Enolase catalyzes the interconversion of d-glyceric acid 2-phosphate and enolpyruvic acid phosphate, and, as a participant of Embden-Meyerhof glycolysis and fermentation, is widely distributed in living cells. The enzyme was first purified by Warburg and Christian (1), who demonstrated its metal activation and made the initial studies of the kinetics and the thermodynamics of the enolase reaction. More recently, Malmström (2-6) has extended that work, with special emphasis on the effect of the activating metals. Previous studies on enolase have employed synthetic dl-glyceric acid 2-phosphate as the substrate, and the interpretation of results has been complicated by this fact. A recent unequivocal synthesis leading to pure d-GA2P (7) makes available both substrates of enolase as the pure natural isomers.

The enolase system is particularly useful for the study of the mechanism of metal activation. The equilibrium is near enough to 1 so that both the forward and the reverse reaction can be studied, the enzyme is activated by at least seven different metals, and the interconversion of the substrates may be followed spectrophotometrically.

This paper describes in detail the factors affecting the ultraviolet absorption characteristics of enolpyruvic acid phosphate so that the assay method can be made more precise. The binding constants for complexes between the substrates and several metal ions were determined, and the apparent equilibrium constants at different metal and hydrogen ion concentrations were related to the metal binding and the pK values of the ionizable groups in the substrates.
EXPERIMENTAL

Materials—The trisodium salt of d-GA2P was synthesized by the method of Ballou and Fischer (7), and PAP was prepared according to the procedure of Baer and Fischer (8). The resulting water-insoluble and light-sensitive silver barium salt was converted to the new, stable, water-soluble cyclohexylammonium salt, as described below. Enolase was prepared as the crystalline mercury salt according to the method of Warburg and Christian (1), starting with brewers' yeast (generously supplied by the Goebel Brewing Company, Oakland, California). The activity of the purified enzyme was comparable with the values in the literature. The dialyzed mercury-free solution was stored in small samples (0.05 to 0.1 ml.) at $-4^\circ$. When used, each sample was diluted with distilled water and the dilute solution was kept on ice during the experiment and stored in the refrigerator. When treated in this way, the activity of the dilute samples remained undiminished for several days.

All the other chemicals used were either commercial samples of highest purity or synthetic compounds, recrystallized before use.

Preparation of Cyclohexylammonium Enolpyruvic Acid Phosphate—1.9 gm. of a twice crystallized sample of silver barium enolpyruvic acid phosphate (8) in a 40 ml. centrifuge tube were suspended in 15 ml. of water, and 1.0 N hydrochloric acid (4.36 ml.), equivalent to the silver ion, was added. The mixture was stirred for 5 minutes, then the silver chloride was centrifuged and the supernatant fluid decanted into a clean 40 ml. centrifuge tube. The silver chloride was washed with 5 ml. of water, and this water was combined with the decanted solution. The barium ions in the solution were then precipitated by the addition of equivalent 1.0 N sulfuric acid (8.75 ml.) and the mixture was again centrifuged. The supernatant fluid was filtered, if necessary, by suction through a Whatman No. 50 paper to remove floating particles of barium sulfate; the filtrate then was brought to pH 8 with cyclohexylamine. This solution was concentrated to dryness in vacuo at a bath temperature of 40$^\circ$, giving a white crystalline residue. The crude cyclohexylammonium salt was redissolved in 15 ml. of warm water (60$^\circ$), and the solution was diluted with acetone to turbidity. After 3 hours at 5$^\circ$, more acetone was added to complete crystallization, and the mixture was left overnight at 5$^\circ$. The crystalline product was collected on a Büchner funnel and washed with acetone. The crystals were dried in air, and then in a vacuum desiccator over phosphorus pentoxide for 1 day. The yield was 1.35 gm. The mother liquor was concentrated to dryness and the residue was redissolved in 5 ml. of warm water and diluted with acetone. After 20 hours at 5$^\circ$, a second crop of 0.5 gm. was obtained. The total yield was 1.85 gm.
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(91 per cent). A sample, recrystallized and dried as above, slowly decomposed between 155–180°.

$C_{21}H_{44}O_{4}P_{2}N_{4} (465)$. Calculated. N 9.01, P 6.65

Found. " 9.24, " 6.48

The biological purity of this compound has been tested by measuring the oxidation of reduced diphosphopyridine nucleotide in the presence of adenosine diphosphate, pyruvic phosphokinase, and lactic dehydrogenase. 1 mole of the enolpyruvic acid phosphate resulted in the oxidation of 0.97 mole of reduced nucleotide. Therefore, it is felt that the silver barium salt (Baer procedure) and the cyclohexylammonium salt are of equal purity, and that the ultraviolet absorption characteristics recorded in this paper (Table III, and Figs. 1, 2, and 3) more nearly approximate those for the pure compound than the values recorded previously (1). The cyclohexylamine salt is preferred because of its solubility and stability and may be used as such in most enzymatic studies, although removal of the amine can be easily accomplished by treatment with the appropriate ion exchange resin.

Apparatus and Methods—The pH of all solutions was determined with a Beckman pH meter or a Leeds and Northrup pH indicator, both with the usual calomel and glass electrodes.

The titration experiments were carried out with the automatic recording apparatus of Neilands and Cannon (9). The gas phase was $N_2$ and the temperature 25°. The compounds to be titrated were converted into the free acids by treatment with Dowex 50 (H+) and titrated at a concentration of 1 μmole per ml. in a solution containing tetra-$n$-propylammonium iodide to give constant ionic strength, and with 0.5 $N$ tetra-$n$-propylammonium hydroxide as the base. The $pK_a'$ values were read directly from the titration curves. The use of the tetraalkylammonium salt and base has been recommended by Smith and Alberty (10) for the determination of binding constants from titration data. They showed that the alkali metals will bind phosphate esters to a considerable extent and thus complicate the study of the binding of other metals, whereas the larger tetraalkylammonium ions do not form complexes significantly with the esters.

The binding constants were estimated according to the method of Smith and Alberty (10, 11), who showed that at constant ionic strength the shift in the apparent dissociation constant of an acid, caused by the addition of metal, is related to the metal concentration and the binding constant for the acid-metal complex in the following way:

$$pK_a' \text{ (no metal)} - pK_a' \text{ (metal)} = \log (1 + D [M])$$

(1)
where $D$ designates the binding constant or stability constant ($D = [AM]/-[A][M]$), $[A]$ the acid concentration, and $[M]$ the metal concentration.

The determinations of the apparent equilibrium constant were based on the ultraviolet absorption of PAP (1). Most of the equilibrium determinations were performed starting with PAP. The optical density at start and at equilibrium was determined with a Beckman DU spectrophotometer, and the apparent equilibrium constant was obtained directly from these values after proper correction for the absorption of the enzyme and buffer. When the equilibrium was approached from GA2P, the extinction coefficient for PAP for each set of conditions was determined.

### RESULTS AND DISCUSSION

**Titration of GA2P and PAP**—The apparent dissociation constants of GA2P and PAP at ionic strength 0.1 and 0.4 are shown in Table I. Since the method is not reliable below pH 2, the dissociation of the primary phosphate group is not included. The free acids were titrated in 5 ml. aliquots of a 1 mM solution, in a medium containing tetra-$n$-propylammonium iodide to constant ionic strength. The binding constants were determined by substituting metal for TPAI to give the same ionic strength, and then determining the shift in the $pK_a$' values caused by the addition of the metal. The binding constants determined from this shift in $pK_a$' according to Equation 1 are given in Table II. Equation 1 is derived on the assumption that only the totally ionized species of the substrates bind metals. This assumption seems valid in the present case, since the addition of metals caused no significant change in the second dissociation of the substrates. The binding constants did not vary significantly over the ionic strength range from 0.1 to 0.4. The data in Table II show that the assumption made in the past, namely that the metal binding to the two substrates is of equal magnitude (4), is incorrect.
Ultraviolet Absorption of Analytically Pure PAP—The results from our initial measurements of the extinction coefficient of PAP are compared with those of Warburg and Christian in Table III. The more detailed study of the effect of pH and magnesium ion concentration on the extinction coefficient is summarized in Figs. 1 to 3.

In Fig. 1 is given the variation of the extinction coefficient in the physio-

TABLE II

Binding Constants for Complexes of GA2P and PAP with Several Metals
Determined from titration data according to Equation 1.

<table>
<thead>
<tr>
<th></th>
<th>$D_{GA2P}'$ (l per mole)</th>
<th>$D_{PAP}'$ (liters per mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>Magnesium</td>
<td>280</td>
<td>180</td>
</tr>
<tr>
<td>Manganese</td>
<td>1225</td>
<td>560</td>
</tr>
<tr>
<td>Zinc</td>
<td>2500</td>
<td>920</td>
</tr>
<tr>
<td>Cadmium</td>
<td>2500</td>
<td>920</td>
</tr>
<tr>
<td>Cobalt</td>
<td>920</td>
<td>350</td>
</tr>
<tr>
<td>Nickel</td>
<td>760</td>
<td>220</td>
</tr>
</tbody>
</table>

TABLE III

Extinction Coefficients ($E \times 10^{-5}$ Liters per Mole per Cm.) of Enolpyruvic Acid Phosphate in 0.05 M Phosphate Buffer

<table>
<thead>
<tr>
<th>Wave length, m$\mu$</th>
<th>Present investigation</th>
<th>From Warburg and Christian (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 6</td>
<td>pH 7</td>
</tr>
<tr>
<td>220</td>
<td>3.43</td>
<td>3.74</td>
</tr>
<tr>
<td>230</td>
<td>2.18</td>
<td>2.91</td>
</tr>
<tr>
<td>240</td>
<td>0.94</td>
<td>1.44</td>
</tr>
<tr>
<td>250</td>
<td>0.24</td>
<td>0.33</td>
</tr>
<tr>
<td>260</td>
<td>0.06</td>
<td>0.06</td>
</tr>
</tbody>
</table>

logical pH range corresponding to the change from $PAPH^{2-}$ to $PAP^{3-}$. Clearly, the extinction coefficient of the protonated form is less than that of the totally ionized form, and, at any pH at which both species are present, the observed extinction coefficient is determined by

$$E_{\text{observed}} = \frac{E_1 + ([H^+] / K_\alpha') E_2}{1 + [H^+] / K_\alpha'}$$

(2)

where $E_1$ and $E_2$ are the extinction coefficients of $PAP^{3-}$ and $PAPH^{2-}$, respectively, and $K_\alpha' = [PAP^{3-}][H^+]/[PAPH^{2-}]$. 

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A value of 1520 was estimated for $E_1$ from Fig. 1, as $E_{\text{observed}}$ at a high pH, and $E_2 = 675$ was calculated from Equation 2, the experimental data obtained in imidazole buffer and the titration data in Table I being used. The theoretical curve calculated from Equation 2 corresponds well with the experimental points for $E_{\text{observed}}$ in imidazole (Fig. 1). This buffer has the advantage that no metals are introduced, and the slight difference in the data for the two buffers (Fig. 1) is probably due to the presence of potassium in the phosphate buffer. Potassium shifts the

![Fig. 1](http://www.jbc.org/)  
*Fig. 1. The effect of pH on the molar extinction coefficient at 340 m$\mu$ ($E_{240}$) of PAP in 0.05 M imidazole buffer (O) and in 0.05 M phosphate buffer (●). The theoretical curve (solid line) was calculated from Equation 2, the value $pK_s'$ (PAP) = 6.35 from Table I being used.*

$pK_a$ of PAP and also decreases the extinction coefficient of PAP$^{2-}$, as do magnesium and other metals as well (Fig. 2). The variations of $E_{\text{observed}}$ with pH in a solution containing constant concentrations of potassium and magnesium ions were determined, and the data obtained for the effect of pH on the kinetics and the equilibrium of the enolase reaction could be corrected for the extinction coefficient variation under the same conditions. Such a standard curve for the conditions for optimal enolase activity is shown in Fig. 3.

*Equilibrium of Enolase Reaction*—In their original work on the isolation of enolase, Warburg and Christian (1) showed that magnesium, manganese, and zinc activate enolase, and more recently (4) iron has been
added to this list. Other metals such as beryllium, calcium, strontium, cadmium, cobalt, and nickel have been reported to be inactive (4) and

![Graph](image1)

**Fig. 2.** The effect of magnesium and potassium on the molar extinction coefficient at 240 mμ (E₂₄₀) of PAP in 0.05 M imidazole buffer at pH 8.35.

![Graph](image2)

**Fig. 3.** A correction curve for the effect of pH on the molar extinction coefficient at 240 mμ (E₂₄₀) of PAP in the standard assay medium: 0.008 M MgSO₄, 0.4 M KCl, and 0.05 M imidazole buffer.

also inhibitory when added to the activated enolase system (4, 12, 13). As has already been pointed out (4), a metal with less activating effect than magnesium will act as an inhibitor when added to the magnesium-
ENZYME ENOLASE. I

activated system. The reports in the literature of metals being inhibitory to the magnesium-activated enolase reaction should, therefore, not be interpreted to mean that these metals cannot activate the enzyme in the absence of magnesium.

In this work a total of 6 divalent metal ions was found to activate enolase, namely magnesium, manganese, zinc, cadmium, cobalt, and nickel. Iron was not tested. The reaction rate in the presence of cobalt and nickel was very low compared to that in the presence of the other metals, but was still significantly higher than the rate in the absence of metals. The relative activation strength of the different metals is discussed in Paper II (14) of this series.

The effects of pH and metal ions on the equilibrium of biochemical reactions have been discussed by several workers (15–19). If a reaction between two acids, AH and BH, is affected by the hydrogen ion concentration or the metal ion concentration, the over-all apparent equilibrium constant is expressed by

$$K_{\text{apparent}} = \frac{[B]_{\text{total}}^{-} + [B]_{\text{total}}^{-} + [BM]}{[A]_{\text{total}}^{-} + [AH] + [AM]}$$

Here the different concentrations can be expressed in terms of the respective ionization constants ($K_a'$) and binding constants ($D'$):

$$[AH] = \frac{[H^+]|[A^-]|}{K_{aA'}} \quad [BH] = \frac{[H^+]|[B^-]|}{K_{aB'}}$$


and substituting these values in Equation 3, one obtains

$$K_{\text{apparent}} = \frac{[B^-]^{-}}{[A^-]} \times \frac{1 + [H^+]|K_{aB'}' + D_B'[M]}{1 + [H^+]|K_{aA'}' + D_A'[M]}$$

The quantity $[B^-]/[A^-]$ is a constant and represents the pH- and metal-independent equilibrium constant $K_{eq}$, and Equation 3 can thus be written:

$$K_{\text{apparent}} = K_{eq} \frac{1 + [H^+]|K_{aB'}' + D_B'[M]}{1 + [H^+]|K_{aA'}' + D_A'[M]}$$

If more than one metal is involved, the more general form of Equation 4 can be used:

$$K_{\text{apparent}} = K_{eq} \frac{1 + [H^+]|K_{aB'}' + D_{B1}[M_1] + D_{B2}[M_2] + \ldots + D_{Bn}[M_n]}{1 + [H^+]|K_{aA'}' + D_{A1}[M_1] + D_{A2}[M_2] + \ldots + D_{An}[M_n]}$$
where \( D_1, D_2, \) and \( D_n \) are the binding constants for the complexes of the substrates \( A \) and \( B \) with the metals \( M_1, M_2, \) and \( M_n. \)

It was found early in this work that the apparent equilibrium constant for the enolase reaction, expressed as \( K_{\text{apparent}} = \frac{[PAP]}{[GA2P]} \), varied with both the metal concentration and pH. These variations are shown in Figs. 4 to 6. In order to obtain the metal- and pH-independent equilibrium constant, the experimental values for \( K_{\text{apparent}} \), and the ionization and binding constants from Tables I and II, were substituted into the proper form of Equation 5. In this way \( K_{\text{eq}} \) was found to be 6.3.

The solid and broken lines in Figs. 4 to 6 represent the theoretical curves calculated from Equation 5, by use of the value 6.3 for \( K_{\text{eq}} \) and the data in Tables I and II for the \( pK_a' \) values and the binding constants. They coincide quite well with the experimental points. All the calculations were based on an uncertainty of 0.05 in the determination of the \( pK_a' \) values. Bock and Alberty (16) have shown the effect of a variation of this magnitude on the calculated equilibrium constants, and it is obvious that the method is sensitive to titration errors. It is felt, however, that Equation 5 adequately describes the enolase equilibrium.
Fig. 5. The variation of the apparent equilibrium constant for the enolase reaction with pH at two magnesium concentrations (●, 0.001 M MgSO₄; ○, 0.01 M MgSO₄) in 0.05 M imidazole buffer. The corresponding theoretical curves were calculated from Equation 5 using the following data from Tables I and II: pKₐ' (GA₂P) = 7.0, pKₐ' (PAP) = 6.35, Dₐₕₐₐ' (GA₂P) = 280, Dₐₕₐₐ' (PAP) = 180, and led to the value 6.1 for $K_{eq}$. No attempt was made to correct for the change in ionic strength.

Fig. 6. The variation of the apparent equilibrium constant for the enolase reaction with pH at three manganese concentrations (●, 0.001 M MnSO₄; ○, 0.002 M MnSO₄; ⬤, 0.005 M MnSO₄) in 0.05 M imidazole buffer. The corresponding theoretical curves were calculated from Equation 5 using the same pKₐ' values as in Fig. 5 and the binding constants $D_{Mn}$(GA₂P) = 1225 and $D_{Mn}$(PAP) = 560 from Table II, and led to the value 6.3 for $K_{eq}$. No attempt was made to correct for the change in ionic strength.
The equilibrium constants for the enolase reaction reported in the literature vary between 1.4 and 3.9 (1, 20–23), but are all apparent constants, dependent on metal concentration and pH. The higher value for the metal- and pH-independent equilibrium constant determined in this work is, therefore, not in conflict with the older data.

The graphical picture, obtained for different concentrations of magnesium and manganese (Figs. 5 and 6), was also obtained for zinc, cadmium, cobalt, and nickel, and is also the same as Trevelyan et al. (17) found for phosphorylase. It is interesting to note that for each metal there is a pH at which the apparent equilibrium constant is independent of the metal concentration. Utter and Werkman (12) showed that the over-all equilibrium between GA3P and PAP, involving both glyceric acid phosphate mutase and enolase, is independent of the metal concentration. If their study were conducted at, or close to, the pH of the isosbestic point, this could account for their observation. Another possibility is that the metal binding to GA3P and PAP is equal, which in itself has interesting thermodynamic implications.

With the new equilibrium constant, $K_{eq} = 6.3$, $\Delta F_{298}$ for the enolase reaction was found to be $-1090$ calorie per mole, and from the thermal data in Table IV the average value of 3500 calories per mole was obtained for $\Delta H_{298}$. It should be noted that the latter value is not independent of the metal concentration and pH.

### Table IV

**Effect of Temperature on Apparent Equilibrium Constant for Enolase Reaction**

Determined in 0.05 M imidazole buffer containing 0.4 M potassium chloride and 0.008 M magnesium sulfate at pH 7.5.

<table>
<thead>
<tr>
<th>°K</th>
<th>$K_{apparent}$</th>
<th>$\Delta H$, calories per mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>288.0</td>
<td>3.54</td>
<td>3220</td>
</tr>
<tr>
<td>299.5</td>
<td>4.55</td>
<td>3700</td>
</tr>
<tr>
<td>307.5</td>
<td>5.28</td>
<td></td>
</tr>
</tbody>
</table>

**SUMMARY**

The preparation of the cyclohexylammonium salt of enolpyruvic acid phosphate has been described, and some of its properties as a substrate for enolase have been discussed.

From the study of the equilibrium of the enolase reaction it has been shown that both metal ions and hydrogen ions are involved in the over-all stoichiometry of the interconversion of D-glyceric acid 2-phosphate and
enolpyruvic acid phosphate. A mathematical expression for the theoretical variations of the apparent equilibrium constant with pH and metal concentration has been derived, and from the determination of the pH- and metal-independent equilibrium constant has been evaluated and found to be 6.3.

A total of 6 divalent metal ions, magnesium, manganese, zinc, cadmium, cobalt, and nickel, was found to activate enolase, and the effect of all of these metals on the equilibrium of the enolase reaction has been determined.

The new value of the equilibrium constant and studies of the effect of temperature on the equilibrium have yielded the following thermodynamic constants: \( \Delta F_{298} = -1000 \) calorie per mole and \( \Delta H_{298} = 3500 \) calories per mole.

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Finn Wold and Clinton E. Ballou


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