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THE PATH OF CARBON IN PHOTOSYNTHESIS

VIII. THE ROLE OF MALIC ACID

James A. Bassham, Andrew A. Benson and Melvin Calvin

January 25, 1950

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ABSTRACT

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VIII. THE ROLE OF MALIC ACID(*)

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ABSTRACT

Malonate has been found to inhibit the formation of malic acid during short periods of photosynthesis with radioactive carbon dioxide. This result, together with studies which show the photosynthetic cycle to be operating normally at the same time, indicates that malic acid is not an intermediate in photosynthesis but is probably closely related to some intermediate of the cycle.

Absence of labeled succinic and fumaric acids in these experiments, in addition to the failure of malonate to inhibit photosynthesis, precludes the participation of these acids as intermediates in photosynthesis.

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

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It has been suggested that the fixation of carbon dioxide during photosynthesis is initiated by the carboxylation of some two-carbon compound to give phosphoglyceric acid or some closely related three-carbon compound which is then used in two ways (1). Part of it is reduced via a reversal of the well known glycolysis to hexose while the remainder is further carboxylated to give a four-carbon compound which is split and reduced to give two molecules of the two-carbon carbon dioxide acceptor. This second carboxylation might be the Wood-Werkman reaction (2). The enzyme system for this reaction has been found in higher plants by Vennesland, et al. (3). In this case, the expected products would be oxaloacetic acid and perhaps the closely related dicarboxylic acids.

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

(1) Calvin, M. and Benson, A.A., Science, 107, 476 (1948).

(2) Wood, H.G., Werkman, C.H., Hemingway, A. and Nier, A.O., J. Biol. Chem., 139, 365 (1941).

(3) Vennesland, B., Gollub, M. and Speck, J.F., J. Biol. Chem., 178, 301 (1949).

In order to test the validity of the proposed regenerative cycle and elucidate some of its details, an investigation of these acids was undertaken. Malic, succinic and fumaric acids were found to have incorporated radioactive carbon in dark (respiration) experiments suggesting their formation by a reversal of respiration reactions (4), while radioactive malic alone of these acids was found after short periods of photosynthesis with labeled carbon dioxide. Thus, it appears that succinic and fumaric acids are not intermediates in photosynthesis and that malic acid is possibly related both to respiration and photosynthesis.

In order to establish whether malic acid is an actual intermediate in the regenerative cycle or merely a carbon reservoir closely related to some intermediate, an attempt was made to inhibit the formation of malic acid. In addition to the well known inhibition of succinic dehydrogenase, it has been reported that malonic acid inhibits the formation of malic acid from oxaloacetic acid (or from pyruvic acid and carbon dioxide) in animal tissues (5,6). If the formation of malic acid could be inhibited and, at the same time, the operation of the cycle followed by degradation studies, then the participation of malic acid in the cycle could be tested.

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(4) Benson, A.A. and Calvin, M., J. Exper. Botany (in press).

(5) Das, N.B., Biochem. J., 31, 1124 (1937).

(6) Pardee, A.B., and Potter, V.R., J. Biol. Chem., 178, 241 (1949).

METHODS AND MATERIAL

In each pair of experiments a one day growth from one liter of a continuous culture of Scenedesmus (7) yielded 2.0 grams of wet packed cells. One gram of cells was suspended in phosphate buffer (0.007M, pH 4.3) and the other in phosphate buffer and 0.5M malonate (pH 4.3). After varying periods in the dark, (Column A, Table 1) the cells were centrifuged from the buffers, resuspended in 70 ml. phosphate buffer and illuminated in a thin water-jacketed vessel. A stream of air and carbon dioxide was passed through the cell suspension for thirty to forty minutes (Columns B, C and D, Table 1) and then the radioactive sodium bicarbonate solution (100 microcuries, 0.012 millimoles) was added, the flask stoppered and the cells allowed to photosynthesize for thirty to ninety seconds (Column E) before being killed in boiling ethanol. The alcohol extract was then analyzed by paper chromatography and radioautography (8). The percentages of the total fixed activity found in malic acid are given in Column G, Table 1.

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

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(7) Benson, A.A., Calvin, M., Haas, V.A., Aronoff, S., Hall, A.G., Bassham, J.A., and Weigl, J.W., Chapter 19, "Photosynthesis in Plants", Iowa State College Press, Ames, Iowa, 1949, pp 381-401.

(8) Benson, A.A., Bassham, J.A., Calvin, M., Goodale, T.C., Haas, V.A. and Stepka, W., J. Am. Chem. Soc., (in press).

Table 1

EXPERIMENT	Time in Dark Minutes	4% CO_2 96% Air-Light Minutes	1% CO_2 Air-Light Minutes	Air	$C^{14}O_2$ Sec.	Total Radio-activity Fixed, μc.	Percent Malic	Percent Inhibition of Malic	Distribution of Radioactivity in Glyceric Acid Percentages of Starting Activity Carboxyl	alpha	beta	total found	Percent Aspartic Acid
	A	B	C	D	E	F	G	H	I	J	K	L	M
M-1	150	30	10	0	60	5.0	2.41	70	50	25	30	105	1.50
B-1	210	30	10	0	60	7.7	8.14		55	25	27	107	1.68
M-2	255	30	15	0	60	7.7	1.64	66	41	25	29	95	2.13
B-2	315	30	15	0	60	9.0	4.86		44	25	31	99	2.19
M-3	180	40	0	0	30	1.6	0.42	83	73	12	15	100	---
B-3	240	40	0	0	30	2.4	2.48		31	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 = 5500 foot candles for M-1 to B-3; 20,000 foot candles for M-4 and B-4.

Temperature = 22° C.

M-1, M-2, M-3, M-4 are malonate inhibition experiments.

B-1, B-2, B-3, B-4 are the control experiments.

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

recrystallized twice with unlabeled calcium glycerate. The specific activity of the resulting labeled calcium glycerate was determined and the following degradation carried out.

Fifty mg. of calcium glycerate (0.4 millimoles) was placed in a flask with 0.80 ml. 1.0N periodic acid. After two hours, the solution was made slightly alkaline and the volatile contents, including formaldehyde, were distilled *in vacuo* into a solution of dimethyldihydroxyresorcinol where the dimethone compound was isolated and recrystallized.

To the non-volatile residue of sodium glyoxylate, 5 ml. 1.0N periodic acid was added, and after twenty-four 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 supernate 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, alpha, and beta carbons (expressed as percentages of starting radioactivity in Columns I, J and K of Table 1).

The percentages of radioactivity found in the alpha and beta carbon atoms of glyceric acid serve as a measure of the operation of the regenerative cycle since carbon, initially incorporated into the carboxyl groups of glyceric or oxalacetic acid by a carboxylation, must pass through

all the intermediates of the cycle before it can reach the alpha and beta positions. The near equality of the alpha and beta labeling suggests a symmetrical two-carbon intermediate at some point in the cycle although there is some indication of inequality in the labeling of these positions. Further investigation of this distribution of radioactivity is being made together with degradation of more two- and six-carbon compounds.

From Table 1 it is seen that although the radioactivity incorporated in malic acid was appreciably decreased by malonate, the operation of the cycle was as great in the first two and fourth malonate inhibited cell suspensions as in the controls and significantly greater in the third. This can be explained by assuming that malic acid itself is not an intermediate in the cycle but a carbon reservoir closely related to an intermediate (Figure 1).

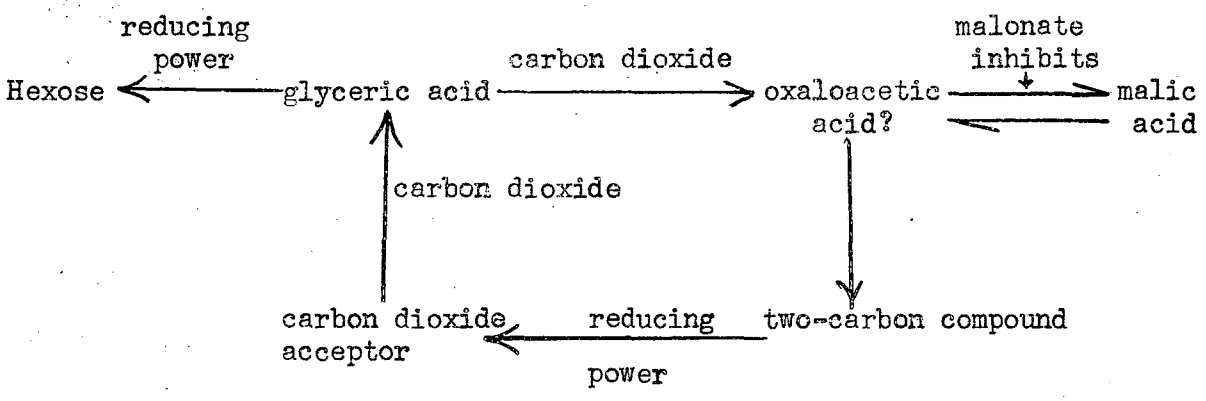

Figure 1

In normal photosynthesis (control experiments) malic acid is rapidly interconvertible with a cycle intermediate (possibly oxaloacetic acid). When radioactive carbon dioxide is introduced and enters the cycle

via carboxylation reactions it is soon found in malic acid. Moreover, since unlabeled malic acid is being converted to a cycle intermediate the percentage of labeled carbon in the alpha and beta positions is less than that which would be obtained without an unlabeled carbon reservoir.

When malonate is introduced, the interconversion of malic acid and the cycle intermediate is inhibited, so less radioactivity is incorporated in malic acid and less unlabeled carbon finds its way into two-carbon carbon dioxide acceptor, thus increasing the labeling of the alpha and beta carbons of glyceric acid.

The operation of the cycle in the presence of sufficient malonate to inhibit the formation of malic acid provides additional evidence that succinic and fumaric acids are not intermediates in the cycle since it has been found that malonate blocks the conversion of succinic acid to fumaric acid in at least some plant tissues (9,10).

There remains the observation that total carbon fixed appears to be decreased some 15 per cent to 35 per cent by malonate. This indicates that malonate inhibits some other reaction by which carbon is incorporated in the plant since the difference in malic is not great enough to account for the difference in total activity fixed.

It may be that the carboxylation of pyruvate to oxalacetate is slightly inhibited as has been reported for animal tissues (6). However,

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(9) Bonner, J. and Wildman, S.G., Arch. Biochem., 10, 497 (1946)

(10) Laties, G., Arch. Biochem., 22, 8 (1949)

this inhibition cannot account for the decrease in malic labeling since aspartic acid, presumed to be formed from oxaloacetic acid by a transaminase system, was found to contain nearly as much radioactivity in the first two inhibition experiments as in the controls. (The amount of labeled aspartic formed in the third pair of experiments was too small to permit accurate counting.)

SUMMARY

Malonate has been found to inhibit the formation of malic acid during short periods of photosynthesis with radioactive carbon dioxide. This result, together with studies which show the photosynthetic cycle to be operating normally at the same time, indicates that malic acid is not an intermediate in photosynthesis but is probably closely related to some intermediate of the cycle.

Absence of labeled succinic and fumaric acids in these experiments, in addition to the failure of malonate to inhibit photosynthesis, precludes the participation of these acids as intermediates in photosynthesis.