Investigation of the reduction of carbon dioxide in plants requires a rapid and general method for separating and identifying a large number of compounds. Paper chromatography is such a method. This paper constitutes a report of progress in the development of experimental procedures in the application of paper chromatography to the separation of compounds formed by plants during short periods of photosynthesis. The compounds involved are important intermediates of plant and animal metabolism. These methods should find application not only in other biochemical problems, but in the control of chemical synthesis as well.

A number of products formed during a short period of photosynthesis with radioactive carbon dioxide have been reported. The classical biochemical methods and ion exchange techniques which were used in those identifications would require considerable effort to separate the large number of compounds involved. The success of paper chromatography in separating two groups of compounds important in photosynthesis, amino acids and sugars, has proved especially useful to distinguish between all compounds containing the radioactive carbon atoms by exposing the paper to a sheet of X-ray film. Radioautography has been used to identify amino acids and the extension to other compounds depends only upon the ability of the method to separate the compound.

Identification of those compounds for which specific color reactions exist is relatively simple. For many compounds found in plant extracts no color reactions are known. The most useful property of organic substances in the identification of tracer amounts of material has been the distribution coefficient between immiscible solvents. It is readily determined and is highly characteristic, especially when measured as a function of pH. It has proved especially useful to distinguish between a number of possibilities for an unknown spot. We have found it convenient to synthesize the suspected compound containing a radioisotope and to determine the chromatographic position by radioautography. Identification is then accomplished by preparation of a mixed chromatogram. The isolated substance eluted from the paper is co-chromatographed with the authentic specimens. Identity of the two samples is evidenced by the appearance of a single spot.

**Chromatography**

Two-dimensional paper chromatograms were prepared in a conventional manner. The solvent flowed downward through the paper suspended from a long stainless steel trough, Fig. 1. To reduce volatilization and decomposition of compounds the paper was dried in a hood without heat.

**Selection of Solvents.**—Phenol, saturated with water at the room temperature, 24°C, was chosen.
as the first solvent and proved satisfactory in moving most of the radioactive components.

The use of lutidine or collidine as a second solvent was prohibited by the instability of many phosphate esters in these bases. Much hydrolysis and formation of immovable material occurred with lutidine and streaking of spots indicated slow decomposition.

The acidic solvent butanol-propionic acid-water was chosen as the second solvent for much of the present work since it effects an optimum separation of a majority of components. By varying the proportion of propionic acid it was possible to change the water content of the organic phase from 20 to 50%. An intermediate composition, approximately 31% water, appeared to give an optimum separation of the compounds involved. Another solvent, butanol-acetic acid-water, has been used and is especially effective in moving the phosphate esters. Such solutions must be freshly prepared, since the composition of the phases changes upon esterification. In several experiments the solvent system, ethylene glycol-diethyl ether-propionic acid (or formic acid)-water provided a solvent with desirable characteristics and without the disadvantage of esterification.

**Butanol-Propionic Acid-Water.**—Fresh solvent is prepared from equal volumes of two solutions, A (1246 ml. of n-butanol and 84 ml. of water) and B (620 ml. of redistilled propionic acid and 790 ml. water). A and B are adjusted to give a single-phase solution which becomes cloudy upon cooling to 22° or 24° below the thermostated room temperature. Stainless steel troughs 24 inches long with a semi-circular cross section 1.5 inches in diameter, Fig. 1, are filled with 90-120 ml. of solvent for two papers (18° X 221/4") The time required for the solvent to traverse the paper varies from fifteen to twenty hours.

**Butanol-Acetic Acid-Water.**—A fresh solution is prepared from 74 ml. of n-butanol, 19 ml. of acetic acid, and 50 ml. of water.

**Preparation of Radioautographs.**—The dried chromatogram is folded to fit the conventional 14" X 17") Eastman No-Screen X-ray film with the origin at one corner. One or two films are then exposed to the chromatogram in a light proof "Eastman X-Ray Exposure Holder" which can be stacked and weighted with a carton of sand to equalize pressure distribution. In the case of C14-Pa2 radograms both sides of the duplicated film are exposed by phosphorus while C14 exposes only the adjacent emulsion. After development of the film only the Pa activity is shown on one side of the film while the other is exposed by both raditions. For documentation it is advisable to use two sheets of film on one side of the chromatogram so that the second sheet shows only Pa activity.

The activity emerging from the paper (Whatman no. 1 filter paper) is ~30% of that detectable if the sample were dried in a thin film on glass or metal and counted directly. The time of exposure of the X-ray film depends upon the intensity of the radiation coming from the paper. An activity of 15,000 disintegrations per minute per sq. cm. produces satisfactory exposures in three to six days.

It has not been possible to reproduce the radio-grams accurately by photography. Very faint radioactive spots are readily discerned when the film is observed by light reflected from a white background. The details of more exposed spots such as the phosphate esters are clearly seen when viewed over a standard X-ray illuminator.

Radioactive ink provides an exact record of the relative position of the film and chromatogram during exposure. Opposite corners of the chromatogram are marked with stamp pad ink containing a non-volatile C14-labeled compound.

**Application of Plant Extracts on Paper.**—The eighty per cent. ethanol extract of algae (1-g. packed cells) or leaves (2 g.) is concentrated in vacuo (less than 20°) to 2 ml. Ten to two hundred microliters of extract is applied to the 1.5-cm. origin circle. An air stream warmed with a flame, when stability of the compounds allows it, rapidly dries the spot by flowing above and below the paper. Infrared lamps cause decomposition and are not satisfactory heat sources.

The capacity of Whatman no. 1 filter paper varies with the type of compound and the solvent. Loads varying from a few μg. for carboxylic acids and up to 1 or 2 mg. for the sugars have been successfully separated.

It is possible to separate simple mixtures on a larger scale by a one dimensional technique. A mixture is applied in a 1.5-cm. wide stripe near the edge of a sheet and separated in an appropriate solvent into two or more bands. As much as 20 mg. of hexoses or 4 mg. of hexose phosphates have been separated in this way.

**Identification**

The majority of compounds so far found to be involved in photosynthesis are well known metabolic intermediates whose physical and chemical properties are known. Earlier work had established the presence of large fractions of the fixed activity in alanine, malic acid, sucrose, and phosphoglyceric acid. When the chromatograms were made it was a relatively straightforward matter to determine which of the forty spots detectable in a radiogram of ninety seconds photosynthesis by Scenedesmus corresponded to these compounds. Identification of the remaining compounds is begun in this work.

The positions of these radioactive compounds are reproducible for extracts of a given plant. However, absolute Rf values for compounds in such mixtures should not be applied universally. The presence of contiguous substances on the paper, whether they be radioactive or not, may disturb equilibrium and movement of spots. For example, inorganic phosphate is found to restrict the movement of adjacent compounds. It is necessary, then, to consider the relative location of known vicinal spots rather than the absolute posi-
tion on the chromatograms. The location of a spot relative to several standards, that is, the pattern of spots, presents the most reliable method of describing chromatographic position. In this paper, the positions of the neutral amino acids, serine and alanine, are taken as reference points. A schematic plot of $R_F$ values of compounds involved in photosynthesis is given in Fig. 2.

The Carboxylic Acids: According to Consden, Gordon and Martin, the $R_F$ of a solute is given by

$$R_F = A_L/(A_L + \alpha A_S)$$

where $A_L$ is the fraction of cross-sectional area occupied by the mobile phase, $A_S$ is the fraction of cross-sectional area occupied by the stationary phase, and $\alpha$, the distribution coefficient of the solute, is the concentration of solute in the stationary phase divided by the concentration of solute in the mobile phase. This expression can be written as

$$R_F = 1/(1 + \alpha A_S/A_L)$$

The term $A_S/A_L$ is a constant for a given paper and set of solvents while $\alpha$ is characteristic of the solute and determines its position on the paper.

The gross distribution of a carboxylic acid between the stationary aqueous phase and the mobile organic phase depends on the distribution coefficient of the undissociated acid and on the degree of dissociation of the acid. The degree of dissociation depends on the $pH$ of the aqueous phase and on the dissociation constant of the acid. Consequently the gross distribution of a given acid, and hence its $R_F$ value, is in general a function of two physical properties of the acid, its distribution coefficient and its dissociation constant(s) and one experimental condition, the $pH$ of the aqueous phase of the chromatogram.

If neutral, non-buffered solvents are employed, the $pH$ of the aqueous phase at a given point depends only on the concentration of carboxylic acid at that point, and as the mobile phase carried the acid past this point there is a decreasing concentration and increasing degree of ionization of the acid with a resultant changing distribution of the acid between phases. The rate of movement of the acid therefore decreases when its concentration decreases and streaking is produced. By using formic acid as a “swamping” acid, they suppressed the ionization of the test acids so that the $R_F$ values depend principally on the distribution coefficients of the free acids.

It is possible to adjust the $pH$ of the aqueous phase by employing buffers. This can be accomplished either by applying an acidic solvent or a buffered solvent. Lugg and Overell have used an acidic solvent to effect the separation of carboxylic acids. By using formic acid as a “swamping” acid, they suppressed the ionization of the test acids so that the $R_F$ values depend principally on the distribution coefficients of the free acids.

It is desirable in two-dimensional chromatography to employ a low $pH$ solvent in one direction and a higher $pH$ solvent in the other direction so that a different order and degree of separation is obtained in the two directions. While it is possible to obtain good separations of a number of carboxylic acids with two-dimensional chromatograms which employ acidic solvents in both directions, the resultant positions of the acids tend to overlap those of amino acids, sugars, and other plant constituents. The use of solvents of differing $pH$ moves the acids to a region of the paper...
apart from the rest of the plant constituents, as shown in Fig. 2. Such “area” separation is desirable in analysis of plant extracts in that it circumvents preliminary fractionations.

The usual method of buffering in the phenol direction has been to apply the buffer to the origin along with the mixture of acids or cell extract. Since the buffer capacity thus obtained is limited, it is necessary to use very small amounts of acids. From one to five micrograms of acid gives satisfactory results. The colorimetric methods described by Lugg and Overell give best results for twenty micrograms or more so the tracer method of detection is preferable for this purpose.

Several carboxylic acids labeled with C\(^{14}\) have been synthesized, and chromatographed to determine their positions with the solvents employed. The resultant \(R_F\) values are given in Fig. 2, and they represent average values obtained from chromatograms buffered at \(pH\) 6-7 at the origin. In addition to malic, succinic, fumaric, and tartaric acids, which were studied by Lugg and Overell, isocitric,\(^{(22)}\) glycolic,\(^{(23)}\) and glyceric acids have been separated.

The relation of \(R_F\) values in an acidic solvent to the distribution coefficients is shown in Table 1. The \(R_F\) values and the distribution coefficients of the acids, of alanine, and of glucose were measured.

**Table I**

**Comparison of Measured and Calculated \(R_F\) Values in Butanol–Propionic Acid–Water Solvent**

<table>
<thead>
<tr>
<th>Solute</th>
<th>(\alpha)</th>
<th>(R_F) (calc.)</th>
<th>(R_F) (measured)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>3.65</td>
<td>0.333</td>
<td></td>
</tr>
<tr>
<td>Fumaric</td>
<td>0.50</td>
<td>0.78</td>
<td>0.72</td>
</tr>
<tr>
<td>Succinic</td>
<td>1.02</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>Glycolic</td>
<td>2.06</td>
<td>0.47</td>
<td>0.51</td>
</tr>
<tr>
<td>Malic</td>
<td>2.40</td>
<td>0.43</td>
<td>0.45</td>
</tr>
<tr>
<td>Isocitric</td>
<td>2.75</td>
<td>0.40</td>
<td>0.41</td>
</tr>
<tr>
<td>Glyceric</td>
<td>2.95</td>
<td>0.38</td>
<td>0.38</td>
</tr>
<tr>
<td>Tartaric</td>
<td>3.20</td>
<td>0.36</td>
<td>0.32</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.75</td>
<td>0.24</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Using the constants of alanine as a standard, the term \(A_\beta/A_1\), was calculated and found to be 0.55. The \(R_F\) values of other solutes can then be calculated according to the expression

\[ R_F = \frac{1}{1 + 0.55\alpha} \]

Comparison of the calculated \(R_F\) values with measured \(R_F\) values shows fairly good agreement and affords additional evidence that paper chromatograms of this type are partition chromatograms.

Inspection of radiograms of cell extracts from plants which had been exposed to C\(^{14}\)-labeled carbon dioxide for short periods indicated several compounds in the carbohydrate region. The synthetic labeled acids were co-chromatographed, one at a time, with the plant extracts. Intensification of an unknown radioactive spot by the authentic compound demonstrated their identity. Plant acids were then eluted individually from the paper and co-chromatographed with the corresponding synthetic compounds. Finally if no separation appeared between unknown and synthetic acid, the unknown was subjected to suitable physical and chemical confirmatory tests.

The acids formed in short term photosynthesis and positively identified so far include malic, succinic, glycolic and fumaric. Those found in respiration experiments (dark fixation of labeled carbon dioxide without preillumination of the plant) include isocitric or citric, succinic, fumaric and malic.

**Synthesis of Succinic and Malic Acids.**—One gram of carboxyl-labeled sodium acetate (specific activity, 6 \(\mu\)c./mg.) was converted to malic and succinic acids by the following series of reactions

\[
\begin{align*}
\text{CH}_2\text{C}^*\text{O}_2\text{Na} \rightarrow & \text{CH}_3\text{C}^*\text{O}_2\text{Et} \\
\text{CH}_2\text{C}^*\text{O}_2\text{Et} \rightarrow & \text{H}_2\text{PtO} + \text{NaOMe}, (\text{CO}_2\text{Et}) \\
\text{EtO}_2\text{C}^*\text{CH}_2\text{CO}_2\text{Et} \rightarrow & \text{H}_2\text{PtO} + \text{EtO}_2\text{C}^*\text{CH}_2\text{CH}_2\text{C}^*\text{O}_2\text{Et} \\
\text{EtO}_2\text{C}^*\text{CH}_2\text{CHOHCO}_2\text{Et} \rightarrow & \text{OH}^+ \text{ Succinic acid} \\
& \text{(1) H}^+ \text{ and malic acid}
\end{align*}
\]

The standard methods were modified when necessary for small-scale synthesis. The mixture of succinic and malic esters was obtained by using unpoisoned PtO\(_2\) catalyst and one atmosphere of hydrogen at room temperature with rapid stirring of a 95% ethanol solution for two hours.\(^{(24)}\) The free acids were separated by fractional ether extraction and recrystallization. The yield of succinic acid was 28% of the theoretical and of malic acid 39% of the theoretical, so that the total yield was 67%. The specific activity of the malic acid was 3.7 \(\mu\)c./mg. and that of the succinic acid was 4.2 \(\mu\)c./mg.

**Synthesis of Fumaric Acid.**—Malic acid was converted to fumaric acid by heating in nitrogen at 140\(^\circ\) for two hours. Fiftieth milligrams of carboxyl labeled malic acid gave 30 mg. (69%) of fumaric acid and 15 mg. unreacted malic acid. The specific activity of the product was 4.3 \(\mu\)c./mg.

**Synthesis of di-Tartaric Acid.**—Fumaric acid was oxidized with sodium chlorate and osmium tetroxide according to the method of Hofmann, et al.\(^{(25)}\)

**Identification of Glyceric Acid.**—The radioactive acid was eluted from the paper with water. Hydrochloric acid was added to give a 1.0 N solution in the graduated centrifuge tube. After ether was added and the phases mixed by forcing one phase through the other with a narrow tipped dropper, the mixture was centrifuged. In 1.0 ml of the 7.0-ml ether phase was found 140 c.p.m. In 0.50 ml of the 1.8 ml aqueous phase was found 1300 c.p.m. The resultant distribution coefficient, 0.005, approximates that of authentic glyceric acid, 0.004-0.008.

Radioactive glyceric acid was prepared by hydrolysis\(^{(24)}\) of phosphoglyceric acid eluted from a number of chromatograms. After heating two days in a sealed evacuated tube at 120\(^\circ\) in 1 N hydrochloric acid the solvent was evaporated to dryness. The product was taken up in water and an aliquot part co-chromatographed with the eluted unknown material. A single spot was obtained.

**Identification of Malic Acid.**—The spot on a chromatogram suspected to be malic acid was eluted and its distribution coefficient between water (\(pH\) 1) and ether was

\(^{(22)}\) We are indebted to Dr. R. E. Stutz for a sample of C\(^{14}\)-labeled isocitric acid.

\(^{(23)}\) Labeled glycolic acid was obtained from Dr. B. M. Tolbert, see “Isotopic Carbon” by Calvin, Heidelberger, Reid, Tolbert and Yankwich, John Wiley and Sons, Inc., New York, N. Y., 1949.
found to be 0.020. The constant for low concentrations of the authentic labeled dl-malic acid is 0.017. The eluate, 20,500 cpm., was co-crystallized with 50.0 mg. of malic acid and found to have a specific activity of 395 c.p.m./mg. unchanged by sublimation in vaccuo. This was dehydrated by heating under nitrogen for 24 hours at 145°. The specific activity of the fumaric acid was 500 ± 50 c.p.m./mg. The distribution coefficient between water (pH 1) and ether was found to be 0.9 (authentic value, 1.0).

Phosphate Esters

The greater part of the activity fixed in short periods of photosynthesis26 lies in the area near the origin (Fig. 3). A characteristic of this area is the presence of three distinct spots which move about the same small distance in the phenol but different distances in the butanol-propionic acid and thus lie one above the other in the butanol direction. As the periods of photosynthesis with C14O2 are shortened, the fraction of activity in this area increases. In five seconds photosynthesis only the uppermost spot in the pattern of three is radioactive. Therefore these compounds include the first and some of the subsequent intermediates in fixation and reduction of carbon by plants.27

This was confirmed by chromatographing the extract of some algae which had photosynthesized for an hour with normal carbon dioxide in the presence of inorganic radiophosphate (P32). The radiogram thus obtained (Fig. 4) was remarkably similar in this area to those obtained from radiocarbon experiments.

Phenol-water.

Phenol-water.  

Fig. 3.—Radiogram of products photosynthesized in ninety seconds by Scenedesmus in C14O2. The algae were rapidly killed by pouring into four volumes of boiling ethanol. Considerable clarity of the original radiogram is lost in photographic reproduction.

It was suspected that the compounds in this area of the chromatogram were phosphate compounds.26 C14O2 was given to the plants during steady-state photosynthesis with ordinary carbon dioxide for the lengths of time indicated.27 Similar radiograms have been obtained from Chlorella, Scenedesmus, geranium and barley.

Yeast Fermentation.—One hundred grams of Type 20-40 Fleischmann dry yeast was suspended in 300 ml. of M/15 potassium monohydrogen phosphate at 37° for three hours. The mixture was centrifuged and to 40 ml. of the supernate was added 1.5 ml. of 50% glucose and 10 mg. sodium pyruvate. After incubation at 32° for thirty minutes vigorous fermentation had started. Then five ml. of potassium dihydrogen phosphate containing 2 millieuries of P32 was added to the fermentation mixture. After three hours incubation approximately 80% of the total phosphorus was organic. The mixture was incubated for an additional three hours after the addition of 2 ml. of 1 M sodium fluoride and 6 g. sodium pyruvate. Absolute alcohol was then added to the fermentation mixture to a
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Fig. 5a,b.—One dimensional radiograms of P-32 labeled yeast fermentation intermediates: solvent, butanol-acetic acid-water. The two mixtures were obtained from ion exchange resin eluate fractions.

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final concentration of 95%. All of the proteins and 94% of the phosphorus compounds precipitated. The precipitate was washed three times with water which extracted over 95% of the phosphorus activity. An aliquot of this water extract gave six major spots on the radiogram.

Separation of Fermentation Products.—The water extract was absorbed on a Dowex A-1 anion exchange resin column in the chloride form. Washing with water removed 2.4% of the phosphate. Elution with 0.1 M sodium chloride separated the remaining phosphates into two distinct fractions. The first fraction eluted from the resin was found to contain fructose-6-phosphate, inorganic phosphate and at least three unidentified compounds. The second fraction contained only fructose-1,6-diphosphate and phosphoglyceric acid, Fig. 5a. The fractions were chromatographed in the butanol-acetic acid solvent and the individual spots of the radiograms were identified by spray tests applied to the paper or by chemical analysis of solutions of the eluted spots.

Identification of Fructose-1,6-diphosphate.—The spot having the lowest Rf value in both the phenol and butanol-acetic acid directions was eluted and identified as fructose-1,6-diphosphate by the following tests. Analysis showed that there were two moles of phosphorus to one mole of fructose. Seven minutes hydrolysis at 100° in 1 N hydrochloric acid converted 25% of the original organic phosphorus to inorganic phosphorus. This seven minute hydrolysate was re-chromatographed, two new radioactive spots of equal intensity appeared. One of the new spots contained only inorganic phosphate; the other spot which contained fructose and no easily hydrolyzable phosphorus was later shown to be fructose-6-phosphate.

Identification of Fructose-6-phosphate.—In the radiograms of samples containing the first radioactive components eluted from the anion exchange column, a spot, Fig. 5b, was obtained in the butanol-acetic acid-water solvent the intensity of which, from sample to sample, corresponded directly to the fructose analysis. The eluted spots contained fructose and phosphate in a 1:1 ratio. The chromatographic position of this compound was identical with the fructose compound obtained by hydrolysis of fructose-1,6-diphosphate. Therefore it must be an acid stable fructose monophosphate, presumably fructose-6-phosphate. A mixed chromatogram of this material with inactive glucose-1-phosphate showed considerable overlapping of the two compounds.

Identification of Phosphoglyceric Acid.—The spot labeled PGA in Fig. 5a gave a negative spray test for easily hydrolyzable phosphate. A solution of the eluted spot contained no fructose and gave no inorganic phosphate when hydrolyzed for six minutes in 1 N hydrochloric acid at 100°. When heated for one hour with 8-naphthoresorcinol in concentrated sulfuric acid, a blue color, as the same as obtained with an authentic sample of 3-phosphoglyceric acid, was observed. The other known products of this fermentation do not give this color.

Phosphoglyceric Acid in Algal Extracts.—Two early experiments indicated that the uppermost of the pattern of three spots shown in photosynthesis radiograms might be the phosphoglyceric acids. The first was a chromatogram of the sodium hydroxide eluate of the weak base anion resin, Duolite A-3, which had been shown to contain largely phosphoglyceric acid radioactivity. The position of the spot obtained corresponded with that of the uppermost of the three spots. The second experiment involved isolation of radioactive barium phosphoglycerate from a small aliquot of the extract from a one-hour photosynthesis in inorganic radiophosphate by co-crystallization with ten milligrams of the authentic barium salt. After three recrystallizations from water the salt was decationized and co-chromatographed with an algal extract (ninety second

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photosynthesis in C\textsuperscript{14}O\textsubscript{2}). Exact correspondence of the P\textsuperscript{32} activity with C\textsuperscript{14} activity in the uppermost spot of the phosphatase area was observed. After the radiophosphorus had decayed to five per cent. of its initial activity, the pattern obtained was identical to that originally apparent and the uppermost spot of the pattern of three which now was made almost entirely by carbon radiation had the identical position and shape of that given by the phosphorus formerly present.

These experiments strongly indicated the identity of the upper spot as phosphoglyceric acid but did not preclude the possibility of a highly active contaminant in co-crystallizations. Synthesis of labeled phosphoglyceric acid by yeast fermentation provided authentic material for co-chromatographing with unknown mixtures.

**Carrier Free Isolation of Phosphoglyceric Acid.**—When the period of photosynthesis in radiocarbon is sufficiently shortened only one major radioactive compound appears and is in the position corresponding to the uppermost of the pattern of three. This radioactive compound was isolated from *Scenedesmus* which had photosynthesized for five seconds\textsuperscript{31} in C\textsuperscript{14}O\textsubscript{2} and was identified as phosphoglyceric acid. The isolation was carried out according to the general method for phosphoglyceric acid with several modifications. It was found that the radioactive material was strongly adsorbed by inactive precipitates removed at PH 7. Thorough washing at PH 10, where the adsorbed anions are apparently displaced from the precipitate, was necessary to remove the activity. When barium chloride was added to an acid solution the precipitate obtained was active, but repeated washing with dilute hydrochloric acid removed the adsorbed activity. When the barium-containing supernatant solution from this acid precipitation was adjusted to PH 10, no barium phosphoglycerate precipitated since the volume of the solution was large enough to retain the amount of material present. Addition of ethanol gave a radioactive precipitate. The fructose-1,6-diphosphate which would be obtained by this procedure was hydrolyzed by repeated warming in dilute acid. The amount of hexose diphosphate in algal extracts must be much smaller than the amount of phosphoglyceric acid since the radioigrams of longer photosyntheses, where the reservoirs of intermediates are saturated with activity, show at least three times as much phosphoglyceric acid.

Repeateable dissolution of the barium salt in 0.05 N hydrochloric acid and re-precipitation from 50% ethanol gave a barium salt whose phosphorus analysis and molybdate-enhanced optical rotation\textsuperscript{31} corresponded closely to that of barium 3-phosphoglycerate.

A mixture of the isolated material (Fraction 11-a) and an equal weight of authentic barium 3-phosphoglycerate\textsuperscript{32} was eluted from an anion exchange resin column which had previously been standardized with 3-phosphoglyceric acid. The standard and the mixed elution curves are shown in Fig. 6a,b. The somewhat low value of the optical rotation together with the barely noticeable displacement of the radioactivity elution curve might be taken to indicate the continued presence in Fraction II-a of a small amount of 2-phosphoglyceric acid of higher specific activity than the 3-phosphoglyceric acid.

We have thus isolated and obtained in a crystalline form at least one-third of the radioactivity fixed in five seconds as barium phosphoglycerate, largely the 3-isomer. It is not unreasonable to suppose that the radioactivity left in the last four supernates (B, C, D, E) from the alcohol crystallizations is phosphoglyceric acid somewhat richer in the 2-isomer. The sum of these, together with that which was actually isolated would account for over 65% of the total carbon fixed in five seconds. This closely corresponds to the distribution observed on a paper chromatogram of the same type of preparation. A small sample of phosphoglyceric acid Fraction II-a was chromatographed separately and came to the expected position.

It should be noted that the results of similar experiments have been reported,\textsuperscript{33,34,35} but labeled intermediates were not identified. Fager\textsuperscript{36} reported that phosphoglyceric acid is not a major product of forty second photosynthetic experiments performed at PH 7.8 at considerably lower light intensities than used in these experiments (PH 6, 10,000 f.c.).

These differences in experimental results appear irreconcilable but may depend on differences in technique, both in cell culture and exposure to radiocarbon and in handling and isolation of the photosynthetic products. The effects of procedural differences, insofar as they can be determined from published data and private communication are being investigated.\textsuperscript{37}

**Phosphoglyceric acid Isolation Procedure.**—Six grams of day-old *Scenedesmus* cells which had photosynthesized in 1.0 g. batches for five seconds was added to 18 g. of normal algae to provide sufficient phosphoglyceric acid for isolation. These algae were killed rapidly with acetic acid-concentrated hydrochloric acid which contain all of the activity of the substrate.}

\textsuperscript{32} Barium-3-phosphoglycerate was obtained through the courtesy of Professor Carl Neuberg, New York University, New York, N. Y.

\textsuperscript{33} S. Ruben, M. D. Kamen and W. Z. Hassid, *This Journal*, 61, 661 (1939); 62, 3443 (1940).


\textsuperscript{35} E. W. Fager, *ibid.*, pp. 423.

\textsuperscript{36} At intensities of 500-foot candles we find that phosphoglyceric acid is a major product of thirty second photosyntheses. Experiments to be published demonstrate that periodate oxidation of hydrolyzed phosphoglyceric acid gives carbon dioxide, formic acid and formaldehyde which contain all of the activity of the substrate.
Fig. 6a, b.—a. Elution curve of authentic 3-phosphoglyceric acid from Dowex A-1 anion exchange resin column; eluant: 0.2 N sodium chloride; b. elution curve of a mixture of equal amounts of authentic and isolated phosphoglyceric acids; ●, total phosphorus analyses; ○, C14 radioactivity. The smaller peak represents inorganic phosphate and hexose monophosphates. The ordinate unit represents 100 µg of phosphorus/ml of eluate and 20,000 cpm/ml of C14 radioactivity.

trifuged and washed three times with water (12, 3.5, and 2.6 ml.) at pH 10. The combined washings and supernate (1.45 × 10^6 c.p.m.) were acidified with 1 N hydrochloric acid and 0.3 ml. of saturated barium chloride was added. The precipitate was washed twice with 1 N hydrochloric acid (1.5 ml.) and discarded. The combined washings and supernate was concentrated to 3.0 ml. and basified to pH 10 with 1 N sodium hydroxide. The small precipitate was discarded.

The supernate was diluted with 1.5 volumes of ethanol and centrifuged. The supernate, A, contained 0.2 × 10^6 c.p.m. The precipitate, dissolved in 2.0 ml. of water (wash contained 0.05 × 10^6 c.p.m.) and was taken up in 4.3 ml. of 0.05 N hydrochloric acid and found to have 1.1 × 10^6 c.p.m. The small residue was centrifuged and discarded.

The acid solution was diluted to 8.6 ml. with ethanol and the precipitate centrifuged. The supernate, B, contained 0.25 × 10^6 c.p.m. The precipitate, dissolved in 2.0 ml. of 0.05 N hydrochloric acid, contained 0.78 × 10^6 c.p.m. Ethanol was again added to a total volume of 5.0 ml. and the precipitate was centrifuged. Supernate, C, contained 0.01 × 10^6 c.p.m. A solution of the precipitate in 1.5 ml. of 0.05 N hydrochloric acid was diluted with ethanol to 3.0 ml. and the precipitate centrifuged. The supernate, D, contained 0.2 × 10^6 c.p.m.

A solution of the precipitate in 0.9 ml. of 0.05 N hydrochloric acid was diluted with 0.5 ml. of ethanol to obtain Precipitate I; 3.9 mg., 0.19 × 10^6 c.p.m. Anal. Calc. for C3H7O5Pb2H2O; P, 8.7. Found: P, 8.6. Molar rotation (as phosphoglyceric acid) in the presence of molybdate ion: −620 ± 50° (authentic value, −750°); specific activity, 4.9 × 10^4 c.p.m./mg.

The supernate from Precipitate I was added to 1.5 ml. of ethanol. Precipitate II, 7 mg., 0.29 × 10^6 c.p.m., and supernate E (0.08 × 10^6 c.p.m.) were obtained. The solution of Precipitate II in 0.05 N hydrochloric acid diluted with 1.5 volumes of ethanol gave Precipitate II-a, containing 8.6% phosphorus and having a specific activity of 4.2 × 10^6 c.p.m./mg. The total activity in supernates B, C, D and E was 0.58 × 10^6 c.p.m. (36% of the total). The activity in Precipitates I and II was 0.48 × 10^6 c.p.m. (30% of the total).

**Easily Hydrolyzed Phosphate Esters.—** Readily hydrolyzed phosphate esters and inorganic phosphate can be recognized on the paper by spraying with suitable reagents to produce molybdenum blue. Glucose-1-phosphate, 3-phosphoglyceraldehyde, and phosphopyruvic acid are easily detectable when three micrograms of phosphorus per square centimeter is present on the paper. Fructose-1,6-diphosphate is variable in its color development. This is probably caused by different degrees of hydrolysis during the spraying and drying processes.

**Spray Test for Phosphates on Chromatograms.—** The paper is sprayed consecutively with the following solu-
activity between water (pH 1) and ether was 0.15-0.18.

When the authentic compound containing P32 was co-chromatographed with an algal extract, the compound containing P32 was co-chromatographed with the presence of glucose-1-phosphate. When the authentic photosynthetic intermediates in the middle one of the three phosphate spots to form radioactive glucose suggested the presence of glucose-1-phosphate. When the authentic compound containing P32 was co-chromatographed with an algal extract, the P32 activity coincided with the C14 radioactive spot.

**Phosphopyruvic Acid.**—An authentic specimen of phosphopyruvic acid was chromatographed and its position detected by molybdenum blue color. A photosynthetic intermediate found in this area was investigated. Acid hydrolysis formed pyruvic acid which was identified by its distribution coefficient between water and ether, solubility and volatility properties and the formation of a dinitrophenylhydrazone which co-crystallized with an authentic specimen.

A radioactive substance was eluted from a chromatogram of five seconds photosynthetic fixation and hydrolyzed one hour in 1 N hydrochloric acid. The active product was then volatile and required addition of alkali for reproducible direct counting. An aliquot part of the hydrolysate was assayed for pyruvaldehyde and the 2,4-dinitrophenylhydrazine. The product, theoretical yield 19.5 mg., was recrystallized several times from pyridine and a 1.44-mg. sample counted on a 11.5 sq. cm. disk gave 165 c.p.m. This indicates that 2,200 c.p.m. or 35% of the original activity is pyruvaldehyde.

The radioactive spot labeled triose phosphate, was eluted from a chromatogram. Pyruvaldehyde 2,4-dinitrophenylhydrazone was prepared from 1320 c.p.m. of eluate, 5.42 mg. of 3-phosphoglyceric acid, 1.44-mg. of 2,4-dinitrophenylhydrazine. The specific activity of the pure osazone (theoretical yield, 7.29 mg.) was 178 c.p.m./mg. This indicated that 1300 c.p.m., or all of the eluate activity, was triose phosphate.

**Amino Acids.**—Identification of radioactive amino acids by superposition of the radiogram on the chromatogram sprayed with ninhydrin has been described. In cases where the original separation is incomplete or where the amount of amino acid is insufficient to produce visible ninhydrin color, the radioactive spot or group of spots is eluted and re-chromatographed with added carriers. The coincidence of shape and location of the radioactive spot with the colored spot produced by an authentic compound on the paper indicates the identity of the amino acid.

**Sugars.**—Sucrose, fructose and glucose are the only sugars which have been identified in the radioactivity. Their chromatographic coordinates in phenol have been determined. The Roe test for fructose and the Tollens test for glucose gave added confirmation of these identifications.

**Summary**

1. Paper chromatography has been employed to separate the radioactive products formed during photosynthesis in C14O2.
2. The method has been used for the separation and identification of carboxylic acids and phosphate esters.
3. The first observed product of carbon dioxide assimilation during photosynthesis has been isolated and shown to be phosphoglyceric acid.

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(38) Pre pared by Drs. H. A. Barker and F. Lipmann.

(39) Obtained through the kindness of Professors H. O. L. Fischer and E. Baer.


(41) Authentic specimens of these radioactive sugars were kindly supplied by Professor W. Z. Hassid.