A New Enzyme for the Interconversion of Pyruvate and Phosphopyruvate and its Role in the C₄ Dicarboxylic Acid Pathway of Photosynthesis

By M. D. HATCH AND C. R. SLACK

David North Plant Research Centre, The Colonial Sugar Refining Co. Ltd.,
P.O. Box 68, Toowoong, Queensl., Australia

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1. An enzyme was isolated from leaves of tropical grasses that catalyses the reversible conversion of pyruvate, ATP and orthophosphate into phosphopyruvate, AMP and pyrophosphate. A requirement for Mg²⁺ could not be replaced by Mn²⁺ or Ca²⁺. 2. By replacing orthophosphate with [³²P]orthophosphate or with arsenate, evidence was provided that the orthophosphate consumed appears in pyrophosphate. 3. Without Mg²⁺ or 2-mercaptoethanol the enzyme was rapidly and irreversibly inactivated. EDTA only partially replaced the requirement for the thiol compound. The enzyme was considerably more unstable at 0° or when frozen than at 22°. Even with the best conditions devised the enzyme lost about 25% of its activity every 3 hr. 4. The activities of the enzyme in leaves of the tropical grasses sugar cane (*Saccharum* hybrid var. Pindar), maize (*Zea mays*) and sorghum (*Sorghum vulgare*) were comparable with their maximum photosynthesis rates. The enzyme was not detectable in leaf extracts from several other plants. 5. Its role in photosynthesis is discussed.

When leaves of tropical grasses are exposed to ¹⁴CO₂ in the light radioactivity is detected first in C-4 of the C₄ dicarboxylic acids oxaloacetate, malate and aspartate. Subsequently the label from these acids appears in C-1 of 3-phosphoglycerate and then in hexose phosphates (Hatch & Slack, 1966; Hatch, Slack & Johnson, 1967). Phosphopyruvate carboxylase is apparently responsible for most of the carbon dioxide fixed by these plants in the light (Slack & Hatch, 1967). A conclusion from these studies was that leaves with this pathway must be capable of rapidly converting pyruvate into the carbon dioxide-acceptor, phosphopyruvate (Slack & Hatch, 1967). The presence of an enzyme system in extracts of the leaves of tropical grasses that catalyses this transformation has been reported (Hatch & Slack, 1967). The enzyme was not detected in leaves in which the Calvin cycle is operative. The stoichiometry we reported for this reaction was the same as that described by Cooper & Kornberg (1965) for an enzyme from *Escherichia coli*:

A similar enzyme has been reported in photosynthetic bacteria (Buchanan & Evans, 1966).

In the present paper the partial purification and properties of the leaf enzyme are described. We now have evidence that, in addition to ATP, ortho-

phosphate is also a reactant for the conversion of pyruvate into phosphopyruvate. AMP and pyrophosphate are the other products. In this paper the name suggested by Cooper & Kornberg (1965), 'phosphopyruvate synthetase', is retained.

MATERIALS

Mature leaves were obtained from plants grown in sunlight, either in the field or in a glasshouse. The plants examined were sugar cane (*Saccharum* hybrid var. Pindar), maize (*Zea mays* var. DS606A), sorghum (*Sorghum vulgare* var. Rio), silver beet (*Beta vulgaris* var. Fordhook Giant), wheat (*Triticum sativum*), oat (*Avena sativa* var. Bentland), sunflower (*Helianthus annuus*) and bean (*Phaseolus vulgaris* var. Canadian Wonder).

The following compounds and enzymes were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.: sodium pyruvate, tricyclohexylammonium phosphopyruvate, ATP, ADP, AMP, NADH₂, NADP, oxaloacetic acid, glutamate aminotransferase, hexokinase, pyruvate kinase, glucose 6-phosphate dehydrogenase and lactate dehydrogenase. Calcium phosphate gel (Hypatite C) was obtained from Clarkson Chemical Co., Williamsport, Pa., U.S.A.

Partially purified phosphopyruvate carboxylase, free of phosphopyruvate synthetase, was prepared from sugar-cane leaves by treating the 40–53%-saturated (NH₄)₂SO₄ fraction on a Hypatite C column at pH 6.8.

A pyrophosphatase preparation, free of non-specific phosphatases, was obtained from oat leaf by fractionation with (NH₄)₂SO₄.

METHODS

Extraction and partial purification of phosphopyruvate synthetase. For the survey of activity in extracts of different plants 4g. of tissue was blended at 0° for 90sec. in an atmosphere of N₂ with 16ml. of 0.1M-tris-HCl buffer, pH 8.3, containing 5mM-2-mercaptoethanol, 5mM-MgCl₂, 2.5mM-pyruvate and 1mM-EDTA. Subsequent steps were conducted at 22°. The homogenate was filtered through fine muslin and the filtrate centrifuged at 10000g for 5min. To remove small-molecular-weight components samples of the supernatant were passed through a column of Sephadex G-25 pre-equilibrated with 50mM-tris-HCl buffer, pH 8.3, containing 5mM-MgCl₂, 5mM-2-mercaptoethanol and 2.5mM-pyruvate. Samples of the extract obtained by filtration through muslin were used for the determination of chlorophyll.

For the purification of the enzyme 100g. of maize or sugar-cane leaf was extracted as described above up to the stage of centrifugation. Subsequent steps were conducted at 22°. The supernatant was treated with saturated (NH₄)₂SO₄ (5°, pH 7.0) and the protein precipitated between 40% and 53% saturation was suspended in 30ml. of 50mM-tris-HCl buffer, pH 8.3, containing 5mM-MgCl₂, 5mM-2-mercaptoethanol and 2.5mM-pyruvate. In subsequent purification steps MgCl₂, 2-mercaptoethanol and pyruvate at the above concentrations were included in all buffer solutions. This preparation was passed down a 180ml. column of Sephadex G-200 pre-equilibrated with 50mM-tris-HCl buffer, pH 7.5. The enzyme emerged about 15ml. after the void volume of 55ml. and was collected in the following 35ml. This fraction was applied to a 32ml. column of calcium phosphate gel (Hypatite C) previously washed with 50mM-tris-HCl buffer, pH 7.5, until the eluate was pH 7.4. After the column was washed with 30ml. of 50mM-tris-HCl buffer, pH 7.5, the phosphopyruvate synthetase was eluted with 50ml. of 50mM-tris-HCl buffer, pH 7.5, containing 30mM-potassium phosphate followed by 50ml. of 50mM-tris-HCl buffer, pH 7.5, containing 40mM-potassium phosphate. The fractions free of phosphopyruvate carboxylase were pooled and the protein was precipitated by the addition of 2vol. of saturated (NH₄)₂SO₄ (5°, pH 7.0). The precipitated protein was dissolved in 3.5ml. of 50mM-tris-HCl buffer, pH 8.3, and applied to a 180ml. column of Sephadex G-200 pre-equilibrated with the same buffer. The first 15ml. after the void volume was discarded and the enzyme, which emerged in the next 20ml., was collected in 4ml. fractions. These fractions were stored at -15° after the addition of 2vol. of saturated (NH₄)₂SO₄, pH 7.0.

As required, the enzyme was isolated from the protein suspension in the 66%-saturated (NH₄)₂SO₄ solution by centrifuging samples and dissolving the precipitate in the 50mM-tris-HCl buffer, pH 8.3, containing 5mM-MgCl₂, 5mM-2-mercaptoethanol and 2.5mM-pyruvate.

Assay of enzyme activity. For most studies the formation of phosphopyruvate from pyruvate was determined by the amount of H¹⁴CO₃⁻ fixed into aspartate in an assay system containing phosphopyruvate carboxylase, aspartate aminotransferase and glutamate. This is referred to below as the coupled assay system. Reaction mixtures contained enzyme, 15mM-sodium pyruvate, 7.5mM-ATP, 2.5mM-potassium phosphate, 20mM-glutamate, 15mM-MgCl₂, 12mM-NaH¹⁴CO₃ (approx. 200000 counts/min./μmole), 6mM-2-mercaptoethanol, 0.2 unit of phosphopyruvate car-

boxylase, 0.5 unit of aspartate aminotransferase and 50mM-tris-HCl buffer, pH 8.3, in a total volume of 0.15ml. All enzyme assays were conducted at 30°. Reactions were stopped by adding 0.02ml. of 20% (w/v) trichloroacetic acid. The procedures for counting samples of the reaction mixture and for determining the specific radioactivity of NaH¹⁴CO₃ were as previously described (Slack & Hatch, 1967). In some experiments the reaction proceeding towards phosphopyruvate synthesis was measured without the coupling system that converted phosphopyruvate into aspartate. The composition of these reaction mixtures was as described above except that phosphopyruvate carboxylase, aspartate aminotransferase and glutamate were omitted. Reactions were stopped either by heating at 100° for 2min. or by the addition of trichloroacetic acid. The phosphopyruvate formed was determined either from the H¹⁴CO₃⁻ incorporated into aspartate after the addition of phosphopyruvate carboxylase, aspartate aminotransferase and glutamate or with the pyruvate kinase-lactate dehydrogenase system previously employed (Hatch & Slack, 1967).

The enzyme was also assayed in the direction of pyruvate formation. Details of the composition of the reaction mixture are provided in the text. The procedure for stopping the reaction and for measuring the pyruvate, ATP and orthophosphate formed were as previously described (Hatch & Slack, 1967).

Inorganic pyrophosphatase assay. The formation of orthophosphate was measured in reaction mixtures containing enzyme, 50mM-tris-HCl, pH 8.3, 2.5mM-sodium pyrophosphate and 10mM-MgCl₂.

Adenylate kinase assay. NADPH₂ formation was measured spectrophotometrically in a system containing the enzyme, hexokinase, glucose, glucose 6-phosphate dehydrogenase and NADP in 50mM-tris-HCl buffer, pH 8.3.

Studies with [³²P]orthophosphate. Reactions were stopped by heating at 100° for 2min. EDTA was added to give a final concentration of 20mM and samples were co-chromatographed with marker compounds on Whatman no. 54 paper with propyl acetate-90% (v/v) formic acid-water (11.5:3, by vol.) (Bielecki & Young, 1963) as the developing solvent. The radioactivity in individual compounds was determined by measuring the areas of peaks traced with a radiochromatogram strip counter.

Other determinations. Pyrophosphate was determined by measuring the increase in orthophosphate after the treatment of samples with pyrophosphatase. Chlorophyll was determined by the procedure of Arnon (1949) and protein by the procedure of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Purification of phosphopyruvate synthetase. The purification procedure described in the Methods section was designed primarily to remove enzymes that interfere with studies of the reaction catalysed by phosphopyruvate synthetase. In crude extracts the activities of several of these enzymes, including phosphopyruvate carboxylase, pyrophosphatase, adenylate kinase and a non-specific phosphatase, were higher than that of phosphopyruvate synthetase. The rejection of fractions containing these

Table 1. *Purification of phosphopyruvate synthetase from maize leaf*

Activity was determined in the direction of phosphopyruvate synthesis by measuring the incorporation of $\text{NaH}^{14}\text{CO}_3$ into aspartate. This procedure and the details of the purification procedure are described in the Methods section. The protein concentration for the Hypatite C fraction is for the pooled fractions concentrated to 3.7 ml.

Fraction	Total units	Concn. of protein (mg./ml.)	Sp. activity (units/mg. of protein)
Supernatant	210	7.2	0.065
40-53%-satd. $(\text{NH}_4)_2\text{SO}_4$	180	15.0	0.25
First Sephadex G-200	108	8.6	0.28
Hypatite C	30	8.6	0.76
Second Sephadex G-200	23	1.1	1.2

Table 2. *Requirements for phosphopyruvate synthesis*

Activity was measured by the incorporation of $\text{NaH}^{14}\text{CO}_3$ into aspartate as described in the Methods section. In the standard assay system the concentration of the reactants were: pyruvate, 12 mM; ATP, 2.5 mM; MgCl_2 , 10 mM; orthophosphate, 2.5 mM. The value of 0.12 mM for orthophosphate in some reactions was the concentration of orthophosphate in 2.5 mM-ATP. No activity was detected when ATP, pyruvate or MgCl_2 was omitted.

Reaction mixture composition	% of activity compared with standard assay system
Standard assay system	(100)
With 2.5 mM-UTP, no ATP	3
With 2.5 mM-GTP, no ATP	2
With 2.5 mM-CTP, no ATP	1
With 5.4 mM- MgCl_2	94
With 2.9 mM- MgCl_2	79
With 1.4 mM- MgCl_2	16
With 5 mM- MnCl_2 , no MgCl_2	4
With 5 mM- CaCl_2 , no MgCl_2	0
With 0.6 mM- P_i	59
With 0.12 mM- P_i	16
With 0.12 mM- P_i and 3.5 mM-arsenate	72
With 0.6 mM- P_i and 2 mM-PP _i	7

enzymes together with the marked instability of phosphopyruvate synthetase militated against obtaining preparations with a high specific activity. The procedure employed resulted in a 17-fold increase in specific activity (Table 1). With either sugar-cane or maize leaf extracts the procedure provided fractions from the second Sephadex G-200 treatment that were free of all the interfering enzymes except pyrophosphatase. However, particularly with sugar cane the first active fractions

that emerged from Sephadex G-200 contained relatively little pyrophosphatase activity.

Requirements for phosphopyruvate synthesis. With the assay system described in Table 2, in which a system was provided to convert phosphopyruvate into aspartate, the reaction rate was linear until one of the reactants was nearly depleted. Optimum activity was observed at pH 8.3 and 60% of this activity at pH 7.7 and 9.0. No phosphopyruvate was formed when ATP, pyruvate or Mg^{2+} was omitted. Since Mg^{2+} is required for phosphopyruvate carboxylase the absolute requirement for Mg^{2+} was demonstrated with the non-coupled assay procedure (see the Methods section). The K_m , calculated from Lineweaver-Burk plots, was 0.11 mM for pyruvate and 0.09 mM for ATP when the non-variable components of the reaction were provided at a saturating concentration. As shown in Table 2 the reaction required orthophosphate for activity, the K_m for this reactant being approx. 0.5 mM.

Little activity was observed when ATP was replaced by UTP, CTP or GTP (Table 2). The proportion of ATP and Mg^{2+} was also a factor affecting enzyme activity. The large decrease in activity observed when the Mg^{2+} /ATP concentration ratio was decreased to less than 1:1 suggested that Mg^{2+} as such is essential for activity. Neither Mn^{2+} nor Ca^{2+} could replace the requirement for Mg^{2+} .

Control experiments in which pyruvate was replaced by phosphopyruvate indicated that the results obtained were not due to effects on the activity of the enzymes added to convert phosphopyruvate into aspartate.

Requirement for orthophosphate. When the concentration of orthophosphate was increased from 0.12 mM to 2.5 mM the rate of phosphopyruvate formation increased sixfold (Table 2). Arsenate at 3.5 mM was almost as effective as 2.5 mM-orthophosphate. In Fig. 1 the rate of phosphopyruvate formation is compared in reactions with and without a system for converting phosphopyruvate into aspartate. The initial rates obtained with both systems were similar, but the rate declined rapidly when phosphopyruvate was not removed. With 3 mM-arsenate the initial rate was lower, but there was only a small decline in rate with time.

The results in Table 3 provide evidence that pyrophosphate is a product of the reaction. Although the sugar-cane enzyme used for the present studies was not entirely free of pyrophosphatase, the amount of pyrophosphate formed was approx. 66% of the phosphopyruvate formed after 20 min. The further deviation from a stoichiometry of unity during the next 20 min. was attributed to the decline in phosphopyruvate synthesis accompanied by continued pyrophosphatase activity.

Fig. 1. Time-course of phosphopyruvate synthesis in the presence of orthophosphate or arsenate. All reaction mixtures contained the partially purified enzyme from sugar cane, 4.0 μ moles of sodium pyruvate and 0.32 μ mole of ATP, but were otherwise as described in the Methods section except that they contained: $\circ$, 0.07 mM-orthophosphate; $\bullet$, 3 mM-orthophosphate; Δ , 0.07 mM-orthophosphate and 3 mM-arsenate; $\blacktriangle$, 3 mM-orthophosphate and the coupling system to convert phosphopyruvate into aspartate. The total volume was 0.22 ml. Other details are described in the Methods section.

Assuming the stoichiometric consumption of orthophosphate the decrease in orthophosphate after 20 min. was approximately that expected if 0.03 μ mole of pyrophosphate was hydrolysed.

Studies with [32 P]orthophosphate provided evidence that orthophosphate appears as one of the phosphate groups of pyrophosphate (Table 4). Considerable incorporation of [32 P]orthophosphate into pyrophosphate was observed only when unlabelled pyrophosphate was included in the reaction mixture. When unlabelled pyrophosphate was not provided the small percentage of labelled orthophosphate incorporated into pyrophosphate remained constant after 10 min. incubation, but the label in ATP increased. The labelled product co-chromatographed with pyrophosphate and was converted into [32 P]orthophosphate when treated with pyrophosphatase. Preparations containing pyrophosphatase did not catalyse the incorporation of label into pyrophosphate when ATP and pyruvate were omitted.

Equilibrium constant. Assuming a stoichiometry of unity for the disappearance of pyruvate and ATP

Table 3. *Orthophosphate and pyrophosphate changes accompanying phosphopyruvate synthesis*

Reaction mixtures contained the partially purified enzyme from sugar cane, 5.9 μ moles of sodium pyruvate, 1.4 μ moles of ATP, 0.43 μ mole of sodium orthophosphate, 2 μ moles of 2-mercaptoethanol, 6 μ moles of $MgCl_2$ and 15 μ moles of tris-HCl buffer, pH 8.3, in a total volume of 0.4 ml. Other details are described in the Methods section.

Incubation time (min.)	Amount (μ mole/reaction mixture)		
	Phosphopyruvate	PP _i	P _i
0	0	0	0.43
20	0.10	0.07	0.37
40	0.12	0.05	0.45

Table 4. *Incorporation of [32 P]orthophosphate into pyrophosphate*

The composition of the reaction mixture was as described in the Methods section for the assay of phosphopyruvate synthetase except that the concentration of orthophosphate (3×10^6 disintegrations/min. of 32 P) was only 0.2 mM. The coupling system to convert phosphopyruvate into aspartate (see the Methods section) was provided as indicated. Other details of the procedure are given in the Methods section. The reaction time was 10 min.

Reaction composition and treatment	% of total 32 P		
	P _i	PP _i	ATP
Without added PP _i	91	4	5
With 0.4 mM-PP _i	51	46	3
With 0.4 mM-PP _i and coupled assay system	69	29	2

and the formation of phosphopyruvate and AMP (Hatch & Slack, 1967) and with the concentrations for phosphopyruvate, orthophosphate and pyrophosphate in Table 3 an estimate for the equilibrium constant was made. The value obtained, approx. 2×10^{-4} , for the reaction proceeding towards phosphopyruvate synthesis was the same after 20 min. and 40 min. (Table 3). Apparently the reaction catalysed by phosphopyruvate synthetase had reached an equilibrium at 20 min. The continued hydrolysis of pyrophosphate between 20 min. and 40 min. would result in a redistribution of the concentration of all the reactants.

Conversion of phosphopyruvate into pyruvate. On the basis of our earlier studies (Hatch & Slack, 1967) we attempted unsuccessfully to demonstrate the conversion of phosphopyruvate into pyruvate in a system containing AMP, orthophosphate and lactate dehydrogenase. However, the conversion proceeded rapidly in a similar system containing pyrophosphate instead of orthophosphate. The

Table 5. *Formation of pyruvate, ATP and orthophosphate from phosphopyruvate, AMP and pyrophosphate*

The reaction mixture contained the partially purified enzyme from sugar cane, 1.7 μ moles of sodium phosphopyruvate, 0.85 μ mole of sodium pyrophosphate, 3 μ moles of $MgCl_2$, 2.5 μ moles of AMP, 3.5 μ moles of 2-mercaptoethanol and 30 μ moles of tris-HCl buffer, pH 8.3, in a total volume of 0.7 ml. Other details of the procedure are provided in the Methods section. No pyruvate was formed when AMP, phosphopyruvate or pyrophosphate was omitted or when orthophosphate replaced pyrophosphate. The values for orthophosphate in parentheses are those obtained by subtracting the orthophosphate formed by the hydrolysis of pyrophosphate.

Incubation time (min.)	Product formed (μ mole/reaction mixture)			
	With complete reaction			P_i formed without added AMP and phosphopyruvate
	ATP	Pyruvate	P_i	
0	0	0	0	0
15	0.18	0.17	0.45 (0.18)	0.27
30	0.29	0.29	0.72 (0.32)	0.40

stoichiometry of product formation was determined in a reaction not coupled to lactate dehydrogenase (Table 5). Neither pyruvate nor ATP was formed when phosphopyruvate, AMP or pyrophosphate was omitted. After correction for orthophosphate formed by the hydrolysis of pyrophosphate the stoichiometry for the production of pyruvate, ATP and orthophosphate was close to unity.

Factors affecting enzyme stability. Little activity was detected in crude leaf extracts unless Mg^{2+} and 2-mercaptoethanol were included in the blending mixture. On several occasions an increase of up to threefold in enzyme activity was observed in crude extracts from leaves of maize and sorghum standing at 22° for 2–4 hr. None of the increased activity was lost by treating the extracts on a Sephadex G-25 column to remove compounds of small molecular weight.

The partially purified enzyme was rapidly inactivated in the absence of Mg^{2+} or 2-mercaptoethanol (Table 6). With the partially purified enzyme, pyruvate had a small stabilizing effect, but was more effective in crude extracts. The enzyme was more stable at 22° than at 0° or when frozen. EDTA was not as effective as 2-mercaptoethanol. Attempts to regain activity by the subsequent addition of missing components or by rewarming preparations kept at 0° were not successful. When stored as a suspension in 66%-saturated ammonium

Table 6. *Factors affecting enzyme stability*

Samples of the partially purified enzyme [stored as a suspension in 66%-saturated $(NH_4)_2SO_4$ solution] were isolated by centrifugation and dissolved in 25 mM-tris-HCl buffer, pH 8.3, containing other components as shown in the Table. Except where otherwise indicated the enzyme solutions were incubated at 22°. At intervals samples of the enzyme solutions were assayed as described in the Methods section. ME, 2-Mercaptoethanol.

Additions to the buffer medium	% of original activity		
	After 5 min.	After 60 min.	After 180 min.
None	5	0	0
5 mM-ME	10	4	0
5 mM- $MgCl_2$	90	21	4
5 mM-ME, 5 mM- $MgCl_2$	100	82	58
5 mM-ME, 5 mM- $MgCl_2$, 5 mM-pyruvate	100	93	76
5 mM-ME, 5 mM- $MgCl_2$, 5 mM-pyruvate at 0°	100	62	28
2 mM-EDTA, 6 mM- $MgCl_2$, 5 mM-pyruvate	100	64	13

sulphate solution at -15° the enzyme lost about 35% of its activity in 5 days.

Distribution of phosphopyruvate synthetase in plants. Phosphopyruvate synthetase was found in leaf extracts of the three tropical grasses examined, maize, sugar cane and sorghum. The respective activities were 1.4, 2.2 and 1.2 μ moles/mg. of chlorophyll/min. Values of 3–4 μ moles/mg. of chlorophyll/min. were recorded for maize and sorghum after the extracts had been kept at 22° for 2–4 hr. With all preparations similar activities were observed with extracts treated on a column of Sephadex G-25 (see the Methods section). No activity was detected in extracts of the stem or root of maize or in leaf extracts of silver beet, wheat, oat, sunflower or bean.

DISCUSSION

The phosphopyruvate synthetase preparation we employed previously (Hatch & Slack, 1967) contained considerable pyrophosphatase activity. Owing to the action of this enzyme and to the addition of orthophosphate to the reaction mixtures with phosphopyruvate carboxylase and ATP the requirement for orthophosphate was not recognized. The results of our present studies accord with a reaction proceeding as follows:

Evidence for this reaction was provided by

studies on the equilibrium and stoichiometry and by experiments in which orthophosphate was replaced by arsenate or [^{32}P]orthophosphate. The large difference in the standard free energy of hydrolysis for ATP to AMP and pyrophosphate (-7.6 kcal./mole) and for phosphopyruvate to pyruvate (-13.6 kcal./mole) at pH 7.0 indicates that the equilibrium for the reaction described in eqn. (1) would strongly favour pyruvate formation. Our estimate of the equilibrium constant of approx. 2×10^{-4} for the reaction proceeding towards phosphopyruvate synthesis would be consistent with the operation of the above reaction, but not of the reaction previously proposed (Hatch & Slack, 1967). The fact that phosphopyruvate synthesis declined rapidly when enzymes were not provided to remove the product, and that this decline was largely prevented when arsenate replaced orthophosphate, was consistent with this conclusion. Presumably an unstable arseno derivative is formed when arsenate is provided and its hydrolysis displaces the equilibrium in favour of phosphopyruvate formation. The fact that arsenate sustained phosphopyruvate formation suggested that orthophosphate does not provide the phosphate group of phosphopyruvate. However, its effect on the rate of the reaction provided additional support for the view that orthophosphate was a reactant rather than an activator.

The role and the fate of orthophosphate was confirmed when pyrophosphate was found to be a reactant. With enzyme preparations with much lower pyrophosphatase activity than those previously employed (Hatch & Slack, 1967), it was possible to demonstrate the production of pyrophosphate along with phosphopyruvate and also the pyrophosphate-dependence of the reverse reaction. Studies with [^{32}P]orthophosphate indicated that orthophosphate provides one of the phosphate groups of pyrophosphate.

The stoichiometry reported by Cooper & Kornberg (1965) for the enzyme from *E. coli* was the same as that found in our initial studies (Hatch & Slack, 1967). Though they did not report any requirement for orthophosphate, their preparation, like ours, contained pyrophosphatase. It appears possible that the bacterial enzyme may catalyse a reaction of the type described in eqn. (1). It is noteworthy that these enzymes share the unusual characteristic of being cold-labile and both are stabilized by Mg^{2+} . Alternatively, if the stoichiometry proposed for the bacterial enzyme is correct then the reactions may only differ in the respect that hydrolysis of an intermediate phosphate ester bond to yield orthophosphate is replaced by phosphorolysis to yield pyrophosphate.

Labelling studies (Hatch & Slack, 1966; Hatch, Slack & Johnson, 1967) and enzyme studies (Slack & Hatch, 1967) are consistent with the view that the initial steps in photosynthetic carbon dioxide fixation in tropical grasses involve the carboxylation of phosphopyruvate followed by the transfer of C-4 of the oxaloacetate thus formed to an acceptor, yielding 3-phosphoglycerate and leaving pyruvate as the other product. To complete the cyclic process pyruvate must be converted into the carbon dioxide acceptor, phosphopyruvate. The view that the latter reaction may be catalysed by phosphopyruvate synthetase was supported by a survey of the occurrence of the enzyme in different tissues. The activity in leaf extracts of tropical grasses was similar to the maximum photosynthesis rates for these leaves (approx. $3\text{ }\mu\text{moles/mg. of chlorophyll/min.}$; Slack & Hatch, 1967), but the enzyme was not detected in stem or root tissue of maize or in leaf extracts of several plants in which the Calvin cycle is operative (Hatch, Slack & Johnson, 1967).

Although the equilibrium for the leaf phosphopyruvate synthetase does not favour phosphopyruvate formation, its initial velocity would be maintained if one or more of the products were removed. In the intact leaf exposed to the light phosphopyruvate would be converted into oxaloacetate. The activity of the enzyme that catalyses this reaction, phosphopyruvate carboxylase, is considerably in excess of the maximum photosynthesis rates in the leaves of tropical grasses (Slack & Hatch, 1967). Enzymes are also available for the transformation of the other products, AMP and pyrophosphate. Separate studies have shown that the activities of adenylate kinase and a specific alkaline pyrophosphatase in the leaves of tropical grasses, expressed per mg. of chlorophyll, were greater than their maximum photosynthesis rates.

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