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AND INTERMEDIATE COMPOUNDS

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PHOTOSYNTHESIS BY ISOLATED CHLOROPLASTS. II. EFFECTS OF ADDITION OF COFACTORS AND INTERMEDIATE COMPOUNDS

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SUMMARY

Using isolated spinach chloroplasts capable of high rates of photosynthesis with $^{14}CO_2$, we have investigated the effects on these rates of the additions of metabolic intermediate compounds of the carbon reduction cycle, of additions of certain cofactors, and the additions of pyrophosphate and of phosphate. Following a preillumination period of 3 min to overcome the induction period, the rates of $^{14}CO_2$ photosynthesis during the first 10 min are essentially the same as would be found for spinach leaves *in vivo*, when allowance is made for the fraction of chloroplasts (30% or less) that have lost their structural integrity. During this 10-min period, no metabolic intermediate compounds or cofactors were found which appreciably stimulated the rate, except that added inorganic pyrophosphate is required to achieve the maximum rate. After 20 min the rates were always less, but could be somewhat sustained by the addition of fructose-1,6-diphosphate, and to a lesser degree by addition of NADP. This stimulation could not be replaced or enhanced (in the highly active preparations) by ribose-5-phosphate. It is proposed that the activity of the carboxylation enzyme, ribulose diphosphate

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carboxylase, is the rate limiting factor after 20 minutes. Thus, it appears that the stimulatory effects of added fructose-1,6-diphosphate and NADP may be exerted in some way on the carboxylation reaction itself rather than in the regeneration of the carboxylation substrate. The rates were not sharply dependent on inorganic phosphate concentration, but the maximum rate was found with about 1.0 m$\underline{M}$ added inorganic phosphate.

Recently conditions have been found under which isolated spinach chloroplasts carry out photosynthesis with $^{14}CO_2$ at rates approaching those found in experiments with whole spinach leaves.[1] These rates are highest during the first 10 to 15 min, and are reasonably high for periods up to 40 min or longer. During this period certain intermediate compounds of the carbon reduction cycle diffuse rapidly out from the chloroplasts into the suspending medium.[2] Also it appears that ATP, formed in the light by photophosphorylation, can diffuse from the chloroplasts, and that ATP can reenter the chloroplasts under certain conditions where it is required for the maintenance of photosynthesis. These findings raise the questions of whether the rates of fixation of $^{14}CO_2$ by the isolated chloroplasts may be limited by inadequate concentrations of metabolites and cofactors within the chloroplasts, and whether the rates could be stimulated by the addition to the suspending medium of metabolic concentrations of these substances.

Rates of light-induced fixation of $^{14}CO_2$ by less active chloroplasts have been shown to be stimulated by fructose-1,6-diphosphate

(Fru-1,6-P_2),[3,4] by ribose-5-phosphate (Rib-5-P)[4,5] and, to a lesser extent, some other intermediate compounds of the photosynthetic carbon reduction cycle.[4,5] It was concluded that intermediate compounds of the cycle can diffuse into chloroplasts and increase the rate of photosynthesis, particularly during the first few minutes of illumination when the added compounds could overcome the "lag" period.[5]

It was not clear whether similar stimulatory effects of added intermediate compounds would be found in studies with chloroplasts capable of approaching in vivo rates of $^{14}CO_2$ uptake. The rate-limiting reactions in such chloroplasts could well be different than the limiting steps in less active preparations. We have now investigated the effects on $^{14}CO_2$ uptake rates of adding various intermediate compounds and cofactors to the more active chloroplasts.

EXPERIMENTAL

Fructose-6-phosphate, 3-phospho-D-glyceric acid, ATP, ADP, AMP, thiamine pyrophosphate, and NADP were obtained from Calbiochem and used without further purification. Dihydroxyacetone phosphate, which comes as the dimethyl ketal, dicyclohexyl-ammonium salt, was prepared just prior to the experiment and the methanol removed by distillation at reduced pressure. The sodium salts of fructose-1,6-diphosphate, ribose-5-phosphate, and D-ribulose-1,5-diphosphate were obtained from Sigma Chemical Co. The ribulose-1,5-diphosphate was claimed to be 77% pure, having 5% inorganic phosphate. There was little difference in the CO_2 fixation response of chloroplasts to this sample as compared to one reprecipitated as the barium salt and resuspended by passing through Dowex 50 resin (H^+ form).

Chloroplasts were isolated from freshly harvested spinach leaves as described earlier.[1] The leaves used in the experiments were from 4 to 6 weeks old. The chloroplasts were suspended in the previously described assay medium,[1] except in the experiments in which the dependence on pyrophosphate and phosphate was studied.

Photosynthesis was carried out at 20°C in round-bottom flasks with a liquid volume of 0.5 ml. The total chloroplast content of chloroplasts added and the specific activity of the $H^{14}CO_3^-$ used in each experiment is given in the figure legends. Light intensity of 32,000 lux was obtained from a bank of "reflector" fluorescent lamps rated at 20 watts but each powered by a 0.6 ampere DC source. The flask contents were gently swirled as described earlier.[1]

During the experiments, aliquot samples (0.05 ml) were removed from the flasks, and the biochemical reactions were stopped by addition of 0.20 ml methanol. Aliquot samples of this methanolic mixture were placed on filter paper, together with three drops of acetic acid, and the mixture was dried. The ^{14}C content of the acid stable compounds was then determined with a Geiger-Muller tube. From the known specific radioactivity of the $H^{14}CO_3$ added, and from the chlorophyll content, the rates of photosynthesis were calculated.[1]

In the experiment testing the effect of pyrophosphate, all conditions were kept as before,[1] except that pyrophosphate was omitted from the assay medium in the control, and added at several different concentrations to individual flasks. The control rates and 0.7 mM and 5.0 mM concentrations are given in the Results; other intermediate concentrations gave intermediate results. The effect of preincubating the chloroplasts in pyrophosphate (prior to illumination) was investigated (see Table I for details).

In the experiment testing the effect of phosphate concentration, all conditions were kept as before,[1] except that two assay media were made up, one without phosphate and one with 2.0 mM phosphate. These media were then combined in various proportions and used in different flasks to give the rates shown in the Results.

RESULTS

The effects of addition of unlabeled metabolic intermediate compounds on the rate of $^{14}CO_2$ uptake are shown in Fig. 1. Only the addition of fructose-1,6-diphosphate (Fru-1,6-P_2) caused a significant increase in rate. This increased rate in the presence of Fru-1,6-P_2 was seen only after 10 min of photosynthesis. In contrast to the results reported by Walker,[4,5] addition of ribose-5-phosphate (Rib-5-P) caused no significant rate enhancement in this experiment.

Addition of 3-phosphoglyceric acid, of dihydroxyacetone phosphate, and of ribulose-1,5-diphosphate (Ribul-1,5-P_2) caused a significant decrease in the rates of $^{14}CO_2$ assimilation during the first 15 min. After that, the rates were comparable to that of the control which by 15 min had declined. The possibility that some of the apparent inhibition by added Ribul-1,5-P_2 was caused by inorganic phosphate or other impurities cannot be ruled out, although some purification of the commercially available Ribul-1,5-P_2 was effected.

The effects of addition of $NADP^+$, ATP, ADP, and AMP on $^{14}CO_2$ uptake are seen in Fig. 2. In this experiment, the usual preincubation period of 3 min was eliminated. Consequently, an induction period of about 3 min is seen in the early rates. The maximum rate (first 10 min) was

very high (195 μmoles CO_2(mg Chl·hr)$^{-1}$ in this experiment. The curve in the presence of thiamine pyrophosphate (not shown) was essentially identical with that with ATP. No large effects were seen, although after 20 min the presence of added $NADP^+$ has the effect of maintaining a slightly higher rate than the control. The differences with other cofactors seem hardly large enough to warrant comment.

The effects of added pyrophosphate at two levels are shown in Fig. 3. The greatest stimulation during the first 10 min was obtained with 5.0 mM pyrophosphate. However, a good rate was sustained for 25 min in the presence of 0.7 mM pyrophosphate, whereas the rates in the control, and especially with 5.0 mM added pyrophosphate had fallen off by that time.

Table I shows the rates of $^{14}CO_2$ fixation following preincubation in the dark with higher amounts of pyrophosphate. The rates depend on the concentration of pyrophosphate in the light only, ranging from 120 - 150 μmoles CO_2(mg Chl·hr)$^{-1}$ with 0.5 mM pyrophosphate to nearly 200 μmoles CO_2(mg Chl·hr)$^{-1}$ with 2.0 mM. However, the rates were not stimulated by preincubation in the dark with pyrophosphate.

The maximal rates obtained (4 to 8 min) with various levels of added phosphate are shown in Fig. 4. Since the medium used for chloroplast isolation[1] contains phosphate, the lowest concentration of phosphate investigated was 0.025 mM. The rate dependence on phosphate concentration was broad, with the maximum rate of 213 μmoles CO_2(mg Chl·hr)$^{-1}$ at about 1.0 mM.

DISCUSSION

Explanation of the changing effects of added intermediate compounds and cofactors is probably related to the changing rate of $^{14}CO_2$ uptake during the 40 min of photosynthesis by isolated chloroplasts as seen in the control experiments. Reasonable interpretations of the observed variation in rate with time can be made on the basis of different causes of rate limitation which can become important at different times.

When the chloroplasts are first placed in the light (without preillumination) at room temperature (after being stored in the dark at 0°C), there is an induction period[6,7] during which the rate, initially low, increases. With chloroplasts prepared and incubated by our methods,[1] this induction period is also seen. The induction period can be partially overcome by the addition of Rib-5-P,[4-7] suggesting that the chloroplasts at first have insufficient sugar phosphates to permit the formation of enough carboxylation substrate, Ribul-1,5-P_2, to support maximum CO_2 uptake.

In the experiment shown in Fig. 1, a preillumination time of 3 min was given the chloroplasts before the labeled bicarbonate was added. Apparently this time was sufficient for endogenous bicarbonate to be reduced and enough Ribul-1,5-P_2 to be formed, for the maximum rate was observed from the start. However, the experiment in Fig. 2 was performed without preillumination, and the induction period is clearly seen. That this induction can be only <u>partially</u> overcome by the addition of Rib-5-P is probably a consequence of the fact that the light activation of the carboxylation reaction itself[8] requires one min or more.[9] We shall refer to the period of about 10 min of maximum rate, following any induction period, as Phase I.

Since no added intermediate compound or cofactor except pyrophosphate caused significant stimulation of the rate during Phase I, it is concluded that with added pyrophosphate the active (probably the intact) chloroplasts are operating at near the maximum rate of which they would be capable *in vivo*. The failure to reach the maximum measured *in vivo* rate[1] of about 250 μmoles CO_2(mg Chl·hr)$^{-1}$ may be due to the fact that only 70 to 90% of the chloroplasts appear to be intact when viewed through the optical micorscope with phase optics.[1] The variation in control between experiments 1 and 2 is attributed to variation in the physiological state in the leaves from which the chloroplasts were isolated. One might be inclined to argue that even though additions did not stimulate during Phase I, the carboxylation enzyme itself (3-phospho-D-glycerate carboxy-lyase (dimerizing), 4.1.1.39) might not be fully activated, were it not for the fact that radiochromatographic analysis of the photosynthetic products in other experiments[2] does not show a rapid accumulation of labeled Ribul-1,5-P_2, the carboxylation substrate, during Phase I. Such an accumulation would be expected if the carboxylation reaction itself were rate-limiting.

The inhibition of $^{14}CO_2$ uptake during Phase I by added 3-phosphoglyceric acid, dihydroxyacetone phosphate, and Ribul-1,5-P_2 cannot be satisfactorily explained at present.

From 10 to 20 min, the rate of $^{14}CO_2$ uptake decreases, leading to Phase II (20 to 40 min), during which the rate is lower but more or less constant. During Phase II the rate is no longer decreased significantly by any of the added intermediate compounds, but is enhanced somewhat by added Fru-1,6-P_2. It also appears that there may be some

enhancement by added NADP. It is noteworthy that the level of Fru-1,6-P_2 actually dropped by 30 min to about one-half the level it had reached in 15 min in other experiments in which radiochromatographic analysis of the products of chloroplast photosynthesis with $^{14}CO_2$ were performed.[2] Since this drop corresponds well with the period when the rate is decreasing, it would appear that a certain level of Fru-1,6-P_2 is required for maximum fixation rate. The role of added NADP in stimulating a decelerating rate may be in holding up an otherwise lagging rate of electron transport to supply electrons for the reduction of phosphoglyceric acid to triose phosphates from which Fru-1,6-P_2 is formed.

The question of how Fru-1,6-P_2 helps to maintain a higher rate remains unanswered. The answer can hardly be that added Fru-1,6-P_2 supplies carbon for the regeneration of the carboxylation substrate, Ribul-1,5-P_2, since neither added Fru-6-P nor Rib-5-P significantly stimulate the rate. Rather we must look for some effect of the Fru-1,6-P_2 on the activity of the carboxylation reaction, other than an effect on the supply of Ribul-1,5-P_2. In fact, it is apparent from kinetic studies of photosynthesis of isolated chloroplasts with $^{14}CO_2$ [2] that the concentration of Ribul-1,5-P_2 increases rapidly during Phase II (see total sample pool in Fig. 8, Ref. 2). Therefore, the activity of the carboxylation enzyme, ribulose diphosphate carboxylase (4.1.1.39), seems to be rate-limiting during Phase II.

The cause of the stimulatory effect of added pyrophosphate has not yet been established. Earlier, in vivo kinetic studies[8] of Chlorella pyrenoidosa in light and in dark with ^{32}P-labeled phosphate showed that there is a metabolic pool of pyrophosphate. In some experiments, this labeled pool was substantial in the light, dropped to a low

level in the dark, and rose rapidly again to a steady-state level in the light, the times required for these light-dark and dark-light transitions being about the same (around one min) as the times required for light activation of the carboxylation and diphosphatase reactions.

This effect, while clearly beyond experimental error in the cases where it was seen, has not proven to be easily reproducible. Pyrophosphate is formed in varying degree during the application of inorganic phosphate to paper chromatograms. Also, pyrophosphate is somewhat unstable in solutions, and may be more so in the mixture of methanol-water and killed algae. Thus, the light-dark changes in level may be masked in some experiments by artifacts.

Pyrophosphate is formed metabolically in vivo in the synthesis of oligomers and macromolecules, proteins, carbohydrates, and nucleic acids. The size of its steady-state metabolic pool will depend on the rates of these reactions and on the pyrophosphatase activity with which the pyrophosphate comes in contact.

The fact that 0.7 mM pyrophosphate, added to isolated spinach chloroplasts, continues to exert a stimulatory effect on the rate for 30 min or more suggests that this pyrophosphate does not come in contact with a highly active pyrophosphatase. Moreover, when ^{32}P-labeled pyrophosphate was added to photosynthesizing isolated spinach chloroplasts,[1] very little ^{32}P was transformed into metabolic products. Even that very small amount could be accounted for as having come from a small amount of labeled phosphate which was released by the slow hydrolysis of pyrophosphate.

The amount of labeled pyrophosphate formed from labeled inorganic phosphate in photosynthesizing spinach chloroplasts has generally been indistinguishable from artifactual pyrophosphate formation, but in at least one case there was an observably higher level in the light than in the dark.[9] Thus, it may be that the level of inorganic pyrophosphate in chloroplasts is part of a natural regulatory mechanism, as yet not understood. The findings that 5.0 mM pyrophosphate caused the highest initial rate, whereas 0.7 mM pyrophosphate was more effective in maintaining a high rate after 10 min, suggest that the optimum natural level is probably 1 mM or less. The higher concentration of 5.0 mM pyrophosphate in the medium would help to bring the level of pyrophosphate inside the chloroplast up to the optimum level more quickly, but after that the higher level could become inhibitory. Moreover, the slow hydrolysis of the pyrophosphate may produce a level of inorganic phosphate higher than the optimum level in the medium. Finally, it is clear, from the results in Table I, that the pyrophosphate exerts its stimulatory effect only when it is present in the light.

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TABLE I

Rates of $^{14}CO_2$ Fixation by Isolated Chloroplasts after Preincubation with Pyrophosphate during Dark Storage

Flask	M x 10^{+3} Pyrophosphate Dark Suspension Media	Light CO_2 Fixation Media	Maximum Fixation rate μmoles ^{14}C(mg Chl·hr)$^{-1}$
1	0	2.0	197
2	0	1.0	169
3	0	0.5	148
4	5	1.0	144
5	5	0.5	122
6	10	1.0	145
7	10	0.5	132
8	20	1.0	141

Chloroplasts were isolated as described earlier,[1] separated into four vessels and suspended with solution B[1] having the amounts of pyrophosphate above and kept in the dark in ice for 30 min. A 25 μl aliquot sample of this suspension was added to the CO_2 fixation media (Solution C)[1] adjusted so that a total of 0.5 μmole pyrophosphate was present in 500 μl. After 3 min illumination at 20°C, 6 μmoles $NaH^{14}CO_3$ (60 μC) was added to start photosynthesis. The amount of chlorophyll in each flask was: 1-3, 32 μg; 4,5, 39 μg; 6,7, 35 μg; 8, 37 μg. The maximum rate of CO_2 fixation was observed between 4 and 8 min.

FIGURE LEGENDS

Fig. 1. Effect of added sugar phosphates on CO_2 fixation. Each flask had 1.0 μmole of a sugar phosphate or 3-phosphoglyceric acid or NaCl (control) with 46 μg of chlorophyll in 500 μl. Six μmoles of $NaH^{14}CO_3$ (60 μC) was added after 3 min of illumination.

Fig. 2. Effects of added cofactors on CO_2 fixation. Each flask had 2.5 μmoles of each cofactor or NaCl (control) and 3.8 μmoles $NaH^{14}CO_3$ (31 μC). The chloroplast suspension (47 μg chlorophyll) was added directly to the flasks to start photosynthesis. Thiamine pyrophosphate (not shown) had the same curve as ATP.

Fig. 3. Effect of pyrophosphate concentration on CO_2 fixation. Each flask having various amounts of pyrophosphate had 37 μg chlorophyll in 550 μl at pH 7.6. Six μmoles of $NaH^{14}CO_3$ (60 μC) was added after 3 min illumination. Intermediate amounts of pyrophosphate had intermediate effects.

Fig. 4. Effect of orthophosphate concentration on rates of CO_2 fixation. Amounts of exogenous orthophopshate were varied and the maximum rate of fixation determined within the first 8 min. Each flask had 40 μg chlorophyll in 500 μl. After 3 min illumination 4.5 μmoles $NaH^{14}CO_3$ (36.5 μC) was added.

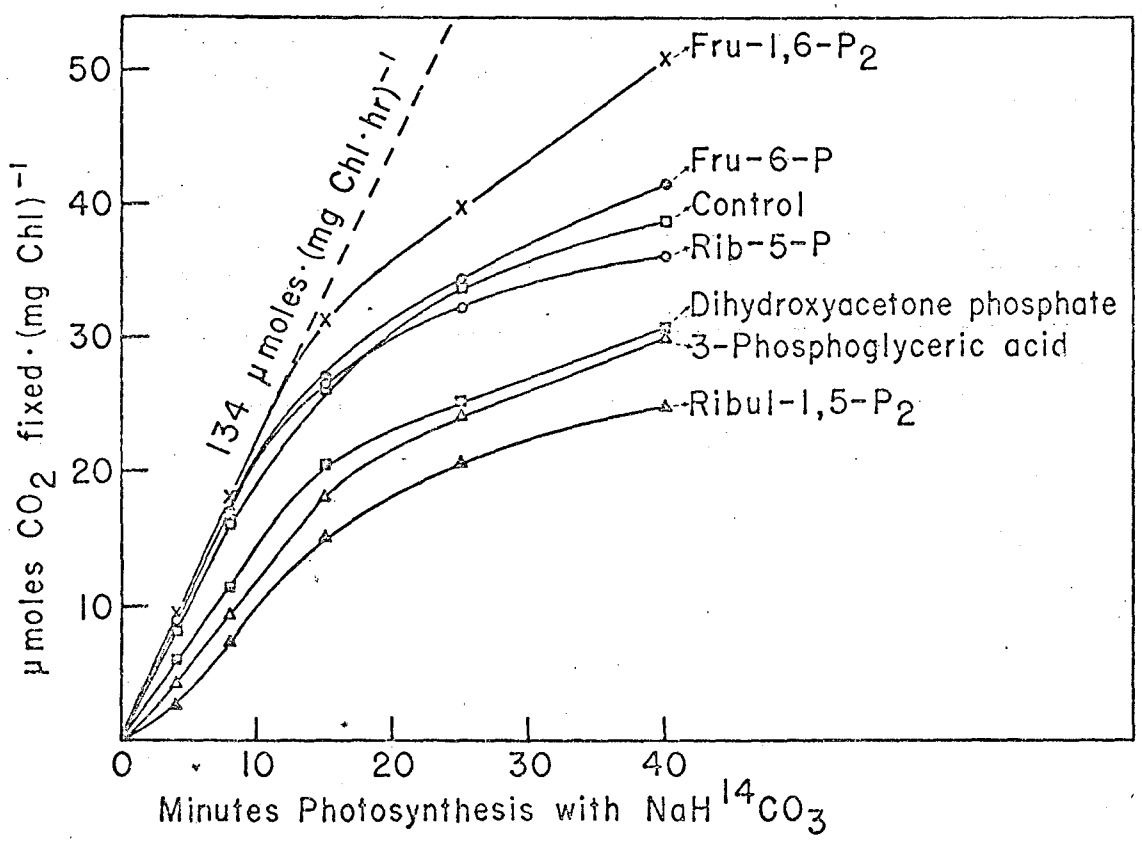

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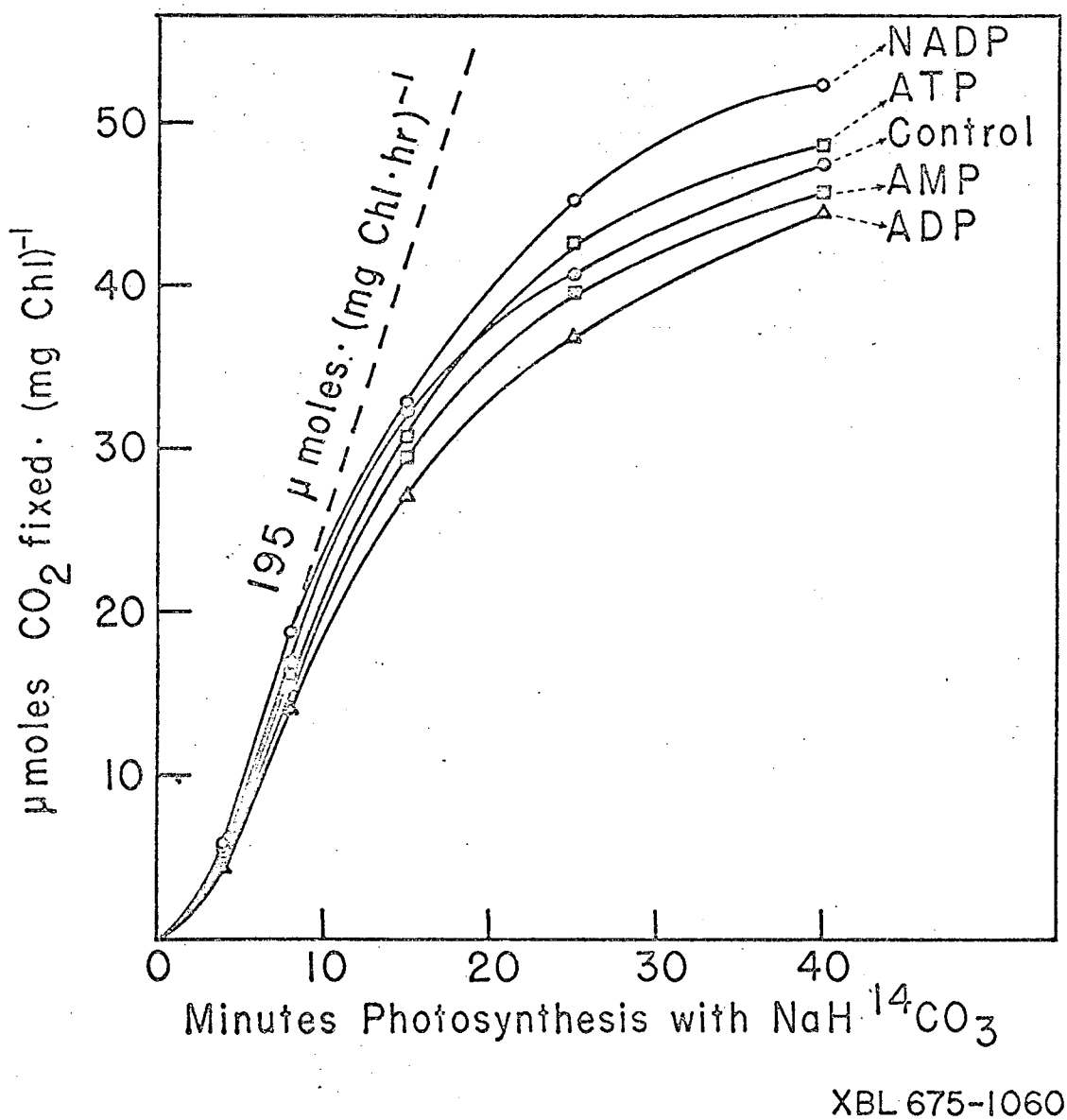
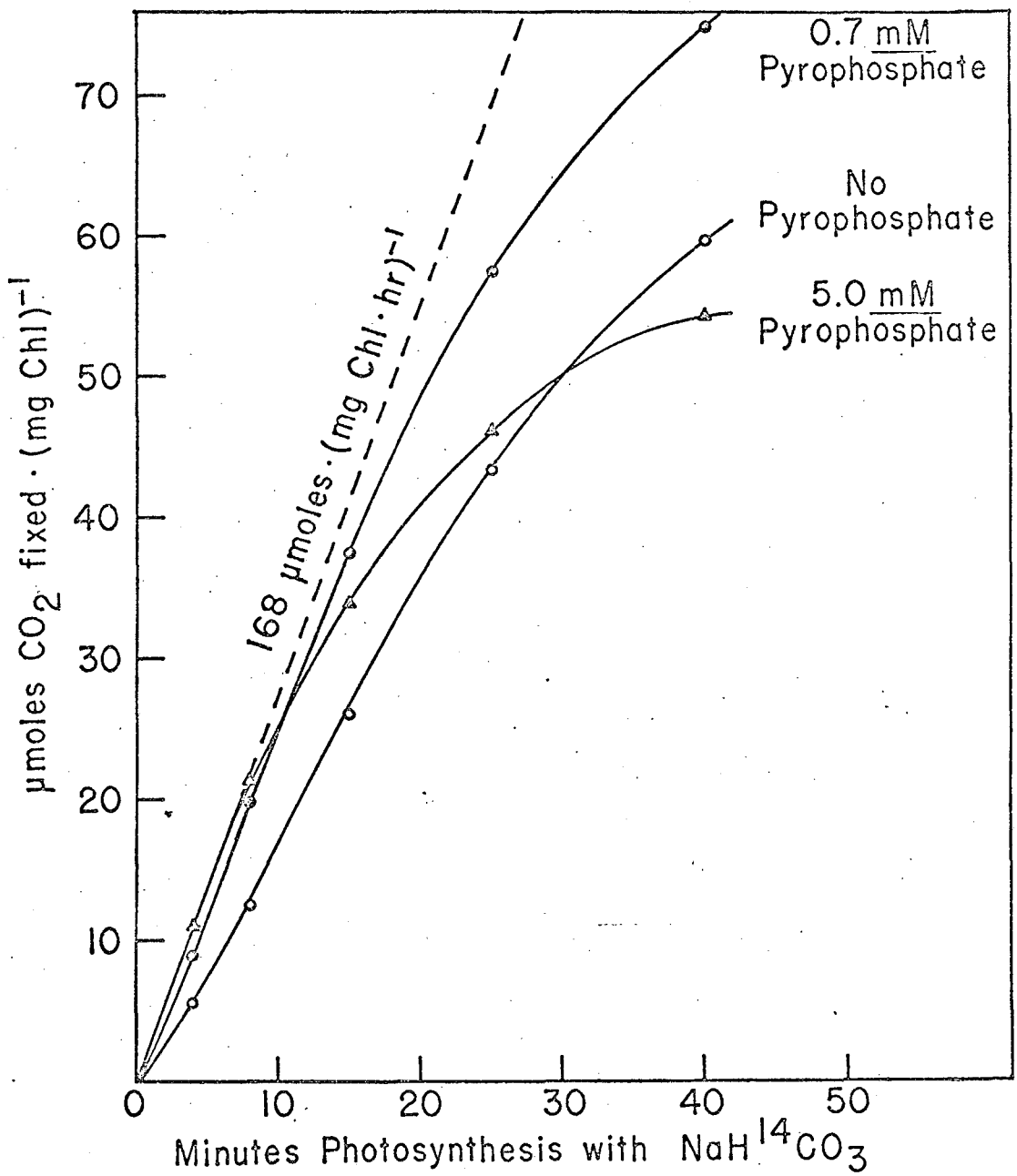
Fig. 1



XBL 675-1060

Fig. 2



XBL 676-1074

Fig. 3

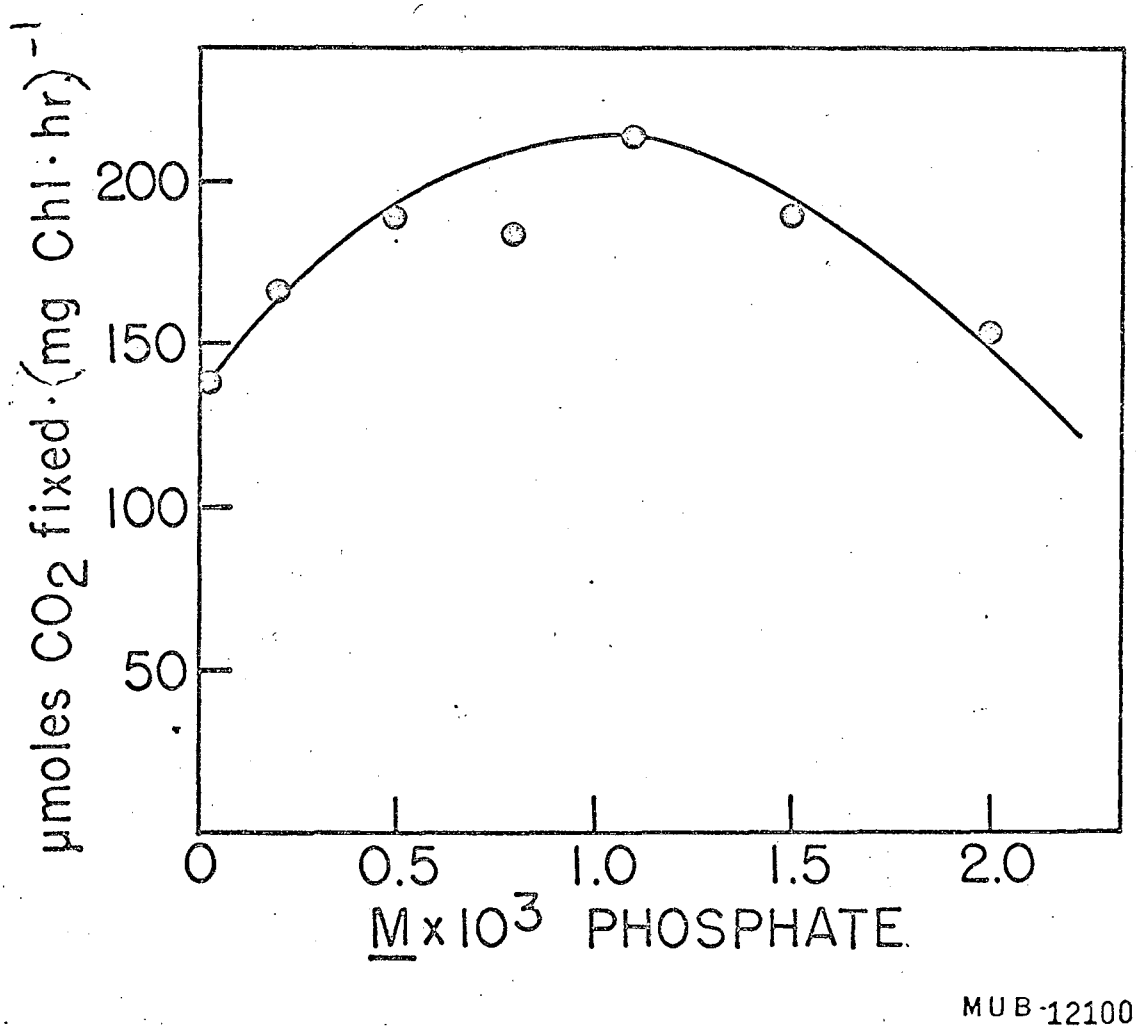

μmoles CO2 fixed · (mg Chl · hr)-1
200
150
100
50
0
0.5
1.0
1.5
2.0
M x 10^3 PHOSPHATE
MUB-12100

Fig. 4