The Pyruvate-Phosphate Dikinase Reaction

THE FATE OF PHOSPHATE AND THE EQUILIBRIUM*

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SUMMARY

An enzyme capable of catalyzing the reaction

\[ \text{AMP} + \text{PP}_1 + \text{P-enolpyruvate} \rightleftharpoons \text{ATP} + \text{Pi} + \text{pyruvate} \]

was obtained from Bacteroides symbiosus cell extracts and 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 \( \gamma \) position of ATP while the label of \(^{32}\text{P}-\text{enolpyruvate} \) was transferred to the \( \beta \) 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 mM for the amebal enzyme and 0.06 mM and 0.1 mM for the bacterial enzyme, respectively.

An enzyme catalyzing the reaction

\[ \text{AMP} + \text{PP}_1 + \text{P-enolpyruvate} \rightleftharpoons \text{ATP} + \text{Pi} + \text{pyruvate} \] (1)

has been reported from three laboratories(1,2). The name now proposed for this enzyme is ATP:pyruvate, phosphate diphosphotransferase. The trivial name pyruvate-phosphate dikinase serves to relate it to pyruvate kinase which catalyzes a similar glycolytic reaction. Hatch and Slack (1) found the enzyme in the leaves of tropical grasses. Evans and Wood found it in extracts from propionibacteria. Both these groups indicated their belief that the enzyme functioned principally in the reverse 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 insightful 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 \(^{32}\text{P} \)-labeled PP\(_1\) 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 \(^{32}\text{P}-\text{enolpyruvate} \) 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.

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 spectro photometrically in sodium phosphate buffer, pH 7, by their absorbance at 259 nm (A_259 15.4 X 10^4).

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 PP\(_1\) and 0.15 mM in Pi. The PP\(_1\) was assayed by linking the amebal dikinase to the oxidation of
the entering solution was 0.1 N HCl. By this gradient successively removed the remainder of the Pi, eluted in the first 65 ml, after which a nonlinear gradient elution was begun. The reservoir then contained 80 ml of the acid and the effluent was monitored at 260 nm with a flow cell and 5-ml fractions were collected. The AMP and most of the Pi was washed with water. Elution was begun with 0.01 N HCl. 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 \( \mu \)moles of P-enolpyruvate. The radioactivity of these pooled fractions was 575,000 cpm per \( \mu \)mole of P-enolpyruvate.

**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 Pi was eluted in the first 65 ml, after which a nonlinear gradient elution was begun. The reservoir then contained 80 ml of the acid and the entering solution was 0.1 N NaCl in 0.01 N HCl. Elution by this gradient successively removed the remainder of the Pi, the P-enolpyruvate, and the PPi, and finally the ATP appeared. When this occurred the eluting solvent was changed to 0.25 N 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°C and then analyzed for phosphate with the AutoAnalyzer with Technicon Method N-4b, adapted from the Fiske SubbaRow procedure.

**Synthesis of \( ^{32}P \)-enolpyruvate**

One millicurie of carrier-free H\(^{32}P\)O\(_4\) 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 POCI\(_3\) 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°C water bath for 20 min. The brown reaction mixture was stirred with a stout glass rod. It became very viscous. The tube was next chilled and 3 ml of water were added with stirring, followed by centrifugation. The supernatant solution was decanted, 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.

**Preparation of Bacterial Dikinase**

*Bacteroides symbiosus* (ATCC 14940) was grown in 1.5-liter lots in the Trypticase-thiomalate-glucose medium previously described (7). Fresh *B. symbiosus* cells, 95 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°C. Ten milligrams of lysozyme (Nutritional Biochemicals) were added to the suspension at 37°C. After 30 min the suspension was heated in a 50°C water bath for 5 min and allowed to stand overnight at room temperature. The viscous suspension was then centrifuged at 30,000 x 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 25,000 x 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

- Other work has shown that centrifugation may immediately follow the lysozyme treatment at 37°C.
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 229 units of the enzyme, specific activity 0.63.

The enzyme was adsorbed on a column of DEAE-cellulose, 2 × 18 cm, which had been washed with 0.5 ml alkali and water and conditioned with the imidazole-EDTA buffer. After adsorption 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 mM 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.

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 ml alkali and water and conditioned with the imidazole-EDTA buffer. After adsorption 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 mM 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.

![Fig. 1](http://www.jbc.org/content/243/20/5468/F1.large.jpg) **Fig. 1.** The effect of ammonium ion on the amebal and bacterial dikinases. O, amebal enzyme; •, bacterial enzyme. The lactate dehydrogenase had been centrifuged and then dialyzed overnight against 100 volumes of 1 mM EDTA, pH 7. Cuvettes contained 50 mM imidazole-HCl buffer, pH 7.0, 1 mM P-enolpyruvate, 1 mM AMP, 0.625 mM PPi, 1 mM MgCl₂, 0.15 mM NADH, 1.25 enzyme units per ml of lactate dehydrogenase, ammonium chloride as indicated, and enzyme. The inset uses the same data for the calculation of the $K_m$ for ammonium ion of the bacterial enzyme.

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 229 units of the enzyme, specific activity 0.63.

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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 previously conditioned in the imidazole-EDTA buffer. It was eluted with the same buffer and the eluate was collected in 3-ml fractions. The fractions containing enzyme were pooled, yielding a solution containing 16.8 mg of protein and 101 units of the enzyme, specific activity 6.6. This enzyme preparation lost only 10% of its activity during 2 weeks at 4°C. It was used in kinetic studies and in the equilibrium studies.

**Standard Assay Methods for Bacterial Enzyme**

_A Chemical Method_—The incubation mixture contained 50 mM imidazole-HCl (pH 6.8), 1 mM AMP, 1 mM P-enolpyruvate, 0.5 mM PPi, 5 mM MgCl₂, 20 mM NH₄Cl, and enzyme in a volume of 2.0 ml. After a 5-min incubation at 25°C the pyruvate formed was assayed by the method of Reynard et al. (8).

**B. Linked with Lactate Dehydrogenase**—Cuvettes contained the incubation mixture of Method A plus 0.15 mM NADH and 1.25 enzyme units per ml of lactate dehydrogenase. The initial rate of oxidation of the nucleotide was determined by spectrophotometric observations at 340 nm.

Method A was used for enzyme preparations prior to their purification on the DEAE-cellulose column; Method B was regularly used thereafter. With the column purified preparations both methods gave nearly equivalent results.

**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 Reeves (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°C 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 $K_m$ 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 $K_m$ 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 concentration 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 μmoles of glycyl-glycine-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 μg 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 cen-
trifugation. The acid was then neutralized with KHCO₃ 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 am-
nomia to 100 ml of 50% aqueous alcohol. Ten micromoles of
Pi, 8 µmoles of PPi, and 0.4 mole of ADP were added to this
eluate which was then loaded onto a Dowex column and chro-
matographed by the standard technique. Radioactivity ap-
peared in the Pi 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 Pi and PPi peaks, but not
with the P-enolpyruvate peak.

Combination of the three ATP peak fractions yielded 0.55
µmole of ATP with 137,000 cpm per µmole. Ninety-seven
per cent of these counts were adsorbed by carbon. Upon 7-min
hydrolysis 96% of the label was liberated as Pi. A sample of
the ATP was incubated with glucose, MgCl₂, 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 Pi was more highly labeled.
The ATP isolated from the column contained 260,000 cpm per
µmole. Seventy-eight per cent of these counts were liberated
as Pi 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 3²P-enolpyruvate into β Position of
ATP—The incubation mixture contained in a final volume of 4
ml: 2.85 µmoles of 3²P-enolpyruvate (298,000 cpm per µmole),
3.6 µmoles of AMP, 5.7 µmoles of PPi, 50 µmoles each of MgCl₂
and NH₄Cl, 250 µmoles of imidazole HCl buffer (pH 7.0), and
0.143 mg of bacterial enzyme, specific activity 6.6. Incubation
was for 45 min at 37°, after which the solution was directly
applied to a Dowex column. Washing of the column was
continued until material absorbing at 280 nm was absent from
the effluent. The standard elution procedure was then begun.

Some of the results of the assays on the column effluent are
presented in Fig. 2. A trace of label appeared in the Pi peak
and more appeared in the position assigned to P-enolpyruvate.
The amount of P-enolpyruvate remaining at the conclusion of
the reaction was insufficient to allow this peak to be evident in
the phosphate analyses. The ATP peak was heavily labeled.
No label appeared to be attributable to AMP, and little or none
to PPi.

Four fractions comprising the ATP peak were pooled and
concentrated on a Diaflo UM-3 membrane. The nucleotide
was twice washed on the membrane with 15-ml portions of water
and concentrated to a volume of 3.5 ml. This solution contained
1.65 µmoles of ATP having 218,000 cpm per µmole. This label
was 99% adsorbed on carbon and 75% of it was liberated as Pi
following a 7-min hydrolysis. After incubation of a sample of
the ATP with glucose, MgCl₂, and hexokinase, 98% of the label
was adsorbed on carbon and 70% was liberated in the 7 min
hydrolysis. When adenylate kinase was incorporated in the
above incubation mixture, only 4% of the label was then ad-
sorbed 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% con-
version of the added nucleotide at equilibrium. These experi-
ments 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 reac-
tion 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
meter. The pH shifts amounted to about 0.15 to 0.2 pH unit.
About 10 min after constant pH was attained a sample was
withdrawn, placed in a boiling water bath for 2 min, centrifuged,
and assayed enzymatically for ATP. All reactions were incu-
bated at 25° and the times required to reach equilibrium ranged
from 20 to 90 min.

When AMP was the added nucleotide the conditions of incuba-
tion were similar, but the pH shifts were toward higher values.
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 μmoles 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 μmoles each of MgCl₂ and NH₄Cl in a final volume of 2.2 ml.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Added nucleotide</th>
<th>Final pH</th>
<th>Final concentration</th>
<th>Assay, ATP</th>
<th>Pyruvate</th>
<th>AMP</th>
<th>PP₃</th>
<th>P-enolpyruvate</th>
<th>K_{obs}</th>
<th>log K'</th>
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<td>1</td>
<td>AMP, 0.82</td>
<td>6.51</td>
<td>mM</td>
<td>0.66</td>
<td>18.8</td>
<td>8.1</td>
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<td>6.91</td>
<td>mM</td>
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<td>AMP, 0.82</td>
<td>7.00</td>
<td>mM</td>
<td>0.69</td>
<td>19.7</td>
<td>4.6</td>
<td>0.16</td>
<td>0.67</td>
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<td>7.06</td>
<td>mM</td>
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<td>7.18</td>
<td>mM</td>
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<td>18.7</td>
<td>7.9</td>
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<td>mM</td>
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<td>17.1</td>
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<td>0.80</td>
<td>0.50</td>
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<td>mM</td>
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<td>17.0</td>
<td>3.7</td>
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<tr>
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<td>AMP, 0.75</td>
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<td>mM</td>
<td>0.23</td>
<td>17.0</td>
<td>2.5</td>
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<td>mM</td>
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<td>mM</td>
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<td>1.5</td>
<td>1.2</td>
<td>0.28</td>
<td>1.31</td>
<td>0.93</td>
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The ATP concentration was determined by enzymatic assay after equilibrium had been reached.

Calculation of Equilibrium Results—The final concentrations of all substrates except ΔTP 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; PP₃, 1.04; P-enolpyruvate, 0.96; ATP, 0.00; Pₐ, 18.2; pyruvate, 7.41. At equilibrium the concentration of ATP was found to be 0.00 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, PP₃, and P-enolpyruvate, 0.00; ATP, 0.62; P₉, 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 Δlog K_{obs}/ΔpH = 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 PP₃ and Pi 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. The solution was titrated with NaOH with a Gilson microburette and a line-operated pH meter with calomel and glass 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 PP₃ occurred at pH 6.65.

SODIUM PHOSPHATE was similarly titrated and the point corresponding to 1.5 moles of NaOH per mole of Pi occurred at pH 6.65.

**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
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 PPi and ATP. The present work shows that this nucleotide is labeled in the y position by $^{32}\text{Pi}$. It further shows that P-enolpyruvate does not become labeled from $^{32}\text{Pi}$, 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 Wood1 with enzyme from the propionibacteria. Based on the extent of label in the $^{32}\text{P}$-enolpyruvate the radiochemical purity of the $^{32}\text{Pi}$-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 Pi to be equal to the fourth ionization constant for PPi, actually, to the second constant for MgPPi. Thus, in the investigated pH range four of the substrates are fully ionized and the other two having equal pH 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 PPi. This strongly implicates MgPPi as the reactive species. Our findings suggest that the ionic species involved in the reaction are represented by Equation 2.

\[
2\text{H}^+ + \text{AMP}_2^2 + \text{MgPPi}_2^2 + \text{P-enolpyruvate}_3^2 \rightleftharpoons \text{MgATP}_2^2 + \text{Pi}_2^- + \text{pyruvate}^- \tag{2}
\]

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 bound 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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REFERENCES
