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Ernest O. Lawrence  
Radiation Laboratory

TWO-DIMENSIONAL HIGH-VOLTAGE, LOW-TEMPERATURE PAPER  
ELECTROPHORESIS OF  $^{14}\text{C}$ -LABELED PRODUCTS OF  
PHOTOSYNTHESIS WITH  $^{14}\text{CO}_2$

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Wolfgang Rohr and J. A. Bassham

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## TWO-DIMENSIONAL HIGH VOLTAGE, LOW TEMPERATURE PAPER ELECTROPHORESIS

OF  $^{14}\text{C}$ -LABELED PRODUCTS OF PHOTOSYNTHESIS WITH  $^{14}\text{CO}_2$ Wolfgang Pohr<sup>1</sup> and J. A. BasshamLawrence Radiation Laboratory, University of California,  
Berkeley, California

## INTRODUCTION

Paper chromatography in combination with radioautography has proved to be a highly useful analytical tool in elucidating the carbon reduction cycle of photosynthesis as it is now known (1,2). However, several attempts (3,4,5) have failed to produce unequivocal evidence for the existence of the six-carbon beta-keto-acid diphosphate (2-carboxy-3-keto-pentitol-1,5-diphosphate), which has been proposed (1) to be the first real reaction product of  $^{14}\text{CO}_2$  fixation. Such a compound, as well as the unidentified early  $^{14}\text{CO}_2$  fixation product reported by Shkolnik and Doman (6) would be highly labile.

It is possible that the analysis by ordinary two-dimensional paper chromatography, mostly employed in past studies, is not sufficiently mild because of time required, temperature at which performed, and reactivity of solvents during chromatography. Other problems sometimes encountered in the separation of sugar phosphates and related compounds by paper chromatography could prevent one from finding small amounts of the proposed beta-keto acid diphosphate. These problems include salt effects (causing streaking), "double-spotting" due to solvent phase separation, etc.

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1. NATO Fellow, University of Heidelberg, Germany, 1962-1964.

It seemed worthwhile to attempt to find conditions for the separation of early photosynthesis products by high-voltage-electrophoresis at low temperatures, because the method should be faster and milder than paper chromatography. Also, since it depends on different molecular properties, it might supplement chromatographic procedures.

Galmich has reported the separation of products of photosynthesis for short times with  $^{14}\text{CO}_2$  by one-dimensional paper electrophoresis at  $0^\circ\text{C}$  (7). He found no new substances likely to be intermediates in the early steps of  $\text{CO}_2$  reduction during photosynthesis.

In view of these reported results, in which incomplete resolution of photosynthetic products was achieved, we felt that two-dimensional electrophoresis at two different pH values might be <sup>more</sup> informative. Two-dimensional electrophoresis has been employed by Durrum (8) and by Mead (9) for the separation of amino acids, and by Strain (10) for the separation of inorganic ions and some non-biochemical organic compounds. Other two-dimensional electrophoreses have been reported, but not for the type of mixture in which we are interested.

We have found it necessary to incorporate several techniques different from those of these earlier studies in order to achieve separation of photosynthetic intermediate compounds. Essential to our method are the following features: The paper is supported horizontally in an inert solvent which is cooled by specially-designed cooling coils, resulting in the elimination of local heating effects. Following chromatography in the first dimension, the widths of the areas occupied by compounds are decreased during the application of the second buffer.

Buffers have been chosen to achieve a maximum separation of a wide range of biochemical intermediates. Through the application of relatively high voltage (2.5-4.0 K volts) rapid separation is achieved. Various other features are discussed under "Methods".

#### MATERIALS AND METHODS

The apparatus. A suitable dissipation of the generated heat was accomplished by immersing the paper (wet with buffer) in a bath of decahydronaphthalene (decalin) between two cooling coils. The cooling coils were supplied independently with a circulating cooling mixture (equal volumes of water and ethylene glycol) from an insulated tank by means of two pumps. The contents of the tank were kept at  $-8$  to  $-10^{\circ}$  by a refrigeration unit.

The actual apparatus consists of a square box of Lucite ( $66.4 \times 66.4 \times 11.4$  cm, inside measurements), having two buffer vessels placed at opposite sides. Each buffer vessel capacity is approximately 1800 ml when filled to the edge (buffer vessel measurements:  $46.8 \times 7.7 \times 5.7$  cm). The paper sheet lies horizontally above a square plate of Lucite ( $48.2 \times 48.3 \times 0.65$  cm) that contains 13 rows of 13 holes (2.9 cm in diameter). The paper is actually supported by 12 rows of 12 glass beads (5 mm) fixed in the space between the holes, thus giving point support as well as free circulation of the cooling decalin to the paper (Fig. 1).

Above and below the paper and its support are cooling coils, the upper of which is mounted to the cover of the box. The cover also bears the two platinum electrodes. The cover itself can be raised and lowered by a pulley. Nitrogen pressure is used to push the decalin from a glass container below the apparatus up through a valve and into the system,

after which the valve is closed. The valve is opened to drain the box. The temperature at various points near the paper is measured by 9 thermometers inserted through the cover and almost touching the paper sheet.

Buffers. The two following buffers proved to be suitable for our purposes: First dimension (pH 4.4), 11,760 ml distilled water, 150 ml glacial acetic acid, 90 ml pyridine, and 8 g ethylene diamine tetraacetic acid. Second dimension (pH 2.0), 4500 ml distilled water, 290 ml glacial acetic acid, and 130 ml 25% formic acid.

Papers. Whatman No. 3MI paper was used. This paper has sufficient wet-strength to be handled for chromatography in two dimensions with one drying operation in between.

Photosynthetic  $^{14}\text{CO}_2$  fixation. Bean leaves, as well as algae, were allowed to fix  $^{14}\text{CO}_2$  for a short period (1-30 sec). Subsequent killing and extraction of the material resulted in a suspension that contained the whole material.

Bean leaves. According to size, one single leaf or an excised di- or trifoliate leaf from a bean plant (Phaseolus vulgaris) was placed in an illumination chamber of 80 cc volume and exposed to  $^{14}\text{CO}_2$  as described elsewhere (11). Subsequently, the leaf was plunged into isopentane at  $-160^\circ\text{C}$  and crushed into smaller pieces (10). These were ground to a fine powder in a homogenizer that was cooled with dry ice-acetone. After lyophilization, the dry residue was resuspended in ice water so that the suspension contained the extract of 100 mg fresh weight of the starting material in 0.2 ml.

Algae. Chlorella pyrenoidosa, grown in continuous culture, was harvested and resuspended in  $10^{-3}\text{ M}$   $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$  buffer, giving a 15

(wet packed volume/suspension volume) suspension, 20 ml of which were placed in a simplified "lollipop" that was immersed in a water bath (11). Air, containing 2% CO<sub>2</sub>, was passed through the vessel, which was illuminated from either side by a photospot lamp. After 10-15 min of photosynthesis, the air was replaced by nitrogen for 60 sec. Then, 0.1 ml of NaH<sup>14</sup>CO<sub>3</sub> solution (0.06 M, 1.7 mc/ml) was added with shaking. After a short period of photosynthesis, the algae suspension was run into 80 ml methanol at -20° C. This suspension was then concentrated in vacuo to 1.5 ml at a temperature below 0°.

Electrophoretic technique. The buffer vessels were each filled with 1600 ml of buffer. A sheet of paper (48.4 x 46.4 cm) was dipped into buffer solution, blotted between filter paper, and placed in position under the decalin. The connection of the paper to the buffer vessels was made by paper wicks. These were strips of the same paper, 48.4 cm long and 9 cm wide. They overlapped the edge of the paper sheet by 1 to 1.5 cm.

After 40 min of hydraulic equilibration at 10° C, the system was cooled to 0° C and further equilibrated under the voltage to be used for 20 min.

The cover was lifted and the sample to be analyzed was applied to the origin as a stripe, 2-4 cm long (10 to 25  $\mu$ liters of sample). The cover carrying the electrode, the upper cooling coil, and the thermometers (Fig. 2) was then lowered into position again. The voltage was then applied (conditions given under Fig. 3).

After separation in each dimension, the wicks were removed and the paper (clamped to its support) was taken out and placed in a drying box.

Drying in our chromatography boxes with forced air flow at room temperature requires 1-1/2 to 2 hrs.

For two-dimensional separation, the paper was sprayed with the more acidic buffer after the first drying. Care was taken not to wet a strip 5 to 6 cm wide in which the first separation of compounds had taken place. The wet sections of the paper were blotted again, and the paper was put in position to give a separation at right angles to the first one. After application of the paper wicks, the two buffer fronts start moving together, thus bringing about a concentration of the single zones to single spots. This sharpening of spots after the first dimension seems to be very helpful in obtaining good overall resolution. When the whole strip was completely wet with the buffer, the system was cooled down, and the second run was started.

The radioactive areas of the electrophoretograms were located by radioautography. The compounds were identified by cochromatography and coelectrophoresis, with known compounds as markers.

#### RESULTS

A radioautograph of a two-dimensional electrophoretogram of the products of 20 sec photosynthesis of  $^{14}\text{CO}_2$  by bean leaves is shown in Fig. 3.

As is the case with two-dimensional paper chromatography, it has been possible to separate from a crude extract a large number of compounds from several classes in a single operation. Compounds of similar pK and molecular size are found to move to the same general area of the electrophoretogram. Thus the sugar phosphates are found distributed in a row according to these properties. The resolution of these sugar phosphates

after two-dimensional electrophoresis is somewhat better than is usually achieved with a general two-dimensional paper chromatography.

Despite the mildness of the analytical conditions employed and the excellent resolution achieved, we have been unable to identify any new substances. Moreover, we have not observed on the electrophoretogram any new or unknown radioactive compound which under the usual conditions of two-dimensional paper chromatography gives known compounds such as phosphoglyceric acid.

Similar results (not shown) were obtained upon two-dimensional electrophoresis and radioautography of the products of 20-sec photosynthesis with  $^{14}\text{CO}_2$  by Chlorella pyrenoidosa.

#### DISCUSSION

The method of low temperature, high voltage, two-dimensional electrophoresis described here accomplishes at least as good resolution of complex biochemical mixtures, such as the products of a few seconds' photosynthetic reduction of  $^{14}\text{CO}_2$ , as does two-dimensional paper chromatography. The electrophoretic method requires about two hours of actual running time plus four hours' drying time, whereas the chromatographic method requires 16 to 48 hours' running time plus about 24 hours of drying time. The principal advantage of the electrophoretic technique, however, lies in the milder conditions of analysis. Because of the considerably greater experimental complexity involved in running the two-dimensional electrophoretograms, this method is not likely to supplant chromatography except where the investigator wishes to employ the mildest conditions of analysis, or where the better resolution achieved by electrophoresis is essential.

Our failure to find any new compounds as early products of  $^{14}\text{CO}_2$  fixation by photosynthesis does not eliminate the possibility that such

compounds exist, but it does indicate that any such substances must be extremely unstable to be lost during the mild conditions of analysis. An acid buffer (pH 2.0) was used in the second dimension in order to achieve good resolution of the phosphate compounds. If this buffer, or the drying operation after the first dimension caused the decomposition of some unknown, stable compound, and if all the  $^{14}\text{C}$  was not lost in the process, then we should have found the product of the decomposition in the final chromatogram. For example, ribulose diphosphate is already labeled in the radioautograph shown in Fig. 3. If a beta keto acid were formed in the algae by the carboxylation of this ribulose diphosphate, and if it were subsequently decomposed in the drying operation or in the second chromatography, we should have found a second spot of ribulose diphosphate or of phosphoglyceric acid, or at least a streak in the second dimension. Neither was found, either in this or other experiments. Thus, we may conclude that if such an unstable product of carboxylation is originally formed in the algae, its concentration must be below the limits of detection by the sensitive radioautographic method.

#### SUMMARY

The separation of early products of  $^{14}\text{CO}_2$  reduction during photosynthesis by two-dimensional high voltage, low temperature paper electrophoresis is described. Although excellent resolution was obtained under very mild conditions of analysis, no new products were found.

#### ACKNOWLEDGMENTS

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FIGURES

Fig. 1. Lower cooling coil, plastic support sheet, buffer tanks and electrode from two-dimensional electrophoresis apparatus. For illustration, these parts of the apparatus are shown outside the container. The lower cooling coil is under the plastic sheet (which has holes to permit circulation of cooling liquid). Small glass beads glued to plastic sheet with epoxy resin are for the support of the paper which is not shown. At either end are the plastic buffer tanks and the plastic and platinum electrode assemblies.

Fig. 2. Two-dimensional electrophoresis apparatus in an open (non-operating) position.

The paper can be seen resting on its plastic support sheet and immersed in decalin. The upper part of the assembly shows the upper cooling coils, the electrodes, and the thermometers, all attached to the cover. The upper part of the assembly is raised and lowered by means of a pulley arrangement (not shown). It is kept in this raised position during application of the extract to the origin. During the electrophoresis the upper assembly is lowered until the cover and electrodes are in place. The upper cooling coil is then 1 cm from the paper. The entire apparatus is operated in a fume hood, the doors of which carry interlocks which prevent the application of voltage until the doors are closed.

FIGURES (Cont.)

Fig. 3. Radioautograph of two-dimensional electrophoretogram of products of photosynthesis of  $^{14}\text{CO}_2$  in bean leaves.

Bean leaves of Phaseolus vulgaris were exposed 20 sec to  $^{14}\text{CO}_2$ , killed, and extracted as described in text.  $^{14}\text{C}$ -labeled fumaric, succinic, and citric acids were added to the extract. First dimension, pH 4.4, 75 min at 2.35 kilovolts (53 volts/cm) and 105 mA. Second dimension, pH 2.0, 55 min at 3.35 kilovolts (78 volts/cm) and 90 mA.

Abbreviations: 2-PGA, 2-phosphoglyceric acid; 3-PGA, 3-phosphoglyceric acid; PEPa, phosphoenolpyruvic acid; RuDP, ribulose-1,5-diphosphate; FDP, fructose-1,6-diphosphate; SDP, sedoheptulose-1,7-diphosphate; UDP-Glu, UDP-glucose; UDP-Gal, UDP-galactose; ADP-Glu, ADP-glucose; RMP, ribose-monophosphate; RuMP, ribulose monophosphate; FMP, fructose monophosphate; GMP, glucose monophosphate; SMP, sedoheptulose monophosphate.

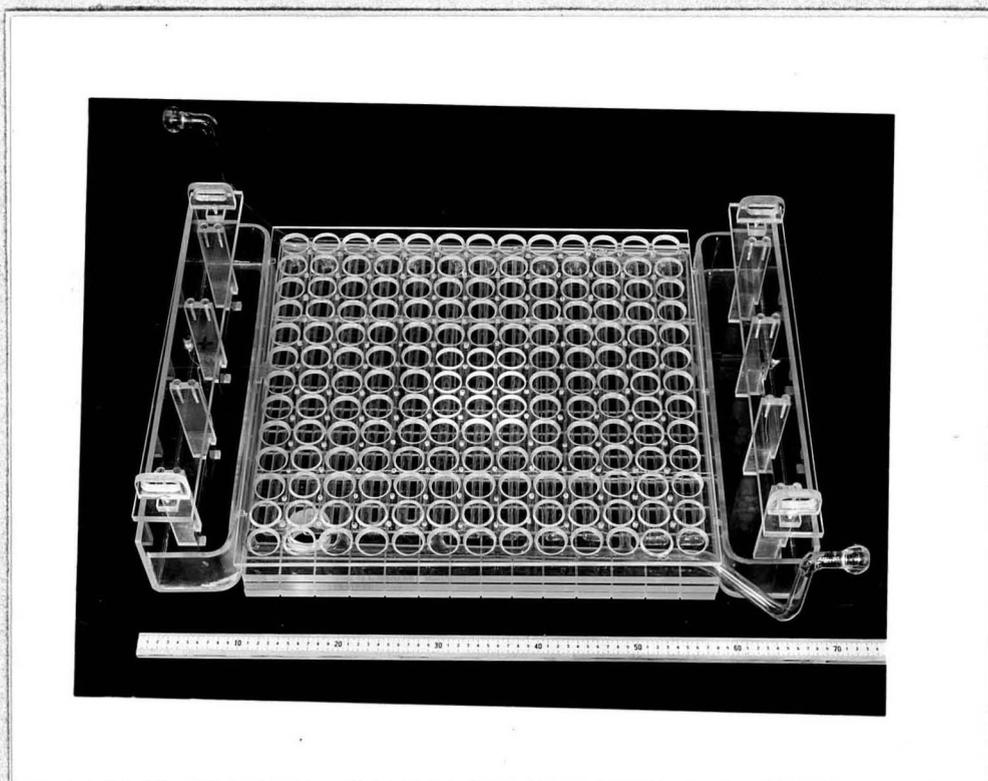


Fig. 1.

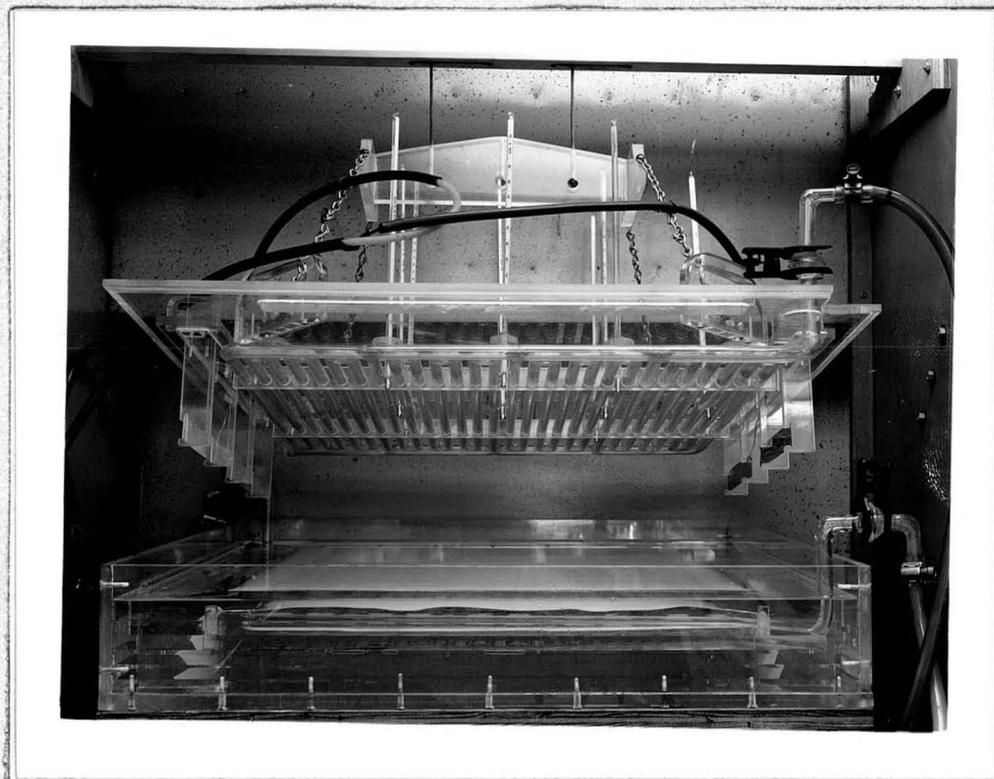


Fig. 2.

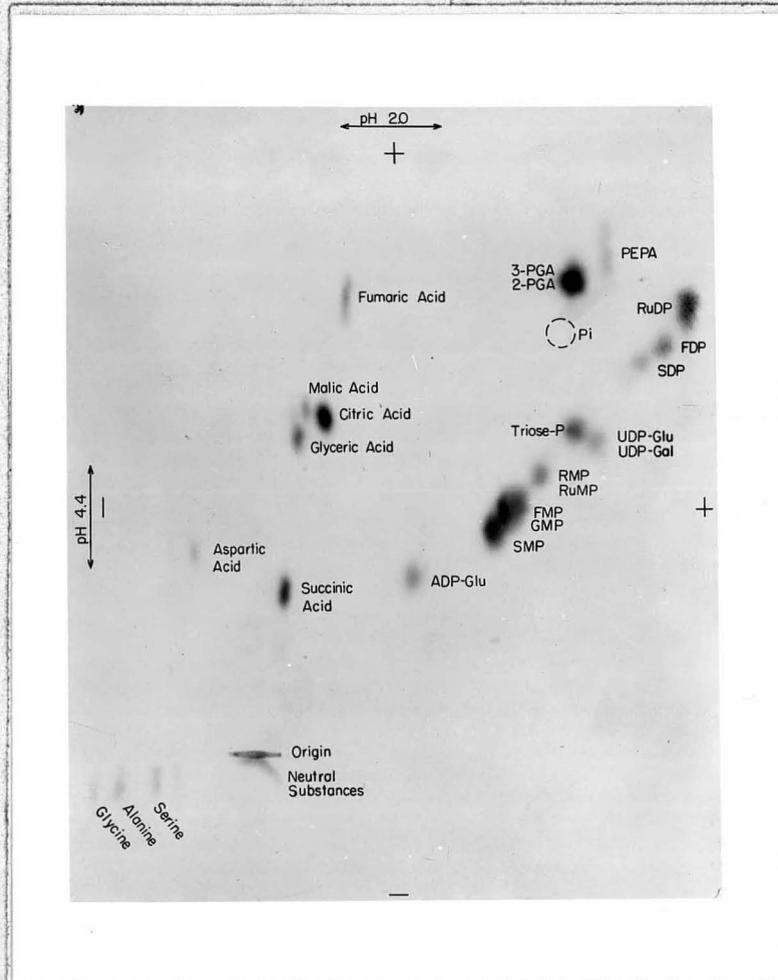


Fig. 3.

