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# THE DISTRIBUTION OF METABOLITES BETWEEN SPINACH CHLOROPLASTS AND MEDIUM DURING PHOTOSYNTHESIS IN VITRO

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SUMMARY

1. The formation of metabolites in the stroma compartment of isolated chloroplasts during carbon fixation, and their export to the medium, have been investigated using improved techniques.

2. Rapid separation of photosynthesising chloroplasts from the medium, accompanied by simultaneous quenching of metabolism was achieved by using silicone oil layer filtering centrifugation under illumination. Metabolites were separated by microscale ion-exchange chromatography. Quantitative determination of each metabolite was based on labelling with $^{32}P$.

3. It was found that fixed carbon was exported from the chloroplasts only as triose phosphate and phosphoglycerate, and to a minor extent, as pentose monophosphate. The main compounds accumulating in the stroma were hexose and heptose monophosphates and phosphoglycerate. A marked decrease in the concentration of inorganic phosphate in the stroma during the first 5 min of illumination was accompanied by a complementary increase in organic phosphate so that the total amount of phosphate within the chloroplasts remained constant.

4. The concentration difference for phosphoglycerate between the stroma and the medium was much higher than for triose phosphate or inorganic phosphate, although all three compounds are transported across the inner membrane of the chloroplast envelope by the same carrier. It was concluded that the efflux of phosphoglycerate was restricted.

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Abbreviations: $P_i$, inorganic phosphate; PGA, 3-phosphoglycerate; GAP, glyceraldehyde phosphate; TP, triose phosphate; DHAP, dihydroxyacetone phosphate; PMP, pentose monophosphate; SBP, sedoheptulose 1,7-bisphosphate; FBP, fructose 1,6-bisphosphate; RuBP, ribulose 1,5-bis-phosphate; HMP, mixture of hexose and heptose phosphates; X, unidentified compound.

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## INTRODUCTION

The phosphate translocator is a specific carrier situated in the inner membrane of the chloroplast envelope [1]. It facilitates a counterexchange of inorganic phosphate, 3-phosphoglycerate and the triose phosphates glyceraldehyde phosphate and dihydroxyacetone phosphate. The physiological function of this translocator is to transport the main products of photosynthesis, triose phosphates and phosphoglycerate, from the stroma into the cytosol in exchange for inorganic phosphate.

The properties of this transport process have previously been studied mainly by adding substances to the medium and measuring their uptake into the sorbitol-impermeable space of the chloroplasts. In this way the specificity of the transport from the external space into the stroma was established [2]. It is the objective of the present work to investigate the specificity of the transport in the opposite direction during $CO_2$ fixation. For this the release of the products of photosynthesis from the chloroplasts was measured.

Pioneering studies on this subject have been carried out earlier by Bassham et al. [3]. Using paper chromatography and electrophoresis for the separation of $^{14}C$-labelled compounds formed from [$^{14}C$]bicarbonate, these authors reported that phosphoglycerate was the main product of $CO_2$ fixation released, followed by triose phosphates, fructose bisphosphate, pentose monophosphate and glycolate, whereas hexose and heptose monophosphates and ribulose bisphosphate were retained in the spinach chloroplasts.

In the present publication this work is extended by the use of improved methodology. For higher sensitivity, the intermediates of the $CO_2$ fixation cycle were radioactively labelled by the incorporation of [$^{32}P$]phosphate present in the medium. With silicone layer filtering centrifugation [4] the illuminated chloroplasts were rapidly separated from the medium, and immediately deproteinized. In this way an instantaneous quenching of the metabolism in the illuminated chloroplasts was achieved. For metabolite assay a microscale ion-exchange chromatography was employed, which had been applied in our laboratory for the separation of mitochondrial metabolites [5]. Although some compounds (hexose and heptose monophosphates) are better separated by the two-dimensional paper chromatography and electrophoresis system utilized by Bassham et al. [3], the system employed here has the advantage of being far less laborious, of accurate peak evaluation and of very high sensitivity. Thus a chloroplast suspension equivalent to 5 $\mu$g chlorophyll is sufficient for an accurate determination of the metabolites in the separated chloroplasts and in the medium. This method is therefore well suited for routine measurements.

## MATERIALS AND METHODS

*Preparation of chloroplasts.* Spinach (*Spinacia oleracea* var. True Hybrid 102, Arthur Yates and Co., N.S.W., Australia) was grown under artificial illumination in aerated nutrient solution similar to the control medium used by Randall and Bouma [6]. Chloroplasts with a high proportion of intact envelope were prepared as described previously [7]. Chlorophyll was measured by the procedure of Arnon [8].

*Reaction media and silicone layer filtering centrifugation.* The basic reaction

medium contained 0.33 M *N*-2-hydroxyethylpiperazine-*N'*-ethane sulphonic acid, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM $NaHCO_3$, 0.5 mM potassium phosphate, pH 7.6, and chloroplasts as indicated. In addition, reaction mixtures for chromatography contained $NaH^{14}CO_3$ (40 $\mu$Ci/ml) and inorganic [$^{32}$P]phosphate (approx. 40 $\mu$Ci/ml). Reaction mixtures intended for measurement of the sorbitol-impermeable space in the chloroplasts contained $^3H_2O$ (5 $\mu$Ci/ml) and [U-$^{14}$C] sorbitol (1 $\mu$Ci/ml). The reaction and centrifugation were done in 400-$\mu$l capacity polypropylene microtubes (Sarstedt, Nümbrecht, W. Germany). Each microtube contained a layer of 10% $HClO_4$ at the bottom (20 $\mu$l), a middle layer of 70 $\mu$l silicone oil (three parts AR 100 to one part AR 150 (Wacker Chemicals, Munich, W. Germany)), and a top layer of 200 $\mu$l of reaction mixture. Each microtube was illuminated at an intensity of 60 W/m$^2$ by a tungsten-halogen light source fitted with an RG 630 cutoff filter (Schott, Mainz, W. Germany) and a Calflex C infrared filter (Balzers, Liechtenstein) at a temperature of 20 °C. Reactions were started by turning on the light and terminated by centrifugation (Beckman Microfuge, Calif., U.S.A.) when the intact chloroplasts passed through the silicone layer into the $HClO_4$ within 2 s. Continuous illumination was maintained until the centrifugation step was completed. Immediately after centrifugation, $HClO_4$ was added to the top layer in each microtube to give a concentration of 0.5 M. The top and bottom layers were then separated by cutting the microtubes at the centre of the silicone layer. The bottom layer was resuspended in 300 $\mu$l water. Both samples were neutralized with $K_2CO_3$, centrifuged, and stored at −15 °C. Precipitation of sugar phosphates and nucleotides during centrifugation of the neutralized extracts was found to be negligible. However, some precipitation of insoluble material containing $^{14}$C activity but not $^{32}$P activity was detected with bottom layer extracts. The $^{14}$C activity in this material, representing starch originating from the intact chloroplasts [9], was determined after separation from the supernatant and washing.

The radioactivity of samples from each layer was analysed in a scintillation counter (Philips, LSA) and the amounts of $^{14}$C and $^{32}$P activity, or of $^{14}$C and $^3$H activity in each layer determined. The sorbitol-permeable and sorbitol-impermeable volumes associated with the intact chloroplasts which were transferred into the lower layer during centrifugation were determined from the relative amounts of [$^{14}$C] sorbitol and $^3H_2O$ in each layer as described previously [4]. From duplicate microtubes which contained $NaH^{14}CO_3$ and $^{32}P_i$ in the reaction mixtures, the distribution of fixed carbon (acid-stable $^{14}$C activity) and of total (organic plus inorganic) phosphate between the two layers was determined. Quantitative analysis of the $^{32}$P-labelled compounds present in these samples was then performed by chromatography.

*Chromatography.* A quantitative analysis of the $^{32}$P-labelled compounds present in each extract obtained from microtubes containing $NaH^{14}CO_3$ and $^{32}P_i$ was performed by ion-exchange chromatography, using the basic procedure described previously [5]. A 200 $\mu$l sample of extract was applied to a column (1.1 mm diameter $\times$1 m long) containing washed formate-anion exchanger (Bio-Rad AG1-X8, minus 400 mesh). The column was eluted first with 1.0 ml chloroform-saturated water, and then with a linear concentration gradient from 0 to 8.4 M formic acid plus 1.5 M ammonium formate, at a constant flow rate of 1.12 ml/h. The radioactivity in the column effluent was monitored continuously by a methane gas-flow Geiger counter (Philips) equipped with a continuous flow cell. The metal foil window of the counter

excluded $^{14}C$ emissions, so that the counts recorded represented $^{32}P$ activity only. In some cases, the effluent from the column was finally collected at 10-min intervals and the radioactivity analysed by liquid scintillation counting to determine the absolute values of the $^{14}C$ and $^{32}P$ activity present.

*Analysis of data and peak identification.* The $^{32}P$ activity of the column effluent, as counts recorded by the gas-flow counter at 4-min intervals, was plotted by a computer after applying corrections for background subtraction and for $^{32}P$ activity decay with time. The resulting chromatogram was highly reproducible. The area under each peak of the chromatogram was measured. From the total peak area and known amount of total phosphorus in the sample, the absolute amount of phosphorus in each peak was computed.

Individual peaks were identified by three criteria. Firstly, the order and approximate position of each compound was known from previous work. From the results of the scintillation counting of column effluent fractions a second chromatogram was prepared showing relative activities of both $^{32}P$ and $^{14}C$. This enabled the carbon/phosphorus ratio of each peak to be calculated. Finally, $^{14}C$-labelled pure samples of each compound were obtained (Amersham, U. K., Boehringer, W. Germany, and New England Nuclear Co., Boston, U.S.A.). Each compound was individually eluted on the column and its position on the chromatogram confirmed by scintillation counting of column effluent. The position of the inorganic phosphate peak was confirmed by chromatography of $^{32}P$-labelled orthophosphate alone. All peaks identified in the results were identified in this manner and exhibited the expected carbon/phosphorus ratio. Further details on the peak identification will be published separately (Portis, A., Lilley, R. McC., Chon, C. J. and Heldt, H. W., in preparation). Since glucose 6-phosphate, glucose 1-phosphate, fructose 6-phosphate and sedoheptulose 7-phosphate eluted from the column together, the resultant combined peak was termed "hexose and heptose monophosphates". Similarly, the peak containing both dihydroxyacetone phosphate and glyceraldehyde 3-phosphate was termed "triose phosphates". The peak containing ribose 5-phosphate was termed "pentose monophosphates" since isomers were probably present. The fructose bisphosphate peak contained also another, not yet identified compound.

After the absolute amounts of each compound in the upper and lower layers had been calculated, the amounts in the lower layer were corrected for those compounds from the external medium which had been carried trough the silicone layer with the chloroplasts. The amount of external medium carried through was represented by the sorbitol-impermeable volume of the chloroplasts. The final figures thus represent the amount of each compound in the sorbitol-impermeable volume of the chloroplasts, that is, within the stroma compartment.

## RESULTS AND DISCUSSION

### *Metabolite levels during $CO_2$ fixation*

In order to measure the overall reaction of $CO_2$ fixation and the formation of the single products a number of parallel choroplast samples were incubated in the light with the bicarbonate and the inorganic phosphate radioactively labelled. A low chloroplast concentration was employed to follow $CO_2$ fixation for a longer time period. This avoided depletion of the phosphate during the experiment. The time

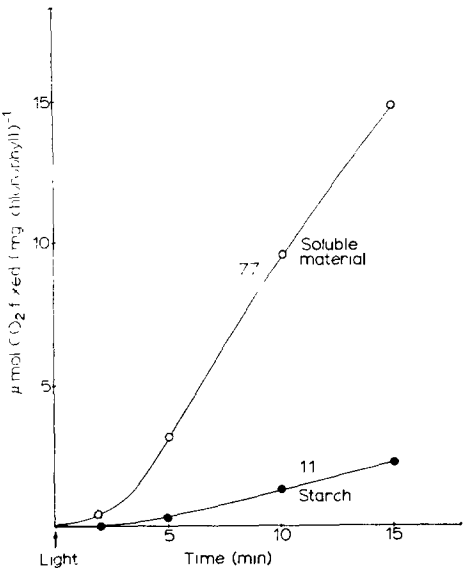


Fig. 1. $CO_2$ fixation by spinach chloroplasts (0.025 mg chlorophyll/ml; $P_i$ concentration, 0.32 mM; other conditions given in Materials and Methods).

course of the $CO_2$ fixation is shown in Fig. 1. After a lag of approx. 3 min a near linear rate of $CO_2$ fixation is maintained; this is typical behaviour for isolated chloroplasts [10]. Since a low concentration of phosphate was employed in the medium, a considerable portion of the fixed carbon was recovered as starch [9]. To distinguish the appearance of the phosphorylated products of $CO_2$ fixation within the chloroplasts from those in the surrounding medium, the illuminated chloroplast suspension was subjected to silicone layer filtering centrifugation. A chromatogram of the $^{32}P$-labelled compounds found in the filtered chloroplasts is shown in Fig. 2A. From these, inorganic phosphate, phosphoglycerate and hexose and heptose monophosphates, are present in largest quantities. In the corresponding medium (Fig. 2B) beside the added inorganic phosphate only triose phosphates and phosphoglycerate are found in substantial amounts.

The concentrations of the $^{32}P$-labelled compounds in both compartments have been evaluated in Table I. The values obtained for the chloroplasts have been corrected for the medium adhering to the outer surface and that contained between the inner and the outer membrane of the chloroplast envelope [4]. Since the thylakoid membrane is impermeable to phosphate and phosphorylated compounds (Chon, C. J., Portis, A. and Heldt, H. W., in preparation), the $^{32}P$-labelled compounds found in the chloroplast fraction can be attributed to the stroma compartment. The very low ratio of triose phosphates to phosphoglycerate (approx. 0.1) in the stroma is largely determined by the equilibrium of these compounds with ATP, ADP, NADPH and NADP (Portis, A., Chon, C. J. and Heldt, H. W., in preparation). Some phosphoglycerate may be also bound to the ribulose bisphosphate carboxylase since a very large amount of this enzyme, equivalent to a concentration of 3.6 mM active sites is

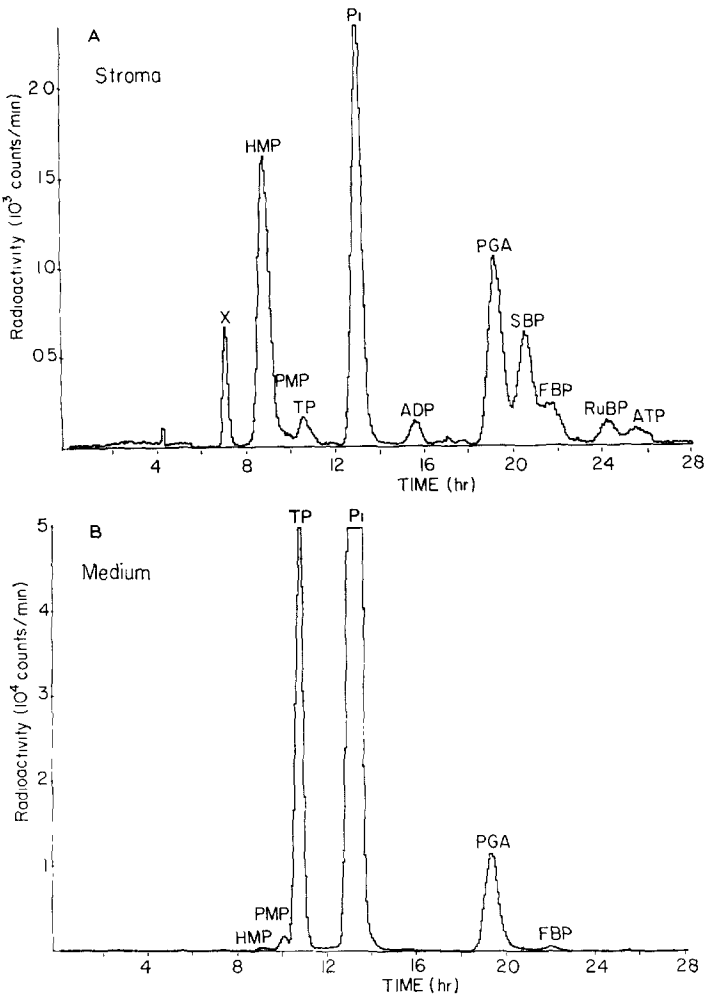


Fig. 2. Ion-exchange chromatogram of $^{32}$P-labelled metabolites in the filtered chloroplasts (A) and the remaining medium (B). Experiment of Fig. 1, samples obtained after 10 min of illumination. For details see Materials and Methods.

contained in the stroma*. In the corresponding medium (Fig. 2B) beside the added inorganic phosphate only triose phosphates and phosphoglycerate are found in large quantities. Here the ratio of triose phosphates to phosphoglycerate (2.3) is high. A considerable difference between these ratios in the stroma and in the external medium is observed. The implications of these findings will be discussed later.

Time courses for the amounts of $^{32}$P-labelled metabolites in the stroma com-

* Spinach leaves contain 5.8 mg fraction 1 protein (ribulose bisphosphate carboxylase) per mg chlorophyll [11]. This enzyme has eight active sites [12] and a molecular weight of 560 000 [13]. With a stroma space of 23 $\mu$l/mg chlorophyll, the concentration of active sites of ribulose bisphosphate carboxylase in the stroma is therefore 3.6 mM.

TABLE I

$^{32}$P-LABELLED METABOLITES AFTER 10 MIN ILLUMINATION. EVALUATION OF FIG. 2

The determination of ADP and the unidentified compound X is based on one labelled phosphate group per molecule, of ATP on 2, and all other compounds on the number of phosphate groups present in the respective molecules.

| Compound | In medium | | In chloroplasts | | Percent in chloroplasts |
|---|---|---|---|---|---|
| | Amount (nmol/mg chlorophyll) | Concentration ($\mu$M) | Amount (nmol/mg chlorophyll) | Concentration (mM) | |
| X | 3.4 | 0.08 | 17.1 | 0.74 | 83.4 |
| Hexose monophosphate | 21.3 | 0.53 | 94.4 | 4.10 | 81.6 |
| Pentose monophosphate | 69.4 | 1.73 | 2.4 | 0.10 | 3.3 |
| Triose phosphate | 1 920 | 48.00 | 7.8 | 0.34 | 0.4 |
| Inorganic phosphate | 10 550 | 263.8 | 134.0 | 5.83 | 1.3 |
| ADP | 0 | 0 | 6.8 | 0.30 | 100 |
| 3-Phosphoglycerate | 829 | 20.72 | 77.0 | 3.35 | 8.5 |
| Sedoheptulose bisphosphate | 0 | 0 | 24.3 | 1.06 | 100 |
| Fructose bisphosphate | 22.7 | 0.56 | 9.0 | 0.39 | 28.4 |
| Ribulose bisphosphate | 0 | 0 | 6.1 | 0.26 | 100 |
| ATP | 0 | 0 | 3.5 | 0.15 | 100 |

partment during the 15 min illumination period are shown in Fig. 3A. The amounts of all the intermediates of $CO_2$ fixation in the stroma increase greatly between 2 and 5 min, corresponding to the transition from the lag phase to a linear rate of $CO_2$ fixation. After that the levels of the intermediates in the stroma remain about constant, and the amounts of triose phosphates and of phosphoglycerate in the medium (Fig. 3C) increase in an approximately linear fashion. Obviously, these compounds are then being exported from the chloroplasts. Furthermore, there is a small amount of pentose phosphates released from the stroma, whereas hexose and heptose monophosphates are virtually retained inside the chloroplasts. The appearance of fructose bisphosphate in the medium is probably the result of enzymatic synthesis from the external triose phosphates by triose phosphate isomerase and aldolase activities derived from broken chloroplasts. Direct measurements have shown that the chloroplast envelope is relatively impermeable to fructose bisphosphate [2].

In Fig. 3B the stroma levels of inorganic phosphate and total phosphates (inorganic plus organic phosphates) are plotted. The rapid decrease in inorganic phosphate during the first 5 min of illumination is accompanied by a complementary increase in bound, organic phosphate as the amounts of the $CO_2$ fixation cycle intermediates build up. For the duration of the experiment the total amount of phosphates remains at a relatively constant level. This indicates that the total efflux of labelled phosphates from the chloroplasts is closely matched by the influx of inorganic phosphate during this period. The phosphate translocator facilitating the transport of inorganic phosphate, triose phosphates and 3-phosphoglycerate across the envelope was shown to catalyze a strict counterexchange [2]. This explains why the total amount of phosphates in the stroma is kept constant. In the experiment of Fig. 3 the concentrations of sedoheptulose bisphosphate in the stroma is comparatively high. In

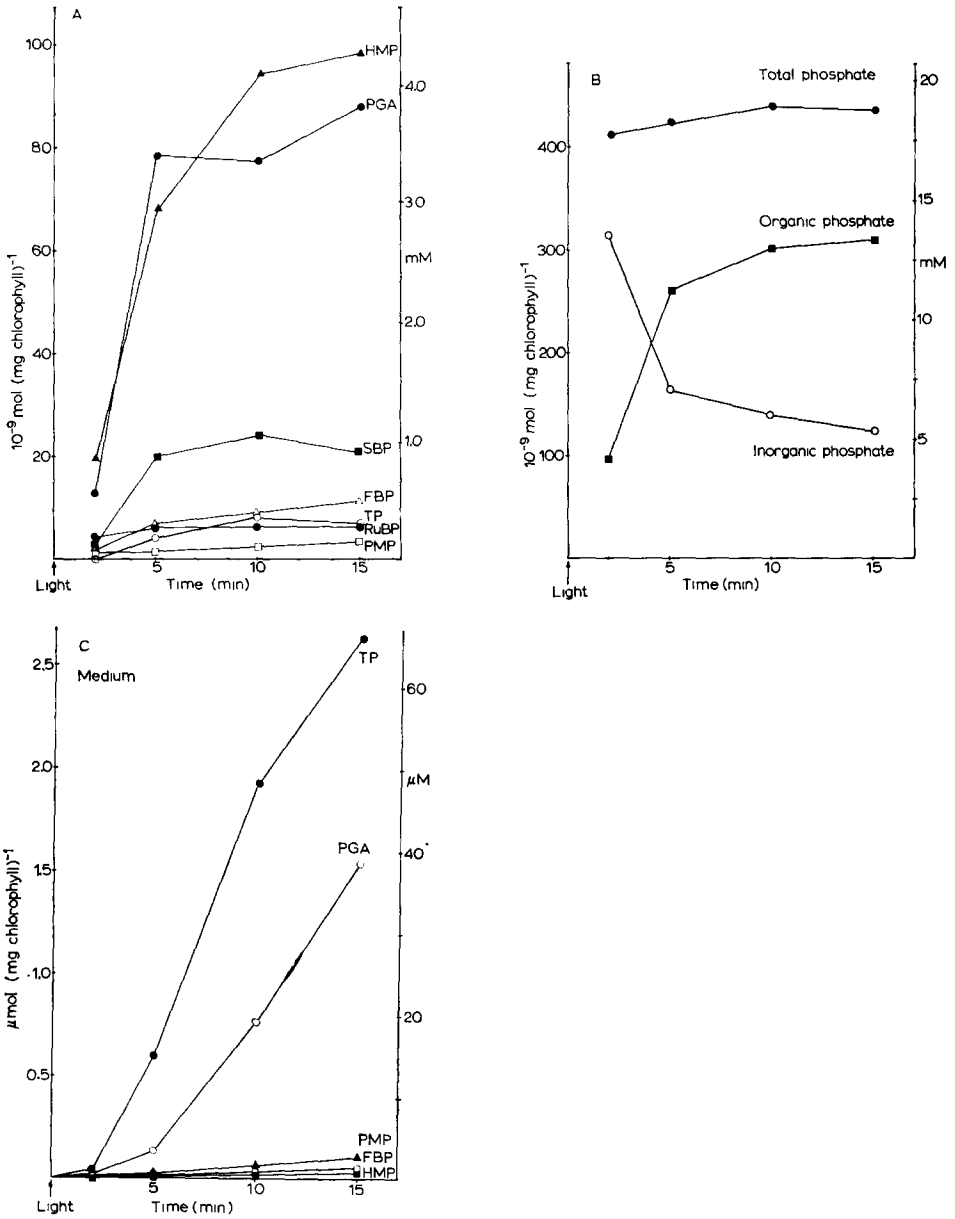


Fig. 3. Distribution of $^{32}P$-labelled metabolites during $CO_2$ fixation. Experiment of Fig. 1. For details see Materials and Methods. (A, B) Metabolites in the stroma. (C) Metabolites in the medium.

other experiments, not shown here, we found that the sedoheptulose bisphosphate concentration in the stroma was largely decreased on the addition of catalase (Portis, A. and Heldt, H. W., unpublished). This indicates that the previously observed inhibition of $CO_2$ fixation by $H_2O_2$, which is reversed by catalase [14, 15] may be at least in part due to an inhibition of sedoheptulose bisphosphatase.

The time course of a second experiment, designed to investigate the distribution of metabolites during the lag phase of $CO_2$ fixation, is shown in Fig. 4. In the dark only $^{32}P$ incorporation into hexose monophosphates is observed, caused probably by the phosphorylysis of starch contained in the chloroplasts [9]. Illumination leads to a rapid rise of the $^{32}P$-labelled phosphoglycerate in the stroma (Fig. 4A). Likewise a rapid initial increase of fructose bisphosphate and triose phosphates is observed, but the concentration of these compounds is decreased again when the maximum rate of $CO_2$ fixation has been reached. The overshoot of fructose bisphosphate demonstrates the light-induced transition of the fructose bisphosphatase from the inactive to the active form (for references see ref. 16). Consequently the level of hexose monophosphate declines at first during this period and increases again when the fructose bisphosphate concentration falls. The relative inactivity of the fructose-bisphosphatase during the early phase of $CO_2$ fixation also causes an "overshoot" of triose phosphates, and this in turn, seems to be the cause for the observed overshoot of ATP (Fig. 4B) during that period. Apparently, during the induction period, the rate of ATP synthesis exceeds the rate of ATP utilisation. When the maximum rate of $CO_2$ fixation is reached, the ATP concentration is decreased again to a very low value. These data indicate that under the conditions of our experiment $CO_2$ fixation may be limited by the rate of ATP synthesis, which is in accordance with earlier findings [17]. It should be noted, however, that our experiments were carried out with saturing concentrations of bicarbonate. With air levels of $CO_2$ the limitations of $CO_2$ fixation may be therefore different.

During the lag period there is a steady increase of the ribulose bisphosphate level observed (Fig. 4B). It reaches its highest value when the rate of $CO_2$ fixation is maximal (see also Fig. 3A). Thus the concentration of ribulose bisphosphate in the stroma seems to reflect the rate of $CO_2$ fixation. Fig. 4C shows the time course of the phosphate level in the stroma. Again the amount of total phosphates remains about constant. The slow rise may be due to an exchange of unlabelled phosphorylated compounds being present in the stroma.

Although the concentration of the triose phosphates in the stroma is relatively low, export of triose phosphates commenced almost immediately after illumination (Fig. 4D). The export of phosphoglycerate, however, did not commence until after 1 min, following the onset of phosphoglycerate accumulation in the stroma.

*Metabolite gradients*

Because the phosphorylated intermediates of $CO_2$ fixation are all formed in the stroma, the permeability of the envelope to these compounds is reflected by the corresponding concentration gradients between the stroma and the external medium (Table II). Since the pool of phosphate and phosphorylated intermediates in the stroma is nearly constant, the gradient of inorganic phosphate across the envelope is dependent on the phosphate concentration in the medium, which was 0.32 mM in experiment 1 and 0.50 mM in experiment 2. The corresponding gradients of triose phosphates were even lower than the gradients of inorganic phosphate, indicating that triose phosphates moved very readily across the envelope. The gradient for pentose monophosphates was also relatively low, concurring with earlier observations that pentose monophosphates are able to cross the envelope [3]. This might be due to transport by the phosphate translocator, which has also a slight affinity for pentose

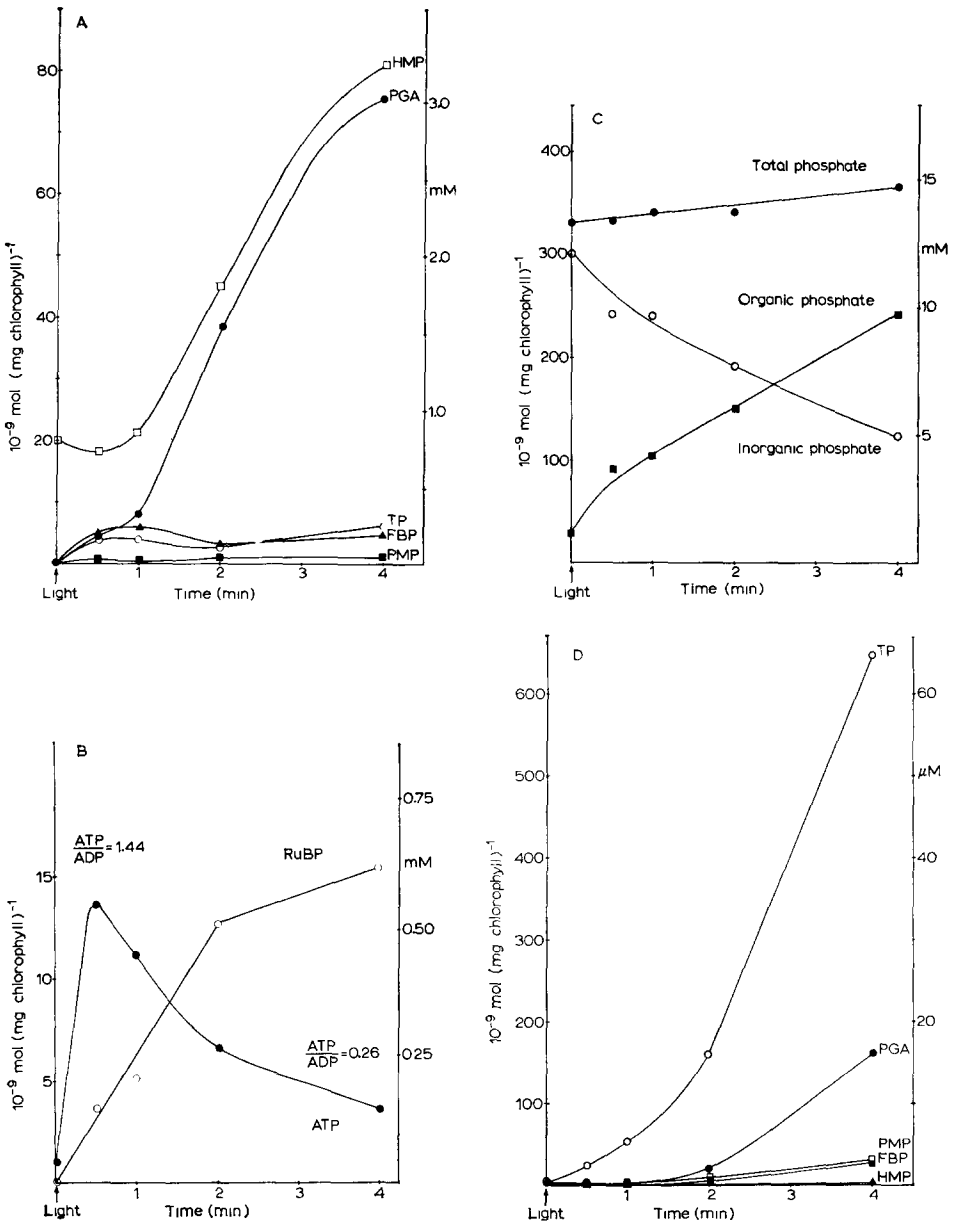


Fig. 4. Distribution of $^{32}P$-labelled metabolites during $CO_2$ fixation by spinach chloroplasts (0.1 mg chlorophyll/ml; $P_i$ concentration, 0.5 mM; other conditions given in Materials and Methods). (A, B, C) metabolites in the stroma. (D) Metabolites in the medium.

monophosphates [18]. Compared with these gradients, the gradient for phosphoglycerate is very high, showing that the exit of phosphoglycerate from the stroma is restricted. This retention of phosphoglycerate in the stroma is unexpected, since it was shown earlier that phosphoglycerate was transported across the envelope by the

TABLE II

CONCENTRATION GRADIENTS AND FLUXES OF METABOLITES FROM THE CHLOROPLAST STROMA TO THE MEDIUM

Expt. 1: evaluation of Fig. 3, gradient 10 min, flux 5–10 min. Expt. 2: evaluation of Fig. 4, gradient 4 min, flux 2–4 min.

| | Concentration gradients (conc. in stroma/conc. in medium) | | Efflux from stroma (μmol/mg chlorophyll per h) | |
|---|---|---|---|---|
| | Expt. 1 | Expt. 2 | Expt. 1 | Expt. 2 |
| Triose phosphate | 7 | 4 | 15.7 | 14.7 |
| Phosphoglycerate | 160 | 190 | 7.5 | 4.2 |
| Pentose monophosphate | 58 | 16 | 0.55 | 0.66 |
| Hexose and heptose monophosphate | 7700 | 5200 | 0.14 | 0.07 |
| Fructose bisphosphate | 700 | 35 | 0.15 | 0.83 |
| Sedoheptulose bisphosphate | ∞ | 1700 | 0 | 0 |
| Ribulose bisphosphate | ∞ | ∞ | 0 | 0 |
| Unidentified compound X | 8700 | 9300 | 0.02 | 0.03 |
| Total organic phosphate | | | 24.2 | 21.4 |
| Inorganic phosphate | 22 | 13 | −25.0 | −22.6 |

same carrier which transports inorganic phosphate and triose phosphates [2]. Since the kinetic constants for the transport of triose phosphates and phosphoglycerate do not differ largely [18], a higher rate of phosphoglycerate export might have been expected. The discrepancy between the kinetic data of phosphoglycerate transport into the chloroplasts (measured in the dark) and the apparent low permeability of the envelope for phosphoglycerate during $CO_2$ fixation, could be explained from previous observations that illumination increased the rate of phosphoglycerate uptake without affecting the transport of inorganic phosphate and triose phosphates [19]. This increase of phosphoglycerate uptake was later found to be due to an increase of the $V$ [20]. It was then attributed to the fact that phosphoglycerate has one more negative charge than inorganic phosphate and the triose phosphates, and that a light dependent alkalisation of the stroma may favour the uptake and restrict the release of phosphoglycerate [19]. This is supported by the observation of Heber (private communication) that the uptake of phosphoglycerate during phosphoglycerate reduction also involves proton uptake. It seems possible that a low permeability of the envelope to protons [21] might limit the efflux of phosphoglycerate. In this way the efflux of phosphoglycerate during $CO_2$ fixation may be largely restricted despite the capability of the phosphate carrier for rapid transport of phosphoglycerate. This enables a preferential export of triose phosphates from the chloroplasts, as required for the synthesis of sucrose in the cytosol.

An alternative explanation, which is difficult to assess at present, is that the concentration of free phosphoglycerate in the stroma is much lower than the total measured concentration due to binding to the high concentration of active sites of ribulose bisphosphate carboxylase. Since the measured concentrations of ribulose bisphosphate were less than 10 % of the estimated 3.6 mM active sites, most of the enzyme present at any time could be bound to its product, phosphoglycerate.

The concentration gradients measured for hexose monophosphates, sedoheptulose and ribulose phosphate are very high. In agreement with earlier investigations [3] this clearly demonstrates that these compounds are not able to permeate the envelope. Also, for fructose bisphosphate, high gradients were observed (experiment 1 in Table II). This concurs with our earlier observation that fructose bisphosphate is not transported across the envelope [2]. In those cases where the gradient of fructose bisphosphate has been found to be lower, this can be explained by the activity of aldolase in the medium derived from broken chloroplasts.

*Rates of metabolite efflux in relation to the total activity of the phosphate translocator*

In summarizing the results of the experiments in Figs. 3 and 4, rates of metabolite efflux from the chloroplasts have been evaluated (Table II). These data clearly show that the fixed carbon is primarily exported in the form of triose phosphates, and some also as phosphoglycerate. In comparison to the observed net efflux of these metabolites from the stroma, the total activity of the phosphate translocator as measured under optimal conditions is much higher (250 μmol/mg chlorophyll per h) [18]. However, as the phosphate carrier is capable of transporting phosphate, phosphoglycerate and triose phosphates in either direction, a very large portion of the transport activity is expended in exchanges that do not result in a net flux. Since the transport catalyzed by the phosphate translocator is an obligatory counter exchange (see also Figs. 3B and 4C) the total fluxes in each direction are equal.

$$V_{P_i}^{\rightarrow} + V_{TP}^{\rightarrow} + V_{PGA}^{\rightarrow} = V_{P_i}^{\leftarrow} + V_{TP}^{\leftarrow} + V_{PGA}^{\leftarrow} \tag{1}$$

(→ represents influx from the external space to the stroma, ← represents efflux.) The net flux, or transport of each compound is the difference between the fluxes in each direction.

The effect of the metabolite concentrations in the stroma on the corresponding fluxes is illustrated by a simple calculation for the flux of dihydroxyacetone phosphate from the stroma to the medium during $CO_2$ fixation. Since the transport of triose phosphates, 3-phosphoglycerate and inorganic phosphate as facilitated by the phosphate translocator, exhibits Michaelis-Menten characteristics, and all these compounds compete for the same carrier [2], the flux of a single compound, e.g. dihydroxyacetone phosphate in the presence of the other metabolites can be calculated from the following equation:

$$V_{DHAP} = \frac{V_{max,DHAP} \cdot \frac{[DHAP]}{K_{DHAP}}}{1 + \frac{[DHAP]}{K_{DHAP}} + \frac{[GAP]}{K_{GAP}} + \frac{[PGA]}{K_{PGA}} + \frac{[P_i]}{K_{Pi}}} \tag{2}$$

Introducing the metabolite concentrations in the stroma, as measured in the experiment of Fig. 4 (mean values of the data measured at 2 and 4 min) with the simplification that dihydroxyacetone phosphate represents 100 % of the triose phosphates (in reality only 98 %) and the corresponding $K_m$ values ($K_{P_i} = 0.28$ mM, $K_{PGA} = 0.14$ mM, $K_{DHAP} = 0.08$ mM (4 °C), $V_{max,DHAP} = 250$ μmol/mg chlorophyll per h (extrapolated to 20 °C) [18]) this calculation yields an efflux of 13 μmol DHAP/mg chlorophyll per h. This value is of similar order to the observed net flux of triose

phosphates in the experiment (Table II). It should be kept in mind, however, that the above calculation is a very simplified treatment of a more complex system. It is based on $K_m$ values obtained at 4 °C and the yet unproven assumption that the $K_m$ values for the transport from the stroma to the external space are the same as those measured for the transport in the opposite direction, and it also does not account for the possibility that some portion of the metabolites in the stroma may be bound there. Furthermore, when considering the simultaneous influx of dihydroxyacetone phosphate from the medium to the stroma the calculated net efflux of dihydroxyacetone phosphate from the stroma is considerably lower.

This model calculation illustrates that the effective rate of dihydroxyacetone phosphate transport is indeed only a small portion of the total carrier activity. It is a prerequisite for $CO_2$ fixation in intact chloroplasts, that the rate of export does not exceed the rate of $CO_2$ fixation, otherwise the stroma would be depleted of the intermediates of $CO_2$ fixation, and the $CO_2$ fixation cycle would come to a stop. This explains why $CO_2$ fixation can be inhibited by the addition of higher phosphate concentrations to the medium [10, 20]. According to Eqn. 2 a high phosphate concentration in the medium would decrease the flux of triose phosphates and of phosphoglycerate from the external medium to the stroma and thus increase the net efflux of these compounds. As a result the concentrations of these metabolites in the stroma would be decreased [9], and the rate of $CO_2$ fixation lowered. Consequently, the inhibition of $CO_2$ fixation should be reversed by a partial inhibition of the phosphate translocator. It has been observed in fact that the inhibition of $CO_2$ fixation by phosphate can be overcome by the addition of pyrophosphate [10, 20] or citrate (Heldt, H. W., unpublished) which are both known to cause a competitive inhibition of the phosphate translocator (Fliege, and Heldt, H. W., in preparation). Details on this matter shall be dealt with in a later publication.

In conclusion, the data shown here demonstrate the importance of the phosphate translocator as a partial step of $CO_2$ fixation by intact chloroplasts. In order to achieve an optimal overall rate of $CO_2$ fixation, rate of transport must be matched to the capacity of $CO_2$ fixation in the stroma.

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