Title

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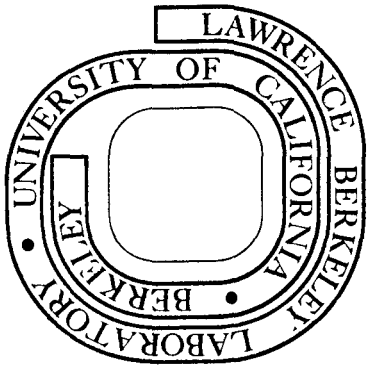

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Running head: Glycolate Formation in *Chlorella*

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Sequence of Formation of Phosphoglycolate and Glycolate in Photosynthesizing *Chlorella pyrenoidosa*[1]

[1]This work was supported by the U. S. Atomic Energy Commission.

[2]Abbreviations: PGA, 3-phosphoglycerate; PG, 2-phosphoglycolate; RuDP, ribulose-1,5-diphosphate; HMP, glucose-6-phosphate plus sedoheptulose-7-phosphate; UDPglucose, uridine diphosphoglucose.

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ABSTRACT

In Chlorella pyrenoidosa which have been photosynthesizing in either 1.5% $^{14}CO_2$ or 0.05% $^{14}CO_2$ in air, gassing with 100% O_2 results in rapid formation of phosphoglycolate which is apparently converted to glycolate. However, only about one-third to one-half of the rate of glycolate formation can be accounted for by this route. The remaining glycolate formation may be the result of the oxidation of sugar monophosphates. The rates of formation of both glycolate and phosphoglycolate are about four times greater with algae that have been photosynthesizing in 1.5% $^{14}CO_2$ than with algae which have been photosynthesizing with air, when the algae are then gassed with 100% O_2.

When some species of green plants photosynthesize with $^{14}CO_2$, and the total CO_2 concentration is not higher than in air, glycolate is prominent among the early labeled products (5). However, its formation occurs later than the formation of 3-phosphoglycerate (PGA) and sugar phosphates (7), which are the first products of CO_2 fixation in photosynthesis and are intermediates of the reductive pentose phosphate cycle of photosynthesis (1). This is also true when ^{14}C-labeled glycolate is formed by isolated spinach chloroplasts photosynthesizing with $^{14}CO_2$ (8). Glycolate formation during photosynthesis is favored by low CO_2 concentration (19), high oxygen concentration (4), and high light intensity (17).

Once formed, glycolate may be metabolized via a variety of pathways [for review, see Tolbert (18)] including paths which lead to photorespiratory CO_2 in higher plants. In some unicellular algae, such as Chlorella pyrenoidosa, glycolate may be excreted into the suspending medium.

The mechanism whereby glycolate is formed from the intermediate compounds of the photosynthetic carbon reduction cycle remains in dispute. Wilson and Calvin (19) suggested that the glycolyl moiety transferred in the transketolase-mediated reactions of the cycle might be oxidized to glycolic acid. Dihydroxyethylthiamine pyrophosphate, the intermediate in the transketolase-catalyzed reaction, can be converted to glycolate

with ferricyanide (6). Moreover, Shain and Gibbs (16) described a reconstituted preparation containing fragments of spinach chloroplasts, transketolase and cofactors which was capable of rapid conversion of fructose-6-phosphate or dihydroxyethyl thiamine pyrophosphate to glycolate in the light.

Since phosphoglycolate is also seen upon the increase of concentration of oxygen with photosynthesizing plants, Bassham and Kirk (4) suggested that the first step in the transformation of sugar phosphates to glycolate could be the oxidation of ribulose-1,5-diphosphate (RuDP) to phosphoglycolate. This would be followed by the conversion of phosphoglycolate to glycolate, which could be mediated by the active glycolate phosphatase found in chloroplasts (15). Recent findings that molecular oxygen competitively inhibits ribulose diphosphate carboxylase, the carboxylation enzyme of the photosynthetic reductive pentose phosphate cycle (12) led to proposals that RuDP is oxidatively split on the enzyme RuDPase by oxygen, giving phosphoglycolate and phosphoglycerate, instead of two molecules of phosphoglycerate normally formed by the carboxylation reaction.

There is considerable evidence that glycolate is formed oxidatively from sugar phosphates (10,11,14,16), by one or the other of these paths, although there is also a question about whether the oxidant is oxygen, a peroxide formed by reaction of oxygen with a primary reductant, such as reduced ferridoxin, or an intermediate in the oxidation of water formed by the photosystem 2 of the photoelectron transport path (16).

The present study was undertaken to assess the importance <u>in vivo</u> of phosphoglycolate as an intermediate in glycolate formation. The kinetic curves for the appearance and disappearance of RuDP, phosphoglycolate and glycolate following the introduction of 100% O_2 to

Chlorella pyrenoidosa previously photosynthesizing with $^{14}CO_2$ have been determined.

EXPERIMENTAL

In some experiments, Chlorella pyrenoidosa were grown in a continuous culture apparatus aerated with 4% CO_2 in air (2). A 0.3% suspension of these algae was withdrawn, the algae were centrifuged from the culture medium, and resuspended in 10^{-4} M KH_2PO_4 plus 10^{-3} M KNO_3 to a v/v concentration of 1%. Sixty ml of this suspension were transferred to the algae vessel of the steady-state apparatus (3). The pH was adjusted to 5.5 with 0.1 M HNO_3 and maintained by periodic automatic addition of 0.1 M HNO_3. The temperature of the algae was kept at 20°C. CO_2 (1.5 to 2%) in air was bubbled through the suspension and the lights were turned on. Cells were preilluminated in this way for about 30 min. During this time the photosynthesis rate was tested and found to be about 18-22 μg-atoms of carbon assimilated per minute per cm^3 wet-packed algae (3).

The gas flow system was closed and $^{14}CO_2$ added so that the concentration of CO_2 was about 1.5% with a specific activity of 15 μC/μmole. The cells were allowed to photosynthesize normally for 11 min, by which time the cycle intermediates had time to become "saturated" with $^{14}CO_2$. After 11 min the system was quickly flushed with 100% oxygen. Samples were taken before the flushing with oxygen and were removed at short intervals after the oxygen flush began.

These samples were killed in methanol and were analyzed by paper chromatography and radioautography (13). Radioactivity in each compound was determined using an automatic gas-flow Geiger counter (13). In four experiments, the period of flushing with O_2 was followed by a

period of flushing with N_2, with times as shown in the figures. In one of these experiments, alternate samples were killed in 80%% methanol as usual, while the remaining samples were killed in 80% methanol to which 100 µl 12 N HCl (per 4 ml methanol) had been added. In another of these experiments, alternate samples were killed in 50% methanol, 27% formic acid, and 23% water.

A second series of experiments were performed using Chlorella pyrenoidosa grown on air not enriched with CO_2. For this purpose the algae are grown in a low-form culture flask on a shaking apparatus in a temperature-controlled bath with illumination through the transparent bottom of the flask (2). A sintered glass bubbler provides for better aeration. Other conditions for culturing and for the exposure to $^{14}CO_2$ in the steady-state apparatus were as described above for the algae grown in 1.5-2% CO_2. Gassing times and regimes are described with the Results.

RESULTS

A comparison of the changes in levels of RuDP, P-glycolate, and glycolate after the introduction of O_2 (Figure 1) shows that the RuDP pool and the phosphoglycolate (PG) pool each increase rapidly in amount and then fall. The PG pool level tends to follow the level of the RuDP pool, and peaks about 90 sec after the peaking of the RuDP pool. This is clearly consistent with the idea that PG formation depends on O_2 and RuDP concentration. Since the only likely fate of PG is its conversion to glycolate, this suggests that some of the glycolate is formed by the sequence RuDP → PG → glycolate.

The formation of glycolate, however, does not seem to be totally dependent on the prior formation of PG. The rate of formation of glycolate seems only partly to depend on the level of PG. Thus, it appears that some of the O_2-stimulated glycolate production may be proceeding independently of the formation of PG. This qualitative impression of the relation between PG level and rate of glycolate production is further borne out by an attempt at evaluation of kinetic rate constants (see Discussion).

Among other effects of the addition of O_2 (and removal of CO_2) were the following:

The levels of 3-phosphoglycerate dropped sharply while the levels of glucose-6-phosphate plus sedoheptulose-7-phosphate (HMP) and of fructose-6-phosphate (not shown) first dropped and then rose (Figure 2).

The level of sucrose begins to decline soon after the addition of O_2, suggesting that this reserve sugar is mobilized to supply carbon to the cells' metabolism (Figure 4). The later rise in levels of hexose monophosphates, following the initial drop, also suggests that carbon flows into the reductive pentose phosphate cycle from endogenous sugars. This appears to be some kind of compensatory regulatory mechanism to keep the chloroplasts "primed" for the return of CO_2. This mechanism keeps the RuDP level from dropping below its steady-state level during the time of the experiment, in spite of the conversion of RuDP to glycolate.

The levels of several amino acids including alanine dropped, while the level of glycine rose (Figure 4). This rise in glycine level may indicate that some of the glycolate is metabolized via glyoxylate to give glycine.

An analysis of these kinetic curves for PG, RuDP, and glycolate concentrations as shown in Figure 1 suggested that the rate of glycolate formation changes substantially during the first 2 minutes of O_2 gassing (Discussion). Therefore, we decided to attempt to evaluate the rate of PG hydrolysis under a set of conditions under which PG formation should have stopped. The sequence of gas changes was altered to include a second change from 100% O_2 to 100% N_2 after 3 min, when phosphoglycolate is still at a high level. Since O_2 is required for the formation of phosphoglycolate from RuDP, we reasoned that the disappearance rate of phosphoglycolate after a few seconds of N_2 flushing would represent the absolute rate of phosphoglycolate hydrolysis. Presumably this rate should be dependent on phosphoglycolate concentration. Provided that the rate constant for the hydrolysis of phosphoglycolate remains constant during the time the algae are with O_2 and the time they are with N_2, the rate of hydrolysis of phosphoglycolate at a given concentration of phosphoglycolate during the N_2 regime should be the same as the rate of hydrolysis during the O_2 regime.

The concentration of phosphoglycolate in this experiment (Exp. 2, Figure 5 and Table I) was the same at 11.3 min as it was at 13.8 min, and the rates of phosphoglycolate disappearance at 13.8 min thus can be compared with the rate of glycolate formation at 11.3 min. The rate of disappearance of phosphoglycolate at 13.8 min was 0.705 μg-atoms per min/cm^3 algae (Table I). The rate of glycolate appearance at 11.3 min was 2.5 g-atoms per minute and represents a lower limit for the rate of formation of glycolate at that time, since there is apparently some metabolism of glycolate, though at a much slower rate than its formation.

In another experiment, with similar conditions to those just described (Exp. 3, Table I), the rate of appearance of glycolate at 11.3 min/and of disappearance of phosphoglycolate at 13.8 min were comparable to the values just given. In Experiments 4 and 5 (Table I) the O_2 was replaced by N_2 after only 2 min. In Experiment 4, the rate of disappearance of phosphoglycolate was somewhat higher, but still well below the rate of appearance of glycolate at 11.3 min, the time of the same PG concentration during O_2 gassing.

No additional PG was detected in samples killed with methanol plus HCl or with methanol and formic acid (27%).

With air-adapted algae, photosynthesizing in 0.05% $^{14}CO_2$, there is a measurable rate of appearance of glycolate and a measurable steady-state level of phosphoglycolate before the addition of 100% O_2 (Figure 6).

After the air and $^{14}CO_2$ is replaced by 100% O_2, the rate of appearance of both PG and glycolate increases. However, the rate of appearance of glycolate is much less than it was in the algae switched from 1.5% $^{14}CO_2$ to 100% O_2. It is clear that the rate of appearance of glycolate after the switch to 100% O_2 is much greater in relation to PG concentration than before (Table II), but this is possibly misleading, since the rate of both formation and utilization of glycolate may change when 0.05% $^{14}CO_2$ in air is replaced by O_2. The data do suggest once again that PG concentration is not very dependent on glycolate concentration.

When algae photosynthesizing in 0.05% $^{14}CO_2$ are switched first to O_2, and then after 3 min to N_2 (Figure 7), it can be seen that the rate of glycolate utilization is in this case significant compared to rate of

appearance of glycolate. Thus, in O_2 glycolate increased at a rate of +0.26 μg-atoms per min cm^3 algae. During the first minute in N_2, the glycolate concentration dropped at a rate of 0.60 μg-atoms per minute. If we assume that this rate of decrease in glycolate during the first minute in N_2 represents a lower limit for the rate of glycolate utilization in O_2, then the total rate of formation of glycolate in O_2 was at least 0.26 -(-0.60) = +0.86 μg-atoms per min cm^3 algae. This is to be compared with a rate of disappearance of PG in the first minute in N_2 of 0.22 μg-atoms per min cm^3 algae.

DISCUSSION

Although the rise and fall in phosphoglycolate concentration follows closely after the rise and fall in RuDP concentration (Figure 1), it is not likely that the rate of PG formation is linearly dependent on RuDP concentration if the enzyme ribulosediphosphate carboxylase is catalyzing the conversion of RuDP to PG in the presence of O_2. The enzyme is normally saturated with respect to RuDP under conditions of normal *in vivo* photosynthesis, and the level of RuDP goes still higher when the O_2 gassing commences, due to removal of CO_2. Thus the rise and fall in PG concentration is taken as an indication of a changing rate of conversion of enzyme-RuDP complex to phosphoglycerate and phosphoglycolate in the presence of O_2.

The increase in rate after the onset of O_2 gassing is easily explainable as being the result of several seconds being required for the effective dissolved concentration of O_2 to rise as a result of the switch from atmospheric O_2 to 100% O_2. The subsequent fall in the level of PG we attribute to a decreased rate of formation of PG resulting from some

change in the activity of the enzyme. It has been found that the carboxylation activity of the enzyme greatly decreases (presumably as the result of conformational change) when the enzyme is presented with RuDP in the absence of CO_2 (9). We suspect that this change, which must occur when the O_2 gassing takes place, also inactivates the enzyme with respect to the oxidative reaction.

Given the low concentration of PG, and the high reported activity of the enzyme glycolate phosphatase (15), it seems reasonable to suppose that the rate of hydrolysis of PG exhibits first order dependence on PG concentration under the conditions of our experiments. This assumption is implicit in our comparison of the rate of disappearance of PG under N_2 in Experiments 2 to 5 with the maximum rates of appearance of glycolate in those experiments. We wish to use this assumption also in the analysis of the PG and glycolate curves in Experiment 1 (Figure 1).

There are two points in Experiment 1, Figure 1, at which the rate of change of PG concentration is at least momentarily zero. These are at 13.5 min, when the PG concentration is 0.98 μg-atoms of ^{14}C, and again at 20 min, when the PG concentration is 0.33 μg-atoms of ^{14}C. Let us make the assumption that at each of these two points the rate of hydrolysis of PG (equal to its rate of formation) is given by k(PG). Let us make the second assumption that glycolate formation is given by K + k(PG). This rate experimentally is +3.00 μg-atoms ^{14}C min^{-1} and +1.9 μg-atoms ^{14}C min^{-1} at 13.5 min and at 20 min respectively. Solving the two simultaneous equations for these two times, we get K = 1.34 μg-atoms ^{14}C min^{-1} and k = 1.7 min^{-1}. In other words, rate of glycolate formation = 1.34 + 1.7 [PG]. Thus there would be formation of glycolate by a pathway independent of

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phosphoglycolate at a rate of 1.34 μg-atoms min^{-1} and formation as the result of PG formation and hydrolysis at rates varying from 1.7 μg-atoms min^{-1} when PG formation is maximal to 0.56 μg-atoms ^{14}C min^{-1} at 20 min.

These values may be compared with the results for Experiments 2 through 5 where the measured rates of PG disappearance varied from 0.4 to 0.9 μg-atoms min^{-1}, and the calculated values for k varied from 2.0 to 2.6 (Table I). Considering these measurements and calculations for Experiments 1 through 5, it appears that under the conditions of O_2 gassing of Chlorella previously photosynthesizing in 1.5% CO_2, glycolate formation takes place both by way of phosphoglycolate (presumed to be formed by oxidation of RuDP) and by an independent pathway, which may be the oxidation of sugar monophosphates. During the first 90 sec of O_2 gassing, the pathway via phosphoglycolate may account for one-half or more of the glycolate formation, but during a subsequent period the rate of formation of the phosphoglycolate decreases, and this route to glycolate accounts for only about a third of the glycolate formation.

It will be noted that no allowance has been made for the conversion of glycolate to other matabolic products, despite the evidence (glycine formation, Figure 4) that such conversion does occur. The actual rate of formation of glycolate is the sum of the rate of its appearance plus the rate of its conversion, so that even less of the total glycolate production can be accounted for in terms of phosphoglycolate hydrolysis.

To summarize, it appears that with Chlorella, photosynthesizing either in 1.5% CO_2 or in air, addition of 100% O_2 results in a rapid formation of phosphoglycolate which is converted to glycolate, but that

only about one-third to one-half of the glycolate formed as a result of O_2 addition is formed by this route. The remaining glycolate formation may be the result of the oxidation of sugar monophosphates. The rates of formation of both phosphoglycolate and glycolate appear to be about four times greater when the algae had been photosynthesizing in 1.5% CO_2 than when they had been photosynthesizing with air.

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Table I. Rate of Appearance of Glycolate in O_2 Compared with Rate of Disappearance of Phosphoglycolate in N_2

In Experiment 2, rate of glycolate appearance was measured at 11.3 min while rate of PG disappearance was at 13.8 min (see Figure 5). Comparable measurements were made in Experiment 3. In Experiments 4 and 5, O_2 was administered for 2 min instead of 3 min. Rates were again measured at points on the curve where PG concentration was the same in O_2 and in N_2. In all cases rate of disappearance of PG was measured where it was maximum. Steady-state rates of CO_2 uptake, prior to O_2 flushing, were from 18 to 23 μmoles CO_2/min cm^3 algae. See text for discussion of k.

Experiment	d(glycolate)/dt [μg-atoms ^{14}C/min cm^3 algae]	d[PG]/dt	$k = \frac{d[PG]}{dt}/[PG]$
2 (Fig. 5)	+2.5	-0.87	2.5
3	+2.7	-0.40	2.0
4	+3.2	-0.90	2.7
5	+3.0	-0.75	2.3

Table II. Rate of Appearance of Glycolate Compared with Level of Phosphoglycolate

Rates are calculated from slopes of curve shown in Figure 6 at the indicated times.

Time (min)	Condition	[PG] [μg-atom $^{14}C/cm^3$]	$\frac{d(Glycolate)}{dt}$ [μg-atom $^{14}C/min \cdot cm^3$ algae]
17	0.05% $^{14}CO_2$ in air	0.100	.0115
21	100% O_2	0.256	.434
34	100% O_2	0.125	.324

FIGURE CAPTIONS

Fig. 1. Effect of 100% O_2 on levels of labeled RuDP, PG, and glycolate in Chlorella pyrenoidosa after photosynthesis with 1.5% $^{14}CO_2$.

Fig. 2. Effect of 100% O_2 on levels of 3-phosphoglycerate (PGA) and sugar monophosphates in Chlorella pyrenoidosa after photosynthesis with 1.5% $^{14}CO_2$.

Sugar monophosphates include glucose-6-phosphate and sedoheptulose-7-phosphate.

Fig. 3. Effect of 100% O_2 on levels of sucrose and uridine diphosphoglucose in Chlorella pyrenoidosa after photosynthesis with 1.5% $^{14}CO_2$.

Fig. 4. Effect of 100% O_2 on levels of alanine and glycine in Chlorella pyrenoidosa after photosynthesis with 1.5% $^{14}CO_2$.

Fig. 5. Effect of 100% O_2 for 3 min followed by 100% N_2 on levels of labeled RuDP, PG, and glycolate in Chlorella pyrenoidosa after photosynthesis with 1.5% $^{14}CO_2$.

Fig. 6. Effect of 100% O_2 on levels of RuDP, PG, and glycolate in Chlorella pyrenoidosa after photosynthesis with 0.05% $^{14}CO_2$.

Fig. 7. Effect of 100% O_2 for 3 min followed by 100% N_2 on levels of labeled RuDP, PG, and glycolate in Chlorella pyrenoidosa after photosynthesis with 0.05% $^{14}CO_2$.

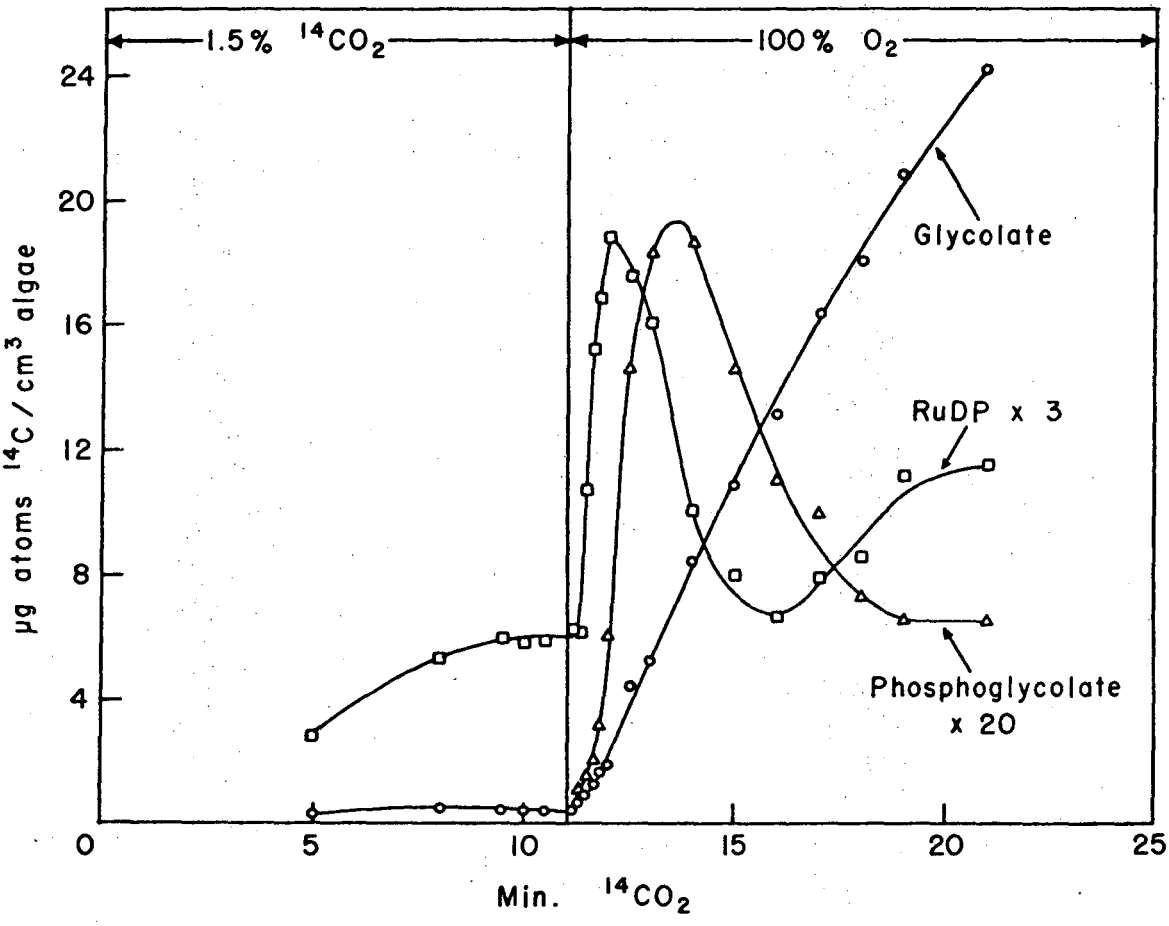

Fig. 1

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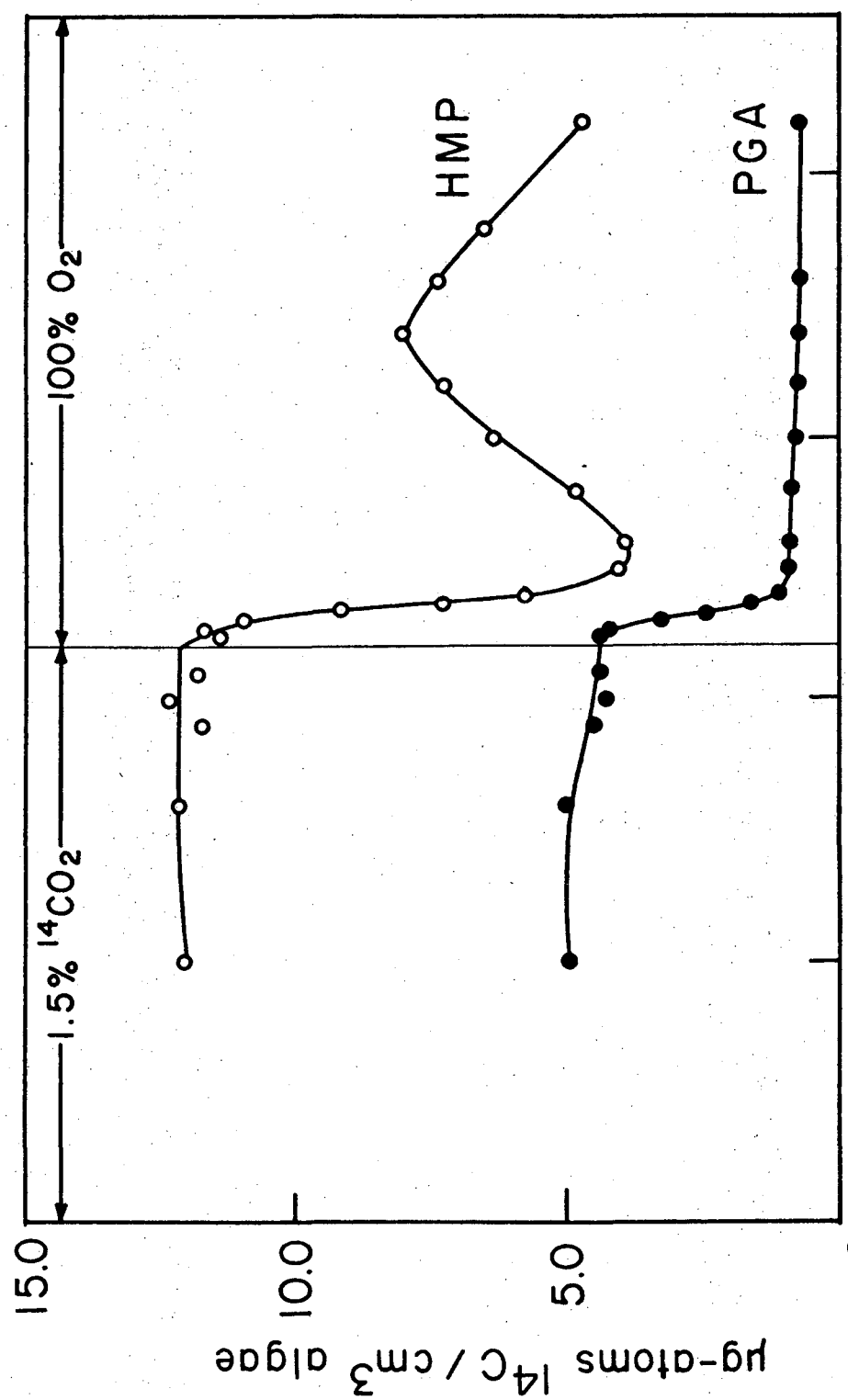

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Fig. 2

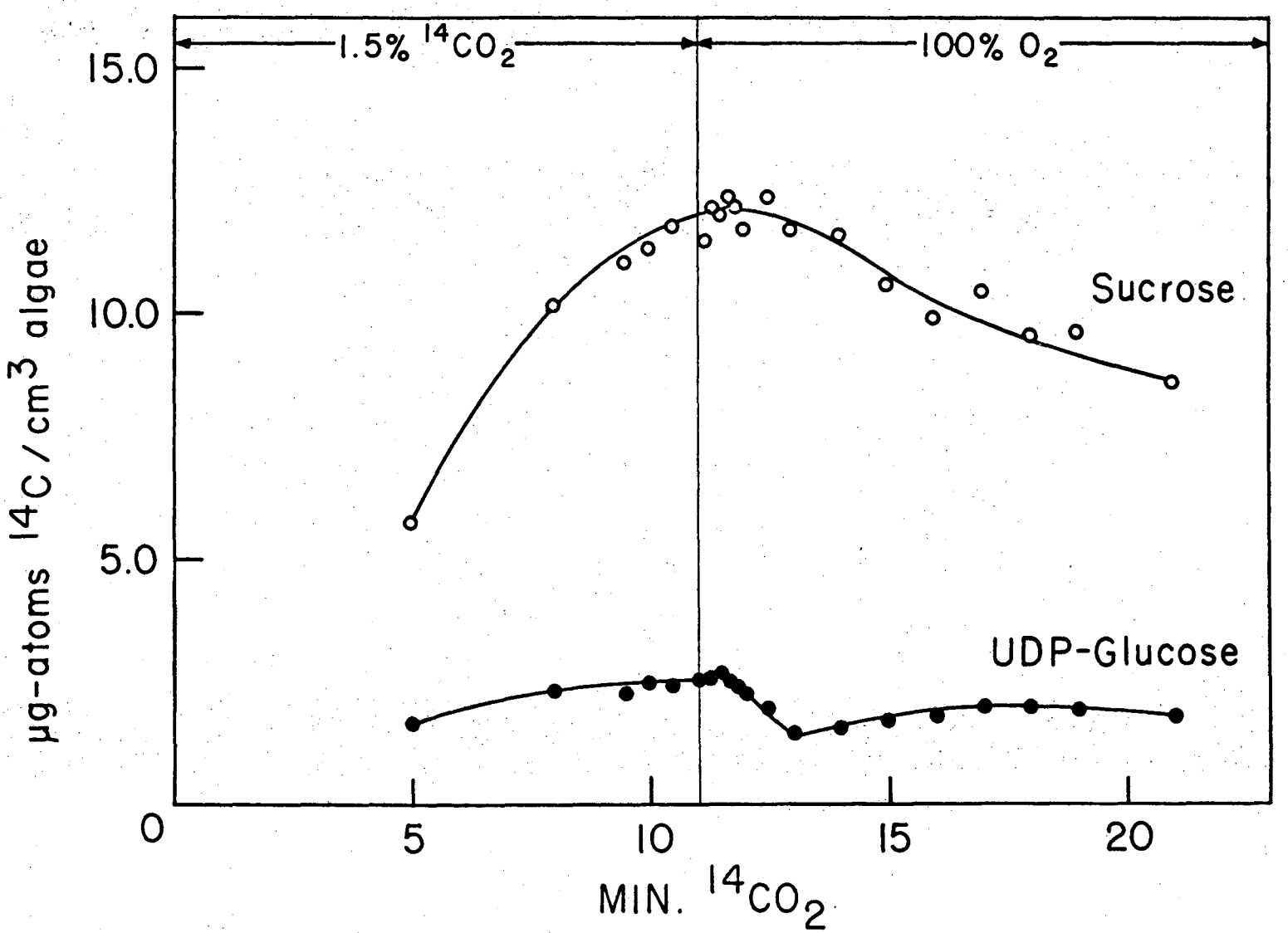

Fig. 3

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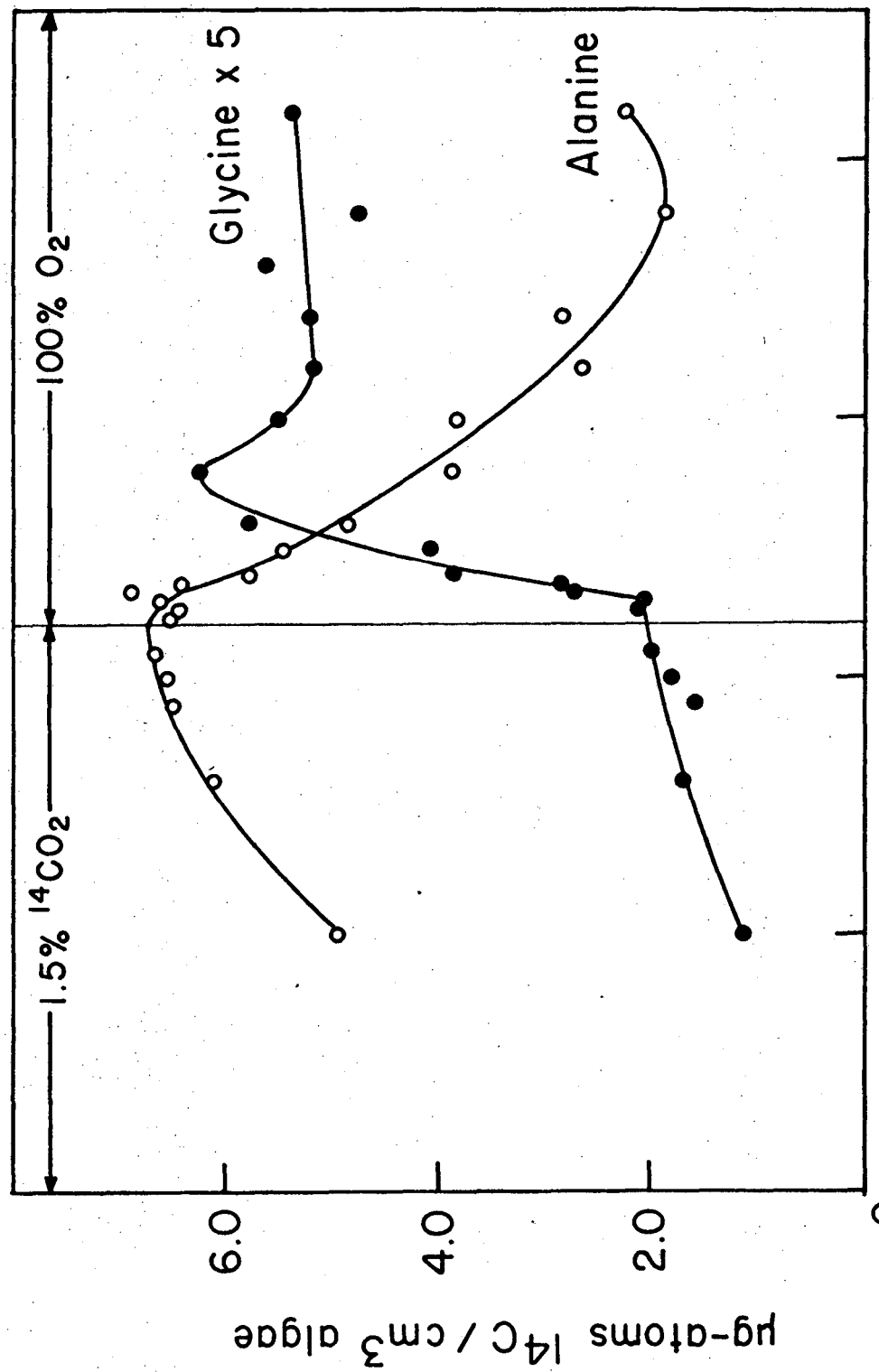

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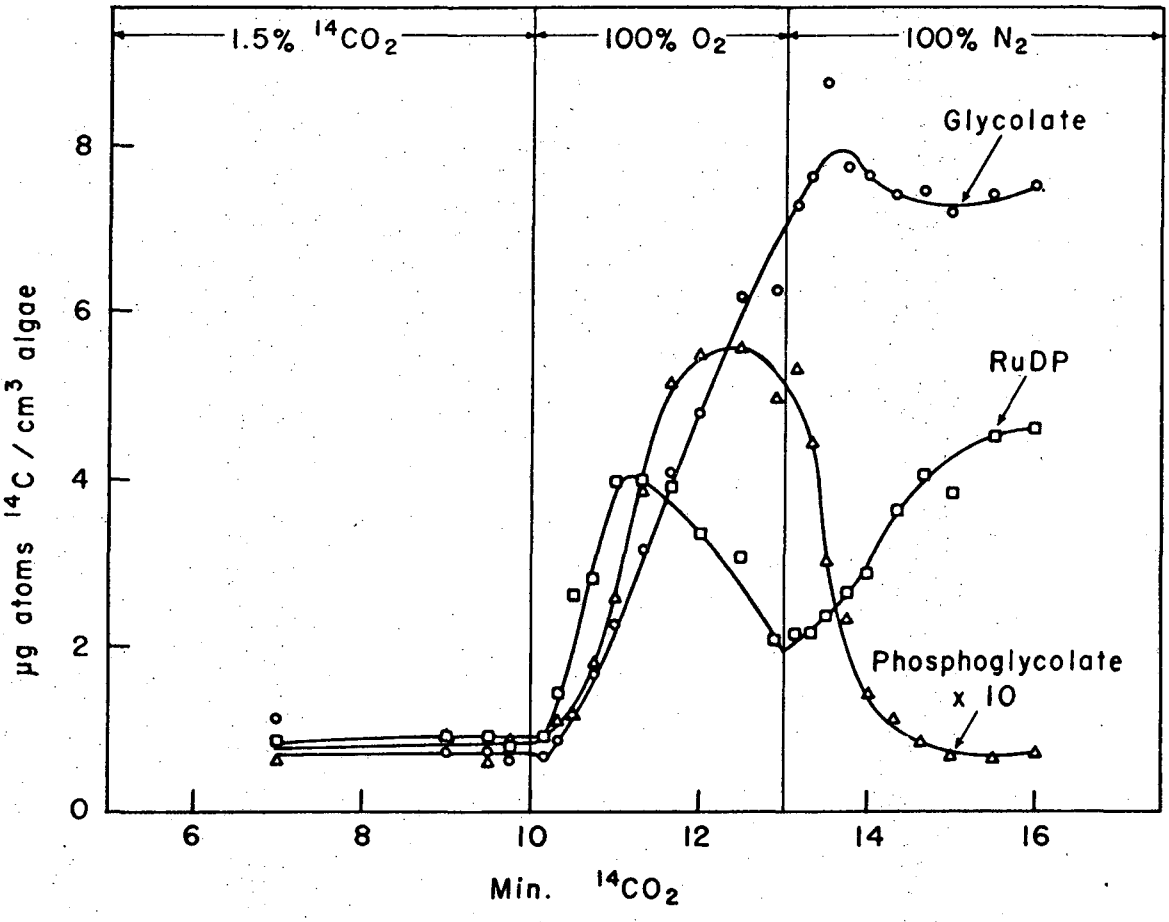
Fig. 4


1.5% $^{14}CO_2$
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100% N_2
Glycolate
RuDP
Phosphoglycolate
x 10
μg atoms ^{14}C / cm^3 algae
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Fig. 5

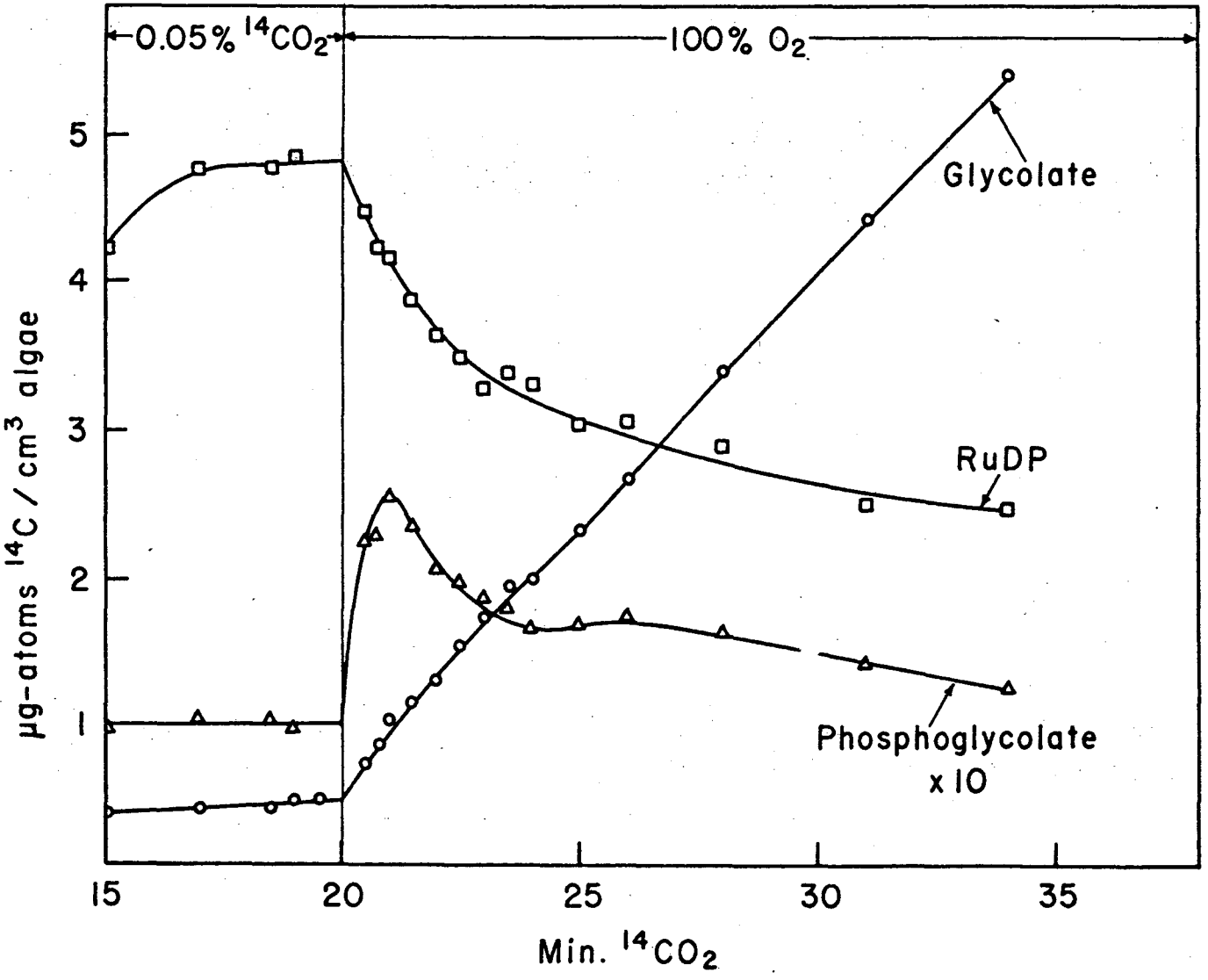

Fig. 6

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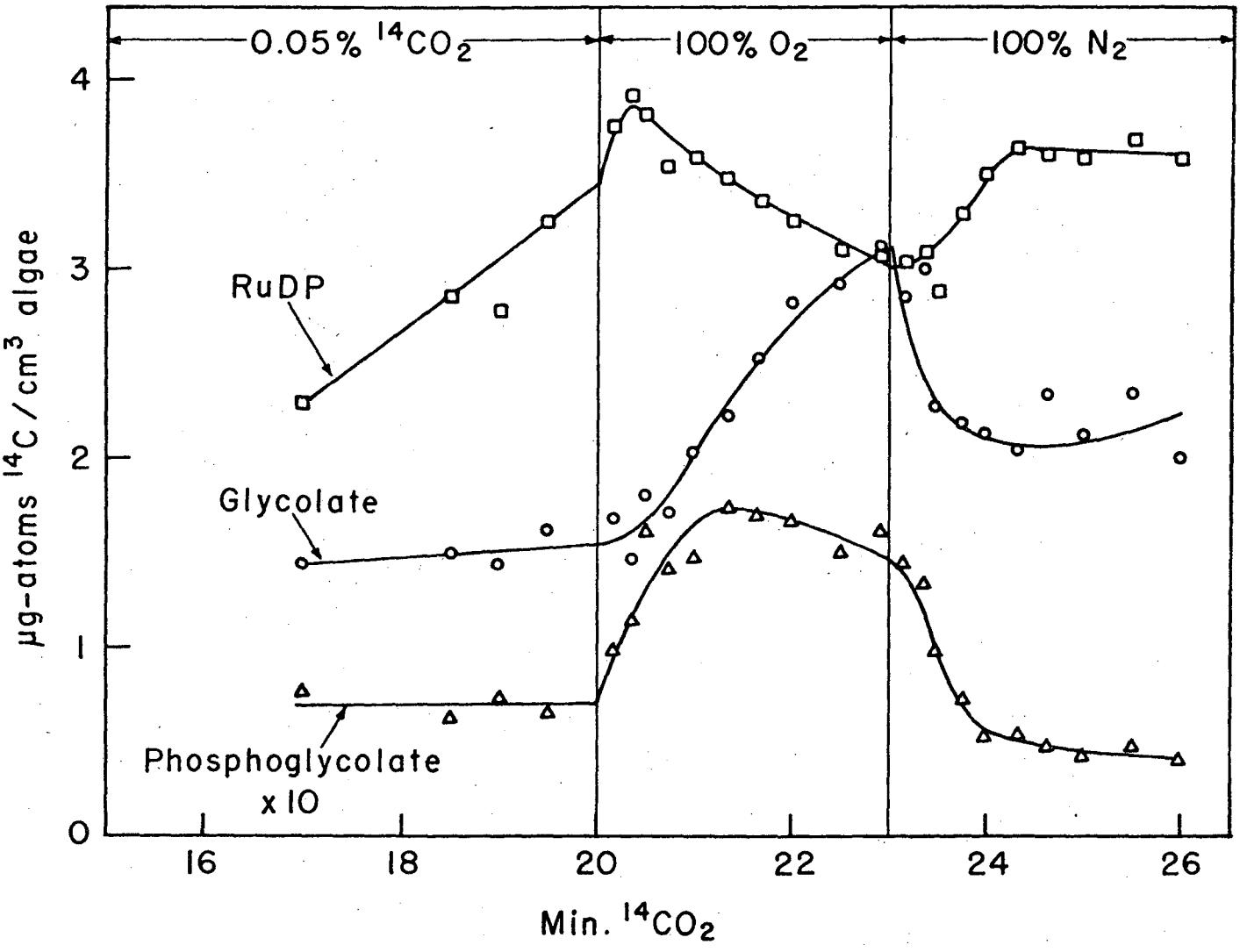

Fig. 7

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