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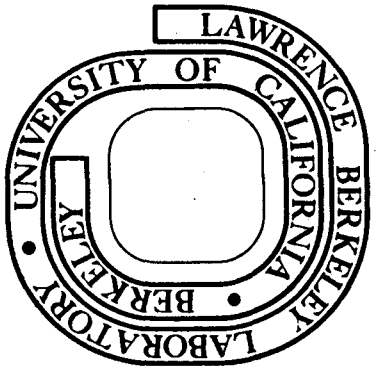

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RIBULOSE DIPHOSPHATE CARBOXYLASE REGULATION IN RECONSTITUTED SPINACH CHLOROPLASTS

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Summary

In a preparation of soluble components from previously isolated spinach chloroplasts, the activity of the carboxylating enzyme of the photosynthetic reductive pentose phosphate cycle, ribulose-1,5-diphosphate carboxylase (E.C.4.1.1.39), is strongly increased by 6-phosphogluconate or by NADPH, and is decreased by ribulose-1,5-diphosphate in the absence of these effectors and CO_2. With the thylakoid membrane system added to these soluble components (reconstituted chloroplast system) plus ferredoxin, the carboxylase is even more strongly activated in the light. This "light" activation appears to be due to reduction of endogenous $NADP^+$ by electrons from the light reactions transferred via ferredoxin, since NADPH alone can activate the purified enzyme while reduced ferredoxin does not activate the enzyme. The regulatory properties of the enzyme in the complete mixture of soluble chloroplast components are compared with those of the isolated enzyme, and their possible physiological significance is discussed.

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Introduction

All green photosynthetic cells capable of oxidizing water and reducing carbon dioxide to sugar phosphate employ the photosynthetic reductive pentose phosphate cycle[1], irrespective of whether or not they also employ the preliminary "tropical grass" or "C-4" cycle found in some higher plant species such as maize and sugar cane[2]. In the reductive pentose phosphate cycle, carbon dioxide is incorporated via the carboxylation of ribulose-1,5-diphosphate (Ribul-1,5-P_2) to give two molecules of 3-phosphoglycerate in a reaction mediated by the enzyme ribulose diphosphate carboxylase (E.C.4.1.1.39). This reaction, like the conversion of fructose-1,6-diphosphate (Fru-1,6-P_2) and sedoheptulose-1,7-diphosphate to their respective monophosphates and the conversion of ribulose-5-phosphate and ATP to Ribul-1,5-P_2 and ADP, has a high negative free energy change under conditions of steady-state photosynthesis and is thus a rate-limiting step in the reductive pentose phosphate cycle[3]. These four steps in the cycle share two other characteristics. On the basis of light-to-dark and dark-to-light transient changes in metabolite levels following photosynthesis with $^{14}CO_2$ in Chlorella pyrenoidosa,[4,5] or after addition of Vitamin K_5 to these algae,[6] each of these steps has been implicated as occurring rapidly in the light but not as rapidly in the dark. A further characteristic of these four reactions is that each is unique to the reductive pentose phosphate cycle, whereas all of the other steps of the reductive pentose phosphate cycle are found as part of either of the oxidative pentose phosphate cycle (hexose monophosphate pathway, 6-phosphogluconate pathway) or glycolysis. Thus, it is not surprising that the activities of the specific enzymes which mediate these steps are the

ones which decrease when the light is turned off or photosynthesis stopped and respiratory metabolism begins[7,8].

Studies with isolated spinach chloroplasts capable of carrying out photosynthesis with carbon dioxide at high rates have provided further evidence for the light-dark regulation of the carboxylase step[9]. Complimentary to the dark inactivation of the reductive pentose phosphate cycle, there is a dark activation of the oxidative pentose phosphate cycle in Chlorella pyrenoidosa[4] and in isolated spinach chloroplasts[6]. This activation of the oxidative pentose phosphate cycle is accomplished by activation of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) in the dark. In the light this enzyme is inactivated by high ratios of $NADPH/NADP^+$ as amplified by concentrations of the metabolite Ribul-1,5-P_2[10].

The mechanism of regulation of the four controlled enzymes of the reductive pentose phosphate cycle has also been studied by investigation of properties of the isolated enzymes. Fructose-1,6-diphosphatase and sedoheptulose-1,7-diphosphatase are reported to be activated by reduced ferredoxin in the presence of a low molecular weight protein factor[11]. Phosphoribulokinase (EC 2.7.1.19) which mediates the conversion of ribulose-5-phosphate and ATP to Ribul-1,5-P_2 and ADP, is activated by dithiothreitol[12]. Presumably in vivo it is activated by a physiological reducing agent, possibly reduced ferredoxin or NADPH.

Recently, Ribul-1,5$_2$ carboxylase has been found to be subject to rather complex regulatory behavior[13]. It was long known that this enzyme is more active following preincubation with magnesium ion and bicarbonate prior to addition of the other substrate, Ribul-1,5-P_2. Careful studies at low levels of bicarbonate (1 mM or less) revealed that the enzyme is converted to an inactive form when exposed to physiological levels of Ribul-1,5-P_2 in the absence of preincubation with both magnesium and

bicarbonate[14]. It was concluded that such treatment resulted in binding of Ribul-1,5-P_2 at an allosteric site which converts the enzyme into a form with weak binding for the other substrate, carbon dioxide. This inactive form can persist for 20 minutes or longer in the presence of both substrates when the concentration of bicarbonate is not higher than 1 mM. Preincubation with 10 mM magnesium ion and 1 mM bicarbonate for several minutes partially prevents this inactivation binding by Ribul-1,5-P_2 and gives a moderately active enzyme, which remains active in the presence of physiological concentrations of Ribul-1,5-P_2 and bicarbonate. Further activation of the enzyme is seen if the preincubation mixture includes, in addition to bicarbonate and magnesium, either 6-phosphogluconate (6-PGluA) in the concentration range of 0.05 to 0.1 mM, or NADPH in the concentration range of 0.5 to 1.0 mM[13,15]. From these and other experimental results it was concluded that there are allosteric binding sites for both 6-PGluA and NADPH which result in activation of the enzyme in such a way that it no longer binds Ribul-1,5-P_2 at the inactivating allosteric binding site. One physiological significance of this complex regulatory behavior was attributed to the need to avoid the oxidative reaction which occurs when oxygen (O_2) binds competitively at the catalytic binding site for CO_2 when CO_2 pressure is very low and oxygen pressure is high. Such a binding can lead to an oxygenase activity for the enzyme in which oxygen attacks Ribul-1,5-P_2 producing phosphoglycolic acid and phosphoglyceric acid[16-19]. Phosphoglycolic acid can be converted to glycolic acid, the proposed substrate for photorespiration[20]. Under some physiological conditions, as when leaves close their stomata during bright illumination under stress of lack of water, the level of CO_2 in the chloroplasts may drop very low while the level of Ribul-1,5-P_2 rises above the normal physiological level. Such conditions can lead to photorespiration. If, under such conditions,

the enzyme binding for both CO_2 and O_2 decreases (K_m increases), photorespiration due to Ribul-1,5-P_2 oxidation can be diminished. The additional activation of the enzyme by 6-PGluA and by NADPH was interpreted in terms of the physiological transition from darkness to light and the requirement under those circumstances of an activated Ribul-1,5-P_2 carboxylase[13].

Recently it has become possible to reconstitute spinach chloroplasts by adding the soluble enzyme system of lysed chloroplasts back to the membrane system which has been previously separated from the soluble components and washed[21]. Such a system is capable of carrying out photosynthetic carbon reduction at an appreciable fraction of the rate of intact leaf photosynthesis. We now find that this is true even if the reconstituted system is exposed to only air levels of CO_2. Thus, it must be that the K_m for CO_2 in this reconstituted system is sufficiently low to permit active rates of photosynthesis with air levels of CO_2. Usually, the K_m levels for the isolated and purified carboxylase enzyme have appeared to be too high for good rates of photosynthesis to occur with air levels of CO_2, although the reported K_m values have been reduced recently as a result of careful adjustment of the conditions of photosynthesis, including the activation by preincubation with magnesium bicarbonate and by an effector such as NADPH or 6-PGluA[15]. However, Bahr and Jensen have reported that with freshly disrupted chloroplasts there is a form of the Ribul-1,5-P_2 carboxylase with an even lower K_m sufficient to accommodate the physiological conditions occurring during photosynthesis in air[22].

This low K_m form of the carboxylase was reported to be rather unstable and was converted to a higher K_m form after some minutes at room temperature. Since we found that photosynthesis in the reconstituted chloroplasts is maintained for up to one hour at substantially high rates, it was of

interest to study the properties of activation of the carboxylase activity in this reconstituted system and to compare the regulatory properties in this system with those of both the isolated enzyme and the apparent activity of the enzyme in whole leaves or whole chloroplasts. In this study we find that many of the properties reported for the isolated and purified enzyme are reproduced in the reconstituted system. However, there are some interesting differences which suggest that the Ribul-1,5-P_2 carboxylase as it exists in the reconstituted system and perhaps in whole chloroplasts has certain specific/different properties from those of the isolated enzyme.

Materials and Methods

Whole spinach chloroplasts were isolated from freshly picked spinach according to methods described previously[23] were then subjected to lysis and the components separated and reconstituted as in our previous report[21]. It should be noted that in the reconstituted system, due to the elimination of the outer chloroplast membrane, the soluble components (proteins, cofactors, metabolites) are diluted into the bulk volume of the flask. In the usual whole chloroplast suspension, the volume of the chloroplasts occupies only about 1% of the total suspension volume. Thus, it is to be expected that there would be undue dilution of soluble components if the ratio between total soluble components and lamellae or membrane system were not increased. It was for this reason that we studied the effects on CO_2 fixation of various ratios of soluble components to membrane system, and found that there had to be a 14:1 increase in this ratio as compared with a whole chloroplast suspension. This means that the soluble components are still diluted by a factor of about 7 as compared with their concentration inside the intact chloroplasts, and so certain constituents, such as ferredoxin, $NADP^+$, and ADP have been added to increase the concen-

tration to a more optimal level[21]. The concentrations of soluble proteins, and many metabolites, however, must still be considerably more dilute in the reconstituted system than they would be in the stroma regions of intact spinach chloroplasts.

Studies with ribose-5-phosphate and an ATP regenerating system.

In order to generate Ribul-1,5-P_2 in some experiments an addition was made to the reconstituted system of ribose-5-phosphate (Rib-5-P, 0.05 mM), creatine phosphate kinase (1 unit), creatine phosphate (2.5 mM) and ATP (2.5 mM). In most cases it was found that given comparable conditions, a higher rate of CO_2 fixation could be achieved with this system than with added Ribul-1,5-P_2. Creatine phosphate, ATP and creatine phosphate kinase were obtained from Sigma Chemical Co. In other experiments, Ribul-1,5-P_2 was obtained as the barium salt from Sigma Chemical Co. and was regenerated with acid Dowex-50W.

Preincubation and incubation.

A variety of preincubation and incubations were carried out using the reconstituted system in small round-bottom flasks with serum stopper caps. These flasks were mounted on the shaking device for chloroplasts studies as described previously[23]. In studies with air levels of CO_2, these flasks have been equipped with small inlet and outlet tubes and connected by means of these tubes and flexible tubing to a manifold which, in turn, was connected to the steady-state apparatus which provides for maintenance of steady-state levels of CO_2 or $^{14}CO_2$ in various mixtures of air or nitrogen[24]. During incubation, the flasks were subjected to a gentle swirling motion. Additions or removals from the reconstituted system can be made by hypodermic needles through the serum stoppers while this motion is momentarily stopped. In all cases, we have employed an atmosphere of nitrogen or nitrogen mixed

with prescribed amounts of CO_2; we have found that with the reconstituted system better sustained rates of photosynthesis are obtained under nitrogen than under air. When appropriate, illumination was achieved by lights which are under the transparent bottom of the shaker apparatus. A constant temperature of 20°C was maintained by recirculating water from a thermostat controlled bath to the shaker bath into which the individual reaction flasks were immersed.

The "preincubation" procedure is defined as follows: to the reaction flasks in a final volume of 0.5 ml were added the soluble chloroplast protein solution (0.36 ml), 6-PGluA or NADPH (concentrations are given in figures), creatine phosphate 2.5 mM, creatine phosphate kinase (1 unit) and Rib-5-P (0.5 mM). The flasks were then flushed for 5 minutes with nitrogen. Then ATP (2.5 mM) was added, to complete the Ribul-1,5-P_2 generating system. In experiments where Ribul-1,5-P_2 was used as the substrate, the Ribul-1,5-P_2 generating system was omitted and the Ribul-1,5-P_2 (0.5 mM) was added at the end of the nitrogen flushing period. After 1 minute, $NaH^{14}CO_3$ (1 mM) was added, or the flasks were opened to the steady-state apparatus and 0.04% $^{14}CO_2$.

In the "non-preincubation" procedure, the same additions were made, except that the complete Ribul-1,5-P_2 generating system, including ATP, was added before the nitrogen flushing period and effectors were added after the nitrogen flushing. In experiments where Ribul-1,5-P_2 was used as a substrate, the Ribul-1,5-P_2 generating system was replaced by Ribul-1,5-P_2.

Assay.

Assay of carboxylation activity was achieved by removing 0.025 ml samples from the reaction flasks, using a hypodermic needle and syringe, and immediately injecting the samples into 0.2 ml methanol to a final concentration of 80%. Ali-

quot samples of this killed material are then added to small vials, acidified and taken to dryness by a nitrogen stream and then taken up in scintillation fluid and counted with scintillation counting.

A variety of treatments, including variation in preincubation time, constituents, etc., were carried out and are reported with the experimental results. Where employed, 6-PGluA was obtained from Sigma Chemical Co. $NADP^+$ and NADPH from Boehringer-Mannheim.

Results

Experiments with soluble components only.

The first experiments were conducted without added chloroplast membranes but with only the soluble proteins plus various added soluble metabolites such as 6-PGluA as effectors. Studies with the purified Ribul-1,5-P_2 carboxylase had shown that while preincubation with 6-PGluA prior to addition of Ribul-1,5-P_2 resulted in increased activation of the enzyme activity, only inhibition was obtained if the 6-PGluA was added at the same time or subsequent to Ribul-1,5-P_2.[13-15] However, with the complete mixture of soluble components from the chloroplasts (Figure 1) the result is different. Even when the 6-PGluA was added after the Ribul-1,5-P_2 generating system (non-preincubation), stimulation in the rate of fixation was seen with levels of 6-PGluA ranging from 0.1 mM up to 0.5 mM. However, when the generation of Ribul-1,5-P_2 was delayed during 5 minutes flushing with N_2, by omitting ATP until after this flushing, the stimulation in the rate of carboxylation by addition of 6-PGluA was much larger. Thus, the relative difference between preincubation with 6-PGluA and non-preincubation is the same for the mixture of soluble enzymes as for the purified Ribul-1,5-P_2 carboxylase, even though with the mixture of enzymes only stimulation is observed. These experiments were carried out with 1 mM sodium bicarbonate.

When experiments were carried out with air levels of CO_2 (0.04%), stimulation was seen with addition of 6-PGluA either with preincubation or with non-preincubation (Table I). In this case, almost the same stimulation was seen during the first 10 minutes, although there was a lag of about 2 minutes in the case with the non-preincubation. If the 6-PGluA was added as long as 10 minutes after the initiation of the reaction by addition of the Ribul-1,5-P_2 generating system (Figure 2), stimulation of the rate was still observed. In fact, after 8 minutes following the addition of 6-PGluA the stimulated rate was even higher than it had been when the 6-PGluA was added before the Ribul-1,5-P_2 generating system.

Since with the Ribul-1,5-P_2 generating system, the concentration of Ribul-1,5-P_2 at any given time is not known, an experiment was carried out with both the Ribul-1,5-P_2 generating system and added 0.5 mM Ribul-1,5-P_2 (in the same flask). In this case also, large stimulation of the carboxylase rate was observed when 6-PGluA was added prior to the Ribul-1,5-P_2 and a smaller stimulation when it was added subsequent to the Ribul-1,5-P_2.

Since in these experiments with the total soluble components from the chloroplasts, some stimulation was seen with 6-PGluA even if it was added after the Ribul-1,5-P_2 generating system, and since a large stimulation was seen if the 6-PGluA was added following 10 minutes of photosynthesis (Figure 2) it was of interest to investigate the time course of stimulation by 6-PGluA in the absence of bicarbonate. An experiment was conducted (with (1 mM sodium bicarbonate) in which 6-PGluA was added at various times relative to the addition of the other components (Table II). In some flasks there was a delay in the addition of bicarbonate until 2 minutes and 8 minutes after the addition of 6-PGluA (Table II). In these cases, the amount of stimulation of the rate increased with time between the addition of

6-PGluA and the addition of sodium bicarbonate, with the stimulation following 8 minutes delay approaching that of the enzyme mixture in which there had been preincubation of the enzymes with 6-PGluA prior to the addition of the Ribul-1,5-P_2 generating system.

In the studies with the isolated carboxylase, some stimulation of the enzyme activity was seen upon preincubation with Fru-1,6-P_2. With the mixture of soluble chloroplast components, stimulation by 0.5 mM Fru-1,6-P_2 was equal or less than the smaller stimulation found with 6-PGluA (0.5 mM) when it was added after the Ribul-1,5-P_2 generating system (non-preincubation). Some stimulation was seen by Fru-1,6-P_2 whether it was added before or after the Ribul-1,5-P_2 generating system, and even smaller stimulation (slightly above the control) was seen with addition of fructose-6-phosphate, either before or following addition of the Ribul-1,5-P_2 generating system.

Experiments with soluble components plus membrane systems.

A much larger activation of the enzyne than any of those reported so far was obtained in the presence of the chloroplast lamellae in the light with added ferredoxin (Figure 3). The initial slope of the carboxylation reaction rate is 35 times greater with ferredoxin and light and lamellae than it is in the dark control. Essentially no further activation is seen with the addition of 6-PGluA to the already highly stimulated rate with light and ferredoxin. If added ferredoxin is omitted, somewhat smaller rates are obtained with light and lamellae in the soluble system only. There is, of course, some ferredoxin already presence since all the soluble components of the chloroplasts are in the material used. In fact, the concentration of ferredoxin in the flask should be approximately one-seventh that which it would be in intact chloroplasts. For comparison

with these rates, the dark rate in the presence of 6-PGluA is also shown and is something less than half the maximum rate obtained with ferredoxin and light. This experiment was carried out with sodium bicarbonate (1 mM), with the Ribul-1,5-P_2 generating system, and with all soluble constituents added during 5 minutes preillumination with nitrogen before the addition of bicarbonate (1.0 mM). No $NADP^+$ was added to the soluble system as is usually done in our reconstitution experiments. There is, of course, some $NADP^+$ present as a consequence of the lysing of the original chloroplasts.

Another demonstration of the effect of light and dark operating via reduced ferredoxin, and perhaps reduced $NADP^+$, is shown in Figure 4. In this case, Ribul-1,5-P_2 was used directly as a substrate rather than the Ribul-1,5-P_2 regenerating system. The stimulated rate (Figure 4)is many times greater than the rate in the dark in the presence of ferredoxin. When the light is turned off, the rate of the carboxylation reaction drops immediately to the dark control rate and remains very low until the light is turned on again, at which point there is an immediate resumption of the stimulated rate in the presence of ferredoxin and light.

Given the previously reported stimulation of the carboxylase activity by NADPH, the data just reported raise the question of whether the stimulation of the carboxylase activity is due to reduced ferredoxin or to NADPH. In any experiment where we employ the soluble components of the lysed chloroplasts, $NADP^+$ plus the enzyme responsible for its reduction by reduced ferredoxin in the light will be present, even though we may omit the added $NADP^+$ usually employed in such reconstitution experiments.

When the soluble enzyme system without lamellae, light, or added ferredoxin is exposed to various concentrations of NADPH, both with preincubation and without preincubation, stimulation of the carboxylase

activity is seen (Table III). In this case, a large stimulation was observed with the NADPH present during a preincubation period and prior to the addition of the Ribul-1,5-P_2 regenerating system. A smaller stimulation was observed with the NADPH was added subsequent to the Ribul-1,5-P_2 regenerating system. The actual activated rate of the carboxylase is considerably less than that observed with lamellae and ferredoxin in the light. However, it must be kept in mind that in this case the NADPH once oxidized is not regenerated, and so it is to be expected that the activation effect may disappear or may be decreased as a consequence of NADPH oxidation.

When purified Ribul-1,5-P_2 carboxylase (obtained from Sigma Chemical Co.) was assayed with Ribul-1,5-P_2, 1 mM sodium bicarbonate and with added ferredoxin and chloroplast lamellae in the light and in the dark, low CO_2 fixation rates were seen and no substantial difference in rate was observed between the light and the dark. From this it would appear that reduced ferredoxin by itself is incapable of activating the isolated enzyme. However, one cannot exclude the possibility that there could be some soluble protein factor in the chloroplast that is required for activation of the enzyme by reduced ferredoxin.

Time course of Ribul-1,5-P_2 inactivation of Ribul-1,5-P_2 carboxylase.

Experiments carried out with only the soluble components of the chloroplasts plus Ribul-1,5-P_2, or Ribul-1,5-P_2 generating system, but without any other added effectors give information about the time required for inactivation of the enzyme by Ribul-1,5-P_2 in the absence of effectors. The supernatant chloroplast fixation plus the ATP generation system was flushed with N_2 for 5 minutes. When ribose-5-phosphate and bicarbonate were then added, there was a very rapid fixation of CO_2 for about 1 minute and then almost no fixation for the remaining time (Fig. 5). In contrast, if the ribose-5-phosphate was added prior to the 5 minutes nitrogen

flushing (to generate Ribul-1,5-P_2), then addition of bicarbonate resulted in a much lower rate of CO_2 fixation. This lower rate was sustained for some period of time. With Ribul-1,5-P_2 present during the 5 minutes nitrogen flushing a similar result was observed, although, in this case, the total fixation during the first couple of minutes was only about half as great. Following that time, the fixation was virtually zero. From these results it is clear that when the carboxylase, mixed with the other soluble components from the chloroplasts, is exposed to Ribul-1,5-P_2 for 5 minutes in the absence of effectors or bicarbonate (under nitrogen), the enzyme activity is greatly reduced. On the other hand, if the enzyme activity, mixed with the soluble components, is exposed for five minutes to nitrogen and the Ribul-1,5-P_2 is added simultaneously with the bicarbonate, the enzyme remains active for 1-2 minutes before it becomes inactivated. Thus, there is a short large fixation followed by inactivation.

Further study of this inactivation of the enzyme following an initial burst of fixation was made by varying the time of addition of bicarbonate relative to the time of addition of Ribul-1,5-P_2 following a period of flushing the soluble components with nitrogen (Table IV). With Ribul-1,5-P_2 added simultaneously with bicarbonate, or either 2 or 5 minutes after addition of bicarbonate, the full initial burst of fixation was seen after which the enzyme was converted to its nearly inactive form, and only slow fixation took place. When the Ribul-1,5-P_2 was added 2 minutes prior to the addition of bicarbonate, about half of the initial burst of fixation took place, after which the enzyme was essentially inactive. With addition of the bicarbonate as long as 5 minutes after the addition of Ribul-1,5-P_2, the enzyme was almost completely inactive and there was no initial burst of CO_2 fixation. The inactivation of the enzyme by Ribul-1,5-P_2 (0.5 mM) in the absence of CO_2 or effectors thus has a halftime of about 2 minutes.

Discussion

Several aspects of regulation of Ribul-1,5-P_2 carboxylase activity previously described for the isolated and purified enzyme are also observed with carboxylase activity in the reconstituted chloroplast system, or in the separated soluble components of chloroplasts. However, there are also some interesting differences.

The role of effectors in activating the enzyme seems to be even more important to the activity of the carboxylase in the total soluble components or reconstituted system than it is with the isolated and purified enzyme. With the isolated enzyme, preincubation with magnesium and bicarbonate was sufficient to permit a substantial activity to be established upon addition of Ribul-1,5-P_2 and much of this activity was maintained for 20 minutes or more without other effectors[14]. With the complete soluble component system, but without added effectors or light and lamellae, exposure of the enzyme activity to Ribul-1,5-P_2 results in inactivation after about 2 minutes in spite of the presence of Mg^{2+} and bicarbonate. Thus it seems clear that with the reconstituted system activity of the carboxylase is dependent upon the presence of either reduced $NADP^+$ or 6-PGluA, although the possibility of activation by reduced ferredoxin is not completely ruled out.

With the isolated enzyme, exposure to 6-PGluA or to a lesser extent Fru-1,6-P_2, during the preincumbation period with 10 mM Mg^{2+} and 1 mM bicarbonate resulted in considerable activation of the enzyme activity during subsequent assay with added Ribul-1,5-P_2. However, addition of either 6-PGluA or Fru-1,6-P_2 simultaneously with Ribul-1,5-P_2 resulted in competitive inhibition. Moreover, NADPH could not activate the isolated enzyme unless it was added during the preincubation with bicarbonate and magnesium.[13-15]

With the enzyme activity in the complete soluble components from the chloroplasts, activation is achieved even if the effector, whether 6-PGluA or NADPH, is added subsequent to the presentation of the enzyme with Ribul-1,5-P_2. The actual extent of the activation seems to vary in a rather complex manner with the order of the various additions and treatments of the system. Such complexity of behavior is perhaps understandable if one remembers that all of the soluble components of the chloroplast are present. Thus, for example, in the light with ferredoxin the carbon reduction cycle is operating and regenerating various metabolic components of the system at any given time. Regardless of these complications, it seems clear that the effectors NADPH, or 6-PGluA, are of great importance in maintaining the activity of the Ribul-1,5-P_2 carboxylase in the reconstituted system. Presumably they are of similar importance in the intact chloroplasts. While it cannot be completely decided that activation in the light is a consequence of NADPH and not of reduced ferredoxin, the simplest assumption, in view of all of the known properties of the enzyme, is that the action of light and of chloroplast membranes in activating the enzyme is via the reduction of $NADP^+$ by reduced ferredoxin. Certainly this seems to be suggested by the failure of reduced ferredoxin to activate the purified isolated carboxylase.

It should be noted that the activation of the carboxylase by addition of reduced ferredoxin or 6-PGluA subsequent to addition of Ribul-1,5-P_2 is dependent upon the presence of bicarbonate and magnesium. Thus, the hypothesis remains tenable that inactivation of the carboxylase by Ribul-1,5-P_2 in the absence of CO_2 or bicarbonate[13] is a mechanism for raising the binding constant for CO_2 and, more importantly, for O_2, as a defense against

photorespiration stemming from the oxidation of Ribul-1,5-P_2 ~~remains tenable~~. It should be noted that in the experiments (Fig. 1 dashed line) where the enzyme was incubated with Ribul-1,5-P_2 in the absence of bicarbonate and the effector was then added prior to addition of bicarbonate, the activation of the enzyme was considerably less than it was when the effectors were subsequently added after bicarbonate addition (Fig. 2). Thus it is clear that presentation of Ribul-1,5-P_2 to the enzyme in the absence of bicarbonate and effectors converts it into an inactive form from which its activity only slowly recovers, even with effectors and bicarbonate and magnesium present. If, as we propose, the binding of both CO_2 and O_2 by the enzyme in vivo is decreased by very low CO_2 pressure and high Ribul-1,5-P_2 concentration, then the activity can be restored with the return of CO_2 at air levels in the presence of either 6-PGluA (in the dark) or NADPH (in the light).

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TABLE I

Activation of ribulose-1,5-diphosphate carboxylase in the soluble chloroplast components by 6-phosphogluconate (0.5 mM) in the presence of 0.04% $^{14}CO_2$.

Assay conditions were the same as described in Fig. 1, except that $NaH^{14}CO_3$ was replaced by a gas mixture of 0.04% $^{14}CO_2$ (specific activity 20 μCi/μmole) and 99.96% nitrogen, which was continuously supplied by the steady-state apparatus.

Treatment	$^{14}CO_2$ fixation (dpm) for	
	2 min	10 min
no 6-PGluA	500	4,000
non-preincubation with 6-PGluA	1,000	16,800
preincubation with 6-PGluA	2,500	22,000

Table II

Time course of stimulation of ribulose-1,5-diphosphate carboxylation in the soluble chloroplast component by 6-phosphogluconate (0.5 mM) as a function of time of addition of bicarbonate.

Preincubation and non-preincubation conditions were the same as described under "Methods". However, the addition of 1 mM $NaH^{14}CO_3$ in the case of non-preincubation with 0.5 mM 6-PGluA was simultaneous with 6-PGluA or was 2 or 8 minutes later.

Treatment	$^{14}CO_2$ fixation (dpm) for		
	2 min	10 min	20 min
no 6-PGluA	2,300	11,500	21,000
preincubation with 6-PGluA	12,000	92,000	136,000
non-preincubation with 6-PGluA: addition of $NaH^{14}CO_3$ after			
0 min	4,500	36,000	58,000
2 min	8,000	60,000	86,000
8 min	10,000	74,000	115,000

Table III

Activation of ribulose-1,5-diphosphate carboxylase in the soluble chloroplast components by NADPH in the presence of 1 mM $NaH^{14}CO_3$ and a Ribul-1,5-P_2 generating system

Treatment	$^{14}CO_2$ fixation (dpm) for		
	2 min	10 min	20 min
no NADPH	7,000	19,000	28,000
preincubation with:			
0.01 mM NADPH	10,000	24,000	40,000
0.1 mM NADPH	13,500	34,000	55,000
0.5 mM NADPH	18,000	41,500	64,000
1.0 mM NADPH	35,000	71,000	86,000
non-preincubation with:			
1.0 mM NADPH	11,500	28,500	45,000

Table IV

Inactivation of ribulose-1,5-diphosphate carboxylase in the soluble chloroplast components by Ribul-1,5-P_2 during varying times of addition of Ribul-1,5-P_2 (0.5 mM) relative to the time of addition of $NaH^{14}CO_3$ (1 mM)

The substrate for the carboxylation reaction was supplied as Ribul-1,5-P_2 (0.5 mM). All effectors were omitted. The additions of Ribul-1,5-P_2 (except for the first line) occurred after the 5 minutes nitrogen flushing period.

Treatment	$^{14}CO_2$ fixation (dpm) for		
	1 min	2 min	10 min
addition of Ribul-1,5-P_2:			
prior to N_2 flushing	200	320	690
5 min prior to $NaH^{14}CO_3$	77	78	78
2 min prior to $NaH^{14}CO_3$	16,600	20,000	20,000
simultaneously with $NaNaH^{14}CO_3$	26,700	34,100	34,700
2 min after addition of $NaH^{14}CO_3$	35,000	35,000	35,500
5 min after addition of $NaH^{14}CO_3$	31,300	32,000	33,000

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FIGURE CAPTIONS

Figure 1

Activation of ribulose-1,5-diphosphate carboxylase in the soluble chloroplast components by 6-phosphogluconate in the presence of 1 mM $NaH^{14}CO_3$ (specific activity 17 μCi/μmole) and the Ribul-1,5-P_2 generating system. 6-Phosphogluconate (0.5, 0.1 mM) was added to the soluble chloroplast protein solution either before addition of the Ribul-1,5-P_2 generating system (preincubation: solid lines; see "Methods") or after the generation of Ribul-1,5-P_2 (non-preincubation: dashed lines). The generation of Ribul-1,5-P_2 from 0.5 mM Rib-5-P was initiated upon addition of ATP.

Figure 2

Activation of ribulose-1,5-diphosphate carboxylase in the soluble chloroplast components by 6-phosphogluconate in the presence of 0.04% $^{14}CO_2$ (specific activity 20 μCi/μmole) and the Ribul-1,5-P_2 generating system.

- ✕ preincubation with 6-PGluA (0.5 mM)
- ○ preincubation with 6-PGluA (0.1 mM)
- △ 6-PGluA (0.5 mM) added after 10 minutes photosynthesis
- □ 6-PGluA (0.1 mM) added after 10 minutes photosynthesis
- ● Control. no 6-PGluA addition

See "Methods" for description of "preincubation".

Figure 3

Activation of ribulose-1,5-diphosphate carboxylase in a reconstituted chloroplast system. Effect of light and 6-phosphogluconate (0.5 mM). In addition to the components of the standard assay procedure described

under "Methods", the reaction mixture contained the following components in a final volume of 0.5 ml: spinach ferredoxin (Fd) 50 µg (added simultaneously with the soluble protein solution); chloroplast lamellae with 29 µg chlorophyll (added after addition of ATP). The reaction mixtures were preilluminated for 5 minutes prior to the addition of 1 mM $NaH^{14}CO_3$. Controls were kept in the dark as indicated. Reaction mixtures with 6-PGluA were all preincubated.

Figure 4

Activation of ribulose-1,5-diphosphate carboxylase in a reconstituted chloroplast system in the presence of continuous light and with periods of light, dark and then light.

The test conditions were the same as in Fig. 3, except that the Ribul-1,5-P_2 generating system is replaced by 0.5 mM Ribul-1,5-P_2. The light-dark and dark-light transitions are indicated by arrows.

Figure 5

Time course of inactivation of ribulose-1,5-diphosphate carboxylase in the soluble chloroplast components by Ribul-1,5-P_2 in the absence of effectors. Ribul-1,5-P_2 or the Ribul-1,5-P_2 generating system (Rib-5-P as primer) were added either prior to the 5 minutes nitrogen flushing period (solid line) or after the flushing period (dashed line). The experiment was carried out with 1 mM $NaH^{14}CO_3$.

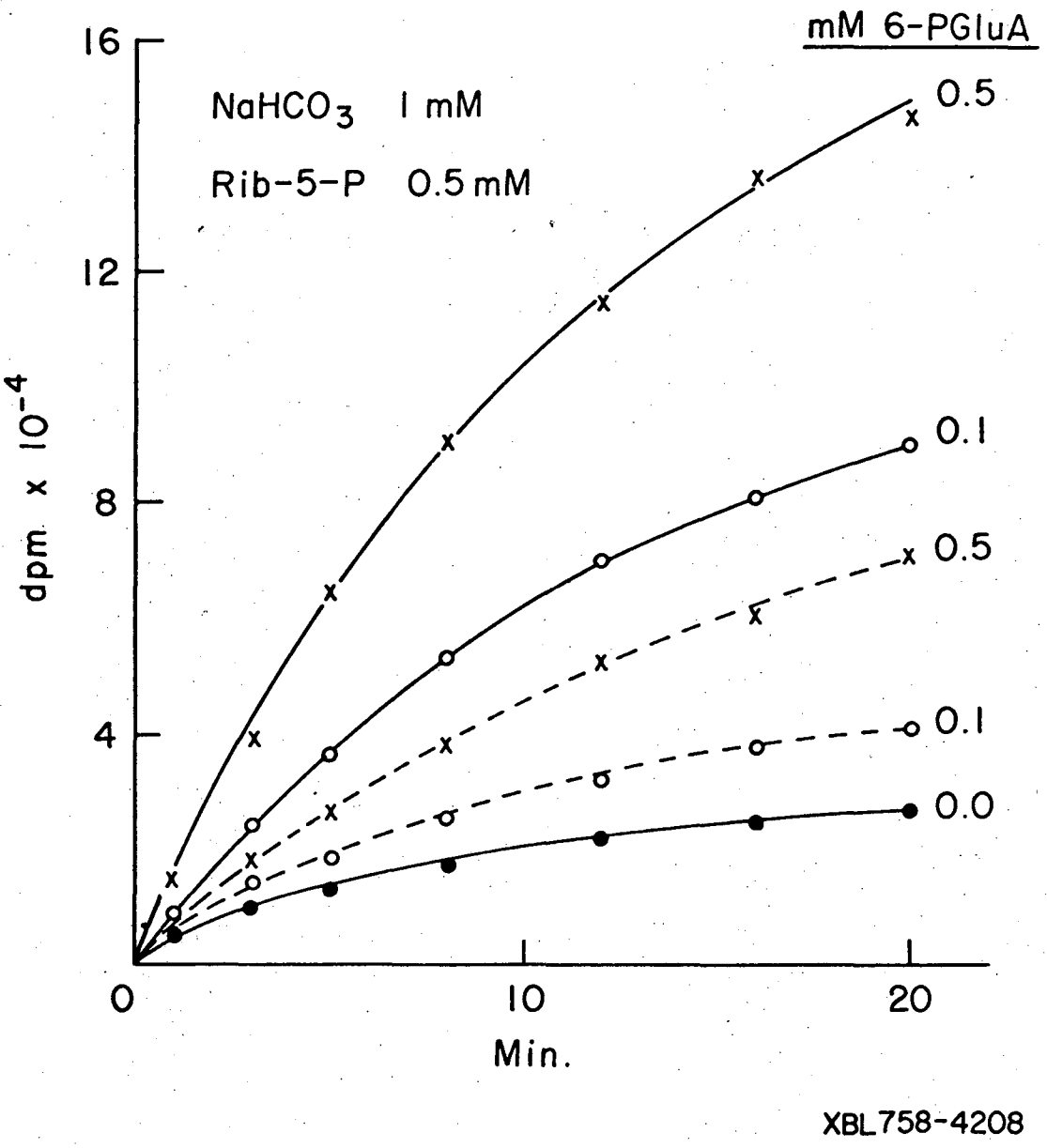
mM 6-PGluA
NaHCO3 1 mM
Rib-5-P 0.5 mM
0.5
0.1
0.5
0.1
0.0
16
12
8
4
0
10
20
dpm x 10-4
Min.
XBL758-4208

Fig. 1

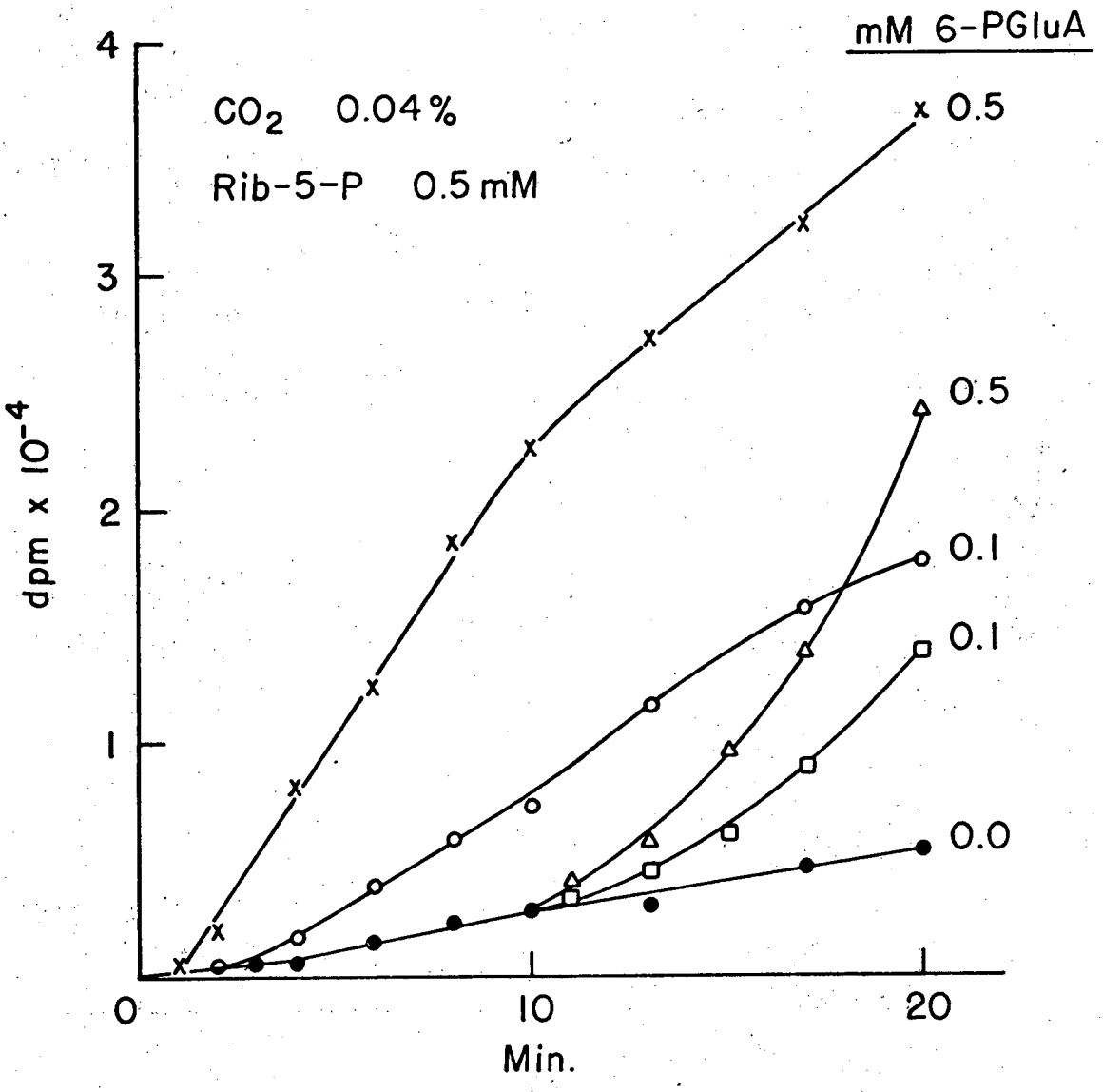

Fig. 2

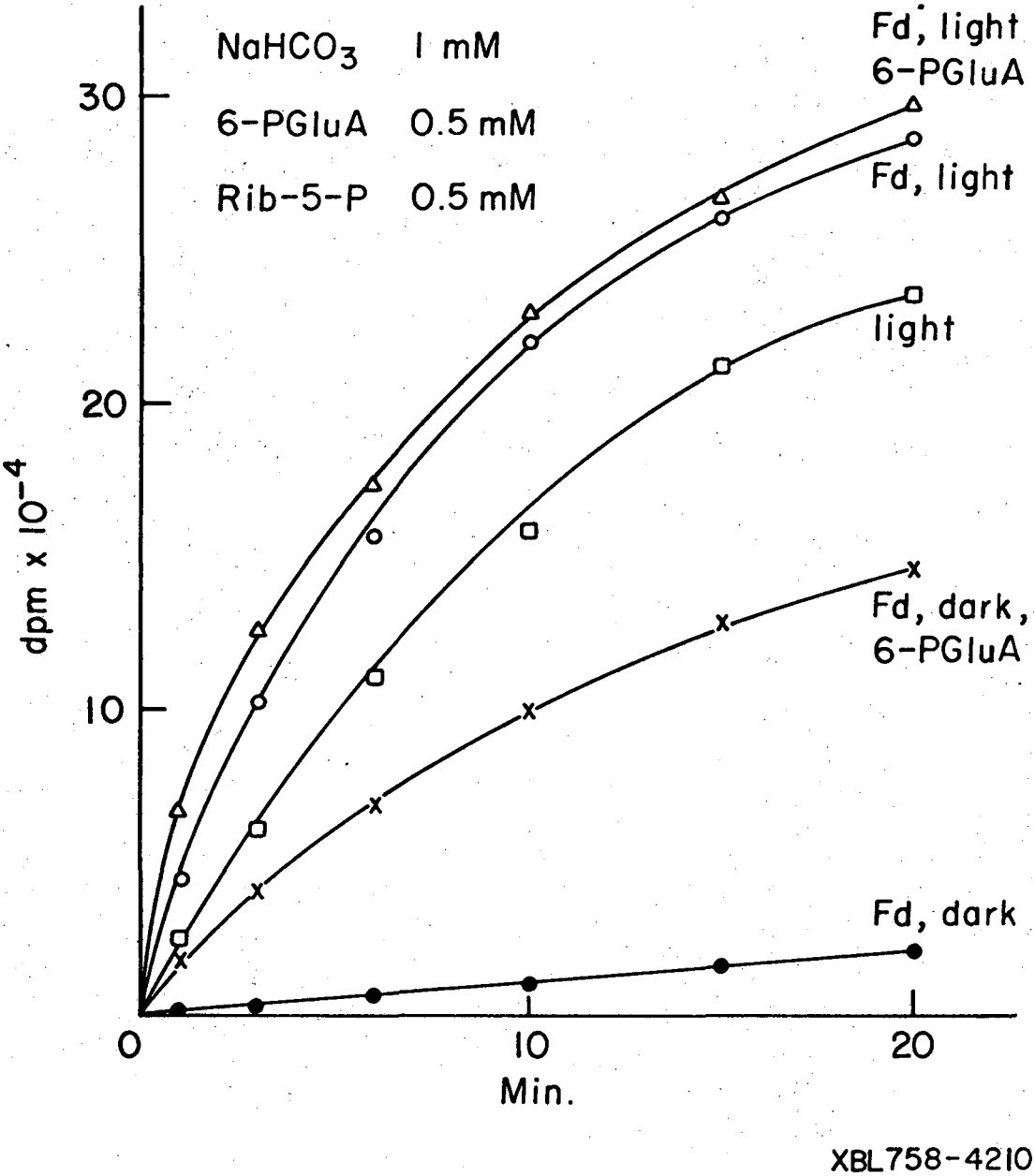
NaHCO3 1 mM
6-PGluA 0.5 mM
Rib-5-P 0.5 mM
Fd, light
6-PGluA
Fd, light
light
Fd, dark,
6-PGluA
Fd, dark
dpm x 10-4
30
20
10
0
10
20
Min.
XBL758-4210

Fig. 3

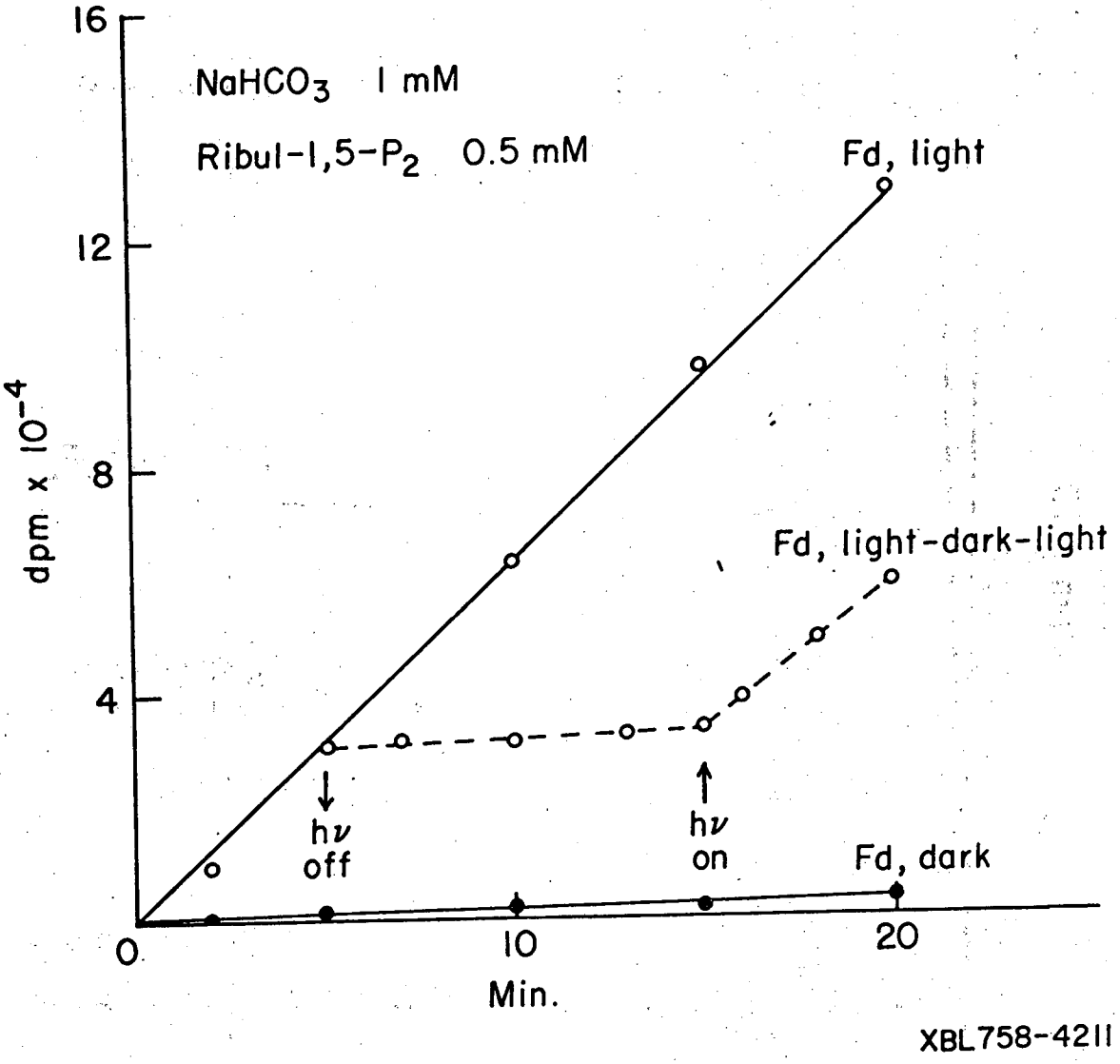
16
12
8
4
0
dpm x 10^{-4}
$NaHCO_3$ 1 mM
Ribul-1,5-P_2 0.5 mM
Fd, light
Fd, light-dark-light
Fd, dark
hν off
hν on
10
20
Min.
XBL758-4211

Fig. 4

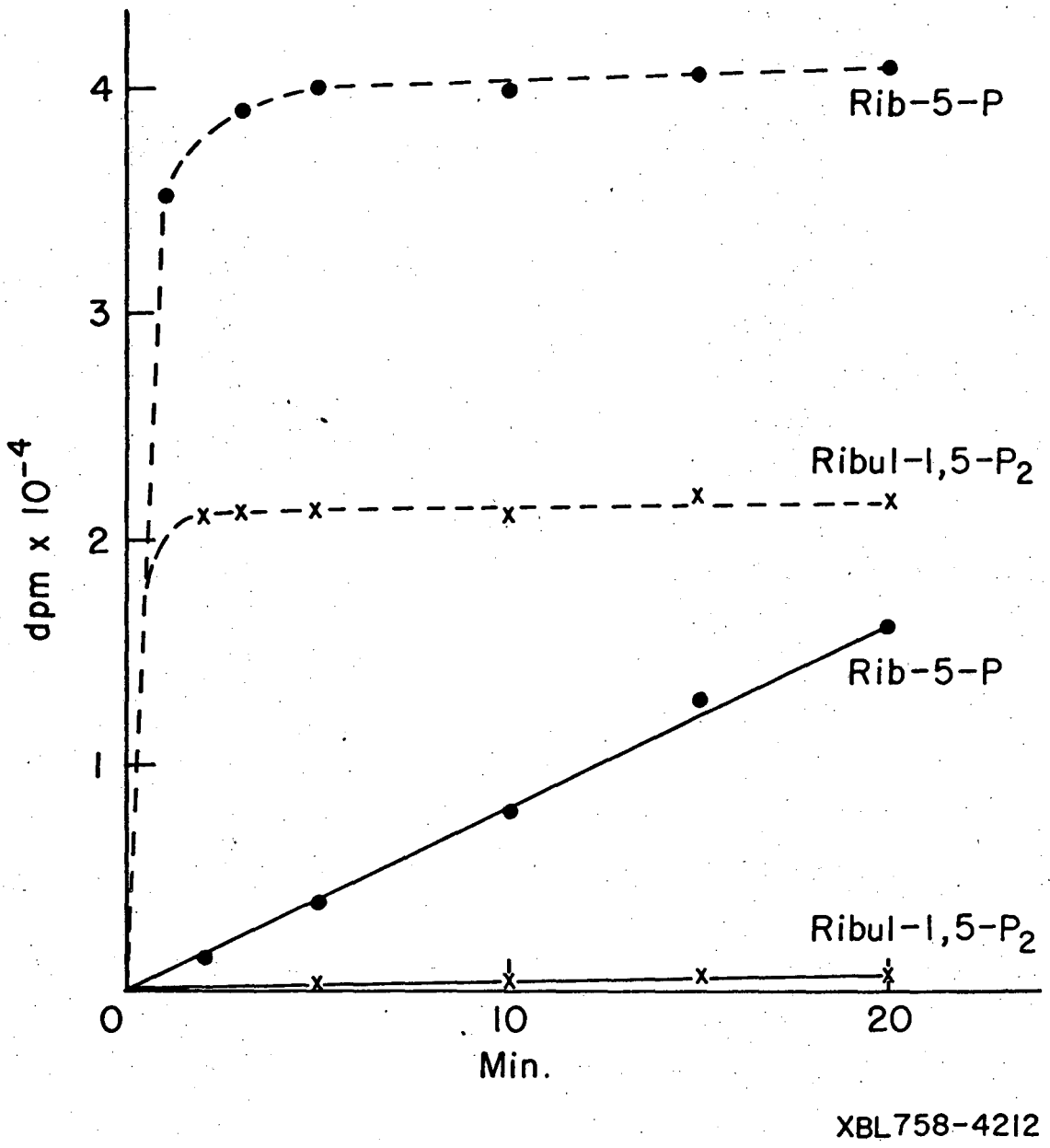
4
3
2
1
0
10
20
Min.
dpm x 10^{-4}
Rib-5-P
Ribul-1,5-P_2
Rib-5-P
Ribul-1,5-P_2
XBL758-4212

Fig. 5

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