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PHOTOSYNTHESIS BY ISOLATED CHLOROPLASTS.
DIFFUSION OF LABELED PHOTOSYNTHETIC INTERMEDIATES
BETWEEN ISOLATED CHLOROPLASTS AND SUSPENDING MEDIUM

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ABSTRACT

The diffusion of ^{14}C and ^{32}P-labeled photosynthetic intermediate compounds from isolated chloroplasts has been investigated. Those intermediate compounds of the photosynthetic carbon reduction cycle lying between the carboxylation reaction and the diphosphatase reaction were found to diffuse rapidly from the chloroplast to the suspending medium. In contrast, those intermediates lying between the diphosphatase reaction and the carboxylation reaction, with the exception of the pentose monophosphates, tend to be retained by the chloroplasts during the 15 minutes or more when the photosynthesis of carbon dioxide by the isolated chloroplasts is most active. An experiment in which changes in the level of ATP were observed on addition of carbon dioxide shows that ATP and ADP, which had diffused from the chloroplasts, can apparently re-enter the chloroplasts and be used metabolically. It is proposed that the diphosphatase reaction plays a general role in metabolic regulation in biosynthesis as well as in the light-dark transition.

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In a previous report[1] we described conditions for obtaining high rates of photosynthesis with isolated chloroplasts. It was apparent in these studies that the labeling of certain intermediates of the carbon reduction cycle, particularly 3-phosphoglyceric acid , dihydroxyacetone phosphate, fructose-1,6-diphosphate (Fru-1,6-P_2), sedoheptulose-1,7-diphosphate (Sed-1,7-P_2), and ribose-5-phosphate (Rib-5-P), were unusually heavily labeled with ^{14}C following photosynthesis by the isolated chloroplasts in the presence of $^{14}CO_2$, as compared with the amount of labeling seen with whole spinach leaves photosynthesizing in the presence of $^{14}CO_2$.

On the basis of data obtained from labeled compounds in "chloroplasts" isolated by the non-aqueous technique from Elodia after in vivo photosynthesis with $^{14}CO_2$, Heber[2] concluded that 3-phosphoglyceric acid and either dihydroxyacetone phosphate or Fru-1,6-P_2, or both, function as transport metabolites during photosynthesis. Also, ATP and ADP were reported to be rapidly translocated between chloroplasts and cytoplasm of the leaf cell.[3] The non-aqueous isolation technique produces particles which have lost the integrity of the outer membrane and have apparently lost some components since they no longer photosynthesize. Also, the cytoplasmic fraction includes considerable material from the chloroplasts. Hence certain assumptions must be made in arriving at the conclusions about metabolite distribution. For example, the material lost from the chloroplasts may not be qualitatively the same as that retained.

Until the achievement of high rates of photosynthesis with isolated chloroplasts with intact membranes,[1] it has not been possible to carry out satisfactory studies of metabolite transport from chloroplasts in vitro. Our preliminary studies[4] of the distribution of photosynthetic intermediate compounds between the chloroplasts themselves and the medium in which they are suspended showed that several intermediate compounds rapidly diffuse or are

transported from the chloroplasts to the suspending medium. In contrast, the hexose and heptose monophosphates are very well retained in the chloroplasts during the first few minutes of photosynthesis when the rate of photosynthesis is most active. It was also found that glycolic acid is rapidly transported from the chloroplast to the suspending medium. These studies have been extended in the present report in order to obtain a picture of the distribution of the individual compounds between the chloroplasts and the suspending medium as a function of time of photosynthesis.

In another experiment, ^{32}P has been introduced in order to ascertain the distribution of ATP between the isolated chloroplasts and their medium. In this experiment, the chloroplasts were first deprived of CO_2 in order to build up a high level of labeled ATP, and then carbon dioxide was added to see what effect this would have on the level of ATP, both within and outside the chloroplasts.

EXPERIMENTAL

Chloroplasts were isolated from freshly grown spinach according to the methods described earlier.[1] The leaves used for the experiments were four to six weeks old. The chloroplasts were suspended in the usual assay medium.[1]

The total volume of the suspension in the first experiment was 1.5 ml containing chloroplasts with 0.213 mg chlorophyll. The flasks containing the chloroplasts were mounted on a shaking frame which imparted a swirling motion to the chloroplast suspension,[1] and were first pre-illuminated for three minutes without added CO_2 or bicarbonate. $NaH^{14}CO_3$ solution was then added to make the solution 0.01 M in bicarbonate ion with a specific activity of 35.2 μC/μM. From time to time, 100 μl samples were withdrawn from the flask and subjected to centrifugation with a Spinco microfuge to separate the chloroplasts as a

pellet from the suspending medium. Pellet and supernatant solutions were separated and killed with 80% methanol as quickly as possible. The entire operation, withdrawing the sample, centrifuging, and killing, required 30 seconds.

We recognized that this period of time is long enough for metabolic changes characteristic of light-dark transients to take place. Therefore, additional unseparated 50 μl samples of chloroplasts were taken and killed directly. The labeling of a compound isolated from these unseparated samples can be compared with the sum of the labeling of the same compound from separated chloroplasts and medium. This comparison gives a measure of the change which occurred during the separation of chloroplasts from medium.

The mixtures of methanol, water, and chloroplasts or suspending medium were analyzed by two-dimensional paper chromatography and radioautography as described earlier.[5] The amount of ^{14}C in each compound in each sample in the pellet and in the supernatant solution was determined as a function of time of sampling.

In a second experiment, chloroplasts were isolated and preilluminated as just described. However, ^{32}P-labeled phosphate was added at the beginning of the pre-illumination period, and ^{14}C-labeled bicarbonate was added after 4 minutes of pre-illumination with the radiophosphorus. One sample was taken just before the addition of $^{14}CO_2$, and other samples were taken periodically after the addition of bicarbonate.

RESULTS

The labeling with ^{14}C of intermediates of the carbon reduction cycle of photosynthesis and of glycolic acid in pellet and supernatant solutions in the first experiment are shown in Figures 1 through 9. In each case the ^{14}C-labeling of the compound found in the supernatant solution is indicated by open circles and the ^{14}C-labeling of the compound found in the pellet is indicated by black dots. The ^{14}C-labeling of the compound found in the samples where the chloroplasts were not separated from the supernatant solution prior to killing are indicated by the squares.

In most cases the labeling of a given compound found in the sample which was not separated is roughly the sum of the labeling found in the pellet and supernatant curves. However, the Sed-1,7-P_2 and Fru-1,6-P_2 labeling was higher in the samples which were not separated than in the sum of the pellet and supernatant. At the same time, the labeling of dihydroxyacetone phosphate was lower in the unseparated sample than the sum of its labeling in pellet and supernatant. Thus it appears that some Sed-1,7-P_2 and some Fru-1,6-P_2 were converted to dihydroxyacetone phosphate during the centrifugation. The level of ribulose-1,5-diphosphate (Ru-1,5-P_2) also apparently dropped during the centrifugation, presumably due to the continued carboxylation reaction. These changes, however, do not affect greatly the general conclusions which can be drawn from the pellet-supernatant curves.

As noted in earlier studies,[4] several intermediates of the carbon cycle and the glycolic acid are found very predominantly in the supernatant solution, indicating that they are not well retained in the pellet. Most marked among these compounds are 3-phosphoglyceric acid (Fig. 1), dihydroxyacetone phosphate (Fig. 2), and Fru-1,6-P_2 (Fig. 3). Also the total pentose monophosphates (Fig. 4), Sed-1,7-P_2 (Fig. 5), and glycolic acid (Fig. 9) are found predominantly in the supernatant solution.

In contrast to the compounds just mentioned, the ^{14}C-labeled fructose-6-phosphate (Fru-6-P) (Fig. 6) and sedoheptulose-7-phosphate (Sed-7-P) (Fig. 7) are found predominantly in the pellet. Ru-1,5-P_2 (Fig. 8) is about equally distributed between pellet and supernatant solution. In other earlier experiments the Ru-1,5-P_2 was found predominantly in the pellets. In this experiment, as already mentioned, the level of Ru-1,5-P_2 was much higher in the unseparated samples, and it is reasonable to suppose that most of this extra Ru-1,5-P_2 which was converted during the centrifugation was in the pellet.

In order to obtain a better idea of the rate of formation of labeled compounds, we took the slope of the various labeling curves for pellet and supernatant and unseparated samples at 3 minutes after addition of ^{14}C and during the period between 5 and 10 minutes after addition of ^{14}C. These rates are listed in Table I.

The results of another experiment, in which both ^{32}P and ^{14}C were used, are shown in Figures 10 and 11. Again, open circles represent labeling of compounds in the solution and black dots represent labeling of compounds found in the pellet. It is apparent that both ATP (Fig. 10) and ADP (Fig. 11) rapidly diffuse between the pellet and the supernatant solution. When $H^{14}CO_3$ is added to preincubated chloroplasts, which were deprived of bicarbonate, the level of ATP drops precipitously in both chloroplasts and supernatant solution.

DISCUSSION

From the distribution of labeled compounds between chloroplasts and the suspending medium, shown in Figures 1 through 9, a general pattern emerges. Compounds lying between the carboxylation reaction of photosynthesis and the diphosphatase reaction are readily released from the chloroplast into the supernatant solution. Compounds which lie between the diphosphatase reaction and

the carboxylation reaction seem to be much better retained in the chloroplasts, except for the pentose monophosphates.

One possible explanation of the observed differential movement of photosynthetic intermediate compounds from isolated spinach chloroplasts to the medium may be found in the control of the flow of carbon from the cycle into biosynthetic pathways. The absence of formation of significant amounts of labeled amino acids and fatty acids or fats in studies so far with isolated chloroplasts photosynthesizing with $^{14}CO_2$[1] suggests that some of the conversion of intermediates of the carbon reduction cycle to other metabolic intermediates required for synthesis of macromolecules in the chloroplasts may occur outside the chloroplasts. Thus the flow of certain intermediate compounds of the photosynthetic carbon reduction cycle from the isolated chloroplasts to the medium may reflect, possibly in an exaggerated way, a normal in vivo flow of these same intermediate compounds to the cytoplasm for subsequent steps in biosynthesis.

Fru-6-P is the biosynthetic starting point for the synthesis of most carbohydrates in the chloroplasts. Fru-1,6-P_2, as well as dihydroxyacetone phosphate, and 3-phosphoglyceric acid, could supply carbon for glycolysis and for the biosynthesis of amino acids and proteins, fatty acids, glycerol phosphate and fats, and various other products required for cell growth and division (see Fig. 12).

In vivo studies with Chlorella pyrenoidosa of the kinetics of the transient changes in levels of intermediate compounds of photosynthesis from light to dark to light have led to the following conclusions:[6] 1) There is a rapid interaction of intermediate compounds of the photosynthetic carbon reduction cycle with intermediate compounds of glycolysis. 2) There is a light activation of the hexose diphosphatase enzyme responsible for the removal of the phosphate group on the number one carbon atom of Fru-1,6-P_2 and Sed-1,7-P_2 (D-fructose-1,6-diphosphate-1-phosphohydrolase, 3.1.3.11), with this enzyme

becoming inactive in the dark. 3) The carboxylation enzyme (3-phospho-D-glycerate carboxy-lyase, dimerizing, 4.1.1.39) also is light activated, and becomes less active in the dark.

Besides the "on-off" regulation of these enzymes between light and dark, there may be a finer regulation of the diphosphatase reaction which would control the flow of carbon in the light from the photosynthetic carbon reduction cycle to the biosynthesis of end products. An additional possibility is that neither carboxylation reaction nor the diphosphatase reaction are fully light activated in the photosynthesizing isolated chloroplasts. If so, either, or both, of these key reactions could become rate-limiting in the operation of the photosynthetic carbon reduction cycle.

In vivo studies of photosynthesis inhibited by octanoate[5] showed that the light-dark regulation could be mimicked by a chemical that interferes reversibly with photophosphorylation, the diphosphatase reaction, and the carboxylation reaction.

A limited rate for the diphosphatase step would be expected to cause a slight accumulation of the intermediate compounds just preceding that step. These are Fru-1,6-P_2, Sed-1,7-P_2, dihydroxyacetone phosphate, and 3-phosphoglyceraldehyde. If these compounds are not retained in high concentrations within the chloroplasts, they would diffuse from the chloroplasts to the medium.

Using the ratio of appearance of labeled compound in supernatant solution to appearance of labeled compound in the chloroplasts, S/P, as an indicator of the degree of differential "export", we see from Table I that this ratio is by far the largest for Fru-1,6-P_2 (35), dihydroxyacetone phosphate (42.5), and 3-phosphoglyceraldehyde. However, the value of S/P for Sed-1,7-P_2 is only 2.0. This could be a consequence of the fact that Sed-1,7-P_2 lies between two reactions catalyzed by hexose diphosphatase. (See scheme in Fig. 12.)

Although it has just been suggested that a rate-limiting hexose diphosphatase reaction may increase the differential movement of some of the photosynthetic intermediate compounds out of the chloroplasts, it is clear that the rate of movement of a given compound from the chloroplast to the medium is not simply a function of its concentration in the pellet. For example, at 3 minutes of photosynthesis, the amount of labeled Fru-6-P in the pellet was 0.05 μmoles ^{14}C, while that of Fru-1,6-P_2 was 0.02 μmoles ^{14}C. The rate of appearance of labeled Fru-1,6-P_2 in the medium was 30 times that of Fru-6-P.

This retention of Fru-6-P, and export of Fru-1,6-P_2 seen with isolated chloroplasts may be similar to, or an exaggeration of, a differential retention and export from chloroplasts to cytoplasm *in vivo*. According to this hypothesis, hexose diphosphatase, *in vivo*, could thus play an important role in the regulation of the flow of carbon from the photosynthetic carbon reduction cycle to biosynthesis of protein and fat, as compared with biosynthesis of carbohydrate.

If utilization in biosynthetic pathways outside the chloroplast is a factor in whether or not any given photosynthetic carbon cycle intermediate compound is readily released from the chloroplasts, then the release of pentose monophosphates may be explainable in terms of their role in ribonucleotide synthesis.

The possibility that ATP can flow in and out of chloroplasts was suggested by *in vivo* studies of the light-dark and dark-light transitions.[6] It was found that intermediate compounds of photosynthesis and glycolysis interact, and that the level of ATP is maintained in the dark at a level comparable to that maintained by photosynthesis in the light. The present study shows that ATP and ADP can flow in and out of the isolated chloroplasts. This finding is significant in connection with the conclusions from *in vivo* experiments[6] that the carboxylation reaction is activated only in the light. According to the formulation of the photosynthetic carbon reduction cycle[7] (see Fig. 12), only ATP is required to convert other sugar phosphates to Ribul-1,5-P_2, the substrate for

the carboxylation reaction. Addition of ATP to chloroplasts which already have a supply of other sugar phosphates should lead to carboxylation in the dark, unless the light-activation is required for isolated chloroplasts. When this experiment was performed, it was found that no CO_2 uptake occurred upon addition of ATP to the isolated chloroplasts, when other conditions were as described in this paper.*

In summary, selected intermediate compounds of the photosynthetic carbon reduction cycle, as well as ATP, are rapidly released from photosynthesizing isolated chloroplasts to the suspending medium. ATP, at least, can also rapidly re-enter the chloroplasts. These results are in general agreement with those reported earlier[2,3] from studies with "non-aqueous" isolated fractions from leaves which had already photosynthesized with $^{14}CO_2$. It is proposed that the rapid flow of certain compounds from the chloroplasts is an indication of the role of these compounds in biosynthetic pathways, which have some steps taking place in the cytoplasm *in vivo*. It is further suggested that the diphosphatase reaction, which converts Fru-1,6-P_2 to Fru-6-P, is mediated by an enzyme which is regulated to provide a control of the flow of carbon to protein and fat synthesis *vis-a-vis* carbohydrate synthesis. This regulation would be in addition to the previously proposed light-dark regulation.[6]

ACKNOWLEDGEMENT

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*Detailed results of this experiment will be published in a separate paper.

Table I

Photosynthesis Rates of Product Formation

Compound	μmoles $^{14}C \cdot (mg\ Chl \cdot hr)^{-1}$						
	3 min after ^{14}C added				7 min after ^{14}C added		
	P Pellet	S Super-natant	S/P	Not separated	P Pellet	S Super-natant	Not separated
3-phosphoglyceric acid	8.3	39.6	4.8	51.0	3.30	54.8	51.8
Total pentose monophosphate	0.21	1.79	8.5	1.70	0.20	2.94	2.70
Dihydroxyacetone phosphate	0.56	23.8	42.5	21.10	0.20	14.4	10.4
Glyceraldehyde P	--	1.69	large	1.11	--	1.27	.86
Ribul-1,5-P_2	0.07	0.03	0.4	0.45	0.07	0.09	0.17
Glycolic acid	0.71	3.94	5.5	4.65	0.06	4.62	4.72
Sed-1,7-P_2	0.06	0.12	2.0	1.06	0.06	0.15	0.64
Fru-1,6-P_2	0.19	6.66	35.0	3.90	0.10	7.92	6.92
Fru-6-P	0.38	0.17	0.4	2.94	0.73	0.27	1.32
Sed-7-P	4.30	0.27	0.06	4.80	4.33	1.02	4.10
Glucose monophosphate	1.60	0.21	0.13	2.04	1.98	1.00	3.46
Other	0.19	--	--	--	0.05	--	--
Total:	16.57	78.28		94.84	11.12	88.50	87.13
Total: Pellet + Supernatant	94.85				99.62		

The figures given for P and S are the slopes of the curves of ^{14}C label vs time for each compound, for pellet and supernatant solution respectively, when the data from which Figures 1 through 9 were derived were plotted on an expanded scale. The ratio S/P gives the relative rate of appearance of ^{14}C labeled compound in supernatant solution as compared with pellet.

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FIGURE CAPTIONS

Figs. 1-9. Distribution of ^{14}C-labeled compounds between chloroplast pellet and supernatant solution during photosynthesis with isolated chloroplasts in the presence of ^{14}C-labeled bicarbonate. Open circles denote ^{14}C-labeling of compound in supernatant solution, closed circles denote ^{14}C-labeling of compound found in the pellet, and open squares denote ^{14}C-labeling of compound in the samples that were not subjected to centrifugation. The identity of the specific compound is indicated in the figure.

Fig. 10. Distribution of ^{32}P-labeling of ATP between pellet and supernatant solution during photosynthesis with isolated chloroplasts. Open circles denote ^{32}P-labeling of compound in supernatant solution, and closed circles denote ^{32}P-labeling of compound in the pellet.

Fig. 11. Distribution of ^{32}P-labeling of ADP between pellet and supernatant solution during photosynthesis with isolated chloroplasts. Open circles denote ^{32}P-labeling of compound in supernatant solution, and closed circles denote ^{32}P-labeling of compound in the pellet.

Fig. 12. Photosynthetic carbon reduction cycle and formation of fats, proteins and carbohydrates in photosynthesis. The sites of light-dark regulation[6] are shown by the parallel lines. Abbreviations: DPT, diphosphothiamine; Fru-6-P, fructose-6-phosphate; Fru-1,6-P_2, fructose-1,6-diphosphate; P, phosphate; P_2-ase, hexose diphosphatase; Rib-5-P, ribose-5-phosphate; Ribul-1,5-P_2, ribulose-1,5-diphosphate; Sed-7-P, sedoheptulose-7-phosphate; Sed-1,7-P_2, sedoheptulose-1,7-diphosphate.

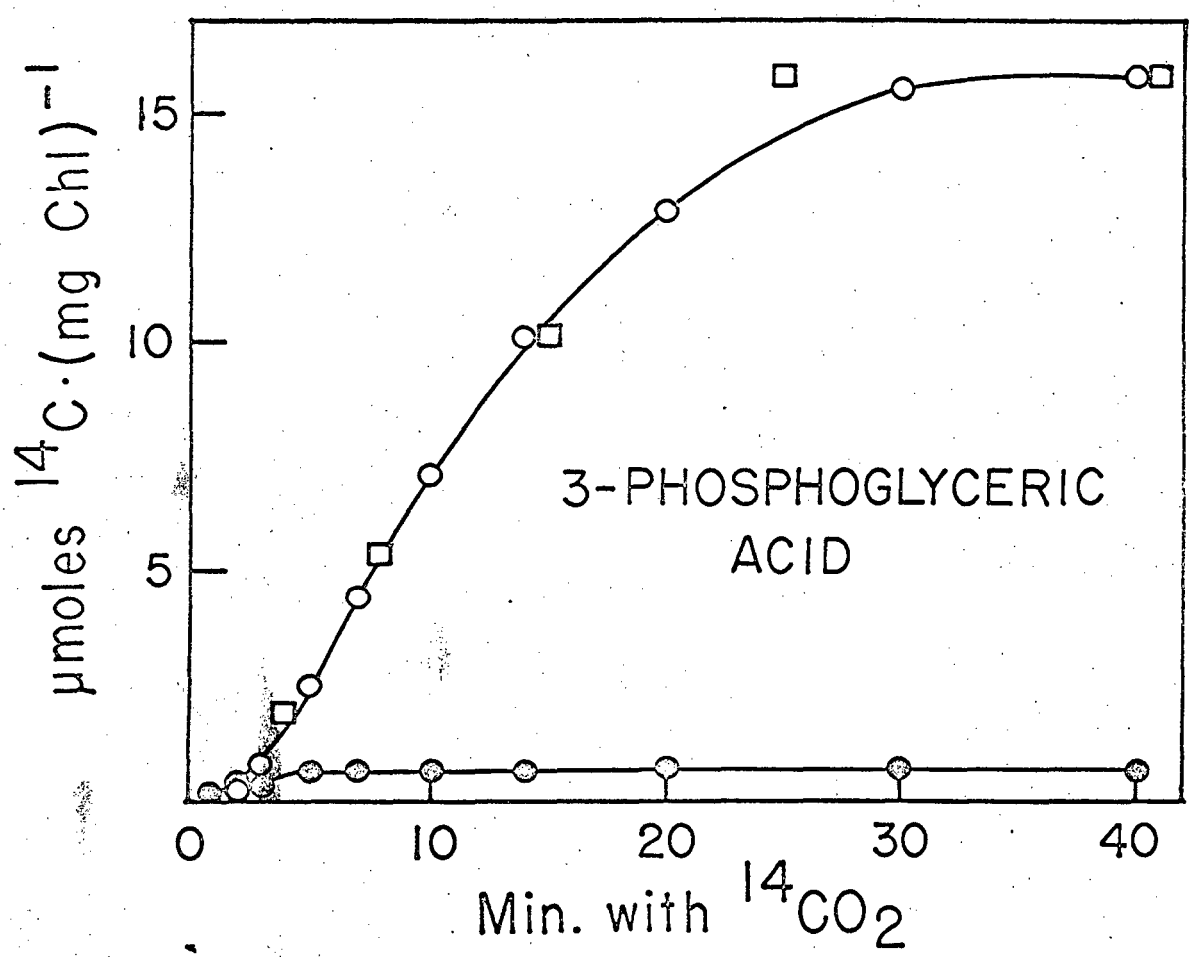

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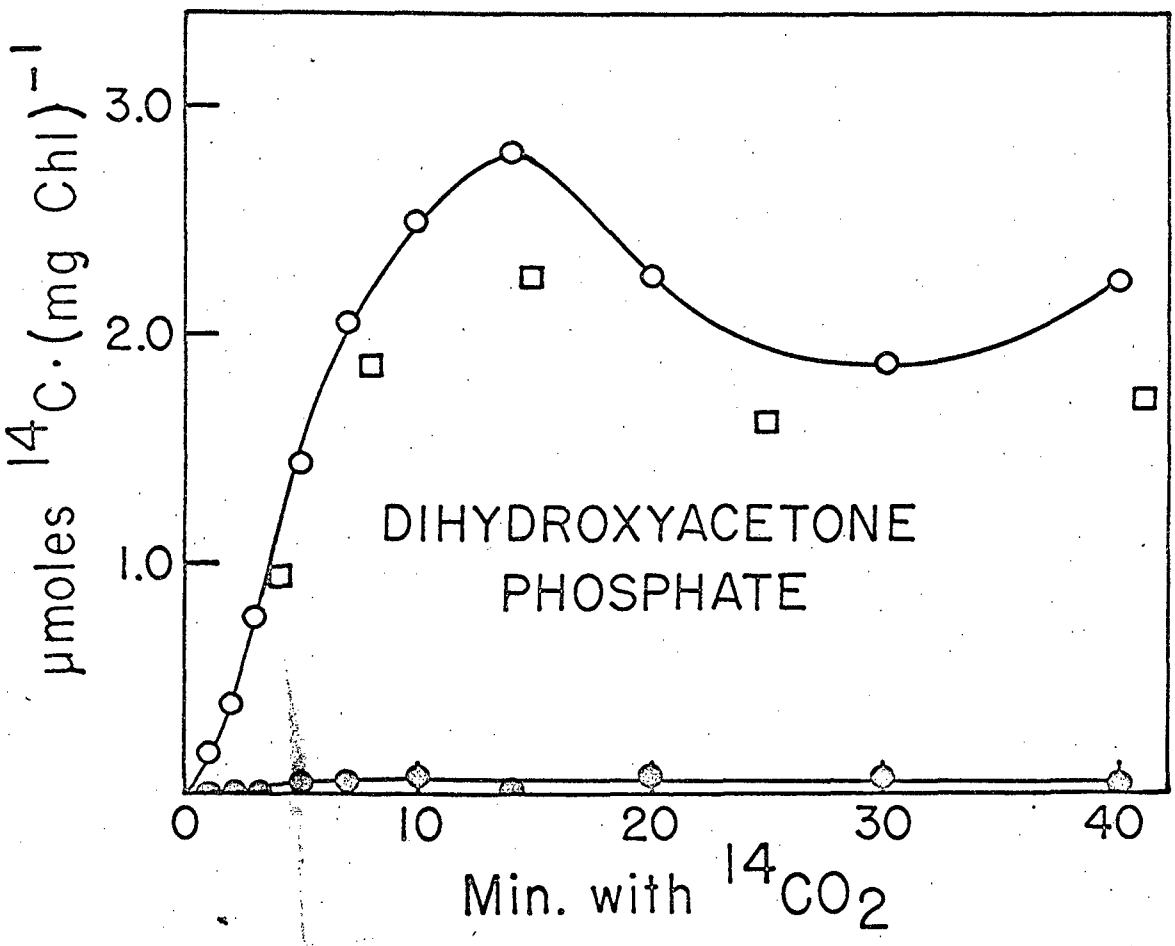
Fig. 1



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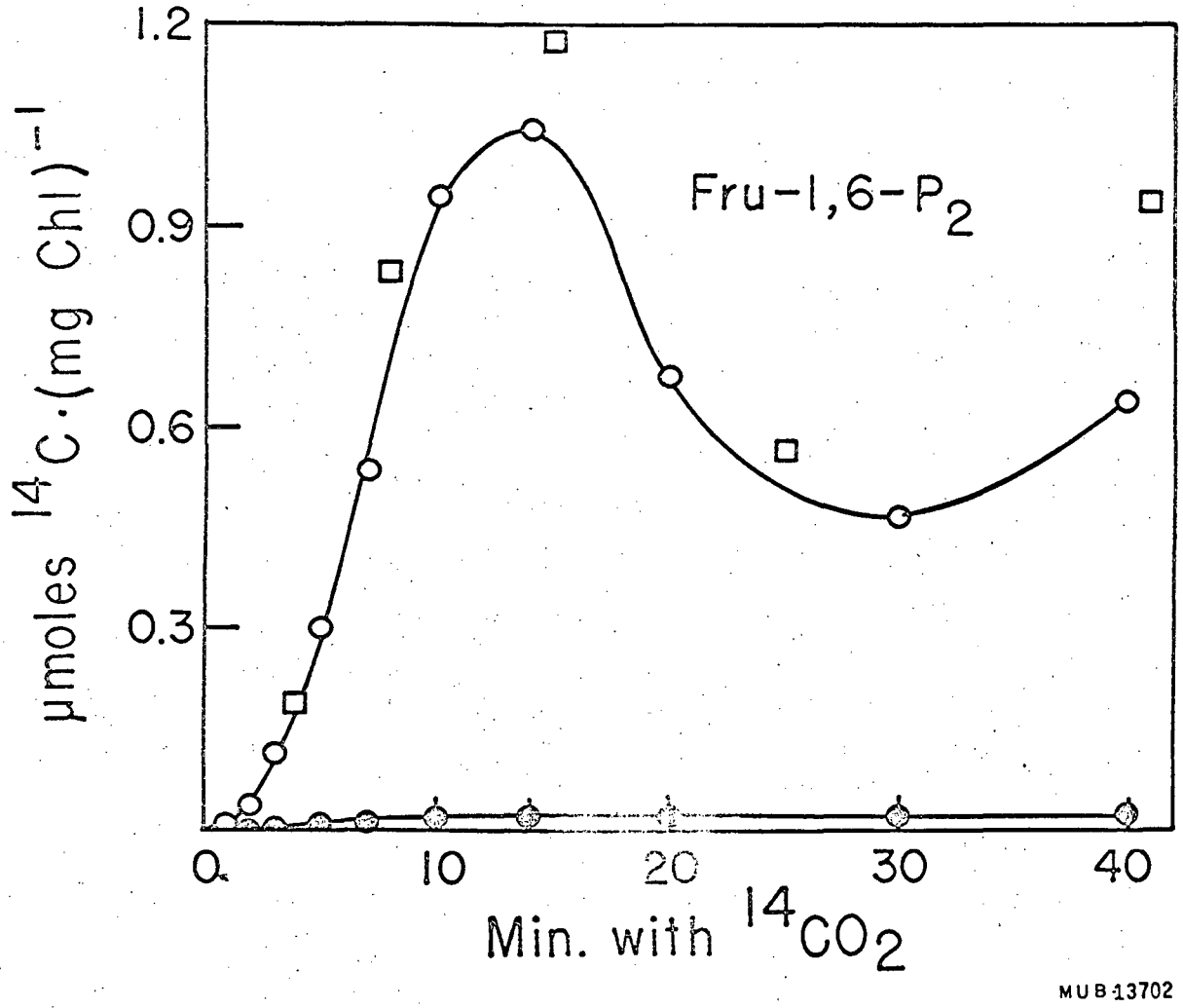

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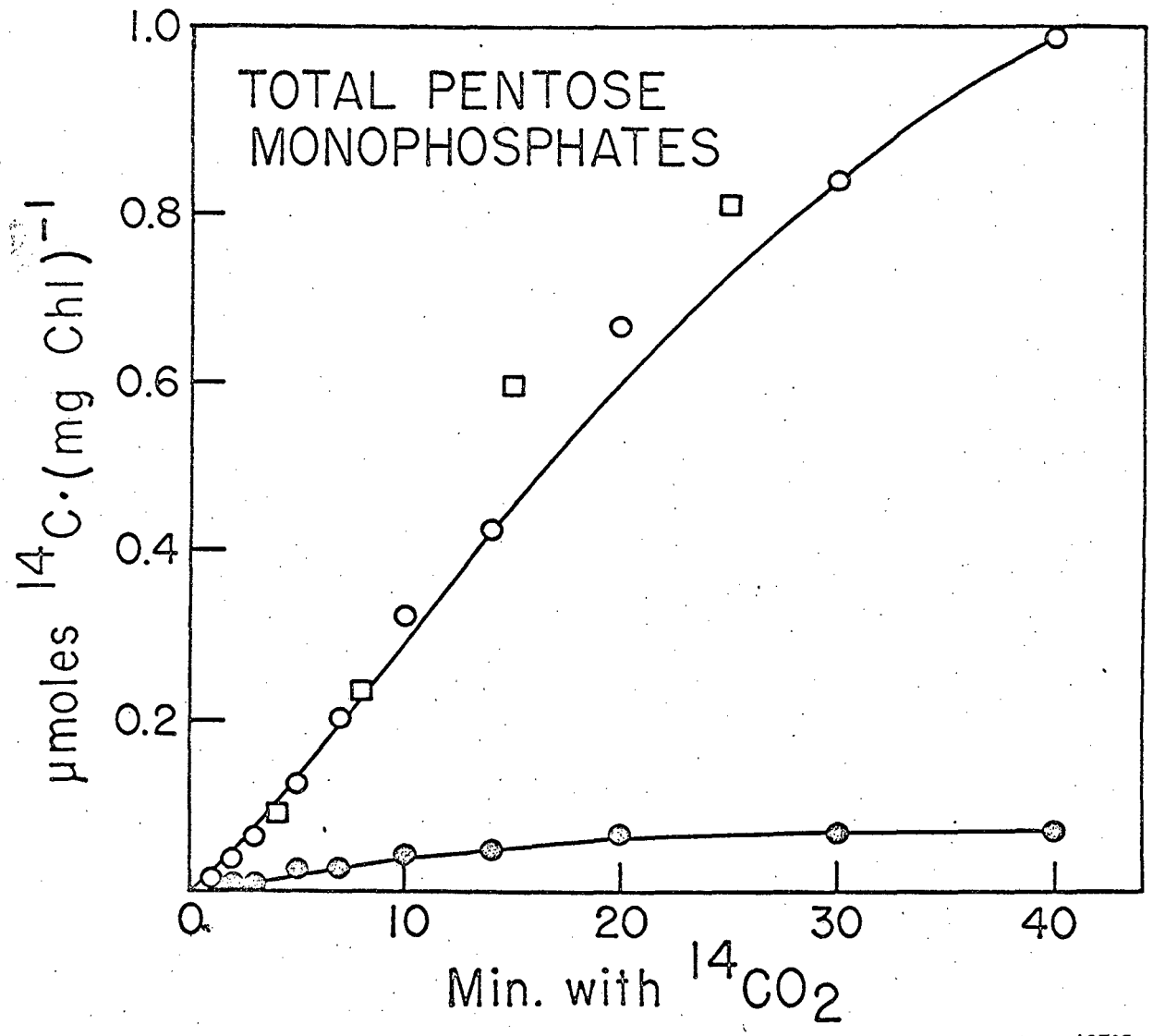

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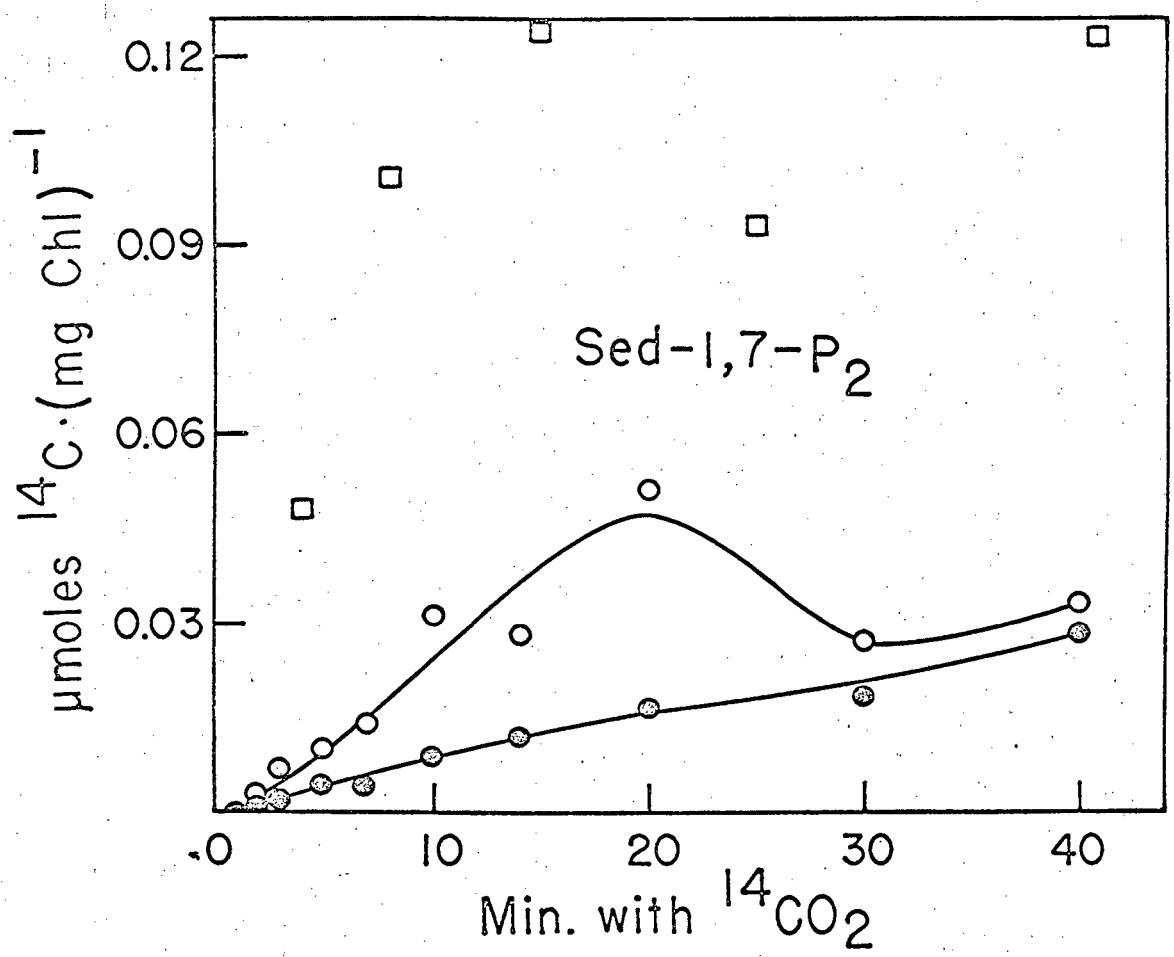

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Fig. 5

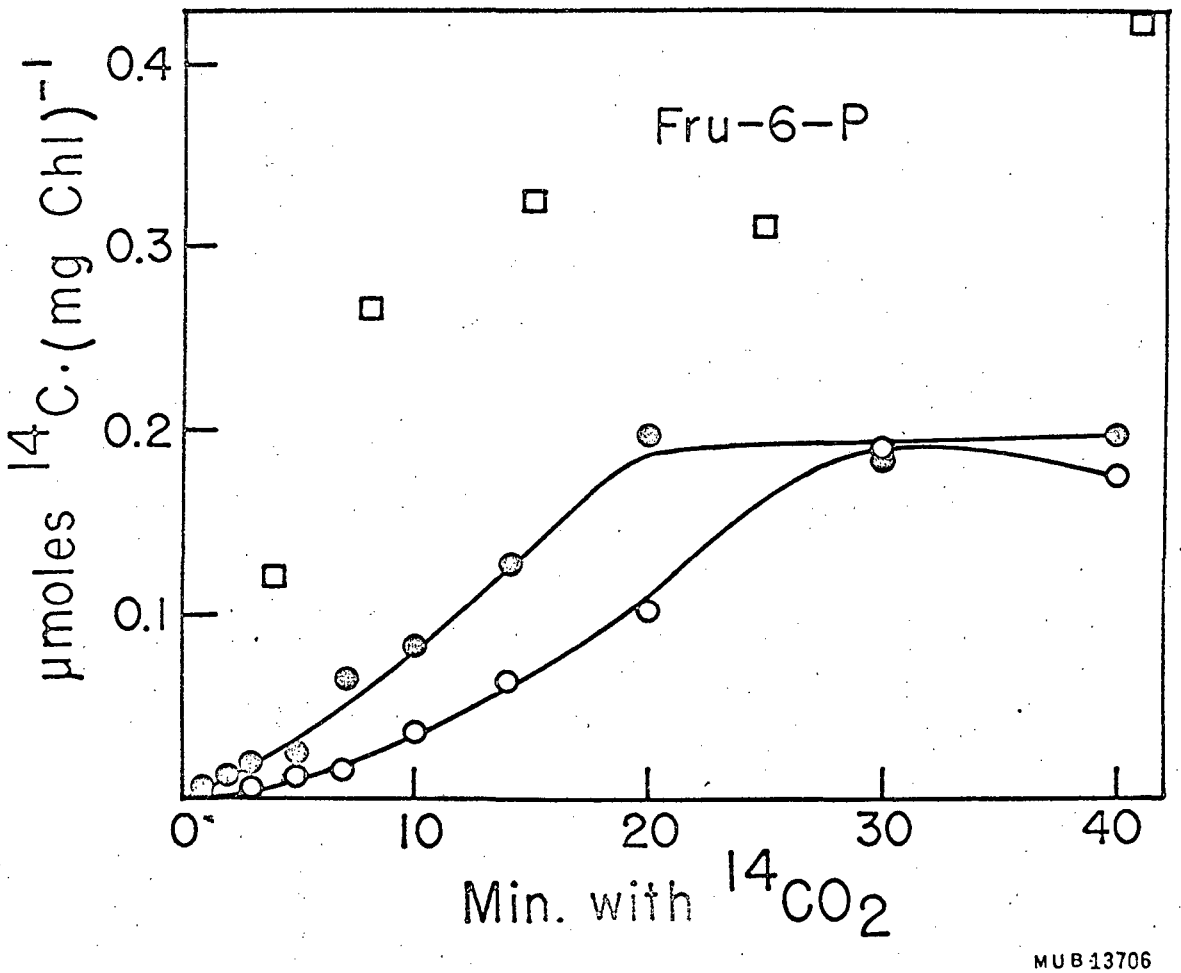

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Fig. 6

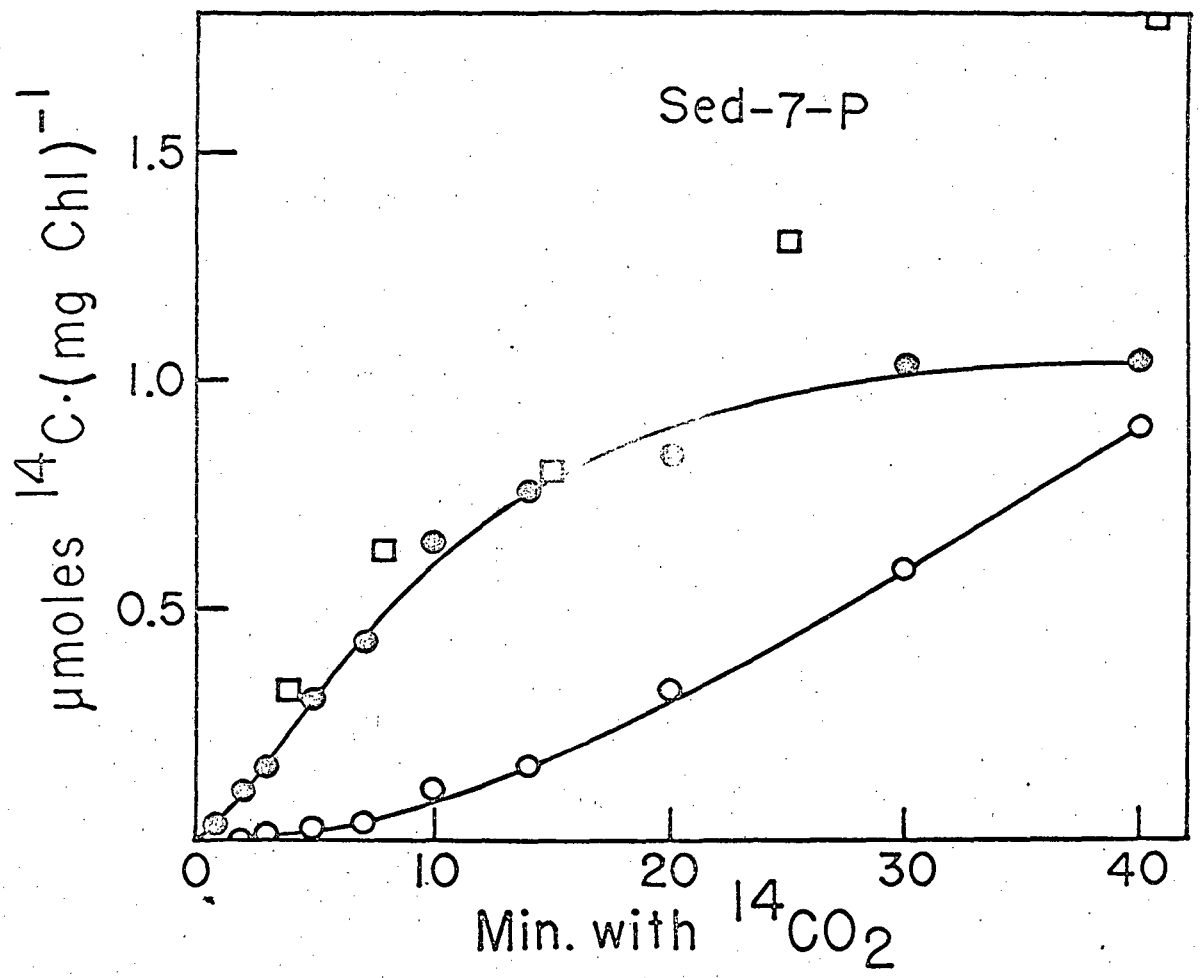

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Fig. 7

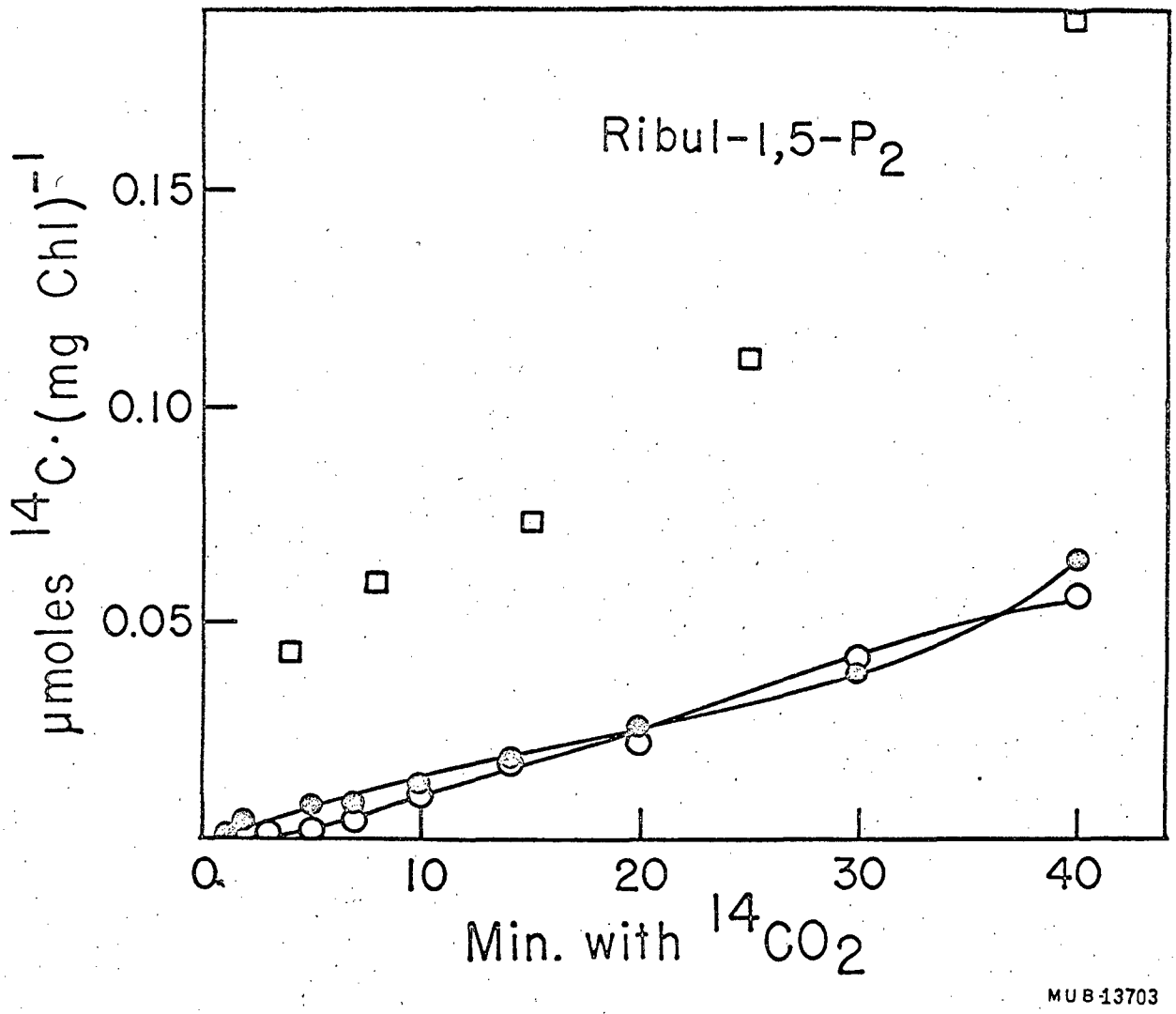

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Fig. 8

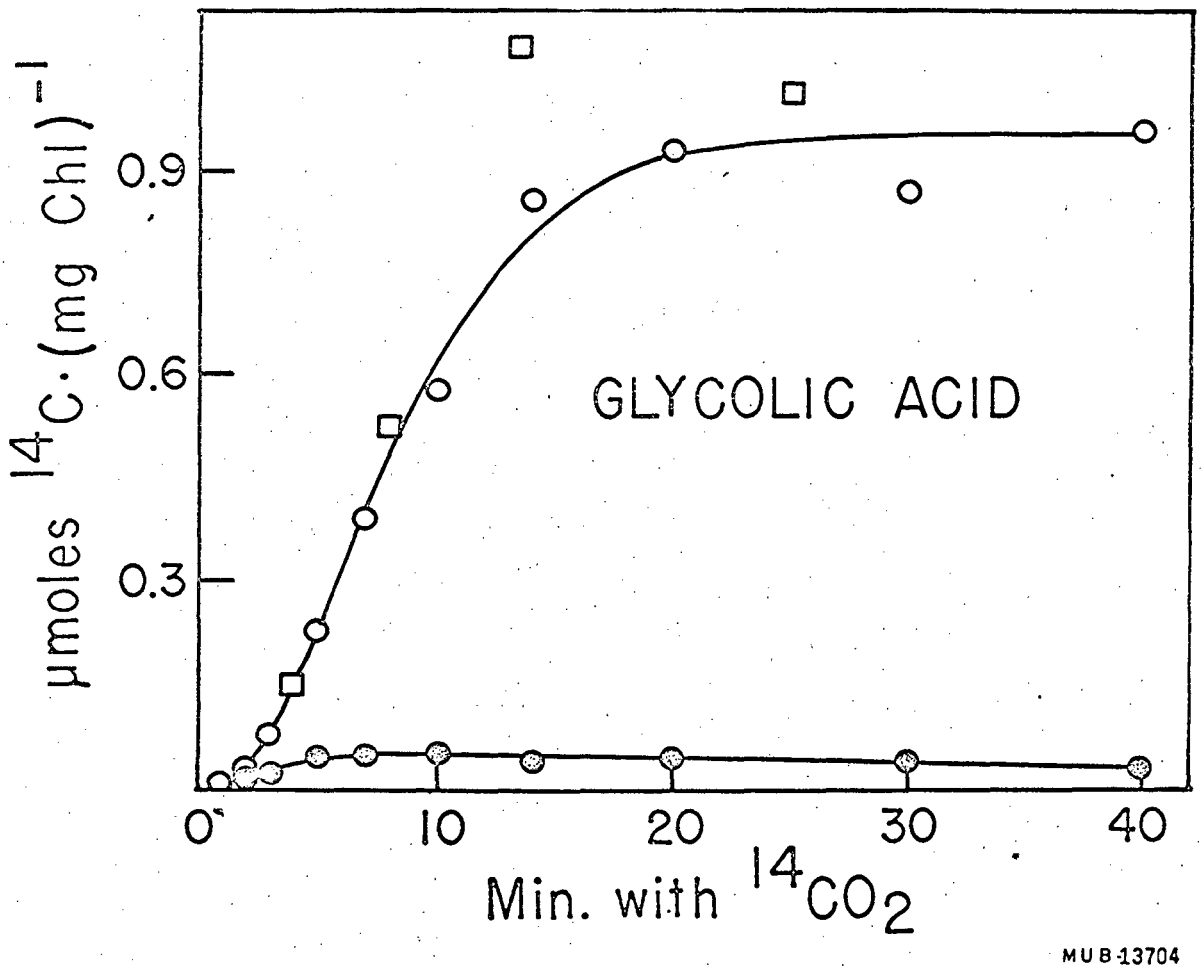

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Fig. 9

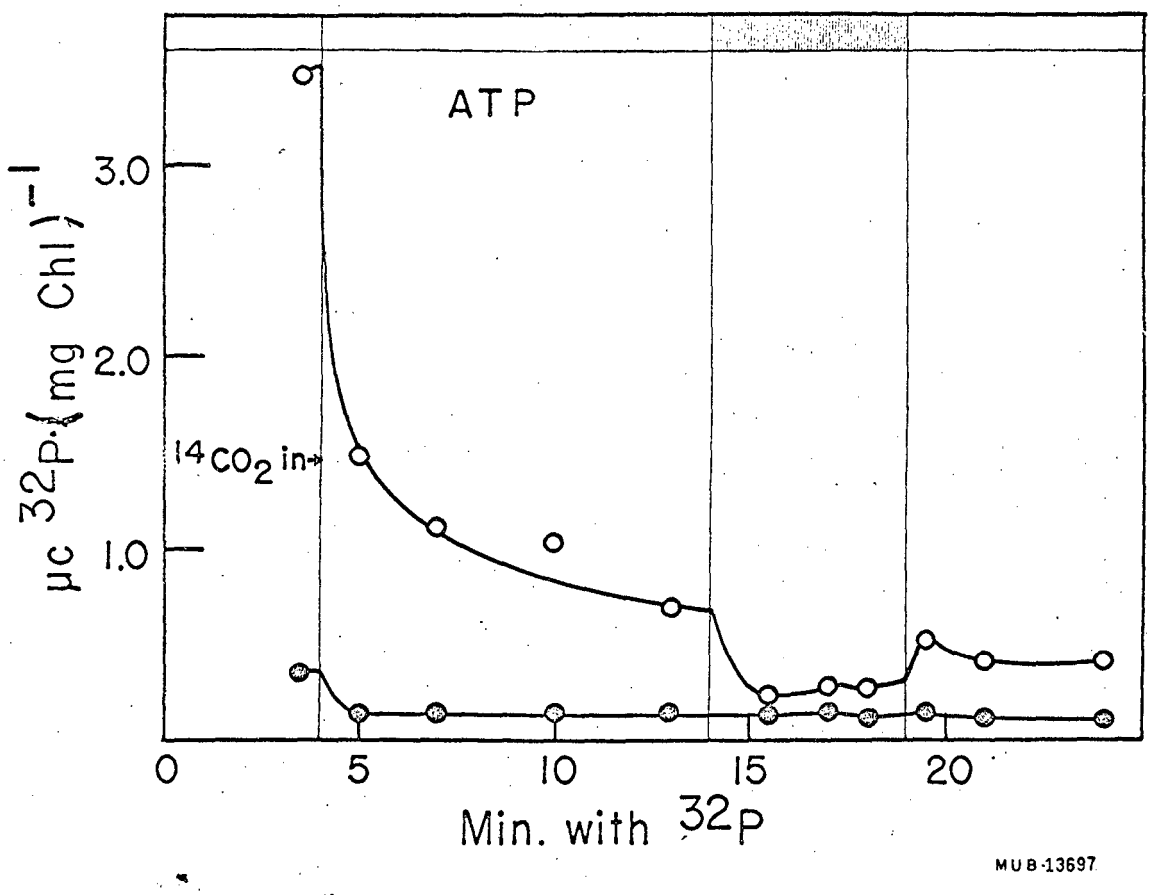

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Fig. 10

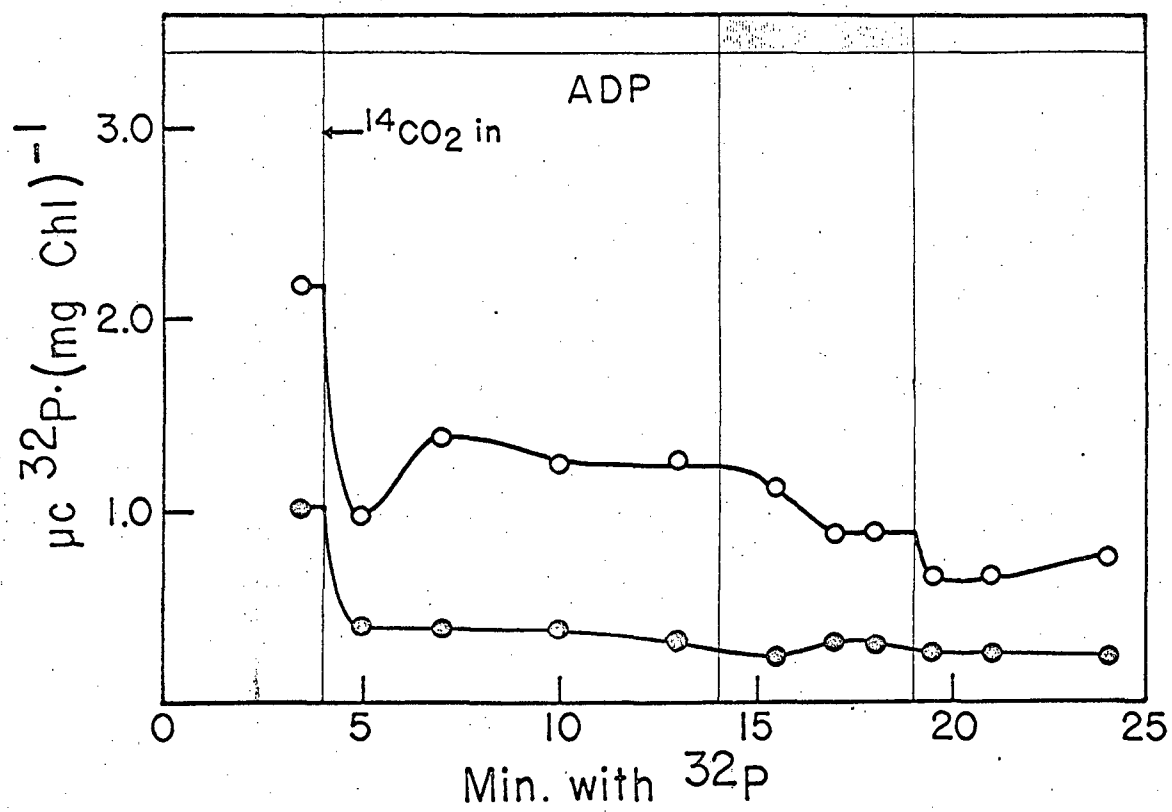

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Fig. 11

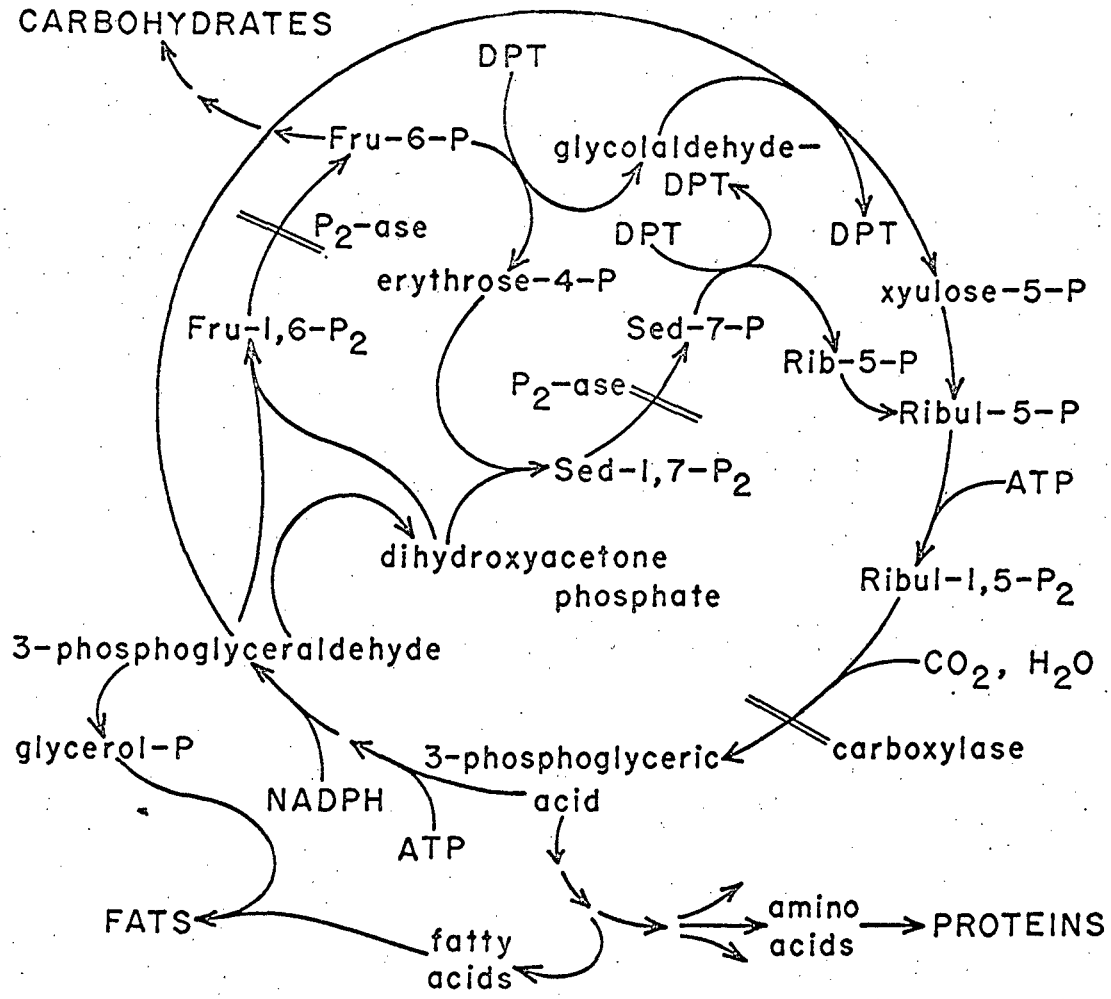

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Fig. 12

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