THE PATH OF CARBON IN PHOTOSYNTHESIS

VIII. THE RÔLE OF MALIC ACID*

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(Received for publication, January 27, 1950)

Exposure of various plants to C^{14}-labeled carbon dioxide under a number of different conditions both in the light and in the dark, followed by killing the plants and analysis of the labeled compounds formed by the plants, has led to the following experimental results (1).[1]

1. When the length of exposure of an actively photosynthesizing plant to labeled carbon dioxide in the light was shortened until nearly all the labeled carbon fixed by the plant was found in one compound, that compound was found to be phosphoglyceric acid (2). For example, when the green alga *Scenedesmus* was allowed to photosynthesize 5 seconds with labeled carbon dioxide and then killed, analysis showed that, of the total activity fixed and stable under the conditions of chromatographic analysis employed, 87 per cent was incorporated in phosphoglyceric acid, 10 per cent in phosphoenolpyruvic acid, and 3 per cent in malic acid.

2. As longer exposures to labeled carbon dioxide in the light are permitted (15 to 60 seconds), radioactivity is found not only in the above compounds, but also in aspartic acid, alanine, serine, glycine, glycolic acid, hexose diphosphate, triose phosphates, sucrose, and several as yet unidentified phosphorus-containing compounds (3, 4).

3. After still longer periods of exposure a large number of additional compounds are found to be labeled. These include succinic, fumaric, citric, and glutamic acids, glucose, fructose, and a number of amino acids (3–5).

4. When plants were exposed to $C^{14}O_2$ immediately following a period of illumination in the absence of carbon dioxide, the labeled products were found to be nearly the same as in the short exposures (15 to 60 seconds) in the light, although the proportion of radiocarbon found in malic acid, aspartic acid, and alanine to the total radiocarbon fixed was somewhat greater (5).

5. When the exposure to radioactive carbon dioxide in the dark did not

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

[1] See Calvin and Benson (2) for a general discussion of previous experimental results and conclusions derived therefrom.

follow soon after a period of illumination, the labeled products (95 per cent of total) were malic, succinic, fumaric, citric, glutamic, and aspartic acids, and alanine (5).

6. 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 and 6 positions was found equal to that in the 2 and 5 positions (6). Degradation of phosphoglyceric acid (by methods described below) and of alanine demonstrated that the greatest labeling was in the carboxyl carbon.

In an attempt to correlate and explain these experimental results the following proposals have been made.

A. Most of the labeled products formed in the short periods of light or following preillumination are formed not at all or much more slowly in the dark without preillumination. Such compounds are considered to be primary (not dependent upon the prior formation of sugars) products of photosynthesis. Into this classification fall the compounds listed under 1 and 2 above (2).

B. Those compounds found to be labeled in the dark non-preillumination experiments are formed at no appreciably greater rate in the light (with the exceptions of malic and aspartic acids and alanine); hence such compounds (listed under (5) above) are considered to be formed by exchange in the reversible respiration reactions (5).

C. Similarity of products from light experiments and dark preillumination experiments indicates that the reactions involving carbon dioxide reductions are "dark reactions" and that reducing power, generated in the light, has a half life of several minutes (5, 7, 8).

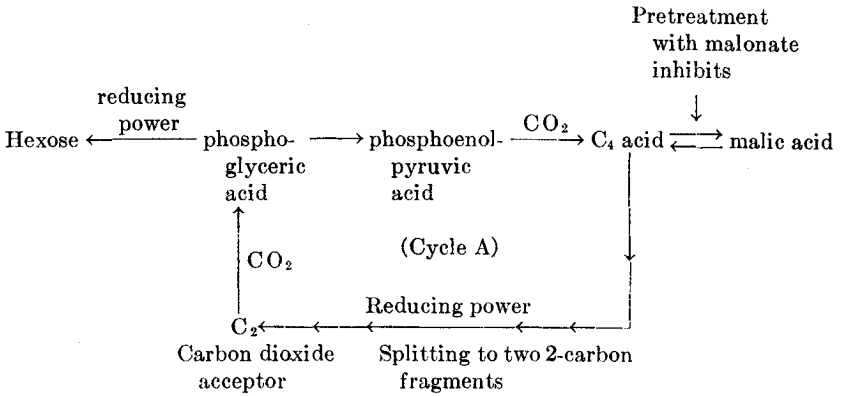
D. It is proposed that phosphoglyceric acid is formed by the carboxylation of a 2-carbon compound and that this reaction is involved in photosynthetic carbon dioxide assimilation. This is suggested by the fact that phosphoglyceric acid is the first isolable compound and that its label first appears in the carboxyl group (2).

E. Part of the phosphoglyceric acid is believed to be reduced through the reversible reactions of glycolysis to give triose phosphates, hexose phosphates, and sucrose (2).

F. Another part of the phosphoglyceric acid is believed to give phosphoenolpyruvic acid which could then be carboxylated as in the Wood-Werkman reaction (9) to give oxalacetic acid from which in turn malic and aspartic acids may arise. The enzyme system for such a carboxylation has been found in higher plants by Vennesland *et al.* (10).

G. There remains the necessity for continuously generating the 2-car-

bon compound which, according to the above proposals, is carboxylated to give phosphoglyceric acid. One possible way in which a C_2 compound might be formed would be a C_1 to C_1 condensation. This possibility is being investigated but is considered somewhat unlikely for several reasons. First, very little radioactivity has been found in 2-carbon compounds in the very short term experiments. Secondly, the phosphoglyceric acid is predominantly carboxyl-labeled (95 per cent of the total label of the molecule in 5 seconds light with *Scenedesmus*). Furthermore, only extremely small amounts of radiocarbon have been found in formaldehyde and formic acid after 2 minutes in the light. The absence of labeled, reduced C_1 compounds, together with the improbability of a direct coupling of 2 carbon dioxide molecules and the absence of any labeled oxalic acid in these experiments, argues against the C_1 to C_1 condensation.



The other alternative for the formation of a 2-carbon compound is for a 2-carbon fragment to be split from a larger molecule. The only larger molecules found to be labeled in the very short term photosynthesis experiments were the 3- and 4-carbon acids, phosphoglyceric, phosphoenolpyruvic acid, and malic acid, while at the same time a small but significant labeling of the α- and β-carbons of phosphoglyceric acid was found. Since the sum of the radioactivity found in the 3- and 4-carbon compounds in such short term experiments was equal within experimental error to the total non-volatile activity fixed during the experiment, it appears that all appreciably labeled compounds were detected by the methods of analysis employed. The splitting of a 3-carbon compound would result in either a profitless decarboxylation or in the formation of formaldehyde or formic acid, neither of which has been found to be labeled significantly even in longer experiments. Consequently, the most likely

regenerative mechanism would appear to be the splitting of a 4-carbon, dicarboxylic acid (*cf.* (F) above) to give 2 molecules of 2 carbons each which would be converted to the 2-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, splitting of a C_4 compound to two C_2 compounds, and reduction of the C_2 compound. This proposed cycle will be designated as Cycle A throughout this paper. Thus far, no experimental evidence has been obtained which would contradict the existence of the proposed Cycle A.

In attempting to find evidence for or against this cycle several dicarboxylic acids have been considered as possible intermediates in the Cycle A. Succinic and fumaric acids, tentatively suggested in earlier papers, appear more likely to be respiration intermediates than photosynthetic intermediates according to the reasoning in section (B). However, malic acid, because of its 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.

In addition to the well known inhibition of succinic dehydrogenase by malonic acid, it has been reported that malonic acid inhibits the reduction of oxalacetic acid to malic acid (or the formation of malic acid from pyruvic acid and carbon dioxide) in animal tissues (11, 12). If the formation of malic acid could be appreciably inhibited and if, at the same time, some way could be found to measure the operation of the proposed cycle, then the participation of malic acid in the cycle could be tested.

Regardless of whether or not the proposed Cycle A exists, if the proposals in (D) and (E) are correct, then in order to be a photosynthetic intermediate between carbon dioxide and carbohydrate malic acid would have to be a precursor to the 2-carbon carbon dioxide acceptor and consequently a precursor to the α- and β-carbon atoms of phosphoglyceric acid. Therefore, by degrading phosphoglyceric acid and measuring the labeling of its α- and β-carbon atoms both in experiments in which labeled malic acid is formed without inhibition and in experiments in which the formation of labeled malic acid is inhibited it should be possible to determine whether malic acid is an intermediate in photosynthesis.

Methods and Material

In each pair of experiments a 1 day growth from 1 liter of a continuous culture of *Scenedesmus* (6) yielded 2.0 gm. of wet packed cells. 1 gm. of cells was suspended in phosphate buffer (0.007 M, pH 4.3) and the

other in phosphate buffer and 0.05 M sodium malonate[2] (pH 4.3). After various periods in the dark (Column A, Table I) the cells were centrifuged from the buffers, resuspended in 70 ml. of malonate-free phosphate buffer, and illuminated in a thin water-jacketed vessel. The removal of the cells from the malonate buffer prior to the exposure to labeled carbon dioxide was necessitated by the procedure for analyzing the cell constitu-

TABLE I

Effect of Pretreatment of Algal Suspensions with Malonate

Experiment No.	Time in dark	4 per cent CO_2-96 per cent air and light	1 per cent CO_2-air and light	Air and light	$C^{14}O_2$ and light	Total radioactivity fixed	Per cent of fixed radioactivity		Per cent inhibition of malic acid	Distribution of radioactivity in glyceric acid, per cent of starting activity fixed as PGA				Per cent of total radioactivity fixed in aspartic acid
							PGA*	Malic acid		Carboxyl	α	β	Total	
	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)	(I)	(J)	(K)	(L)	(M)	(N)
	min.	*min.*	*min.*	*min.*	*sec.*	*μc.*								
M-1	150	30	10	0	60	5.0	19	2.41	70	50	25	30	105	1.50
B-1	210	30	10	0	60	7.7	17	8.14		55	25	27	107	1.68
M-2	255	30	15	0	60	7.7	22	1.64	66	41	25	29	95	2.13
B-2	315	30	15	0	60	9.0	28	4.86		44	25	31	99	2.19
M-3	180	40	0	0	30	1.6	30	0.42	83	73	12	15	100	
B-3	240	40	0	0	30	2.4	35	2.48		81	7	10	98	
M-4	210	30	0	10	90	14.0		0.45	97	50 ~10†	50 ~10†			3.86
B-4	270	30	0	10	90	15.9		13.90		55 ~10†	45 ~10†			5.93

Light intensity = 2500 foot-candles for Experiments M-1 to B-3, 10,000 foot-candles for Experiments M-4 and B-4. Temperature 22°. Experiments M-1, M-2, M-3, and M-4, malonate inhibition; Experiments B-1, B-2, B-3, and B-4, the controls (no malonate).

* Phosphoglyceric acid.

† In Experiments M-4 and B-4 alanine rather than glyceric acid was degraded.

ents after killing. Had the sodium malonate been present when the cell extract was concentrated and applied to the paper the chromatogram would have been overloaded by the large quantity of sodium malonate. Streams of air with different percentages of carbon dioxide were passed through the cell suspension for 30 to 45 minutes (conditions as in Columns B, C, D, Table I, performed in that order) and then the radioactive sodium bicarbonate solution (100 μc., 0.02 mM) was added, the flasks

[2] Identical experiments with radioactive malonate demonstrated that the concentration of malonate within the cells (or adsorbed on the cell surface) was 0.015 M at the time of the analysis of photosynthetic products.

stoppered, and the cells allowed to photosynthesize for 30 to 90 seconds (Column E) before being killed in boiling ethanol. The alcohol extract was then analyzed by paper chromatography and radioautography (4). The percentages of the total fixed activity found in malic acid are given in Column H, Table I.

The phosphoglyceric acid obtained from the cell extract was hydrolyzed in acid and the hydrolysate rechromatographed to give a single spot of glyceric acid. The glyceric acid was eluted from the paper and recrystallized twice with unlabeled calcium glycerate. The specific activity of the resulting labeled calcium glycerate was determined and the following degradation carried out.

50 mg. of the labeled calcium glycerate (0.4 mM) were placed in a flask with 0.80 ml. of 1.0 N periodic acid. After 2 hours, the solution was made slightly alkaline and the volatile contents, including formaldehyde, were distilled *in vacuo* into a solution of dimethyldihydroresorcinol from which the dimethone compound of formaldehyde was isolated and recrystallized.

To the non-volatile residue of sodium glyoxylate, 5 ml. of 1.0 N periodic acid were added, and after 24 hours the volatile contents were distilled into a carbonate-free sodium hydroxide solution. Barium chloride solution was then added, the barium carbonate precipitate centrifuged, washed, and dried, and the supernatant solution was acidified and steam-distilled to collect the formic acid. The steam distillate was neutralized with barium hydroxide and concentrated to dryness. The barium formate was recrystallized from water and alcohol. The specific activities of the barium carbonate, barium formate, and dimethone compound were determined, and, with the theoretical yields, gave the total radioactivities of the carboxyl, α- and β-carbons (expressed as percentages of starting radioactivity in Columns J, K, and L of Table I).

The results are given in Table I. It is seen that pretreatment with malonate caused an appreciable decrease in the radioactivity incorporated in malic acid (66 to 97 per cent) and a much smaller decrease in total activity fixed (12 to 35 per cent). The labeling of aspartic acid was not appreciably decreased in two experiments and was decreased 35 per cent in a third experiment. The per cent of the total labeling of the phosphoglyceric acid molecule found in the α- and β-carbons was as great or greater in the experiments in which malic labeling was decreased by malonate pretreatment of the cells as in the experiments in which no malonate pretreatment was employed. No labeled succinic or fumaric acid was detected in any of the experiments.

These results indicate that the malonate pretreatment may have decreased slightly the rate of the carboxylation of C_3 compound to give C_4 dicarboxylic acid, as may be evidenced by the decrease in total radiocarbon fixed and by the decrease in aspartic acid labeling in one case, but

that the formation of malic acid is much more strongly inhibited by the malonate pretreatment.

The undiminished labeling of the α- and β-carbons of phosphoglyceric acid at the same time that malic acid labeling is greatly decreased indicates that malic acid is not a precursor of the α- and β-carbon atoms of phosphoglyceric acid. If the proposal is correct that phosphoglyceric acid is an intermediate in photosynthesis and is reduced through reversible glycolysis reactions to carbohydrate, then malic acid cannot be a photosynthetic intermediate.

The absence of labeled succinic and fumaric acid in the above experiment indicates that these two acids are not precursors of the α- and β-carbon atoms of phosphoglyceric acid and hence by the above reasoning not photosynthetic intermediates.

SUMMARY

Pretreatment of algal suspensions with malonate, followed by short periods of photosynthesis with radioactive carbon dioxide, has been found to inhibit the formation of labeled malic acid.

Degradation of phosphoglyceric acid formed at the same time shows no decrease in per cent labeling of the α- and β-carbon atoms over that obtained with cells not pretreated with malonate.

These results suggest that malic acid is not a precursor of the α- and β-carbons of phosphoglyceric acid in photosynthesis.

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