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NADPH/NADP$^+$ RATIOS IN PHOTOSYNTHESIZING RECONSTITUTED CHLOROPLASTS

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SUMMARY

Levels of reduced and oxidized triphosphopyridine nucleotides have been determined in reconstituted spinach chloroplasts and compared with levels in whole isolated chloroplasts during photosynthesis and darkness. The ratio of NADPH/NADP$^+$ reaches values slightly above 1.0 at the beginning of photosynthesis, less than half the ratio attained with whole chloroplasts. Nonetheless these lower ratios are sufficient to maintain high rates of photosynthetic carbon dioxide fixation and reduction, which are comparable in the reconstituted chloroplasts to the rates found with whole chloroplasts. As with whole chloroplasts there is a decline in the ratio of NADPH/NADP$^+$ as a function of time of photosynthesis. The effect of addition of bicarbonate (6 mM) in causing a transient drop in the ratio of NADPH/NADP$^+$ is described and discussed in terms of the reversibility of the reduction of 3-phosphoglycerate to triose phosphate. The ratio NADPH/NADP$^+$ can be improved by the addition of more lamellae either before or during the course of photosynthesis, and this improvement in ratio is accompanied by an improved rate of CO_2 fixation or a more sustained rate of CO_2 fixation with time of photosynthesis. The importance of NADPH/NADP$^+$ ratio not only to the reduction of 3-phosphoglycerate to triose phosphate but also to the activation of the ribulose-1,5-diphosphate carboxylase-mediated step is discussed.

INTRODUCTION

Conversion of carbon dioxide to sugar phosphates by the soluble enzymes during photosynthesis requires ATP and NADPH, which are regenerated by photoelectron transport and photophosphorylation reactions in the internal chloroplast membranes. Thus, these carriers of reducing equivalents and chemical energy are the direct links between the photochemical and the synthetic reactions of photo-

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synthesis. Early experiments showed that isolated chloroplast membranes are capable of reducing $NADP^+$ photochemically [1–3]. Furthermore, illuminated chloroplasts could bring about the reduction of carbon dioxide to the level of sugar phosphates [4]. Also, $NADP^+$ could be reduced in broken chloroplasts in the dark when a source of electrons in the form of hydrogen, bacterial hydrogenase and soluble ferredoxin was added [5].

From knowledge of the enzymatic reactions of the reductive pentose phosphate cycle [6], it was recognized that NADPH was required for the reduction of 3-phosphoglyceric acid to glyceraldehyde 3-phosphate, which includes the step mediated by $NADP^+$-dependent triose-phosphate dehydrogenase [7]. There are indications that NADPH and $NADPH/NADP^+$ ratios play an important role in the regulation of enzymes of the reductive and oxidative pentose phosphate cycle. With the oxidative cycle, the key regulated step, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) is controlled by the ratio $NADPH/NADP^+$, as mediated by ribulose 1,5-diphosphate (Rib-1,5-P_2) [8]. Moreover, studies with ribulose-1,5-diphosphate carboxylase (EC 4.1.1.39) have demonstrated that NADPH is a powerful effector for this enzyme [9, 10]. Thus, it appears that the level of NADPH may play an important role in controlling the initial step for fixation of carbon dioxide.

Heber and Santarius [11] reported that in isolated spinach chloroplasts there is a rapid increase in the $NADPH/NADP^+$ ratio during the first 30 s of light, followed by a return to its original level after 3 or 4 min and a further decline when the light was turned off. In contrast, we reported that $NADPH/NADP^+$ ratios in whole isolated spinach chloroplasts have steady values of 0.3 in the dark but reach values of up to 2.5 in the light and are maintained at those high values for as long as 10–20 min [8]. This difference can be attributed mainly to improvements in the techniques for the isolation of whole chloroplasts [12–14] and improvements in the method of determining the levels of reduced and oxidized triphosphopyridine nucleotides in biological materials.

In recent years, a number of investigations have been made of biochemical properties of reconstituted chloroplast systems, to learn more about the interaction of membrane bound system with the soluble enzymes [15–18]. Bassham et al. [16] were able to demonstrate operation of the entire reductive pentose phosphate cycle from 3-phosphoglycerate to ribulose 1,5-diphosphate in such a system. In this method [16], the previously isolated chloroplasts are disrupted by lysis, and the separated membranes and soluble components are reconstituted with certain added co-enzymes which allow photosynthesis to proceed in vitro. In this preparation it is possible to add various cofactors and other chemicals without concern for a permeability barrier imposed by an outer chloroplast membrane. Therefore, it becomes possible to study directly the role of NADPH and $NADP^+$ in regulating the operation of the reductive pentose phosphate cycle of photosynthesis. It is necessary, however, to add additional ferredoxin and $NADP^+$ to these preparations in order to have sufficient concentrations of electron transport cofactors.

MATERIALS AND METHODS

Reconstituted chloroplast system. Spinach chloroplasts were isolated according to methods described previously [12]. The chloroplasts were disrupted by lysis.

The membrane fraction and the soluble components were separated by centrifugation. The membranes were washed and were then recombined with soluble components in a suspension in which the soluble components were increased relative to the membrane components by a ratio of 14 : 1 compared with whole chloroplasts [16]. In some experiments (described below) the amount of soluble components relative to the lamellae was reduced by a factor of 4 (to a ratio of 3.5).

Materials. ADP, NADP$^+$, 3-phosphoglycerate, glucose and hexokinase were purchased from Cal-Biochem.

Fixation of $^{14}CO_2$. The incubation with NaH^{14}CO$_3$ (6 mM; spec. act. 17 Ci/mol) and subsequent determination of rate of photosynthesis by incorporation of ^{14}CO$_2$ into acid-stable compounds were as described previously [12, 16].

Assay of NADPH and NADP$^+$. The samples for assaying NADPH and NADP$^+$ were removed by hypodermic needle from the incubation flasks and were killed in HCl for determination of NADP$^+$, or in NaOH for determination of NADPH. They were then immediately assayed according to the previously described method [8].

Chlorophyll determination. Chlorophyll was determined according to Vernon [19].

The detailed conditions for incubation of reconstituted chloroplasts are described in each case with the data.

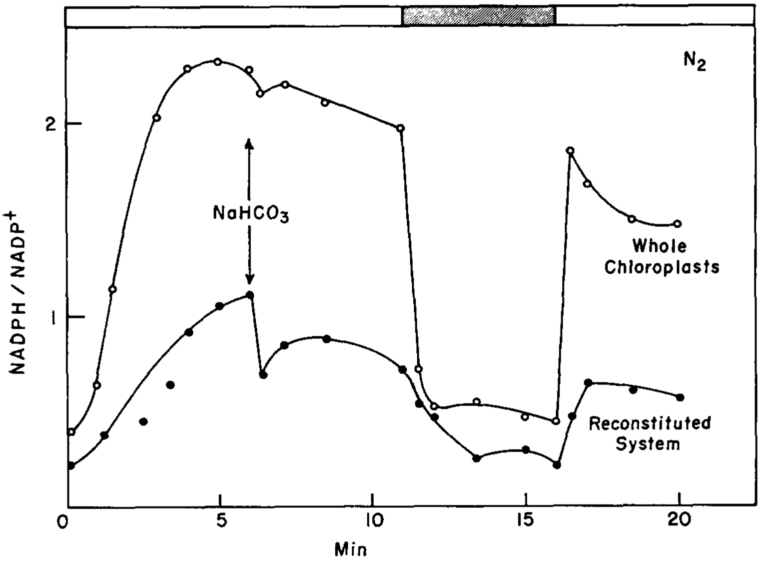

Fig. 1. NADPH/NADP$^+$ ratios in whole chloroplasts and in a reconstituted chloroplast system during preillumination followed by a light-dark-light period. Addition of NaH^{14}CO$_3$ (6 mM, specific activity 17 Ci/mol) and the length of the dark period are indicated by an arrow and a dark bar, respectively. The experiment was run under nitrogen. 3-phosphoglycerate (1 mM) was provided as primer of photosynthetic intermediates in the reconstituted chloroplast system. The total amount of triphosphopyridine nucleotides was 50 nmol/mg chlorophyll (whole chloroplasts) and 2.1 μmol/mg chlorophyll (reconstituted chloroplast system).

RESULTS

Comparison of reconstituted system with whole chloroplasts

With photosynthesizing whole chloroplasts, the ratio of NADPH/NADP$^+$ typically rises above 2 and only slowly declines with time. When the light is turned off with whole chloroplasts, the ratio drops very rapidly to less than 0.5. When the light is turned on, the ratio again rises to high values (Fig. 1). The ratio is much lower with the reconstituted system, barely reaching values of 1.0. Addition of sodium bicarbonate (6 mM) results in very little change in the NADPH/NADP$^+$ ratio for whole chloroplasts but causes a substantial initial drop in the ratio for the reconstituted system. Towards the end of a 20 min period of photosynthesis with a reconstituted system, values of the ratio may be as low as 0.5. It is important to note that the rate of CO_2 fixation is essentially as high on the basis of fixation per mg of chlorophyll in the reconstituted system as in the whole chloroplasts. For the experiment giving the data shown in Fig. 1, for example, the rate with whole chloroplasts was 129 μmol CO_2 fixed/mg chlorophyll per h, whereas for the reconstituted system the rate was 117 μmol CO_2 fixed/mg chlorophyll per h. Thus it seems clear that, under

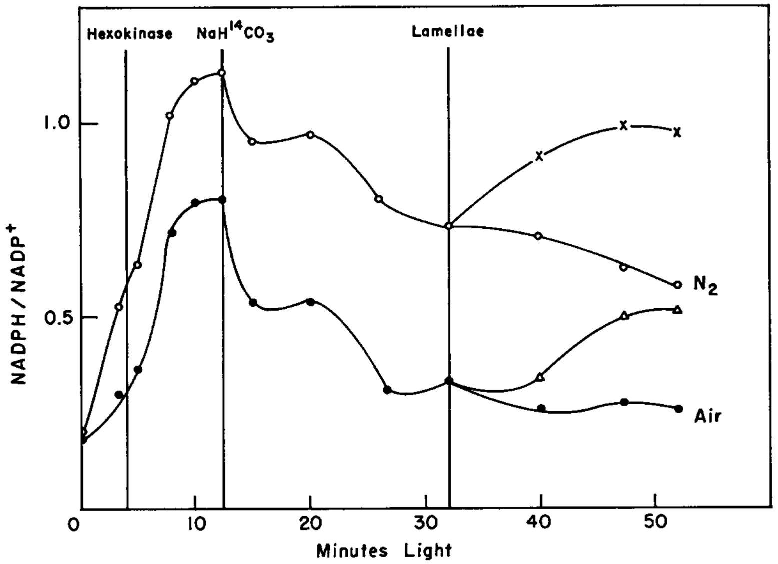

Fig. 2. The effect of $NaH^{14}CO_3$ (6 mM) addition and increased concentration of chloroplast lamellae after 20 min photosynthesis on the NADPH/NADP$^+$ ratio in a reconstituted chloroplast system. The system was provided with glucose (0.5 mM) and hexokinase (100 units) as a primer system instead of 3-phosphoglycerate. Glucose was added just before the preillumination period; the time of addition of hexokinase and $NaH^{14}CO_3$ are indicated in the figure. The chlorophyll content for the first 32 min illumination was 53 μg/ml reaction mixture, and after 20 min photosynthesis it was increased 4 times. "1 × chloroplast lamellae" refers to our usual reconstituted system [16] in which the ratio of soluble components in the system to lamellae is 14 times greater than in the original chloroplasts. With "4 × chloroplast lamellae", this ratio is 3.5. (○) NADPH/NADP$^+$ ratios under nitrogen (1 × chloroplast lamellae); (×) NADPH/NADP$^+$ ratios under nitrogen (4 × chloroplast lamellae); (●) NADPH/NADP$^+$ ratios under air (1 × chloroplast lamellae); (△) NADPH/NADP$^+$ ratios under air (4 × chloroplast lamellae).

the physiological conditions employed, the reconstituted system is able to carry out all of the reactions of the photosynthetic carbon reduction cycle at high rates despite the relatively lower NADPH/NADP$^+$ ratios.

Effect of increasing the amount of lamellae relative to soluble components

Since the ratio NADPH/NADP$^+$ was considerably lower in the reconstituted system than in chloroplasts, we investigated the effect of adding additional internal chloroplasts membranes or lamellae in order to increase the rate of electron transport from the light reactions to the reduction of NADP$^+$. In an experiment employing glucose and hexokinase as primer, additional lamellae sufficient to increase the amount of chlorophyll by a factor of 4 were added 20 min after the initiation of photosynthesis by addition of bicarbonate (Fig. 2). With the reconstituted system either under an atmosphere of nitrogen or under an atmosphere of air (where lower ratios of NADPH/NADP$^+$ and rates of photosynthesis are observed) the addition of lamellae resulted in a substantial increase in the ratio NADPH/NADP$^+$ over a period of time. Although the total fixation rate increased upon the addition of lamellae (Fig. 3), the actual fixation rate calculated on a basis of per mg chlorophyll would, of course, be much decreased, since the amount of chlorophyll had been increased by a factor of 4. Thus, there might have been some dependence of the CO_2 fixation rate on the ratio, NADPH/NADP$^+$, although it must be noted that additional lamellea

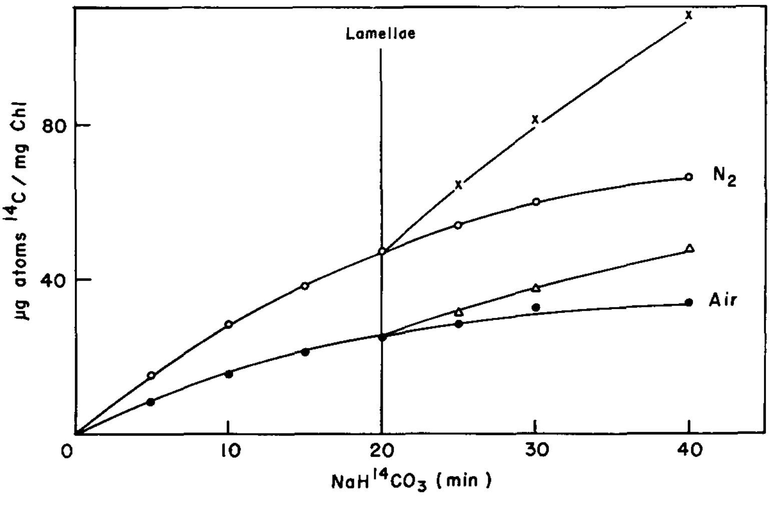

Fig. 3. Kinetics of CO_2 fixation by a reconstituted chloroplast system under air and nitrogen and after increase of the chloroplast lamellae during photosynthesis. Samples for the determination of CO_2 fixation were taken from the same reaction mixtures used for the pyridine nucleotide measurements in Fig. 2. "1 × chloroplast lamellae" refers to our standard reconstituted system [16] in which the ratio of soluble components to lamellae is 14 times greater than in the original chloroplasts. With "4 × chloroplast lamellae" this ratio is 3.5. $^{14}CO_2$ fixation rate is given in terms of μgatoms ^{14}C fixed per mg chlorophyll in the usual reconstituted system (14:1). The rate for "4 × chloroplast lamellae" is 0.25 that shown on a basis of ^{14}C fixed per mg chlorophyll. (○) NADPH/NADP$^+$ ratios under nitrogen (1 × chloroplast lamellae); (×) NADPH/NADP$^+$ ratios under nitrogen (4 × chloroplast lamellae); (●) NADPH/NADP$^+$ ratios under air (1 × chloroplast lamellae); (△) NADPH/NADP$^+$ ratios under air (4 × chloroplast lamellae).

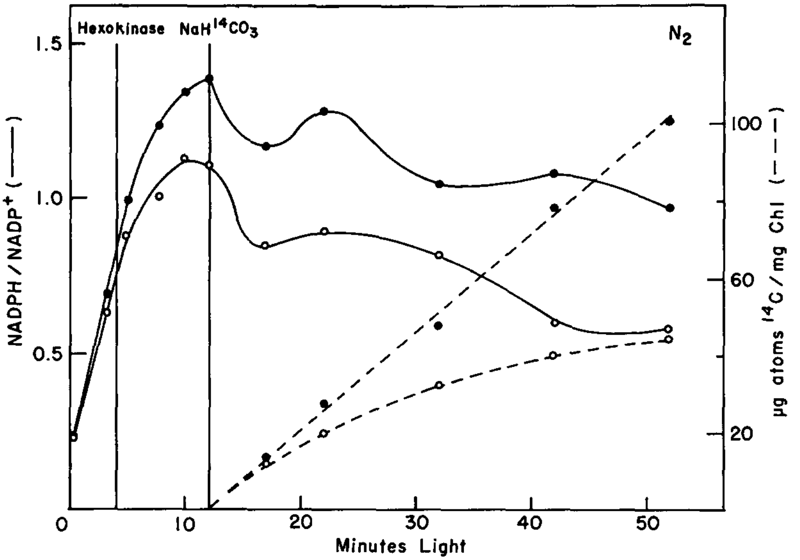

Fig. 4. Similar experiment to that described in Fig. 2; however, the amount of chloroplast lamellae was increased 4 times at the beginning of the light period rather than later. CO_2 fixation was measured simultaneously with NADPH/NADP$^+$ ratios and is shown by dashed lines. Solid lines indicate NADPH/NADP$^+$ ratios. The experiment took place under a nitrogen atmosphere. "1 × chloroplast lamellae" refers to our standard reconstituted system [16] in which the ratio of soluble components to lamellae is 14 times greater than in the original chloroplasts. With "4 × chloroplast lamellae" this ratio is. 3.5. $^{14}CO_2$ fixation rate is given in terms of μgatoms ^{11}C fixed per mg chlorophyll in the usual reconstituted system (14: 1). The rate for "4 × chloroplast lamellae" is 0.25 that shown on a basis of ^{14}C fixed per mg chlorophyll. (○), 1 × chloroplast lamellae; (●), 4 × chloroplast lamellae.

also increased the rate of photophosphorylation. When the lamellae were increased by a factor of 4 from the beginning of the experiment, then both the NADPH/NADP$^+$ ratio and the $^{14}CO_2$ fixation rate were maintained at higher levels throughout the course of the experiment (Fig. 4).

Effect of glucose/hexokinase or 3-phosphoglycerate as primers

In experiments with the reconstituted system, it is necessary to provide a primer of photosynthetic metabolites in order to achieve high rates of CO_2 fixation [16]. In our earlier work we have usually employed 3-phosphoglycerate as a primer, since this is a product of the carboxylation reaction. An alternative primer is glucose together with hexokinase. Photophosphorylation occurring in the light prior to the addition of sodium bicarbonate produces ATP, which in turn converts the glucose to glucose 6-phosphate. This compound is subsequently converted to various other sugar phosphates including Rib-1,5-P_2, the substrate for the carboxylation reaction.

The sudden drop in the NADPH/NADP$^+$ ratio upon the addition of sodium bicarbonate (Fig. 1) was of interest, since it does not occur significantly with whole chloroplasts, and since the only utilization of NADPH known to occur in the reductive pentose phosphate cycle is during the reduction of 3-phosphoglycerate to glyceraldehyde 3-phosphate. In experiments with [^{14}C]glucose as a primer, the 3-phosphoglycerate concentration was less than 0.050 mM just prior to the addition

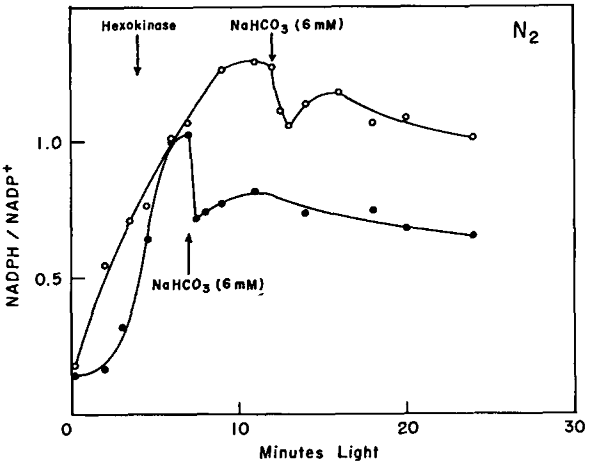

Fig. 5. Kinetics of NADPH/NADP$^+$ ratios in a reconstituted chloroplast system with either 3-phosphoglycerate (1 mM) or glucose (0.5 mM)/hexokinase (100 units) as primer. The 3-phosphoglycerate primer system was preilluminated for 7 min and the glucose/hexokinase primer system was preilluminated for 12 min before the addition of $NaHCO_3$ (6 mM). The flasks were kept under nitrogen. (○) 3-phosphoglycerate; (●) glucose/hexokinase.

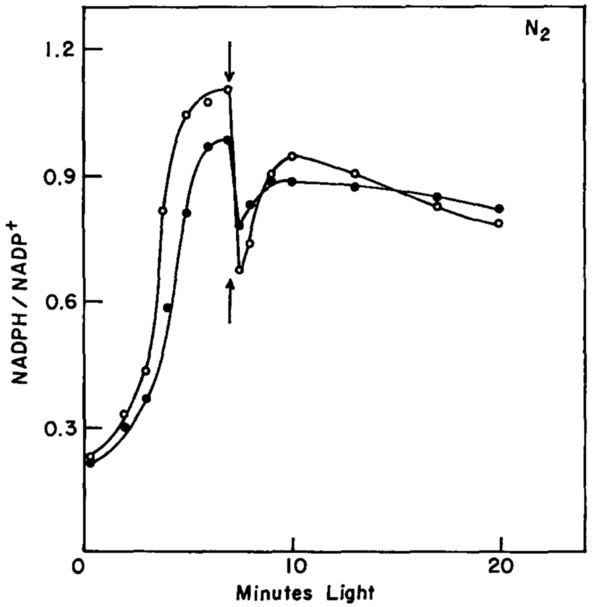

Fig. 6. Changes in the NADPH/NADP$^+$ ratios of an illuminated reconstituted chloroplast system upon addition of $NaHCO_3$ (6 mM) or 3-phosphoglycerate (1 mM). (●) 3-Phosphoglycerate (1 mM) present since beginning of preillumination period; addition of $NaHCO_3$ (6 mM) indicated by arrow. (○) No 3-phosphoglycerate present during preillumination period; addition of 3-phosphoglycerate (1 mM) indicated by arrow; no addition of $NaHCO_3$.

of sodium bicarbonate. When 1 mM 3-phosphoglycerate was used as a primer, its concentration was reduced prior to the addition of bicarbonate but is higher than when glucose was used as a primer. Addition of sodium bicarbonate to the reconstituted system with glucose as a primer should cause a large relative change in phosphoglycerate concentration, and in turn there might be a larger effect on the NADPH/NADP$^+$ ratio. However, the NADPH/NADP$^+$ ratio actually declines somewhat less rapidly upon bicarbonate addition when glucose was employed as a primer than with 3-phosphoglycerate (Fig. 5).

This point was further investigated by carrying out an experiment (Fig. 6) in which in one case no primer at all was employed and in the other case 1 mM 3-phosphoglycerate was employed. After 7 min light, 1 mM 3-phosphoglycerate was added to the flask with no primer, but no bicarbonate was added. In the other flask, with 3-phosphoglycerate as a primer already present, 6 mM bicarbonate was added at 7 min. In both cases there was a sharp drop in the ratio NADPH/NADP$^+$ with the drop being about twice as much in the case with 3-phosphoglycerate added. Therefore, a sudden increase in the concentration of 3-phosphoglycerate does result in an increased utilization of NADPH for the reduction of the added 3-phosphoglycerate. The question remains, however, as to why there is a sudden drop in NADPH/NADP$^+$ level upon addition of sodium bicarbonate to a reaction mixture which already contains a high concentration of 3-phosphoglycerate.

The amount of CO_2 fixed during the first 30 s following addition of bi-

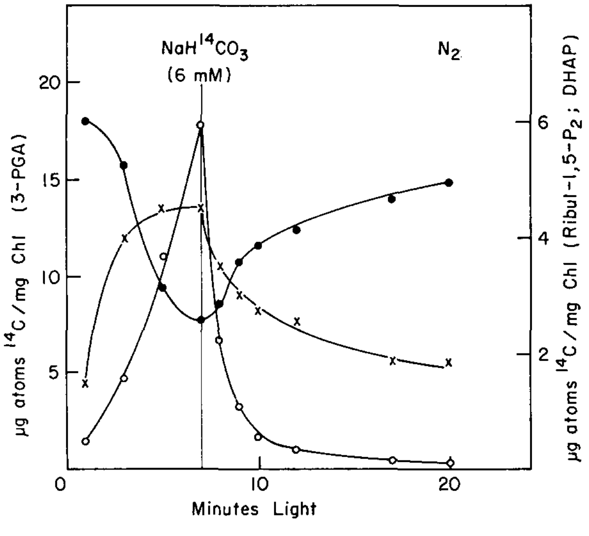

Fig. 7. Pool size changes of Rib-1,5-P_2, 3-phosphoglycerate and dihydroxyacetone phosphate during preillumination and upon addition of NaH^{14}CO$_3$ (6 mM; specific activity 17 Ci/mol) in a reconstituted chloroplast system. 3-Phospho[^{14}C]glycerate (1 mM, specific activity 51 Ci/mol) as the primer was introduced to the reaction flask prior to the preillumination period. Since 3-phosphoglycerate has three carbon atoms per molecule, the specific activity of carbon in 3-phosphoglycerate was 17 Ci/gatom, which was the same as the specific activity of the NaH^{14}CO$_3$. Thus, ^{14}C contents of Rib-1,5-P_2 and dihydroxyacetone phosphate provide measures of pool sizes if we neglect endogenous, unlabeled carbon in these pools. (○) Rib-1,5-P_2; (●) 3-phosphoglycerate (3-PGA); (×) dihydroxyacetone phosphate (DHAP).

carbonate produced about 20 % as much 3-phosphoglycerate as was already present, but the actual concentration of 3-phosphoglycerate rose by only a small amount (Fig. 7). While we do not usually observe the concentration of 3-phosphoglyceraldehyde directly, owing to its very low concentration, we know from many experiments that the reaction mediated by triosephosphate isomerase (EC 5.3.1.1) is highly reversible and that we can estimate the concentration of glyceraldehyde 3-phosphate as 0.05 that of dihydroxyacetone phosphate. It is interesting that the level of dihydroxyacetone phosphate drops very rapidly upon the addition of sodium bicarbonate. Although the triose phosphates are formed much more rapidly following the addition of sodium bicarbonate, a large effect on triose phosphate concentration is produced by the accelerated conversion of dihydroxyacetone phosphate via the sugar phosphates of the cycle to the carboxylation substrate Rib-1,5-P_2, which declines very rapidly in concentration upon the addition of 6 mM bicarbonate (Fig. 7). The addition of bicarbonate is expected to lower the concentration of ribulose 1,5-diphosphate, and it may be that this in turn would accelerate the conversion of pentose monophosphates to ribulose 1,5-diphosphate. This accelerated removal of pentose monophosphates (ribose 5-phosphate and xylulose 5-phosphate) could accelerate the utilization of triose phosphase, since the reactions of glyceraldehyde 3-phosphate with either fractose 6-phosphate or sedoheptulose 7-phosphate leading to formation of pentose monophosphates are reversible [20].

Since the reduction of 3-phosphoglycerate to triose phosphates is a highly reversible (2-step) sequence [20], the accelerated conversion of triose phosphates to Rib-1,5-P_2 upon bicarbonate addition is in turn reflected in accelerated 3-phosphoglycerate reduction. Thus, at 3-phosphoglycerate concentrations of at least 0.05 mM, the rate of NADPH utilization depends more on this acceleration of the cycle (due to utilization of triose phosphate) than on the absolute concentration of 3-phosphoglycerate. In other words, a change of 20 % in 3-phosphoglycerate concentration has a smaller effect on the net rate of reduction of 3-phosphoglycerate than a 50 % change in triose phosphate concentration.

DISCUSSION

Although the sustained ratio of NADPH/NADP$^+$ is lower in the reconstituted chloroplast system than in the whole chloroplasts, the supply of NADPH is sufficient to maintain high rates of CO_2 fixation during illumination of the system. We may conclude that the levels of NADPH in the reconstituted system are sufficient to fully activate the carboxylase enzyme [10]. We are unable to explain why the ratio of NADPH/NADP$^+$ appears to reach a saturation value of about 1.1 in reconstituted chloroplasts and 2.3–2.5 in whole chloroplasts in the absence of sodium bicarbonate addition. Presumably there is either some back reaction which oxidizes the NADPH to NADP$^+$ or some regulatory mechanism which limits the maximum extent to which the NADP$^+$ can be reduced. With respect to back reactions, it is noteworthy that with whole chloroplasts the maximum NADPH/NADP$^+$ ratio was relatively unaffected by the atmosphere of nitrogen or air, whereas with the reconstituted system there is a large effect of nitrogen as compared to air. Of course, with air levels of CO_2 there is already some utilization of NADPH for photosynthesis due to the fixation of this CO_2 and the production of NADP$^+$. The difference in the NADPH/NADP$^+$

TABLE I

CONCENTRATIONS OF NADPH AND NADP$^+$ IN WHOLE ISOLATED SPINACH CHLOROPLASTS AND IN RECONSTITUTED CHLOROPLASTS

Values for whole isolated chloroplasts in the light [8] were NADPH, 32 nmol · mg^{-1} chlorophyll and NADP$^+$, 12 nmol · mg^{-1} chlorophyll. 1 ml whole chloroplast suspension contained 50 μg chlorophyll, and an estimated 0.01 ml internal volume. Thus, there were 1.6 nmol NADPH and 0.6 nmol NADP$^+$ in 1 mol suspension, or 160 nmol and 60 nmol per 1 ml of internal volume, giving concentrations of 0.160 mM and 0.060 mM for NADPH and NADP$^+$, respectively, inside the whole chloroplasts. In preparing the reconstituted system, the $0.16+0.06 = 0.220$ mM endogenous, NADPH+NADP$^+$ was diluted 6.94-fold, giving 0.0317 mM NADPH+NADP$^+$. An additional 0.1 mM NADP$^+$ was added before illumination, giving 0.1317 mM in all. After illumination under N_2, but before $H^{14}CO_3^-$ addition (see Fig. 6), the NADPH:NADP ratio was about 1.0, so the concentration of each was 0.0659 mM.

	NADPH (mM)	NADP$^+$ (mM)
Whole chloroplasts	0.160	0.060
Reconstituted chloroplasts	0.066	0.066

ratio remains high, however, and in fact increases after the addition of sodium bicarbonate (6 mM). Thus, it seems clear that the principle difference between the ratios with nitrogen and with air must be ascribed to oxidation of electron carriers by O_2. Perhaps, even under nitrogen, the maximum ratio attained for NADPH/NADP$^+$ is limited by the oxygen produced by photosynthesis. It remains unexplained as to why the intact chloroplasts should be less susceptible to this phenomenon than the reconstituted system.

In comparing the ratios of NADPH/NADP$^+$ between whole chloroplasts and the reconstituted system, one needs to consider both the total amount of the cofactors as compared with the chlorophyll and also the actual concentration available to the enzymes. In whole chloroplasts, the internal volume of the chloroplast occupies about 0.01 of the total volume of the chloroplast suspension. This small volume contains the entire soluble components, including the enzymes. For whole chloroplast experiments, in which the chlorophyll content is equivalent to 0.05 mg of chlorophyll per flask, although the amount of NADPH in the flask is very small, the concentration inside the chloroplast is estimated as 0.16 Mm (Table I). With the reconstituted system, where we initially add 0.1 μmol of NADP$^+$ per 1 ml of flask contents, and where just before addition of bicarbonate the ratio of NADPH/NADP$^+$ is 1, the concentration of NADPH in the flask is 0.066 mM. Thus, the actual concentration of NADPH available to the soluble components in the reconstituted system is about 0.4 that available in the intact chloroplasts at the time just before addition of bicarbonate.

In order to test the reversibility of the reactions leading to the reduction of 3-phosphoglycerate to triose phosphate in this reconstituted system, we employed the methods described by Bassham and Krause [20] to calculate the actual free energy changes at various times during the course of the experiments, which gave the data shown in Figs. 6 and 7. The physiological standard free energy change, $\Delta G^{0\prime}$, for the two-step reactions converting 3-phosphoglycerate, ATP and NADPH to glyceraldehyde 3-phosphate, NADP$^+$, ADP and inorganic phosphate is +4.3 kcal.

With *Chlorella pyrenoidosa* photosynthesizing under steady-state conditions, it was calculated that the actual free energy change, ΔG^s, was -1.6 kcal. In the experiment with the reconstituted chloroplasts just described, ΔG^s was estimated as about -0.5 kcal just prior to addition of bicarbonate (at 7 min). At the 30 s point following addition of bicarbonate (7.5 min), the calculated value for the ΔG^s was almost zero, and then became about -0.5 kcal at 20 min. Thus, it is clear that, as is the case with photosynthesizing *C. pyrenoidosa*, the two reactions constituting the reduction of 3-phosphoglycerate and mediated by 3-phosphoglycerate kinase (EC 2.7.2.3) and triose-phosphate dehydrogenase (EC 1.2.1.12) are highly reversible and not rate-limiting steps in the reconstituted chloroplast system. This does not mean that these steps could not be regulated as between light and dark as has been suggested elsewhere [21], but does indicate that these enzymes are not being subjected to change in activity due to allosteric regulation in the light.

From the data given earlier, it seems clear that with the other metabolite concentrations found in the reconstituted system relatively low ratios of NADPH/NADP$^+$ (0.5) are adequate for the reduction of 3-phosphoglycerate to triose phosphate, and subsequently regeneration of Rib-1,5-P_2. In addition to its function as a reductant, NADPH is now known to be an important effector for ribulose-1,5-diphosphate carboxylase [9, 10]. Thus, an adequate ratio of NADPH/NADP$^+$ is important for the activation of the carboxylation reaction.

From the results with addition of lamellae to the reconstituted system after a period of 20 min photosynthesis (Figs. 2 and 3) and the experiment where the amount of lamellae has been increased from the beginning, it is clear that with the levels of sodium bicarbonate which we have commonly employed (6 mM), the rate of reduction of NADP$^+$ to NADPH is sometimes unable to keep up with the needs of the soluble enzyme systems after some minutes of photosynthesis. By increasing the proportion of lamellae, this rate can be boosted and the rate of CO_2 fixation and the level of NADPH reduction restored. Of course, when this is done, the actual rate of fixation as compared with chlorophyll content of the system will be much lower because of the greater chlorophyll content now employed. In other studies, where we have employed air levels of CO_2 throughout the course of the experiment, the ratio of lamellae to soluble components used in the present experiment has been sufficient to sustain a nearly constant NADPH/NADP$^+$ ratio and constant rate of photosynthesis for up to 1 h.

The decline in the NADPH/NADP$^+$ ratio with time in some experiments is the consequence of the deterioration of the thylakoid system during an extended period of in vitro photosynthesis. It has been reported [22] that with isolated spinach chloroplasts, photosynthesis in the light in the presence of a glutathione buffer leads to extensive swelling of the thylakoids accompanied by a decreased capacity for photophosphorylation. In examination of our thylakoid system by electron microscopy before and after a period of photosynthesis in the light, we have seen swelling of the thylakoids with time. In the experiments shown in Figs. 6 and 7, the ratio ATP/ADP declined from about 5 : 1 to about 2 : 1 during the 20 min of photosynthesis with sodium bicarbonate. Thus, both the decline in phosphorylation potential and the ratio NADPH/NADP$^+$ may be affected by the thylakoid swelling in the light. It would appear that in order to achieve even more steady-state conditions with the reconstituted system, including constant potentials of NADPH/

NADP$^+$ and ATP/ADP, further improvements, perhaps in the buffer system or other conditions, will be required to overcome this problem of thylakoid swelling.

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