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J. A. Bassham

June 1949

Berkeley, California

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Radiation Laboratory
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ABSTRACT

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1. Methods have been developed for the separation, isolation, identification, and degradation of the carboxylic acids formed in the reduction of carbon dioxide during photosynthesis.

2. These methods have been applied and the results indicate that the primary reduction of carbon dioxide during photosynthesis takes place via two carboxylations of a two carbon acceptor followed by splitting and reduction of the resulting four-carbon acid to two new molecules of acceptor. One molecule of acceptor is then used to continue the reductive cycle and the other is used in photosynthesis.

3. The participation of 2-PGA, phosphoenolpyruvate, phosphoenoloxalacetate, and glycollate in the cycle are discussed and a tentative cycle is proposed.

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* The work described in this paper was sponsored by the Atomic Energy Commission.

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I. Introduction

The reactions which comprise the process of photosynthesis appear to be at least as numerous and complex as those of any other biological process. By employing the results of studies not only of photosynthesis in green plants but also of photosynthetic bacteria and of other biological systems, Van Niel (1) has presented a strong argument for the division of these reactions into two major classes. These are the photolysis of water:

$$2\ H_2O + 2\ B \xrightarrow{\text{Light}} 2\ BH_2 + O_2,$$

and the reduction of carbon dioxide:

$$CO_2 + 2\ AH_2 \xrightarrow{\text{Light}} (CH_2O) + H_2O + 2\ A.$$

While AH_2 and BH_2 may be identical, it seems feasible to include a third

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class of reactions for the transfer of reducing power:

$$2\ BH_2 + 2\ A \longrightarrow 2\ AH_2 + 2\ B.$$

Combination of the three classes of reactions then gives us the general statement of photosynthesis:

$$H_2O + CO_2 \xrightarrow{\text{Light}} (CH_2O) + O_2,$$

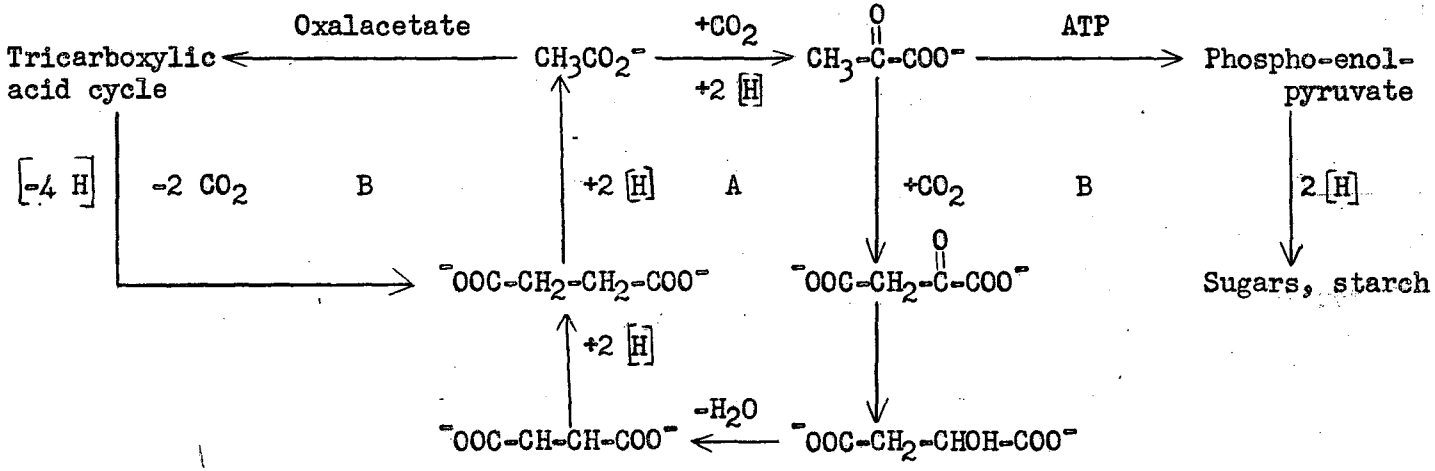
if we consider the products to be of the carbohydrate reduction level.

While very little is known about the details of the photolysis of water and the transfer of reducing power, there is at present a rapid accumulation of knowledge regarding the reduction of carbon dioxide in photosynthesis. This information has been obtained by employing the tracer technique. Using C^{11}, Ruben, et.al. (2,3,4,5) studied the uptake of carbon dioxide by plants both in the light and in the dark. The dark fixation products as well as those from short periods of photosynthesis were found to be largely water soluble and some components exhibited the properties of carboxylic acids, thus lending support to the proposals of Liebig (6), Thimann (7), Ochoa (8) and others that carboxylic acids commonly found in plants are actually intermediates in photosynthesis. The failure of Ruben and coworkers to find any of these acids among the labeled products is understandable in view of the difficulty of working with C^{11}, which has a half life of only 20.5 minutes. Many of the techniques that have been developed for the isolation of intermediates containing the long-lived C^{14} which have repeatedly demonstrated the presence of such acids as fumaric, malic, and succinic, would be inapplicable with C^{11}. The discovery of C^{14} (half life - 5000 years) by Ruben

and Kamen (9) made possible a more detailed investigation of the path of carbon in photosynthesis, but the war, coupled with Ruben's untimely death, interrupted the work. In 1946 the investigation was taken up by Calvin, et.al. (10,11,12,13,14,15) at the University of California, Radiation Laboratory. This study has resulted in the identification of a large number of compounds formed by fixation and reduction of carbon dioxide by Chlorella and other plants. The compounds isolated thus far fall into three general classes: the carboxylic acids, the amino acids, and a group composed of sugars, sugar phosphates, and compounds such as phosphoglyceric acid closely related to sugars through glycolysis. Most of the compounds isolated have been found both in dark fixation of carbon dioxide after a period of illumination of the plant and in short periods (2 seconds to 90 seconds) of photosynthesis. On the basis of early results, Calvin and Benson (10) proposed the tentative path shown in Figure 1 for the reduction of carbon dioxide in photosynthesis. While recent studies have suggested modifications in some details of this cycle, the basic principle of a regenerative two, three, and four carbon carboxylic acid cycle plus the formation of carbohydrates via a reversal of the glycolytic path has been supported by all the subsequent work. The details and modifications of this scheme will be discussed in a later section.

The purpose of the author has been to identify the carboxylic acids involved in the regenerative cycle and to elucidate the details of their formation. In order to apply the tracer method to a study of

Figure 1.


Oxalacetate
+CO2
ATP
Tricarboxylic acid cycle
CH3CO2-
+2 [H]
CH3-C-COO-
Phospho-enol-pyruvate
[-4 H]
-2 CO2
B
+2 [H]
A
+CO2
B
2 [H]
-OOC-CH2-CH2-COO-
-OOC-CH2-C-COO-
Sugars, starch
+2 [H]
-H2O
-OOC-CH-CH-COO-
-OOC-CH2-CHOH-COO-

this nature, it was found necessary to develop several techniques. First a good method of separating and identifying the intermediates of photosynthesis formed with tracer carbon had to be found. This problem was complicated by the relatively large number of labeled intermediates, by the instability of some of the compounds, and by the very minute quantities formed. Thus, while the great sensitivity of the tracer method may detect the presence of a very small amount of a compound, the usual methods of identification -- melting point, quantitative analysis of its elements, etc. -- are quite inapplicable.

Secondly, it was found that the isolation and identification of tracer amounts of the photosynthetic intermediates was greatly facilitated if one had available the suspected compounds, labeled with C^{14}. Consequently, a number of these compounds were synthesized with C^{14}.

Finally, once a compound is isolated and identified, a way must be found to degrade it one carbon atom at a time and thus locate the position of C^{14} within the molecule. This method is particularly useful in studying the regenerative cycle since it demonstrates the derivation of one intermediate from another, enables one to measure the rate of turnover of compounds in the cycle, and affords information regarding the formation of carbon-carbon bonds by carboxylation. The development and use of these methods will be discussed in the following sections.

II. Separation, Isolation, and Identification of Photosynthetic Intermediates

A. Preliminary Fractionation

The carboxylic acids were separated from the other products of carbon dioxide reduction by the methods described by Calvin and Benson (11). One day old cultures of algae, Chlorella pyrenoidosa, or Scenedesmus, as well as leaves from Barley and other plants were employed in various experiments. After a short period of photosynthesis or dark reduction with $C^{14}O_2$, the plants were killed with boiling ethanol, extracted with hot 80% ethanol, and the insoluble material removed by filtration. The filtrate was concentrated at reduced pressure and room temperature, and the concentrated solution brout to pH 6 with dilute alkali. The solution was continuously extracted with ether for six hours to remove lipids. The aqueous layer was acidified to pH 1 and extracted continuously with ether for fifteen hours. This ether extract, which contains the carboxylic acids, is then concentrated at reduced pressure and room temperature.

B. Silica Gel Separation

In the earlier work, a silica gel column was employed to separate the carboxylic acids. The methods described by Isherwood (16) were used for the preparation of the silica gel and the detection of the acid fractions. In addition to some of the acids previously reported, keto-glutaric acid was studied and found to come through the column in about the same range as succinic acid when a mobile phase composed of 90% chloroform and 10% n-butanol was employed. The apparatus used is shown in Figure 2.

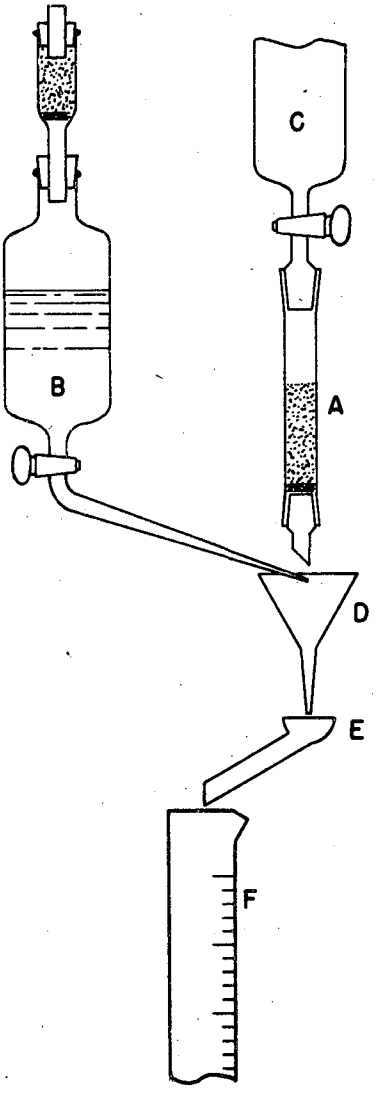


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Figure 2

A. Silica Gel Column

B. Indicator Reservoir

C. Developing Solvent Reservoir

D. E. Mixing Funnels

F. Receiver

The procedure for analyzing for acids containing C^{14} was as follows. Three grams of dry silica gel were mixed with 2 ml. 0.5 N H_2SO_4 and the mixture run through a 90 mesh sieve. About 20 ml. of 10% butanol-chloroform solution was added to the dry powder, the mixture poured into the column, and the solvent permitted to run through. The pH 1 ether extract from preliminary separation was evaporated to dryness at room temperature, about 10 mg. of carrier acids added, and the mixture taken up in 200 of n-butanol. Chloroform was then added until the total volume was 2.0 ml. and any insoluble material was removed by centrifugation. After the solution had run in, three 2-ml. portions of 10% butanol-90% chloroform solution were added, each being allowed to run into the silica gel. The column above the silica gel was then carefully filled with 10% butanol-chloroform solution and a reservoir of this developing solution attached. The rate of flow of solvent through the column, about 30 ml. per hour was maintained by applying a pressure of 1 to 4 pounds per sq. in. of nitrogen as needed. The flow from the silica gel column was combined in the mixing funnels with a slower flow (0.1 as much) of indicator solution. (The indicator solution was made by dissolving 20 mg. thymol blue in 2 ml. of 0.1 N NaOH and 100 ml. of CO_2 free H_2O). When there is too little organic acid to detect in the solvent coming off the column, the aqueous phase is blue while the organic phase is colorless. As soon as a detectable amount of acid begins to come through, the aqueous phase turns yellow and the organic phase pink. The various acidic fractions as well as the intermediate cuts were collected in separate receivers,

concentrated at room temperature, and aliquots counted.*

After about 110 ml. of the 10% butanol-chloroform solution had passed through the column, the developing solvent was changed to 20% butanol-chloroform in order to elute from the silica gel acids with greater solubility in water relative to their solubility in the organic phase. Finally the column was washed with 100% butanol in order to remove as much as possible of any remaining acids.

The results of a typical experiment are shown in Table 1. It should be kept in mind in considering the data presented therein, that while the absence of activity in a given acid fraction indicates that the acid has not been a product of the reduction of labeled carbon dioxide in that particular experiment, the presence of activity in a given fraction does not prove that the carrier acid is the same as the labeled product. Thus in the example shown, the activity which was found in the first fraction was not acetic acid, since any labeled acetic acid would have been removed in the concentration of the pH 1 extract.

In order to demonstrate the identity of the activity in a given fraction, it was necessary to acidify the concentrated fraction and once again continuously extract with ether. The ether extract was

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* Unless otherwise specified, all counts referred to in this paper were made with very thin samples (less than 0.1 mg./cm^2) so that self absorption could be neglected. These thin plates were made with the turntable apparatus developed by A. A. Benson. (17).

then evaporated to dryness, and the residue was recrystallized, usually after adding an additional measured amount of carrier. Several recrystallizations were carried out, the specific activity being determined after each recrystallization. When the specific activity is unchanged after a recrystallization, the activity originally present in the acid is obtained from the specific activity and the total amount of carrier added. For example, if 40 mg. of malic acid were added to fraction 8, shown in Table 1, and if 10 mg. of malic acid had been added to the original mixture of acids, the total carrier added would be 50 mg., which can be taken as the total amount of malic acid present since the acid from the plant is of negligible weight. If the specific activity were found to be 4000 counts/mg. the total malic acid activity would be 200,000 cts/min. or 95% of fraction 8. Because of the danger of co-crystallization, even with repeated recrystallizations, a derivative should then be prepared, recrystallized and its specific activity obtained. In the case of malic acid, dehydration (see Section III C) gives fumaric acid which is easily separated and purified. The fumaric acid thus formed must have a specific activity of 134/116 or 1.15 that of malic acid.

During the time when the silica gel method of separation was employed, most of the experiments involved fixation of labeled carbon dioxide in the dark, after a period of illumination and carbon dioxide starvation, in which case most of the carboxylic activity appears as malic acid. However, the silica gel method could be applied to the

separation of other acids which have been found since then, and would have been, had it not been for the advent of an easier and more satisfactory method which will be described in the following section.

C. Paper Chromatographic Separation

The methods which had been described previously (18,19) for the separation of amino acids by paper chromatography were applied by Stepka, Benson, and Calvin (12) to the separation of amino acids obtained from plants which had been permitted to reduce carbon dioxide labeled with C^{14}. All amino acids present were detected by the ninhydrin color test while those containing C^{14} were detected by scanning the paper with a Geiger counter tube and, more accurately, by making a radioautograph. A superposition of the transparent radioautograph on the original chromatogram which has been sprayed with a ninhydrin solution to develop the color with the amino acid spots then suffices to demonstrate which amino acids were labeled with C^{14} during the course of the experiment.

It was found that if the alcohol extract from the whole cells was concentrated and applied to the paper and the chromatogram developed in the usual way, good separation of a number of labeled compounds other than amino acids was obtained. The presence of these compounds is indicated by darkening of the radioautograph film in positions not occupied by amino acids. When tests are found which identify these compounds, it becomes possible to accomplish an almost complete separation, isolation, and identification of a large number of biological substances

in one operation.

The method used in preparing the chromatograms is as follows. The solution or mixture to be analyzed is concentrated to 0.5 ml. or less and applied with a dropper to a circle 15 mm. in diameter near the corner, two inches from each edge of a piece of Whatman No. 1 filter paper, 18 x 22-1/2 inches. A one inch fold is made along the long edge nearest the starting spot or "origin" and the fold is placed in a long shallow trough where it is held by a glass rod weight. The paper passes over a horizontal bar slightly higher than the edge of the trough which is supported in a frame within a vapor-tight wooden box and the box is closed. The troughs and boxes used are shown in Figure 3. The box is kept in a room in which the air temperature is thermostated at 25°C. The solvent advances down the paper with a straight front and reaches the bottom edge in 16 to 20 hours. The paper is then removed from the trough and hung in a drying room.

After the paper is free of phenol the entire process is repeated with a different solvent and with the flow across the paper at a right angle to the direction of flow of the first solvent. While collidine and lutidine have been used very successfully as a second solvent for amino acid separations (18,19), it was found by Calvin, Benson and Stepka that a solvent composed of a n-butanol, propionic acid, and water is more satisfactory for separation of some of the other products of carbon dioxide reduction in plants. In order to avoid the slow formation of butyl propionate, the solvent is prepared just before it

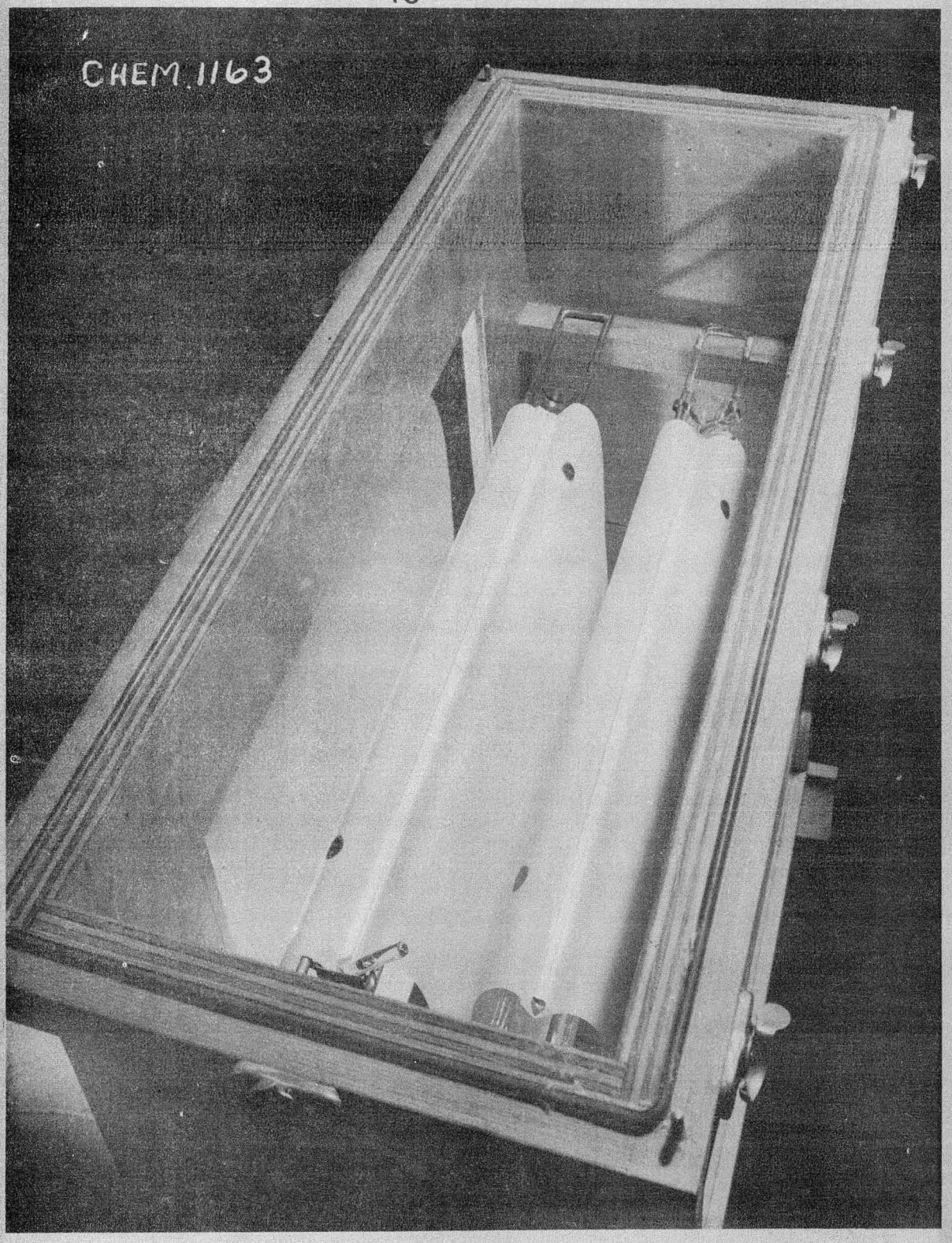

FIGURE 3

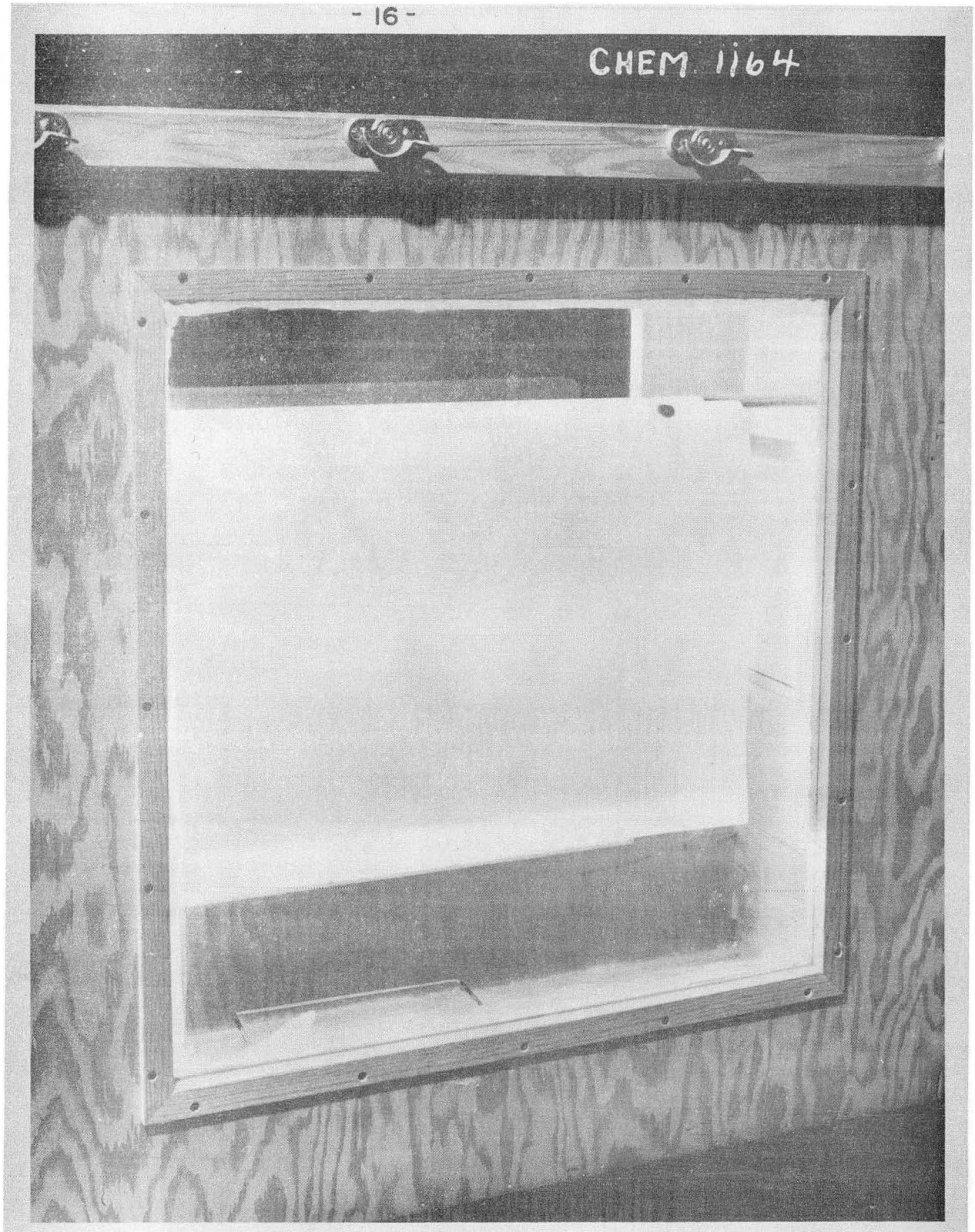

FIGURE 3

is used from equal volumes of two solutions, one composed of 1246 volumes of n-butanol and 84 volumes of water, the other composed of 620 volumes of propionic acid to 790 volumes of water.

After the chromatogram has been freed of the second solvent in the drying room, the paper is usually scanned with a Geiger tube to ascertain the approximate locations of radioactive compounds. The paper is then placed against a sheet of Eastman No-Screen X-ray film and the film exposed for one to thirty days, depending on the amount of radioactivity and the number of compounds in which it is distributed. The film is then developed and the original paper may be sprayed to identify the amino acid "spots". Radioautographs of typical chromatograms are shown on pages 54-56.

The other spots must then be identified in different ways. The phosphate esters have been identified by Calvin, Benson and Haas (15) by a variety of methods, including syntheses by plants and by yeast fermentations with radioactive phosphorus, P^{32}, hydrolysis of unknown phosphorus-containing spots and chemical tests with the products, and phosphate color tests.

As previously mentioned, most of the carboxylic acids can be removed from a pH 1 aqueous phase by continuous ether extraction. This property of limited solubility in ether serves to distinguish the organic acids as a class from the other compounds encountered in this work and also provides a characteristic physical constant for each acid. This constant is the ether-water distribution coefficient, D, which will be defined

here as the concentration of organic acid in the ether phase divided by the concentration in the aqueous phase, at pH 1, where the organic acid has been equilibrated between the two phases. Thus it is possible to cut out a spot from a paper chromatogram, elute the substance from the paper with a couple drops of water, acidify the eluate with a little hydrochloric acid, add ether, equilibrate, count aliquots from the two phases, and in this way determine the distribution coefficient of tracer amounts of C^{14}-labeled acids. This can then be compared with the distribution coefficients of known acids, and the identity of a substance occupying a given spot is thereby limited to a very few compounds.

Labeled succinic acid had been isolated from Chlorella pyrenoidosa by Calvin and Benson (11) in early studies of dark reduction of labeled carbon dioxide, and the presence of succinic and malic acids had been demonstrated by the silica gel method in later experiments; therefore these acids were among the first to be studied on the paper chromatograms. For this purpose these acids and some closely related acids were synthesized with C^{14} labels at high specific activity so that they might be applied to the papers and their positions found. The syntheses of these acids will be discussed in section III. Malic, succinic, fumaric oxalacetic, tartaric, glycolic, glyceric and isocitric* acids have all been applied to the papers and the positions of all except oxaloacetic, which decomposes and streaks on the paper, have been determined.

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* The author is indebeted to Dr. R. E. Stutz for supplying some isocitric acid labeled with C-14.

The labeled acids were first applied one at a time, each with serine and alanine, to different chromatograms and their positions relative to these standards determined. They were then applied two, three and four at a time until all their positions relative to each other were known.

It was then possible to view a chromatogram of unknown plant constituents and predict from its position which spot might be one of the organic acids being investigated. The suspected acid at several times the activity of the unknown spot is then added to the cell extract and the mixture applied to a new paper. If the unknown substance is identical with the added acid, no additional spot will appear on the second chromatogram and the investigated spot will contain the sum of its activity on the first paper and the added activity.

Another technique used to identify an unknown spot, is to elute the spot from the paper, add a known labeled acid and apply the mixture to a new paper. If the two compounds are identical, only one spot will be obtained.

It was found that the positions of these acids vary somewhat more from experiment to experiment than do the positions of neutral amino acids and sugars. In order to understand the movement of a given acid on a paper chromatogram it is necessary to consider briefly the theory of movement of a solute in a partition chromatogram.

According to Consden, Gordon and Martin (18) the R_F of a solute is given by

$$R_F = \frac{A_L}{A_L + \alpha A_S}$$

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 α, the distribution coefficient of the solute, is the concentration of solute in the stationary phase divided by the concentration of solvent in the mobile phase. This expression can be written as

$$R_F = \frac{1}{1 + \alpha A_S/A_L} .$$

The term A_S/A_L is a constant for a given paper and set of solvents while α 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 carries the acid past this point there is a decreasing concentration of acid and increasing degree of ionization 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. (20).

This effect can be avoided by maintaining the pH of the aqueous phase at a constant value. This is accomplished either by employing an acidic solvent or a buffered solvent. Lugg and Overell (20) 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 acids so that the R_F values depend principally on the distribution coefficients of the free acids.

It is possible to maintain the pH of the aqueous phase at a higher level by employing buffers. This can be accomplished either by applying the buffer to the origin and allowing the water-saturated mobile solvent to carry it along, or by dissolving the buffer in the solvent beforehand. The R_F value then depends on both the distribution coefficient and the dissociation constant of the carboxylic acid.

It is desirable in two-dimensional paper chromatography to employ a low pH solvent in one direction and a higher pH solvent in the second 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 (20), 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 Figure 5. Such "area" separation is desirable in analysis of plant extracts in that it circumvents preliminary fractionations.

The butanol-propionic acid solvent served as the acidic solvent and water-saturated phenol was employed as the other solvent. 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. The paper is run first in phenol so the buffer is carried along the paper for a short distance in the same path as the acids. Since the buffer capacity thus obtained is limited, it is necessary to use very small amounts of acids. From one to five micrograms of acids 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.

As would be expected, the R_F values decrease with increasing pH of the buffer and even with buffers of the same pH, some variation is obtained due perhaps to variations in movement of the buffer on the paper. For purposes of separation and identification, however, the relative rather than the absolute positions of the acids is adequate. The R_F values reported are the average of those obtained from a number of chromatograms on which the buffer applied to the origin was from pH 6 to pH 7.

The R_F values of the carboxylic acids as well as the values for other compounds studied by members of this laboratory are shown in Figure 4.

The relation of R_F values in the acidic solvent to the distribution coeffeicients is shown in Table 2. The distribution coeffeicients of the

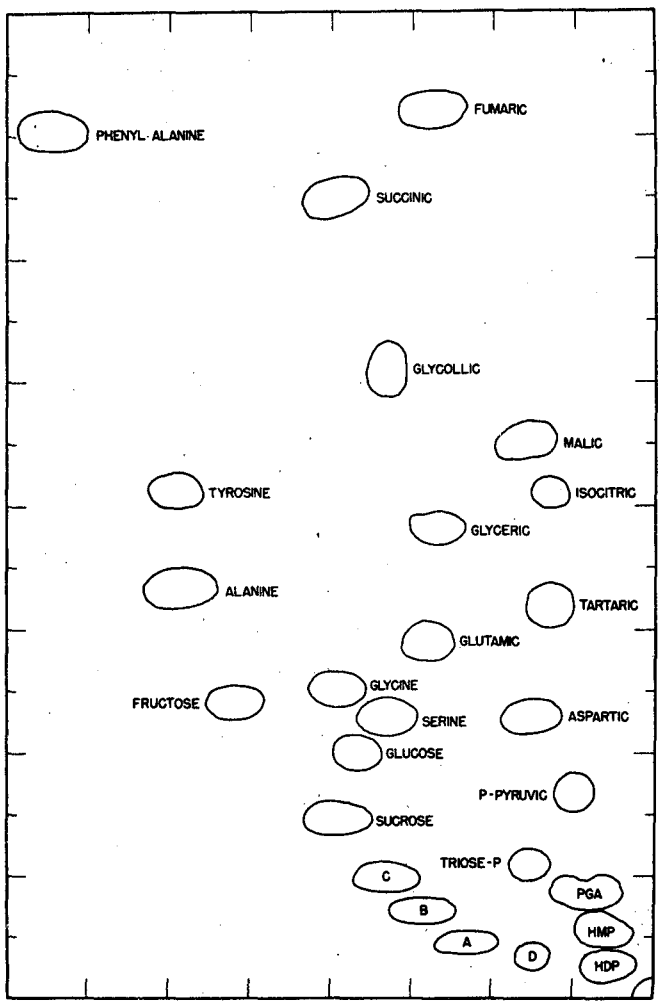

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Figure 4

solutes between butanol-propionic acid solvent and water were determined in a manner similar to the determination of ether-water distribution coefficients already described. The volumes of organic solvent and water used were so adjusted that the final volumes of organic phase after equilibration would be about two times that of the water phase. This was done in order to approximate the conditions on the paper where the ratio of aqueous phase to organic phase is found to be 0.55 according to the calculation below. The necessity for approximately duplicating the ratio existing on the chromatogram arises from the fact that some of the propionic acid is extracted into the aqueous phase and the composition and hence the solvent properties of the remaining organic phase is thus dependent on the volume ratio of the two phases, since this ratio determines how much of the propionic acid is extracted.

The R_F values and distribution coefficients of alanine and glucose were also measured and alanine was used as a standard, its constants being employed to calculate A_S/A_L.

$$A_S/A_L = (1-R_F)-R_F\alpha$$
$$= (1-.333).333 \times 3.65$$
$$= 0.55$$

The R_F values of the other compounds studied were then calculated according to the expression:

$$R_F = \frac{1}{1 + 0.55\ \alpha}$$

Table II

Comparison of Measured and Calculated R_F Values

in Butanol-Propionic Acid-Water Solvent

Solute	α	R_F (calculated)	R_F (measured)
Alanine	3.65	---------	0.333
Fumaric	0.50	0.78	.72
Succinic	1.02	.64	.65
Glycollic	2.06	.47	.51
Malic	2.40	.43	.45
Isocitric	2.75	.40	.41
Glyceric	2.95	.38	.38
Tartaric	3.20	.36	.32
Glucose	5.75	.24	.20

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.

D. Identification of Labeled Compounds

In addition to the techniques already discussed it is often desirable to confirm the identity of a compound by some additional method. The conversion of malic acid to fumaric acid previously mentioned is an example of this type of test. The usual procedure is to add to the tracer substance a weighed amount of the inactive compound. This mixture is recrystallized and the specific activity checked. The purified material is then converted to another compound which is purified and its specific activity measured, as well as its distribution coefficient. If the distribution coefficient of the activity now agrees with that of the derivative the proof of the identity of the original compound is unequivocal.

Succinic acid has been converted to succinic-dihydrazide (see Section IV-A) and the specific activity of this derivative checked. Other derivatives which might be prepared with the acids studied include tartaric acid from fumaric acid and various high molecular weight esters from each of the acids.

III. Syntheses of Intermediates with C^{14}

A. Malic and Succinic Acids

The synthesis of malic and succinic acids labeled with carbon fourteen from carboxyl-labeled sodium acetate has been carried out through the following reactions:

(1) $2\ CH_3C^{14}O_2Na + (C_2H_5)_2SO_4 \longrightarrow 2\ CH_3C^{14}O_2H_5 + Na_2SO_4$

(2) $CH_3C^{14}O_2C_2H_5 + C_2H_5O_2C\text{-}CO_2C_2H_5 \xrightarrow{NaOCH_3} C_2H_5O_2C\text{-}CO\text{-}CH_2C^{14}O_2C_2H_5$

(3) $C_2H_5O_2C\text{-}CO\text{-}CH_2C^{14}O_2C_2H_5 + H_2 \xrightarrow{Pt} C_2H_5O_2C\text{-}CHOH\text{-}CH_2C^{14}O_2C_2H_5$ and $C_2H_5O_2C\text{-}CH_2CH_2C^{14}O_2C_2H_5$

(4) $2\ H_2O + C_2H_5O_2C\text{-}CHOH\text{-}CH_2C^{14}O_2C_2H_5 \xrightarrow[(2)\ H^+]{(1)\ OH^-} HO_2C\text{-}CHOH\text{-}CH_2C^{14}O_2H + 2\ C_2H_5OH$

and

$2\ H_2O + C_2H_5O_2C\text{-}CH_2CH_2C^{14}O_2C_2H_5 \xrightarrow[(2)\ H^+]{(1)\ OH^-} HO_2C\text{-}CH_2CH_2C^{14}O_2H + 2\ C_2H_5OH$

The carboxyl-labeled sodium acetate was obtained from the Bio-Organic Group of the Radiation Laboratory and was prepared from radioactive barium carbonate by the methods described by Tolbert (21).

Conversion of $CH_3C^{14}O_2Na$ to $CH_3C^{14}O_2C_2H_5$

One gram of sodium acetate labeled in the carboxyl position with 6.0 millicuries of radiocarbon was placed in a 20 ml. ampule with a long neck and 5 ml. of freshly redistilled diethyl sulfate plus 10 % concentrated H_2SO_4 were added. The ampule was evacuated and then filled with dry nitrogen at atmospheric pressure. It was then sealed and the lower part

containing the reactants immersed in an oil bath at 160° to 175° for two hours, with occasional gentle shaking, care being taken to avoid splashing the reactants up the sides. The ethyl acetate produced refluxes, condensing in the cool neck of the ampule. At the end of this time, the ampule was cooled to room temperature, then immersed in liquid nitrogen. The neck was opened and quickly attached to a U-tube connected to a vacuum system. Both the receiver on the other arm of the U-tube and the ampule were immersed in liquid nitrogen and the system evacuated. The U-tube was then closed off from the vacuum line and the liquid nitrogen bath removed from the ampule. Several hours were allowed for the ethyl acetate to distill into the receiver which was then closed off, removed from the liquid nitrogen bath and detached from the U-tube. Some diethyl sulfate distills over with the product but is not detrimental to the next reaction since it reacts with the catalyst, sodium methoxide to give sodium sulfate and methyl ethyl ether:

$$(C_2H_5)_2SO_4 + 2\ NaOCH_3 \longrightarrow 2\ C_2H_5OCH_3 + Na_2SO_4$$

Conversion of $CH_3C^{14}O_2C_2H_5$ to $C_2H_5O_2C\text{-}CO\text{-}CH_2C^{14}O_2C_2H_5$

Reaction 2 is a modification of a general procedure, described by Adickes and Andresen (22) for the syntheses of α-keto acids and involves a Claisen condensation of diethyl oxalate and a fatty acid ester, in this case, ethyl acetate. The reaction was carried out in a 120 ml. three-necked flask fitted with a mercury seal stirrer and reflux condenser equipped with a $CaCl_2$ drying tube. About 40 ml. dry ether was distilled into the flask and 1.4 grams of sodium methoxide plus 2 ml. diethyl oxalate

quickly added and the third neck stoppered. The flask was warmed with a water bath until the ether refluxed and the solution became pale yellow. The solution was allowed to cool and the ethyl acetate tube was attached to the third neck of the flask. The ethyl acetate was gently distilled into the reaction mixture and the tube was cooled and warmed several times to insure flushing of all the ethyl acetate vapor into the reaction flask, ether vapor providing the flushing gas. The third neck was again stoppered and the flask warmed on the water bath at 37°C with stirring for sixteen hours.

After cooling, the reaction mixture was transferred to a flask containing 50% H_2SO_4 kept colder than 0°C by occasional immersion in an acetone and solid CO_2 bath. After stirring the contents of the flask, the ether layer was removed and the acid-aqueous phase extracted several times with ether. The combined ether fractions were concentrated at 0°C at reduced pressure. The crude ester mixture was used directly in the next step of the synthesis.

Conversion of $C_2H_5O_2C\text{-}CO\text{-}CH_2C^{14}O_2C_2H_5$ to $C_2H_5O_2C\text{-}CHOH\text{-}CH_2C^{14}O_2C_2H_5$ and $C_2H_5O_2C^{14}CH_2CH_2C^{14}O_2C_2H_5$

Reaction 3 was carried out in a small three-necked flask fitted with a gas tight induction stirrer and a hydrogen inlet tube, which is connected through two three-way stopcocks to a hydrogen tank, an aspirator and a 500 ml. gas buret with a leveling bulb. This apparatus is shown in Figure 5.

The crude esters from the previous reaction were diluted with 20 ml. 95% ethanol and placed in the reaction flask with 400 mg. PtO_2.

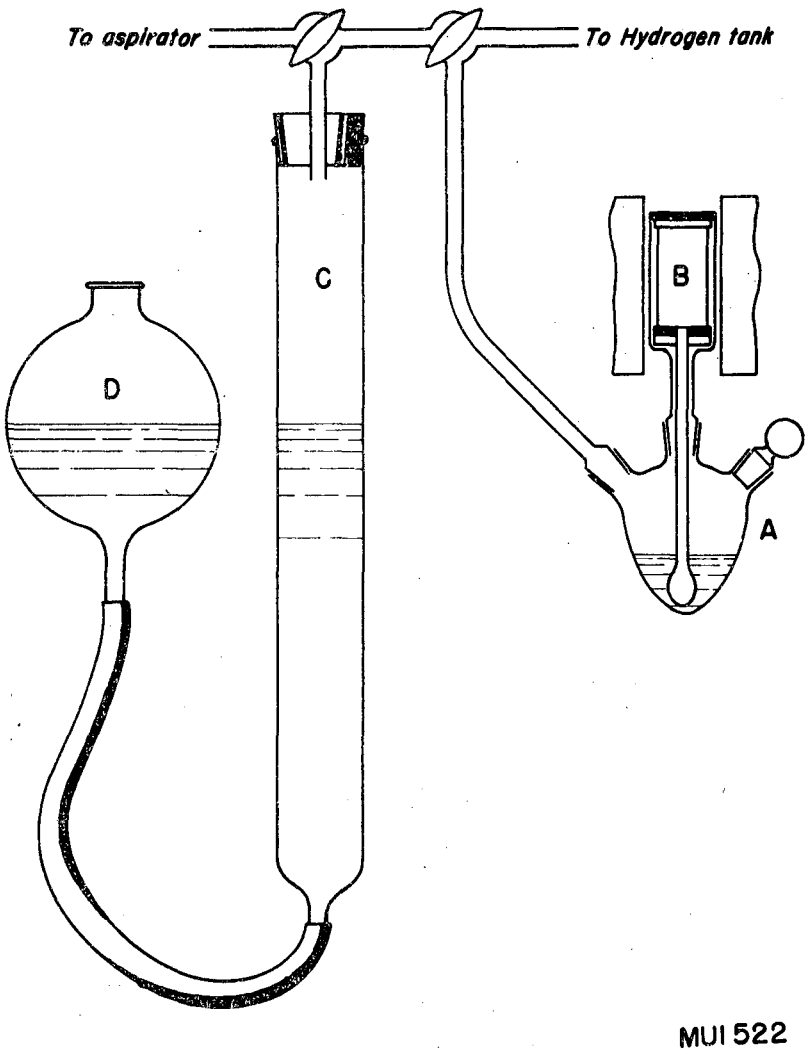


Figure 5

Hydrogenation Apparatus

A. Reaction Vessel

B. Induction Stirrer

C. Gas Buret

D. Leveling Bulb

Assuming 100% yield from sodium acetate, there were 12.20 millimoles of diethyl oxalacetate which, together with 1.765 millimoles of PtO_2, would use up 15.73 millimoles of hydrogen gas or 382 cc. at 23°C, 1 atmosphere pressure, if diethyl malate were the only reduction product.

The flask was stoppered and the system filled with hydrogen by reducing the pressure by means of the aspirator until the ethanol just began to boil, closing the stopcock to the aspirator and admitting hydrogen to the system. This procedure was repeated about five times, after which the water in the gas buret was displaced by hydrogen to 450 cc., the internal pressure of the system was adjusted to one atmosphere with the leveling bulb, and the stopcock to the hydrogen supply closed. The stirrer was then started and the uptake of hydrogen followed with the gas buret. The reaction was carried out at room temperature (23°C). In one hour the hydrogen uptake was 328 cc. and in two hours the hydrogen uptake had stopped with 372 cc. having been absorbed.

Conversion of the Esters to the Free Acids

The flask was then opened, the solution removed from the catalyst with a dropper, the catalyst rinsed and the rinsings and solution of products combined and shaken 12 hours with 100 ml. 0.5 N NaOH solution at room temperature. The mixture neutralized, concentrated at reduced pressure, and transferred to a 100 cc. centrifuge cone, where 8 ml. of 1 M $CaCl_2$ solution was added, precipitating calcium oxalate. The supernatant solution was removed with a syringe and combined with several rinsings of the calcium oxalate precipitate. Counting of the precipitate showed no appreciable percentage of the starting activity.

The solution of free acids was acidified with 3 ml. 1 N H_2SO_4 and continuously ether extracted overnight. The ether extract was then evaporated to dryness at room temperature and reduced pressure. The crude residue contained a mixture of malic and succinic acids.

A small amount of the mixture was applied to a chromatogram and the succinic and malic spots identified. No other spots were produced.

Of the 1140 mg. of mixed product obtained, 861 mg. or .755 of the yield were taken up in 0.1 N HCl and separated by repeated fractional ether extractions. If M_n is the fraction of a compound remaining in the aqueous phase after n ether extractions, $\frac{V_2}{V_1}$ the ratio of the ether volume to the water volume in each extraction and D the distribution coefficient, then M_n is given by

$$M_n = 1/(1 + D \frac{V_2}{V_1})^n.$$

For succinic acid, D = 0.15 and for malic acid, D = 0.017. If $V_2/V_1 = 2$, the fraction of succinic acid remaining in the aqueous phase after 10 extractions will be 0.0726 and that of the malic acid will be 0.730. The combined ether extracts can then be extracted with suitable volumes of 0.1 N HCl to remove malic acid preferentially from the ether phase. The resulting intermediate fraction can then be subjected to the same two processes. When the aqueous solutions contained more than 15 parts of malic acid to one part of succinic and the ether solution contained the opposite proportion of these acids, the solutions were evaporated to dryness at reduced pressure and the acids each recrystallized from ethyl acetate and toluene solution. The mother liquors were combined with the

intermediates from the ether extractions and the entire process of ether extraction and recrystallization repeated. A yield of 308 mg. succinic acid or 28.4% of theoretical and 481 mg. malic acid or 39% of theoretical was obtained, so that the total yield was 67.4% from sodium acetate. The specific activity of the malic acid was 3.68 μc./mg. and that of the succinic acid was 4.17 μc./mg. In the case of malic acid only the carboxyl carbon adjacent to the hydroxy-substituted carbon atom is labeled while in succinic acid the label is equally distributed between the two carboxyl groups since the molecule is symmetrical.

B. Oxalacetic Acid

Crude diethyl oxalacetate prepared according to the procedure described above, was dissolved in 30 ml. ether and extracted with 2 ml. portions of 10% K_2CO_3 solution until addition of a drop of 1% $FeCl_3$ solution to a small sample of the ether solution plus 50% ethanol no longer gave the deep red color of a positive enol test. The aqueous solution was then neutralized and acidified at 0°C with dilute sulfuric acid and extracted several times with ether. The combined ether extracts were concentrated at reduced pressure and the ester dissolved in concentrated hydrochloric acid at 0°C. The solution was kept at 5°C for three days. The hydrochloric acid and alcohol were evaporated in vacuo at 0°C. The residue was recrystallized from acetone and toluene. The yield of pure acid was 27%, based on sodium acetate.

C. Fumaric Acid

Carboxyl-labeled fumaric acid was prepared in the following way.

A small ampule containing 49.7 mg. carboxyl-labeled malic acid having a specific activity of 3.68 μc./mg. was evacuated and filled with dry nitrogen at atmospheric pressure. The ampule was sealed and immersed in an oil bath at 140° to 150°C for two hours. After cooling, the ampule was opened and the fumaric and malic acid mixture transferred to a 2 ml. centrifuge tube. The fumaric acid was recrystallized from water, centrifuged, washed, dried and weighed. The yield was 29.5 mg. or 68.5% of theoretical, and 13.0 mg. or 26% of the malic acid was recovered. The specific activity of the fumaric acid is 4.25 μc./mg. The product gives only one spot on a chromatogram and its distribution coefficient is 1.0. The label is distributed equally between the two carboxyl carbons, each having one half the specific activity of the labeled carboxyl carbon in malic acid.

D. Tartaric Acid

Carboxyl-labeled fumaric acid was converted to carboxyl-labeled dl-tartaric acid by oxidation with sodium chlorate in the presence of osmic acid.

$$3\ HO_2C^{14}CH{=}CH{-}C^{14}O_2H + 3\ H_2O + ClO^-_3 \xrightarrow{OsO_4} 3\ HO_2C^{14}CHOH{-}CHOH{-}C^{14}O_2H + Cl^-$$

The procedure of Milas and Terry (23) was modified slightly for preparation of a small quantity of tartaric acid.

To a solution of 1.6 g. of sodium chlorate and 50 mg. C^{14}-labeled fumaric acid dissolved in 2.0 ml. H_2O at 50°C was added 200 λ of 1% osmic acid solution. The solution was warmed on a water bath at 50° for nine hours at which time it was cooled and the osmic acid removed

by extraction with benzene. An excess of barium chloride solution was added and the precipitate of barium tartrate centrifuged, dried and weighed. The barium tartrate was decomposed by warming with a calculated amount of dilute sulfuric acid for 1/2 hours, after which the barium sulfate was removed by centrifugation and the supernatant solution concentrated to about 200 λ.

A drop of alcohol was added and the solution cooled to 0°C. The tartaric acid crystallized out and was centrifuged and washed a couple of times with a drop of cold 50% alcohol, then dried and weighed. A yield of 15.0 mg. or 23% was obtained. The product gave only one spot on a paper chromatogram and had the proper distribution coefficient, 0.004.

E. Glycollic, Glyoxylic and Oxalic Acids

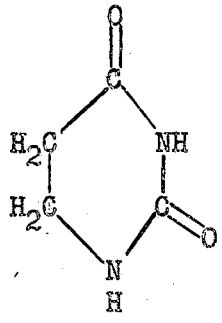
Carboxyl-labeled glycollic acid was obtained from the Bio-Organic Group of the University of California Radiation Laboratory and was prepared by monochlorination of carboxyl-labeled acetic acid followed by hydrolysis (24).

The syntheses of glyoxylic and oxalic acids is contemplated in the near future. Chlorination of labeled acetic acid to dichloroacetic and subsequent hydrolysis would give glyoxylic acid while tri-chlorination of acetic acid and hydrolysis would yield oxalic acid. An alternative preparation of glyoxylic acid is the oxidation of tartaric acid by periodate (25).

IV. Degradations

A. Succinic Acid

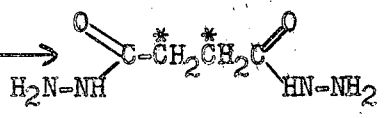
The first organic acid isolated from algae which had reduced labeled carbon dioxide was succinic acid and therefore it was this acid which was first degraded. It was believed that pyrolysis of a salt of succinic acid to produce decarboxylation, and measurement of the activity in the resulting carbon dioxide might not provide an unequivocal measurement of the carboxyl-carbon activity due to the possibility of exchange reactions at high temperatures. The Hoffman degradation is apparently not applicable since it has been reported that treatment of succinic diamide with potassium hypobromite solution produces a compound of the formula $C_4H_6O_2N_2Br_2$ which on treatment with alkali gives β-lactyl urea,

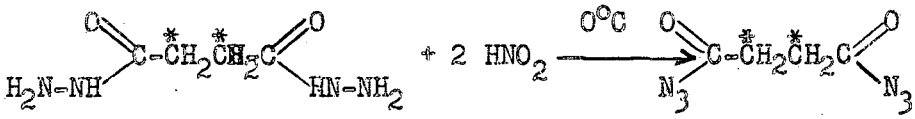


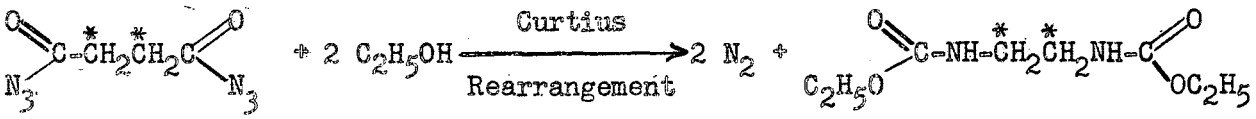
(26)

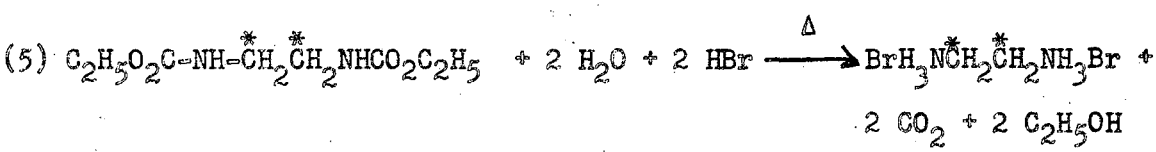
A satisfactory degradation of succinic acid was found in the Curtius Reaction (27) and the following series of reactions have been carried out.

(1) $HO_2C\text{-}\overset{*}{C}H_2\overset{*}{C}H_2CO_2H + 2\ CH_2N_2 \longrightarrow 2\ N_2 + CH_3O_2C\text{-}\overset{*}{C}H_2\overset{*}{C}H_2CO_2CH_3$

(2) $CH_3O_2C\text{-}\overset{*}{C}H_2\overset{*}{C}H_2CO_2CH_3 + 2\ N_2H_4 \longrightarrow$  $+ 2\ CH_3OH$

(3) 

(4) 

(5) 

Succinicdihydrazide

Reaction 1 was carried out in a 60 ml. conical flask into which was placed 280 mg. of labeled succinic acid and 10 ml. ether. A solution of diazomethane in ether was added with shaking until the yellow color of diazomethane persisted. The solution was allowed to stand several hours after which the ether and excess diazomethane were removed by evaporation at 0°C and reduced pressure. Throughout the procedure the usual safety precautions were observed in working with diazomethane. The ester was distilled from the residue *in vacuo* into a small liquid nitrogen trap, after which it was transferred with a dropper and several rinsings with ethanol into a 30 ml. conical flask.

Reaction 2, the conversion of diethyl succinate to succinic dihydrazide was carried out according to the procedure of Schäfer and Schwan (28), which was adapted to small-scale synthesis. To the ester-alcohol solution was added 300 mg. 85% hydrazine hydrate. The flask was fitted with a small reflux condenser and the reaction mixture refluxed on a steam bath for five hours. The reaction mixture was evaporated to dryness at reduced pressure and the residue of crude succinic dihydrazide taken up in water and recrystallized. A 90% yield, based on

succinic acid was obtained. The specific activity was measured and found to agree with that calculated from the specific activity of the succinic acid used.

Ethylenediurethan

The succinic dihydrazide, dissolved in 5 ml. 2.0 N HCl, was transferred to a small flask equipped with a mechanical stirrer and immersed in an ice salt bath. About 20 ml. ether was added and then 450 mg. $NaNO_2$ dissolved in 5 ml. water was added dropwise. In this way most of the ether soluble produce, succinic-diazide, was extracted as formed from the reaction mixture. When all of the $NaNO_2$ solution had been added, the stirring was continued a few minutes, then stopped and the ether layer removed. The aqueous layer was extracted several times with ether and the ether extracts combined. Absolute alcohol was added to the ether solution and most of the ether removed by evaporation. The alcoholic solution was then refluxed on a water bath overnight. The solution was then evaporated to dryness at reduced pressure and the residue taken up in water and recrystallized. After drying, the product, ethylenediurethan, was further purified by sublimation in vacuo and a yield of 117 mg. (36% from succinic acid) was obtained. The specific activity was determined.

Hydrolysis of Ethylenediurethan

A solution of 104 mg. of ethylenediurethan in 5 ml. of 85% hydrobromic acid was refluxed for two hours in a 30 ml. two-necked flask equipped with a reflux condenser and a nitrogen inlet tube. During this time a slow stream of nitrogen carried the evolved carbon

dioxide through the condenser into a sodium hydroxide bubbler from which it was recovered as barium carbonate, 210 mg. The blank for the volume of sodium hydroxide used was previously determined at 7 mg. of barium carbonate and the theoretical yield from reaction 5 was 1.02 millimoles of carbon dioxide or 201 mg. of barium carbonate, so the actual yield was nearly quantitative. The barium carbonate was counted as a 1.0 to 2.0 mg./cm^2 layer on a thin aluminum disk. Sample preparations and corrections for self-absorption were in accordance with the procedures described in "Isotopic Carbon" page 118 (17).

The solution of ethylenediamine dihydrobromide in the reaction flask was evaporated to dryness and methanolic potassium hydroxide was added to the residue. After the methanol was evaporated, the ethylene diamine was distilled in vacuo and converted to ethylene diamine dihydrochloride by addition of methanolic hydrogen chloride. The solution was concentrated until the product crystallized out. The salt was then recrystallized from water-methanol, centrifuged, dried and weighed. A yield of 52 mg. (77% of theoretical from ethylene diurethan) was obtained and the specific activity was determined. The entire purification process (distillation, etc.) was then repeated and the specific activity again measured and found to be unchanged. Specific activities as low as 6.0 disintegrations per minute ($2.7 \cdot 10^{-9}$ mc.) per mg. were measured. In some cases the activity of the methylene carbons was as low as 2.6% of the total succinic activity (29).

B. Malic Acid

Malic acid was degraded by oxidation with chromic acid.

$$HO_2C\text{-}\overset{*}{C}H_2\overset{**}{C}HOH\text{-}CO_2H + 4/3\ HCrO_4^- + 16/3\ H^+ \rightarrow 2\ CO_2 + \overset{*}{C}H_3\overset{**}{C}O_2H + 13/3\ H_2O + 4/3\ Cr^{+++}$$

The carboxyl groups yield two molecules of carbon dioxide and a molecule of acetic acid is obtained from the alpha and beta carbon atoms. Degradation of the acetic acid could then be performed by decarboxylation.

$$2\ \overset{*}{C}H_3\overset{**}{C}O_2Ba_{1/2} \xrightarrow[1/2\ \text{hour}]{400^\circ} \overset{*}{C}H_3C\text{-}\overset{*}{C}H_3 + Ba\overset{**}{C}O_3 \qquad (30)$$

To date only the degradation to acetic acid and carbon dioxide has been carried out.

Oxidation of Malic Acid

To a solution of 100 mg. C^{14}-labeled malic acid in 10 ml. of 1.0 N sulfuric acid in a 100 ml. flask equipped with nitrogen inlet bubbler, reflux condenser, and dropping funnel was added 20 ml. of 0.15 M chromic acid solution over a period of two hours. The flask was heated on a steam bath during this time and a slow stream of nitrogen carried the evolved carbon dioxide into a sodium hydroxide bubbler.

Addition of barium chloride to the sodium hydroxide solution precipitated barium carbonate which was washed, dried, weighed and counted. The weight of barium carbonate, after correction for sodium hydroxide blank gives a yield of 96%.

Acetic acid was obtained by steam distillation of the reaction mixture. The steam distillate was titrated with barium hydroxide, and then evaporated to dryness at reduced pressure. The barium acetate was recrystallized from water and its specific activity determined. From the specific activity of the barium acetate and its theoretical yield

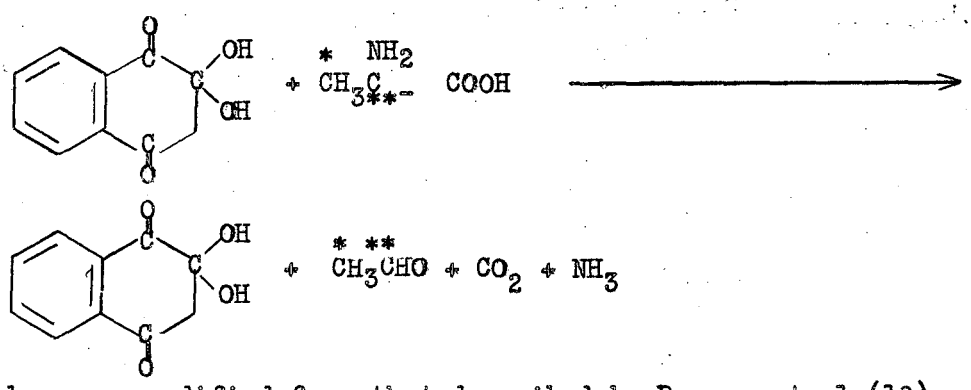
(100 mg.), the activity in the α and β carbon atoms of the malic acid can be determined without interference from the carboxyl activity.

Aspartic acid has been degraded by exactly the same procedure.

$$HO_2C\text{-}\overset{**}{C}HNH_2\overset{*}{C}H_2CO_2H + 4/3\ HCrO_4^- + 19/3\ H^+ \longrightarrow 2\ CO_2 + \overset{*}{C}H_3\overset{**}{C}OOH + NH_4^+ + 4/3\ Cr^{+++} + 10/3\ H_2O$$

C. Alanine

The decarboxylation of alanine with ninhydrin was carried out using the evacuated U-tube method of Van Slyke.



The procedure was modified from that described by Benson et.al.(13) to give the activity both of the carboxyl-labeled carbon and the sum of the alpha and beta carbons in one experiment. The carbon dioxide was collected as barium carbonate and the acetaldehyde was converted to its 2,4-dinitrophenylhydrazone.

$$CH_3CHO + (NO_2)_2\ C_6H_3NHNH_2 \longrightarrow (NO_2)_2\ C_6H_3NHN\text{-}CHCH_3 + H_2O$$

To 5 ml. of an aqueous solution of 15.0 mg. of C^{14}-labeled alanine and 150 mg. citrate buffer (pH 2.5) in a 30 ml. flask was added 200 mg. ninhydrin at 0°C and the flask quickly closed with a tube leading to a closed stopcock. The flask was immersed in liquid nitrogen and after several minutes a vacuum pump was attached to the stopcock tube, the stopcock opened, and the system evacuated. The stopcock was again closed, the flask removed from the liquid nitrogen and after warming to

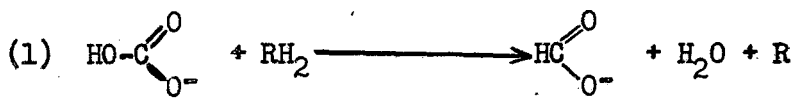
room temperature, placed on a steam bath for thirty minutes. The flask was again cooled and attached through the stopcock to a U-tube. On the other arm of the U-tube was placed a 30 ml. flask containing a 2 ml. solution of 22 mg. (2/3 the theoretical requirement) of 2,4-dinitrophenylhydrazine in glacial acetic acid. A stopcock, attached to the U-tube at its center, led to a vacuum pump. With both stopcocks closed, both flasks were immersed in liquid nitrogen. After several minutes both stopcocks were opened and the system evacuated. The stopcock to the vacuum pump was then closed and the ninhydrin reaction flask (F-1) removed from liquid nitrogen and warmed on a water bath until all the liquid inside the flask had distilled into the 2,4-dinitrophenylhydrazine flask (F-2). With both stopcocks closed, the contents of F-2 were warmed on a water bath at 70°C for several minutes, and meanwhile, F-1 was replaced with a third flask, F-3, which contained 15 ml. 6 N NaOH. Both flasks were again immersed in liquid nitrogen and the system evacuated. The stopcock to the vacuum pump was closed and F-2 warmed, its volatile contents distilling into F-3. Finally F-3 was warmed to room temperature with occasional shaking, removed from the U-tube and its contents transferred to a centrifuge tube. Barium chloride solution was added and the precipitated barium carbonate was centrifuged, dried, weighed and counted.

The residue of acetaldehyde 2,4-dinitrophenylhydrazone in F-2 was recrystallized twice from glacial acetic acid, dried and its specific activity determined. From the theoretical yield and the specific activity, the percentage of alanine activity in the alpha and beta carbons can be calculated.

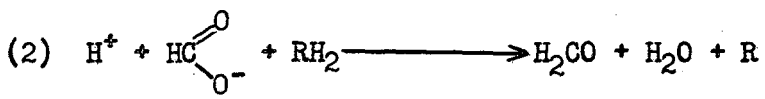
V. Application of Methods

A. Introduction

The reduction of carbon dioxide during photosynthesis might proceed through either of two initial steps. The first possibility is the direct reduction of bicarbonate to formic acid or formaldehyde.

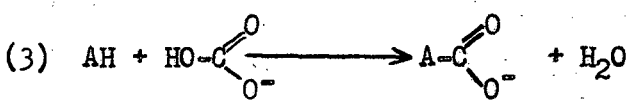


(1) $HO\text{-}C(=O)(O^-) + RH_2 \longrightarrow HC(=O)(O^-) + H_2O + R$

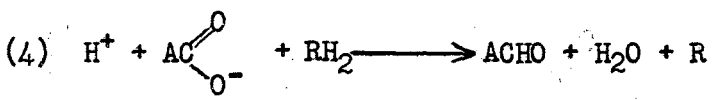


(2) $H^+ + HC(=O)(O^-) + RH_2 \longrightarrow H_2CO + H_2O + R$

A second possibility is the carboxylation of a carbon dioxide acceptor followed by reduction of the carboxylated compound.



(3) $AH + HO\text{-}C(=O)(O^-) \longrightarrow A\text{-}C(=O)(O^-) + H_2O$



(4) $H^+ + AC(=O)(O^-) + RH_2 \longrightarrow ACHO + H_2O + R$

(5) $ACHO + RH_2 \longrightarrow [CH_2O] + AH + R$

The first of these two schemes, particularly the formation of formaldehyde, first proposed by Baeyer (31) in 1870, had been studied by a great many workers prior to the development of tracer methods, but the results reported were inconclusive and contradictory (32). Since the advent of tracer carbon, when it became possible to detect much smaller amounts of photosynthetic intermediates, the one carbon compounds have been looked for and have not been found. (33) (3).

At the same time, several three and four carbon compounds have been found to appear, labeled with radioactive carbon after nearly every experiment involving reduction of labeled carbon dioxide by plants. The repeated occurrence of these compounds, under conditions of carbon dioxide reduction suggests that carbon dioxide is first incorporated by carboxylation of a C_2 or C_3 acceptor into a three or four carbon carboxylic acid which is subsequently reduced. The reduced carbon must then be used to synthesize carbohydrates or other photosynthetic products and the acceptor must be regenerated. There are two ways in which this might occur. First, the acceptor might retain its identity throughout the cycle, merely holding the carbon dioxide during reduction and then passing the reduced carbon on to another system. That this is not the case is indicated by degradation studies, which show that the early products of carbon dioxide fixation quickly become labeled in each carbon position when labeled carbon dioxide is used.

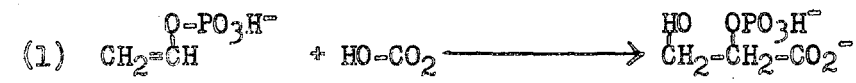
The second way in which the acceptor can be regenerated is by carboxylation, followed by reduction and finally by splitting into two molecules of acceptor. One molecule of acceptor can then be used to continue the carbon dioxide reduction cycle. The other molecule of acceptor, or some member of the cycle derived from it, can then be used for carbohydrate synthesis.

An obvious corollary to this scheme is that the number of carboxylations per cycle must equal the number of carbon atoms in the acceptor. Thus, the identification of three and four carbon products of carbon dioxide reduction which appear to be formed before any other products provides a strong indication that two carboxylations are involved in the cycle and that the first acceptor of carbon dioxide is a two carbon compound. This was further veri-

fied by Calvin and Benson who found that the first stable compound to appear labeled with tracer carbon in very short periods of photosynthesis was a three carbon compound, 2-phosphoglyceric acid (13,14). As the time of photosynthesis increased, other products of photosynthesis appear and in 90 seconds photosynthesis with Scenedesmus the observable products are 2-phosphoglyceric acid, 3-phosphoglyceric acid, phosphoenolpyruvic acid, glucose-1-phosphate, glucose-6-phosphate, fructose-1,6-diphosphate, fructose-6-phosphate, sucrose, aspartic acid, serine, alanine, malic acid, glycollic acid, and small amounts of fumaric and succinic acids and glycine (12,13,14).

The formation of sugars and sugar phosphates points to a formation of carbohydrates by a reversal of the glycolytic path, while the formation of 2, 3 and 4 carbon carboxylic acids and amino acids indicates the operation of a two, three and four carbon cycle to regenerate the carbon dioxide acceptor. The problem, therefore, is to determine which of the observed compounds are actually members of the cycle and which are not actually intermediates but are rapidly formed from intermediates.

Since 2-phosphoglyceric acid appears to be the first stable product of carbon dioxide reduction, one might take as a starting point the carboxylation of some acceptor to form 2-PGA. This acceptor might be some unstable compound such as vinyl phosphate, in which case the carboxylation could be the addition of bicarbonate ion to the double bond, probably through the agency of an enzyme.

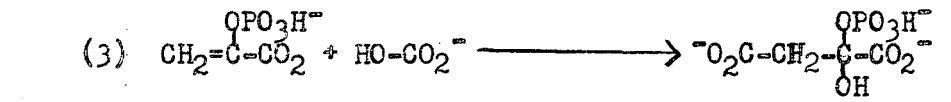


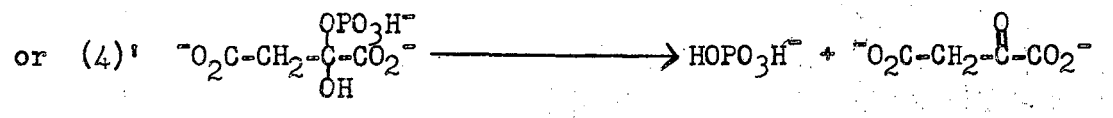
(1) $CH_2{=}CH(O{-}PO_3H^-) + HO{-}CO_2 \longrightarrow CH_2(HO){-}CH_2{-}CO_2^-(OPO_3H^-)$

For every two molecules of 2-PGA formed, one could go to 3-PGA and eventually

be reduced to carbohydrate, while the other could undergo the well-known dehydration to phosphoenolpyruvate:

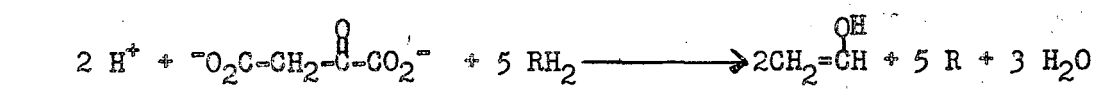
Now if a second molecule of bicarbonate ion adds to the double bond, in the opposite direction (because of enzyme specificity, or perhaps repulsion of the carboxyl group by the two acid groups on the α-carbon), and another dehydration occurs, the product would be phosphoenoloxalacetic acid.



or (4)' 

The carboxylation of pyruvic acid to oxalacetic acid is the well-known Wood-Werkman reaction (34).

The reduction and splitting of oxalacetate to two molecules of vinyl phosphate or a two carbon compound of the same reduction level requires ten equivalents of reducing agent.



This differs from the previously proposed cycle; page 4, in which oxalacetate was reduced to acetate which was then reductively carboxylated to give pyruvate. The early formation of 2-PGA is evidence in favor of the present scheme.

There are several paths by which the reductive splitting of oxalacetate to vinyl phosphate could occur, the difference depending on the point in the series of reductions that the splitting occurs. Oxalacetate might be reduced through malate and fumarate to succinate which could split reductively to acetate and the acetate could then be further reduced. An alternative is the hydrolytic splitting of oxalacetate to glycollate and glyoxylate and reduction of these two-carbon compounds. A third possibility is the hydrolytic splitting of malate to two molecules of glycollate. Other possibilities include the oxidation of oxalacetate to dihydroxymaleate and splitting to two molecules of glyoxylate.

Because malate was always found in photosynthesis experiments together with small amounts of fumarate and succinate and because enzyme systems which bring about the transformations of these acids from one to another were known to exist, it was tentatively proposed that the cycle was completed by the following reactions:

(5) $^{-}O_2C-CH_2-\overset{O}{\overset{\|}{C}}-CO_2^{-} + RH_2 \longrightarrow {}^{-}O_2C-CH_2-CHOH-CO_2^{-} + R$

(6) $^{-}O_2C-CH_2-CHOH-CO_2^{-} \longrightarrow H_2O + {}^{-}O_2C-CH=CH-CO_2^{-}$

(7) $^{-}O_2C-CH=CH-CO_2^{-} + RH_2 \longrightarrow {}^{-}O_2C-CH_2-CH_2-CO_2^{-} + R$

(8) $^{-}O_2C-CH_2-CH_2-CO_2^{-} + RH_2 \longrightarrow 2\ CH_3CO_2^{-} + R$

(9) $H^+ + CH_3CO_2^- + H_2PO_4^- + RH_2 \longrightarrow CH_2{=}CHOPO_3H^- + R + 2\ H_2O$

B. Degradation Studies*

If the cycle composed of reaction 1 to 9 is in operation at the start of an experiment involving photosynthetic uptake of labeled carbon dioxide, the labeling of the various compounds formed will proceed at a rate depending on the rate of movement of carbon through the cycle, and for a few turns of the cycle, the labeling will be unequal among the several carbon atoms of each molecule. This is demonstrated in Figure 6 where the labeled positions after one turn of the cycle are marked with two asterisks and those labeled after two turns of the cycle are marked with one asterisk.

Of course, the actual situation is not that of complete conversion of all the molecules of one intermediate to the next with each turn of the cycle but rather is analogous to a series of reservoirs through which is "pumped" a stream of carbon with two inlets (carboxylations) and one outlet (formation of carbohydrates). The "pumping" energy is the reducing energy formed by the photochemical reaction. When radiocarbon is admitted through the inlets it spreads from pool to pool, becoming more dilute with each step. Thus the concentration of labeled carbon which reaches the inner, reduced position of the carboxylic acids after a period of photosynthesis is dependent on the aggregate concentration of the intermediates and the rate of "turning" of the cycle and may also depend on the concentrations of other reservoirs, not intermediates in photosynthesis, but in rapid equilibrium

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* All the alanine degradations mentioned in section V-B were performed by Dr. A. A. Benson.

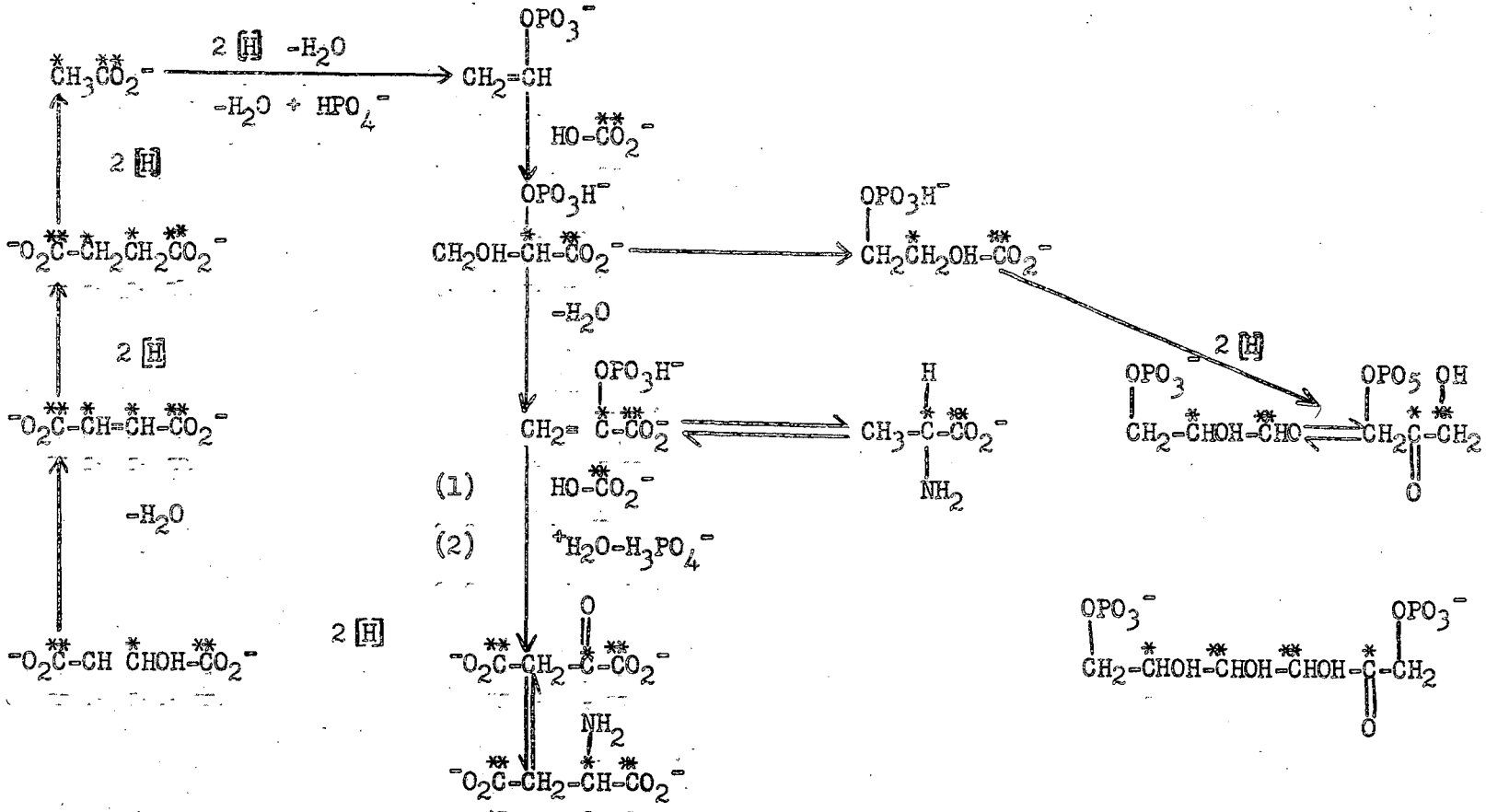


Figure 6

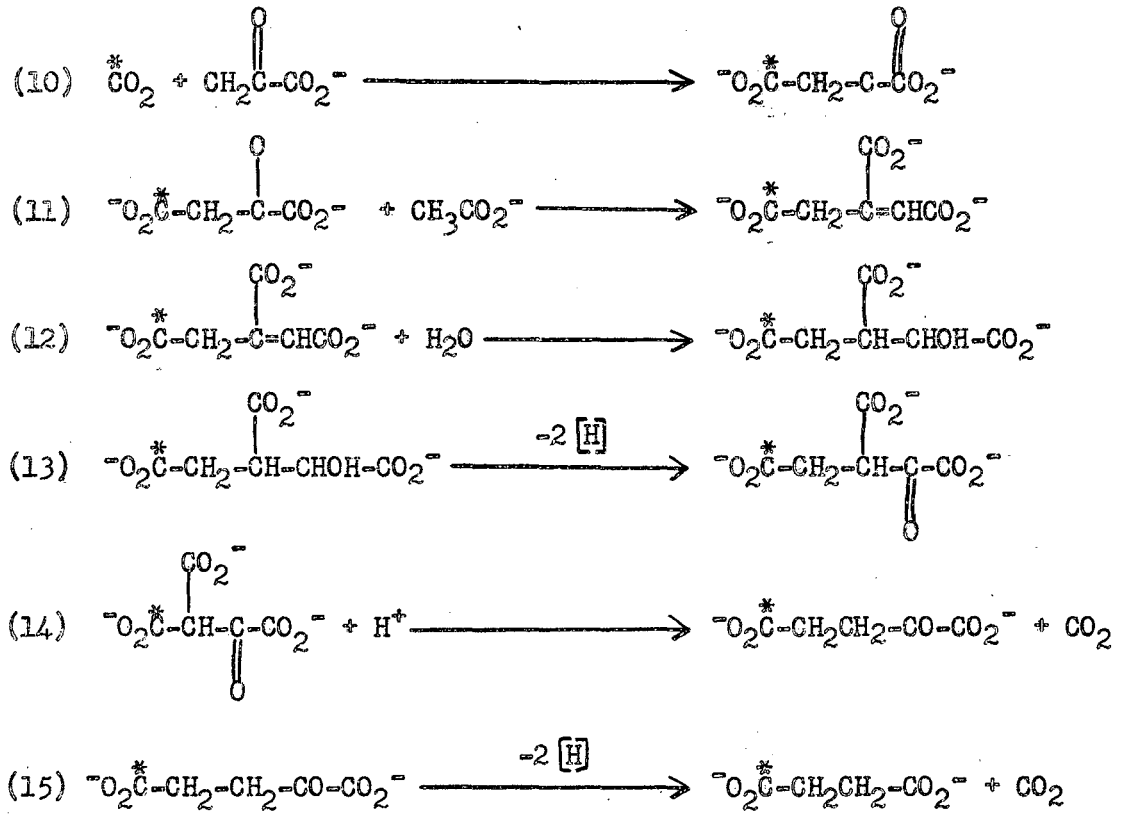
with intermediates.

One method of testing the operation of the cycle is to degrade a suspected intermediate and determine the distribution of activity within the molecule. The first such degradation was carried out with succinic acid obtained from Chlorella after a period of dark reduction of labeled carbon dioxide following illumination. It was found that 2.6% of the radioactivity was present in the methylene carbons of the succinic acid. Thus the operation of a cycle was demonstrated in dark reduction, but not necessarily the participation of succinic acid in the cycle, since it could be merely derived from a photosynthetic intermediate. Glucose obtained from a similar experiment was degraded by other members of the laboratory (35), and 24% of the activity found in other than the 3 and 4 positions, thus again indicating the operation of a cycle during dark reduction. Degradation of alanine from the same experiment demonstrated that 89% of the activity was in the carboxyl group, 10% in the α-carbon and less than 1% in the β-carbon. Alanine is thought to be in equilibrium with pyruvate through the transaminase system.

In another dark fixation experiment following a period of illumination, malic acid was degraded and 0.86% of the activity found in the methylene carbons. In the same experiment 2% of the alanine activity was found in the α and β carbon atoms.

When dark fixation of labeled carbon dioxide was carried out without a period of preillumination, no activity was found in the methylene carbon atoms of succinic acid, indicating that the incorporation of carbon dioxide into the molecule was entirely by a respiratory mechanism. Such a mechanism

might be the Wood-Werkman carboxylation followed by the usual tricarboxylic acid respiration cycle:



The distribution of labeled carbon in succinic acid from dark fixation experiments following illumination is probably the result of both respiration fixation and reductive fixation but it is to be noted that the reductive fixation is many times greater than the respiration fixation during the first two minutes following illumination (11).

When Scenedesmus are allowed to photosynthesize with radioactive carbon dioxide for 30 seconds, and the malic acid is isolated and degraded, 6.5% of the activity is found in the α and β carbons and 93.5% is in the carboxyl carbons. Aspartic acid from the same experiment shows 96% in the carboxyl groups and 4% in the other two positions. Aspartic acid is presumed to be in equilibrium with oxaloacetic acid through the transaminase system.

Barley which had photosynthesized for 75 minutes with labeled carbon dioxide yielded succinic acid which when degraded was found to contain 37% of its radioactivity in the methylene carbons and 63% in the carboxyl carbons.

C. Malonate Inhibition Experiments

Another method of testing the validity of a proposed cycle or series of conversions is to use a poison or inhibitor which is known to block one or more steps of the series. A specific set of conditions is designed to test the operation of the cycle, and two experiments are performed, one with inhibitor and one without. The results of the two experiments are compared and the differences are interpreted in the light of the known effects of the inhibitor.

An inhibitor which could be used to test the series of hydrogenations from oxaloacetate to succinate was found in malonate. Quastel and Wooldridge (36) and others (37) demonstrated that malonate inhibited the oxidation of succinate both in certain bacteria and in brain and muscle tissue. They interpreted the inhibition by malonate as being due to a competition with succinate for succinic dehydrogenase.

Goszy and Szent-Gyrögyi (38) found that respiration in pigeon breast muscle was inhibited by malonate, and this finding led in later work to the postulation by Szent-Gyrögyi and co-workers of a hydrogen carrier system consisting of fumarate and succinate with succinic dehydrogenase. They believed this system to be specifically poisoned by malonate. They found that respiration in malonate poisoned tissue was restored by the addition of fumarate.

The malonate inhibition of both succinic and malic dehydrogenases has been studied by Das (39) who found that both enzymes were inhibited by

malonic acid. He further reported the succinic dehydrogenase was inhibited by oxalacetate even more strongly than by malonate. Lactic dehydrogenase was also found to be inhibited by malonate. Finally, Jowett and Quastel (40) found that the breakdown of acetoacetate is inhibited by malonate. The relations of these acids and enzymes are shown in Figure 7.

There are three conditions common to all these reactions. First, the compounds involved are all three- and four-carbon carboxylic acids. Secondly, the enzyme catalyzed reaction in each case may be considered as an addition to a double bond, α-β to the carboxyl group. Finally, each reaction involves the addition of either two hydrogen atoms or of a hydrogen atom and a hydroxyl group to the double bond.

The enzyme in each case may contain two functional parts. The first might be a protein with a spatial arrangement of its components which permits it to form a complex specifically with three- or four-carbon carboxylic acids. This part of the enzyme might be unable to distinguish between these several acids and would form complexes with any of them, including malonate. The fraction of enzyme complexed with any particular acid species would then be merely a function of the various acid concentrations.

The second portion of the enzyme is the one which gives its specificity and probably has as its function the aligning or drawing together of the hydrogen (or water) acceptor with the corresponding donor. In the case of hydrogen transfer, if the donor is a carboxylic acid, then the acceptor may be some coenzyme in its oxidized state. For example, malic dehydrogenase requires coenzyme I, diphosphopyridinenucleotide (DPN).

The use of malonate as an inhibitor has been extended to plants by

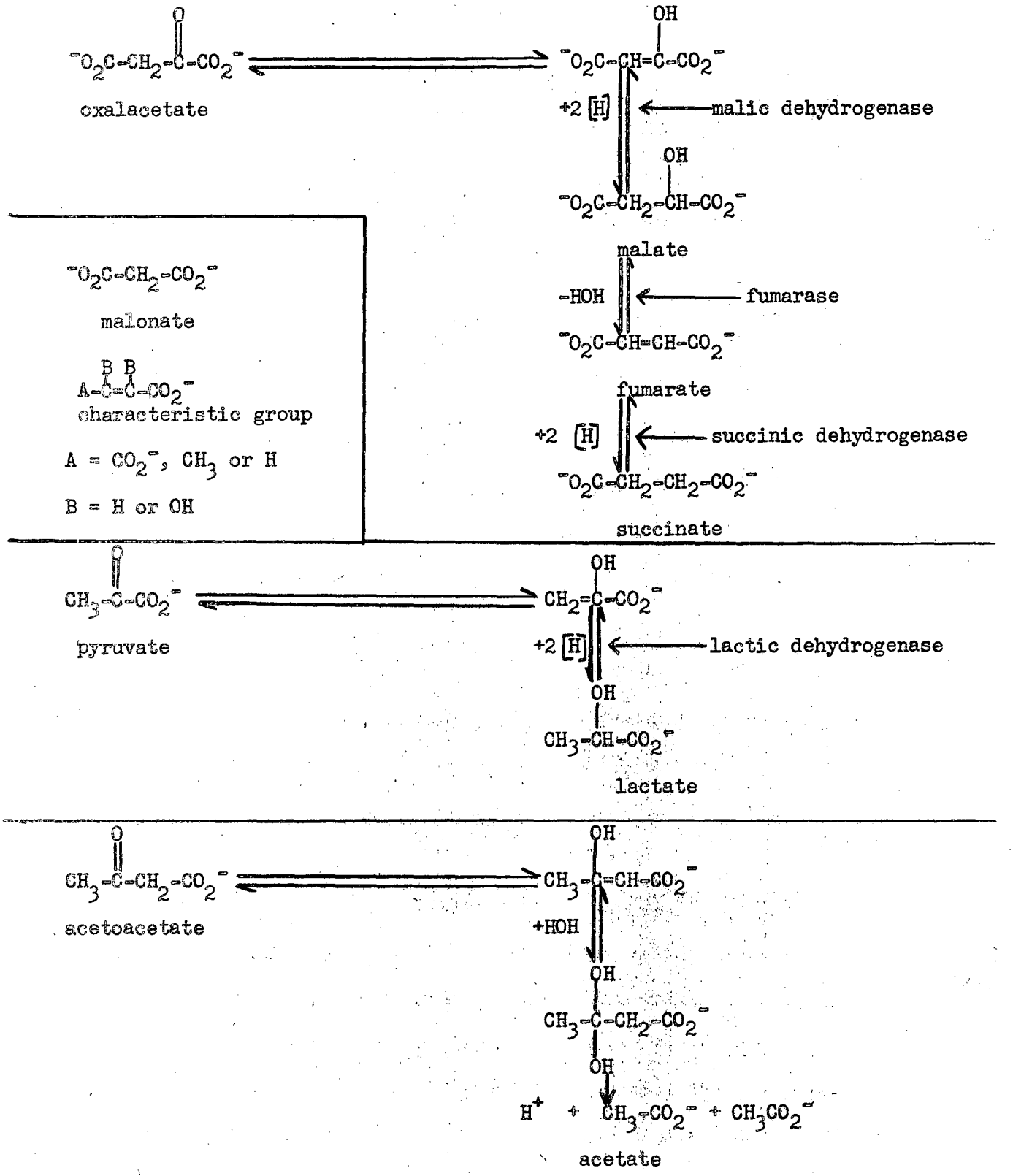

oxalacetate
malic dehydrogenase
malate
fumarase
fumarate
succinic dehydrogenase
succinate
malonate
characteristic group
A = CO2-, CH3 or H
B = H or OH
pyruvate
lactic dehydrogenase
lactate
acetoacetate
acetate

Figure 7

various workers. Bonner has studied the effect of malonate on respiration in spinach leaves (41) and found that the respiration could be inhibited by as much as 90% by infiltrating into the leaf a solution containing 5 mg. malonate per ml. He interprets this result as indicating that respiration in spinach leaves proceeds through the tricarboxylic acid cycle (page 38).

In order to study the effect of malonate on photosynthesis it was necessary to devise a way of introducing the malonate into a plant suitable for tracer studies, and then with the plant photosynthesizing, allow reduction of labeled carbon dioxide by the plant to take place.

The green algae, Scenedesmus, were used for the purpose. A 24 hour growth from a continuous culture was centrifuged and 1.3 cc. wet, packed cells were obtained. These cells were divided into two equal portions and one portion was suspended in 50 ml. water. The other portion was suspended in a sodium malonate buffer, pH 4.3, which contained 5 mg. malonic acid per ml. Both suspensions were kept in the dark at room temperature. After three and one-half hours, the cells in malonate buffer were centrifuged and resuspended in 100 ml. water with a small amount of fumarate buffer. The suspension was placed in a thin, water-jacketed vessel which was illuminated from both sides. A rapid stream of 4% CO_2 in air was passed through the solution for 30 minutes to hasten the end of the induction period and start the algae photosynthesizing. A stream of unenriched air was then bubbled through the suspension for 10 minutes. At the end of this time, the air stream was stopped, a solution containing C^{14} labeled sodium bicarbonate rapidly run into the flask from a syringe, and the flask quickly closed. The flask was shaken under illumination for 90 seconds, at the end of which time a large stopcock in the bottom was opened, the top stopper removed, and the

cell suspension rapidly run into a beaker containing 400 ml. boiling absolute ethanol.

The resulting 80% ethanol suspension was counted and filtered after 30 minutes and the clear, green filtrate counted and then concentrated at reduced pressure and room temperature. The total activity fixed by the cells was 14 μc. and of that, 80% was carried into the alcohol extract. The final volume of the extract was 2.0 ml. and of this, 100. aliquots were applied to papers and chromatographed.

The treatment of the second portion of cells was exactly the same except for the absence of malonate inhibitor and the fact that they were left in the dark three hours longer before they were started photosynthesizing. The non-inhibited cells fixed 16 μc. of radiocarbon and 76% of this was carried into the alcohol extract.

The radioautographs of the chromatograms prepared from the inhibited and non-inhibited cells are shown in Figures 8, 9 and 10, together with one in which synthetic labeled malic and succinic acids were added to the extract from the malonate-inhibited cells. As can be seen, the most pronounced difference is the diminution of malic acid in the inhibited cells. Thus, it appears that the inhibition of the reduction of oxalacetate to malate was successful. Since very little succinate or fumarate was obtained in either case it was impossible to measure the inhibition of succinic dehydrogenase. Because the formation of malate has been very greatly reduced, the operation of the regenerative cycle should be greatly reduced also, if malate were a member of the cycle. Alanine degradation provides a convenient means of testing the operation of the cycle and considerable activity was obtained in alanine in each experiment, so the alanine spots were eluted and the de-

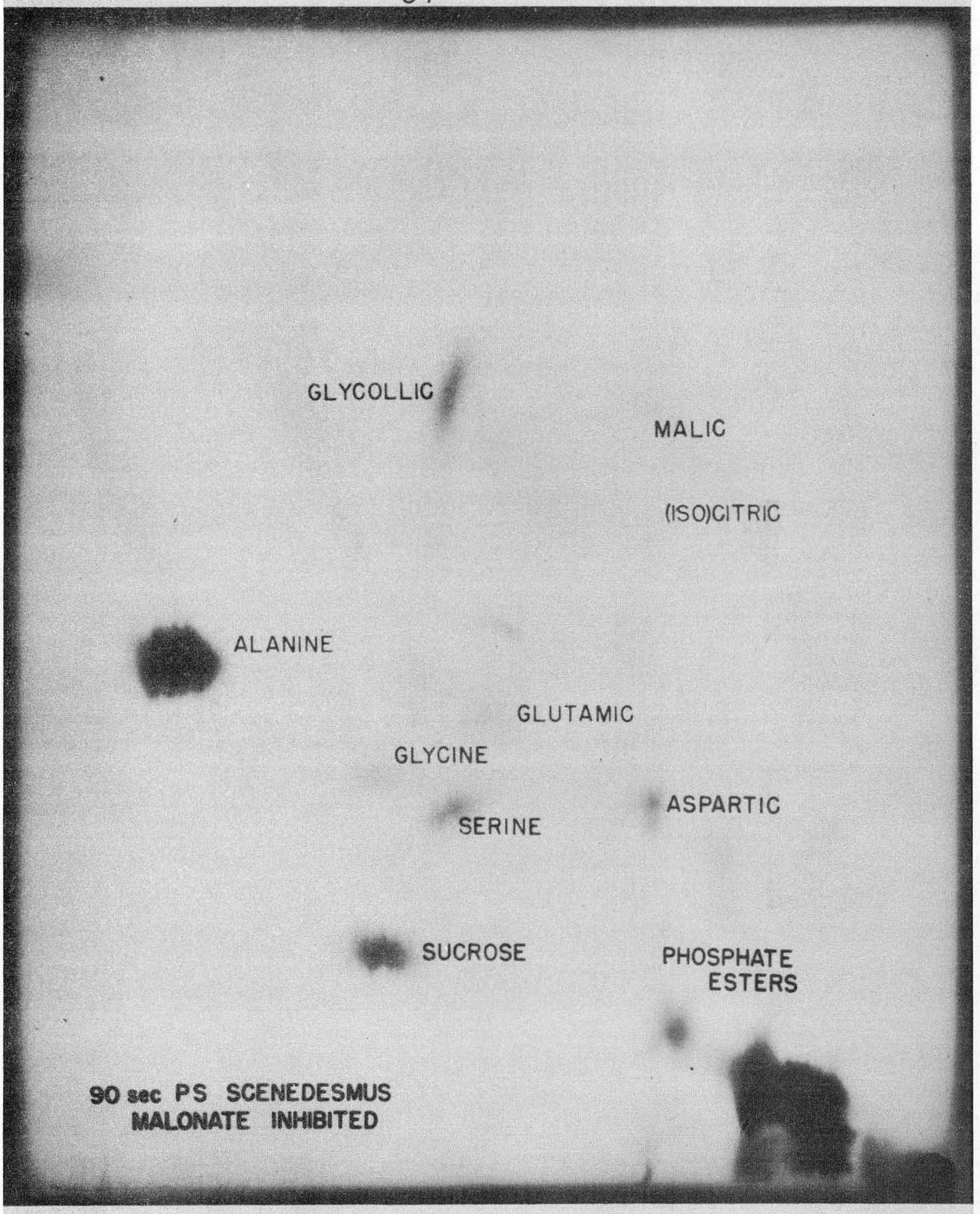

GLYCOLLIC
MALIC
(ISO)CITRIC
ALANINE
GLUTAMIC
GLYCINE
ASPARTIC
SERINE
SUCROSE
PHOSPHATE
ESTERS
90 sec PS SCENEDESMUS
MALONATE INHIBITED

FIGURE 8

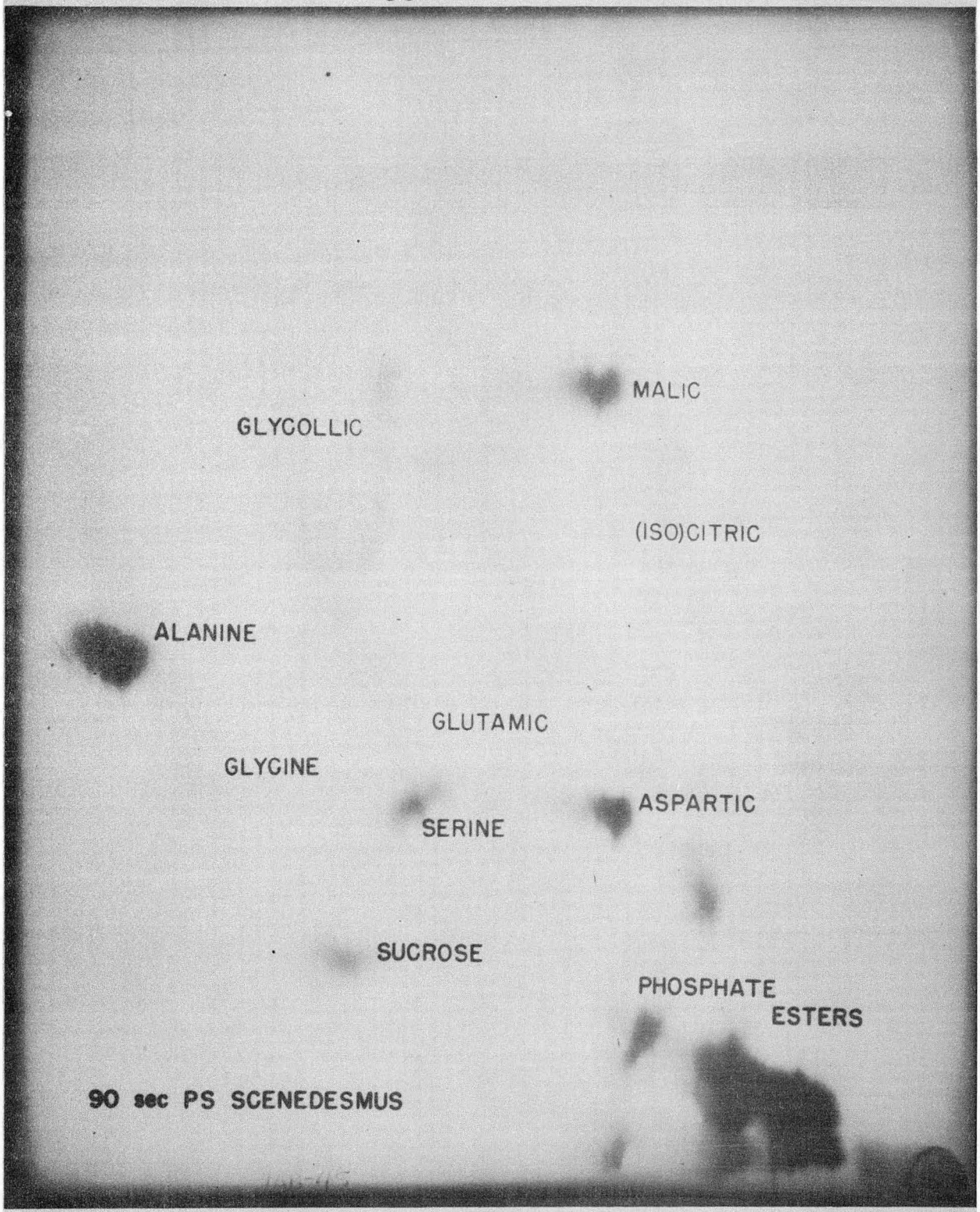

MALIC
GLYCOLLIC
(ISO)CITRIC
ALANINE
GLUTAMIC
GLYCINE
ASPARTIC
SERINE
SUCROSE
PHOSPHATE
ESTERS
90 sec PS SCENEDESMUS

FIGURE 9

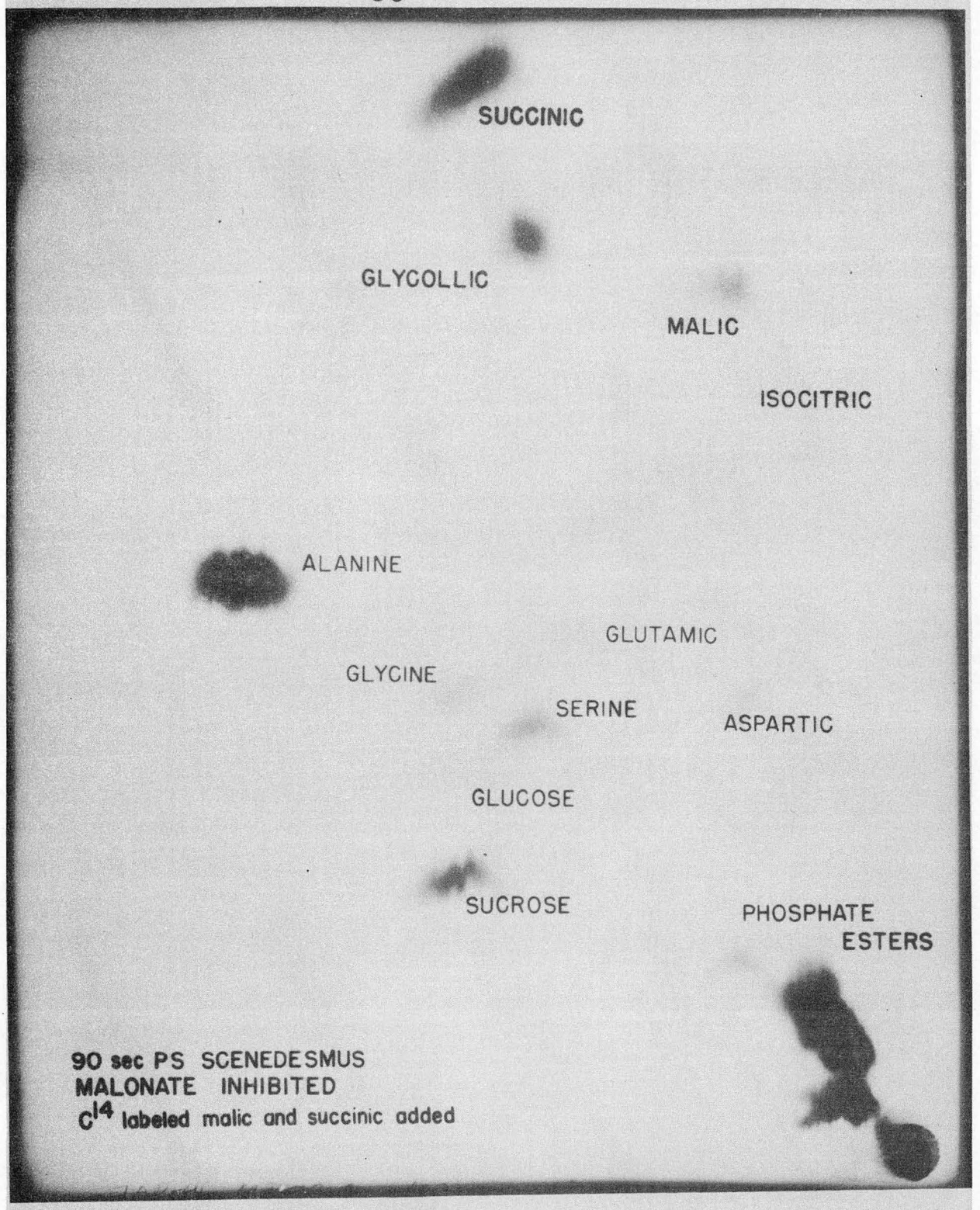

SUCCINIC
GLYCOLLIC
MALIC
ISOCITRIC
ALANINE
GLUTAMIC
GLYCINE
SERINE
ASPARTIC
GLUCOSE
SUCROSE
PHOSPHATE
ESTERS
90 sec PS SCENEDESMUS
MALONATE INHIBITED
C14 labeled malic and succinic added

FIGURE 10

gradation with ninhydrin was carried out.

It was found that in the alanine obtained from the malonate-inhibited cells, 33% of the total activity was in the α and β carbons, whereas in the alanine from non-inhibited cells, the α and β carbons contained 44% of the activity. Thus it appears that the regenerative cycle is operating nearly as well in the malonate-inhibited cells as in the normal cells. It is strongly indicated, therefore, that malic is not a member of the regenerative cycle, or at least that there is an alternate cycle not involving malate and that this alternate cycle is the principal one in operation in photosynthesizing Scenedesmus. It is further indicated that neither fumarate nor succinate are members of the regenerative cycle since the most likely path by which they would be formed would be by reduction of oxalacetate through malate.

It should be noted that malonate inhibition did not increase the amounts of the phosphate esters, alanine, serine, sucrose or glycollic acid. In another malonate inhibition experiment in which the cells were suspended in malonate buffer only one hour instead of three, the amount of malate decreased as in the experiment shown but the amount of glycollate actually increased appreciably. The possible significance of this result is considered in the following section.

VI. Discussion and Summary

A. Discussion

By applying the methods which have been developed for separation, isolation, identification and degradation of products of carbon dioxide reduction in photosynthesizing plants it should become possible eventually to carry out a detailed analysis of the path of carbon in photosynthesis. The applications performed thus far represent a beginning of this analysis and serve to indicate the way in which this work might be continued.

By systematically varying the time of labeled carbon dioxide reduction in plants from preillumination to very short photosynthesis periods to perhaps several minutes photosynthesis and by isolation and degradation of the products, one can watch the relative rate of introduction of carbon fourteen into all the carbon positions of all the intermediates. With this data the actual path of carbon will be traced from the time it enters the first fixation product until the time it emerges as carbohydrate and as other storage products in the plants. At the same time, inhibition studies can serve to limit the number of alternative paths which must be investigated and perhaps to indicate the correct scheme.

The final part of the path to carbohydrates is already becoming known through the work of Calvin and Benson. Much work remains to be done on the photosynthesis of fatty acids and some of the amino acids. But the real foundation of plant synthesis is the reduction of carbon dioxide to the fundamental carboxylic acids from which are synthesized all the products of photosynthesis.

The proposal of a three- and four-carbon carboxylic acid cycle has been supported by all the experiments performed in this laboratory whereas the

alternatives which include direct reduction to formaldehyde, C_1-C_1 addition, etc., have received no experimental support. That the tricarboxylic acid cycle is in operation in respiration is indicated by the formation of considerable amounts of isocitric, glutamic, fumaric, succinic and malic acid in dark, non-preilluminated fixation experiments, but by the same reasoning, a reversal of this cycle in photosynthesis is excluded as a possibility by the absence of appreciable quantities of these acids with the exception of malic in photosynthesis experiments. The relative increase in malic acid during photosynthesis as compared with the other Krebs Cycle acids indicates that malic acid is formed by some path other than that cycle. The inhibition of malic acid formation by malonate during photosynthesis indicates that malic acid is formed by reduction of oxaloacetic acid. Although malic acid is ruled out as a member of the regenerative cycle by the same experiment, the rapid appearance of malic acid during photosynthesis indicates that it lies very close to some actual member of the cycle. Although oxaloacetic acid itself has not been isolated in our experiments, its presence is indicated by the rapid formation of labeled aspartic acid in short term reductions of radioactive carbon dioxide.

Consequently, it is tentatively suggested that oxaloacetic acid is a member of the regenerative cycle. Further, the formation of 2-PGA as the first stable product of carbon dioxide reduction seems to place it as a member of the cycle. Phosphoenolpyruvate has been identified as an early product also so that it is possible to list at least these three consecutive members of the cycle with some confidence.

The mechanism by which oxaloacetate or some other four-carbon acid is split and the resulting fragments reduced to vinyl phosphate or its equivalent

remains unknown. Since the reduction of oxalacetate through malic acid is apparently not part of the regenerative cycle, the splitting probably takes place at a reduction level close to that of oxalacetate. The reduction levels at which splitting might occur are shown in Figure 11.

No significant amount of tartrate has been found in short term photosynthesis experiments so far, so it may be that some phosphate ester of tartrate is involved in the actual splitting to two carbon fragments. Such an ester would be quite stable and only very slowly hydrolyzed as compared with the labile, easily hydrolyzable esters of the phosphoenol type. For example, hydrolysis of the phosphate ester, 2-PGA, is known to require heating in acid on a steam bath for several days. Consequently, such an ester can be an intermediate in carbon reduction without the appearance of the free acid in short term experiments.

Glycollate has been found in a number of short term experiments and frequently appears in higher concentration (of activity) than malic acid. The concentration of glycollate is not decreased in malonate inhibition experiments and in one such experiment is actually increased. If malate and glycollate are formed from oxaloacetate by s eparate paths, the inhibition of malate formation might be expected to increase the amount of glycollate formed, provided the formation of glycollate is not also inhibited.

The appearance of small amounts of glycine in short term reductions might be taken as an indication of the formation of labeled glyoxylate. Whether glyoxylate would be formed by oxidation of glycollate or by splitting of either phosphotartrate or phosphohydroxymaleate cannot be decided at present.

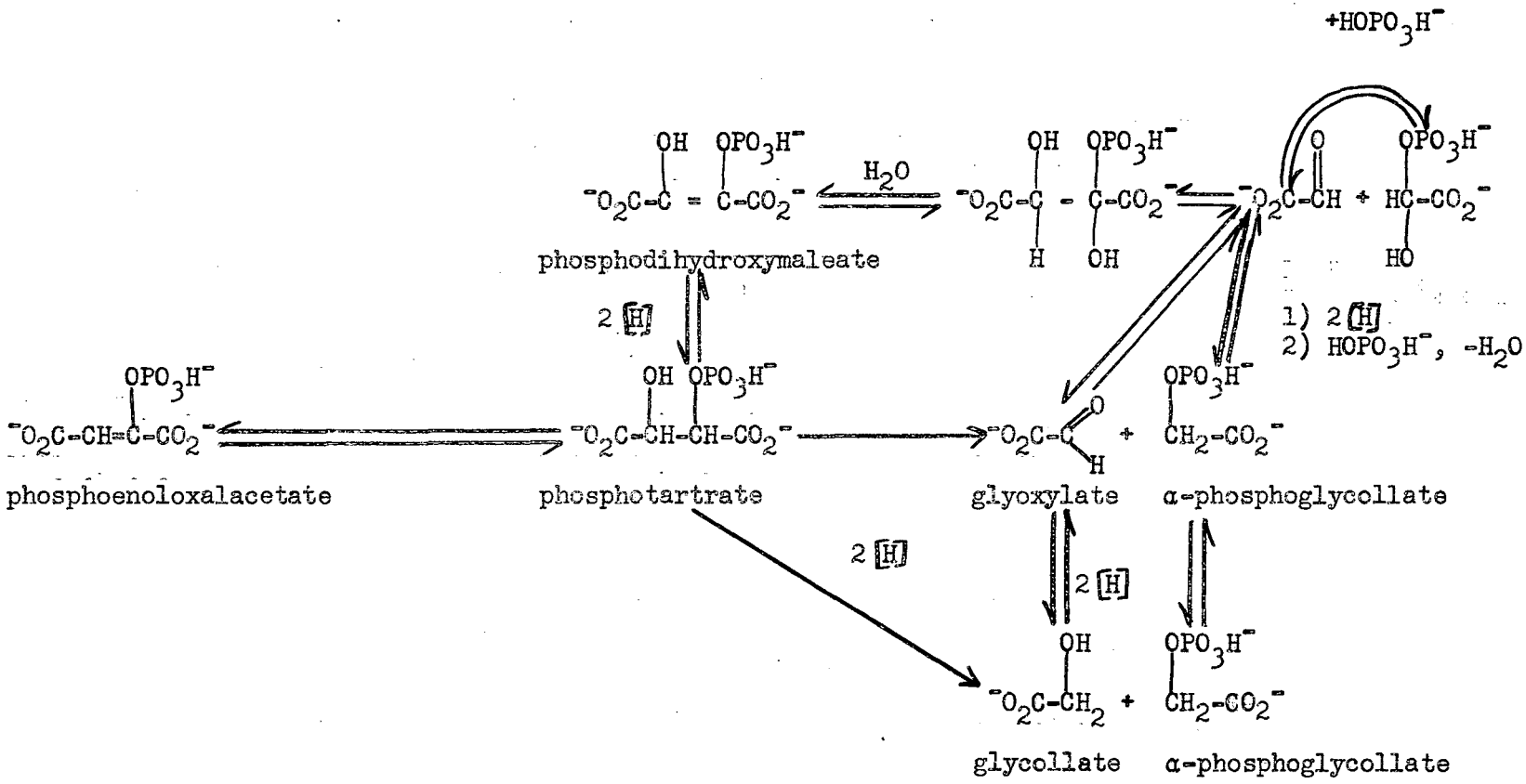

+HOPO3H⁻
H2O
phosphodihydroxymaleate
2 [H]
1) 2 [H]
2) HOPO3H⁻, -H2O
phosphoenoloxalacetate
phosphotartrate
glyoxylate
α-phosphoglycollate
2 [H]
2 [H]
glycollate
α-phosphoglycollate

Figure 11

A possible path for the splitting of the four-carbon acids might be the oxidation of phosphotartrate to phosphodihydroxymaleate and splitting of the latter to glyoxylate and phosphoglyoxylate. The oxidation of phosphotartrate might be malonate-inhibited but not necessarily since the phosphate esters may require a different enzyme system than the unsubstituted carboxylic acids.

The reduction of glycollate to vinyl phosphate may occur through a series of hydrogenations, phosphorylations and dehydrations. A possible path for these reductions is shown in Figure 12. Since none of the compounds intermediate between glycollate and 2-PGA have been isolated, the mechanism shown must be considered as very tentative, and is presented only in order to indicate the kind of transformations which may take place and to provide a basis for the thermodynamic calculations which are to follow. As in all the reactions presented so far, these reactions involve only the formation of phosphate esters and anhydrides, enolizations and additions to carbon-carbon double bonds.

Although little experimental evidence is available to substantiate the proposed mechanism of carbon reduction from glycollate to vinyl phosphate, it is of interest to consider the energy relations of this mechanism as well as those of the proposed carboxylation reactions.

In Table III are listed a number of free energies of known and proposed compounds involved in carbon dioxide reduction. The values followed by the letter B are obtained from Borsook (42), while those labeled C were calculated by the author. The standard state is 1.0 molar aqueous solution, 298° K., pH 0. The methods by which approximate calculations of the free energies of some of the compounds were made are discussed in Appendix I.

The reactions, starting with the carboxylation of vinyl phosphate are listed in the order of their positions in the cycle:

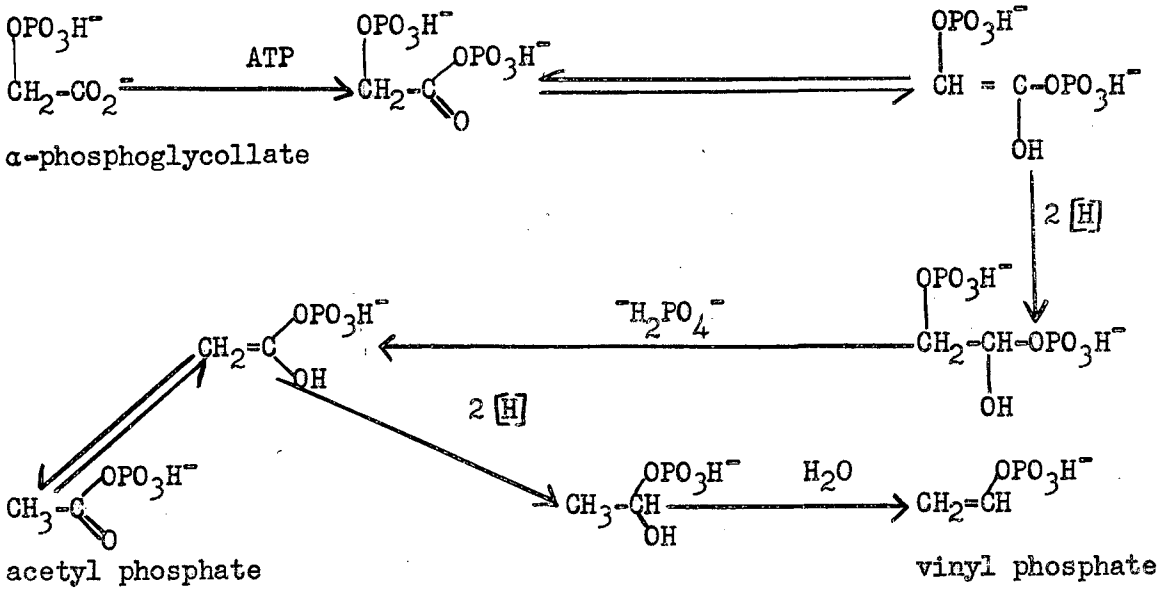
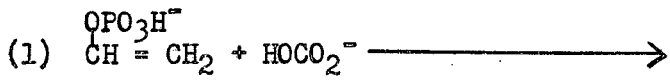
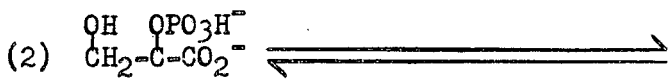
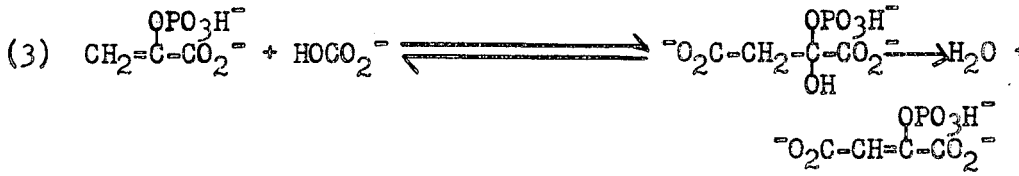

ATP
α-phosphoglycollate
2 [H]
$^{-}H_2PO_4^{-}$
2 [H]
H_2O
acetyl phosphate
vinyl phosphate

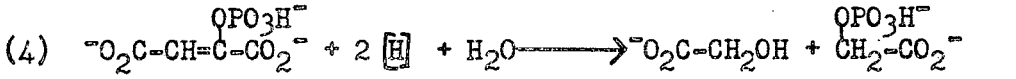
Figure 12

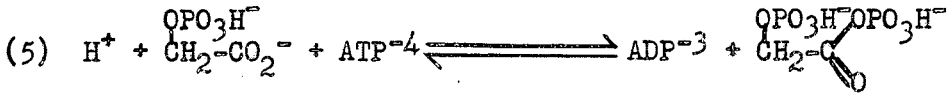
(1)  $\Delta F = +7,770$

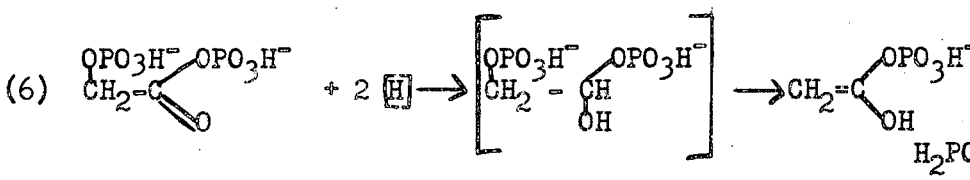
(2)  $\Delta F = -550$

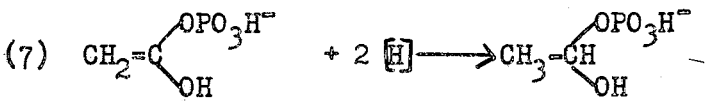
(3) 

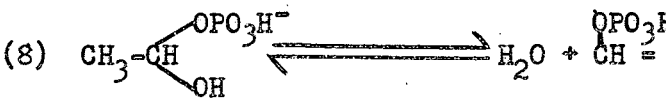
$\Delta F = +5,770$

(4)  $\Delta F = -3,310$

(5)  $\Delta F = 0$

(6)  $\Delta F = +26,300$

(7)  $\Delta F = +2,300$

(8)  $\Delta F = -500$

No free energy value is assigned to the reducing agent which would supply the hydrogen atoms (denoted by 2 H) in reactions 4, 6 and 7, but it is seen that a reducing agent of considerable strength is required in step 6. At pH 7, a two electron reducing agent with a potential of 0.16 v would be required and the standard potential (pH 0) would be 0.57 v. Such a strong reducing agent has not been found in other biological systems and would undoubtedly be unique to photosynthesizing plants.

Table III

FREE ENERGIES OF SOME CARBON COMPOUNDS

		298° K.	1.0 M aqueous
H_2O		-56,690 calories	B
H^+ (pH 7)		-9,560	C
$HO-CO_2^-$		-140,230	B
(P)		$\Delta F_{H_2PO_4^-} - \Delta F_{H_2O}$	
$CH_3CO_2^-$		-90,130	B
CH_3CHO		-31,650	B
$CH_2OH-CO_2^-$		-118,710	C
$CH_3CO-CO_2^-$		-107,900	B
$^-O_2C-CH_2-CO-CO_2^-$		-186,140	B
$CH_2OH-CHOH-CO_2^-$		-156,340	C
$CH_2(OPO_3H^-)-CH(OH)-CO_2^-$	(P)	-154,060	C
$CH_2OH-CH(OPO_3H^-)-CO_2^-$	(P)	-153,110	C
$CH_2=C(OPO_3H^-)-CO_2^-$	(P)	-96,970	C
$CH_2(OPO_3H^-)-CO_2^-$	(P)	-116,430	C
$CH_2(OPO_3H^-)-C(OPO_3H^-)=O$	2(P)	-105,430	C
$^-O_2C-CH=C(OPO_3H^-)-CO_2^-$	(P)	-175,140	C
$CH_2=CH-OPO_3H^-$	(P)	-20,650	C
$CH_3-C(OPO_3H^-)=O$	(P)	-79,130	C
$CH_3-CH(OH)(OPO_3H^-)$	(P)	-76,840	C

The free energy required for the photolysis of water at pH 7,

$$H_2O + n\,h\nu \longrightarrow 1/2\ O_2 + 2\ H^+ + 2\ e^-$$

is 37,570 calories and if a reducing agent of sufficient strength to bring about reaction 6 is formed at the same time,

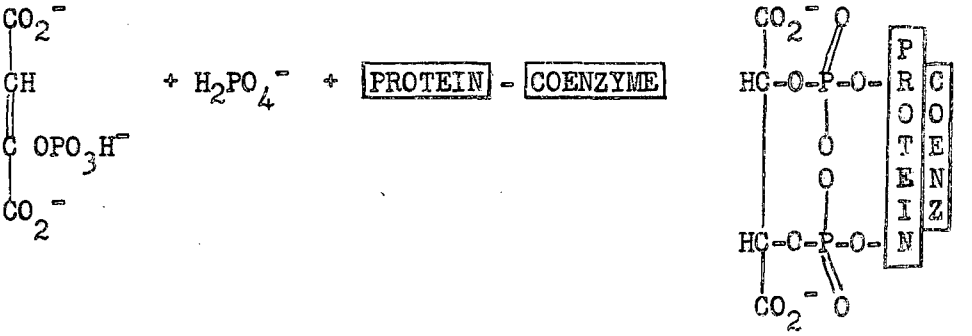
$$H_2O + R + n\,h\nu \longrightarrow 1/2\ O_2 + RH_2 \qquad \Delta F = 0$$

then the minimum light energy required would be about 64,000 calories. Since the energy of one einstein of light of 7000 Å wave length is 40,670 calories, two quanta would be required for the formation of one molecule of reducing agent and the photolysis of one water molecule.

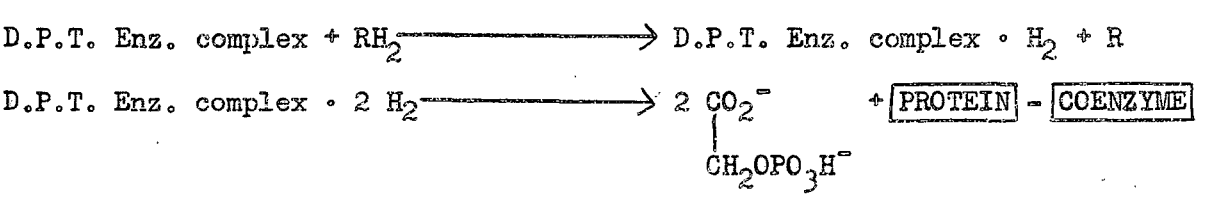
It is to be noted that the carboxylations operate against a positive free energy and it may be that some enzymatic energy transfer system is required to supply the necessary free energy for these reactions. Kinetic studies of carbon dioxide reduction in plants (43) indicate that some complex is formed with bicarbonate ion or carbon dioxide prior to carboxylation to 2-PGA and that the reaction proceeds with a negative free energy of about 5700 calories. This energy in addition to the energy required in carboxylation might be supplied by the release of a high energy phosphate bond in some way or by some other energy transfer system.

Another point at which energy might be required is the reduction and splitting of oxaloacetate to glycollate. Although the free energy of the overall reaction 4 is -3,310 calories, the actual mechanism of this reaction is not indicated and it is possible that unstable intermediates are involved. The formation of these intermediates may require a supply of energy from the photochemical process. This possibility is supported by the fact that although aspartic acid is formed during dark respiration, indicating

the formation of some oxaloacetic acid, no glycollate was found in such experiments. Glycollate is found when photochemical reducing power is available, both in preillumination and photosynthesis experiments. The actual mechanism may be the reduction of oxalacetate to an unstable product which splits spontaneously to glycollate.

(9) 

Diphosphotarate-enzyme complex



The free energy calculations indicate that there is no serious energy block in the proposed mechanism of carbon dioxide reduction. They also show at what point a high energy reducing agent is required. As more information is obtained regarding the actual intermediates involved, it may be found that the actual mechanism is more efficient energetically.

B. Summary

1. Methods have been developed for the separation, isolation, identification, and degradation of the carboxylic acids formed in the reduction of carbon dioxide during photosynthesis.

2. These methods have been applied and the results indicate that the primary reduction of carbon dioxide during photosynthesis takes place via

two carboxylations of a two carbon acceptor followed by splitting and reduction of the resulting four-carbon acid to two new molecules of acceptor. One molecule of acceptor is then used to continue the reductive cycle and the other is used in photosynthesis.

3. The participation of 2-PGA, phosphoenolpyruvate, phosphoenoloxalacetate and glycollate in the cycle are discussed and a tentative cycle is proposed.

APPENDIX I

Calculations of Free Energies of Some Organic Compounds

Because the free energies of some of the compounds involved in the path proposed for carbon dioxide reduction in photosynthesis were not known, approximate calculations were made so that the energy required for the several steps in the scheme could be estimated.

As a starting point, the free energies of glyceric acid and glycollic acid were required together with the free energies of formation of phosphate esters and "high energy" phosphate anhydrides.

According to Lipmann's (44) calculation for the reaction

$$CH_2OH\text{-}CHOH\text{-}CO_2^- \longrightarrow H_2O + CH_3\text{-}CO\text{-}CO_2^-,$$

$$\Delta F = -8250 \text{ calories.}$$

The free energy of glycerate therefore is - (-8250) - 56,690 - 107,900 = -156,340 calories.

Lipmann also calculated the energy of hydrolysis of phosphoglycerol as -2280 cal. so that the free energy of 3-PGA may be estimated as Ⓟ -156,340 + 2280 = Ⓟ -154,060 cal., where Ⓟ equals the free energy of $H_2PO_4^-$ minus the free energy of water.

The free energy differences between 2-PGA, 3-PGA and phosphoenolpyruvate can be calculated from the equilibrium concentrations, namely 59% 3-PGA, 12% 2-PGA, 29% PEP by the relation

$$\Delta F = -RT\ln K.$$

The resulting values are:

$$3\text{-PGA} \rightleftharpoons 2\text{-PGA} \qquad \Delta F = +950 \text{ cal.}$$

$$2\text{-PGA} \rightleftharpoons PEP + H_2O \qquad \Delta F = -550 \text{ cal.}$$

Consequently,

ΔF of 2-PGA = (P) -154,060 + 950 = (P) -153,110 cal.

and

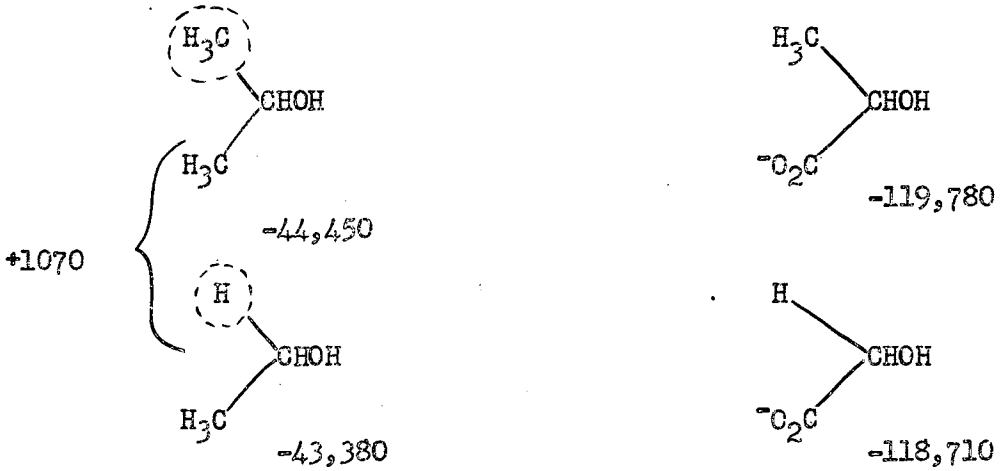
ΔF of PEP = (P) -153,110 + 56,690 -550 = (P) -96,970 cal.

Since ΔF for pyruvate is -107,900 cal., the energy liberated on hydrolysis of the high energy phosphate bond is, in this case,

-107,900 - 96,970 = -10,930 or - 11,000 cal.

The energy liberated by a secondary phosphate ester (2-PGA) is -2280-950 or about -3200 cal.

The free energy of glycollate was estimated from the free energies of isopropyl alcohol, lactate, and ethyl alcohol.



The substitution of -H for $-CH_3$ on a secondary alcohol should produce approximately the free energy change in the two cases.

The free energies of the remaining phosphate esters and anhydrides were calculated by simply assuming that the free energy of formation of each primary ester is -2280 cal., each secondary ester -3200 cal., and each anhydride or "high energy" phosphate bond -11,000 cal.

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