Plant Physiol. (1966) 41, 143-147

Rate of Glycolate Formation During Photosynthesis at High pH [1]

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Received June 4, 1965.

Summary. The products of $C^{14}O_2$ fixation by *Chlamydomonas* and *Chlorella* were studied under conditions most favorable for glycolate synthesis. The highest percentage of the C^{14} was incorporated into glycolate in the pH range of 8 to 9. After 1 to 2 minutes as much as 40 % of the C^{14} was found in glycolate products and only a trace of C^{14} was present as phosphoglycerate. Below pH 8 the rate of photosynthesis was much faster, but only a small percent of the C^{14} was incorporated into glycolate in 1 or 2 minutes, while a high percent of the C^{14} accumulated in phosphoglycerate. C^{14} labeling of glycolate even at pH 8 or above did not occur at times shorter than 10 seconds. During the first seconds of photosynthesis, nearly all of the C^{14} was found in phosphoglycerate and sugar phosphates. Thus glycolate appears to be formed after the phosphate esters of the photosynthetic carbon cycle.

Washing *Chlamydomonas* with water 2 or 3 times resulted in the loss of most of their free phosphate. When a small aliquot of $NaHC^{14}O_3$ was added to washed algae in the absence of this buffering capacity, the pH of the algal medium became 8 or above and much of the fixed C^{14} accumulated in glycolate.

The formation of glycolate-C^{14} by algae during $C^{14}O_2$ fixation is promoted by many factors (1,3,8–13). Low CO_2, high O_2 partial pressure, and high light intensity all favor glycolate accumulation. In addition, as indicated in this report, algae suspended in medium at pH 8 or higher incorporate a high percentage of the newly fixed $C^{14}O_2$ into glycolate. The results are consistent with the rapid excretion of glycolate at pH values of 5 or above (9).

Since the biosynthetic route of glycolate formation has not been elucidated (8), it is possible that glycolate synthesis is indicative of a pathway of CO_2 fixation other than phosphoglycerate formation from ribulose diphosphate. An alternate pathway has been implicated from data on CO_2 fixation by algae under stress conditions of low manganese (7) or low CO_2 concentration (12). However, there is no indication of a separate CO_2 fixation pathway for glycolate synthesis in any of the steady state photosynthetic experiments from Calvin's group (1, 3, 6). Nevertheless, most of the latter experiments were run under conditions which did not favor glycolate formation. Consequently, we have repeated time rate studies with algae on glycolate-C^{14} formation under conditions most favorable for rapid glycolate labeling.

Methods

Algae, as obtained from the Indiana University collection, were *Chlamydomonas reinhardtii* Dangeard + strain (No. 89), *Chlamydomonas reinhardtii* Dangeard-strain (No. 90), and *Chlorella pyrenoidosa* Chick (No. 395). The *Chlamydomonas* cultures were grown in a phosphate rich medium adjusted to pH 6.8 which contained on a per liter basis 2 g NH_4NO_3, 0.2 g $MgSO_4 \cdot 7H_2O$, 25 mg $CaCl_2$, 5 mg/liter Fe as sequestrene NaFe, 1.53 g K_2HPO_4, 0.87 g KH_2PO_4 and 2 ml Hoagland Trace nutrients. *Chlorella* cultures were grown in media V of Norris et al. (5). Temperature was maintained at about 20° and light intensity at 1000 ft-c from daylight fluorescent bulbs. Since low CO_2 promotes glycolate formation, the cultures were aerated with air enriched with 0.2 to 0.5 % rather than with 4 % CO_2 as previously used.

Inoculation consisted of diluting 100 ml of culture with 900 ml of fresh nutrient and the algae were harvested after 1 or 2 days when they were in a rapid phase of growth, which is another condition favoring glycolate production. The algae were removed from the medium by centrifuging at 1000 × *g* for 3 minutes. A 1 % v/v suspension of algae was prepared in water or in 0.001 M potassium phosphate and adjusted to the designated pH. If the algae were to be washed they were resuspended in water and held for 3 to 5 minutes with aeration and then recentrifuged. The latter procedure was designated as the first washing, and it was sometimes repeated to obtain algae washed 2 or 3 times.

[1] Supported by NSF grant G24036 and published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal article no. 3652.

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$C^{14}O_2$ fixation experiments were performed on aliquots in a lollipop or flattened test tubes immersed in a 20° water bath. Photoflood lamps provided an illumination of about 3000 ft-c to each side of the algae culture. An equilibration period of 10 minutes in the light and with aeration was used prior to the addition of $NaHC^{14}O_3$ (16 % C^{14}). For every 10 ml of 1 % algal suspension, 0.015 ml of 0.05 M $NaHC^{14}O_3$-$NaC^{14}O_3$ (pH about 9) was added. This amount of $NaHC^{14}O_3$ provided a constant rate of photosynthesis for 2 minutes at 6.5 to 7 or for 10 minutes at pH 8.5. At designated times aliquots were drained off through a stopcock into hot methanol which was then boiled and unused $C^{14}O_2$ was removed by flushing with $C^{12}O_2$. Aliquots were counted for fixed C^{14} in a liquid scintillation counter.

Samples were reduced to a small volume at about 30° under vacuum in a Gyrorotary shaker. Two-dimensional paper chromatograms and radioautographs (2) were made of aliquots in order to determine the percentage distribution of the C^{14} among the products. After the second solvent development of the chromatogram with butanol-propionic acid-water, the paper was air dried for 18 hours and then sprayed with 1 M Na_2CO_3 to convert the acids on the paper to their sodium salt. Glycolic acid is somewhat volatile, however less than 10 % of added tracer amounts of glycolic-C^{14} acid was loss in the total chromatographic procedure. A major loss of glycolic acid from the chromatograms during radioautography (9) was prevented by converting it to the nonvolatile sodium salt.

Experiments of 2 to 4 seconds duration were run by rapidly injecting with a large syringe 5 ml of diluted $NaHC^{14}O_3$ (100 μc) into 20 ml of algae in a large lollipop. Another operator forced in 50 ml of hot methanol from a second syringe which provided instant mixing and killing.

Experiments of about 1-second duration in 3000 ft-c of light were run by mixing a $NaHC^{14}O_3$ solution with an algal suspension as the suspension flowed down a glass tube before reaching a beaker of hot methanol. The algae were being drained from a lollipop where steady state photosynthesis had been maintained. By means of dyes it was ascertained that the $NaHC^{14}O_3$ and algal suspension were mixed together for only 1 second before reaching methanol.

Results

Effect of Washing the Algae and Addition of Phosphate Buffer. A reproducable amount of C^{14} fixation from the $NaHC^{14}O_3$ by either *Chlamydomonas* or *Chlorella* was obtained if unwashed algae were resuspended in water or if the algae were washed 1 to 3 times and then resuspended in 3.3×10^{-3} M phosphate at pH 7 (table I). When either strain of *Chlamydomonas* were washed 3 times with water and resuspended in water, the rate of $C^{14}O_2$ fixation was about 12 to 15 % of the rate which was obtained by unwashed cells resuspended in water or washed cells resuspended in phosphate buffer.

Complete restoration of the photosynthetic capacity by phosphate suggested that loss of phosphate during washing was critical either for pH control or for metabolic processes. The loss of orthophosphate and of total phosphorus from the *Chlamydomonas* after successive washings was quantitatively determined. The algae were removed from their growth medium by centrifugation and resuspended in water (1 volume algae to 100 volumes of water). After trichloracetic acid disruption and precipitation of the algae, the medium contained about 400 μg orthophosphoric acid per 100 ml. The total phosphorus after sulfuric acid digestion of the cells was at least 3 times greater. When the algae were washed once, the orthophosphate reservoir dropped to a low level of less than 10 % that found in the unwashed cells. The decrease in total phosphorus upon washing the algae was mainly caused by loss of orthophosphate, although some loss of organic phosphorus was indicated. Upon repeated washing, the total phosphorus gradually decreased. Thus *Chlamydomonas*, when removed from their growth medium and resuspended (1 % v/v) in water, had a buffering capacity of 40 μm phosphate, while washed cells did not possess this buffering capacity to neutralize the added $NaHC^{14}O_3$.

Although the orthophosphate of the algae was reduced drastically by 3 washings, the rate of $C^{14}O_2$ fixation could be fully restored by numerous substances other than phosphate so long as the compounds added possessed buffering capacity between pH 5 and 8. Serine was nearly as effective as phosphate in restoring the rate of CO_2 fixation (table I) and other amino acids, phosphate esters, and Tris buffer were also effective. Thus the loss of phosphate from washing the algae 3 times was not so severe as to restrict the participation of phosphate in metabolic reactions involved in the fixation of CO_2.

The photosynthetic rate for *Chlorella* in bicarbonate (pH 8.–8.5) is much less than when sufficient CO_2 is present at lower pH values (4). Likewise when the pH of our *Chlamydomonas* suspensions in 0.001 M phosphate was greater than 8, the rate of C^{14}

Table I. *Effect of 3 Washings with Water on $C^{14}O_2$ Fixation by* Chlamydomonas

The 1 % algal suspension in a designated medium fixed $NaHC^{14}O_3$ for 10 minutes. Unwashed cells were removed by centrifugation from the growth medium and resuspended in the photosynthetic medium. See methods for preparation of washed cells.

Photosynthetic medium	Total c/s C^{14} fixed per ml algae	
	Unwashed cells	Washed cells
Water	2700	546
3.3×10^{-3} M phosphate (pH 7)	2960	3100
3.3×10^{-3} M serine (pH 7)	...	2100

Table II. *Effect of pH on Rate of $NaHC^{14}O_3$ Fixation by* Chlamydomonas

A 1 % algal suspension in 0.001 M phosphate was used.

Initial pH	Final pH	Total C^{14} fixed: c/s per ml algae/10 min
7.0	7.4	1953
7.5	7.9	938
8.0	8.3	375
8.9	8.5	300

fixation decreased to a low but significant value of about 12 to 15 % of that obtained at pH ranges between 6 and 7.5 (table II). When a small amount of $NaHC^{14}O_3$-$NaC^{14}O_3$ was injected into an algal suspension which had been washed 3 times, the final pH of the algal medium was that of the added bicarbonate-carbonate. Algae centrifuged from their growth medium and resuspended in water (1 % suspension) lost some phosphate but retained an amount sufficient to maintain the pH of the medium below 7.5 in the presence of the small aliquot of added $NaHC^{14}O_3$ and $NaC^{14}O_3$. Thus, a good rate of photosynthesis persisted. Even algae washed once and resuspended in water retained enough phosphate to automatically maintain pH in a physiological range.

Effect of pH of Medium on Glycolate Labeling. When photosynthesis experiments were run at pH 9, *Chlamydomonas* fixed about 15 % as much C^{14} as at pH 6 to 7 (table III). The distribution of the C^{14} among the products was also affected by pH. At pH values above 8.0 a high percentage of the C^{14} was found in glycolate, glycine, malate and aspartate. Correspondingly the presence of C^{14} in phosphate esters decreased and particularly little C^{14} was found in phosphoglycerate or ribulose diphosphate. Similar distribution of C^{14} was obtained with 3 times washed algae which had been resuspended in 0.001 M phosphate and adjusted to pH 8.5.

Rate of C^{14} Labeling of Glycolate. The rate of C^{14} labeling of a compound was used by Calvin et al. (3) to show that phosphoglycerate was the first labeled product of $C^{14}O_2$ fixation. We have repeated this type of experiment with *Chlamydomonas* and *Chlorella* using all the known conditions for maximum C^{14} incorporation into glycolate. The results of an experiment with *Chlamydomonas* which show much C^{14} label in glycolate products are presented in figure 1. The best results with *Chlorella* indicated much less glycolate labeling after short periods of time. No C^{14} labeled glycolate products (glycolate, glycine, and serine) were detected in experiments run for 1, 2, or 4 seconds. Only after 10 seconds of photosynthesis did C^{14} label first appear in glycolate products. The proportion of C^{14} in glycolate products increased in this experiment to 40 % of the total after 2 minutes. At pH 8 to 8.5 we generally obtain 20 to 40 % of the total C^{14} in glycolate products after 2 to 5 minutes of photosynthesis. After 10 minutes of photosynthesis with *Chlamydomonas* as much as 70 % of the C^{14} in glycolate products have been observed, provided the culture was

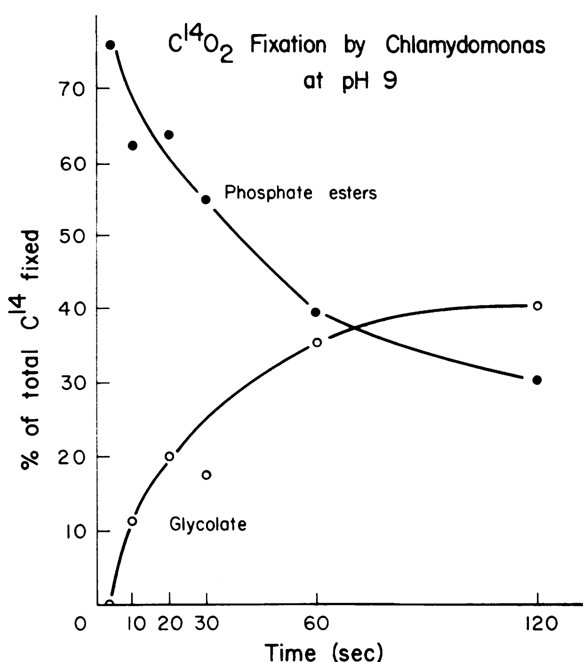

FIG. 1. Rate of glycolate formation by *Chlamydomonas*.

Table III. *Effect of pH on Distribution of C^{14} among Products of Photosynthesis by* Chlamydomonas

The *Chlamydomonas* were washed 3 times and then a 1 % algal suspension was prepared in either 0.001 M phosphate or in water at pH 8.8.

	Algae in 0.001 phosphate at pH 7		Algae in water at pH 8.8	
	30 sec	60 sec	30 sec	60 sec
Total c/s fixed per ml algae	15,780	30,880	2430	4930
Chromatographic area	%	%	%	%
P-glycerate	24.2	18.1	Trace	Trace
Sugar phosphates	65.3	67.1	54.5	39.7
Glycolate	Trace	Trace	13.0	15.2
Serine + glycine	1.4	3.0	9.6	12.2
Malate	5.1	5.2	13.9	17.7
Aspartate	3.1	2.7	5.3	8.5
Others	0.9	3.9	3.8	6.9

Table IV. *Products from 1 Second of $NaHC^{14}O_3$ Fixation by* Chlamydomonas r. (+)

A 1 % algal suspension in 0.001 M phosphate at pH 8.0 was used.

Products	Expt 1	Expt 2
	%	%
P-glycerate	84	88
Malate	16	8
Others	0	4
Total fixation as c/s per ml algae	4738	3686

maintained between pH 8.5 to 9. In experiments run for less than 10 seconds most of the C^{14} was present in P-glycerate and sugar phosphates. For 1 second experiments over 80 % of the total C^{14} was present in P-glycerate (table IV). The phosphate ester spots from these short experiments were hydrolyzed by phosphatase and no glycolate-C^{14} was found which indicated that P-glycolate also was not labeled.

Discussion

By the concept that time-rate studies can establish a sequence A → B → C → D → etc., the data in figure 1 indicate that glycolate is formed after the phosphate esters of the photosynthetic carbon cycle. Even though as much as 40 % of the $NaHC^{14}O_3$ was incorporated into glycolate after 2 minutes of photosynthesis at pH 9, at shorter periods of time C^{14} incorporation into glycolate dropped to zero while the components of the photosynthetic carbon cycle were still substantially labeled with nearly all of the C^{14} fixed. The possibility remains, however, that components A, B, C of a second CO_2 fixation pathway are not stable, and that an initial slow rate of glycolate labeling reflects only that it is not the first component of an alternate pathway.

The large increase at pH 9 in the percent of the total C^{14} which was incorporated into glycolate-C^{14} does not mean that the total amount of glycolate-C^{14} had increased greatly at the higher pH. The rate of $C^{14}O_2$ fixation was much lower at the higher pH (table II, III). Preliminary estimates indicated that nearly the same total amount of C^{14} was incorporated into glycolate products after 10 minute experiments at either pH 6.5 or at pH 8.5.

Warburg and Krippahl (10) reported a 92 % yield of glycolate from CO_2 fixation by *Chlorella* at pH 4.3 after 1 or 2 hours. In contrast we have been studying the rate of C^{14}-labeling of glycolate during only the first 1 or 2 minutes. In these short time periods little or no glycolate was labeled at pH 4 to 5. Experiments with *Chlamydomonas* lasting 1 to 2 hours indicated the production of large amounts of glycolate (unpublished). Further if *Chlamydomonas* were provided with $C^{14}O_2$ at pH 4 to 6, they began to produce C^{14}-labeled glycolate, glycine, and serine only after 5 to 10 minutes. Thus, we have not studied extensively the rate of glycolate-C^{14} formation at lower pH values, because it was slower, although ultimately C^{14} was incorporated into these reservoirs. The reason for a faster rate of glycolate production at pH 9 is not known. Glycolate production may be favored by an obligate anionic exchange at the high pH (9). Also uninhibited glycolate production at pH 9 may occur by a route of CO_2 fixation different from P-glycerate formation from ribulose diphosphate plus CO_2 which may not occur above pH 8. However our experiments of shorter time periods than 10 seconds did not suggest any route of CO_2 fixation even at pH 9 other than P-glycerate formation. Above pH 8 labeled P-glycerate and sugar phosphates were still formed, but they did not accumulate. Instead glycolate, glycine and serine accumulated in a time sequence which suggested that the glycolate products arose from the phosphate esters of the photosynthetic carbon cycle.

The cause for deterioration of the photosynthetic capacity of washed algae has been attributed to many factors, including physical damage and loss of minerals. In $NaHC^{14}O_3$ experimentation the need for some buffer control also seems to be a very important factor, for activity was readily restored when the washed algae were buffered at pH values below 8. The unwashed or once washed *Chlamydomonas* when resuspended in water control their own environmental pH to some extent by excreting phosphate. This loss of internal phosphate was not severe enough to limit the photosynthetic rate. The claim that a higher percentage of the C^{14} was incorporated into glycolate by washed *Chlamydomonas* in the absence of phosphate buffer is unjustified (8). In the absence of the phosphate the pH of the medium had been increased by the added $NaHC^{14}O_3$, and the higher pH and not the absence of phosphate will explain the increase in percent of C^{14} in glycolate.

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