Pyruvate, Phosphate Dikinase from *Bacteroides symbiosus*

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(Received 9 July 1971)

1. An improved method is given for preparation of pyruvate, phosphate dikinase from *Bacteroides symbiosus*. 2. The bacterial enzyme is stable, free from interfering enzyme activities, and does not require thiol compounds to maintain stability during storage or assay. 3. New direct assays of enzyme activity are based on acid evolution or consumption as measured at constant pH in a pH-stat. 4. The optimum rate of reaction in the direction of pyruvate formation occurs at about pH 6.4; in the direction of phosphoenolpyruvate formation, it is at pH 7.2-7.8. 5. Newly determined substrate K_m values for the enzyme are: AMP, 3.5×10^{-6} M; ATP, 1×10^{-4} M; pyruvate, 8×10^{-5} M; P_i , 6×10^{-4} M. 6. K^+ may substitute for NH_4^+ in activating the reaction catalysed by the *B. symbiosus* enzyme. 7. In the direction of pyruvate formation the bivalent metal ion requirement of the enzyme is fulfilled by salts of nickel, manganese, magnesium and cobalt. In the other direction only magnesium salts were effective. 8. The nucleotide specificity of the enzyme is strictly limited to the adenine nucleotides. CTP and ITP strongly inhibit the reaction in the direction of phosphoenolpyruvate formation.

The enzyme now called by the trivial name pyruvate, phosphate dikinase was first reported by Hatch & Slack (1968) in plant sources. Reeves (1968) found the enzyme in *Entamoeba histolytica*. Reeves, Menzies & Hsu (1968), Evans & Wood (1968) and Benziman & Palgi (1970) identified it in bacterial sources. Reeves *et al.* (1968), working with the enzyme from *Bacteroides symbiosus*, established that label from [^{32}P] P_i appears in the γ -position of ATP and labelled phosphate from phosphoenol pyruvate appears in the β -position. They also found the observed equilibrium constant in the direction of pyruvate formation to be 1140 at pH 7 and that, over a wide pH range, it varied directly with the square of the H^+ concentration. This indicates that two hydrogen ions are consumed or produced in the forward or reverse reactions, respectively. The reaction that occurs in the presence of Mg^{2+} was written as follows:

Andrews & Hatch (1969) and Evans & Wood (1971) have undertaken studies on the mechanism of action of dikinase by using isotope-exchange reactions. These workers noted certain difficulties with enzyme prepared from plant sources and propionibacteria. The enzyme was cold-labile and required the presence of thiol compounds during storage and assay.

The present paper reports further work on the preparation and properties of the enzyme from *B. symbiosus*. Enzyme from this source is very stable, does not require thiols, and is readily prepared in good yield in a state free from interfering enzyme activities. Additionally, methods of assay are introduced that are based on H^+ consumption or evolution, according to the above equation. These methods avoid the restrictions imposed by linked-enzyme systems and they offer advantages over fixed-term assays of enzyme products.

MATERIALS

Bacteroides symbiosus (A.T.C.C. 14940) was grown anaerobically in 400-litre lots at the Fermentation Plant of the Department of Biochemistry, University of Georgia, Athens, Ga., U.S.A. The medium contained (g/l): Tryptone (Difco-0123) (20), D-glucose (10), yeast extract (Difco) (2), NaCl (2.5), anhydrous K_2HPO_4 (1.5), mercaptosuccinic acid (1.5), L-cysteine hydrochloride (0.79) and NaOH to pH 7.0. Incubation was for 16-20 h at 37°C. Cells were harvested on a Sharples supercentrifuge, frozen in solid CO_2 , and stored at -60°C until used. The yield of wet cell paste was approx. 5.7 g/l.

Nucleoside mono- and tri-phosphates and the pyridine nucleotides were obtained from P-L Laboratories, Milwaukee, Wis., U.S.A. Sigma Chemical Co., St Louis, Mo., U.S.A., supplied hexokinase, phosphoenolpyruvate (tricyclohexylammonium salt) and DEAE-cellulose. Lysozyme was from Nutritional Biochemicals Inc., Cleveland,

Ohio, U.S.A. Bio-Gel P-300 was from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Mercaptosuccinic acid and pyruvic acid were from Eastman Chemical Co., Rochester, N.Y., U.S.A. The pyruvic acid was distilled before use. Glucose 6-phosphate dehydrogenase and lactate dehydrogenase were from Boehringer und Sohne, Mannheim, Germany. Inorganic pyrophosphatase was prepared from *Escherichia coli*. Crude enzyme was prepared by the method of Blumenthal, Johnson & Johnson (1967). It was concentrated by vacuum dialysis against 10 mM-tris-HCl buffer, pH 7.2, containing 1 mM-MgSO₄. It was purified by chromatography on a column containing 50 g of Sephadex G-25 (Pharmacia), by elution with the same buffer. The peak fractions of enzyme from the column were used. The inorganic pyrophosphatase was free from non-specific phosphatases.

DEAE-cellulose was prepared for column use by allowing 20 g to sink in 1 litre of 1 M-NaOH. Then 750 g of cracked ice and 250 ml of conc. HCl were added, with cooling and stirring. The suspension, diluted to 4 litres with cold water, was washed by decantation until the free acid concentration fell below 0.01 M. Concentrated solutions of imidazole-HCl buffer, pH 7, NH₄Cl, NaCl and EDTA (sodium salt) were then added to final concentrations of 20, 20, 75 and 1 mM, respectively, and the suspension was adjusted to pH 7 with NaOH.

METHODS

Enzyme assays were done at 25°C. The enzyme purification procedures were done at 0–4°C except where otherwise noted. The buffer contained 20 mM-imidazole base adjusted to pH 7 with HCl, 20 mM-NH₄Cl and 1 mM-EDTA (sodium salt). All modifications of this buffer are specifically noted in the text.

Extraction and purification of B. symbiosus dikinase. Frozen cells (107 g) were thawed and washed by centrifugation with 1.5 litres of 0.15 M-NaCl. The packed cells were resuspended in 2 litres of buffer containing 5 mM-EDTA (sodium salt). The suspension was incubated at 37°C while 100 mg of separately dissolved crystalline lysozyme was added with stirring. Cell lysis was apparent within 15 min. After 35 min the viscous suspension was chilled in an ice bath and a solution containing 10 g of streptomycin base, as the sulphate, was added dropwise with stirring. The suspension was centrifuged for 10 min at 9000g and the sediment was discarded. To the supernatant solution was added 450 g of solid (NH₄)₂SO₄/l, with stirring. The precipitate was collected by centrifugation and redissolved in 204 ml of buffer.

To the above solution was added sufficient 4 M-potassium acetate to make the concentration 0.05 M with respect to acetate. The solution was chilled in an ice bath while 2 M-acetic acid was added dropwise with stirring until pH 4.9 was attained. The precipitate was removed by centrifugation and discarded. The supernatant solution was quickly re-adjusted to pH 7 with NaOH.

A column was prepared in the cold-room from 40 g of the pretreated DEAE-cellulose. It was washed with two column volumes of the buffer containing 75 mM-NaCl. The dark-yellow enzyme solution was then applied to the column and, after washing with 500 ml of the buffer containing NaCl, elution was begun with a non-linear

gradient achieved by allowing buffer containing 0.5 M-NaCl to flow into the closed reservoir containing 600 ml of 75 mM-NaCl-buffer. The effluent was collected in fractions of 36 ml. Enzyme was eluted immediately after the highly coloured material. Fractions 19–23 comprising most of the enzyme peak were combined and dialysed against buffer.

The dialysed solution from the first column was placed on a second column prepared from 20 g of DEAE-cellulose, similarly preconditioned. The enzyme solution was applied and the column washed with 300 ml of the 75 mM-NaCl-buffer. Enzyme was eluted with a linear gradient prepared by allowing 600 ml of 0.4 M-NaCl in buffer to flow into an open reservoir containing an equal volume of 75 mM-NaCl-buffer. Effluent was collected in 32 ml fractions. Enzyme appeared in fraction 14, immediately after a slightly coloured material. Three fractions comprising the enzyme peak were combined, and enzyme was precipitated by stirring in solid (NH₄)₂SO₄ (45 g/100 ml). The suspension was centrifuged in the cold and most of the supernatant solution was withdrawn and discarded. The sediment was suspended in the remaining supernatant solution (11.5 ml) and stored in the refrigerator. Portions of this suspension were centrifuged. The sediment was dissolved in buffer, and dialysed twice against 300 vol. of buffer. Such enzyme is referred to as enzyme from the second (NH₄)₂SO₄ precipitation.

A 4.5 ml portion of the above suspension was centrifuged. The sediment was dissolved in buffer and dialysed against 100 vol. of buffer. This solution was placed on a column prepared from 10 g of Bio-Gel P-300 that had previously been conditioned with 500 ml of buffer. The sample volume was 6 ml. It was eluted with buffer and collected in 6 ml fractions. Enzyme appeared in fractions 17–25. Enzyme activity and E_{280} were parallel across the enzyme peak. The Bio-Gel column was prepared and used at room temperature. This enzyme is referred to below as column-purified enzyme.

Enzyme assays. Assay A. The standard enzyme assay was slightly modified from that described by Reeves *et al.* (1968). Cuvettes contained 50 mM-imidazole-HCl buffer, pH 6.8; 5 mM-MgCl₂; 20 mM-NH₄Cl; 1 mM-AMP; 1 mM-phosphoenolpyruvate; 0.2 mM-NADH₂; 4 units of lactate dehydrogenase; and about 0.01 unit of enzyme in a volume of 0.39 ml. After monitoring the change in extinction at 340 nm for a few moments reaction was started by the addition of 0.01 ml of 50 mM-sodium pyrophosphate. The final rate of extinction change was corrected for any rate noted before the addition of the PP_i. Such corrections were not needed with enzyme preparations obtained after the stage of the second DEAE-cellulose-column step. One unit of enzyme activity is defined as the amount that would effect the oxidation of 1 μ mol of NADH₂/min under the conditions of the assay. The molar extinction of NADH₂ was taken to be $6.22 \times 10^3 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-2}$ at 340 nm. This assay was used to measure enzyme activity during the purification steps. Specific activity is defined as units of enzyme/mg of protein.

Assay B. Reaction in the direction of pyruvate formation was assayed on a Sargent pH-stat equipped with two Sargent S-30070-10 combination electrodes. One of the electrodes was connected to the pH-stat and the other to an expanded-scale pH-meter. This arrangement facilitated adjustment of the contents of the reaction vessel to

the desired pH and also provided independent monitoring of the pH during reaction. The complete reaction mixture contained 10 mM-MgCl₂, 10 mM-NH₄Cl, 1 mM-AMP, 1 mM-phosphoenolpyruvate, 1 mM-PPi, and enzyme in a volume of 5 ml. Enzyme or one of the substrates was withheld until temperature equilibration had occurred, the desired pH was reached, and the control rate of acid consumption was established. Reaction was then initiated by addition of the missing component. Titration was done automatically with 20 mM-HCl from the 0.25 ml burette system and the observed rate was corrected by subtracting the control rate, if any. When working in the normal mode with this burette one chart division equals 1 μ l of titrant; in the expanded-drive mode one chart division equals 0.2 μ l. The instrument contains temperature sensing and regulating devices and provides stirring by a magnetically operated Teflon-coated bar.

Assay C. Reaction in the direction of phosphoenolpyruvate formation was assayed on the pH-stat by using the same manipulative procedures except that the burette now contained 14 mM-NaOH (CO₂-free). The complete reaction mixture contained 5 mM-MgCl₂, 10 mM-NH₄Cl, 1 mM-ATP, 0.5 mM-pyruvate, 2 units of *E. coli* inorganic pyrophosphatase, and enzyme in a volume of 5 ml.

Protein determinations. In crude enzyme preparations protein was determined by the spectrophotometric method of Warburg & Christian (1941) by using the equation proposed by Layne (1957). After the second (NH₄)₂SO₄ precipitation enzyme samples were dialysed against water and dried to constant weight in an oven at 105°C.

RESULTS

Purification of *B. symbiosus* dikinase. The purification scheme described in the Methods section effectively removed activities of those enzymes that might interfere with the reaction studied. Enolase, inorganic pyrophosphatase and adenosine triphosphatase were not detected after the second DEAE-cellulose-column treatment. The very low NADH₂ oxidase activity remaining (insufficient to affect the standard assay) was further decreased by passage through the Bio-Gel column. The results of

the enzyme purification steps are given in Table 1. In spite of the fact that overall purification was only 15-fold, the final product showed no evidence of gross heterogeneity on analytical ultracentrifugation or electrophoresis. The specific activity of the final product is identical with that of the preparation reported by Reeves *et al.* (1968) after adjustment to the same basis for protein determination.

The enzyme suspension from the second ammonium sulphate precipitation was stored in the refrigerator for 3 months with no loss of activity. Enzyme in buffer from the Bio-Gel column stored in the refrigerator at a protein concentration of 1.45 mg/ml lost 22% of its activity in 3 months. The same low rate of loss of activity was observed with enzyme concentrated by vacuum dialysis to a protein concentration of 15 mg/ml.

Evidence that two hydrogen ions are involved in the dikinase reaction. When NADH₂ and an excess of lactate dehydrogenase were added to the reaction mixture specified in assay B, the initial rate of acid consumption catalysed by dikinase was increased. This persisted until all the added NADH₂ was oxidized, after which the rate reverted to the normal value. Fig. 1 reproduces a pH-stat tracing of such an experiment in which the initial rate of 422 nmol/min decreased to 276 nmol/min on depletion of the NADH₂. By making the reasonable assumption that exactly 1 mol of H⁺ is consumed/mol of pyruvate reduced one may calculate from these results that the parallel dikinase-catalysed reaction consumed 1.96 mol of H⁺/mol of pyruvate formed.

Optimum pH values for forward and reverse reactions. The pH optimum for the forward reaction (pyruvate formation) was investigated by both the spectrophotometric and the pH-stat assay systems. By both methods the optimum was found to be a rather sharp peak in the region of pH 6.4 (Fig. 2). To study the reverse reaction (phosphoenolpyruvate formation) assay C was employed.

Table 1. *Purification of pyruvate,phosphate dikinase from B. symbiosus*

Activity was determined by assay A. Purification steps are those described in the Methods section. Of the material from the second (NH₄)₂SO₄ precipitation 40% was taken for the Bio-Gel column. The results are recalculated assuming that the entire sample had been used. Protein and specific activity values are based on spectrophotometrically determined protein concentrations through the second DEAE-cellulose column; thereafter, on dry weight of the protein after dialysis against water.

Step	Total enzyme (units)	Total protein (mg)	Specific activity (units/mg of protein)
First supernatant	2260	6760	0.33
First (NH ₄) ₂ SO ₄ pptn.	1990	4120	0.48
pH 4.9 and first DEAE-cellulose column	1335	1780	0.75
Second DEAE-cellulose column	930	300	3.1
Second (NH ₄) ₂ SO ₄ pptn.	828	245	3.4
Bio-Gel column	778	156	5.0

Fig. 1. Reproduction of a pH-stat tracing showing the effect of NADH_2 plus lactate dehydrogenase on the rate of acid consumption in the forward dikinase reaction. The titration vessel initially contained 1 mM-phosphoenolpyruvate, 1 mM- PP_i , 1 mM-AMP, 20 mM- NH_4Cl , 10 mM- MgCl_2 , 0.5 μmol of NADH_2 , and 20 units of lactate dehydrogenase in a volume of 4.95 ml adjusted to pH 6.4. To initiate the reaction 37 μg of column-purified enzyme in 0.05 ml of buffer was added at zero time. The position of the curve was adjusted on the ordinate scale to compensate for acid consumption by buffer components added with the enzyme. No consumption of acid was observed before addition of enzyme. The initial rate of 422 nmol/min changed when the NADH_2 was depleted (arrow) to 276 nmol/min.

In preliminary studies it was found that, because of the unfavourable equilibrium constant, the initial reaction rate was not sustained for long. The incorporation of inorganic pyrophosphatase into the reaction mixture of assay C facilitated the determination of the initial reaction rate by greatly extending the duration of its linearity (Fig. 3). The reaction in this direction was found to possess a broad pH optimum extending from pH 7.2 to 7.8 (Fig. 4).

Substrate K_m values for the forward and reverse reactions. Reeves *et al.* (1968) reported K_m values of 0.1 and 0.06 mM for pyrophosphate and phosphoenolpyruvate respectively at pH 6.8. In the present work the K_m for AMP, the third substrate of the forward reaction, was found to be 3.5 μM at the same pH. For this experiment it was necessary to use exceptionally long cuvettes and an assay system based on measurements of the rate of ATP formation. These are described in the legend to Fig. 5.

The K_m values for the substrates of the reverse reaction were determined by using the pH-stat. Assay C (see the Methods section) was modified by lowering the concentration of the substrate under investigation and, at the lowest substrate concentrations, by doubling or quadrupling the volume of the reaction mixture. The K_m values

Fig. 2. Effect of pH on the dikinase reaction in the direction of pyruvate formation. Curve A, ●, Spectrophotometrically determined rates of pyruvate formation. Assay A was used with the following modifications. The concentrations of MgCl_2 and lactate dehydrogenase were doubled, and the pH of the imidazole buffer was that shown on the figure. Curve B, ○, pH-stat determinations with assay B as given in the Methods section. Amount of acid consumed ($\mu\text{mol}/\text{min}$) was divided by 2 to determine μmol of product formed/min. The same column-purified enzyme preparation was used in both sets of experiments.

were obtained by making double-reciprocal plots of the velocity and substrate-concentration results. Four to six different concentrations of each substrate were employed which, in each instance, spanned the observed K_m value. The values found were: ATP, 0.1 mM; pyruvate, 0.08 mM; P_i , 0.6 mM. These results were determined with enzyme from the second ammonium sulphate precipitation.

Requirement for K^+ or NH_4^+ . Reeves *et al.* (1968) had demonstrated an NH_4^+ requirement for the *B. symbiosus* enzyme. The concentration of ammonium chloride producing one-half maximum activity was 2.5 mM at pH 6.8. Evans & Wood (1971) found that the univalent cation requirement for enzyme from propionibacteria was satisfied by either NH_4^+ or K^+ . The present experiments showed that K^+ is also effective in the reaction catalysed by the *B. symbiosus* enzyme. One-half maximum velocity was attained with 20 mM-potassium chloride at pH 6.8. The maximum velocity achieved with

Fig. 3. Reproduction of pH-stat tracings showing the effect of inorganic pyrophosphatase on the dikinase reaction in the direction of phosphoenolpyruvate formation. A. The composition of the reaction mixture was that of assay C described in the Methods section. B. The reaction mixture lacked *E. coli* inorganic pyrophosphatase. In both experiments the reaction was initiated at 3 min by the addition of 0.01 ml of enzyme from the second ammonium sulphate step containing 142 μ g of protein in buffer. No correction has been applied to compensate for the small acid equivalent of the added enzyme solution.

potassium chloride was about 85% of that with ammonium chloride. Sodium chloride in concentrations up to 50 mM did not initiate enzyme action in the absence of both of the effective cations. These studies were made by substituting various concentrations of sodium or potassium chloride for the ammonium chloride specified in assay A. Univalent cation requirements for the reaction in the direction of phosphoenolpyruvate formation have not yet been determined. Enzyme for this work was dialysed against buffer lacking ammonium chloride.

Bivalent metal ion requirements for the dikinase reaction. The bivalent metal ion requirements were studied on the pH-stat with the results listed in Table 2. At 1 mM concentrations nickel sulphate, manganous chloride, magnesium chloride and cobaltous chloride appeared to be increasingly effective in promoting the forward reaction. Only magnesium chloride was effective, among those tested, in the reverse reaction. Manganous and cobaltous salts caused precipitation under both

Fig. 4. Effect of pH on the dikinase reaction in the direction of phosphoenolpyruvate formation. The rate determinations were made on the pH-stat by using assay C given in the Methods section. The enzyme was the same as that used in the experiments of Fig. 2. In computing μ mol of products formed/min, μ mol of NaOH consumed/min was divided by 2.

sets of reaction conditions. The results in Table 2 show a pronounced optimum concentration for magnesium chloride in the direction of phosphoenolpyruvate formation. No effect of equal magnitude has been reported for enzyme from another source.

Nucleotide specificity of the dikinase reaction. The nucleotide requirements for the dikinase reaction were assayed on the pH-stat at pH 6.4 for the forward reaction and at pH 7.4 for the reverse reaction. The results of this study are listed in Tables 3 and 4.

DISCUSSION

Pyruvate,phosphate dikinase functions in the gluconeogenic direction in photosynthetic grasses, in lactate-grown propionibacteria, and in *Acetobacter xylinum* grown on medium containing succinate or pyruvate (Benziman & Eizen, 1971). It functions in the glycolytic direction in *Ent. histolytica* and, presumably, also in *B. symbiosus* cells growing on glucose. In either direction there are three substrates and three products. Enzyme from two sources exhibit a univalent cation requirement in addition to that for a bivalent metal ion shared by all the known dikinases. Clarification of the kinetic properties of pyruvate,phosphate dikinase is a task of considerable magnitude. The present work and results previously published from this and other laboratories comprise only the beginning of this undertaking. The present method for the partial purification of enzyme from *B. symbiosus* has been

Fig. 5. Effect of AMP concentration on the forward dikinase reaction. Cuvettes contained 50 mM-imidazole-HCl buffer, pH 6.8, 2.5 mM-glucose, 0.3 mM-NADP, 10 mM-MgCl₂, 20 mM-NH₄Cl, 1.0 mM-phosphoenolpyruvate, 1.25 mM-PP_i, AMP as indicated, 10.4 units of hexokinase, 9 units of glucose 6-phosphate dehydrogenase and 0.75 μ g of column-purified dikinase in a volume of 10 ml. The cuvettes were polarimeter tubes of 200 mm light-path. They were monitored on a Zeiss PMQ spectrophotometer. The insert on the figure shows a reciprocal plot of the same results; the K_m is 3.5 μ M.

Table 2. *Bivalent metal ion requirement for the B. symbiosus dikinase reaction*

The pyruvate formation reactions were conducted at pH 6.4 by employing assay B, except that the bivalent salt listed in the Table replaced the MgCl₂ normally specified for this assay. Each reaction employed 75 μ g of column-purified enzyme. The phosphoenolpyruvate formation reactions were conducted at pH 7.4 by employing assay C. The bivalent salt listed in the Table replaced the MgCl₂ normally specified for this assay. Each of these reactions used 142 μ g of enzyme from the second (NH₄)₂SO₄ precipitation.

Bivalent metal salt	Pyruvate formation, acid consumed (nmol/min)	Phosphoenolpyruvate formation, acid produced (nmol/min)
None	0	0
MgCl ₂ , 1 mM	137	21
MgCl ₂ , 2 mM	362	—
MgCl ₂ , 2.5 mM	—	88
MgCl ₂ , 5 mM	500	101
MgCl ₂ , 7.5 mM	—	55
MgCl ₂ , 10 mM	540	23
MgCl ₂ , 15 mM	510	—
NiSO ₄ , 1 mM	24	—
MnCl ₂ , 1 mM	110	0
CoCl ₂ , 1 mM	196	0
Ca(NO ₃) ₂ , 10 mM	0	—
MgCl ₂ + Ca(NO ₃) ₂ , each 10 mM	0	—
ZnSO ₄ , 1 mM	0	—

found to be considerably more convenient and reproducible than that described by Reeves *et al.* (1968). The addition of streptomycin to the lysozyme homogenate circumvented a problem in

packing the sediment on centrifugation. The pH 4.9 treatment contributed little to the purification and, perhaps, could be omitted. The stability of refrigerated enzyme solutions is

Table 3. *Nucleotide requirement for the pyruvate-formation reaction catalysed by B. symbiosus dikinase*

For these experiments assay B was modified to contain 20 mM-NH₄Cl and the nucleotide as specified in the column at left in the Table. Each experiment employed 75 μ g of column-purified enzyme in a volume of 5 ml. The pH was maintained at 6.4 by the addition of 20 mM-HCl. After monitoring the reaction for a sufficient interval 1 mM-AMP was added to the reaction vessels containing one of the four last nucleotides.

Nucleotide present at start	H ⁺ consumed (nmol/min)	
	Initially	After addition of 1 mM-AMP
AMP, 1 mM	470	—
dAMP, 1 mM	129	—
dAMP, 0.2 mM	102	—
IMP, 1 mM	0	490
UMP, 1 mM	0	490
GMP, 1 mM	0	490
CMP, 1 mM	0	490

Table 4. *Nucleotide requirement for the reverse reaction catalysed by B. symbiosus dikinase*

Initially each reaction vessel contained 0.5 mM-pyruvate, 2 mM-P_i, 10 mM-NH₄Cl, 5 mM-MgCl₂, 2 units of *E. coli* inorganic pyrophosphatase, 142 μ g of enzyme from the second (NH₄)₂SO₄ precipitation, and nucleotide as specified in the Table in a volume of 5 ml. After monitoring the reaction for a sufficient period of time 1 mM-ATP was added to those vessels that did not contain it. The pH was maintained at 7.4 by the addition of 14 mM-NaOH.

Nucleotide present initially	NaOH consumed (nmol/min)		Inhibition of ATP reaction (%)
	Initially	After addition of 1 mM-ATP	
ATP, 1 mM	105	—	—
dATP, 1 mM	0	62	40
GTP, 1 mM	0	106	0
UTP, 1 mM	0	134	0
ITP, 1 mM	0	0	100
ITP, 0.1 mM	0	63	40
CTP, 1 mM	0	0	100
CTP, 0.1 mM	0	62	40

excellent at all stages of purification, but enzyme does not survive freezing and thawing in buffer.

Hatch & Slack (1968) obtained 2.1 units of enzyme/g of maize leaves. The specific activity of their most highly purified product was 1.2 units/mg of protein. Both values were determined at pH 8.3 in the direction of phosphoenolpyruvate formation. One presumes that their definition of unit activity was the same as given in their later paper (Hatch & Slack, 1969). By assaying at pH 8.2 Benziman & Palgi (1970) obtained 19 units from 30 g of *A. xylinum* (dry weight); highest specific activity 0.88. By assaying activity in the other direction at pH 6.7, 7.0, or 6.8, respectively, the following results have been reported. Evans & Wood (1971) obtained 1.8 units of activity/g of lactate-grown propionibacteria, highest specific activity 1.7. Reeves (1968) obtained 1.65 units/ml of packed amoeba cells, highest specific activity 0.84. Reeves *et al.* (1968) obtained from *B. symbiosus* 27 units/g

of cells, and in the present investigation the yield was 21 units/g, highest specific activity 5 (based on dry wt. of protein). Although the above enzyme yields are based on different assay methods, existing evidence indicates that the enzyme is more abundant in *B. symbiosus* than in the other recognized enzyme sources. This may reflect the absence of pyruvate kinase in this organism.

Direct assays of enzyme activity have been limited to term experiments in which one of the products was assayed. In the direction of pyruvate formation a linked assay involving oxidation of NADH₂ in the presence of lactate dehydrogenase is suitable for kinetic studies. The enzyme-linked assay of ATP formation is also satisfactory. In the direction of phosphoenolpyruvate formation all the linked assay systems in use have disadvantages. Most involve carboxylation of phosphoenolpyruvate to oxaloacetate, which imposes a requirement for a bicarbonate buffer. An ingenious method by

Benziman & Palgi (1970) uses lactate, lactate dehydrogenase and NAD to supply pyruvate, but this method places restrictions on the pyruvate concentrations in the assay mixture. The direct assay of enzyme activity on the pH-stat appears to offer an improved method for the investigation of kinetic properties of the enzyme working in the direction of phosphoenolpyruvate formation.

The usefulness of the pH-stat assay methods depends, to a great extent, on the validity of calculating the number of mol of substrate consumed and products formed from the measurement of the acid formed or utilized. The ionic form of the dikinase reaction equation given in the introduction section implicates 2H^+ /mol of substrate, but whether or not exactly 2 mol of H^+ are contributed to or withdrawn from the system depends on the acid dissociation constants for each of the six substrates under the conditions prevailing in the reaction medium. Reeves *et al.* (1968) discuss the reasons for the ratio between H^+ and substrate being approx. 2 over a wide pH range. Their results were based on a study of the reaction equilibrium. A computer analysis of their published results has yielded a more exact value for the ratio: 1.94 ± 0.19 (95% confidence limit). The kinetic approach to this problem attempted in the present work gave a value of 1.96 for this ratio. It should be pointed out that the approximate conversion factor of 2, as used in the present studies, is applicable only to situations where the Mg^{2+} concentration is sufficient to complex essentially completely both the ATP and PP_i in the assay system.

As is evident from Fig. 2, the spectrophotometric assay gives a value which is 20% greater at pH 6.4 than that obtained from the direct pH-stat assay. This discrepancy might be due to the linking enzyme that removes pyruvate in the spectrophotometric assay system or to the greater ionic strength of the spectrophotometric assay medium. Experiments designed to test one or the other of these possibilities seemed to indicate that neither explanation was valid. A third possibility is that imidazole buffer exerts a small activating effect on the dikinase. Evans & Wood (1971) gave evidence that this may be so for the enzyme from propionibacteria.

Figs. 2 and 4 allow a direct comparison of reaction rates catalysed by the same enzyme preparation in both the pyruvate-formation and phosphoenolpyruvate-formation directions. Considering only the direct pH-stat assays it is evident that, at their respective pH optima, the velocity in the pyruvate direction/velocity in the phosphoenolpyruvate direction ratio is about 3.5. Andrews & Hatch (1969) report a value of 0.17 for this ratio when enzyme from sugar-cane leaves was assayed at pH 8.3. The results of Benziman & Palgi (1970) give values for this ratio of 2.2 and 0.2 at pH 6.5 and

8.2, respectively. For the *B. symbiosus* enzyme the $V_{\text{optimum pH}}/(\text{product of the substrate } K_m)$ ratios favoured the direction of pyruvate formation by three orders of magnitude.

Evans & Wood (1971) noted that the reported substrate K_m values are in general agreement irrespective of the enzyme source. The four values reported in the present work are consistent with this observation, but the situation may change as the effect of pH on K_m is explored. The K_m values on the plant enzyme were determined at pH 8.3 (Hatch & Slack, 1968; Andrews & Hatch, 1969). Those for enzyme from propionibacteria were determined at pH 6.7 in the direction of pyruvate formation and at pH 6.8 in the other direction (Evans & Wood, 1971). Those for enzyme from *B. symbiosus* were observed at pH 6.8 in the direction of pyruvate formation and at pH 7.4 in the other direction. Those for enzyme from *A. xylinum* (Benziman & Palgi, 1970) were determined at pH 6.5 and 8.2 in those directions, respectively. The two values reported for enzyme from *Ent. histolytica* (Reeves, 1968) were determined at pH 7.

Adenine nucleotides are the preferred substrates for enzyme from all sources. However, the pattern of inhibition by nucleotides is quite different among the different enzymes. Andrews & Hatch (1969) report no inhibition by 1 mM-ATP on the pyruvate formation reaction catalysed by a plant enzyme whereas Reeves (1968) found strong inhibition of the amoebal enzyme by 0.22 mM-ATP. Evans & Wood (1971) found slight inhibition of the phosphoenolpyruvate formation reaction by GTP and a more severe effect by GMP in the other direction. These findings with enzyme from propionibacteria contrast sharply with those of the present investigation in which ITP and CTP were strong inhibitors in the direction of phosphoenolpyruvate formation whereas no tested nucleoside monophosphate was inhibitory in the other direction. Calcium salts inhibited the *B. symbiosus* enzyme as they did that from propionibacteria.

The enzymes also have different pH optima. Hatch & Slack (1968) found pH 8.3 to be optimum for the plant enzyme in the direction of phosphoenolpyruvate formation. The optimum pH for reaction in the other direction has not been reported for enzyme from plant sources. Evans & Wood (1971) found optima between pH 6.5 to 7 for reaction in either direction catalysed by enzyme from propionibacteria. Benziman & Palgi (1970) found optima at pH 6.5 and 8.2 in the direction of pyruvate formation and phosphoenolpyruvate formation, respectively. The present studies indicate for the enzyme from *B. symbiosus* an optimum at pH 6.4 for reaction in the direction of pyruvate formation and a broad optimum from pH 7.2 to 7.8 for the other direction.

This work was supported in part, by Grants GM-14023 and AI-02951 from the National Institutes of Health. Mr D. Alford assisted in the Bio-Gel column purification experiment.

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