Glycolic Acid Labeling During Photosynthesis with $^{14}CO_2$ and Tritiated Water

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Summary. *Chlorella pyrenoidosa* were allowed to photosynthesize for short periods of time in the presence of $^{14}CO_2$ and HTO. Analysis of tritium and ^{14}C labeling of photosynthetic intermediate compounds showed that the T/^{14}C ratio of glycolic acid was comparable to that of intermediate compounds of the photosynthetic carbon reduction cycle when photosynthesis was performed in nearly 100 % oxygen and only slightly higher under steady-state conditions. It is concluded that formation of labeled glycolic acid as a consequence of its proposed hydrogen transport role in photosynthesis is quantitatively of limited importance compared to the net synthesis of glycolic acid from CO_2.

Although it has been known since 1950 that glycolic acid is among the early labeled products formed during photosynthesis (1, 2) with $^{14}CO_2$, the details of its formation and utilization remain unresolved. It has been suggested that glycolic acid may be formed from sugar phosphate intermediates of the photosynthetic carbon reduction cycle through some mechanism involving the transketolase reaction (3), possibly through an oxidation of the glycolaldehyde-thiamine pyrophosphate addition compound (4). An alternative view is that glycolic acid is formed by a more direct reduction and condensation of CO_2 (5), although intermediate compounds and enzymes for this pathway have not yet been identified. Zelitch (6) reported ^{14}C-labeling data with glycolic acid and 3-phosphoglyceric acid in support of the direct reductive formation of glycolic acid from CO_2, whereas Hess and Tolbert (7) reported data which are consistent with glycolic acid formation from sugar phosphates derived from 3-phosphoglyceric acid.

It has been proposed that glycolic and glyoxylic acids might play roles in the transport of hydrogen from photochemically reduced cofactors, either to intermediates of photosynthesis (8), or between chloroplasts and other parts of the cell (9). Glycolic acid would give up its hydrogen for reductive reactions and its oxidized form, glyoxylic acid, would be reduced at the expense of a photochemically produced reducing cofactor. This proposal can be tested by allowing plants to photosynthesize with $^{14}CO_2$ and tritiated water (HTO). Provided the rate of the hydrogen transport reaction were significant compared to the biosynthesis of glycolic acid from CO_2, one might expect a significantly higher ratio of tritium to ^{14}C in glycolic acid than in other intermediates of the photosynthetic carbon reduction cycle, after a short period of photosynthesis with both tracers. Also, one would expect the T/^{14}C ratio of glycolic acid to drop with time of photosynthesis with the tracers, coming in time to some constant value as the glycolic acid molecules become saturated with both tracers. However, experiments with these 2 tracers probably would not provide data which could discriminate between 2 routes of net synthesis from CO_2 to glycolic acid.

In other published experiments with tritium and $^{14}CO_2$, the ratio of tritium to ^{14}C in glycolic acid was found to be equal to the ratio in the total for all labeled compounds, but higher than the ratio in glucose and fructose (10). However, short periods of photosynthesis by algae were preceded by preincubation in the dark with both tritiated water and $^{14}CO_2$, so that initial photosynthetic labeling rates were not determined. In earlier studies of photosynthesis with tritiated water (11), 1.4 % of the total incorporated tritium was found in glycolic acid, but because of the great darkening of the glycolic acid area of a radioautogram prepared from the labeled *Chlorella,* it appeared that glycolic acid was a major tritium-labeled product. We now know that much of this darkening of the film is due to evaporation of the volatile glycolic acid onto the film, which causes a great exaggeration in the film darkening for the glycolic acid spot.

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Materials and Methods

Early attempts to perform photosynthesis experiments in the presence of HTO and $^{14}CO_2$ gave uncertain and variable results. The principal problem in those studies was in obtaining accurate counting results for both tritium and ^{14}C. In order to obtain reliable counting results it was necessary to raise the ratio of tritium to ^{14}C in the incubation mixture to a rather high level, and this is reflected in the following experimental procedure (used in experiments 1, 2).

A suspension of *Chlorella pyrenoidosa* (1.5 ml, 4 % v/v) was made up in 1 M KH_2PO_4 adjusted to pH 5 with HCl, and was preilluminated for 30 minutes in the presence of 2 % CO_2 in air. The light path through the algal suspension was 0.25 cm, and the suspension was illuminated from both sides with incandescent photospot lamps giving about 15,000 ft-c incident intensity. The light passed through infra-red absorbing glass in rapidly circulating water, and the algae vessel was kept in a water bath at 20° during preillumination. The suspension was then flushed for 1 minute with O_2, after which 1.5 ml HTO (58 mc/ml) and 100 μl $H^{14}CO_3^-$ (1.5 mc/ml) were added. The algae were again briefly flushed with O_2 and the vessel was stoppered. Samples were taken at the times indicated in the tables, and each sample was killed in 4 volumes of methanol.

The resulting mixture was taken nearly to dryness in vacuo to remove unincorporated tritium. Five to 10 ml of 80 % (v/v) methanol was added and the mixture again taken nearly to dryness. This procedure was repeated 4 times in all in order to remove tritium from exchangeable positions.

The residue of killed plant material was then taken up in water and methanol, and an aliquot sample was applied to the origin of Whatman No. 1 filter paper for chromatography. These chromatograms were developed in 2 directions. The first solvent was made up of 840 ml liquefied phenol (Mallinkrodt, about 88 % phenol, 12 % H_2O), 160 ml water, 10 ml glacial acetic acid, and 1 ml 1.0 M EDTA (12). The second solvent was made up of equal volumes of *n*-butanol-water (370:25 v/v) and of propionic acid-water (180:220 v/v) (13). The chromatograms were developed just long enough (24 hr in the first solvent, 12 hr in the second) for the solvent front to reach the edge of the paper.

After drying from the second solvent, a radioautograph was prepared to determine the position of the radioactive compounds on the paper, and the radioactive areas of the paper containing the tritium and ^{14}C-labeled compounds were eluted into vials for scintillation counting. To 0.3 ml or less of aqueous eluate, 18 ml of water miscible scintillation counting liquid (14) was added. The sample vials were counted automatically by the scintillation counter (Packard Instruments, Series 3000). Automatic external standardization was used.

In the experiment just described, high levels of oxygen were used in order to accentuate the formation of glycolic acid. In the following experiment (3), different conditions were used in order to determine the labeling of glycolic acid and other photosynthetic intermediates under conditions more closely approximating normal steady-state photosynthesis.

A 4.5 % suspension of *Chlorella pyrenoidosa* was preilluminated for 1 hour in air, after which $^{14}CO_2$ and HTO were simultaneously introduced in a closed system. The HTO (1.0 C in 1 ml) was dropped into the lollipop from a bulb, while $^{14}CO_2$ (50 μc/μmole) was added from a gas system of about 150 cc volume. The $^{14}CO_2$ pressure was kept relatively constant at about 0.1 %. Three aliquot samples were taken, and the remaining work-up was identical to that described previously.

Results

The results of experiments 1, 2, and 3 are shown in tables I, II, and III, respectively. Perhaps the most striking result is the high ratio of T/^{14}C in glutamic acid as compared with other photosynthetically labeled compounds in experiments 1 and 3. The T/^{14}C ratio of glutamic acid drops with time of photosynthesis. Earlier kinetic studies (15, 16) had shown that during steady-state photosynthesis of $^{14}CO_2$ the ^{14}C-labeling of glutamic acid is delayed, due perhaps to the number and pool sizes of intermediates lying between the photosynthetic carbon reduction cycle and glutamic acid synthesis. Studies with ^{14}C and ^{15}N showed that the formation of glutamic acid is the principal route of entry of ammonium ion into bound amino acid groups during steady-state photosynthesis (17). Thus, the direct reductive amination of α-ketoglutaric acid to form glutamic acid introduces tritium into glutamic acid immediately, whereas the multistep synthesis from CO_2 to glutamic acid delays the ^{14}C-labeling of the glutamic acid carbon skeleton.

To a lesser extent, the delayed ^{14}C incorporation into malic acid and alanine, compared with the final reductive step in their formation, is indicated by the higher T/^{14}C ratios of these compounds in the third experiment, which was carried out under nearly steady-state conditions. From these results, it would appear that T/^{14}C may be a meaningful yardstick for the comparison of rates of reductive formation and of carbon skeleton synthesis from CO_2 of photosynthetic intermediate compounds.

In experiments 1 and 2, the ratio of T/^{14}C of glycolic acid is quite comparable to that of glyceric acid-3-P, which in experiment 1 was roughly similar to that of other intermediates of the carbon reduction cycle, allowing for differences in the ratios of non-exchangeable hydrogen to carbon

Table I. *Labeling of Metabolic Intermediate Compounds During Photosynthesis with $^{14}CO_2$ and HTO*

Compound	dpm ^{14}C		dpm T		T/^{14}C	
	2.5 min	8 min	2.5 min	8 min	2.5 min	8 min
Glycerate-3-P	1750	2300	625	685	0.36	0.30
Hexose and heptose monophosphates	11,700	13,300	5190	6760	0.44	0.51
Diphosphates-1	4580	10,400	2450	4210	0.53	0.41
Diphosphates-2	4860	9870	1830	2030	0.38	0.21
Glycolic acid	6180	65 900	2130	15,000	0.34	0.23
Malic acid	1680	5620	895	2620	0.53	0.47
Aspartic acid	3300	8180	1970	4070	0.60	0 50
Glycine	1920	3870	510	970	0.27	0.25
Serine	6610	16,000	1980	4520	0 30	0.28
Sucrose	9540	39,000	980	6040	0.10	0.16
Glutamic acid	2100	9210	6290	17,400	3 00	1.90
Alanine	5100	6440	2440	3060	0.48	0.48

Conditions as described in text for experiment 1.

Table II. *Labeling of Metabolic Intermediate Compounds During Photosynthesis with $^{14}CO_2$ and HTO*

Compound	dpm ^{14}C			dpm T			T/^{14}C		
	1 min	2.5 min	8 min	1 min	2.5 min	8 min	1 min	2.5 min	8 min
Glycerate-3-P	2220	2000	1230	177	220	185	0.08	0.11	0.15
Glycolic acid	8350	21,200	92,100	585	1490	12,000	0.07	0.07	0.13

Conditions as described in text for experiment 2.

Table III. *Labeling of Metabolic Intermediate Compounds During Photosynthesis with $^{14}CO_2$ and HTO*

Compound	dpm ^{14}C			dpm T			T/^{14}C		
	50 sec	135 sec	250 sec	50 sec	135 sec	250 sec	50 sec	135 sec	250 sec
Glycerate-3-P	21,000	30 400	38,600	4890	8270	11.800	0.23	0 27	0.31
Diphosphates	57,400	93,600	109,000	16,400	35 700	41,000	0.28	0.39	0.41
Monophosphates	41 800	88,300	77,100	16 800	44 000	34 300	0.40	0.50	0.44
Malic acid	2380	16 600	23,100	1830	32 900	32,500	0.77	1.98	1.41
Glycolic acid	3060	6650	6500	1380	4500	5030	0.45	0.68	0.77
Alanine	13 900	52 500	55 500	13 600	52 800	57,600	0.98	1.00	1.04
Glycine	2470	11.100	11,900	1190	6050	6730	0.48	0 56	0.56
Serine	10 600	45 100	62,000	4190	16.800	26,100	0.40	0 37	0.42
Glutamic acid	945	8930	24,500	35 300	108,000	147,000	37.3	12.1	6.03
Sucrose	3830	83 100	230 000	1230	27 600	81,300	0 32	0 33	0.35

Conditions as described in text for experiment 3.

in the various compounds. Thus, in the presence of nearly 100% oxygen, glycolic acid formation from $^{14}CO_2$ via the photosynthetic carbon reduction cycle or an unknown path directly from $^{14}CO_2$ is quantitatively much more important than glycolic acid formation from glyoxylic acid via a hydrogen transport mechanism.

In the steady-state experiment 3, T/^{14}C in glycolic acid does appear to exceed the T/^{14}C in glyceric acid-3-P and other intermediates of the carbon cycle by a factor of about 2. This result suggests that, under the conditions of the experiment, the reductive formation of glycolic acid from an unlabeled carbon compound (presumably glyoxylic acid) might be approximately equal to the rate of formation of glycolic acid from $^{14}CO_2$. Even so, the ratio does not drop with time. The data are inconsistent with a shuttle mechanism in which there are many oxidations of glycolic acid and reductions of glyoxylic acid for each synthesis of glycolic acid from carbon dioxide.

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