Studies on the Mechanism of Activation and Inactivation of Pyruvate, Phosphate Dikinase

A POSSIBLE REGULATORY ROLE FOR THE ENZYME IN THE C₄ DICARBOXYLIC ACID PATHWAY OF PHOTOSYNTHESIS

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1. The activity of pyruvate, P_i dikinase in leaves of maize and *Amaranthus palmeri* rapidly falls on transferring illuminated plants to darkness. Illumination of dark-treated plants results in an immediate rapid increase in activity of the enzyme, the final activity reached being dependent on the intensity of the incident light. 2. Activation of the enzyme in extracts of dark-treated maize leaves after gel filtration on Sephadex G-25 requires a thiol and P_i. The P_i requirement for activation can be replaced by arsenate. Activation of the enzyme is inhibited by AMP and GMP and possibly also by ADP and ATP. Activation of the enzyme after gel filtration on Sephadex G-200 also requires a heat-labile component that is excluded by Sephadex G-25. 3. The active enzyme isolated from illuminated leaves is inactivated by ADP in the presence of a thiol, the rate of inactivation being very much faster in air than in an oxygen-free atmosphere. Reactivation of the ADP-inactivated enzyme requires a thiol, P_i and a component excluded by Sephadex G-25 but considerably retarded by Sephadex G-200. 4. The active enzyme is rapidly and irreversibly inactivated in the absence of a thiol. Inactivation is accelerated by both sodium diethyldithiocarbamate and tetraethylthiuram disulphide, and the enzyme inactivated by these reagents is completely reactivated by incubation with dithiothreitol. This reactivation does not require P_i. The inactive enzyme from dark-treated leaves is stabilized by diethyldithiocarbamate and can be partially activated by dithiothreitol alone; complete reactivation requires both dithiothreitol and P_i. 5. The enzyme activity is markedly inhibited by the thiol reagents *p*-chloromercuribenzoate, γ -(*p*-arsenophenyl)-*n*-butyrate and an equimolar mixture of arsenite and 2,3-dimercaptopropan-1-ol. 6. The processes of activation and inactivation observed *in vitro* are discussed in relation to the regulation of pyruvate, P_i dikinase activity in the leaf.

One of the reactions of the C₄ dicarboxylic acid pathway of photosynthesis (Hatch & Slack, 1966; Hatch, Slack & Johnson, 1967; Johnson & Hatch, 1968) is the conversion of pyruvate into phosphoenolpyruvate. An enzyme catalysing this conversion by the reaction as follows:

was partially purified from sugar-cane leaves and identified in leaves of other tropical grasses (Hatch & Slack, 1968) and species of two dicotyledonous families (Johnson & Hatch, 1968), but was not detected in several species in which the Calvin cycle operates. The enzyme was originally referred to as phosphopyruvate synthase, but has been renamed

pyruvate, P_i dikinase, as suggested by Reeves (1968), to distinguish it from the enzyme described by Cooper & Kornberg (1967). The suggestion that pyruvate, P_i dikinase operates in the C₄ dicarboxylic acid pathway (Hatch & Slack, 1968) is supported by the findings that the enzyme is localized in mesophyll chloroplasts (Slack, 1969) and that in the leaf the enzyme is inactivated by darkness and reactivated on illumination (Slack, 1968). These rapid changes in activity suggest that the activity of pyruvate, P_i dikinase may regulate the rate of carbon dioxide fixation via the C₄ dicarboxylic acid pathway. The aim of the present studies was to provide information from studies *in vitro* about the mechanisms involved in the activation and inactivation of the enzyme.

MATERIALS

Plants were grown at 28° in a naturally illuminated glass-house and transferred to growth rooms at 28° when a specific light-intensity was required. Illumination was provided by six Philips 400 W HPL lamps and the intensity measured with a selenium cell. The species studied were maize (*Zea mays* var. DS606A), sugar cane (*Saccharum* hybrid var. Pindar) and *Amaranthus palmeri*.

The following compounds and enzymes were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.): sodium pyruvate, ATP, ADP, AMP, GTP, GDP, UDP, IDP and aspartate aminotransferase. Dithiothreitol was supplied by Calbiochem (Los Angeles, Calif., U.S.A.). Sodium diethyldithiocarbamate was AnalaR grade (British Drug Houses Ltd., Poole, Dorset) and was recrystallized from ethanol before use. Tetraethylthiuram disulphide was prepared from sodium diethyldithiocarbamate by the method of Braun (1902) and dissolved in 5% (v/v) propane-1,2-diol before use (Hellerman, Coffey & Neims, 1965). Phosphopyruvate carboxylase was partially purified from sugar-cane leaves by the procedure described by Hatch & Slack (1968). Pyruvate, P_i dikinase was partially purified by the method of Hatch & Slack (1968) up to the second gel-filtration step with Sephadex G-200.

METHODS

Preparation of leaf extracts. Plants received specific treatments before harvest depending on the type of experiment. For reactivation studies plants were placed in darkness at 28° for either 3 or 16 hr. before harvest, and the leaves were harvested in darkness, wrapped in foil and transferred to the laboratory. When an extract containing active enzyme was required leaves were harvested from illuminated plants in the glass-house. For studies of changes in activity in the leaf in response to light-intensity the extract was prepared and passed through Sephadex G-25 in the growth room to minimize the time between harvest and extraction. By this procedure a Sephadex-treated extract was obtained within 5 min. of harvesting the leaves.

Lamina tissue from young fully-expanded leaves was ground with sand in 4 vol. (w/v) of medium containing (final concentrations) 100 mM-tris-HCl buffer, pH 8.3, 10 mM-MgCl₂, 10 mM-dithiothreitol and 1 mM-EDTA in a chilled pestle and mortar. The homogenate was filtered through Miracloth (Chicopee Mills Inc., Broadway, N.Y., U.S.A.), passed through a Sephadex G-25 column equilibrated with 50 mM-tris-HCl buffer, pH 7.9, and 10 mM-MgCl₂; omissions and additions to this mixture were as described in the Results section. Extracts prepared from dark-treated leaves were passed through Sephadex at 5° and the protein eluate was used immediately. The Sephadex treatment was performed at 22° for studies involving the active enzyme; and, except in certain specified experiments, the Sephadex G-25 column was equilibrated with 10 mM-dithiothreitol in addition to the reagents described above. The chlorophyll content of extracts was determined before Sephadex G-25 treatment by the method of Arnon (1949).

Partial purification of the inactive form of the enzyme on Sephadex G-200. Extracts of dark-treated leaves, or ADP-inactivated enzyme, were passed through a Sephadex G-25 column equilibrated with 50 mM-tris-HCl buffer, pH 7.9, containing 10 mM-MgCl₂ and 5 mM-dithiothreitol,

and the protein eluate was applied to a Sephadex G-200 column (360 mm. × 25 mm., void volume 45 ml.) equilibrated with the buffer mixture used for the Sephadex G-25 column. The column was eluted at 22° at 1 ml./min. and 4 ml. fractions were collected and kept under N₂ at 22° before assay.

Preparation of activating fraction. This fraction was prepared from leaves of maize plants kept in darkness for 16 hr. before harvest. The leaves were homogenized and filtered as described above, the extract was passed through a Sephadex G-25 column equilibrated with only 50 mM-tris-HCl buffer, pH 7.9, and the protein eluate was kept at 0° for 4 hr. In this procedure the pyruvate, P_i dikinase was irreversibly inactivated.

Assay of enzyme activity. Reaction mixtures contained sodium pyruvate (2.2 μmoles), ATP (1.1 μmoles), K₂HPO₄ (0.37 μmole), sodium glutamate (3.0 μmoles), MgCl₂ (2.2 μmoles), NaH¹⁴CO₃ (1.7 μmoles, approx. 200 000 c.p.m./μmole), dithiothreitol (1.5 μmoles), 0.2 unit of phosphopyruvate carboxylase plus 0.5 unit of aspartate aminotransferase, tris-HCl buffer, pH 8.3 (7.0 μmoles), and enzyme extract in a total volume of 0.15 ml. The amount of enzyme used was adjusted to give a linear reaction rate for at least 10 min. Assay mixtures were incubated at 30° for 5 min., then 0.04 ml. of 10% (w/v) trichloroacetic acid was added to each. The procedures for measuring the radioactivity of samples of acidified reaction mixtures and for determining the specific radioactivity of the NaH¹⁴CO₃ were as described by Slack & Hatch (1967). A unit of enzyme activity represents that which incorporates 1 μmole of H¹⁴CO₃⁻ into aspartate in 1 min.

Glyceraldehyde phosphate dehydrogenase, ribulose diphosphate carboxylase, phosphopyruvate carboxylase, pyrophosphatase and adenylate kinase were assayed as described previously (Slack & Hatch, 1967; Hatch & Slack, 1968).

RESULTS

Activation and inactivation of the enzyme in leaves

Changes in activity in response to changes in the intensity of the incident light. It was found by Slack (1968) that the activity of pyruvate, P_i dikinase decreases rapidly when illuminated leaves of *Amaranthus palmeri* are darkened and increases again when dark-treated leaves are exposed to full sunlight (approx. 12 000 foot-candles). The results presented in Fig. 1 indicate that the activity of the enzyme can change in response to relatively small changes in the intensity of the incident light. There was an immediate increase in activity when dark-treated leaves were illuminated at 500 foot-candles to an activity about eight times that in the darkened leaves. A further fourfold increase in light-intensity resulted in a rapid doubling of activity and the activity was decreased when the light-intensity was lowered again to 500 foot-candles.

The above results, and those described by Slack (1968), were observed when plants previously grown under a natural 24 hr. light-dark cycle were kept in the dark during the period of light. A separate experiment showed that similar changes in

Fig. 1. Effect of light-intensity on activity of pyruvate, P_i dikinase in leaves of *Amaranthus palmeri*. Plants previously grown at 30° under natural illumination were placed in darkness at 30° at 9.30 a.m. At 11 a.m. a number of leaves were harvested and subsampled in darkness and an extract was prepared and assayed as described in the Methods section. The plants were then illuminated at 500 foot-candles ($\bullet$), subsequently at 2000 foot-candles ($\circ$) and finally again at 500 foot-candles ($\bullet$), and the enzyme activity was determined at intervals.

activity occur and are maintained during a 24hr. light-dark cycle. Maize and *Amaranthus palmeri* plants, previously grown at 28° in a glass-house, were subjected to a 24hr. light-dark cycle (dark period 5.30 p.m.–9.00 a.m.) for 7 days and the activity of pyruvate, P_i dikinase was determined at intervals over a 36hr. period as described in the legend to Fig. 1. In leaves of both species the activity was 0.1–0.2 unit/mg. of chlorophyll before illumination, increased to 1.5–1.8 units/mg. of chlorophyll during the first hour in light and remained at this level during the period of illumination, declining to 0.2 unit/mg. of chlorophyll during the first hour in darkness. At 9.00 a.m. the following day the activity was 0.1 unit/mg. of chlorophyll, and this again increased to approx. 1.8 units/mg. of chlorophyll after illumination. During this period there was no significant change in the measured activities of NADP-specific glyceraldehyde phosphate dehydrogenase, ribulose diphosphate carboxylase, phosphopyruvate carboxylase, adenylate kinase and pyrophosphatase.

Activation of enzyme in extracts

Requirements for activation. The activity of pyruvate, P_i dikinase in extracts from dark-treated

Fig. 2. Enzyme activation in extracts from dark-treated maize leaves. Crude extracts were prepared and treated on Sephadex G-25 as described in the Methods section. Crude extract ($\circ$) was incubated at 25° with medium containing (final concentrations) 25 mM-tris-HCl buffer, pH 7.9, 10 mM-MgCl₂ and 10 mM-dithiothreitol. Sephadex-treated extracts were incubated as above ($\blacksquare$) or without dithiothreitol ($\square$). Further additions to the incubation medium used for Sephadex-treated extracts were 0.08 mM-K₂HPO₄ ($\triangle$), 1.5 mM-K₂HPO₄ ($\blacktriangle$) and 3.0 mM-sodium arsenate ($\bullet$). Activity was determined at intervals during incubation.

maize leaves was only about 5% of that obtained in extracts from illuminated leaves, indicating that approx. 95% of the enzyme was present in an inactive form. Considerable activation of this inactive enzyme occurred when crude extracts were incubated at 25° , provided that Mg²⁺ and dithiothreitol were present (Fig. 2). No activation was observed with the protein eluate obtained by treating crude extracts on Sephadex G-25, but the capacity for activation in these extracts was restored by 1.5 mM-potassium phosphate (dipotassium hydrogen phosphate). In certain experiments the rate of activation was slightly faster when 2 mM-pyruvate was present in addition to 1.5 mM-potassium phosphate, but in the absence of potassium phosphate pyruvate produced no activation. A maximal rate of activation was observed at 0.6 mM-potassium phosphate; at 0.08 mM the rate was decreased by 40%. Although the rate of activation was decreased the final activity attained was similar to that with 1.5 mM-potassium phosphate. Other potassium salts such as the chloride and the sulphate did not restore the capacity for activation when added to Sephadex-treated extracts

in place of the phosphate. However, 3.0mM-sodium arsenate was found to be as effective as 1.5mM-potassium phosphate; at 0.7mM-sodium arsenate the activation rate was decreased by approx. 30%. The results presented have been corrected for arsenate inhibition of pyruvate, P_i dikinase activity, amounting to about 30% with 0.5mM-arsenate in the assay system employed. The enzyme activated in the presence of either P_i or arsenate retained activity after passage through Sephadex G-25 to remove these reagents. It is noteworthy that the activity attained per unit weight of chlorophyll in Sephadex-treated extracts, after activation of the enzyme, was comparable with or greater than the activity in extracts prepared from leaves illuminated in full sunlight (see Figs. 6 and 7).

In the absence of a reducing agent slight activation was observed in crude extracts, but none occurred in Sephadex-treated extracts. Dithiothreitol at a concentration of 5mM was optimum for both the rate of activation and final activity reached, but at 0.5mM both the rate and final activity were decreased. With 4mM-lipoate the rate of activation was about half that observed in the presence of 5mM-dithiothreitol. However, the rate of activation and the final activity reached in the presence of 5mM-sodium ascorbate was similar to that observed with 5mM-dithiothreitol.

The active enzyme is irreversibly inactivated in the absence of Mg^{2+} (Hatch & Slack, 1968); hence it was difficult to investigate the possibility of a Mg^{2+} requirement for activation. However, when the small amount of active enzyme in extracts prepared from dark-treated leaves was inactivated by treatment on Sephadex G-25 in the absence of magnesium chloride a considerable proportion of the potential activity was recovered when magnesium chloride and potassium phosphate were subsequently added. There was no increase when only potassium phosphate was supplied. It would appear that the inactive form of the enzyme is more stable in the absence of Mg^{2+} than is the active enzyme.

Effect of pH, temperature and oxygen on activation. Maximum rates of activation were obtained when the pH of the extract was between 7.5 and 8.3. The rate of activation was faster at 30° than at 25°, but a greater final activity was reached at the lower temperature. At 20° the rate was less than half that at 25°. In the presence of 5mM-dithiothreitol the rate of activation was similar when extracts were incubated in either air or nitrogen; however, the final activity attained was greater in a nitrogen atmosphere. Hence, to optimize the final activity obtained, activation studies were performed at pH 7.9 at 25° under nitrogen.

Stability of the inactive form of the enzyme obtained from dark-treated leaves. The results described

Fig. 3. Stability of the inactive form of the enzyme. Extracts were prepared from dark-treated maize leaves and passed through a Sephadex G-25 column without dithiothreitol as described in the Methods section. The protein eluate was then incubated immediately at 25° in the presence of either 10mM-dithiothreitol and 1.5mM- K_2HPO_4 (■), 1.5mM- K_2HPO_4 to which 10mM-dithiothreitol was added after 40min. (○) or 10mM-dithiothreitol (●) to which 1.5mM- K_2HPO_4 was added after 15min. (□) and 40min. (▲) incubation. The enzyme was assayed at intervals.

above indicate that the inactive form of the enzyme isolated from dark-treated leaves can be readily activated under appropriate conditions. To obtain information about this inactive form of the enzyme we studied the conditions that prevented it from becoming irreversibly inactive. The inactive form of the enzyme is stable for short periods at 5° in the absence of dithiothreitol; the rate of activation and final activity were the same when crude extracts were chromatographed either on Sephadex G-25 in the presence of dithiothreitol or on Sephadex G-25 in the absence of dithiothreitol but with the thiol subsequently added to the protein eluate. However, when Sephadex-treated extracts were incubated at 25° for 40min. before the addition of dithiothreitol no activation occurred (Fig. 3). Even in the presence of dithiothreitol the initial rate of activation declined as the period of incubation before addition of P_i increased. Because of this lower rate it was difficult to establish whether the treatment also decreased the final activity attained. We have noted in several experiments that dithiothreitol was more effective in stabilizing the inactive enzyme in leaf extracts from plants kept in darkness for only 3hr. than from plants kept in darkness for 16hr. before the preparation of

Table 1. *Inhibition of enzyme activation by adenine nucleotides*

Sephadex G-25-treated extract, prepared from dark-treated leaves as described in the Methods section, was incubated with medium containing (final concentrations) 25 mM-tris-HCl buffer, pH 7.0, 10 mM-dithiothreitol and 10 mM-MgCl₂. Nucleotides and K₂HPO₄ were added as indicated and the rate of activation was measured. Controls with K₂HPO₄ at 2.5 mM and 0.65 mM gave the same rate of activation. Other details are described in the Methods section.

Nucleotide	Concn. of P _i (mM)	P _i /nucleotide molar ratio	Inhibition (%)
0.6 mM-AMP	2.5	4.2	62
0.6 mM-AMP	0.65	1.1	82
0.125 mM-AMP	2.5	20.0	29
0.125 mM-AMP	0.65	5.2	61

extracts. At present we can offer no explanation for this observation.

An unexpected observation was that both sodium diethyldithiocarbamate and tetraethylthiuram disulphide maintained the enzyme in a form capable of activation. After 45 min. incubation with either compound at 30 μ M the enzyme was completely reactivated by the subsequent addition of dithiothreitol and P_i. About half the activity was recovered by adding dithiothreitol alone.

Effect of nucleotides on activation. AMP inhibited the rate of activation, and the inhibition was competitive with P_i (Table 1). ADP and ATP were also inhibitory, but adenosine had no inhibitory effect. The leaf extracts used for these studies contained adenylate kinase and phosphatases capable of hydrolysing ATP and ADP to AMP. The adenylate kinase activity was sufficient to establish an equilibrium mixture of nucleotides within 2 min. with the amount of ADP supplied, and the phosphatases present were capable of hydrolysing about half the ATP and ADP supplied in 10 min. Consequently it is difficult to determine whether all three nucleotides are directly effective as inhibitors. Interpretation of the data is further complicated by the fact that ADP and ATP facilitate the inactivation of active enzyme (cf. Fig. 7). Unlike ADP and ATP, AMP does not cause inactivation of the active enzyme, so that this nucleotide at least is clearly an inhibitor of the activation process. At concentrations of 0.75 mM, and in the presence of 1 mM-potassium phosphate, GMP inhibited activation only slightly less than AMP, and GDP only slightly less than ADP, but GTP inhibited only 50% as much as ATP. IMP, IDP, UMP and UDP had no effect.

Activation of inactive enzyme after partial purification by gel filtration on Sephadex G-200. Extracts were subjected to gel filtration on Sephadex G-200

Fig. 4. Activation of enzyme in fractions obtained after gel filtration on Sephadex G-200. Maize leaves were obtained from plants kept in darkness for 3 hr. Extracts were prepared and treated on Sephadex G-200 as described in the Methods section. The preparation of the activating fraction is also described in the Methods section. Pyruvate, P_i dikinase activity in the fractions collected off the Sephadex G-200 column was determined immediately (○), after 2.5 hr. incubation at 25° with 1.5 mM-K₂HPO₄ (●) and after 2.5 hr. incubation with 1.5 mM-K₂HPO₄ and the activating fraction (Δ). The emergence peak of the activatable enzyme (▲) was determined from the difference between the activity before and after incubation with 1.5 mM-K₂HPO₄ and activating fraction.

to determine whether the molecular weights of active and inactive enzyme were similar, and whether a large-molecular-weight component besides the inactive enzyme was necessary for activation. Leaves were kept in darkness for 3 hr. before the preparation of extracts since, as mentioned above, this treatment gave a more stable inactive enzyme. The leaf extract was first treated on a small Sephadex G-25 column to remove P_i rapidly and was then applied to a Sephadex G-200 column. Since it had been previously determined that the chlorophyll-protein complex present in the extract was completely excluded by the gel, fractions were collected from the start of the elution of chlorophyll-containing material. In the experiment described in Fig. 4 the small amount of active enzyme present was eluted with a peak at approx. 23 ml. after the void volume. There was no increase in activity in samples of any fraction after

Fig. 5. Effect of extract concentration on kinetics of activation. Extracts were prepared from leaves dark-treated for 16 hr., passed through Sephadex G-25 as described in the Methods section and incubated at 25°. The incubation mixtures contained (final concentrations) in a total volume of 0.4 ml.: 50 mM-tris-HCl buffer, pH 7.9, 10 mM-MgCl₂, 10 mM-dithiothreitol, 1.5 mM-K₂HPO₄ and 0.33 ml. (●), 0.22 ml. (○), 0.10 ml. (■), 0.05 ml. (□) or 0.025 ml. (▲) of extract.

incubation with P_i for 3 hr. Under the same conditions there was complete activation of enzyme in extracts treated on Sephadex G-25 (see Fig. 2). However, the activity increased several-fold when fractions were incubated with P_i together with a Sephadex G-25-treated extract in which pyruvate, P_i dikinase had been irreversibly inactivated. This extract, referred to as the 'activating fraction', was ineffective after being boiled for 2 min. before use. The amount of inactive enzyme in each fraction, represented by ▲ symbols in Fig. 4, was calculated from the difference between the activity before and after incubation with P_i and 'activating fraction'. The mobility of this form of the enzyme on a Sephadex G-200 column was similar to that of the active enzyme with a peak at about 21 ml. However, there was an asymmetrical distribution of inactive enzyme about this peak. This phenomenon has been observed in several experiments and was more pronounced when the amount of activation obtained was less than in Fig. 4. It appears unlikely that the asymmetry represents trailing of the inactive enzyme on the column, since the active enzyme was eluted as a symmetrical peak. An alternative explanation is that the inactive enzyme may be present in two forms, the major one having

the same molecular weight as the active enzyme and the minor component a greater molecular weight.

Kinetics of activation. In several experiments the rate of activation was constant until activation was almost complete, this being particularly common with extracts of leaves kept in darkness for 16 hr. In others a biphasic curve of activation was obtained consisting of two essentially linear components, the initial rate being greater. Subsequent studies showed that the rate of activation and the shape of the activation curve were dependent on the concentration of the extract (Fig. 5). Since the results are expressed as activity per unit of chlorophyll, one would expect all rates to be the same if the rate of activation were decreased in direct proportion with dilution. However, dilution was accompanied by a disproportionately large decrease in the rate of activation. On the other hand the final activity reached was similar with the different dilutions, except with the most dilute extract in which the activity was still increasing at the termination of the experiment. Dilution also resulted in a change in the kinetics of activation from a single rate of activation with the most concentrated extract to a biphasic type of activation on dilution. The proportion of the inactive form of the enzyme that was activated at the faster rate decreased with increasing dilution. It is noteworthy that the change to a lower rate of activation occurred after a similar period of incubation with all dilutions. This could conceivably represent some change in the configuration of the inactive form of the enzyme that was independent of concentration.

Activation of the enzyme in leaf extracts of other species. The results obtained with leaf extracts of sugar cane were essentially the same as those described for maize both with respect to the effects of dithiothreitol and diethyldithiocarbamate on the stability of the inactive enzyme and in the requirement for dithiothreitol and P_i for activation. AMP also inhibited activation in a manner similar to that described for maize. However, activation of the enzyme in leaf extracts of *Amaranthus palmeri* required the presence of pyruvate in addition to P_i and dithiothreitol. In this respect *Amaranthus palmeri* differs from maize and sugar cane, which showed a negligible pyruvate requirement for activation. Compared with the maize enzyme the inactive enzyme from *Amaranthus* was more stable in the absence of dithiothreitol and less stable in the absence of P_i.

Inactivation of active enzyme in extracts

Inactivation in the absence of thiols and reactivation of the inactivated enzyme. Extracts prepared from illuminated maize leaves as described in the Methods

Fig. 6. Inactivation of active enzyme by sodium diethyldithiocarbamate in the absence of a thiol. Extracts were prepared from illuminated maize leaves as described in the Methods section. K_2HPO_4 was added to give a final concentration of 2mM and the extract was kept under N_2 at 22° for 2 hr. This extract was passed through a Sephadex G-25 column, equilibrated with the buffer mixture as described in the Methods section without dithiothreitol, and 0.45 ml. of the protein eluate was incubated at 30° with either 0.05 ml. of water (●) or 0.05 ml. of 10mM-diethyldithiocarbamate (○). After 30 min. either 0.01 ml. of water and 0.01 ml. of 200mM-dithiothreitol (□ and △) or 0.01 ml. of 50mM- K_2HPO_4 and 0.01 ml. of 200mM-dithiothreitol (■ and ▲) were added to 0.18 ml. samples of the incubation mixtures.

section and subsequently passed through Sephadex G-25 to remove dithiothreitol rapidly lost activity (Fig. 6). Activity was not recovered by incubation with either dithiothreitol or with dithiothreitol plus P_i . The presence of substrates such as 2mM-potassium phosphate and 5.0mM-sodium pyruvate or disulphides such as GSSG, cystine or oxidized lipoate did not alter the rate of inactivation and did not maintain the enzyme in a reactivatable form. However, sodium diethyldithiocarbamate accelerated the rate of inactivation and the dithiocarbamate-inactivated enzyme was completely reactivated by dithiothreitol. The reactivation did not require P_i (Fig. 6). Tetraethylthiuram disulphide and diethyldithiocarbamate were equally effective as inactivators of the enzyme and in maintaining the enzyme in a reactivatable form. In other experiments chelating agents such as EDTA, 1,10-phenanthroline or 2,2'-bipyridyl decreased the rate of inactivation, but in no instance did they maintain the enzyme in a reactivatable form.

Inactivation of active enzyme by nucleotides and the reactivation of inactivated enzyme. In the absence of a thiol both 1mM-ATP and 1mM-ADP accelerated the irreversible inactivation of the enzyme, although UDP, IDP, GDP, AMP and adenosine were without

Fig. 7. Inactivation of active enzyme by ADP and reactivation in the presence of P_i . Extracts were prepared from illuminated maize leaves and incubated in the presence of P_i as described in Fig. 6, and passed through Sephadex as described in the Methods section. To 0.9 ml. samples of the protein eluate were added 0.1 ml. of 100mM-dithiothreitol and either 0.1 ml. of water (●) or 0.1 ml. of 20mM-ADP (○), and the mixtures were incubated at 30°. After 35 min., 0.5 ml. of the mixture containing ADP was passed again through a Sephadex G-25 column, equilibrated with the mixture described in the Methods section but containing also 10mM-dithiothreitol, and 0.18 ml. samples of the protein eluate were incubated with either 0.02 ml. of water (□) or 0.02 ml. of 20mM- K_2HPO_4 (■).

Table 2. *Inhibition of activation of ADP-inactivated enzyme by AMP*

Extract prepared from illuminated maize leaves was incubated with P_i and passed through Sephadex G-25 equilibrated with buffer mixture containing 5mM-dithiothreitol as described in the Methods section. The protein eluate was incubated with 2.5mM-ADP until 90% of the enzyme had been inactivated, then passed again through Sephadex as described in Fig. 7. Samples of the protein eluate were incubated with 2.5mM- K_2HPO_4 with and without AMP at the concentrations specified. The initial rates of reactivation were determined.

Concn. of AMP (mM)	P_i /AMP molar ratio	Inhibition (%)
0.25	10	34
1.0	2.5	60
2.5	1.0	79

effect at this concentration. An unexpected finding was that ADP produced inactivation even in the presence of 10mM-dithiothreitol, and after prolonged incubation the enzyme was often reactivated again. Fig. 7 shows the ADP-induced inactivation followed by reactivation, and demonstrates a P_i

requirement for reactivation of the ADP-inactivated enzyme. After treatment of the ADP-inactivated enzyme on a Sephadex G-25 column no reactivation was observed. However, when 2mM-potassium phosphate was provided the enzyme was reactivated initially at a faster rate than in the extract not treated on the Sephadex G-25 column. The apparent incomplete reactivation of the enzyme in the Sephadex-treated extract is attributable to dilution of the extract during gel filtration. As shown in Table 2 the P_i -dependent reactivation of ADP-inactivated enzyme was inhibited by AMP. ATP also facilitated inactivation in the presence of dithiothreitol, but in contrast with the result obtained with ADP there was an initial lag period in the rate of inactivation. This suggests that only ADP facilitates inactivation and that ATP was effective only after conversion into ADP by phosphatases present in the extract.

The above studies of ADP-mediated inactivation were conducted in air, since in the presence of dithiothreitol the active enzyme is stable. In a separate experiment the rates of ADP-mediated inactivation in both air and oxygen-free nitrogen were compared and it was found that the rate of ADP-mediated inactivation in a nitrogen atmosphere was only about 20% of that in air. Addition of 2mM-EDTA to treatments in air also decreased the rate of inactivation.

Activation of ADP-inactivated enzyme after partial purification on Sephadex G-200. After gel filtration on Sephadex G-200 activation of the inactive form of the enzyme in extracts of dark-treated leaves required not only P_i but also a Sephadex G-25 extract, termed the 'activating fraction' (Fig. 4). It was therefore decided to determine whether the ADP-inactivated enzyme had a similar requirement after partial purification on Sephadex G-200. The results presented in Fig. 8 show that activation of the ADP-inactivated enzyme required an 'activating fraction' and that the molecular weight of this inactive form of the enzyme was similar to that of the active enzyme.

Inhibition of pyruvate, P_i dikinase by thiol reagents. The effect of certain thiol reagents on the activity of the enzyme in extracts of illuminated maize leaves are shown in Table 3. The enzyme was assayed in the system linked to phosphopyruvate carboxylase and aspartate aminotransferase. No effect of the thiol reagents, at the concentrations described in Table 3, was observed in control assay systems to which phosphoenolpyruvate was added. *p*-Chloromercuribenzoate and γ -(*p*-arsenophenyl)-*n*-butyrate were equally effective inhibitors of the enzyme. In the experiment described the enzyme was incubated for 20min. with the thiol reagents in the absence of substrates before assay. However, it was found that varying the length of the pre-

Fig. 8. Activation of ADP-inactivated enzyme after gel filtration on Sephadex G-200. Extract prepared from illuminated leaves was incubated with P_i and passed through Sephadex G-25 as described in Fig. 6. Dithiothreitol was added to the protein eluate to give a final concentration of 10mM and a 3.8ml. sample of this was incubated at 30° with 0.2ml. of 50mM-ADP until 95% of enzyme was inactivated. The mixture was then passed through a Sephadex G-25 column and 3.0ml. of the protein eluate and 1.0ml. of the eluate from the first Sephadex G-25 column, containing active enzyme, were passed through a Sephadex G-200 column as described in the Methods section. The fractions collected were assayed immediately (●) and again after incubating 0.2ml. of each fraction with 0.015ml. of 100mM-MgCl₂, 0.01ml. of 50mM-K₂HPO₄ and 0.01ml. of 20mM-dithiothreitol and either 0.05ml. of water (○) or 0.05ml. of activating fraction (Δ) prepared as described in the Methods section.

incubation period between 5 and 30min. had little effect on the amount of inhibition obtained. Sodium arsenate produced only slight inhibition at a concentration of 1mM, but the effectiveness of this reagent as an inhibitor was markedly enhanced by the presence of an equimolar amount of 2,3-dimercaptopropan-1-ol.

DISCUSSION

The present studies indicate that pyruvate, P_i dikinase exists in the chloroplast in two interconvertible forms, one active and the other inactive, and that the amount of active enzyme present at any time bears a relationship to the light-energy available for photosynthesis. During darkness the

Table 3. *Inhibition of pyruvate, P_i dikinase by thiol reagents*

The extract was prepared from illuminated leaves and passed through Sephadex G-25 as described in the Methods section. Thiol reagents at the concentrations specified were incubated at 30° for 20 min. with medium containing, in a total volume of 0.05 ml., tris-HCl buffer, pH 8.3 (10 μ moles), MgCl₂ (1.5 μ moles), EDTA (0.05 μ mole), sodium glutamate (0.5 μ mole), NaH¹⁴CO₃ (1 μ mole, 210000 c.p.m./ μ mole) and enzyme extract at a concentration of 0.23 mg. of protein/ml. The reaction was started by adding 0.025 ml. of a mixture containing ATP (0.5 μ mole), sodium pyruvate (1.0 μ mole) and K₂HPO₄ (0.25 μ mole), and 0.01 ml. of a mixture of a phosphopyruvate carboxylase and aspartate aminotransferase. Assays were performed as outlined in the Methods section. Arsenite and 2,3-dimercaptopropan-1-ol were present in equimolar amounts.

Reagent	Concn. (μ M)	Inhibition (%)
<i>p</i> -Chloromercuribenzoate	1.5	32
<i>p</i> -Chloromercuribenzoate	15	98
γ -(<i>p</i> -Arsenophenyl)- <i>n</i> -butyrate	1.5	25
γ -(<i>p</i> -Arsenophenyl)- <i>n</i> -butyrate	15	91
Arsenite	1000	17
Arsenite + 2,3-dimercaptopropan-1-ol	30	72
Arsenite + 2,3-dimercaptopropan-1-ol	150	80

enzyme is predominantly in the inactive form and is converted into active enzyme as the light-intensity increases, the final ratio of active to inactive enzyme being dependent on the intensity of the incident light. It is possible that the activity of the enzyme in dark-treated leaves is considerably lower than reported here and by Slack (1968), since some activation undoubtedly occurs during the period between the preparation of the extract and the removal of P_i by gel filtration.

Our original suggestion (Hatch & Slack, 1968) that pyruvate, P_i dikinase is a thiol protein is substantiated by the finding that the enzyme is inhibited by low concentrations of certain thiol reagents. Both arsenite, in the presence of an equimolar amount of 2,3-dimercaptopropan-1-ol, and γ -(*p*-arsenophenyl)-*n*-butyrate, which bind strongly to vicinal thiol groups (Webb, 1966), are potent inhibitors of the enzyme. Therefore the active enzyme apparently contains one or more pairs of adjacent thiol groups that are essential for activity. It appears probable that activation and inactivation of the enzyme *in vivo* involve the reduction and oxidation of these groups. However, the inactive enzyme from dark-treated leaves apparently also contains oxidizable thiol groups, since this form becomes irreversibly inactive in the absence of a thiol.

The rapid inactivation of the enzyme by sodium

diethyldithiocarbamate and tetraethylthiuram disulphide cannot be attributed to the metal-ion-chelating properties of these reagents, since other chelating agents decreased, rather than accelerated, the rate of inactivation. We suggest that inactivation by these reagents involves the formation of a stable inactive complex with thiol groups on the enzyme, since the inactivated enzyme was completely reactivated by incubation with dithiothreitol. Lindahl & Åkerström (1965) have discussed possible reactions between thiol and disulphide groups and these reagents. They suggest that the most likely reaction would be between a thiol group and a thiuram disulphide forming a mixed disulphide with the release of dithiocarbamate. Supporting evidence for this suggestion is that tetraethylthiuram disulphide and *p*-chloromercuribenzoate were equally potent inhibitors of the thiol enzyme D-amino acid oxidase (Hellerman *et al.* 1965). However, we were unable to demonstrate that tetraethylthiuram disulphide was more effective than the corresponding dithiocarbamate, or that the two compounds were readily interconverted under the conditions employed. One possible explanation of these findings is that both thiuram disulphides and dithiocarbamates form a mixed disulphide with the enzyme, the reaction with dithiocarbamates requiring the prior oxidation of an adjacent pair of thiol groups on the enzyme. Reactivation of the enzyme by dithiothreitol is envisaged as the splitting of the mixed disulphide with the release of active enzyme and dithiocarbamate.

The studies *in vitro* strongly suggest that activation and inactivation of the enzyme in the leaf involve processes controlled by P_i and adenosine phosphates. Activation of the inactive enzyme isolated from dark-treated leaves requires P_i, a thiol and a heat-labile large-molecular-weight component. The effect of dilution of extracts on the kinetics of activation is also explicable in terms of the requirement, for activation, of a large-molecular-weight component other than the inactive enzyme. Activation was inhibited by AMP and possibly by ADP and ATP. The effects of the latter compounds may have been due to the fact that they are hydrolysed to AMP in extracts and that they inactivate the active form of the enzyme.

ADP facilitated the rapid inactivation of active enzyme even in the presence of dithiothreitol. It is possible that this process also requires the presence of a large-molecular-weight component, since no inactivation was observed when a partially purified preparation of pyruvate, P_i dikinase was incubated with ADP. Reactivation of the ADP-inactivated enzyme required P_i, a thiol and a large-molecular-weight component. In all these properties the ADP-inactivated enzyme resembles the inactive

enzyme obtained from dark-treated leaves. We are therefore led to the conclusion that the concentration of ADP in the chloroplast has a regulatory function in controlling the amount of active enzyme.

Available evidence does not support the possibility that activation and inactivation involves the association and dissociation of an enzyme- P_i complex, since partial activation of inactive enzyme, stabilized by sodium diethyldithiocarbamate, occurred in the presence of dithiothreitol alone. In addition, arsenate replaced P_i as a requirement for activation and the arsenate-activated enzyme was stable after free arsenate had been removed.

We have entertained two possibilities with regard to the nature of the large-molecular-weight component, present in the 'activating fraction', that is required for P_i -dependent activation. One possibility is that this component is an enzyme that catalyses the activation process, and the other that it is a sub-unit of the enzyme that is released during inactivation. The present evidence favours the former possibility. We found that the initial rate of activation of inactive enzyme, after partial purification by gel filtration, was proportional to the amount of 'activating fraction' present, whereas the final activity reached was largely independent of the amount of 'activating fraction'.

Our present studies do not unequivocally support the view that inactivation and activation involves the oxidation and reduction of adjacent thiol groups on the enzyme. The requirement for a thiol or other reducing agents may simply reflect the relative instability of both the active and inactive enzyme in the absence of these reagents. On the other hand, although ADP-facilitated inactivation occurs in the presence of dithiothreitol, we found that the rate of inactivation was decreased by approx. 80% when reaction mixtures were incubated under oxygen-free nitrogen compared with reactions conducted in air. This suggests that inactivation is an oxygen-dependent process facilitated by ADP, which presumably involves the oxidation of thiol groups. Scheme 1 summarizes our present ideas about the processes involved. We suggest that the proportion of active to inactive enzyme present in the chloroplast is determined by the prevailing concentrations of adenosine phosphates and P_i and possibly by the redox status of an intermediate in the photo-electron-transport system. These in turn would be controlled by the intensity of the incident light via photophosphorylation and photo-reduction. It is probable that the processes are more complex than presented here, since we have

Scheme 1. Schematic representation of the processes of activation and inactivation of pyruvate, P_i dikinase. The process leading to the irreversible inactivation of both active and inactive enzyme *in vitro* apparently includes an O_2 -dependent oxidative reaction involving thiol groups.

obtained some information suggesting that inactivation in the leaf may involve dimerization of at least part of the enzyme complement. The fact that activation of the enzyme in extracts of *Amaranthus palmeri* required pyruvate in addition to P_i and dithiothreitol suggests that the mechanism may not be identical in all species in which the C_4 dicarboxylic acid pathway operates.

Further elucidation of the reactions involved must await methods for stabilizing and purifying the inactive enzyme and for purifying the other protein components essential for activation and inactivation.

REFERENCES

- Arnon, D. I. (1949). *Plant Physiol.* **24**, 1.
- Braun, J. L. von (1902). *Ber. dtsh. chem. Ges.* **35**, 52.
- Cooper, R. A. & Kornberg, H. L. (1967). *Proc. Roy. Soc. B*, **168**, 263.
- Hatch, M. D. & Slack, C. R. (1966). *Biochem. J.* **101**, 103.
- Hatch, M. D. & Slack, C. R. (1968). *Biochem. J.* **106**, 141.
- Hatch, M. D., Slack, C. R. & Johnson, H. S. (1967). *Biochem. J.* **102**, 417.
- Hellerman, L., Coffey, D. S. & Neims, A. H. (1965). *J. biol. Chem.* **240**, 290.
- Johnson, H. S. & Hatch, M. D. (1968). *Phytochemistry*, **7**, 375.
- Lindahl, P. E. B. & Åkerström, S. (1965). *Lantbrukshögsk. Ann.* **31**, 459.
- Reeves, R. E. (1968). *J. biol. Chem.* **243**, 3202.
- Slack, C. R. (1968). *Biochem. biophys. Res. Commun.* **30**, 483.
- Slack, C. R. (1969). *Phytochemistry* (in the Press).
- Slack, C. R. & Hatch, M. D. (1967). *Biochem. J.* **103**, 660.
- Webb, J. L. (1966). *Enzyme and Metabolic Inhibitors*, p. 617. New York: Academic Press Inc.