The Pyruvate-Phosphate Dikinase Reaction

THE FATE OF PHOSPHATE AND THE EQUILIBRIUM*

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SUMMARY
An enzyme capable of catalyzing the reaction
AMP + PPi + P-enolpyruvate = ATP + P1 + pyruvate
was obtained from Bacteroides symbiosus cell extracts and was purified from interfering enzyme activities. It was strongly stimulated by ammonium ion. In this respect and in other physical properties the bacterial enzyme differed from that previously obtained from Entamoeba histolytica. In a complete reaction system the enzyme transferred label from orthophosphate to pyrophosphate and to the γ position of ATP while the label of 32P-enolpyruvate was transferred to the β position of ATP. The observed equilibrium constant catalyzed by the bacterial enzyme was directly dependent upon the square of the hydrogen ion concentration. At pH 7.0 its value was 1140. Apparent K_m values for P-enolpyruvate and pyrophosphate were 0.07 mM and 0.1 μM for the amebal enzyme and 0.06 mM and 0.1 mM for the bacterial enzyme, respectively.

MATERIALS AND METHODS
Commercial samples of AMP and ATP were purified by chromatography on a Dowex 1 (Cl) column following the procedure of Cohn (5). The purified ATP was concentrated and washed from excessive amounts of salt by its retention on a Diaflo UM-3 membrane filter obtained from the Amicon Corporation, Lexington, Massachusetts. Its purity was confirmed by rechromatography. The nucleotides were assayed spectrophotometrically in sodium phosphate buffer, pH 7, by their absorbance at 259 nm (A_m 15.4 × 10^5).

Hexokinase, glucose-6-P dehydrogenase, lactate dehydrogenase, pyruvate kinase, and adenylate kinase (myokinase) were obtained as suspensions in concentrated ammonium sulfate from Boehringer Mannheim. The enzyme suspensions were centrifuged and the sedimented enzymes were dissolved in 1 mM sodium EDTA, pH 7.

The stock solution of sodium pyrophosphate (Baker's analytic reagent) was 57.3 mM in PPi and 0.15 mM in P1. The PPi was assayed by linking the amebal dikinase to the oxidation of direction (gluconeogenesis) in the tissue or organism investigated. Reeves (2) independently encountered a similar enzyme in Entamoeba histolytica, an organism lacking pyruvate kinase (EC 2.7.1.40). He suggested that here the new enzyme must function in the glycolytic pathway.

Hatch and Slack (1) and Slack in a subsequent communication (3), referred to the new enzyme as phosphopyruvate synthetase, or synthase, a name previously applied by Cooper and Kornberg (4) to a different enzyme. Reeves referred to it as pyruvate-phosphate ligase. However, upon gaining an insight into the enzyme mechanism it appeared that another name would be more suitable.

The reaction of Equation 1 involves five phosphorus-containing substrates. In their initial report Hatch and Slack (1) showed that the enzyme converts 32P to labeled PPi and ATP. We have confirmed this finding and studied the position of the label in the ATP. Also studied was the fate of the label transferred from 32P-enolpyruvate to ATP. Since the enzyme is believed to function under physiological conditions in either the forward or reverse directions an investigation of its equilibrium constant was undertaken. In this we were aided by the finding of a source of stable enzyme amenable to its isolation from interfering enzyme activities.
NADH by lactate dehydrogenase. The assay conditions were those of the standard assay method for the ameloblast dimerase with suitable controls, except that the concentrations of PP were made limiting. The P was assayed by the method of Berenblum and Chain as modified by Lindberg and Ernster (6). Sodium pyruvate was freshly dissolved in water and assayed by NADH oxidation catalyzed by lactate dehydrogenase.

Enzymatic assays for ATP were made in Tris-HCl buffer, pH 8. The cuvettes contained 50 mM Tris, 4 mM glucose, 2 mM MgCl₂, 0.3 mM NADP, hexokinase, glucose-6-P dehydrogenase, water, and sample. Absorbs were measured before the addition of the sample to the assay cuvette and to a suitable control and again at the completion of the reaction. Enzymatic assays for P-enolpyruvate were made in imidazole-HCl buffer, pH 7. The cuvettes contained 50 mM buffer, 50 mM KCl, 1 mM ADP, 1 mM MgCl₂, 0.2 mM NADH, lactate dehydrogenase, pyruvate kinase, water, and sample. Absorbs were measured as for the ATP assays.

Adenylate kinase was assayed by the method used in the earlier report (9). Enolase was assayed by linking it with pyruvate kinase and lactate dehydrogenase. The cuvettes contained 63 mM glycerylaldehyde (pH 7.5), 3 mM MgCl₂, 0.2 mM NADH, 50 mM KCl, 1 mM ADP, 1 mM 2-phosphoglycerate, 2.5 enzyme units per ml of lactate dehydrogenase, and pyruvate kinase, water, and enzyme. ATPase was assayed by substituting ATP for ADP and P-enolpyruvate for 2-phosphoglycerate in the above system. Inorganic pyrophosphatase was assayed in imidazole-HCl buffer, pH 7, and in Tris-HCl buffer, pH 8. The incubation mixture contained 50 mM buffer, 1 mM Pi, 2 mM MgCl₂, water, and enzyme. Incubation was for 30 min at 25° and the liberated Pi was determined (6).

Adsorption of labeled nucleotides onto acid-washed carbon (Norite A) was from 0.01 N acid solutions. Both centrifuge tube and small column techniques were used with equivalent results. The carbon completely adsorbed 60 pmoles of ATP per g, and 100 to 200 pmole of carbon were used in each experiment. The 7-hydroxydehydrogenase were conducted in a briskly boiling water bath in the presence of 1 N sulfuric acid. After hydrolysis, ammonium molybdate was added and the solutions were five times extracted with equal volumes of isobutyl alcohol-benzene (1:1). Aliquots of this combined extract were evaporated on planchets for counting, and the counts were taken to represent the total acid-liberated ³²P.

Radioactivity was assayed on planchets with a Nuclear-Chicago 12-47 gas flow detector with a Micromol window. Counting efficiency was approximately 50%. All samples of consequence were counted to 1,000 counts, most to 10,000 counts or more. Counts were corrected for background.

Separation of Enzyme Substrates on Dowex Columns

A standard procedure was adopted for the separation and identification of the five phosphorus-containing substrates of the enzyme reaction. Columns containing 2.5 ml of Dowex 1 (Cl), 200 to 400 mesh, were loaded with the sample at pH 7 and washed with water. Elution was begun with 0.01 N HCl. The effluent was monitored at 200 nm with a flow cell and 5-ml fractions were collected. The AMP and most of the P₁ was eluted in the first 65 ml, after which a nonlinear gradient elution was begun. The reservoir then contained 50 ml of the acid and the entering solution was 0.1 M NaCl in 0.01 N HCl. Elution by this gradient successively removed the remainder of the P₁, the P-enolpyruvate, and the P-P, and finally the ATP appeared. When this occurred the eluting solvent was changed to 0.25 M NaCl in 0.01 N HCl which removed the remainder of the ATP in a sharp peak. Aliquots of the collected samples were hydrolyzed in 1 N sulfuric acid for 2 hours at 90-95° and then analyzed for phosphate with the AutoAnalyzer with Technicon Method N-4b, adapted from the Fiske SubbaRow procedure.

Synthesis of P-enolpyruvate

One milliliter of carrier-free ⁵⁷⁶PO₄ in 0.01 N HCl (New England Nuclear) was lyophilized in a glass-stoppered centrifuge tube. To the residue were added 168 mg (1.00 mmole) of POCI₃ and the stoppered tube was allowed to stand in an ice bath for 30 min. Then 0.3 ml of freshly distilled quinoline and 150 mg of freshly distilled pyruvic acid (1.7 mmoles) dissolved in 0.5 ml of quinoline were added with cooling. The tube was placed in a 70° water bath for 20 min. The brown reaction mixture was stirred with a stout glass rod. It became very viscous. The tube was then chilled and 3 ml of water were added with stirring, followed by centrifugation. The supernatant solution was centrifuged, adjusted to pH 8 with 30% NaOH solution, and centrifuged. The aqueous layer was collected, 6 ml of methanol were added to it, and then centrifugation was repeated. The supernatant fluid was transferred to a flask containing 25 ml of water, 4 drops of 6 N HCl, and 0.5 g of Norite A. After standing for 1 hour the suspension was filtered, neutralized with ammonia, and loaded onto a standard Dowex 1 (Cl) column. The first 65 ml of eluate (0.01 N HCl) were led directly to a waste vessel. The salt gradient was begun at 65 ml and was made steeper than usual by using only 30 ml of acid in the reservoir. The effluent was collected in 5-ml fractions and monitored at 230 nm. Radioactivity and absorbance peaks occurred in Fraction 7.

By enzymatic assay the three peak fractions contained a total of 27 pmoles of P-enolpyruvate. The radioactivity of these pooled fractions was 575,000 cpm per pmole of P-enolpyruvate.

Preparation of Bacterial Dikinase

Bacteroides suminum (ATCC 14040) was grown in 1.5-liter lots in the Trypsinase-thiogalactose-glucose medium previously described (7). Fresh B. suminum cells, 9.5 g, were suspended in 200 ml of 0.04 imidazole-HCl buffer, pH 7, containing 1 mM EDTA. Ten milligrams of lysozyme (Nutritional Biochemicals) were added to the suspension at 37°. After 30 min the suspension was heated in a 50° water bath for 5 min and allowed to stand overnight at room temperature.² The viscous suspension was then centrifuged at 30,000 × g for 20 min and the supernatant solution, 137 ml, was decanted. This crude extract was assayed under conditions described below. It contained 753 mg of protein and 260 units of the dikinase, specific activity 0.35. To it were added 33 g of solid ammonium sulfate, with stirring and cooling. After standing for 15 min in an ice bath the precipitate was removed by centrifugation for 10 min at 20,000 × g and discarded. To the supernatant solution were added 26 g of ammonium sulfate. After standing as before this precipitate was collected by centrifugation, and the supernatant solution was discarded. The precipitate was dissolved in 1 mM sodium EDTA, pH 7, and dialyzed for 5 hours against 500 ml of the

² Other work has shown that centrifugation may immediately follow the lysozyme treatment at 37°.
EDTA solution. It was then dialyzed overnight against 500 ml of an imidazole-EDTA buffer containing 0.01 M imidazole-HCl, pH 7, and 1 mM EDTA. After dialysis the solution contained 361 mg of protein and 220 units of the enzyme, specific activity 0.63.

The enzyme was adsorbed on a column of DEAE-cellulose, 12 cm, which had been previously conditioned in the imidazole-EDTA buffer. After desorption of the enzyme the column was washed with 150 ml of the buffer. It was then successively eluted with 150-ml portions of the buffer containing 0.1, 0.2, and 0.3 M ammonium sulfate. The enzyme was eluted by the last solution which was collected in 5-ml fractions. The fractions containing enzyme were pooled and concentrated to 5 ml on a Diaflo UM-1 membrane (Amicon). This solution contained 28.2 mg of protein and 107 units of the enzyme, specific activity 3.8.

The concentrated enzyme solution from the above step was loaded onto a column of Sephadex G-200, 2.5 x 30 cm, which had been washed with 0.5 N alkali and water and conditioned with the imidazole-EDTA buffer. After desorption of the enzyme the column was washed with 150 ml of the buffer. It was then successively eluted with 150-ml portions of the buffer containing 0.1, 0.2, and 0.3 M ammonium sulfate. The enzyme was eluted by the last solution which was collected in 8-ml fractions. The fractions containing enzyme were pooled and concentrated to 5 ml on a Diaflo UM-1 membrane (Amicon). This solution contained 28.2 mg of protein and 107 units of enzyme, specific activity 3.8.

Enzyme from extracts of Entamoeba histolytica was purified through the Biogel P-300 column and assayed by the standard method as described by Reesow (2). This standard assay method differs from Method B, above, in using the buffer at pH 7.0, 0.625 mM PPi, 1 mM MgCl2, and no added ammonium chloride. In the amebal enzyme preparation used for some of the present studies the ratios of adenylate kinase and enolase activities to that of the dikinase were 0.02 and 0.1, respectively.

Amebal Dikinase

Enzyme from extracts of Entamoeba histolytica was purified through the Biogel P-300 column and assayed by the standard method as described by Reesow (2). This standard assay method differs from Method B, above, in using the buffer at pH 7.0, 0.625 mM PPi, 1 mM MgCl2, and no added ammonium chloride. In the amebal enzyme preparation used for some of the present studies the ratios of adenylate kinase and enolase activities to that of the dikinase were 0.02 and 0.1, respectively.

Enzyme Units

One unit of enzyme is defined as the amount required to produce 1 μmole of pyruvate per min at 25° under the applicable standard assay condition. Specific activity refers to units of enzyme per mg of protein. The protein was determined by the method of Lowry et al. (9) with crystalline bovine serum albumin standard.

RESULTS

Comparison of Bacterial and Amebal Enzyme—Unlike the amebal enzyme, the bacterial enzyme is strongly activated by ammonium ions. Some findings showing this difference between the two enzymes are illustrated in Fig. 1. This difference dictated the different assay conditions for the bacterial and amebal enzymes. The bacterial enzyme preparations were more stable at refrigerator temperature, but did not withstand freezing and thawing as well as the amebal enzyme. The former appeared to have no requirement for thiol in the storage fluid.

The apparent Kₘ values for PPi and P-enolpyruvate were 0.1 and 0.07 mM for the amebal enzyme and 0.1 and 0.06 mM for the bacterial enzyme, respectively. These values were determined by modifying the appropriate standard assay method so that the investigated substrate was present at rate-limiting concentrations. The spectrophotometric assay methods used were inadequate to determine the Kₘ for AMP. This value appeared to be less than 0.01 mM in the case of each enzyme.

The crude bacterial extracts, like those from E. histolytica, appeared to be entirely lacking in the ordinary pyruvate kinase. Extracts were tested for the formation of pyruvate from P-enolpyruvate in the presence of ADP and magnesium ions under a variety of conditions, without finding evidence of reaction. The tested conditions included varying the potassium ion concentrations between 10⁻⁴ M and 10⁻¹ M.

Incorporation of ³²P into γ Position of ATP—The incubation mixture contained in a final volume of 1 ml: 63 amoles of glycylglycine-NaOH, pH 8.0; 2 μmoles of ATP; 5 μmoles of sodium pyruvate; 2.5 μmoles of MgCl₂; 2 μmoles of ³²P (743,000 cpm per μmole); 0.4 μmole of P-enolpyruvate; and 0.27 mg of column...
purified amebal enzyme, specific activity 0.5. After incubation at 37° for 1 hour, it was chilled and 0.5 ml of cold 6% perchloric acid was added. The precipitated protein was removed by centrifugation. The acid was then neutralized with K$_2$CO$_3$ and the precipitated salt was removed by centrifugation. The supernatant solution was acidified and the nucleotides were adsorbed on 150 mg of carbon. The carbon was washed free of soluble label and the nucleotides were eluted with a total of 70 ml of a solution prepared by adding 1 ml of concentrated ammonia to 100 ml of 50% aqueous alcohol. Ten micromoles of P$_i$, 5 pmoles of PP$_i$, and 0.4 mole of ADP were added to this eluate which was then loaded onto a Dowex column and chromatographed by the standard technique. Radioactivity appeared in the P$_i$ and the ATP peaks. No label was associated with the AMP or ADP peaks. The nonadsorbed products from the treatment with carbon were also chromatographed on Dowex. Label was found associated with the P$_i$ and PP$_i$ peaks, but not with the P-enolpyruvate peak.

Combination of the three ATP peak fractions yielded 0.55 pmole of ATP with 137,000 cpm per pmole. Ninety-seven per cent of these counts were adsorbed on carbon. Upon 7-min hydrolysis 96% of the label was liberated as P$_i$. A sample of the ATP was incubated with glucose, MgCl$_2$, and hexokinase (Tris-HCl buffer, pH 8.0), after which 9% of the counts were adsorbed on carbon and 12% of the label was liberated by a 7-min hydrolysis.

A similar preparation of labeled ATP was prepared with the bacterial enzyme, except that here 10 mM concentrations of ammonium chloride and magnesium chloride were used in the incubation mixture, and the substrate P$_i$ was more highly labeled. The ATP isolated from the column contained 260,000 cpm per pmole. Seventy-eight per cent of these counts were liberated as P$_i$ by the 7-min hydrolysis. After incubation with glucose and hexokinase as above only 6% of the counts were liberated by the hydrolysis.

**Incorporation of Label from $^{32}$P-enolpyruvate into $\beta$ Position of ATP—**The incubation mixture contained in a final volume of 4 ml: 2.98 pmoles of $^{32}$P-enolpyruvate (298,000 cpm per pmole), 3.6 pmoles of AMP, 5.7 pmoles of PP$_i$, 50 pmoles each of MgCl$_2$ and NH$_4$Cl, 250 pmoles of imidazole-HCl buffer (pH 7.0), and 0.143 mg of bacterial enzyme, specific activity 6.6. Incubation was started at 65 ml and the strong salt elution began at 170 ml (see "Materials and Methods" for details). The pH shifts amounted to about 0.15 to 0.2 pH unit. Unbuffered or lightly buffered solutions (see legend to Table I), were used so that the course of the reactions could be monitored on an expanded scale pH meter. The pH shifts amounted to about 0.15 to 0.2 pH unit. Unbuffered or lightly buffered solutions (see legend to Table I), were used so that the course of the reactions could be monitored on an expanded scale pH meter. The pH shifts amounted to about 0.15 to 0.2 pH unit. Unbuffered or lightly buffered solutions (see legend to Table I), were used so that the course of the reactions could be monitored on an expanded scale pH

**Fig. 2. Dowex column chromatography of the $^{32}$P-enolpyruvate reaction product.** A, effluent absorbance; B, phosphate analyses on effluent fractions after hydrolysis; C, radioactivity counts on effluent fractions (PEP, P-enolpyruvate). The gradient elution was started at 65 ml and the strong salt elution began at 170 ml (see "Materials and Methods" for details).

When adenylate kinase was incorporated in the above incubation mixture, only 4% of the label was then adsorbed on carbon and 2% of the label was liberated upon 7-min hydrolysis.

**Equilibrium Studies**—Equilibrium studies were made with column-purified nucleotides. Either AMP or ATP was used, resulting in equilibrium being approached from the forward or reverse direction, respectively. The other substrates and the initial pH were deliberately poised to produce 40 to 50% conversion of the added nucleotide at equilibrium. These experiments used bacterial enzyme, specific activity 6.6. This enzyme preparation was tested for, and found to be free from, ATPase, adenylate kinase, enolase, and NADH oxidase under conditions which should have revealed these activities if present to the extent of 0.1% that of the dikinase.

When ATP was the added nucleotide its initial concentration was determined by enzymatic assay of an aliquot from the reaction mixture prior to the addition of a known volume of the dikinase. The enzyme was then added and the reaction resulted in a gradual shift to a lower pH. Unbuffered or lightly buffered solutions (see legend to Table I), were used so that the course of the reactions could be monitored on an expanded scale pH

When AMP was the added nucleotide the conditions of incubation were similar, but the pH shifts were toward higher values.
Pyruvate-Phosphate Dikinase

TABLE I

Determination of observed equilibrium constant ($K_{obs}$) for bacterial dikinase

Experiments 1 to 7 had no nonsubstrate buffer; 8 contained 21 mM imidazole-HCl; 9 to 11 contained 23 mM Tris-HCl. Experiments 5, 7, and 8 contained 0.572 mg of enzyme protein and 20 pmoles each of MgCl₂ and NH₄Cl in a final volume of 2.4 ml. The other experiments used 0.286 mg of enzyme protein and 20 pmoles each of MgCl₂ and NH₄Cl in a final volume of 2.2 ml.

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<th>Experiment No.</th>
<th>Added nucleotide</th>
<th>Final pH</th>
<th>Assay, ATP</th>
<th>Calculated</th>
<th>$K_{obs}$</th>
<th>log $K'$</th>
</tr>
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<td>m.M</td>
<td>m.M</td>
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<td>18.8</td>
<td>8.1</td>
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<td>1.2</td>
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Fig. 3. Plot of $K_{obs}$ against pH from the equilibrium data of Table I. The solid line has been given the theoretical slope for the value, $\Delta \log K_{obs} = 2\Delta \text{pH}$. ○, experiments with AMP as the initial substrate; △, ATP.

The ATP concentration was determined by enzymatic assay after equilibrium had been reached.

Calculation of Equilibrium Results—The final concentrations of all substrates except ATP were calculated by correcting their known initial concentrations for the extent of reaction indicated by the difference between the initial and final concentrations of ATP, according to the stoichiometry of Equation 1. For example, with AMP as substrate in Experiment 1 of Table I the initial concentrations (millimolar) were: AMP, 0.82; PP₄, 1.04; P-enolpyruvate, 0.96; ATP, 0.00; P₇, 18.2; pyruvate, 7.41. At equilibrium the concentration of ATP was found to be 0.66 mm. Each of the other substrate concentrations was then corrected by this amount, upward or downward according to Equation 1. An example with ATP as initial substrate is Experiment 9 on Table I. In this case the initial concentrations were: AMP, PP₄, and P-enolpyruvate, 0.00; ATP, 0.62; P₇, 18.2; and pyruvate, 2.94. At equilibrium the ATP concentration had diminished by 0.39 mm. This quantity was subtracted from or added to the known initial concentrations to give the final values listed in the table.

The results of the equilibrium studies are presented in detail in Table I. The value $K_{obs}$ is the observed equilibrium constant based upon Equation 1. It is calculated without regard to the hydrogen ion concentration or to the fractional concentrations of the reactive ionic species. Its relationship to pH is illustrated in Fig. 3 in which the slope of the solid line has been given the theoretical value $\Delta \log K_{obs}/\Delta \text{pH} = 2$. The close approximation of this line by the experimental points indicates that the dikinase reaction involves 2 hydrogen ions. The values for $K'$ were calculated from the relationship $K' = K_{obs}/[H^+]^2$ and the values for log $K'$ are listed in the table.

Potentiometric Titration of PP₄ and P₇ in Presence of MgCl₂—Sodium pyrophosphate, 0.1 mmole, was acidified with HCl and diluted to 15 ml. Magnesium chloride was added to a final concentration of 0.01 M. This solution was titrated with NaOH with a Gilmont microburette and a line-operated pH meter with calibrated glass and calomel electrodes. Beckman pH 7.0 buffer was used to standardize the instrument. The moles of alkali added were plotted against the pH. Inflections at the point of addition of the second and fourth equivalents of alkali were evident. The point corresponding to the consumption of 3.5 moles of NaOH per mole of PP₄ occurred at pH 6.95, Sodium phosphate was similarly titrated and the point corresponding to 1.5 moles of NaOH per mole of P₇ occurred at pH 6.95.

DISCUSSION

The likelihood that the new dikinase plays a role in the glycolytic pathway is strengthened by its being found in two organisms which lack the ordinary pyruvate kinase. That it may also be involved in gluconeogenesis, as suggested by Hatch and Slack (1), is evident from the findings now reported on its equilibrium constant. In the physiological range of pH the

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The text provides a detailed analysis of the equilibrium constants for bacterial dikinase, including the determination of $K_{obs}$ and the calculation of $K'$ based on pH changes. It also includes a discussion on the potential roles of the dikinase in glycolysis and gluconeogenesis.
constant is not so great as to impose a formidable energy barrier to the reverse reaction.

Our studies with \[^{32}\text{P}\] confirm the reported finding by Hatch and Slack (1) that this label becomes incorporated into PP\(_i\) and ATP. The present work shows that this nucleotide is labeled in the \(\gamma\) position by \[^{32}\text{P}\]. It further shows that P-enolpyruvate does not become labeled from \[^{32}\text{P}\] \(\beta\)-acetate, nor does label appear in the form of ADP when enzyme free from adenylate kinase is used.

When \[^{32}\text{P}\]-enolpyruvate was the labeled substrate we obtained \(\beta\)-labeled ATP with no significant amount of label being transferred to the other substrates. These product relationships are consistent with the exchange results reported by Evans and Wood with enzyme from the propionibacteria. Based on the extent of label in the \[^{32}\text{P}\]-enolpyruvate the radiochemical purity of the \[^{32}\text{P}\] labeled ATP was about 80%. The most plausible explanation for this less than theoretical yield is the existence of a labeled impurity in the synthetic \[^{32}\text{P}\] enolpyruvate.

The observed equilibrium constant with bacterial enzyme varied directly with the square of the hydrogen ion concentration. Thus, the \(K_{\text{obs}}\) at pH 7.0 was 1140 while at pH 8.0 it was about 10. The linearity of the observed points of Fig. 3 and the fact that the calculated values for log \(K'\) are essentially constant over the pH range from 6.5 to 8.4 may be dependent on the following circumstances. (a) In this pH range the acidic protons of the substrates pyruvate, MgATP, and P-enolpyruvate are almost completely dissociated. (b) At the magnesium ion concentration used in the equilibrium experiments our potentiometric titrations showed the second ionization constant for P\(_i\) to be equal to the fourth ionization constant for PP\(_i\), (actually, to the second constant for MgPP\(_i\)). Thus, in the investigated pH range four of the substrates are fully ionized and the other two having equal pK values for their reactive species appear one in the numerator and the other in the denominator of the equilibrium equation. The effect of pH changes upon the substrates cancel and the \(K_{\text{obs}}\) values reflect the participation of 2 hydrogen ions in the reaction.

The participation of 2 hydrogen ions in the forward reaction requires that all 4 protons be dissociated from the PP\(_i\). This strongly implicates MgPP\(_i\) as the reactive species. Our findings suggest that the ionic species involved in the reaction are represented by Equation 2.

\[
2\text{H}^+ + \text{AMP}^2^- + \text{MgPP}^0^- + \text{P-enolpyruvate}^0^- \rightleftharpoons \text{MgATP}^2^- + \text{P}^+^- + \text{pyruvate}^4^- \tag{2}
\]

This equation is proposed with reservation regarding the ionic species of P enolpyruvate. The work of Wold and Ballou (10) shows that at the concentrations prevailing in our experiments about 60% of the P-enolpyruvate would be bond to magnesium. Further experimentation at other magnesium ion concentrations will be required to disclose whether the reactive species is the free or the bound P-enolpyruvate.

Hatch and Slack (1) reported an equilibrium constant for the leaf enzyme at pH 8.3. Their value was calculated for the reverse direction of the reaction. Recalculation of their value for the forward direction yields a \(K_{\text{obs}}\) which is three orders of magnitude greater than that of the \(B.\ symbiosus\) enzyme at the same pH. We are unable to offer an explanation for the different \(K_{\text{obs}}\) values found for the leaf and the bacterial enzymes.

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