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PHOTOSYNTHETIC CARBON METABOLISM

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Early in this century, it was thought that photochemical decomposition of carbon dioxide gave oxygen and reduced carbon.[1] In 1931, van Niel[2] formulated photosynthesis as a transfer of hydrogen from water to carbon dioxide in higher plants, and from other hydrogen donors to carbon dioxide in photosynthetic bacteria. The photochemical decomposition of water to give oxygen received further support when Hill and Scarisbric[3] found that illuminated chloroplasts evolve oxygen when supplied with a suitable electron acceptor. Then, in 1941, Ruben and coworkers[4] showed that oxygen evolved during photosynthesis agreed in isotopic composition with the oxygen of water rather than with that of carbon dioxide. With the discovery of radiocarbon, Ruben and coworkers[5,6] could prove that the fixation of carbon dioxide proceeds in the dark to an intermediate compound which is subsequently transformed in the light.

The Calvin Cycle

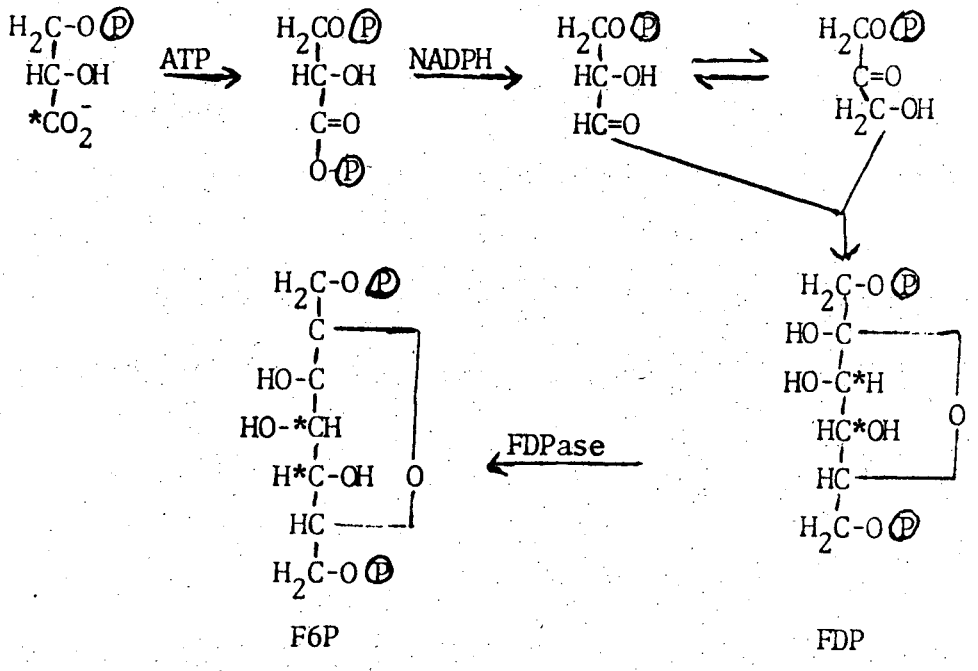
From 1946 to 1953, Calvin and coworkers used carbon-14 as a tracer to follow the path of carbon fixation and reduction in photosynthesis.[7-11] They used two-dimensional paper chromatography and radioautography to analyze the ^{14}C-labeled intermediate compounds formed in the green cells.

The first ^{14}C-labeled, stable compound was identified as 3-phosphoglyceric acid (PGA), labeled in the carboxyl group (according to the results of chemical degradation).[7] This compound was formed in the dark following preillumination.

$$X + {}^*CO_2 \rightarrow \begin{array}{l} H_2C\text{-}O\textcircled{P} \\ | \\ HC\text{-}OH \\ | \\ {}^*CO_2^- \end{array}$$

3-phosphoglycerate (PGA)

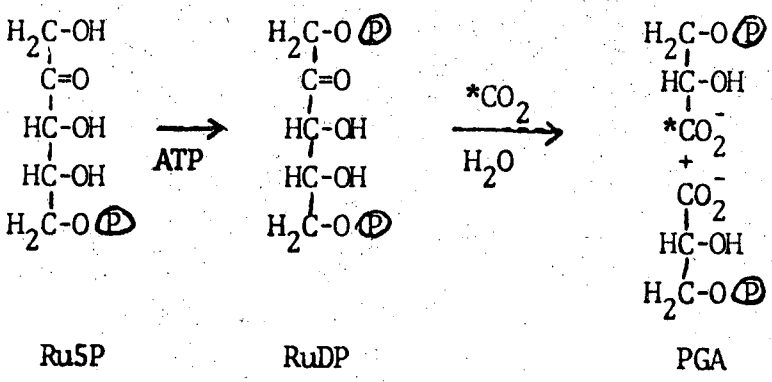
Other compounds labeled during the first 5 sec of photosynthesis included sugar phosphates with 3 to 7 carbon atoms. The hexose phosphates formed during a few seconds of photosynthesis were labeled predominantly in carbon atoms 3 and 4. Thus, the pathway of carbon from PGA to fructose-6-phosphate could be formulated as a reversal of well known reactions of glycolysis. Activation of the carboxyl group of PGA with adenosine triphosphate (ATP) to give phosphoryl PGA is followed by a reduction with NADPH (a two-electron carrier) to give triose phosphates which then isomerize and condense with each other to give fructose diphosphate (FDP) and eventually fructose-6-phosphate.



Chemical degradation of the C_5 and C_7 sugars, isolated after a few seconds of photosynthesis in the presence of $^{14}CO_2$, showed that a series of dismutations and condensations leads to the conversion of 5 molecules of triose phosphate to 3 molecules of pentose phosphate.[8] The carboxylation reaction substrate was revealed by a different type of experiment.

The algae were allowed to photosynthesize for several minutes with $^{14}CO_2$ under steady-state conditions. After about 5 min, the intermediate compounds of the primary photosynthetic pathway were later found to be completely labeled with ^{14}C; that is, the radioactivity in a pool of a given compound no longer increased. The ^{14}C content of each pool of compounds then represented the actual concentration of that compound within the cells.

An environmental condition then was changed; for example, the light was turned off. Samples taken rapidly after this perturbation revealed on subsequent analysis the changes in concentration accompanying the perturbation. In the case of the light to dark transient, it was found that the level of radioactive PGA rose very rapidly, as expected upon interruption of the supply of photochemically produced cofactors required for its reduction to triose phosphate (Figure 1).[10] The level of ribulose-1,5-diphosphate (RuDP) dropped rapidly, suggesting that RuDP is formed from ribulose-5-phosphate (Ru5P) by a reaction utilizing ATP from the light. The carboxylation of RuDP to give 2 molecules of 3-PGA clearly proceeded in the dark. When the light was left on, but the level of CO_2 was reduced, RuDP increased and PGA decreased as expected.[11]



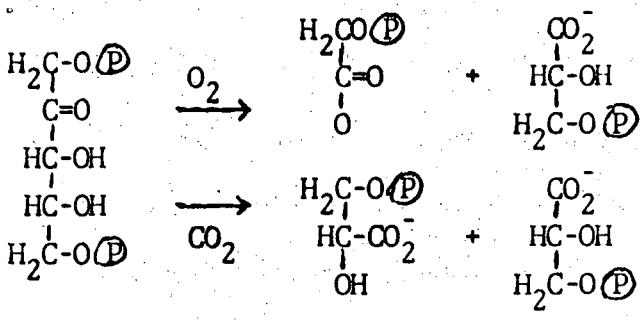
The photosynthetic carbon reduction cycle was formulated by 1953, as shown in Figure 2.[8,9] Carboxylation of three RuDP molecules gives 6 molecules of PGA, which can be reduced to 6 molecules of triose phosphate by reactions using 6 ATP molecules and 6 NADPH molecules. Only 5 triose phosphate molecules are needed to regenerate 3 Ru5P molecules which are then converted to 3 RuDP molecules with 3 more ATP molecules. The triose phosphate molecule gained from the reduction of 3 CO_2 molecules may be condensed with triose phosphate to give sugar monophosphates and eventually carbohydrates, including starch, sugar, and cellulose. Alternatively, the triose phosphate molecules can be converted to glycerophosphate for fat synthesis or transformed via PGA and phosphoenolpyruvate along many well known biochemical pathways to give various amino acids, fatty acids, and other molecules needed by the cell.

Photorespiration and C-4 Pathways

The chemical lability of RuDP, which makes possible its carboxylation, may be the key to the puzzling phenomenon of photorespiration. Warburg discovered in 1920 that excess oxygen inhibits the rate of photosynthesis.[12] A key compound in photorespiration is glycolic acid. The rate of photorespiration and the production of glycolic acid in most green plants are stimulated by low levels of CO_2, high levels of oxygen, high light intensity, and elevated temperature.[11,13-16]

In 1962, we found that when Chlorella pyrenoidosa are exposed to $^{14}CO_2$ in the presence of either oxygen, CO_2-free air, or nitrogen, the inhibition of photosynthesis, the production of glycolic acid and of phosphoglycolic acid, and the decrease in the carboxylation substrate, ribulose diphosphate, are well correlated with the presence of oxygen

(Table I).[16] We suggested that the RuDP-carboxylation enzyme complex may be attacked by oxygen instead of by carbon dioxide to give phosphoglycolic acid and PGA instead of 2 molecules of PGA as would be produced by the reaction with carbon dioxide.



Once formed, phosphoglycolate would be hydrolyzed by glycolate phosphatase, an active enzyme found in chloroplasts.[17]

Another possibility mentioned was the oxidation of glycolaldehyde thiamin pyrophosphate intermediate of the transketolase reaction ($*C_2$ in Figure 2), thereby lowering the level of the pentose monophosphates and eventually of ribulose diphosphate. The oxidant in this case may be an intermediate formed in photochemical reaction 2 in the lamellae (the light reaction which splits water) rather than molecular oxygen.[18] It is possible that both mechanisms operate in photosynthetic cells. Perhaps the formation of glycolate from pentose monophosphates may be the normal enzymic biosynthetic pathway, while the oxidation of RuDP or of RuDP-enzyme complex could be the mechanism of photorespiration.

One may speculate that through the geological ages the pressure of carbon dioxide in the atmosphere has declined as a consequence of photosynthesis and the removal of carbon in the form of fossil fuels and carbonate depositions. With this decline in CO_2, plants have increased their amount of ribulose diphosphate carboxylase so that it now constitutes

about half of the soluble protein in the chloroplasts. Even so, the steady-state concentration of ribulose diphosphate in plants growing in air has become very high. Recently Ogren and Bowes[19] have shown that with purified ribulose diphosphate carboxylase oxygen competes with CO_2 in the reaction with ribulose diphosphate.

Thus, plants have evolved an enzyme to convert phosphoglycolate to glycolate,[17] and a metabolic pathway to deal with the glycolate that is formed.[20] This glycolate pathway is found outside the chloroplast, and results in the conversion of the glycolate to CO_2 and to 3-carbon compounds, serine and glycerate, which can be reincorporated into the plant's metabolism. The conversion of photosynthetic product back to CO_2 is a wasteful process which to some extent determines the light saturation point for many plants. Some plants have proceeded further in the evolutionary pathway and have developed a mechanism for overcoming the wasteful release of CO_2 during photosynthesis. This mechanism seems to have evolved independently in several species of tropical grasses, such as corn and sugar cane, and in various unrelated species, including some dicotyledons.[21]

Kortschak, Hartt, and Burr[22] found in 1965 that in sugar cane, newly incorporated $^{14}CO_2$ first appears in 4-carbon acids, particularly malic and aspartic acids, before it appears in PGA and other intermediates of the Calvin cycle. After further study, Hatch and Slack[21,23,24] and coworkers discovered a cyclic pathway (Figure 3) which is known as the C-4 cycle. In plants with this cycle, chloroplasts in the mesophyll cells near the surface of the leaf are different from the chloroplasts in the parenchyma vascular bundle cells deeper inside the leaves. In

the mesophyll chloroplasts, pyruvic acid is converted with ATP to give phosphoenolpyruvic acid (PEPA) which then undergoes carboxylation to give oxalacetic acid (OAA). This C-4 acid is reduced with NADPH to give malic acid or, alternatively, is converted to aspartic acid by transfer of an amino group from glutamic acid (which is formed by reductive amination of alpha keto glutaric acid. Both malate and aspartate are thought to be translocated from mesophyll cells to parenchyma cells via plasmadesmata which are clearly visible in electron micrographs.[25] In the vascular bundle chloroplasts the C-4 acids are oxidatively decarboxylated, yielding pyruvate, CO_2 and NADPH. The pyruvate presumably is translocated back to the mesophyll cells, while the CO_2 is then incorporated via the Calvin cycle to give PGA and carbohydrates. This cycle requires ATP and NADPH for operation, as discussed earlier. In some C-4 plants, however, the bundle sheath chloroplasts contain only single lamellae; that is, they do not contain grana.[26,27,28] Woo et al.[29] found that such plants are incapable of carrying out photoelectron transport, leading to the splitting of water, oxygen evolution, and $NADP^+$ reduction. Their single, unappressed lamella apparently are only capable of cyclic phosphorylation, leading to the production of ATP. Two NADPHs are required per CO_2 fixed in the Calvin cycle, whereas only one NADPH per CO_2 is supplied in malate decarboxylation. The additional NADPH might be translocated from the mesophyll cells by some type of shuttle mechanism such as the movement of dihydroxyacetone phosphate (DHAP) from mesophyll to parenchyma, the oxidation of DHAP to PGA in the parenchyma cell, and the return of the PGA to the mesophyll cells.

As already mentioned, photorespiratory CO_2 arises from the metabolism of glycolate which in turn may be generated by the attack of oxygen on the

RuDP carboxylase enzyme complex. In plants with but one type of chloroplasts, photoelectron transport which produces oxygen from the oxidation of water and the Calvin cycle with its susceptible ribulose diphosphate and carboxylation enzyme are located in the same small space.

The arrangement of C-4 plants makes possible the avoidance of photorespiratory evolution of CO_2 by two mechanisms. One which has been widely recognized is that the CO_2 generated by metabolism of glycolate in the parenchyma cells must pass out past the mesophyll cells where it can be refixed by the C-4 pathway before leaving the leaf.

The other, and perhaps more important mechanism, may be the separation of the site of oxygen evolution from the site of RuDP carboxylase, leading to a reduced rate of RuDP oxidation to phosphoglycolic acid.

Metabolic Regulation of Carbon Metabolism

Since several intermediate compounds of the Calvin cycle are used as starting points for biosynthesis, some points of metabolic regulation of reactions of the cycle and of paths leading from it are required. The techniques of suddenly interrupting the steady-state photosynthesis and observing the transient effects on metabolite concentrations, mentioned earlier, have been very valuable in locating sites of metabolic regulation.[30] A few examples from many studies will illustrate the method.

When the light-dark steady-state experiment with algae is followed by turning the light on again, additional interesting transient changes are observed.[31] The level of fructose-1,6-diphosphate (FDP) very suddenly increases for about 30 sec when the light comes on, and then, just as suddenly, decreases (Fig. 4). After several oscillations the level settles down to the light steady-state level. We interpret the initial

rise as indication that the enzyme (FDPase) which converts FDP to F6P was inactive in the dark and remained inactive during the first 30 sec of light at a time when rapid reduction of PGA to triose phosphates (which condense to make FDP) took place. Then the FDPase becomes activated by whatever mechanism is operating in the light, and the FDP is for a time more rapidly hydrolyzed than formed. Finally, as the cycle reaches its full steady-state rate due to activation of the various control points, the steady-state levels of FDP and other intermediates are reached.

Similarly, light activation of the carboxylation enzyme (which converts RuDP and CO_2 to PGA) was observed, both with algae and with isolated spinach chloroplasts (Fig. 5). The activations of these enzymes and of phosphoribulokinase (which converts Ru5P with ATP to RuDP) are not direct photochemical activations, since reversible inactivations (Fig. 6) can be seen even with the light on when chemical inhibitors are added which reversibly inactivate photosynthesis.[32,33] Rather, it appears that the mechanism of activation of these regulated enzymes may involve the levels of Mg^{++} ion, pH, and the ratio of reduced to oxidized cofactors (such as $NADPH/NADP^{+}$).[30]

One clue to the purpose of these regulated enzymes was found in the appearance of 6-phosphogluconic acid in the dark (Fig. 7) and its disappearance in the light. This metabolite, which is a marker for the oxidative pentose phosphate cycle, can be made to appear in Chlorella even with the light on when vitamin K_5 is added to the photosynthesizing algae suspension.[33] Vitamin K_5 is readily oxidized by air and light. In its oxidized form, it apparently accepts electrons from the photochemical electron transport system of the chloroplasts, thereby diverting

the electrons from the reductive steps of the reductive pentose phosphate cycle. Since 6-phosphogluconic acid appears upon the addition of vitamin K_5, even with isolated chloroplasts,[33] it is clear that the oxidative pentose phosphate cycle can operate inside the chloroplasts. One purpose of the light activation of carboxylase, FDPase, and phosphoribulokinase and their inactivation in the dark would appear to be to permit the blocking in the dark of reactions unique to the reductive pentose phosphate cycle so that the oxidative pentose phosphate cycle can operate in the chloroplasts (Figure 2). This oxidative cycle would produce NADPH, which together with ATP from the mitochondria could then be used for biosynthesis in the dark inside the chloroplasts. As a corollary to this postulate, the oxidative pentose phosphate cycle must be prevented from operating in the light, presumably through the inactivation of glucose-6-phosphate dehydrogenase.

Studies with unicellular algae in which growth has been synchronized so that all cells in a given culture are growing or dividing at the same time, have indicated that these same regulatory sites may be involved in the allocation of carbon from the reductive pentose phosphate cycle to biosynthetic pathways leading to synthesis of carbohydrate, fat and protein.[34,35] As these algae pass from a stage of rapid growth to one of cell division, their needs for the synthesis of protein and fat as compared with the synthesis of carbohydrates apparently change.

Looking at the photosynthetic carbon reduction cycle (Figure 2), we can see that the compounds required for protein and fat synthesis are derived from PGA and triose phosphate, which lie after the carboxylation reaction and before the fructose diphosphatase reaction. On the

other hand, the compound required for carbohydrate synthesis, fructose-6-phosphate, lies after the FDPase reaction and before the carboxylase reaction. Since the carboxylase reaction and the FDPase reaction are the two rate-limiting steps in the Calvin Cycle, a small change in the ratio of the activities of these two enzymes can cause carbon to accumulate either in the half of the cycle between carboxylation and FDPase or in the other half of the cycle after FDPase and before carboxylation. Thus the regulatory mechanisms can accumulate reduced carbon at the point where it is needed for subsequent biosynthesis.

Additional regulatory steps are required on the pathways leading from the cycle to control more accurately the amount of carbon withdrawn for biosynthesis. The sites of two such regulatory points in Chlorella pyrenoidosa have been revealed by studies of the effects of adding ammonium ion.[36] As might be expected, the addition of ammonium ion in 1 mM concentrations to algae cells results in an increased synthesis of amino acids and proteins. When ammonium ion was added during photosynthesis by algae, several dramatic changes in the rates of flow of carbon compounds through the metabolic pathways were observed. First of all, sucrose synthesis almost completely stopped, and starch synthesis declined somewhat. At the same time, there was an increased rate of synthesis of fats and a greatly increased rate of synthesis of amino acids.

The main reason for the increased amino acid formation became apparent when we examined the changes in steady-state levels of phosphoenopyruvic acid and of pyruvic acid. The conversion of PEPA to pyruvate is the key reaction along the path leading from PGA to the synthesis of

amino acids and fatty acids. Immediately after the addition of ammonium ion the level of PEPA dropped, while the level of pyruvate rose (Fig. 8). From these changes in level, and from the known free energy change accompanying the conversion of PEPA to pyruvate, it is possible to calculate that the ratio of the forward over the back reaction changed by a factor of four.

We have not yet demonstrated similar regulatory sites in the leaves of higher plants, but from our past experience with regulation in the reductive pentose phosphate cycle, it is reasonable to expect that similar points of metabolic regulation will be found on the side paths leading to carbohydrate and to protein and fat synthesis. Pyruvate kinase, of course, is a well known site of metabolic regulation in many other types of living cells.

Since we have been able to alter the amount of regulation in algae with small amounts of chemicals, eventually it may become possible to spray the leaves of plants with a suitable but non-toxic chemical, and induce an increased rate of protein synthesis. The extraction of soluble protein from green plant leaves as a nutritional supplement may become an economic possibility. Thus, any increase in protein synthesis in green leaves could be a useful application of fundamental knowledge about metabolic regulation in green cells. Aside from such speculative possibilities, increased knowledge about photosynthetic pathways of carbon metabolism and their regulation seems likely to be useful both to a basic understanding of cellular metabolic dynamics and to determining mechanisms whereby plants respond to natural and unnatural environmental stimuli.

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Table I. Effect of Oxygen on Photosynthesis with $^{14}CO_2$

	(μc ^{14}C/gm algae)		
	O_2	CO_2-free air	N_2
Total ^{14}C fixed	508.0	642.7	732.9
Glycolic acid	48.2	9.2	2.0
Phosphoglycolic acid	3.4	2.1	1.7
Ribulose diphosphate	58.4	91.0	87.6
Other sugar diphosphates	0.3	0.5	1.3
Phosphoglyceric acid	20.1	25.3	40.6

FIGURE CAPTIONS

Figure 1. Light-dark and High CO_2-low CO_2 Transient Changes in Levels of ^{14}C-Labeled Photosynthetic Intermediates.

Idealized curves based on studies by Calvin and Massini[10] and Wilson and Calvin.[11]

RuDP, ribulose-1,5-diphosphate; PGA, 3-phosphoglyceric acid.

Figure 2. The Reductive Pentose Phosphate Cycle (Calvin Cycle) of Photosynthesis.

Solid lines indicate reactions of reductive cycle, dashed lines reactions of oxidative cycle. Open arrows indicate positions of enzymes activated in light, dark; blunt arrow indicates position of enzyme activated in dark.

GAld3P, glyceraldehyde-3-phosphate; FDP, fructose-1,6-diphosphate; F6P, fructose-6-phosphate; DHAP, dihydroxyacetone phosphate; E4P, erythrose-4-phosphate; SDP, sedoheptulose-1,7-diphosphate; S7P, sedoheptulose-7-phosphate; R5P, ribose-5-phosphate; Xu5P, xylulose-5-phosphate; Ru5P, ribulose-5-phosphate; RuDP, ribulose-1,5-diphosphate; G6P, glucose-6-phosphate; 6-PGluA, 6-phosphogluconate; $*C_2$ is actually enzyme-bound thiamine pyrophosphate-glycolaldehyde, an intermediate in the two reactions mediated by transketolase.

Figure 3. The C-4 Acid Cycle of Photosynthetic Carbon Fixation.

Figure 4. Transient Changes in Levels of FDP and DHAP during Light-dark and Dark-light Transitions.

FIGURE CAPTIONS (Cont.)

Figure 5. Transient Changes in Levels of RuDP during Light-dark-light Transition in Spinach Chloroplasts.

Figure 6. Transient Changes in Levels of FDP and SDP with Octanoic Acid. Addition and Removal.

Inhibition of photosynthesis by Chlorella pyrenoidosa was caused by addition of octanoic acid at pH 5 and was relieved by raising the pH of the medium to 7.

Figure 7. Changes in Level of 6-Phosphogluconate in Light and Dark.

Figure 8. Changes in Levels of PEPA and of Pyruvate in Photosynthesizing Chlorella pyrenoidosa with Added NH_4.

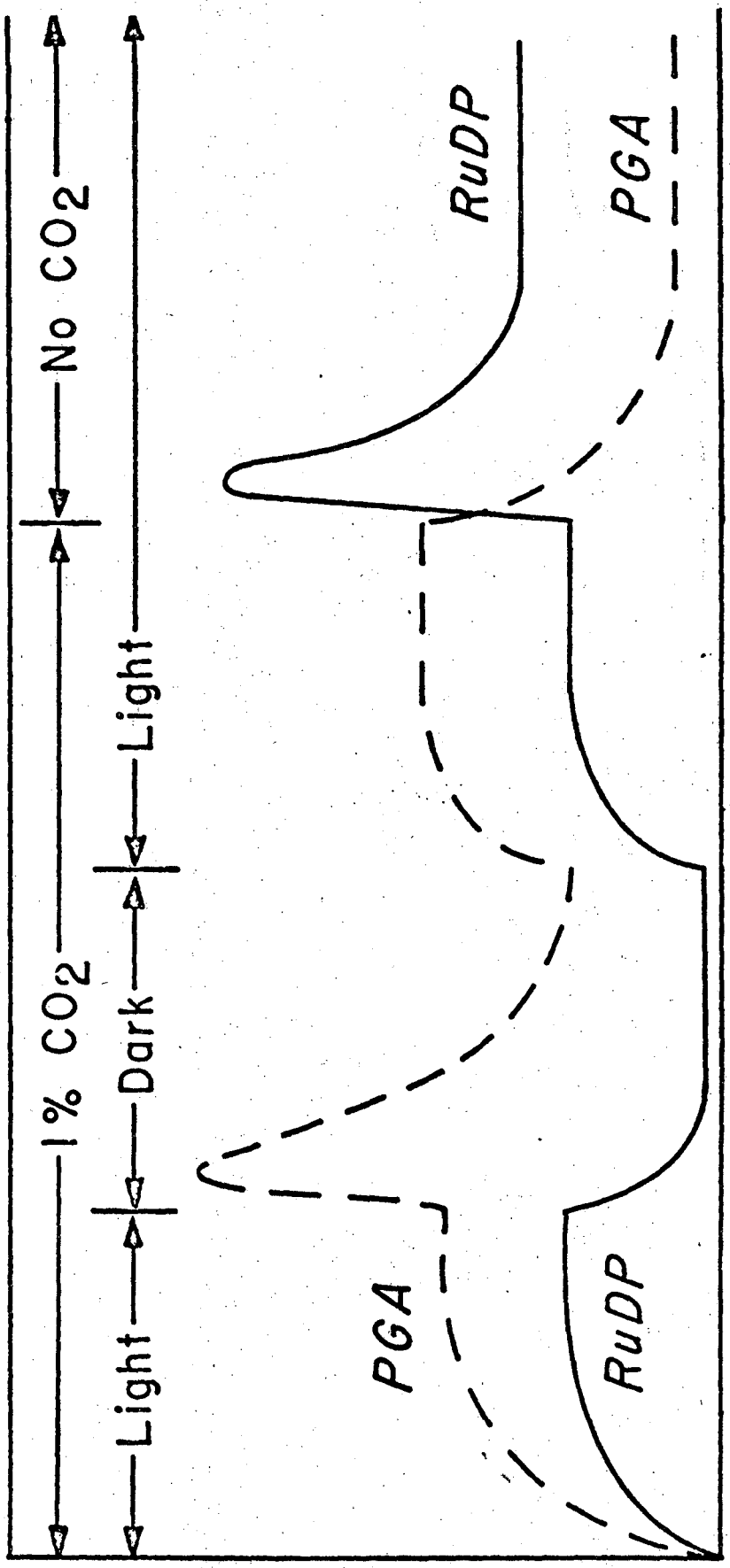

Fig. 1

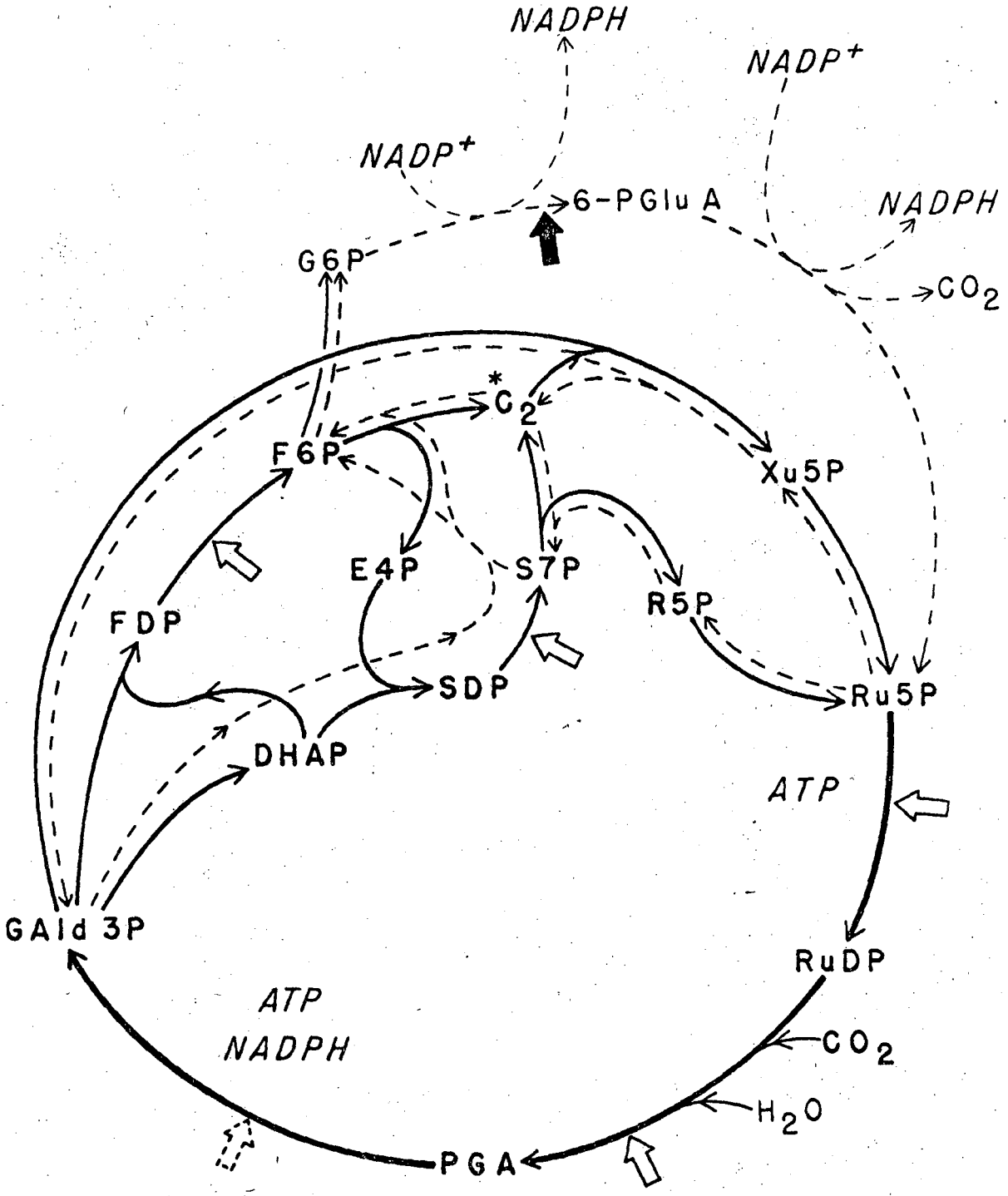

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Fig. 2

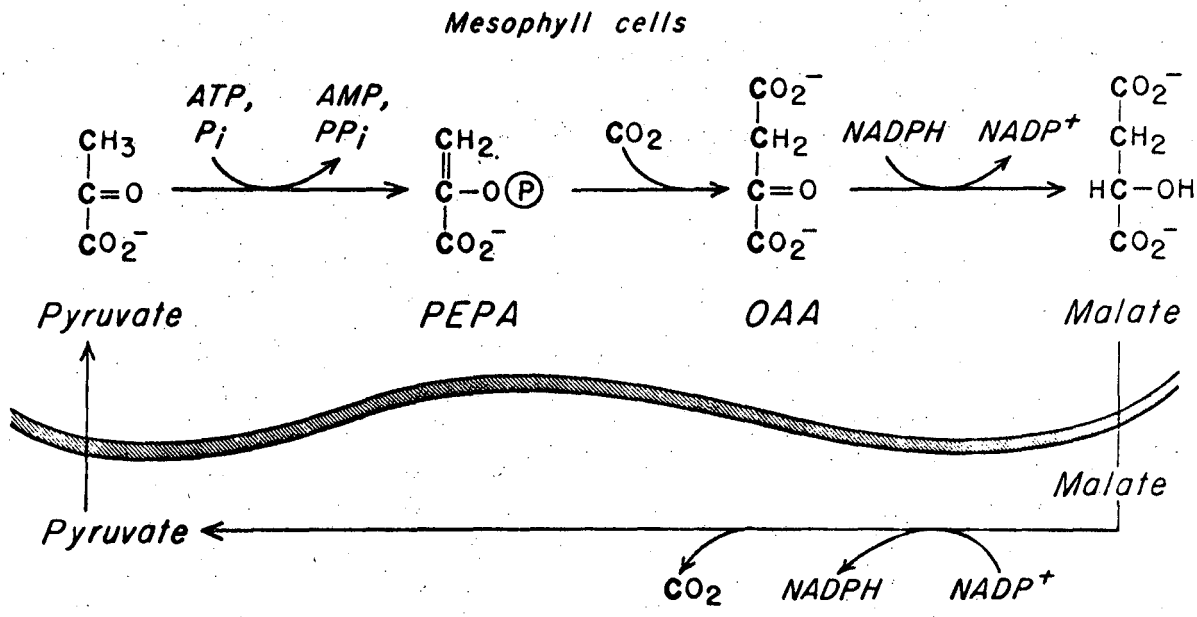
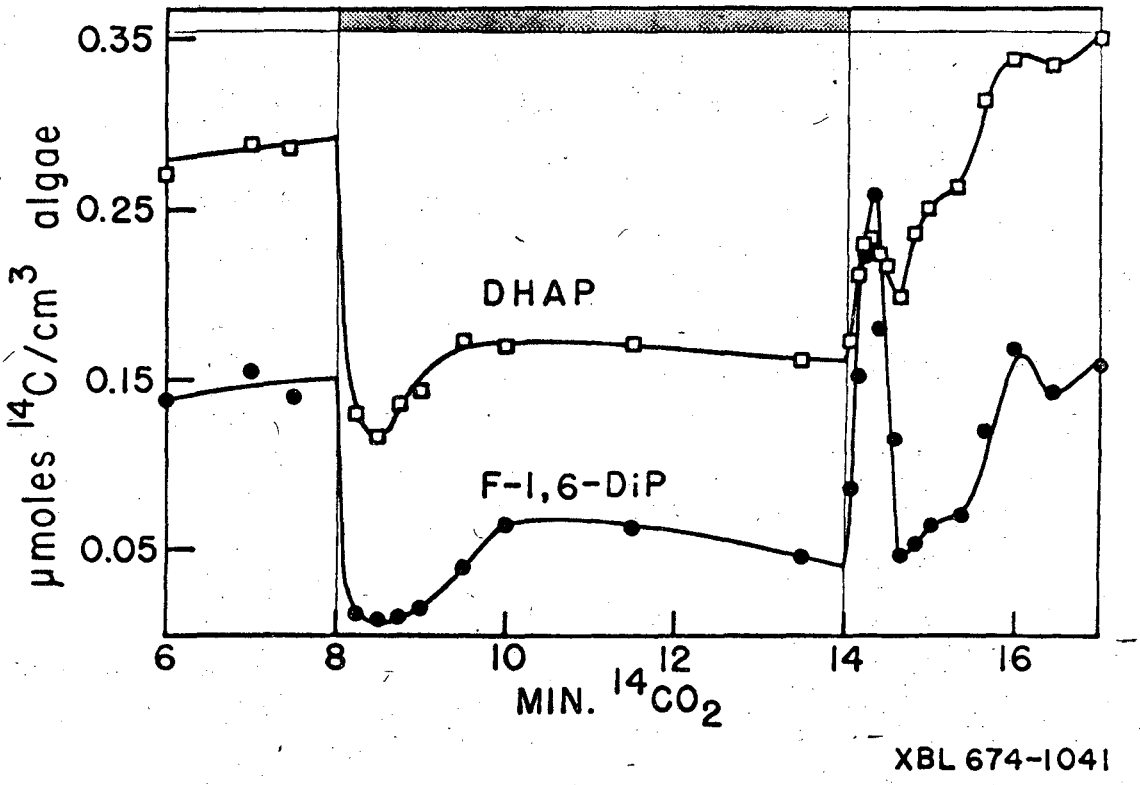

Mesophyll cells
ATP, Pi
AMP, PPi
CO2
NADPH
NADP+
Pyruvate
PEPA
OAA
Malate
Malate
Pyruvate
CO2
NADPH
NADP+
to Calvin Cycle
Parenchyma (vascular bundle) cells
XBL714-5113

Fig. 3


0.35
0.25
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μmoles $^{14}C/cm^3$ algae
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F-1,6-DiP
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8
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16
MIN. $^{14}CO_2$
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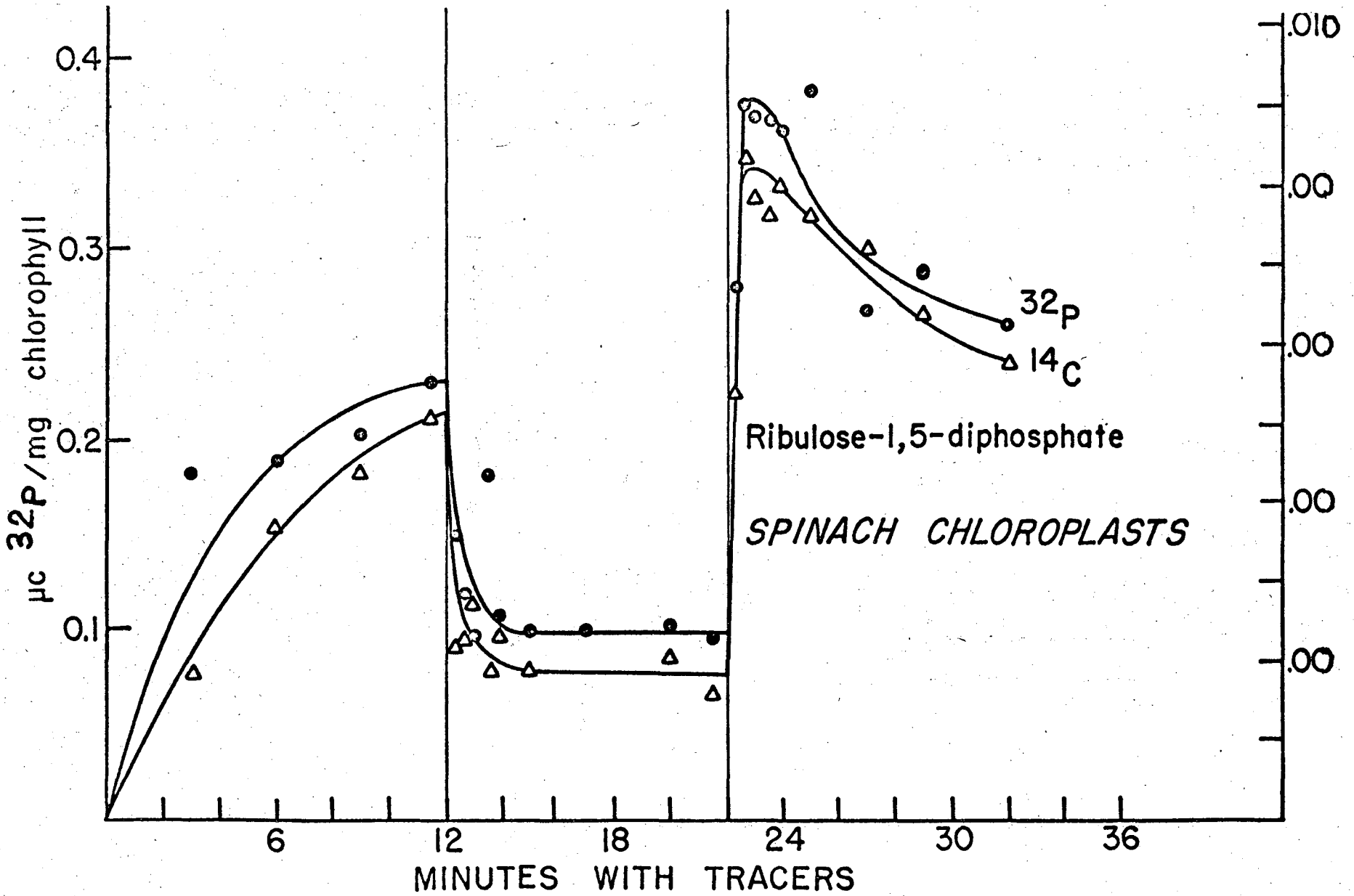
Fig. 4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6
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MINUTES WITH TRACERS

Fig. 5

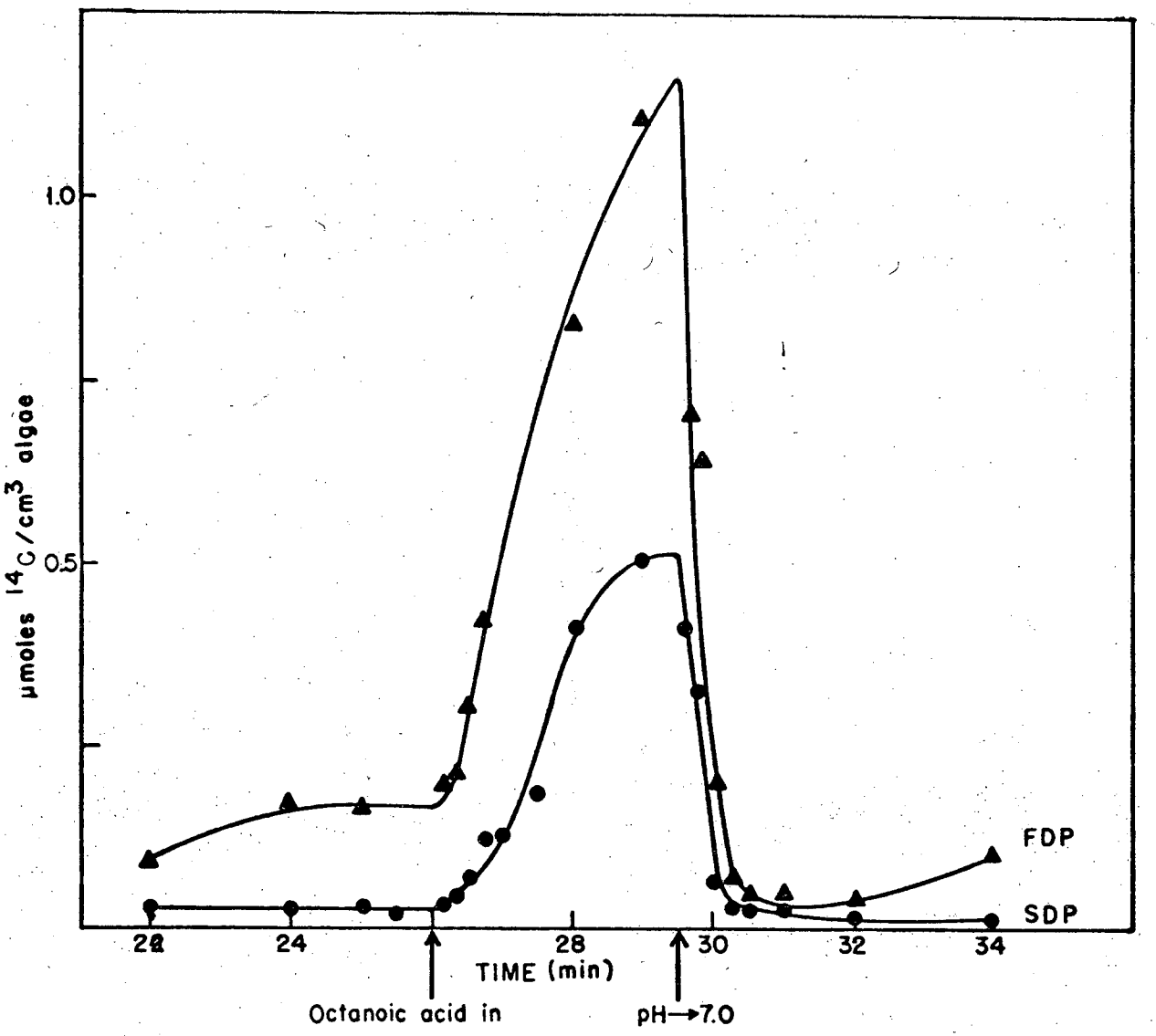

Fig. 6

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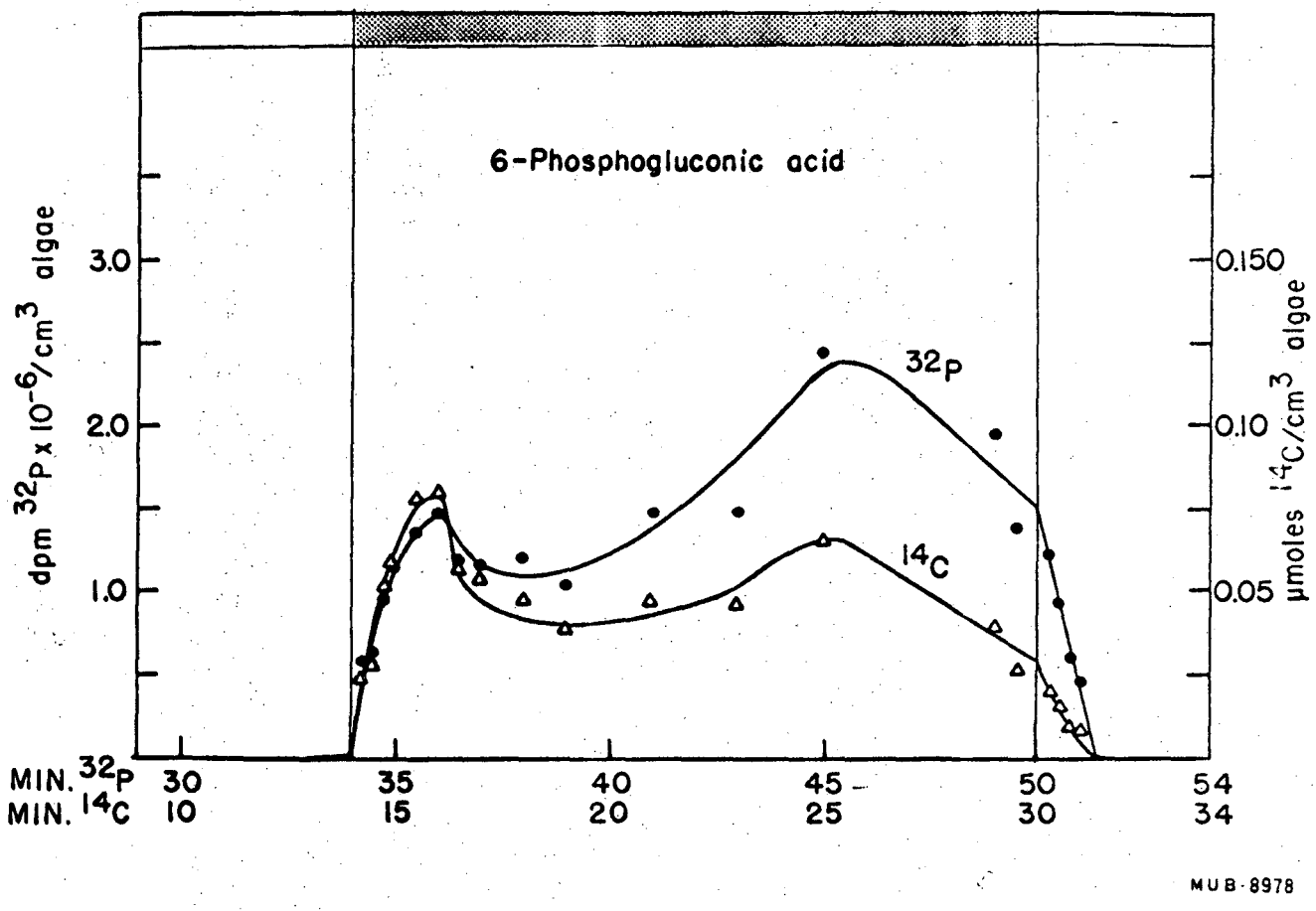
6-Phosphogluconic acid
dpm ^{32}P x 10^{-6}/cm^3 algae
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2.0
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^{32}P
^{14}C
0.150
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0.05
µmoles ^{14}C/cm^3 algae
MIN. ^{32}P 30 35 40 45 50 54
MIN. ^{14}C 10 15 20 25 30 34
MUB-8978

Fig. 7

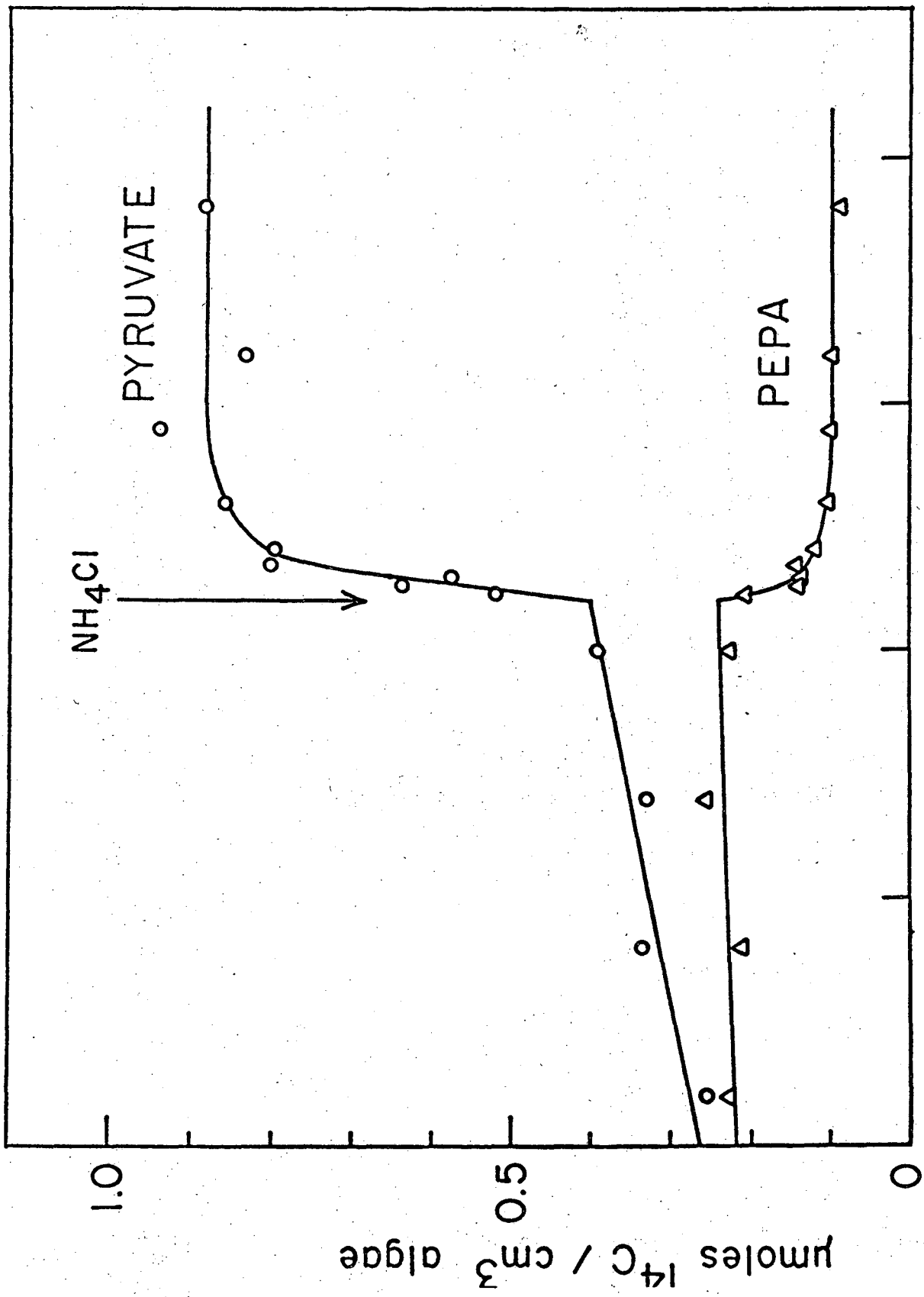
μmoles ^{14}C / cm^3 algae
1.0
0.5
0
10
15
20
25
30
MIN. $^{14}CO_2$
NH_4Cl
PYRUVATE
PEPA

Fig. 8

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