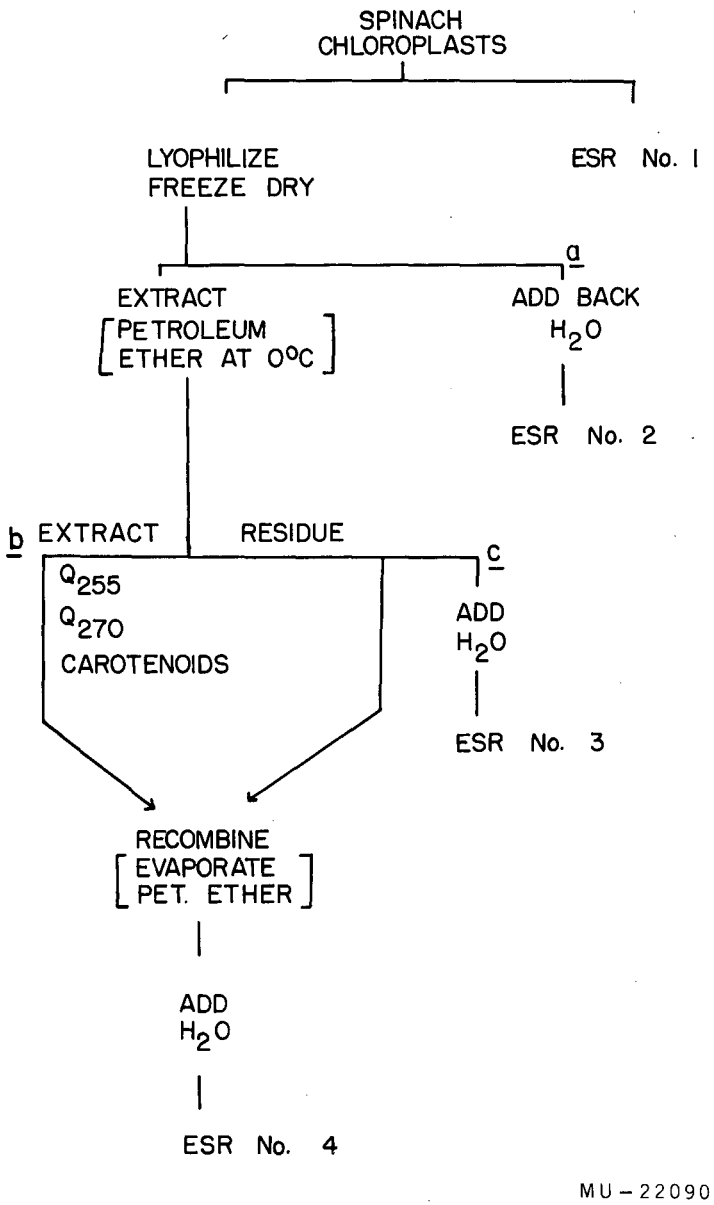



Fig. 16. Diagram of ESR Experiment.

Besides the ESR runs, #1 through #4, the samples were investigated at points a, b, and c, Fig. 16, for free-radical content. For point "a" the freeze-dried material was placed on a quartz tube in air. No signals either light or dark, were observed.

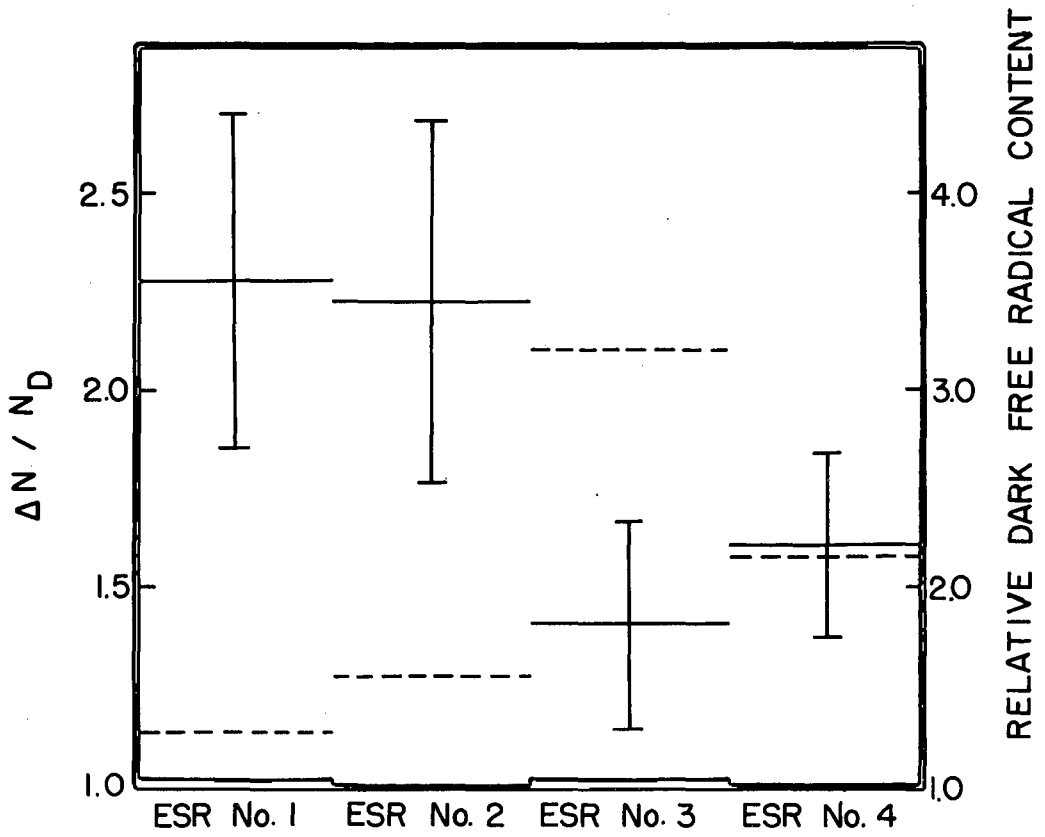
This experiment differed from the others in having less material in the ESR cavity (by a factor of about two). At point "b" no dark or light signals were observed. At point "c" a dark signal of approximately the same amplitude as in ESR #3 was observed. No light-induced signal was observed at this point.

The results of the experiments (10 repetitions) are shown in Figs. 17 and 18. For reasons given below, we have plotted on the left in Fig. 17 the fractional photoinduced change in free-radical content of the sample, i.e., $\Delta N/N_0$ = (Number of spins in light – Number of spins in dark)/(Number of spins in dark) for the various ESR stages of Fig. 16. The fluctuations in our results are large, and are almost certainly not statistical. More probably they are due to the variation of uncontrolled parameters which affect the equilibrium number of spins. However, to show the variation obtained, the fluctuations are treated as statistical and a probable error of the mean is calculated. These errors are designated by the vertical lines in Fig. 17. As a reference the relative number of free radicals existing in the dark is plotted on the right in Fig. 17. The fluctuations observed in this parameter are approximately the same as for $\Delta N/N_0$. In parallel with the ESR part of the experiment, the Hill reactivity was followed through on two occasions; once qualitatively, and once quantitatively. The quantified results of this reaction are plotted in Fig. 18. The qualitative experiment agreed with the results shown in Fig. 18.

In four of the experiments the decay of the ESR photosignal was followed. For ESR #1 there was one decay time of approximately 1/2 hr. Freeze drying with readdition of water (ESR #2) changed this pattern. In each case a short decay time of approximately 10 to 20 sec was induced, in addition to the long decay time of approximately 1/2 hr. The decay at #3 was erratic. On two occasions the decay pattern was that of ESR #1, and on the other two it was that of ESR #2. Approximately the same times were involved. The decay of ESR #4 followed the scheme of ESR #2 (with approximately the same times)except on one occasion, when it decayed with one time constant of several minutes.

It is well known that broken bonds and structural vacancies often lead to observable free-radical signals. Thus, it might be expected that extracting a material would provide more sites for trapping radical species. In particular, if one is observing the equilibrium number of free radicals one would expect this number to increase with the number of trapping sites. The amplitude of the dark ESR signal is an indication of the number of trapping sites in the sample. For this reason we have plotted the light effect in Fig. 17 as the fractional photoinduced increase in free-radical content over that existing in the dark.

It is difficult to compare our results directly with Bishop's because he does not report the effect of adding back the unchromatographed extract




MU-22091

Fig. 17. ———— Fractional photoinduced change in sample free-radical content

---------- Relative dark free-radical content of sample.

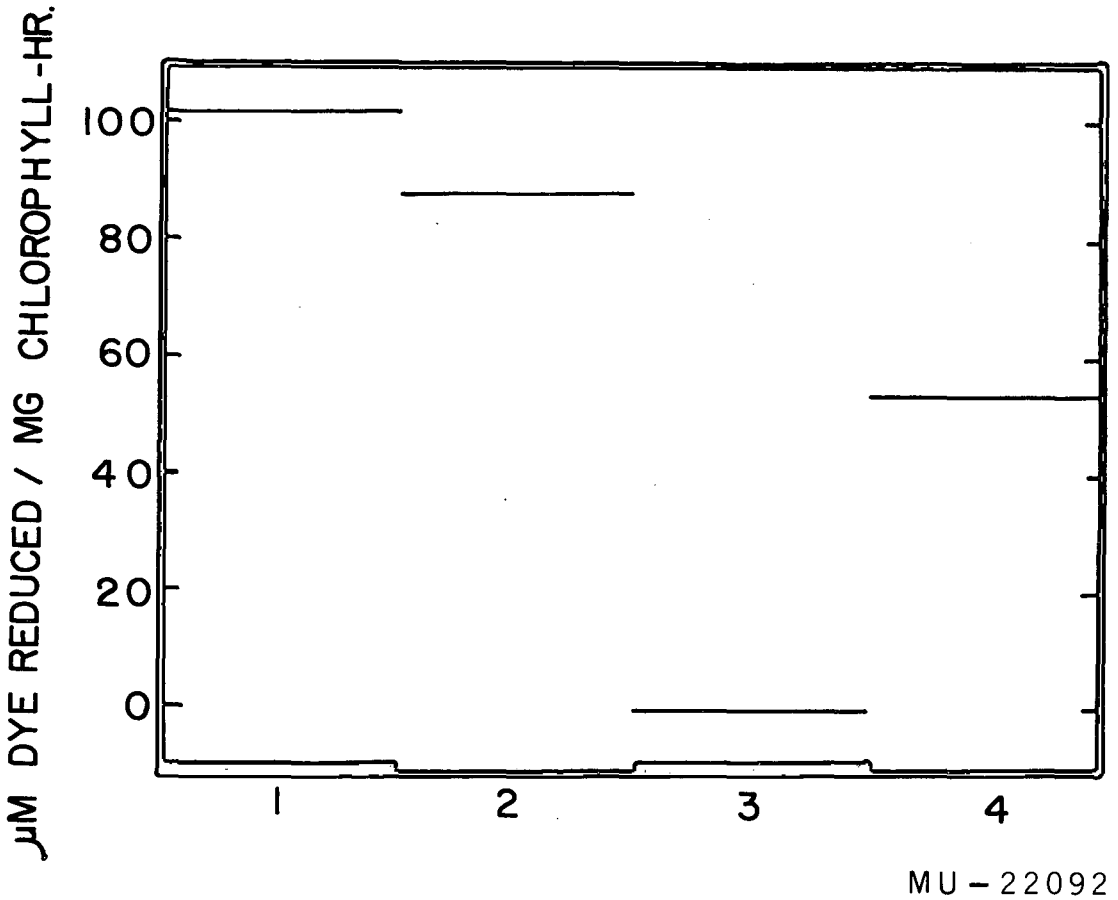



Fig. 18. Hill reactivity at the four ESR stages shown in Fig. 17.

to the chloroplast residue. However, one can compare our ESR results (Fig. 17) with our Hill reactivity results (Fig. 18). It is seen that $\Delta N/N_0$ and the Hill reactivity follow the same general trend, whereas the relative number of radicals existing in the dark goes as the inverse of the Hill reactivity. The equilibrium type of ESR experiment reflects the loss of Hill reactivity as a loss of structure in the chloroplast. That the structure was altered is evidenced by the amplitude of the dark signal, and, more positively, by the induction of different photosignal decay times at the various stages of the experiment. The large fluctuations in our results prevent us from drawing definite conclusions. The reason for the large fluctuations is not understood. It is seen that they are as large in the freshly prepared chloroplasts (ESR #1) as in the subsequent stages of the experiment. It would appear that growth conditions as well as the first stages of preparation of the chloroplasts are important in determining $\Delta N/N_0$ .

This experiment has failed to establish a positive connection between the observed free radical and the extracted pigments or the Hill reactivity as such. There are relatively large light effects in the ESR #3 stage where the Hill reactivity is near zero. This exposes one flaw in the experiment: the use of equilibrium ESR amplitudes. Thus, even if the light effect is small at stage #3, there are many more traps available, so that the resulting equilibrium ESR amplitude may be quite large. A quantum-efficiency-type experiment is suggested, but at the time this experiment was done lack of instrumental sensitivity prohibited such an experiment. Instrumental sensitivity has since been increased, and would probably be sufficient, except for the large sample fluctuations. The experiment will not be pursued until the reasons for these fluctuations are better understood.

## VIII. THE FIXATION OF CARBON-14 IN ISOLATED CHLOROPLASTS

Ulrich Heber

Until now the highest fixation rates obtained in isolated chloroplasts have been not more than about 2% of the rates observed in the intact cell. There seem to be two different possibilities to explain this observation: (a) the insoluble, energy-transferring system of the chloroplasts is damaged during the course of the isolation procedure or (b) there is a loss of soluble enzymes and cofactors due to leaching during isolation. It would seem feasible to check the first possibility by examination of the fixation pattern with both $C^{14}$ and $P^{32}$, and the second one by supplying soluble enzymes and cofactors to isolated chloroplasts.

Successful attempts have been made by Holm-Hansen and co-workers[1] to improve the fixation rate of chloroplasts by addition of "sap" enzymes. The preparation, however, was a crude mixture of both chloroplastic and cytoplasmic material. Therefore it is difficult to exclude the interaction of enzymes which really do not occur in the chloroplasts *in vivo*. It would seem useful to extend the experiments to pure preparations of chloroplast enzymes. We tried to perform such experiments. These experiments are summarized below.

I. In order to obtain evidence as to whether there is a leaching of enzymes during the course of isolation, we compared the content of soluble proteins in chloroplasts obtained in two different procedures: (a) Chloroplasts were isolated from aqueous sucrose containing buffer according to Holm-Hansen and co-workers.[1] (b) Chloroplasts were isolated after freeze drying of the leaves from a nonaqueous medium consisting of petrol ether and carbon tetrachloride, and the purity of the isolated chloroplasts was checked.[2,3] Results are given in Table V.

It is obvious that nonaqueous chloroplasts contain far more soluble proteins than aqueous chloroplasts. It is extremely likely that this is due to leaching of the aqueous chloroplasts during the isolation. Examination in the ultracentrifuge showed that the loss is not limited to the rather low molecular Fractions II and III, but occurs also in Fraction I. It should be mentioned that the "aqueous" chloroplasts in our case are not washed, as is usually done. The loss after washing would be even greater than indicated in Table V.

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[1] O. Holm-Hansen, N.G. Pon, K. Nishida, V. Moses, and Melvin Calvin, Physiol. Plant 12, 475 (1959).

[2] U. Heber, Ber. deut. botan. Ges. 70, 371 (1957).

[3] U. Heber, Z. Naturforsch. 15b, 100 (1960).

Table V. Comparison of the content of soluble proteins in chloroplasts, obtained from aqueous and nonaqueous Media

| Chloroplasts from isolation procedure | Spinacia oleracea | | Vicia faba | | Tetragonia expansa | |
|---|---|---|---|---|---|---|
| | aq. | nonaq. | aq. | nonaq. | aq. | nonaq. |
| Chlorophyll (g/100 g protein) | 8.75 | 6.15 | 12.02 | 4.80 | 7.22 | 5.10 |
| Soluble protein (% total protein) | 28.9 | 55.4 | 16.5 | 57.0 | 41.3 | 71.5 |
| Fraction I (% of total protein) | 18.1 | 26.8 | 3.12 | 33.4 | 21.2 | 35.6 |
| Fraction II (% of total protein) | 4.36 | 6.35 | 3.70 | 6.8 | 7.7 | 9.9 |
| Fraction III (% of total protein) | 6.45 | 22.20 | 9.66 | 16.9 | 12.3 | 27.3 |

II. After obtaining evidence about the loss of proteins during the isolation of chloroplasts in aqueous media, we considered it likely that these preparations lacked soluble enzymes. Therefore we added protein, both from frozen-dried chloroplasts and from whole tissue of different plant species, to "aqueous" chloroplasts of spinach, and performed carbon-fixation experiments as described by Holm-Hansen.[1] Results are given in Table VI. Nonaqueous chloroplasts have completely lost the ability to perform the light-dependent energy-transferring reaction of photosynthesis, and show (as well as whole tissue) no difference between light and dark experiments. The remarkable high dark fixation probably is due to the fact that no loss of polar cofactors could occur in the isolation procedure. Therefore, the system works as long as energy requirements are fulfilled.

The fixation rate of aqueous chloroplasts is different and depends on the ability of the isolated chloroplasts. There is, however, in every case a large increase after the addition of 3.5 mg nonaqueous chloroplasts, irrespective of plant species from which they are obtained. This increase is higher than the one obtained by Holm-Hansen and co-workers after addition of 25 mg "sap" proteins, and may sometimes be thirty times the fixation of aqueous chloroplasts. The dark fixation rates of aqueous and nonaqueous chloroplasts are additive, as expected.

Nonaqueous whole tissue of spinach stimulates aqueous chloroplasts as well as nonaqueous chloroplasts do. Whole nonaqueous tissue of Tetragonia or Vicia, however, does not show this effect. In addition, these extracts apparently inhibit the light reactions. The fixation pattern of the combination of aqueous spinach chloroplasts, nonaqueous and Vicia tissue, is the same in light and dark and does not contain reduced $C^{14}$.

III. As pointed out earlier in this report, there are two possible reasons for the low carbon fixation rate of isolated chloroplasts: a loss of enzymes during the isolation procedure, and damage to the energy-receiving and -transferring system. We have shown that a loss of enzymes is at least partly responsible for low carbon reduction, but even after the addition of the lacking enzymes, the fixation still did not increase as much as expected. The question remains whether there is, besides the loss of protein, a damage in the carbon-reducing system. The pattern of the fixation products now can give further information: A low ratio of reduced carbon to carbon uptake indicates lack of reducing power. We found, indeed, as much as 70% of the fixed carbon in phosphoglyceric acid, phosphoenolpyruvic acid, and aspartic acid, and less than 20% in sugar phosphates. There are two possible explanations for this observation:

(a) The carbon cycle doesn't operate at all and the fixation occurs just into a large pool of ribulose phosphate from the nonaqueous chloroplasts, giving rise to great amounts of PGA due to the carboxydismutase reaction.

(b) The carbon cycle operates very well but is blocked by lack of cofactors, especially ATP or TPNH or both.

Table VI. Fixation rate of aqueous spinach chloroplasts with and without supplementary enzymes from chloroplasts and whole tissue of different plant species (in $10^6$ cts/mg chlorophyll per min). Conditions as in Holm-Hansen, 1959.

| Addition | | 5 mg aq. chloroplasts, alone | 5 mg aq. chloroplasts + 3.5mg nonaq. chloroplasts | 3.5 mg nonaq. chloroplasts alone | 5 mg aq. chloroplasts + 4.4 mg nonaq. whole tissue | 4 4 mg nonaq. whole tissue alone |
|---|---|---|---|---|---|---|
| Nonaqueous | L[a] | 0.22 | 10.1 | 2.5 | 13.1 | 2.2 |
| spinach | D | 0.15 | 2.79 | 2.5 | 2.40 | 2.2 |
| Nonaqueous | L | 4.26 | 12.1 | 0.4 | 8.8 | 0.21 |
| spinach | D | 0.22 | 0.61 | 0.4 | 0.22 | 0.21 |
| Nonaqueous | L | 1.16 | 6.65 | 1.03 | 0.76 | 1.14 |
| *Vicia faba* | D | 0.20 | 1.35 | 1.23 | 0.86 | 1.14 |
| Nonaqueous | L | 0.15 | 3.30 | 0.12 | 0.40 | 0.11 |
| *Tetragonia expansa* | D | 0.15 | 0.47 | 0.12 | 0.36 | 0.11 |

[a] L = Light; D = Dark

A decision can be given in a double labeling experiment with both $P^{32}$ and $C^{14}$. This was done in cooperation with E. Tyskiewicz, and we confirmed the earlier results. Less than 5% of the fixation was obtained in sugar phosphates in spite of big differences in light and dark. But PGA, the main compound, was nearly as heavily labeled with phosphate as with $C^{14}$. ATP was available in excess and therefore we conclude that the carbon cycle is operating very well indeed. The limiting rate of the reaction probably is the reduction of PGA due to lack of TPNH. The purpose of further experiments is to get even better fixation of isolated chloroplasts by improving conditions for better TPN reduction.

# IX. DETECTION OF CARBOXYLIC ACIDS ON PAPER CHROMATROGRAMS

Josephine Hill and J.A. Bassham

In our laboratory we have frequently found the method of detection of carboxylic acids on paper chromatrograms by means of acid-base indicator sprays to be unreliable. Often, when a chromatrogram was sprayed following the development in our usual solvent system (phenol-water and butanol—propionic acid—water) a large area of the paper chromatogram appeared to have an acidic background which made impossible the detection of many biologically important compounds.

The acids which we were particularly interested in detecting are products of photosynthesis and respiration. Most of these acids (e.g., malic, citric, glyceric, fumaric, and α-ketoglutaric) have first ionization constants somewhere in the range of $10^{-3}$ to $10^{-5}$. It seemed advisable to try acid-base indicators which undergo color changes between pH 3 and 5. After testing a number of indicators, we could find no better choice than bromphenol blue (yellow, pH 3.0; blue, pH 4.6) and bromcresol green (yellow, pH 3.8; blue, pH 5.4). These indicators already had been widely used for this purpose.

The amounts of acid which we chromatographed and detected varied from 50 μg for relatively nonvolatile acids to 200 μg for more volatile acids such as glycolic acid.

We also tried other solvent systems and found phenol-water (our usual first solvent) followed by ethyl acetate—acetic acid—water (3:1:1)[1] more satisfactory for this purpose than our usual two-dimensional system. However, the acid background, though diminished, still showed up.

We next investigated the effect of washing the paper prior to chromatography. All studies were made with Whatman No. 4 filter paper. When this paper is sprayed without washing and before any chromatography, it is found to have a neutral to alkaline background (light blue with bromphenol blue). After several combinations of wash solutions had been tried, the following sequence of washing solutions was found to be of some help. First, 2 N acetic acid was used, then distilled and de-ionized water, and finally 5 N ammonium hydroxide.[2]

The method used in washing papers consists of setting 12 papers in a chromatographic trough, 6 on each side, with the edges folded under the middle bar. Four troughs containing a total of 48 papers are placed in a chromatographic box. Wash solution is poured into each trough and is absorbed rapidly by the papers. The wash solution is replenished in the troughs until the papers appear somewhat saturated. Then the chromatographic boxes are closed. In about an hour, or whenever the wash solution needs to be replenished, the troughs are refilled. The total amount of wash solution used is about 2 liters per box. The wash solution is allowed to run to the bottom edge and drip off the papers. After about 6 hours' washing, the

[1] J.E. Loffler and E.H. Reichl, Mikrochim. Acta 79 (1953).

[2] F.A. Isherwood and C.S. Hanes, Biochem. J. 55, 824 (1953).

papers are dried, usually overnight, before each successive washing stage. The papers are thoroughly dried after the last washing.

Although considerable improvement in reliability of acid detection by spraying was achieved through the described washing of the papers, and by using ethyl acetate—acetic acid—water as the second developing solvent, difficulties were still encountered. These difficulties were that the paper after development would have either a background that was uniformly too acid or too alkaline. This problem was partially solved by preparing a number of solutions of bromphenol blue with pH adjusted to 3.0, 4.5, 5.0, 6.0, 6.5, and 7.0, and of bromcresol green at more alkaline pH (7.5, 8.0, and 8.5). The choice of the best spray solution can then be made by spraying a test strip along one edge of the paper chromatogram. An even better method of making this choice is to chromatograph in one dimension test spots of the two indicators. This chromatography is carried out in the same box and with the same solvents as the chromatography of the acids to be sprayed (the second solvent if the chromatography is two-dimensional). From the color of these test spots, one can estimate the background pH and choose the best indicator spray solution.

By means of these methods, the reliability of the detection by indicator sprays has been considerably improved, but the method is still by no means infallible, and further investigation of the problem would seem to be advisable.

By the above methods, two-dimensional chromatographic maps were constructed of organic acids chromatographed with phenol-water (saturated solution at 25° C) in one direction, and ethyl acetate—acetic acid—water (3:1:1) in the other direction. One map was prepared for an acid mixture whose pH at the origin was 4.2, and another for a mixture at pH of 6 (Figs. 19 and 20). The acids were buffered with ammonium phosphate, pH 4.2 or 6.0, before they were applied to the origin of the paper. All buffer solutions were made 0.52 M with respect to total phosphate. With each 200 μg of acid to be chromatographed, approximately 5.16 micromoles of phosphate buffer was mixed in the acid solution. Mixtures of two or three acids were buffered and chromatographed. Radioactive malic acid, eluted from chromatograms of algae extracts of steady-state experiments, was always used as a radioactive marker. The markers and the nonradioactive acids were chromatographed two-dimensionally and located by radioautographs and acid-base indicator sprays. $R_f$'s of the nonlabeled acids were measured and corrected according to the $R_f$ of radioactive malic acid, which appeared on each chromatogram.

Measured, calculated, and corrected $R_f$'s are shown in Table VII. It was observed that acids buffered at pH 6 had a greater tendency to streak than those buffered at pH 4.2, especially in the phenol-water solvent. In determining the $R_f$ an attempt was made to pick the center of the acid area which seemed to have the greatest density of acid color.

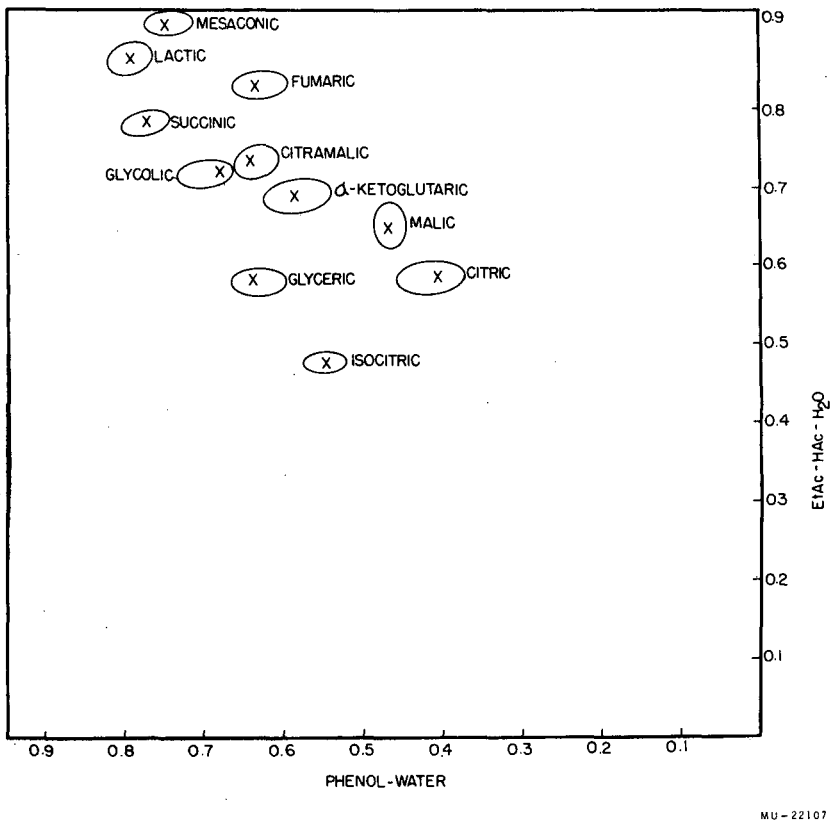


Fig. 19. $R_f$'s of organic acids initially buffered at pH 4.2.

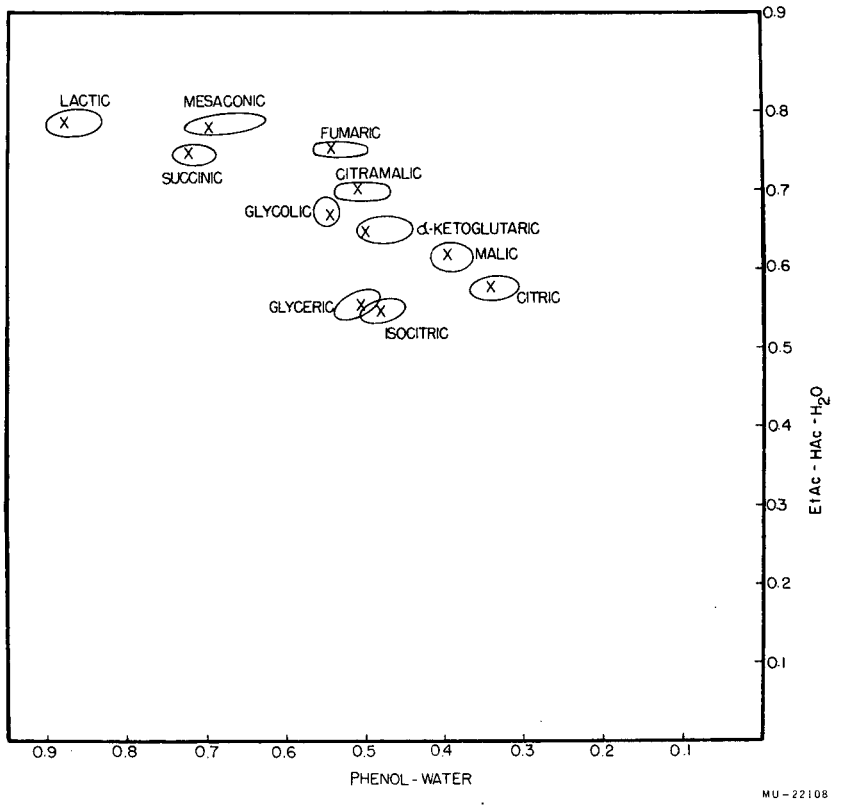


Fig. 20. $R_f$'s of organic acids initially buffered at pH 6.

Table VII. $R_f$'s of acids on paper chromatography

| Acids | Initially Buffered at pH 4.2 | | Initially Buffered at pH 6 | |
|---|---|---|---|---|
| | Developing solvent | | Developing solvent | |
| | Phenol-water | EtAc-HAC-water | Phenol-water | EtAc-HAC-water |
| Citric | 0.41 | 0.59 | 0.34 | 0.58 |
| Malic | 0.47 | 0.65 | 0.41 | 0.63 |
| Isocitric | 0.57 | 0.48 | 0.48 | 0.55 |
| α-ketoglutaric | 0.59 | 0.69 | 0.50 | 0.65 |
| Glyceric | 0.63 | 0.58 | 0.51 | 0.56 |
| Citramalic | 0.64 | 0.73 | 0.52 | 0.70 |
| Fumaric | 0.67 | 0.83 | 0.54 | 0.76 |
| Glycolic | 0.68 | 0.72 | 0.55 | 0.68 |
| Mesoconic | 0.75 | 0.88 | 0.70 | 0.78 |
| Succinic | 0.78 | 0.78 | 0.72 | 0.75 |
| Lactic | 0.81 | 0.86 | 0.89 | 0.79 |

# X. AN ATTEMPT TO IDENTIFY A SUPPOSED ATP PRECURSOR

E. Tyszkiewicz

## Introduction

At present there is evidence that in the living cell the first stable product of phosphorylation is adenosine triphosphate,* although in a very short time other compounds, such as sugar mono- and diphosphates or triose phosphates, are formed.

The aim of this work is to study photosynthetic phosphorylation where ATP is the main product formed after a short time. Heretofore experiments with whole chloroplasts have given no clue of the site of ATP formation. Several investigators proposed the following mechanism for the formation of the ATP precursor:

$$Pi + X \rightarrow PiX ,$$

$$PiX + ADP \rightarrow ATP + X ,$$

but without proof. However, Kahn and Jagendorf[1] obtained evidence for its existence.

## Experimental Procedure

An attempt to show the existence and to identify such a compound was made, using $P^{32}$ in sonically ruptured spinach chloroplasts. Chloroplasts are isolated according to the method of Park et al.,[2] except that phosphate buffer was replaced by tris buffer, pH 8. As shown in Table VIII, the rate of phosphorylation is maximum at this pH. Arnon et al.[3] showed that it dimishes very rapidly above pH 8.3.

If the ADP is the acceptor of the phosphate donated by the unknown PiX, one can expect this compound to accumulate if the phosphorylation reaction is done in the absence of ADP.

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*The following abbreviations are used in this text: ADP, adenosine diphosphate; ATP, adenosine triphosphate; Pi, orthophosphate, ; TPN, triphosphopyridine nucleotide; TPP, thiamine pyrophosphate; GSH, glutathione; EDTA, ethylene diamine tetraacetic acid.

[1] J.S. Kahn and A. T. Jagendorf, Biochim. Biophys. Res. Comm. May 1960.

[2] R.B. Park, N.G. Pon, K.P. Louwrier, and M. Calvin, Biochim, Biophys. Acta 42, 27-33 (1960).

[3] M.B. Allen, F.R. Whatley, and Daniel I. Arnon, Biochim. Biophys. Acta 27, 16-23 (1958).

Table VIII. Rate of phosphorylation as a Function of pH.

| | pH of sucrose, tris buffer, and cofactor solutions. | | | |
|---|---|---|---|---|
| | 7.3 | 7.5 | 7.7 | 8.0 |
| $PO_4$ esterified (%) | 6.0 | 6.9 | 14.8 | 22.3 |
| ATP formed (%) (% of P esterified) | 82.9 | 79.9 | 87.4 | 86.8 |

Each reaction mixture contained (amount in μM): $MgCl_2$, 4.0; $MnSO_4$, 1.6; ADP, 1.0; TPN, 0.02; TPP, 1.4; ascorbate, 2.5; GSH, 1.3 $K_2HP^{32}O_4$, 1.0; fragmented chloroplasts, 1.0 ml. Total volume 1.15 ml. Incubation time: 5 min in light, followed by methanol killing (final volume: 5 ml). The precipitate was extracted with 20% methanol and water. All washings were combined and an aliquot was chromatographed on Whatman No. 4 paper, in the following solvents: 1st dimension, 14 hr in isobutyric acid, 100; $NH_4OH$ 1 N. 60; EDTA 0.1 M, 1.4; 2nd dimension, 16 hr in butanol, 468; propionic acid, 200; water, 312.

Such a reaction has been carried out, and the results in Table IX show that when ADP is absent from the reaction mixture, an unidentified phosphorylated compound is formed in a greater amount than in the presence of ADP.

Under air and in dark this compound is also formed but in different amounts. However, when chloroplasts were previously killed by boiling, it was not formed, indicating that the latter is closely connected with an enzymatic process.

In order to characterize this compound, it was eluted from chromatograms, and hydrolyzed in HCl 1 N for 7 min at 100°. These conditions are sufficient to hydrolyze the terminal high-energy phosphate bonds of ADP and ATP. Another eluate was hydrolyzed with 0.2 M NaOH in methanol for 1.5 hr at 39°, to determine whether a phospholipid or its degradation product was formed. In both cases, the compound was not hydrolyzed. The compound was then treated with acid phosphatase (partially purified "Polydase S") at 37° overnight. The compound was completely dephosphorylated; radioactive orthophosphate was the only product detected by paper chromatography.

When the compound was fed to the sonicated chloroplasts in the presence of all the cofactors (including ADP), 64% of the compound was converted into orthophosphate after 5 min under air atmosphere, and 23% under nitrogen for the same time. This agrees with the results obtained previously that under nitrogen atmosphere after 5 min reaction, more of this compound is formed from $P^{32}O_4$ than under air atmosphere.

Table IX. Distribution of $P^{32}$ among ATP, ADP, and unknown compound in presence or absence of ADP

| | Pi esterified (%) | |
|---|---|---|
| | with ADP | without ADP |
| ATP | 49.1 | 2.3 |
| ADP | 22.4 | 2.2 |
| Unknown | 22.5 | 92.3 |
| Total phosphate esterified[a] | 6.0 | 2.4 |

[a]% of inorganic $P^{32}$ transformed into organic phosphate.

The reaction conditions were the same as indicated in Table VIII, except for the amount of $^{32}$Pi (carrier free), and the reactions were performed under nitrogen. Incubation time: 5 min in light.

The cochromatography of the compound with the following gave a negative answer: glycerol phosphate (α or β), phosphoenolpyruvate, d-phosphohydroxypyruvate, dihydroxyacetone phosphate, glyceraldehyde phosphate, inositol monophosphate, phosphoserine, phosphothreonine, phosphoethanol amine, pyridoxal phosphate, flavin monophosphate, phenol phosphate, p-nitrophenyl phosphate, carboxyphenol phosphate, pyrophosphate, or any mono- or diphosphate sugar.

Because the compound was easily phosphatized, the easiest way to identify it would be to label it with $C^{14}$. Thus a spinach leaf was fed with $NaHC^{14}O_3$ solution for 21hr through the petiole. The leaf was ground and extracted as described above for chloroplasts. The chromatogram of the extract showed no $C^{14}$ labeling. Another leaf fed simultaneously with $P^{32}O_4$ solution labeled the unknown compound with $P^{32}$. Another attmept was made to incorporate radiocarbon from $C^{14}O_2$ into this compound in the steady-state apparatus,[4] using a spinach leaf. The experiment was conducted in an atmosphere containing 2% $CO_2$ and 14 mC of $C^{14}$. The leaf was illuminated for 14 hr with an intermittent dark time of 10 hr. Even under these conditions the compound was not labeled.

Different methods of "killing" were studied. After exposure of chloroplasts to phosphate for 5 min, different agents such as trichloroacetic acid (final concentration 3% in the reaction mixture), ethanol, n-propanol, and n-butanol were all used separately and at a final concentration of 80%.

[4] J. A. Bassham and M. Kirk, Biochim. Biophys. Acta 43, 447 (1960).

Liquid nitrogen in the presence of some inhibitors ($F^-$, $CN^-$, $HSO^-_3$, etc.) was also tried. Not one of these methods formed the unknown compound in the chloroplasts. It is concluded therefore that methanol is responsible for its presence. It is likely that an unspecified phosphate agent donated its phosphoryl group to methanol. The use of $C^{14}$-labeled methanol should establish this point.

The unknown phosphorylating agent remains to be identified, however. With the use of $CH_3O^{18}H$ the labeled atom may be transferred to the agent. These studies are now in progress.

# XI. CONVERSION OF A LIQUID SCINTILLATION DETECTOR UNIT FOR ROOM-TEMPERATURE OPERATION

Fritz Woeller and Fred Vogelsberg

Liquid scintillation counters for soft β emitters have generally been operated with the multiplier phototubes below $0^{\circ}$. The purpose of this cooling has been to lower the thermionic noise from the photocathodes. There are, however, some disadvantages. Cooling of the sample solution results in decreased solubilities of assay materials and narrows the choice of scintillators. Furthermore, phase separations are more likely at the lower temperature and can easily escape observation. In addition, extra time must be used in chilling the samples before they are counted. A current trend exists to overcome the need for lowered temperature.

The objective has been to convert our counter to room-temperature operation at a reasonable sacrifice in the efficiency-to-background relation, and at the same time to design a simple mechanical device for the counting of samples contained in the usual 20-ml Wheaton vials.

The cutaway drawing (Fig. 21) depicts the new detector unit that we are now using for room-temperature liquid scintillation counting. The cell compartment, custom-built here, is embedded in lead brick shielding, and the whole set is mounted on an open bench without further protection from light. The voltage supply to the tubes is continuous, i.e., the amount of stray light entering during sample changes is small enough to make an interlock unnecessary. A face-to-face arrangement of the coincidence-operating phototubes has been retained.

Table X compares some typical data obtained by counting at room temperature with those obtained by counting at $-12^{\circ}$. Discriminator levels were chosen to approximate the same values for backgrounds at the two temperatures used. Currently, two selected, early DuMont 6292 tubes are satisfactory for our purposes. Newer types of photomultiplier tubes have not been tried yet.

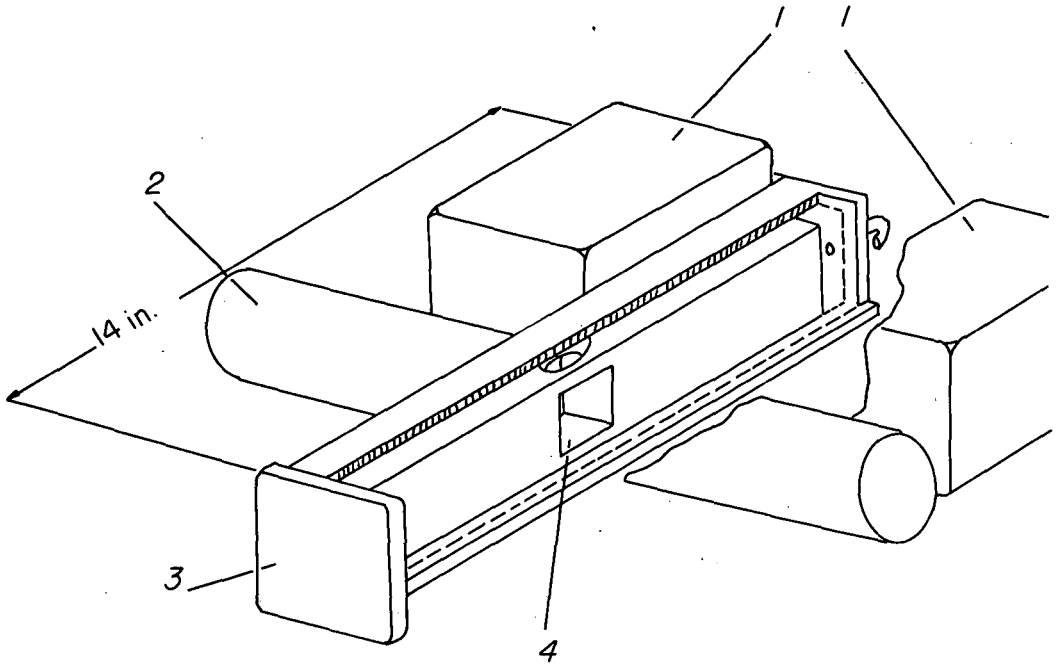


Fig. 21. Lighttight sample changer for liquid scintillation counting.

Table X. Comparison of liquid scintillation counting at two temperatures

| | Temperature | Background (cpm) | Max. eff[a] (%) | $H_2O$ eff[b] (%) |
|---|---|---|---|---|
| Isotope: | -12 | 28 | 78 | |
| C-14 | room temp. | 28 | 75 | |
| Isotope: | -12 | 90 | 37 | 22 |
| H-3 | room temp. | 80 | 26 | 11 |

[a] Maximum efficiency = the counting efficiency obtained by using PPO-POPOP in stock reagent-grade toluene

[b] $H_2O$ efficiency = counting efficiency for a solution containing 0.5 ml of water in 10 ml of toluene-dioxane-ethanol solution (naphthalene-PPO-POPOP)

# XII. FURTHER STUDIES ON THE MECHANISM BY WHICH $D_2O$ PRODUCES STERILITY IN MALE MICE

Ann M. Hughes

(In collaboration with Dr. Laurel Glass, Department of Anatomy, University of California Medical School, San Francisco)

We have previously reported the examination of eggs obtained from female mice mated with $D_2O$-treated males.[1] The examinations made at that time were with the aid of a regular high-power microscope. We have now made some preliminary observations of mouse eggs, using an ultraviolet light microscope in an effort to determine whether the $D_2O$-treated sperm are entering the eggs and whether fertilization occurs.

Male Swiss mice have been given 30% $D_2O$ in their drinking water as in previous experiments. Female Swiss mice were "superovulated" by injecting with pregnant mare serum and human chorionic gonadotropin. (The purpose of superovulating the females is to make them ovulate at a given time. It is possible then to obtain eggs of a known age.) The $D_2O$-treated male mice (and control males) were individually mated with superovulated females at the time of ovulation. Ovulation time was adjusted to give eggs of 10 to 15 hr or 36 to 45 hr of age. These males were also mated weekly with 2 or 3 females which were allowed to go through 10 to 16 days of pregnancy.

Eggs were obtained from the females by flushing the oviducts as previously reported. The eggs were then stained with acridine orange and examined under the uv microscope. DNA-containing material (i.e., nuclei of cells) fluoresces pale green and RNA-containing material fluoresces orange. Thus the egg nucleus and the sperm head, or later the sperm pronucleus, fluoresce green.

Although our results are only preliminary, we can report that after 5 to 8 weeks' $D_2O$ treatment, some of the males were capable of fertilizing eggs (i.e., either pronuclei were seen in 10-to-15-hr eggs, or 2-, 3-, and 4-cell eggs were seen at 36 to 45 hr), but were not capable of producing viable embryos at 10 to 17 days. In one or two cases, 10-to-15-hr eggs from a female mated to a given male showed pronuclei, while 36-to-45-hr eggs from a female mated to the same male the same week were degenerating, suggesting that in some cases, at least, the sperm are capable of fertilizing the egg, but degeneration occurs in the next 24 hr.

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