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# CAPACITIES OF PEA CHLOROPLASTS TO CATALYSE THE OXIDATIVE PENTOSE PHOSPHATE PATHWAY AND GLYCOLYSIS

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**Key Word Index**—*Pisum sativum*; Leguminosae; pea; chloroplasts; enzymes; oxidative pentose phosphate pathway; glycolysis.

**Abstract**—The aim of this work was to measure the capacities of pea (*Pisum sativum*) shoot chloroplasts to catalyse the oxidative pentose phosphate pathway and glycolysis. Of the total activities in the unfractionated homogenates, appreciable proportions of those of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and phosphofructokinase, and smaller but significant proportions of those of phosphopyruvate hydratase and pyruvate kinase were recovered in crude preparations of chloroplasts, and co-purified with intact chloroplasts on sucrose gradients. The activities in the chloroplasts showed considerable latency that was closely correlated with chloroplast integrity. Phosphoglyceromutase activity in the above preparations of chloroplasts did not exceed that expected from cytoplasmic contamination. The mass–action ratio for phosphoglyceromutase in illuminated isolated chloroplasts differed markedly from the enzyme's equilibrium constant. Isolated chloroplasts converted 2-phosphoglycerate to pyruvate. The enzyme activities of the chloroplasts were compared with the rates of respiration and starch breakdown in pea leaves in the dark. It is concluded that in the dark chloroplasts could metabolize all the products of starch breakdown and catalyse much of the respiration of pea shoots via the oxidative pentose phosphate pathway and/or glycolysis as far as 3-phosphoglycerate. It is suggested that pea shoot chloroplasts lack phosphoglyceromutase but contain some phosphopyruvate hydratase and pyruvate kinase.

## INTRODUCTION

The aim of this work was to determine the capacities of pea shoot chloroplasts to catalyse the oxidative pentose phosphate pathway and glycolysis. Chloroplasts contain glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 6-phosphogluconate dehydrogenase (EC 1.1.1.44) [1, 2], and phosphofructokinase (EC 2.7.1.11) [3, 4]. The presence of these enzymes in chloroplasts, together with those of the reductive pentose phosphate pathway, means that, in qualitative terms, chloroplasts should be able to catalyse the complete oxidative pentose phosphate pathway, and glycolysis at least to 3-phosphoglycerate. In order to assess the quantitative significance of the above, we need to know the capacities of the chloroplasts to catalyse the two pathways of carbohydrate oxidation, and the relationship between these capacities and both starch breakdown and respiration in the intact leaf. This knowledge is not available: no one has reported measurements of the capacities of both pathways from chloroplasts from leaves in which carbohydrate oxidation has been characterized. We have already established the pathways of carbohydrate oxidation [5] and of starch breakdown [6] in pea leaves. We now report the relative activities of phosphofructokinase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in pea chloroplasts. These enzymes were chosen because they are specific to carbohydrate oxidation, as opposed to synthesis, and because the first two are likely to regulate their respective pathways.

The extent to which chloroplasts contain the glycolytic enzymes for the conversion of 3-phosphoglycerate to pyruvate is not known. Labelling experiments [7] suggest that these enzymes are not in the chloroplast. More recent studies have revealed small amounts of the enzymes in chloroplast preparations and have shown that such preparations can convert $CO_2$ to fatty acids [8]. However, it is not clear whether the above properties were due to enzymes within chloroplasts or to cytoplasmic contamination. The most convincing evidence is that of Murphy and Leech [9, 10] who showed that the ability of chloroplast preparations from spinach to convert $^{14}CO_2$ to fatty acids was diminished by the presence of unlabelled 3-phosphoglycerate, phosphoenolpyruvate and pyruvate, but not by centrifugation on a stepped gradient of sorbitol. However, it is not clear whether the centrifugation did remove cytoplasmic contaminants; the concentrations of the added non-radioactive intermediates were very high (50 mM), and the rates of labelling of the fatty acids varied appreciably. Thus we have determined whether pea shoot chloroplasts contain phosphoglyceromutase (EC 2.7.5.3), phosphopyruvate hydratase (EC 4.2.1.11) and pyruvate kinase (EC 2.7.1.40).

Our initial experimental approach was to determine the proportions of the enzymes in the unfractionated homogenate that were sedimented with chloroplasts. We paid particular attention to the quality of the chloroplasts. We then investigated whether the sedimented activities were within the isolated chloroplasts. We did

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this by determining whether the activities co-purified with intact chloroplasts on sucrose gradients, and by measuring latency. The latter approach relies on the inability of the substrates or products to cross the chloroplast envelope. If the enzymes were within isolated chloroplasts, assay of their activity in preparations of intact chloroplasts should give low values in comparison to those obtained with preparations of lysed chloroplasts. We isolated chloroplasts and compared enzyme activities in samples kept intact throughout the assay and in samples completely lysed before assay. The difference between the values found for intact and lysed chloroplasts is expressed as a percentage of the activity in the lysed chloroplasts to give what we call the latency.

## RESULTS

The detailed properties of the crude preparations of chloroplasts have been described [6]. On average, 67% of the chloroplasts were intact, the $CO_2$-dependent uptake of oxygen in the light was 90–100 μmol/mg chlorophyll per hr, and the yield of intact chloroplasts was 25–30%. We took the following precautions to ensure that our measurements of enzymes were reliable. The assay for each enzyme in both the unfractionated homogenate and the crude chloroplast preparation was optimized with respect to the concentration of each component, and the pH, of the reaction mixture. At least 91% of the activity of pyruvate kinase was shown to be dependent upon added ADP. If phosphoglyceromutase in the chloroplast preparation was assayed conventionally by coupling to NADH oxidation via lactate dehydrogenase, about half of the observed oxidation was found to be independent of added lactate dehydrogenase. We think that this was due to glyceraldehydephosphate dehydrogenase supported by ATP produced by chloroplast adenylate kinase (EC 2.7.43) [11] or possibly by the added pyruvate kinase. The first suggestion is supported by the fact that 1 mM arsenate, which would be expected to inhibit the formation of triose phosphate, abolished the activity that was independent of lactate dehydrogenase. The latter activity was also abolished by including in the assay 0.23 mM AMP and excess adenylate kinase. We adopted the latter procedure for all our routine assays. We confirmed that this modified assay was satisfactory by showing that it gave similar values, in all the fractions that we examined, to those obtained by the three other assays (methods II–IV) that we developed.

We investigated whether the chloroplast preparations contained anything else that interfered with the enzyme assays. We did this by making a mixture of either the unfractionated homogenate or the 6000 *g* supernatant with a preparation of lysed chloroplasts. We then showed that for each enzyme the activity found in the mixture was within 10% of the value predicted from measurements made on the separate components of the mixture. Finally, we investigated whether the activities of the enzymes in the chloroplast preparations could be enhanced either by dialysis for 4 hr against 30 mM glycylglycine, pH 7.5, or by lysis followed by centrifugation at 100000 *g* for 30 min. No effects were found except that the activity of phosphofructokinase was almost doubled. The latter effect was not found in either the unfractionated homogenate or the 6000 *g* supernatant. Thus chloroplasts were lysed and centrifuged before being assayed for phosphofructokinase.

The extent to which the different enzymes sedimented with the crude preparations of chloroplasts is shown in Table 1. Ribulose bisphosphate carboxylase (EC 4.1.1.39) and glyceraldehydephosphate dehydrogenase (NADP) (EC 1.2.1.9) were used as markers for chloroplasts, and phosphoenolpyruvate carboxylase (EC 4.1.1.31) for cytoplasm. We emphasize the following. First, there was close agreement between different experiments in respect of both the absolute activities of the enzymes and their distribution. Second, for each enzyme the activity in the chloroplast preparation plus that in the 6000 *g* supernatant corresponded to that in the unfractionated homogenate. Thus significant losses of enzyme activity did not occur during the fractionations. Third, the chloroplast fraction contained a considerable proportion of the dehydrogenases of the pentose phosphate pathway, and a lesser but still appreciable proportion of phosphofructokinase. Finally, the proportions of phosphopyruvate hydratase and pyruvate kinase in the chloroplast fractions significantly ($P < 0.001$) exceeded that of phosphoenolpyruvate carboxylase. This was not true of phosphoglyceromutase ($P > 0.4$).

The extent to which the enzymes in the crude preparations were recovered in chloroplasts purified by density gradient centrifugation is shown in Fig. 1, which illustrates a typical fractionation. Chlorophyll and glycer-

Table 1. Activities of enzymes of carbohydrate oxidation in chloroplast preparations from pea shoots

| Enzyme | No. of preparations assayed | Enzyme activity (nkat per mg chlorophyll) Unfractionated homogenate | Sediment | Percentage of activity in unfractionated homogenate recovered in sediment | sediment + supernatant |
|---|---|---|---|---|---|
| Glucose-6-phosphate dehydrogenase | 3 | 20.0 ± 3.8 | 5.5 ± 0.3 | 13.9 ± 1.0 | 105 ± 2 |
| 6-Phosphogluconate dehydrogenase | 3 | 28.5 ± 4.5 | 8.0 ± 0.3 | 13.8 ± 0.3 | 103 ± 1 |
| Phosphofructokinase | 4 | 10.0 ± 2.0 | 1.8 ± 0.3 | 8.5 ± 0.02 | 99 ± 2 |
| Phosphoglyceromutase | 6 | 181.7 ± 33.5 | 2.3 ± 0.7 | 0.5 ± 0.08 | 101 ± 5 |
| Phosphopyruvate hydratase | 6 | 103.3 ± 19.2 | 6.8 ± 1.3 | 3.0 ± 0.20 | 103 ± 2 |
| Pyruvate kinase | 6 | 50.0 ± 5.3 | 3.7 ± 0.5 | 3.6 ± 0.70 | 96 ± 3 |
| Phosphoenolpyruvate carboxylase | 6 | 3.3 ± 0.7 | 0.05 ± 0.02 | 0.6 ± 0.01 | 102 ± 3 |
| Ribulose bisphosphate carboxylase | 3 | 5.7 ± 1.8 | 6.3 ± 3.0 | 30.1 ± 2.72 | 123 ± 5 |
| Glyceraldehydephosphate dehydrogenase (NADP) | 5 | 50.0 ± 5.3 | 40.3 ± 6.8 | 28.2 ± 1.40 | 92 ± 5 |

Homogenates were centrifuged up to 6000 *g* to yield a crude preparation of chloroplasts and a supernatant. Values are means ± s.e.

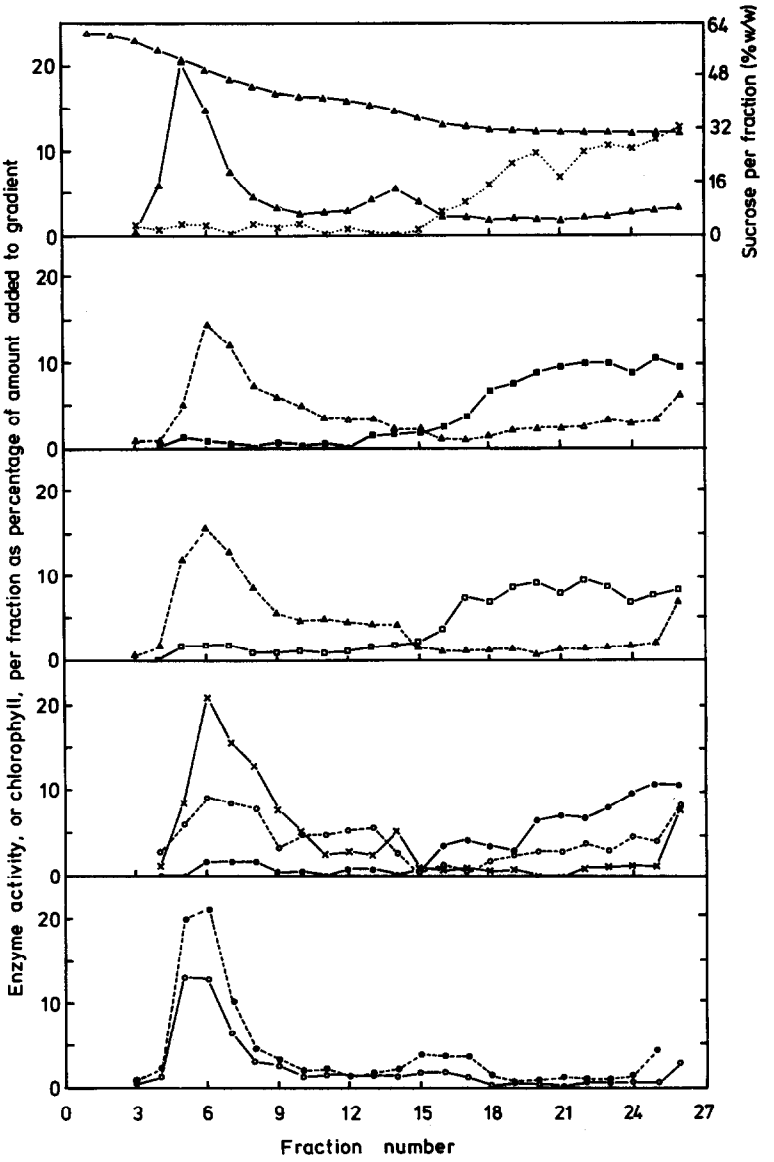


Fig. 1. Enzyme activities of purified chloroplasts. Portions of a crude preparation of chloroplasts were layered on to a sucrose gradient and centrifuged. Fractions of 1.5 ml were collected and assayed for: sucrose (△——△), chlorophyll (▲——▲), phosphoenolpyruvate carboxylase (×----×), glucose-6-phosphate dehydrogenase (△----△), catalase (■——■), 6-phosphogluconate dehydrogenase (▲----▲), cytochrome oxidase (□——□), phosphofructokinase (×——×), phosphopyruvate hydratase (○----○), phosphoglyceromutase (●——●), glyceraldehydephosphate dehydrogenase (NADP) (●----●), and pyruvate kinase (○——○).

aldehydephosphate dehydrogenase (NADP) were used as markers for chloroplasts; and cytochrome oxidase (EC 1.9.3.1), catalase (EC 1.11.1.6) and phosphoenolpyruvate carboxylase as markers for mitochondria, peroxisomes and cytoplasm, respectively. Comparison of the activities added to the gradients with the sum of the activities recovered in the fractions showed that for each enzyme the latter value was 90–110% of the former except for pyruvate kinase where the figure was 60%. The major peak of chlorophyll was due to intact chloroplasts and the minor peak to broken chloroplasts. Minimal proportions of the activities of the mitochondrial, peroxisomal and cytoplasmic enzymes were recovered with the intact chloroplasts. The central feature of the data in Fig. 1 is that, of the enzyme activities added to the gradient, the following percentages were recovered with the intact chloroplasts: glyceraldehydephosphate dehydrogenase (NADP), 60; glucose-6-phosphate dehydrogenase, 50; 6-phosphogluconate dehydrogenase, 40; phosphofructokinase, 59; phosphopyruvate hydratase, 35; pyruvate kinase, 37; phosphoglyceromutase, 2–4; phosphoenolpyruvate carboxylase, 2–4.

Except for phosphoglyceromutase, the enzyme activities in the crude preparations of chloroplasts showed considerable latency. We measured the latency of the enzymes in crude preparations of chloroplasts in which we varied the degree of lysis by suspending the chloroplasts in different concentrations of sorbitol (Fig. 2).

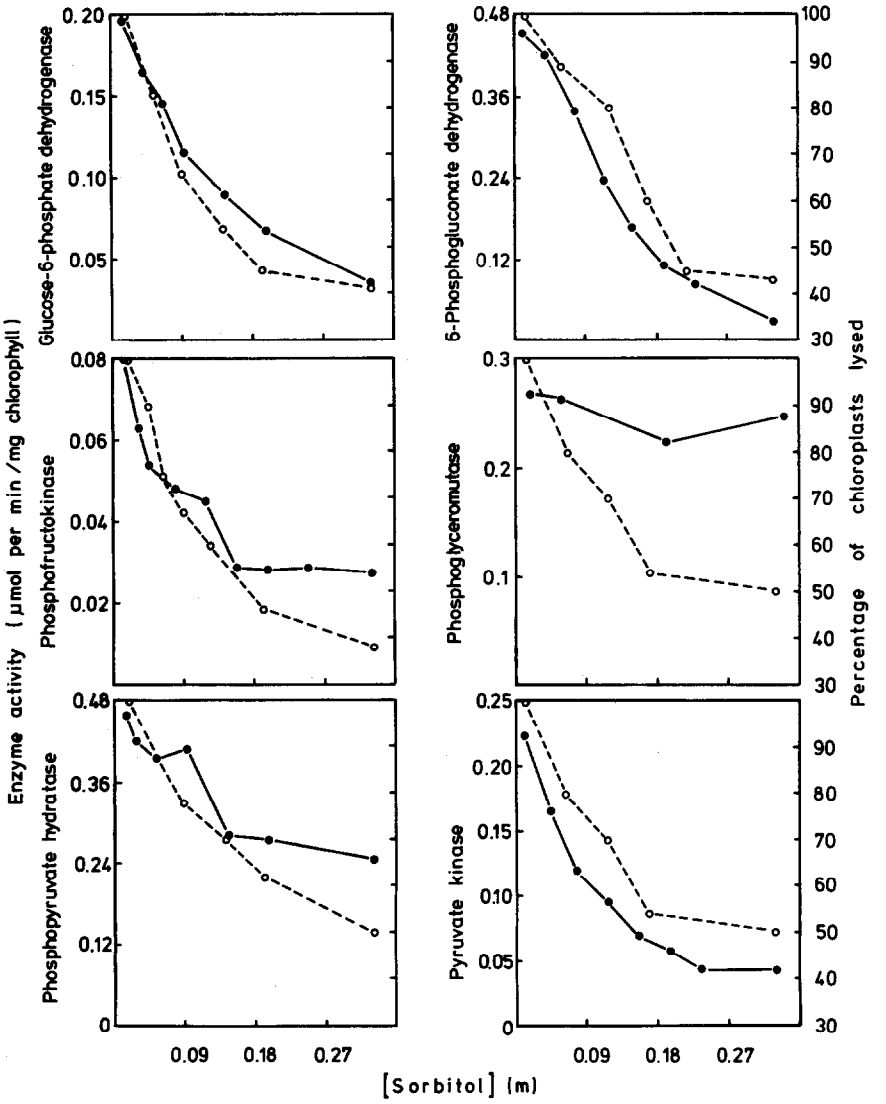


Fig. 2. Relationship between enzyme activity and the extent of lysis of chloroplasts. Samples of crude preparations of chloroplasts were added to reaction mixtures appropriate for the measurement of enzyme activity (●——●), and the extent of lysis (○——○), and in which the sorbitol concentration was varied as shown on the abscissae. The reaction mixtures were incubated at 25° for 5 min and then, by addition of the appropriate substrates, the enzyme activities and the extent of lysis were measured.

There was a very close correlation between latency and chloroplast integrity for all the enzymes except phosphoglyceromutase.

The above experiments are sufficiently decisive for the two dehydrogenases and phosphofructokinase, but further evidence is needed to decide whether chloroplasts contain phosphoglyceromutase, phosphopyruvate hydratase and pyruvate kinase. First, we investigated phosphoglyceromutase. This enzyme catalyses an equilibrium reaction. Illumination of isolated chloroplasts from pea shoots causes a significant increase in 3-phosphoglycerate in the chloroplasts and in the medium [12]. If phosphoglyceromutase is in the chloroplast then the above increase would be expected to be accompanied by one in 2-phosphoglycerate. Thus after illumination the mass-action ratio, 2-phosphoglycerate:3-phosphoglycerate, should be close to the enzyme's equilibrium constant (0.1–0.2). We determined whether this was so.

The above, and subsequent, experiments involved measurements of glycolytic intermediates. We checked the reliability of these measurements. For each check we incubated a crude preparation of chloroplasts at 25° for 10 min as described for the experiments reported in Table 2. Then we divided the chloroplast suspension into halves and centrifuged each at 6000 *g* for 15 sec. We then added $HClO_4$ to a final concentration of 1.41 M, to each of the resulting sediments and supernatants. The $HClO_4$ added to one sediment and to one supernatant contained measured amounts of 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate and pyruvate. These amounts were comparable to those present in the sediment and supernatant, respectively. Comparison of the amounts of these compounds in the different fractions showed whether the added compounds had survived the processes of killing, extraction and assay. For both sediment and supernatant such estimates of

Table 2. Mass-action ratios of 2-phosphoglycerate:3-phosphoglycerate in illuminated suspensions of chloroplasts

| | Ratios | |
|---|---|---|
| | Chloroplasts | Medium |
| Unwashed chloroplasts | 0.017, 0.040<br>0.040 | 0.060, 0.045<br>0.071 |
| Washed chloroplasts | 0.007, 0.006<br>0.007 | 0.012, 0.009<br>0.007 |

Crude preparations of chloroplasts were incubated in the light at 25° for 12 min and then centrifuged at 6000 *g* for 15 sec to give chloroplasts (sediment) and medium (supernatant) which were analysed separately. Each value for the chloroplasts and the corresponding one for the medium is from a separate preparation.

recovery ranged from 87 to 111% of the amounts added.

The amounts of 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate and pyruvate in freshly isolated chloroplasts were, respectively, 5.1 ± 1.4, 0.7 ± 0.1, 0.9 ± 0.1 and 3.8 ± 1.0 nmol per mg chlorophyll (means ± s.e. of values from three extracts). The mass-action ratios for 2-phosphoglycerate:3-phosphoglycerate after 12 min incubation in the light are in Table 2. These ratios differ markedly from the equilibrium constant and show a substantial excess of 3-phosphoglycerate. The ratios were further from equilibrium in the chloroplasts than in the suspending medium, and in washed than in unwashed chloroplasts. Washing removed 75% of the phosphoglyceromutase activity of the crude preparations of chloroplasts.

As a further test of whether the chloroplasts contained phosphoglyceromutase, we compared $H^{14}CO_3^-$ and acetate-[2-$^{14}$C] as precursors of fatty acids in isolated chloroplasts. Acetate-[2-$^{14}$C] labelled the chloroform-soluble substances much more readily than did $H^{14}CO_3^-$ (Table 3). The fluxes into fatty acids from acetate-[$^{14}$C] and $H^{14}CO_3^-$ were 0.32 and 0.011 μmol acetate/mg chlorophyll per hr, respectively. Most of the incorporation from acetate-[$^{14}$C] was into fatty acids in both the chloroplasts and the medium. Of the incorporation from $H^{14}CO_3^-$ only a third of that in chloroplasts was into fatty acids although the figure was 90% for the medium.

Finally, we investigated whether phosphopyruvate hydratase and pyruvate kinase were in the chloroplasts by determining whether isolated chloroplasts could convert 2-phosphoglycerate to pyruvate. We did this by supplying 2-phosphoglycerate to illuminated chloroplasts and measuring whether pyruvate accumulated in either the chloroplasts or the medium (Table 4). There was a significant increase in pyruvate in the chloroplasts but not in the medium. Evidence that this synthesis of pyruvate was not due to cytoplasmic contamination was obtained by showing that the addition of phosphoenolpyruvate to the chloroplasts did not lead to a comparable increase in pyruvate. This phosphoenolpyruvate was added at the concentration found in the medium at the end of the incubation in 2-phosphoglycerate.

Table 3. Conversion of acetate-[2-$^{14}$C] and $H^{14}CO_3^-$ to fatty acids by chloroplasts isolated from pea shoots

| Parameter | Acetate-[2-$^{14}$C] | $H^{14}CO_3^-$ |
|---|---|---|
| Rate of labelling of chloroform-soluble material (μmol/mg chlorophyll per hr) | 0.60, 0.36 | 0.02, 0.05, 0.02 |
| Percentage of chloroform-soluble material retained in chloroplasts | 9.60, 12.0 | 12.3, 13.2, 18.1 |
| Distribution of $^{14}$C within the chloroform-soluble material ($^{14}$C per fraction as % total) | | |
| Chloroplasts | | |
| Total fatty acids | 83 | 33 |
| Esterified fatty acids | 43 | 27 |
| Free fatty acids | 50 | 6 |
| Medium | | |
| Total fatty acids | 92 | 91 |
| Esterified fatty acids | 1 | 2 |
| Free fatty acids | 90 | 88 |

Crude preparations of chloroplasts were incubated in the light at 15° for 30 min, and then separated into chloroplasts and medium as described in Table 2. The two fractions were analysed separately.

Table 4. Conversion of 2-phosphoglycerate to pyruvate by isolated chloroplasts

| Addition to chloroplast suspension | Pyruvate content (as % of control) | |
|---|---|---|
| | Chloroplasts | Medium |
| 1.2 mM 2-Phosphoglycerate | 235, 347, 213 | 112, 133, 116 |
| 0.06 mM Phosphoenolpyruvate | 105, 106 | 88, 100 |

Washed chloroplasts were incubated in the light at 25° for 10 min and were then separated into chloroplasts and medium as described in Table 2. Each value for the chloroplasts and the corresponding one for the medium is from a separate preparation in which control values were obtained from replicate samples of chloroplasts incubated without additions.

## DISCUSSION

The rate of photosynthesis and the integrity of the chloroplasts that we prepared compare favourably with those of other preparations from peas [13]. The purified chloroplasts were not significantly contaminated by cytoplasm, peroxisomes or mitochondria (Fig. 2). The enzyme assays were carefully optimized for each fraction. Apart from some inactivation, during density gradient centrifugation, of pyruvate kinase, known to be a labile enzyme [14], there were no significant losses of enzyme activity during the different fractionation procedures. Thus we argue that the results of our fractionations are a reliable indication of the capacities of pea shoot chloroplasts to catalyse the reactions studied.

Appreciable fractions of the total activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and phosphofructokinase were found in the crude preparations of chloroplasts. Much of this activity co-purified with intact chloroplasts on density gradients, and a high degree of latency was found for each enzyme. We conclude that in pea shoots considerable proportions of these enzymes are in the chloroplasts. The data in Table 1 suggest values of *ca* 40% for the two dehydrogenases and 25% for phosphofructokinase.

Our results also suggest that *ca* 10% of the phosphopyruvate hydratase and pyruvate kinase of pea shoots are in the chloroplasts. The activities of these enzymes in crude preparations of chloroplasts exceeded that expected from cytoplasmic contamination. Significant proportions of these activities were recovered with purified chloroplasts, and both enzymes showed latency that was closely related to chloroplast integrity. Additional evidence for the presence of these enzymes in chloroplasts is provided by the facts that isolated chloroplasts contained significant amounts of the enzymes' substrates and products, and converted 2-phosphoglycerate to pyruvate.

All the data that we obtained for phosphoglyceromutase indicate that this enzyme is absent from pea shoot chloroplasts. The activity in the crude preparations did not exceed that expected from cytoplasmic contamination, was not latent, and remained at the top of the gradient rather than co-purifying with the intact chloroplasts. Additional evidence for the enzyme's absence from chloroplasts is provided by the facts that isolated chloroplasts did not achieve the expected equilibration between 3- and 2-phosphoglycerate, and had such a limited capacity to convert $^{14}CO_2$ to fatty acids that it may reasonably be attributed to cytoplasmic contamination. The lack of phosphoglyceromutase in isolated chloroplasts could have been caused by leakage of the enzyme from the chloroplasts during their isolation. Although this possibility is not eliminated by our results, it seems unlikely because the chloroplasts were intact, functional and retained the other enzymes which we studied. Thus leakage of phosphoglyceromutase would have to have been very specific. We suggest that chloroplasts of young pea shoots do not contain significant amounts of phosphoglyceromutase. Whether this is true of chloroplasts in general cannot be assessed until chloroplasts from leaves at different stages of development and from different species have been examined in more detail.

Our qualitative conclusion is that pea shoot chloroplasts can catalyse the complete oxidative pentose phosphate pathway, but glycolysis only as far as 3-phosphoglycerate. Further metabolism of the latter via glycolysis would have to involve export to the cytoplasm. Our results indicate that at least some of the 2-phosphoglycerate formed from 3-phosphoglycerate could be returned to the chloroplast for metabolism to pyruvate. The mechanism whereby 2-phosphoglycerate enters the chloroplast (Table 4) is not known. Although the phosphate translocator transports it much less effectively than it does 3-phosphoglycerate [15], the rate may still be enough to meet the demands *in vivo*.

Quantitative consideration of our data shows that the absolute activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and phosphofructokinase (Table 1) exceed the estimates of the rates at which pea leaves oxidize carbohydrate, 0.7–1.6 μmol hexose/mg chlorophyll per hr [5], and breakdown starch, 0.5–0.7 μmol hexose/mg chlorophyll per hr [6]. As these are the likely rate-limiting enzymes, this indicates that in pea shoots in the dark a substantial fraction of the total carbohydrate oxidation, and the metabolism of all the products of starch breakdown, could occur in the chloroplast via the oxidative pentose phosphate pathway, or the top half of glycolysis, or some combination of these routes. Examination of the relative activities of the above enzymes in chloroplasts (Table 1) and in leaves [5] indicates that the relative activity of the oxidative pentose phosphate pathway may be higher in the chloroplasts than in the leaf as a whole.

## EXPERIMENTAL

*Materials.* Isotopes were from the Radiochemical Centre, Amersham; substrates, cofactors and enzymes from Boehringer, Mannheim, except for fructose-6-phosphate which was from Sigma. Peas (*Pisum sativum* L. cv Kelvedon Wonder), sown and germinated as in ref. [5], were grown at 25° in natural light supplemented with artificial light of 16-hr photoperiod and intensity of $53 \times 10^{-3}$ J/cm²/min at the soil surface.

*Chloroplasts.* These were prepared, basically as described in ref. [6], from the shoots of 6- to 8-day-old plants. The usual extraction medium was 0.33 M sorbitol, 50 mM $KH_2PO_4$, 50 mM $Na_2HPO_4$, 5 mM $MgCl_2$, 0.1% (w/v) NaCl, 0.2% (w/v) sodium isoascorbate and 0.1% (w/v) bovine serum albumin. The isoascorbate was omitted for the chloroplasts used in the experiments in Tables 2–4; for chloroplasts placed on the sucrose gradient 50 mM glycylglycine, pH 7.0, replaced the $KH_2PO_4$ and $Na_2HPO_4$. The filtrate obtained by passing the initial homogenate through muslin and cotton wool is called the unfractionated homogenate and was centrifuged up to 6000 *g* to give the crude preparations of chloroplasts and the 6000 *g* supernatant. Washed chloroplasts were obtained by resuspending crude preparations of chloroplasts (1–3 mg chlorophyll) in 15 ml 0.33 M sorbitol, 50 mM HEPES, pH 7.6, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA and 0.1% (w/v) bovine serum albumin. The resulting suspension was centrifuged at 4000 *g* for 30 sec and the sediment was washed again in the same way before being resuspended in the experimental medium. The methods used to purify chloroplasts from the crude preparation by density gradient centrifugation, to measure $CO_2$-dependent oxygen evolution, chloroplast intactness and chlorophyll were as in ref. [6].

*Measurements of enzymes.* The unfractionated homogenate and the 6000 *g* supernatant were assayed without further treatment. The 6000 *g* pellet was resuspended in 3–4 ml $H_2O$, homogenized in an all glass homogenizer for 1 min and the homogenate was made up to the composition of the extraction medium to give the crude preparation of lysed chloroplasts

which was used at once for the assay of all enzymes except phosphofructokinase. For the latter, the lysed chloroplast preparation was centrifuged at 100000 *g* for 30 min and the supernatant was assayed. The relationship between chloroplast integrity and the latency of the enzymes was determined as in ref. [6].

All fractionations were done at 2–4° and the extracts and fractions were kept at 2° until assayed spectrophotometrically at 25°. The procedures and reaction mixtures (3.0 ml) were as follows: glucose-6-phosphate dehydrogenase as for pea leaves in ref. [5]; glyceraldehydephosphate dehydrogenase (NADP), phosphoenolpyruvate carboxylase, cytochrome oxidase and catalase as for the cell fractions in ref. [6]; 6-phosphogluconate dehydrogenase [16], 50 mM glycylglycine (pH 8.2), 13 mM $MgCl_2$, 0.3 mM NADP, 1.0 mM 6-phosphogluconate; phosphofructokinase [17], 50 mM glycylglycine (pH 7.25), 1 mM ATP, 0.1 mM NADH, 2 mM cysteine, 0.5 mM $MgCl_2$, 0.135 unit aldolase, 0.03 unit triosephosphate isomerase, 0.36 unit glycerol-3-phosphate dehydrogenase, 10 mM fructose-6-phosphate; phosphopyruvate hydratase [18], 50 mM glycylglycine (pH 7.4), 30 units lactate dehydrogenase, 9 units pyruvate kinase, 0.23 mM ATP, 0.15 mM NADH, 20 mM $MgSO_4$, 12 mM KCl, 3.3 mM 2-phosphoglycerate; pyruvate kinase [14], 50 mM glycylglycine (pH 7.5), 1 mM ADP, 0.1 mM NADH, 10 mM $MgCl_2$, 50 mM KCl, 3 units lactate dehydrogenase, 0.5 mM phosphoenolpyruvate; ribulose bisphosphate carboxylase [19], 50 mM HEPES (pH 7.8), 5 mM ATP, 1 mM dithiothreitol, 10 mM KCl, 1 mM EDTA, 15 mM $MgCl_2$, 2.5 units glyceraldehydephosphate dehydrogenase (NAD), 3.8 units 3-phosphoglycerate kinase, 1 unit phosphocreatine kinase, 1 unit ribose-5-phosphate isomerase, 0.25 mM ribose-5-phosphate. Phosphoglyceromutase was measured in 4 ways: all assay mixtures contained 50 mM glycylglycine (pH 7.4), 5 mM $MgSO_4$, 12 mM KCl and 0.3 mM 2,3-diphosphoglycerate. The specific components were: method I, the routine assay [18], 0.23 mM ADP, 0.15 mM NADH, 30 units lactate dehydrogenase, 9 units pyruvate kinase, 0.4 unit phosphopyruvate hydratase, 0.25 mM AMP, 2 units adenylate kinase, 3.3 mM 3-phosphoglycerate; method II, 0.15 mM NADH, 5 mM $KHCO_3$, 0.42 unit phosphopyruvate hydratase, 0.2 unit phosphoenolpyruvate carboxylase, 10 units malate dehydrogenase, 3.3 mM 3-phosphoglycerate, the 2-phosphoglycerate formed was converted to oxaloacetate which was measured with the malate dehydrogenase; method III, 0.4 unit phosphopyruvate hydratase, 3.3 mM 3-phosphoglycerate, phosphoenolpyruvate formation was determined from measurements of $A_{240}$; method IV, 0.15 mM NADH, 0.25 mM ATP, 5 mM dithiothreitol, 200 units triosephosphate isomerase, 9 units 3-phosphoglycerate kinase, 2.4 units glyceraldehydephosphate dehydrogenase (NAD), 3.3 mM 2-phosphoglycerate, the formation of 3-phosphoglycerate was measured by following NADH oxidation.

*Measurements of substrates.* For measurements of the amounts in freshly isolated chloroplasts, crude preparations were immediately made 1.41 M with respect to $HClO_4$. For determination of the mass-action ratios (Table 2) and the conversion of 2-phosphoglycerate to pyruvate (Table 4), crude preparations of chloroplasts (0.8–3.6 mg chlorophyll) were resuspended in and incubated in 3–6 ml 0.33 M sorbitol, 50 mM HEPES (pH 7.6), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.1% (w/v) bovine serum albumin, 5 mM PPi, 0.1 mM Pi, 0.8 mM ATP and 5 mM $KHCO_3$ (incubation medium) in an Erlenmeyer flask in light of intensity 1.06 J/cm$^2$/min at the surface of the glass. At the end of the incubation the suspensions were centrifuged at 6000 *g* for 15 sec and the sediment and supernatant were separately made 1.41 M with $HClO_4$. After the addition of $HClO_4$, the different fractions were kept at −5° for 12 hr and then centrifuged at 4000 *g* for 1 min. The pellet was washed twice with 0.5 ml portions of 1.41 M $HClO_4$ that had been neutralized with 5 M $K_2CO_3$. The supernatant, and the above washings, were combined, neutralized with 5 M $K_2CO_3$ and then centrifuged at 4000 *g* for 2 min. The resulting supernatant was then assayed spectrophotometrically for the substrates as in ref. [20].

*Labelling of fatty acids.* Crude preparations of chloroplasts (0.5–0.7 mg chlorophyll) were resuspended and incubated in 6 ml 0.33 M sorbitol, 50 mM HEPES (pH 7.6), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.1% (w/v) bovine serum albumin, 0.25 mM Pi, 5 mM PPi, 1 mM ATP, 1.2 mM CoA, and 10 mM $KHCO_3$ to which was added either 42 μCi $NaH^{14}CO_3$ (sp. act. 58.5 mCi/mmol) or 8.9 μCi sodium acetate-[2-$^{14}$C] (sp. act. 0.74 mCi/mmol). The suspensions were incubated for 30 min at 15° and were then separated into chloroplasts and medium by centrifuging at 6000 *g* for 15 sec. Then the chloroplasts and the medium were each made 3 M with respect to $HCO_2H$. Chloroform-soluble material, extracted as in ref. [9], was fractionated by TLC on Si gel 60 $F_{254}$ with hexane–$Et_2O$–$HCO_2H$ (50:10:1) as in ref. [21]. Phospholipid fractions were eluted with $Et_2O$. Phospholipids, mono-, di- and triglycerides were saponified as in ref. [22]. $^{14}$C was determined as in ref. [5].

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