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The Path of Carbon in Photosynthesis

X. Carbon Dioxide Assimilation in Plants

M. Calvin, J. A. Bassham, A. A. Benson, V. Lynch,

C. Ouellet, L. Schou, W. Stepka, and N. E. Tolbert

April 1, 1950

Berkeley, California

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THE PATH OF CARBON IN PHOTOSYNTHESIS

X. CARBON DIOXIDE ASSIMILATION IN PLANTS(*)

M. Calvin, J.A. Bassham, A.A. Benson, V. Lynch, C. Ouellet,

L. Schou, W. Stepka, and N.E. Tolbert

Radiation Laboratory and Department of Chemistry

and Division of Plant Nutrition

University of California, Berkeley

ABSTRACT

The conclusions which have been drawn from the results of $C^{14}O_2$ fixation experiments with a variety of plants are developed in this paper. The evidence for thermochemical reduction of carbon dioxide fixation intermediates is presented and the results are interpreted from such a viewpoint.

The relative rates of appearance of the first observed products of carboxylation reactions of photosynthesis (phosphoglycerate and malate) have been shown dependent upon experimental conditions. The cyclic sequence of reactions required for regeneration of the postulated C_2 carbon dioxide acceptor is discussed in the light of accumulated evidence. Such evidence obtained from degradation of probable intermediates is

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(*) The work described in this paper was sponsored by the U.S. Atomic Energy Commission.

For publication in The Proceedings of the Society of Experimental Biology.

reduction. By employing the results of studies not only of photosynthesis in green plants, but also of photosynthetic bacteria and other biological systems, van Niel had presented a strong argument for the division of these reactions into two major classes. The first group of reactions involves the primary absorption of light energy and its conversion to chemical energy and the employment of this energy to decompose water, forming oxygen and reducing power. The other group of reactions is comprised of those processes by which this reducing power converts carbon dioxide to the various organic compounds formed in photosynthesis.

Further confirmation of the proposal that carbon dioxide reduction is accomplished entirely by dark reactions was obtained through preillumination studies conducted in this laboratory (1-4). In these experiments, green algae Chlorella and Scenedesmus were first illuminated in the absence of carbon dioxide and then allowed to fix $C^{14}O_2$ in the dark. When the cells were killed and analyzed, the C^{14}-labeled products were found to be the same as those formed when the algae were allowed to fix $C^{14}O_2$ in the light for short periods and then killed immediately. Moreover, these experiments indicated that the reducing power had a half-life of several minutes in the dark. With the point of view engendered by these results, the efforts in this laboratory have been directed toward the revelation of the mechanism of the utilization of this energy in carbon dioxide reduction.

Normal photosynthesis is a steady-state process. The rates of the individual reactions involved are highly dependent upon external factors such as light intensity, carbon dioxide pressure, temperature and nutrient

conditions. An intermediate between carbon dioxide and protein, fat, and carbohydrate will exist in amounts dependent upon the relative rates of its formation and conversion. It is seen, then, that the amounts of isolable intermediates may vary considerably and that one might expect rather low concentrations of many important metabolites.

Experiments with high specific activity $C^{14}O_2$ have allowed a great number of observations on this hitherto almost impemetrable system of reactions. The advantages of such a method lie first in its sensitivity; intermediates of concentrations of less than 10^{-6} M may be readily determined in a few milligrams of plant material. Secondly, addition of labeled carbon to a plant in steady-state photosynthesis with $C^{12}O_2$ allows one to follow the path of carbon in the normal reduction of carbon dioxide. When labeled carbon dioxide ($C^{14}O_2$) is added to that being absorbed by the plant, Figure 1, the reservoirs of the intermediate products are consecutively labeled with C^{14}. The specific radioactivity of each reservoir increases to a maximum (equal to that of the initial carbon dioxide) prior to that of any subsequent intermediate. Analysis of the products of photosynthesis in $C^{14}O_2$ by two-dimensional paper chromatography has allowed separation of most of the low molecular weight products. When the plant is exposed to radiocarbon for shorter periods it is found that fewer polymeric products are formed. As the time is decreased, the amount of radioactivity incorporated into the fats, protein and carbohydrate approaches zero, and only the intermediates of hexoses and amino acid synthesis are detectable. Figure 2 shows the relative rate of appearance of radioactivity in intermedidates

soluble in aqueous alcohol.

Exposure of various plants to C^{14}-labeled carbon dioxide under a variety of conditions, both in the light and in the dark, followed by killing the plants and analysis of the labeled compounds formed has led to the following experimental results and conclusions.

Products of Short Photosynthesis.- The length of exposure of an actively photosynthesizing plant to labeled carbon dioxide in the light was shortened until all the labeled carbon fixed by the plant was found in a few compounds (3,5,6). These compounds were found to be phosphoglyceric acid, phosphopyruvic acid, malic acid and sometimes glyceric acid. For example, when the green alga Scenedesmus was allowed to photosynthesize at 10,000 foot candles for five seconds, analysis showed that 87% of the activity was incorporated in phosphoglyceric acids, 10% in phosphopyruvic acid, and 3% in malic acid. Radioactive products of five second and ninety second photosynthesis by Scenedesmus in $C^{14}O_2$ are shown in the radiograms of Figure 3. These are radioautographs of paper chromatograms of the products extracted from 50 mg. of packed cells.

Identification of Phosphoglyceric Acid.- Acid hydrolysis of phosphoglyceric acid which was isolated chromatographically produced glyceric acid. The glyceric acid was identified by subjection to periodate oxidation. All of the radioactivity initially present in the glyceric acid could be accounted for in the expected products of periodate oxidation, carbon dioxide, formic acid and formaldehyde. Phosphoglyceric acid was identified independently by its isolation from Scenedesmus which had photosynthesized for five seconds in $C^{14}O_2$. Over 65% of the fixed activity was so isolated,

and the product characterized as the barium salt by its solubility, specific rotation, phosphorus analysis, and ion exchange resin adsorption properties (6). Phosphoglyceric acid had previously been identified as a major constituent of the products of 30 second photosynthesis by Scenedesmus by its adsorption properties on anion exchange resins (1). The phosphoglycerate so isolated was hydrolyzed to glyceric acid which was identified by physical properties, distribution coefficient, acid strength, anion resin adsorption properties, and by conversion to the p-bromphenacyl ester (1,4).

Sequence of the Intermediates in Photosynthesis.- As longer exposures to $C^{14}O_2$ in the light are permitted, fifteen-sixty seconds, radioactivity is found not only in the above compounds but also in aspartic acid, alanine, serine, glycine, glycolic acid, and triose phosphates, hexose phosphates, hexose diphosphate, sucrose and several other as yet unidentified phosphorus-containing compounds. (Figure 4) Radioactivity accumulated more slowly in succinic, furmaric, citric and glutamic acids, glucose, fructose and a number of amino acids (threonine, phenylalanine, glutamine, asparagine, tyrosine). It should be noted that appearance of a labeled compound in the light and not in the dark indicates that that compound is a product but not necessarily an intermediate of photosynthesis.

The identity of a number of these phosphate esters with those involved in glycolysis suggested that the synthesis of sucrose is accomplished through a reversal of glycolysis. When sucrose isolated from Chlorella which had photosynthesized for 30 seconds in $C^{14}O_2$ was

hydrolyzed, the specific activity of the fructose was twice that of the glucose moiety. This reeult is that expected from a reversal of the glycolytic sequence. Further support for this suggested mechanism of sucrose synthesis is found in the identity of the distribution of C^{14} in the 3,4, the 2,5 and the 1,6 carbons of hexose with that in the carboxyl, alpha and beta carbons, respectively, of glyceric acid (Table 1).

The proteim of the insoluble products formed during 60 second photosynthesis by Scenedesmus contains radioactive amino acids in approximately the same proportion as those found in the soluble products. The proteins synthesized in longer times show that the proportion of amino acid constituents differs greatly from that of the free amino acids. The insoluble products synthesized by barley in five minutes consist largely (>95%) of polyglucose compounds; those of Scenedesmus are about half polysaccharide and half protein, of which alanine and aspartic acid are the major constituents.

Effect of Light Intensity on Early Products.- As the light intensity is decreased the number of products formed in 30 seconds decreases until at 400 foot candles the principal products are the three carbon compounds as is seen in the radiograms (Figure 5). These are phosphoglyceric acid and phosphopyruvic acid. It has not yet been possible to differentiate 2- and 3-phosphoglycerate with certainty on paper chromatograms. The collection of information available is consistent with the possibility that the major compound in the 400 foot candle radiogram is 3-phosphoglycerate, while that below and to the right of it is 2-phosphoglycerate. Separate experiments

performed at high light intensity (4000 foot candles) and low temperature (2°C) showed reversal of radioactivity accumulation in this pair of compounds. At low temperature one might expect the equilibrium between 2- and 3-phosphoglycerate to be slowly attained, hence the prior labeling in the 2-isomer. At higher temperature and low light intensity the greater amount of radioactivity in the 3-isomer is attributed to its rapid formation from the less stable 2-isomer.

The radioactivities determined by counting of radioactive areas of the chromatograms defined by the radiograms in Figure 5 are given in Table 2.

Dark Fixation Products.- Two types of dark fixation of labeled carbon were observed. The first type was obtained when plants were exposed to $C^{14}O_2$ immediately following a period of illumination in the absence of carbon dioxide (Figure 10a), in which case the labeled products as well as their rates of formation were found to be nearly the same as in short exposures (15-60 seconds) in the light(Figure 10b). The proportion of radiocarbon fixed was appreciably greater (3). Depletion of the malic acid (C_4) and alanine (C_3) reservoirs by preillumination (reduction to hexose) resulted in their restoration with labeled compounds as soon as a source of carbon dioxide became available.

The second type of fixation was obtained when the exposure to radioactive carbon dioxide in the dark did not follow soon after a period of illumination (Figure 10c). A much slower rate of fixation of radiocarbon (one-tenth to one one-hundreth the rate) was observed and the labeled

products (95% of the total) were malic, succinic, fumaric, citric, glutamic and aspartic acids, and alanine (3). These compounds are believed to be labeled by fixation of carbon dioxide through reversibility of the common carboxylation reactions. The effect of light on the labeling of some of these compounds will be discussed later.

Those compounds labeled in the light and in preillumination experiments only are considered products of photosynthesis, while alanine, malic acid, and aspartic acid, labeled slowly in non-preilluminated dark experiments and much more rapidly in light and preilluminated dark experiments, are considered to be products of both photosynthesis and reversible respiration reactions.

Degradation Studies

Degradation of hexose formed during short periods of photosynthesis with labeled carbon dioxide revealed that the highest percentages of labeled carbon were in the 3 and 4 positions, the next highest in the 2 and 5 positions and the least in the 1 and 6 positions. In some cases, labeling of the 1,6 positions was found equal to that in the 2,5 positions (4,8). Degradation of phosphoglyceric acid and of alanine demonstrated that the greatest labeling was in the carboxyl groups. Exceptions to this distribution (4a) have been considered caused by brief photosynthesis of exchange with $C^{12}O_2$ immediately before killing the plant. This resulted in decreased carboxyl labeling and 3,4 labeling in hexoses. The results of a number of degradations are given in Table 1. It is seen that as the length of exposure of the plant to $C^{14}O_2$ is shortened, the proportion of radiocarbon in the

carboxyl group of glyceric acid to the total radiocarbon in the molecule becomes large in the case of the preillumination experiment. This result suggests that phosphoglyceric acid which is the first isolable product of photosynthesis is formed by a carboxylation of some C_2 compound.

Malic and aspartic acids from short-term photosynthesis with $C^{14}O_2$ have been degraded and again most of the labeling has been found in the carboxyl groups. This distribution of activity, together with the early appearance of labeled malic acid and phosphopyruvic acid, suggests that phosphoglyceric acid is converted to phosphopyruvic acid which then is carboxylated as in the Wood-Werkman reaction (9) to give oxaloacetic acid from which, in turn, malic and aspartic acids would arise. The enzyme system for such a carboxylation has been found in higher plants by Vennesland et. al. (10). Thus, there would be two carboxylations involved in carbon dioxide reduction in the light, one a C_1 to C_2 addition and the other a C_1 to C_3 addition.

Additional evidence for these two fixation mechanisms was obtained from comparison of tracer studies at high and low light intensities. While the predominant labeled product of short exposures to $C^{14}O_2$ at high light intensity (400 to 10,000 foot candles) is phosphoglyceric acid, the principal labeled product at low light intensities (50 foot candles and lower) is malic acid (11). This variation in products is thought to be the result of variation with light intensity of the concentrations of the respective carbon dioxide acceptors.

is known to be present in these experiments, and from triose phosphate one obtains some pyruvaldehyde under the conditions of analysis of the plant extract. Commercial pyruvaldehyde contains appreciable amounts of acetaldehyde and formaldehyde which probably are decomposition products. Labeled pyruvic acid might give some formic and acetic acids.

Another argument against C_1-C_1 condensation can be based on the absence of appreciable radioactivity in two-carbon compounds in short-term experiments. Glycine and glycolic acid are not found to be significantly labeled in short experiments (one second barley photosynthesis showed no detectable glycolate or glycine), and this evidence is in agreement with the fact that the alpha and beta carbon atoms of glyceric acid possess only 5% of the total label of the glyceric acid molecule in these experiments. If the C_2 compound were formed by condensation of C_1 compounds, present in very small concentrations, then the C_2 compounds should become labeled very rapidly.

Finally, there is good evidence that the labeled carbon which is eventually incorporated in the C_2 compound must first be incorporated in a C_3 or C_4 compound. If plants are illuminated in $C^{14}O_2$ for short periods under conditions where the C_3 and C_4 compounds are normally found to be labeled and then are illuminated for an additional period in the absence of carbon dioxide, there is found to be a disappearance of C_3 and C_4 compounds and an accumulation of labeled glycolic acid and glycine. This suggests the close relationship between the latter two compounds and the C_2 carbon dioxide acceptor. This carbon dioxide

acceptor must be formed as a direct result of the photochemically produced reducing power and must itself accumulated in the absence of carbon dioxide. This explains why the formation of phosphoglyceric acid is the light sensitive carboxylation.

If glycolic acid and glycine, accumulated by illumination in the absence of carbon dioxide (Figure 11a,b) are assumed to be derived from or are precursors of the C_2 acceptor molecule, one must conclude that the C_2 acceptor is not the product of C_1 reduction and C_1-C_1 condensation. The formation of such a C_2 compound by direct reduction of carbon dioxide and C_1-C_1 addition would be highly dependent upon the amount of carbon dioxide available, and conditions of low carbon dioxide pressure would not lead to the observed great increase in C_2 compounds.

If the C_1-C_1 condensation is dismissed as the mechanism of C_2 formation, there is left the alternative of splitting the C_2 compound from a larger molecule. The only larger molecules found to be labeled in the very short-term photosynthesis experiments were the C_3 and C_4 acids, phosphoglyceric acid, phosphopyruvic acid and malic acid, while, at the same time, a small but significant labeling of the alpha and beta carbons of phosphoglyceric acid was found. Since the sum of the radioactivity found in the C_3 and C_4 compounds in such short term experiments was equal, within experimental error, to the total activity fixed during the experiment, it appears that all appreciably labeled compounds were detected by methods of analysis employed. The splitting of a C_3 compound would result in either a profitless decarboxylation or in the formation of formaldehyde

or formic acid, neither of which have been found to be labeled significantly even in longer experiments. Consequently, the most likely regenerative mechanisms would appear to be the cleavage of a C_4 dicarboxylic acid to give two C_2 molecules which would be converted to the two-carbon carbon dioxide acceptor. Thus, there would be a regenerative cycle consisting of C_1 to C_2 addition, C_1 to C_3 addition, and splitting of a C_4 compound to two C_2 compounds. This proposed cycle will be designated as "cycle" A in this paper. Thus far, no experimental evidence has been found which would contradict the existence of the proposed cycle.

Varner and Burell (12) report experiments in which Bryophyllum leaves were exposed to $C^{14}O_2$ in the dark and then exposed to light in an atmosphere free of $C^{14}O_2$. Degradation of malic acid formed in one of these experiments gave 21% of the total labeling of the molecule in carbon atoms 2 and 3, 34% in carbon 1 (alpha carboxyl), and 45% in carbon 4 (beta carboxyl). Degradation of glucose from starch in the same experiments gave for th 3,4 carbon atoms 52% and for the 2,5, and 1,6 positions (total for four atoms) 48%. Varner and Burrell concluded that the conversion of malic acid to carbohydrate does not take place via the cycle A mechanism described above, since this mechanism should produce hexose predominantly labeled in the 2,5 positions rather than the 3,4 positions. They further concluded that the labeling found could be accounted for by a reversal of the Wood-Werkman reaction. Unfortunately, although it is stated that the plants are exposed to light in an atmosphere free of $C^{14}O_2$, no mention was made as to whether or not unlabeled carbon dioxide

was excluded at the same time. If carbon dioxide was excluded, then cycle A, by itself, of course, could not operate since it involves a carboxylation. Even if carbon dioxide were not excluded during illumination it would not be surprising if Bryophyllum, which stores carbon in a large reservoir of malic acid in the dark, should, upon illumination, convert this carbon to phosphoglyceric acid via the reversible Wood-Werkman reaction. It is unlikely that this plant would depend upon its natural environment, notably deficient in carbon dioxide, to supply sufficient carbon dioxide for conversion of malic to carbohydrate entirely through cycle A. It seems likely that the carbon dioxide, temporarily "freed" by the Wood-Werkman decarboxylation never actually escapes the cell, but rather is used immediately in the carboxylation of C_2 compound and possibly in other carboxylation reactions not related to photosynthesis. If only C_2 to C_1 carboxylation is involved, then for each two malic acid molecules decomposed via the Wood Werkman reaction, one is cleaved via cycle A. If, in Varner and Burrell's experiments, the combination cycle A and Wood-Werkman reaction mechanism described above were operating, the resulting distribution of labeling in hexoses would be 53% in the 3,4 position and 47% in the 2,5 plus 1,6 positions. If only the Wood- Werkman transformation were involved, these figures would be 62% and 38%, respectively. Consequently, the Bryophyllum experiments are by no means in contradiction to the proposed cycle.

In attempting to elucidate details of cycle A, several dicaracids have been considered as possible intermediates. Succinic and fumaric acids, tentatively suggested in earlier papers, appear more likely to be respiration intermediates than photosynthetic intermediates, since their specific activities increase only slowly during photosynthesis. In

fact, in some cases, alpha, beta labeled glyceric acid and 2,5 plus 1,6 labeled hexose have been found in the complete absence of any labeled succinic acid. However, malic acid, because of its more rapid labeling in the light, seemed a possible intermediate in the proposed cycle.

In order to ascertain whether malic acid might be such an intermediate, an attempt was made to inhibit its formation during short periods of photosynthesis (7). Scenedesmus was pretreated with sodium malonate buffer in the dark, and resuspended in malonate-free buffer in the light. Finally, after a suitable adaptation period in the light the actively photosynthesizing cells were exposed to $C^{14}O_2$ for short periods. It was found on analysis of the cell constituents that although total fixation of labeled carbon was decreased only slightly (12-35%) over that fixed under similar conditions by non-malonate pretreated cells, the radiocarbon incorporated as malic acid was strongly decreased (60-97%). The other products of this short term exposure were relatively unchanged. Moreover, degradation of glyceric acid from the malonate treated cells and untreated cells showed a labeling of the alpha and beta carbon atoms which was not decreased by malonate pretreatment. This result is interpreted as indicating that malic acid is not itself an intermediate between carbon dioxide and the alpha and beta carbon atoms of glyceric acid in photosynthesis. Consequently, if the conclusion that phosphoglyceric acid is an intermediate in carbohydrate photosynthesis is correct and if carbohydrate is formed from

phosphoglyceric acid by a reversal of glycolysis reactions, then malic acid is not an intermediate in photosynthesis. The role of malic acid appears, therefore, to be that of a carbon reservoir, readily derived from an intermediate in photosynthesis.

Since neither malic acid, fumaric acid nor succinic acid appears to be an intermediate in cycle A, the four-carbon compound which is split to two C_2 fragments must be either a four carbon dicarboxylic acid or some other four carbon compound that can be derived from oxaloacetic acid without first being converted to malic acid. There are four such dicarboxylic acids with the terminal carboxyl groups as would be expected from the proposed carboxylation mechanism. Oxaloacetic acid itself might cleave hydrolytically to give one molecule of glycolic acid and one molecule of glyoxylic acid. Tartaric acid, the hydration product of oxaloacetic acid, would give the same products. Dihydroxymaleic acid, which might be formed by oxidation of oxaloacetic acid (or tartaric acid) could give two molecules of glyoxylic acid. Diketosuccinic acid could cleave hydrolytically to one molecule of oxalic acid and one molecule of glyoxylic acid.

Of the various possibilities the two most plausible seem to be the cleavage of tartaric acid, analogous to glycolytic splitting of hexose, and the cleavage of dihydroxymaleic acid by a reversal of the benzoin condensation. The benzoin type reaction is known to occur in certain organisms which form acetoin from acetaldehyde. Thus far, we have been unable to demonstrate the presence of labeled tartaric acid

in short term experiments with labeled $C^{14}O_2$.

An alternative mechanism for the cleavage of a four carbon molecule involves the preliminary reduction of one or both of the carboxyl groups of the 4 carbon acid followed by subsequent cleavage of the product. An analogy for such a reduction is found in the reduction of 1,3-diphosphoglyceric acid to 1,3-diphosphoglyceraldehyde. A very similar medhanism could be postulated for the reduction of 1,3-diphosphotartaric acid to 1,3-diphosphotartaric acid aldehyde.

This product could either be split to phosphoglycolic acid and phosphoglyoxal or further reduced to diphosphotartaric dialdehyde which could then be cleaved to phosphoglycolaldehyde and phosphoglyoxal. This mechanism, although as yet unsubstantiated by any experimental evidence, is made attractive by the close analogies for all the reactions involved that can be found in the reactions of glycolysis. Thus, the cleavage of diphosphotartaric dialdehyde bears a close resemblance to the splitting of 1,6-fructose diphosphate by aldolase.

The various paths from C_4 to C_2 fragments described above are shown in Figure 7. For the sake of simplicity, only the non-phosphorylated forms are shown. Whatever the mechanism of the cleavage, the products presumably would be reduced to the C_2 carbon dioxide acceptor, either vinyl phosphate or glycol phosphate.

If the cleavage is at the dicarboxylic acid level, then glyoxylic acid and glycolic acid might be intermediates in this reduction, but if the splitting is at the dialdehyde level these two carbon acids are formed by

side reactions. The latter mechanism gains support from the degradation studies of glycolic acid and glyceric acid obtained from 15 second exposure of barley leaves of $C^{14}O_2$ in the light (Table 1). Since more than one-half the label of the glyceric acid was in the carboxyl group it is to be expected that after another carboxylation with $C^{14}O_2$ at least two-thirds of the label of the 4 carbon intermediates was in the two carboxyl groups. If the C_4 acid were split directly to two C_2 acids, the carboxyl group of the C_2 acid would arise from the carboxyl groups of the C_4 acid and should carry two-thirds of the label of the C_2 molecule. If the cleavage took place at the dialdehyde level, there is the possibility of obtaining symmetrically unlabeled products which could then be oxidized to symetrically labeled glycolic acid. The distribution of radiocarbon found in the glycolic acid is in accord with the latter mechanism.

However, it should be noted that a number of experiments have been reported both from this laboratory and from others (4,8,12) in which the 2,5 carbon atoms of a hexose do not have the same specific activity as the 1,6 carbon atoms. This implies that there exists routes in which the C_2 fragment maintains its unsymmetrical labeling throughout the cycle.

The formation of labeled glycolic acid during short periods of photosynthesis with $C^{14}O_2$ was found to be dependent on the partial pressure of oxygen. Thus, in corresponding lengths of exposure to $C^{14}O_2$, the percentage of total fixed activity found in glycolic acid was about ten times greater when the atmosphere surrounding the plant contained 20% oxygen than when the plant was exposed in an atmosphere containing 1% oxygen. This effect

might be explained in at least two ways. The oxygen might be used in the oxidation of oxaloacetic acid to a more oxidized acid if the latter is an intermediate in cycle A. In this case the operation of cycle A would be accelerated by the increase in oxygen pressure described above.

If the cleavage of C_4 compound occurs at a lower reduction level, then the initial C_2 cleavage products might be more reduced than glycolic acid and the oxidation of these reduced compounds would be favored at increased oxygen concentrations. In this case, the formation of C_2 carbon dioxide acceptor would be decreased by increased oxygen. It may be possible in future experiments to measure the rate of formation of C_2 carbon dioxide acceptor in the presence of high and low oxygen pressures by degrading the labeled phosphoglyceric acid and thus discover which of the above explanations of oxygen-enhanced glycolic acid formation is the more probable.

Relation of Respiration to Photosynthesis

The processes involved in respiration in animal tissues and in yeasts have been shown to be operative in plant tissue (13). Evidence that the major oxidative and decarboxylation reactions are in accord with the existence of a tricarboxylic acid cycle is rapidly accumulating.

The rate of respiration is approximately one-twentieth that of photosynthesis at saturating light intensities, but a vast number of experiments have been reported at intensities near the compensation point. Toward the end of understanding the relationships between the chemical reactions of photosynthesis and respiration we have performed a number of experiments (3).

Dark Respiration. As described earlier, the series of reversible reactions ending in decarboxylations has been shown to involve the intermediates of the Krebs tricarboxylic acid cycle, aspartate (oxalacetate), citrate, isocitrate, glutamate, alpha-ketoglutarate, succinate, fumarate and malate. Aspartic acid, malic acid and alanine become labeled with C^{14} both in photosynthesis and in dark $C^{14}O_2$ fixation experiments. It is possible that the reservoris of these compounds are common to both photosynthesis and respiration.

A series of experiments were performed with Scenedesmus and barley leaves in which the products of short (30 seconds) photosynthesis were partially respired in the dark immediately afterwards. Algae rapidly converted the phosphate esters involved in sucrose synthesis to glutamate, succinate, fumarate and citrate. The steady decrease in radioactivity in the cells showed that $C^{14}O_2$ was being lost by decarboxylations. The compounds observed to be most susceptible to respiration in these experiments were glycolic acid and the phosphate esters. One hour of dark respiration in air diminished the amount of labeled phosphate esters to about 10% of its value immediately following 30 seconds photosynthesis. After 18 hours dark respiration the products closely resembled those found by dark exchange of $C^{14}O_2$ in the respiratory intermediates. Barley leaves have not produced observable amounts of labeled succinate and fumarate, even after extended periods of dark respiration. Labeled glutamic acid appears only after one hour of respiration by barley compared to its appearance in copious amounts in algae after two minutes. Radioactivity in glutamine invariably

parallels that of glutamate, although equilibrium between the two is not rapidly attained. If the gradual disappearance of radioactive hexose, triose, and glyceric acid phosphates in barley leaves involves succinate and fumarate as intermediates, the concentration of these acids must be very low.

In five minutes of photosynthesis barley leaves synthesize sucrose as the major product. Sucrose synthesized by 30 seconds of steady-state photosynthesis in $C^{14}O_2$ is not appreciably respired in the dark after an hour. During this dark time the amounts of radioactivity in phosphorylated intermediates of sucrose synthesis is rapidly and greatly diminished without notable decrease in sucrose radioactivity. When intact barley seedlings are allowed to photosynthesize 30 seconds followed by 18 hour dark respiration in air, the amount of radioactive sucrose remaining in the leaves is almost negligible. The roots, on the other hand were found to contain the active citrate, malate, alanine, phosphoglycerate and some sucrose. In these experiments, the carbon of sucrose was transferred to the roots where it appeared in respiratory products.

Light Respiration.- When algae or barley leaves photosynthesize (approximately 8000 foot candles) in $C^{14}O_2$ for 30 seconds and then, in one case, are illuminated in carbon dioxide-free air for a short time and, in another case, allowed to respire in the dark for the same length of time, the amounts of labeled tricarboxylic acid cycle intermediates formed in the light respiration experiment are much smaller than those

found in the dark respiration experiment. Anaerobically in the light the absence of respiration intermediates is even more striking. Such experiments must be done in the absence of $C^{12}O_2$ since the latter rapidly displaces the C^{14}-labeled products of the preceeding photosynthesis which are converted to polymeric products.

The major effect of aerobic illumination in the absence of carbon dioxide is the formation of glycolic acid and glycine. Up to 30% of the fixed radioactivity may be converted to glycolic acid in this way (Figure 8). Thus, glycolic acid rapidly accumulates in the light and very rapidly disappears in the dark(3). The possibility (remaining to be investigated) is that glycolic acid oxidation, catalyzed by an enzyme system widely distributed in green plants (14) and demonstrated in colorless algae (15) represents an alternative oxidative mechanism in the higher plants.

The inhibition of respiration by light was demonstrated by van Niel (16) in acetate metabolism in Rhodospirillum rubrum. The stability of photosynthetic intermediates toward oxidation in the light is also in accord with the kinetic results of Weigl (17) who worked with barley leaves. Weigl observed a ten-fold increase in specific radioactivity of respiratory carbon dioxide produced by immediately previously photosynthesized intermediates upon cessation of illumination. The carbon dioxide respired during photosynthesis then came from relatively non-radioactive sources. It is possible that the physical site of light respiration may be quite different from that of photosynthesis.

These results mean that light does at least affect respirability of intermediates of sucrose synthesis (3). The fact that some compounds

are found to be intermediates in both respiration and photosynthesis suggests that the same molecules of the compounds may be involved in both processes. This is not necesarily the case since the processes may be physically separated. The inhibition of the respiration of newly photosynthesized materials by light would seem to indicate a close interaction between the two processes. This interaction is at present best interpreted in terms of a reduction of the steady-state concentrations of respiratory intermediates which are used as photosynthetic intermediates in the light. This leads to a lower rate of appearance of newly incorporated carbon in the tricarboxylic acid cycle in the light.

Inhibition of respiration by illumination is dependent upon the light intensity. The experiments referred to above involved intensities of 10,000 foot candles. As the light intensity is decreased, the rates of appearance of radiocarbon in respiratory intermediates and in photosynthetic intermediates approach each other. Figure 9 shows the relationship of the rate of formation of radioactive respiratory intermediates and related reservoirs of sucrose synthesis (including alanine, aspartate and malate.)

Feeding Experiments. - A number of feeding experiments with both algae and barley have been performed. The interpretation of such experiments is complicated by such questions as the penetration of the substance into the cell and the fact of complex internal organization within the cell entailing the possibility of different sites of metabolism for the same substance. However, the feeding of labeled acetate to Scenedesmus had led to a definite result. In the dark, Figure 10a, acetate is respired through the expected

tricarboxylic acid cycle intermediates. In the light, Figure 10b, whether carbon dioxide is present or not, acetate is converted to fats in addition to the tricarboxylic acid cycle intermediates. Only a small fraction of the acetate consumed is converted to sucrose or its precursors. It is thus apparent that reducing power resulting from the photochemical reactions may be used to convert acetate directly into fats.

Poisoning Experiments.- A number of poisoning experiments have been performed using those poisons previously employed in the study of photosynthesis. In addition to the difficulties encountered in the interpretation of the feeding experiments there is the question of the number of sites and the relative degree to which they are affected by a given poison.

When photosynthesis in Chlorella suspended in 1.5×10^{-4} M iodoacetamide is inhibited as much as 90% as measured by $C^{14}O_2$ fixation, the absolute rate of sucrose synthesis (as measured by the amount of C^{14} in sucrose) is not decreased but is increased at lower degrees of inhibition. No abnormal phosphoglyceric acid accumulation occurs in the presence of iodoacetamide. (18).

In view of the known action of iodoacetamide on other enzyme systems beside triose phosphate dehydrogenase it is possible that these other systems are more sensitive to this poison. Such selective action might possibly result in the blocking of other paths of carbon utilization and lead to a more rapid sucrose synthesis at certain iodoacetamide concentrations. Alternatively, sucrose may be synthesized by a path not involving triose phosphate, although this in contradiction to the present accumulated information.

Cyanide and hydroxylamine are strong inhibitors of photosynthesis. We have shown that these poisons do not prevent the initial carboxylation step with the formation of phosphoglyceric acid. Scenedesmus (Gaffron D-3 strain) were placed aerobically in the light without carbon dioxide for 30 minutes in order to increase the concentration of the C_2 acceptor. The poison was then given to the algae one minute before adding $C^{14}O_2$. In this length of time the poison becomes effective but the light-generated C_2 acceptor will not have completely decomposed. Phosphoglyceric acid and malic acid are formed in large amounts and little of the C^{14} appears in the other compounds normally formed during photosynthesis, such as the phosphate esters and sugars (Table 3).

It has been suggested that hydroxylamine inhibits the oxygen liberating state of photosynthesis. This oxygen liberation step need not occur simultaneously with the first carboxylation, and the formation of phosphoglyceric acid in the presence of hydroxylamine verifies this suggestion. The formation of the glutamic acid and also of succinic, fumaric and citric acid during one minute photosynthesis in the presence of hydroxylamine is much greater than in an equivalent period of either dark fixation or carbon dioxide or of uninhibited photosynthesis. This indicated that hydroxylamine may reverse the light inhibition of respiration and therefore the rate of appearance of newly assimilated carbon in the respiratory intermediates.

Effect of pH on Carbon Dioxide Fixation.- In an attempt to influence the rates of the enzymatic processes and consequently the proportions of the produts, a number of one minute photosynthesis experiments were carried out on suspensions of Scenedesmus which had been photosynthesizing for 10 minutes at 3000 foot candles in M/300 phosphate solutions of

various pH values ranging between 1.6 and 11.4. The rate of fixation of $C^{14}O_2$ remained approximately normal between pH 4 and 9; it was still 50% or more at pH 2 and 10, but it fell abruptly above pH 10.5. The depressed rates observed were not further affected by letting the cells stand for 30 minutes; moreover, the normal rate could be restored by bringing the pH back to 7.

Radiochromatography of the samples revealed no striking differences between the nature and relative amounts of the radioactive products formed at those different pH values, except for malic acid and sucrose. As shown in Figure 11, on passing from pH 1.6 to pH 11.4 the percentage of malic acid increases from 5 to 25%, while that of sucrose decreases from about 7 to 0%. Phosphopyruvic acid seems to follow malic acid in that the absolute amounts of both these compounds show a sharp maximum at pH 9. On the whole, the general pattern does not break down even at extreme pH values. On the other hand, there are some compensation effects, such as a larger production of acidic material (malic) to counteract the alkalinity.

SUMMARY

The conclusions which have been drawn from the results of $C^{14}O_2$ fixation experiments with a variety of plants are developed in this paper. The evidence for thermochemical reduction of carbon dioxide fixation intermediates is presented and the results are interpreted from such a viewpoint.

The relative rates of appearance of the first observed products of carboxylation reactions of photosynthesis (phosphoglycerate and malate) have been shown dependent upon experimental conditions. The cyclic sequence of reactions required for regeneration of the postulated C_2 carbon dioxide acceptor is discussed in the light of accumulated evidence. Such evidence obtained from degradation of probable intermediates is tabulated.

Respiration and photosynthesis have been found interrelated. There are compounds common to both systems. Light has an inhibitory affect upon respiration of immediately previously formed intermediates of hexose synthesis. This effect has been recognized as a function of light intensity. It has also been shown pertinent in acetate metabolism in algae.

Preliminary observations on the effects of inhibitors and abnormal conditions upon the course of carbon dioxide fixation interpreted on the basis of present information.

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C^{14} Distribution in Photosynthetic Products

Plant	B	C	B	C	S	B	B	B	S	S	S	S	S	S	S	B	B	B	B
Condition	PI	PI	PS	PS	PS	PS	PS	PS	PS	PS	PS	PS	PS	PS	PS	PS	PS	PS	PS
Time (sec)	120D	1800D	2	5	5	15	30	60	30	90	90 MI	30	30 MI	60	60 MI	60	60 MI	15	30	40
Foot Candles ($\times 10^{-3}$)	(Dark) 10	(Dark) 10	10	10	10	10	1	10	10	10	10	3	3	2.7	2.7	2.7	2.7	10	10	10
Glyceric																				
COOH	96		85	95	95	49	75	44				81	73	51	48	44	43	56		
CHOH	2.6			3	2.5	25	6	30				7	12	24	24	25	27	21		
CH_2OH	1.7		15	2	1.2	26	9	25				10	15	25	28	31	30	23		
Alanine																				
COOH		89				67		48		56	~50									
$CHNH_2$		10				30		44		44	~50									
CH_3		0.5						<5												
Glycolic																				
COOH																		50±5	48	47
CH_2OH																		50±5	52	53
Sucrose																				
C_3-C_4		76				52		37	87											
C_2-C_5		17				25		34	7											
C_1-C_6		7				24		32	6											
Malic Acid																				
Both (-COOH)									93.5											
CHOH-CH_2-									6.5											
Aspartic Acid																				
Both (-COOH)									96											
-$CHNH_2$-CH_2									4											

PI - Preilluminated, PS - Photosynthesis, MI - Malonate pretreated

TABLE 2

Effect of Light Intensity on Early Products

Fraction of radioactive carbon fixed expressed in percent, in products of steady state photosynthesis in 4% CO_2 in air by Scenedesmus -D_3 in thirty seconds.

Light Intensity[a] (foot candles)	400	800	4000	8000
Total C^{14} fixed[b] c.p.m. x 10^{-6}	0.36	0.65	3.6	3.7
3-phosphoglyceric acid	55	33	10	12
2-phosphoglyceric acid[c]	3	17	5	5
phosphopyruvic acid[d]	12	10	5	5
triosephosphates	1	1.3	1.6	1.5
hexose phosphates	15	17	69	62
malic acid	3.0	4.1	4	6.1
aspartic acid	1.8	2.5	1.5	2.0
alanine	1.7	1.2	1.5	1.6
serine	--	.3	.3	.5
glycine	--	.2	.2	.4
glycolic acid	--	.3	.2	.8
sucrose	--	.3	.5	1.0
fats	--	--	.6	2.4
succinic acid	0.5	--	.2	0.1
fumaric acid	--	--	.2	0.15
citric acid	2	1.2	.5	0.6

TABLE 3

Effect of Cyanide and Hydroxylamine on $C^{14}O_2$ Fixation by Scenedesmus During Photosynthesis

Compound	Per Cent of Total Soluble C^{14} Fixed		
	No Inhibition	5×10^{-3}M Hydroxylamine (75% Inhibition)	3×10^{-4}M Cyanide (95% Inhibition)
phosphoglyceric	16.1	7.7	23.4
glyceric	4.0	2.9	0.0
triose and hexose PO_4	36.0	1.0	7.3
malic	24.0	62.1	26.3
glutamic	0.5	2.2	0.0
aspartic	9.0	0.0	5.6
alanine	3.6	0.0	16.1
succinic	0.8	5.3	1.2
fumaric	0.8	1.9	0.8
citric (iso)	0.8	6.3	2.3
sucrose	0.1	0.0	0.0

Figure Captions

Figure 1.

Figure 2. $C^{14}O_2$ Fixation by Scenedesmus. Total radioactivity fixed, ◯. Radioactivity fixed in 80% ethanol-insoluble products, ●. Light intensity, 500 foot candles. 4% carbon dioxide in air. Temperature, 20°C.

Figure 3. Radiograms of One and Sixty Second Steady State Photosynthesis by Barley Leaves. The paper chromatograms from which these radiographs were prepared were developed horizontally in phenol and vertically in butanol-propionic acid-water solvent (6).

Figure 4. Incorporation of C^{14} in Products of Short Photosynthesis by Scenedesmus. Light intensity, 500 f.c. Temperature 20°C. The radioactivity as defined by the radioautograph was determined directly on the series of paper chromatograms. Large thin window geiger-müller tubes (K. G. Scott type) were used for radioactivity determination. The products chromatographed include only those soluble in 80% ethanol.

Figure 5. Effect of Light Intensity Upon Products of Thirty Seconds Photosynthesis by Scenedesmus. Aqueous ethanol extracts of approximately equal amounts of cells were used in these radiograms. The two major compounds in the 400 f.c. radiogram were identified as phosphoglyceric acid and phosphopyruvic acid. The spot with increased radioactivity in the 800 f.c. radiogram was also identified as phosphoglyceric acid.

Figure 6. Dark and Photosynthetic $C^{14}O_2$ Fixation by Chlorella.

a. Two minutes dark fixation by Chlorella which had been preilluminated in nitrogen five minutes at 5,000 f.c.

b. Thirty seconds steady state photosynthesis, 8,000 f.c.

c. Forty-five minutes dark $C^{14}O_2$ fixation by non-preilluminated cells.

Figure 7.

Figure 8. Products Synthesized by Sugar Beet Leaf During Five Minutes Followed by Two Minutes Aerobic Illumination. Glycolic acid represents almost thirty percent of the 50% ethanol-soluble radioactive products.

Figure 9. Effect of Light Intensity on Incorporation of $C^{14}O_2$ into Photosynthesis Products and Some Respiration Products. The lower curves include radioactivity in succinic, fumaric, citric and glutamic acids only. The curves for photosynthesis include the remainder of the 80% ethanol-soluble products. It should be noted that the time as well as the radioactivity scales are different in the two plots. The relative slopes, however, are quite different.

Figure 10 a,b. Assimilation of Acetate-1 C^{14} by Scenedesmus. Algae were given labeled acetate for thirty minutes (8.5×10^{-4} Molar, pH 4) immediately after steady state photosynthesis (8,000 f.c.). Half of the cells were killed in 80% ethanol in the dark, 10a. The remainder was illuminated ten minutes, 8,000 f.c., and killed, 10b. Considerable unused labeled acetate remained after the thirty minute dark period. During the whole experiment, 4% carbon dioxide in nitrogen passed through the cell suspension.

Figure 11. Effect of pH on Fraction of Sucrose and Malic Acid in Products of Sixty Seconds Photosynthesis by Scenedesmus. These figures were obtained by direct counting on paper chromatograms of several series of experiments.

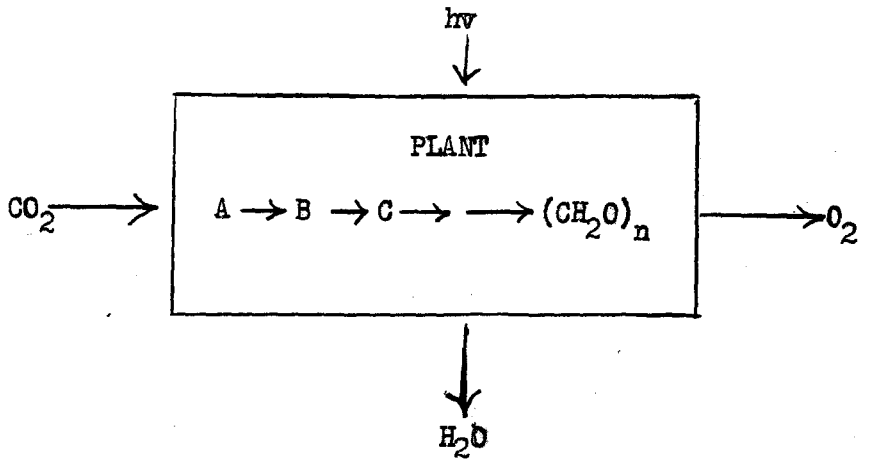

Figure 1.

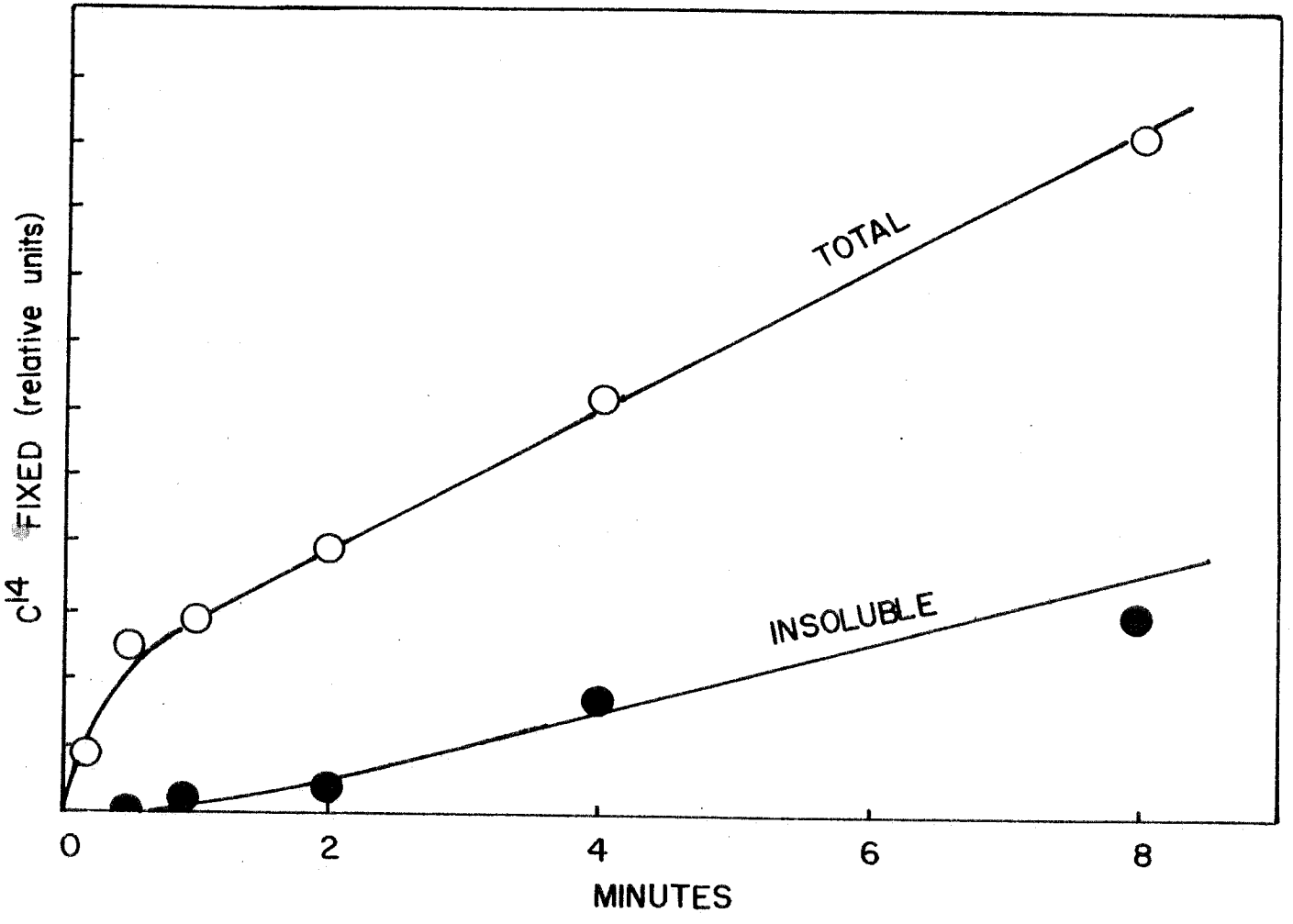

FIG. 2

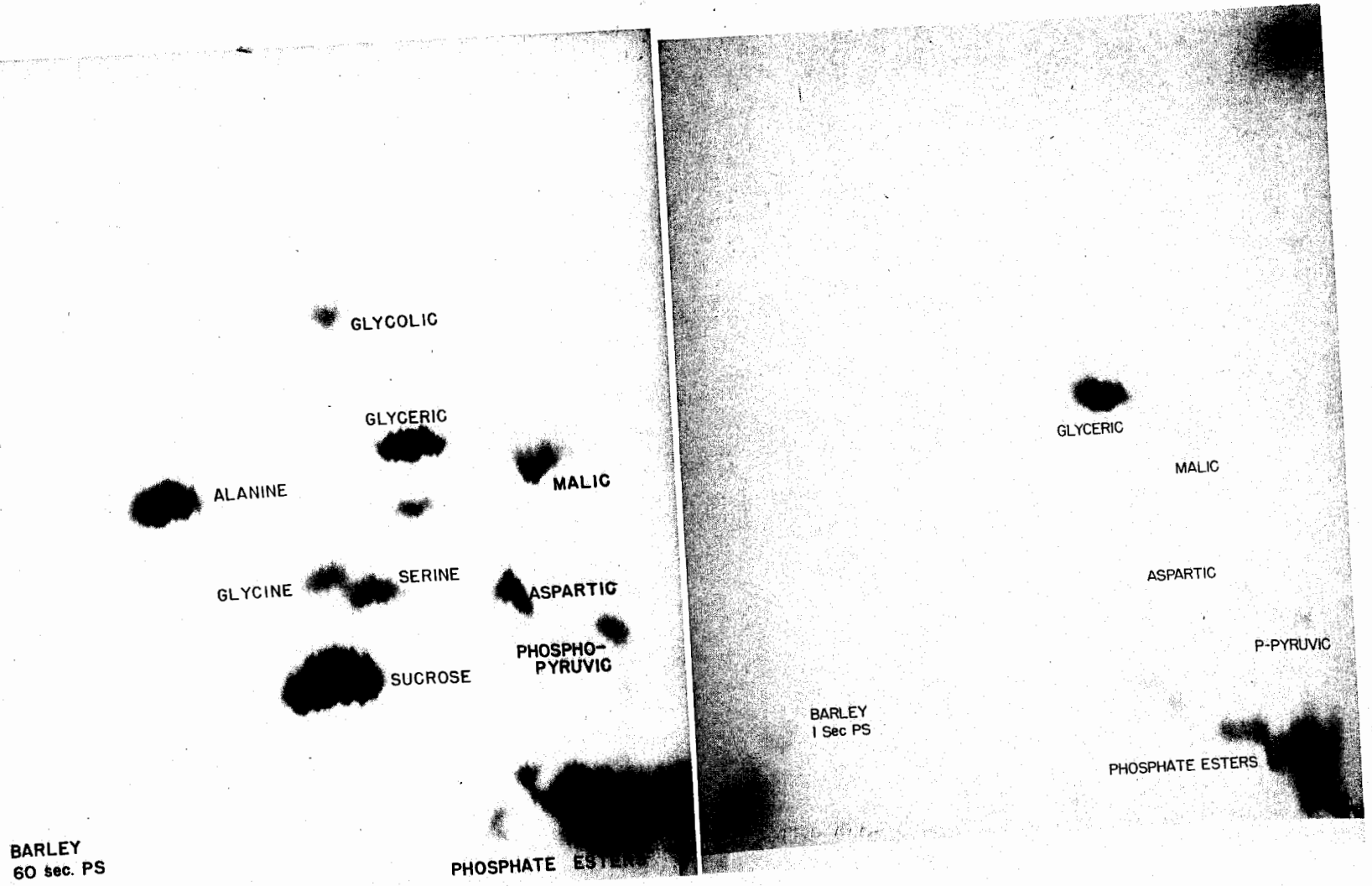
GLYCOLIC
GLYCERIC
ALANINE
MALIC
GLYCINE
SERINE
ASPARTIC
PHOSPHO-
PYRUVIC
SUCROSE
BARLEY
60 sec. PS
PHOSPHATE ESTERS
GLYCERIC
MALIC
ASPARTIC
P-PYRUVIC
BARLEY
1 Sec PS
PHOSPHATE ESTERS

FIG. 3

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