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THE PATH OF CARBON IN PHOTOSYNTHESIS, XI

THE ROLE OF GLYCOLIC ACID

L. Schou, A.A. Benson, J. A. Bassham and M. Calvin

September 11, 1950

Berkeley, California

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ABSTRACT

THE PATH OF CARBON IN PHOTOSYNTHESIS, XI

THE ROLE OF GLYCOLIC ACID*

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September 11, 1950

ABSTRACT

The metabolism of C^{14} labeled glycolic acid by Scenedesmus has been studied using radiochromatographic techniques for the separation and identification of products.

When the pH of the medium was 2.8, appreciable assimilation occurred. The products were identical to those observed in $C^{14}O_2$ photosynthesis.

A major reaction anaerobically in the dark resulted in incorporation of C^{14} in almost equal amounts in the glycine and serine reservoirs. When the algae were illuminated, a diminution in the amount of glycine was observed.

Aerobic and anaerobic glycolic acid assimilation was studied during photosynthesis. The C^{14} level in the sucrose and the intermediates of its

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* This work was sponsored by the United States Atomic Energy Commission.

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synthesis varied with $C^{12}O_2$ pressure in the gas used.

1-C^{14} and 2-C^{14} glycolic acids give similar distribution of radioactivity in the products. Hence, glycolic acid is assimilated by paths other than those involving preliminary cleavage to C_1 compounds.

Phosphoglyceric acid isolated from the products of assimilation of both glycolic acids was degraded and found to be approximately equally labeled in its α and β carbon atoms.

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INTRODUCTION

The participation of glycolic acid in plant metabolism has been considered in the past, but its relationship to the intermediates of carbon dioxide reduction has been obscure.

Kolesnikov (10) reported accumulation of glyoxylic acid in Chlorella during illumination. Anderson (1), Kolesnikov (11) and Clagett, Tolbert and Burris (9,12) studied the non-photosynthetic metabolism of glycolic acid by plant tissues. The presence of glycolic acid among the early photosynthetic intermediates has been observed by Benson and Calvin (4) and by Burris, Wilson and Stutz (6).

The first stable product of carbon dioxide assimilation in photosynthesis has been shown to be carboxyl-labeled phosphoglyceric acid (5,7). Experiments were then designed to force the accumulation of the C_2 precursor of the α and β carbon atoms of this compound by illuminating plants in the absence of carbon dioxide (Benson and Calvin (4)). These conditions resulted in the accumulation of large amounts of glycolic acid and glycine.

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The immediate precursor of the carboxyl labeled phosphoglyceric acid is of the reduction level of acetaldehyde or glycolaldehyde. Consequently, the accumulated glycolic acid appears to be either a precursor of the more reduced C_2 compound or a product of its oxidation.

It has been shown (8) that glycolic acid appears subsequent to carboxyl-labeled compounds in photosynthesis and is not observed in very short periods of photosynthesis (1 Sec. Photosynthesis by Barley). Since glycolic acid accumulates in the absence of carbon dioxide, it cannot arise from the condensation of two C_1 compounds directly formed from CO_2 but rather is probably derived from larger molecules. Since the compounds identified as phosphate esters in radiograms of $C^{14}O_2$ photosynthesis experiments have been found to contain derivatives of glycolic acid*, it is possible that glycolic acid reacts metabolically in a phosphorylated form.

The close relationship of glycolic acid to the α and β carbon atoms of glyceric acid was first observed by Bassham, et.al.(2,8). Even in very short photosyntheses with $C^{14}O_2$ (4 Sec. Photosynthesis by Barley) the carbon atoms of glycolic acid were uniformly labeled. This acid has been degraded by periodate oxidation and the products isolated as barium carbonate and formaldimedon from the carboxyl and α carbon respectively. The carboxyl and α carbon atoms of glycolic acid and the α and β carbon atoms of phosphoglyceric

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* To be published

acid are invariably uniformly labeled. This relationship has now been tested by a study of the conversion of synthetic radiomeric glycolic acids to phosphoglyceric acid during photosynthesis by Scenedesmus.

EXPERIMENTAL PROCEDURE Labeled glycolic acids prepared by Dr. B. M. Tolbert of this laboratory were added in the dark during anaerobic illumination and during photosynthesis in $C^{12}O_2$ by Scenedesmus. Since the absorption of a strong acid such as glycolic acid is generally slow at physiological pH values, the experiments were performed in dilute phosphate buffer at pH 2.8. It has been observed in this laboratory* that photosynthetic ability at low pH (1.5 or less) is neither readily nor irreversibly destroyed and that the products are apparently normal.

Feeding Experiments. -- A suspension of one gram of a two day old culture of Scenedesmus (Strain D_3, Gaffron) in a 1 cm. thick glass vessel (3) containing 50 ml. of .001 M phosphate buffer, pH 2.8 was allowed to photosynthesize in air for 30 minutes with a light intensity of 2000 foot candles from both sides. A temperature of 20°C was maintained by use of adequate infrared absorbers. The cells were allowed a twenty-minute adaptation period in the gas used immediately previous to adding the radioactive glycolic acid. In the dark experiments, the vessel containing the cell suspension was covered with black cloth during the experiment and the funnel was shielded from light during filtration and killing.

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* C. Ouellet -- To be published

Labeled calcium glycolate (4.0 μc/mg) was decationized with Dowex-50. The free acid (3.2 mg., i.e. 0.0008M) was added at zero time and the photosynthesis continued for ten minutes. The cells were then filtered rapidly from the solution in the light and killed by pouring hot absolute ethanol on the filter. By use of filter aids on a funnel equipped with two receivers and a two-way stopcock the killing procedure required 2-5 seconds. The algae were re-extracted with 50% ethanol and hot water. The extracts were concentrated and separated by two-dimensional paper chromatography (5). After extraction, the insoluble materials (protein and polysaccharide) were counted directly and as barium carbonate after combustion. The lipid materials, separated from other soluble compounds in the chromatograms, were eluted and counted directly. The total glycolic acid fixed was then the sum of the three fractions and was tabulated in Table I. From the known specific activity of the glycolic acid the amount assimilated during the experiments was calculated and tabulated in Table I. The products were observed on radiograms of such chromatograms and were identified where necessary by cochromatography with authentic materials. The amount of C^{14} in each of a number of the major products was counted directly on the paper and tabulated in Table II.

Degradation of Phosphoglyceric Acids. -- The radioactive area corresponding to phosphoglyceric acid was eluted from the paper and hydrolyzed for 30 hours in 1.0 N hydrochloric acid. The total hydrolysate was chromatographed and the resultant glyceric acid spot was cocrystallized with 50 mg. of authentic calcium glycerate. The fact that the resulting product

possessed the calculated specific activity serves as added evidence of identity. Glyceric acid was degraded according to the method of Bassham et.al. (2).

Degradation of Glycolic Acid. -- A tracer quantity of C^{14}-labeled glycolic acid, obtained by elution from a paper chromatogram was added to 30.4 mg. of glycolic acid in three ml. of glacial acetic acid in a small flask. The solution was frozen, about 0.5 g. lead tetraacetate was added, and the flask was attached through a stopcock to an inverted U-tube. The system was evacuated, the stopcock closed and the reaction mixture refluxed on a water bath at 90°C for thirty minutes. After cooling, the volatile contents of the flask were distilled through the U-tube into a second flask containing 80 mg. of 2,4-dinitrophenylhydrazine and immersed in liquid nitrogen.

The stopcock was again closed and the second flask warmed until a clear yellow solution was obtained. The first flask was replaced by a third flask containing 5.0 ml. saturated, carbonate-free, sodium hydroxide solution. Both flasks were immersed in liquid nitrogen for a few minutes, the stopcock was opened and the system was evacuated. The liquid nitrogen bath was removed from the second flask and the volatile contents distilled into the third flask. The residue of formaldehyde-2,4 dinitrophenylhydrazone in the second flask was purified chromatographically on silicic acid and the specific activity determined. This specific activity, together with the theoretical yield gives the total activity of the alpha carbon atom. The third flask was warmed to room temperature, and the solution contained

therein yielded, upon addition of barium chloride solution, a precipitate of barium carbonate which was washed, dried, weighed and counted. The product of the specific activity of the barium carbonate and the total yield (slightly greater than theoretical due to introduction of inactive carbon dioxide in reagents and manipulation) gives the total activity of the carboxyl carbon of glycolic acid. These results are tabulated in Table III.

RESULTS AND DISCUSSION

Dark Metabolism of Glycolic Acid

It has been shown (4) that glycolic acid is readily metabolized in the dark by barley leaves and algae. In the experiments described below there is evidence that the assimilation of glycolic acid proceeds by paths other than intermediate conversion to carbon dioxide.

Short Dark Experiments. -- The major products of six to ten minute dark anaerobic assimilation of glycolic acid are glycine and serine. The conversion of labeled glycolic acid to glycine in barley sap has also been observed in this laboratory. The appearance of labeled serine suggests that a reaction similar or identical with the Sakami reaction may occur in plant tissue. (This reaction has also been observed in this laboratory in Scenedesmus which have been fed 1-C^{14} or 2-C^{14} labeled glycine.) Conversion to serine is appreciably faster in the light than anaerobically in the dark where both amino acids appear in like amounts and the ratio of serine to glycine varied from unity to 1.5 in all experiments. No relationship between the nature of the anaerobic flushing

gas mixtures and the serine-glycine ratio was observed. In air the ratio of serine to glycine increased to 7. An appreciable synthesis of sucrose, phosphoglyceric acid and polysaccharide occured in ten minute dark aerobic assimilation. Apparently, energy is derived from oxidative processes and is used for carbohydrate synthesis. Fat synthesis corresponding to about 5% of the soluble products was observed.

In all dark assimilation experiments considerable amounts of unchanged glycolic acid is found in the cell extract. In the light, little excess glycolic was observed in the similarly prepared extracts.

Long Dark Experiments. -- The further metabolic products of glycine, serine and glycolic acid were observed in radiograms of 30 minute to 6 hour dark assimilation experiments with glycolic acid-2-C^{14}. The soluble products formed during 6 hours dark assimilation of glycolic acid-2-C^{14} are given in Table II. It is apparent from the large fraction of glutamic, succinic, fumaric, malic and citric acids that considerable oxidation through the tricarboxylic acid cycle may have occurred. This metabolic course appears similar to that observed in unpublished experiments with Scenedesmus which have been fed 2-C^{14} glycine in the dark for similar periods. Since flushing with inert carbon dioxide did not affect the results, exchange of $C^{14}O_2$ arising from oxidation of the substrate, into the tricarboxylic acid cycle is not likely. The possibility should be pointed out that a C_2 compound related to glycolic acid (glycine) may be condensed through reversible reactions to a C_4 compound and enter the tricarboxylic acid cycle as oxalacetate. Otherwise, it must be further reduced to react as acetate in this cycle.

Light Assimilation Experiments

The products of ten minute and longer periods of illumination were studied and found generally similar to the products of photosynthesis with $C^{14}O_2$. Glycolic acid is converted to cell material (protein, polysaccharides) and fats. The radioactivity in the various reservoirs of soluble products corresponded to that observed when $C^{14}O_2$ is assimilated at this high pH.

The conversion of glycolic acid to serine was observed in the light as well as in the dark and was independent of oxygen or carbon dioxide partial pressures during the experiment. In all cases the ratio of serine to free glycine was very high.

The radioactivity in glutamic acid, which may be taken as a measure of respiration of labeled intermediates via the tricarboxylic acid cycle, was greater in the aerobic experiments. A similar result was observed in the aerobic dark experiment.

When the algae were flushed with $C^{12}O_2$ prior to and during the glycolic acid assimilation, serine, sucrose and polysaccharide (containing glucose) reservoirs accumulated the largest fraction of radioactivity. At the same time, radioactivity in phosphate esters was greatly diminished. This can be attributed to the dilution of C^{14} in the intermediates by the $C^{12}O_2$. The sucrose reservoir, which is much larger, rapidly acquires a greater total of radioactivity, although the specific activity is low.

In experiments without added carbon dioxide photosynthesis can be expected to occur at a slower rate. In air the natural CO_2 concentration was sufficient to allow a moderate accumulation of activity in the sucrose

reservoir. In nitrogen without added carbon dioxide, a much smaller amount of sucrose was formed. Radioactivity in the phosphate compounds, intermediate in sucrose synthesis, was inversely affected. With diminishing dilution by added carbon dioxide the specific activity of these small reservoirs increased and their relative radioactivities probably represents a measure of dilution of assimilated glycolic by carbon dioxide.

In the low carbon dioxide pressure experiments (air and nitrogen) an amount of labeled phosphoglyceric acid, large compared to that of normal photosynthesis was observed. The significance of this observation may well lie in the effect of pH or CO_2 concentration upon the reservoir sizes. The phosphoglyceric acid of the aerobic experiment was degraded and the results are tabulated in Table III.

Carboxyl-Labeled Glycolic Acid Assimilation. -- An identical experiment was performed in air with glycolic acid-1-C^{14}. The distribution of C^{14} in the products, Table II, were similar to that observed in aerobic glycolic acid-2-C^{14} assimilation. The phosphoglyceric acid obtained in this experiment was degraded and the results are tabulated in Table III.

Degradation Results. -- The accumulation of C^{14} in nearly equal amounts in the α and β carbon atoms of phosphoglyceric acid during photosynthesis has now been observed when glycolic acid is the labeled substrate. In Table III it is seen that radioactivity of α and β carbon atoms in both cases are approximately equal. It is possible that the equal α and β labeling in C_3 compounds may arise in a number of ways. However, one possibility, consistent with our

previous observations (8), would be that some symmetrical intermediate or compound existing in rapid equilibrium with an intermediate lies between glycolic acid and the C_2 carbon dioxide acceptor molecule.

SUMMARY

The metabolism of C^{14} labeled glycolic acid by Scenedesmus has been studied using radiochromatographic techniques for the separation and identification of products.

When the pH of the medium was 2.8, appreciable assimilation occured. The products were identical to those observed in $C^{14}O_2$ photosynthesis.

A major reaction anaerobically in the dark resulted in incorporation of C^{14} in almost equal amounts in the glycine and serine reservoirs. When the algae were illuminated, the glycine and glycolic acid radioactivity decreased.

Aerobic and anaerobic glycolic acid assimilation was studied during photosynthesis. The C^{14} level in the sucrose and the intermediates of its synthesis varied with $C^{12}O_2$ pressure in the gas used.

1-C^{14} and 2-C^{14} glycolic acids give similar distribution of radioactivity in the products.

Phosphoglyceric acid isolated from the products of assimilation of both glycolic acids was degraded and found to be approximately equally labeled in its α and β carbon atoms.

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TABLE I

Products of Glycolic Acid Assimilation by Scenedesmus

	10 Min. Light Assimilation				10 Min. Dark Assimilation			6 Hour Dark Assimilation
	N_2	Air	Air*	N_2+1/2% CO_2	N_2	Air	N_2+1/2% CO_2	N_2+1/2% CO_2
C^{14} in Lipids	120,000	76,000	39,000	48,000	3,000	15,000	1,000	10,000
C^{14} in Insoluble protein and carbohydrates	470,000	258,000	113,000	285,000	27,000		30,000	
C^{14} in Water-Soluble Products	350,000	460,000	315,000	115,000	100,000	310,000	81,000	210,000
Total Glycolic Acid fixed (in mg.+).	.71	.60	.35	.34	.099		.085	

C^{14} data are expressed in counts (9 dis./count) per minute.

* Glycolic acid-1-C^{14} Assimilation. All others are Glycolic acid-2-C^{14} experiments.

+ Calculated from C^{14} found in assimilation products and the specific activity of the substrate.

TABLE II

Water Soluble Products of Labeled Glycolic Acid Assimilation by Scenedesmus

Compound	Ten Minute Light Assimilation				Ten Minute Dark Assimilation			Six Hour Dark
	N_2*	Air*	Air+	1/2% CO_2 + N_2*	N_2*	Air*	N_2+1/2% CO_2*	N_2+1/2%CC
Phosphoglycerate	26	19	11	5.3	10	22	17	6
Hexose Phosphates	6.2	6	13	6.9	3	9.4	1.5	3
Triose Phosphates	3.3	2.5	2	2.9	4	2.8	4	1
Phospho pyruvate	6.2	3.9	3		3	6.1		3
Sucrose	2.2	9.5	12	16		2.0		3
Polysaccharides	5.7	7.5	13	18	2	1.5		
Serine	23	8	13	32	37	12	34	3.5
Glycine	1.4	1.5	2	2.9	25	1.7	31	2
Alanine	1.5	3.3	1.5	2.0	7	3.7	9	9
Aspartic	5.9	5.5	7.5	3.1		6.1		3
Glutamic	2.7	8.5	4.5	2.5	3	18	2	40
Glutamine	0	.7	3		1	.9		3
Glyceric	1.9	3	2	1.8	1	5.8	1	
Citric	1.6	4.5	3.5			2.1		2
Succinic	1.3	1.8	0		2	2.7		3
Unk. Spot under Lipids	4.1	10	2	3.6				15
Malic	7.0	8.5	7	3.5	2	3.3	1	8

* Glycolic acid-2-C^{14} assimilation

+ Glycolic acid-1-C^{14} assimilation

TABLE III

C^{14} Distribution in Glycolic and Glyceric Acids

	4 Sec. PS Barley $C^{14}O_2$	10 Min. PS Scenedesmus, Air $C^{14}H_2OH$-COOH	10 Min. PS Scenedesmus, N_2 $C^{14}H_2OH$-COOH	10 Min. PS Scenedesmus, Air CH_2OH-$C^{14}OOH$
Glycolic				
CH_2OH	51	100	100	0
COOH	49	0	0	100
Glyceric				
CH_2OH	6.8	49	48	29
CHOH	6.5	--	56	24
COOH	87	8.6	7.2	46

Percentages given in terms of measured starting activity