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# PROPERTIES OF ENOLASE IN EXTRACTS FROM PEA SEED[1,2]

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Enolase catalyzes the conversion of D-2-phosphoglyceric acid (2-PGA) to phosphoenolpyruvic acid (PEPA). The enzyme was first detected in muscle extract by Lohmann and Meyerhof (5), and later a crystalline mercury derivative was obtained by Warburg and Christian (14). The mercury compound was inactive as an enzyme but active enolase was obtained from the crystalline material by removal of the mercuric ion followed by the addition of $Mg^{++}$, $Mn^{++}$, or $Zn^{++}$ (14). In the presence of phosphate, enolase from yeast (14), *Escherichia coli* (13) or animal tissues (15) is strongly inhibited by fluoride. It is believed that the normal activator of the enolase system is $Mg^{++}$, since the complex formed by magnesium, phosphate, and fluoride is only slightly dissociated.

Bonner and Wildman (1) inhibited respiration in spinach brei more than 90 % by the addition of 1 mg of NaF per ml. This inhibition was completely reversed by the addition of 2.5 mg of sodium pyruvate per ml, indicating the presence of the enolase system in spinach leaf brei. Stumpf (10) and Tewfik and Stumpf (11) provided further evidence for the presence of enolase in higher plants. The breakdown of PGA to acetaldehyde by pea extracts required $Mg^{++}$ for activation and was sensitive to fluoride, indicating the involvement of the glycolytic enzymes known to catalyze these reactions.

The available literature on the enolase system in higher plants is limited, and properties of this system have not been elucidated. It was deemed of value to investigate and characterize the properties of this system in higher plants.

## MATERIALS AND METHODS

PREPARATION OF EXTRACTS: The enzyme extract used in these experiments was prepared from an acetone powder of *Pisum sativum* (Progress No. 9) seed. The seed was soaked in distilled water for five hours at room temperature; then removed from the water and homogenized for three minutes in acetone at −10° C by use of an Omni mixer (Ivan Sorvall, Inc., Norwalk, Conn.). The acetone powder was prepared as previously described by Evans (2). For prepa-

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ration of the extract 1 g of acetone powder was homogenized in 40 ml of 0.05 M Tris (hydroxymethyl-aminomethane-hydrochloride) buffer at pH 7.4 and the mixture centrifuged at 25,000 × G for 10 minutes. The crude extract contained high enolase activity, but phosphoglyceric mutase and pyruvate kinase activity were also present. The extract was purified by $(NH_4)_2SO_4$ fractionation. Sufficient $(NH_4)_2SO_4$ was added to the supernatant to obtain a 30 % saturated solution. After centrifugation for 10 minutes at 25,000 × G, $(NH_4)_2SO_4$ was added to the supernatant until the solution was 55 % saturated. The $(NH_4)_2SO_4$ extract was centrifuged at 25,000 × G for 10 minutes and the precipitate taken up in 10 ml of TRIS buffer at pH 7.4. This extract was dialyzed for 24 hours against 4 liters of cold 0.01 M TRIS buffer at pH 7.4 and used as a source of enolase activity. High enolase activity was found in the purified extract but phosphoglyceric acid mutase, pyruvate kinase, 2-PGA phosphatase, and PEP-carboxykinase activity was not present in the standard reaction mixture. Storage of the extracts for one week at 0° C resulted in 60 % loss of enolase activity. The extracts prepared by the described procedure contained 5 to 6 mg protein per ml as determined by Folin's phenol reagent (6). The $(NH_4)_2SO_4$ fractionated extract was a 7-fold purification over the crude extract.

DL-2-PHOSPHOGLYCERIC ACID: Phosphoglyceric acid was synthesized from β-glycerophosphate (Eastman Chemicals, Rochester, N. Y.) according to the method of Kiessling (4). The product was purified according to procedure outlined by Warburg and Christian (14). After purification less than $10^{-4}$ M free phosphate was present. Heavy metals were removed with a solution of dithizone in $CCl_4$ as described by Malmström (7). The concentration of 2-PGA was determined by hydrolysis of the phosphate group (12), and total phosphorus was determined by the method of Fiske and SubbaRow (3).

STANDARD ASSAY PROCEDURE: Enzyme activity was measured by following the change in optical density of the test solution at 240 mμ as described by Warburg and Christian (14). Five hundredth ml of enzyme solution was added to 1 ml of substrate solution and the O.D. determined every 30 seconds over the desired time range. Since the rate of reaction remained linear for at least 10 minutes under the specified conditions, enzyme activity was calculated as O.D. change in the initial minute at 240 mμ.

The reaction mixture contained the following constituents, expressed as micromoles per ml: 50 TRIS buffer at pH 8.0, 5 2-PGA, 1.0 $MgSO_4$, 30 glycine, and the enzyme extract usually containing 0.1 mg protein. Glycine was included to bind traces of copper (14). The various experiments were replicated three times and the results averaged. Error between determinations was less than 10 %.

A Beckman DU spectrophotometer was used with 1-cm silica cells. The housing of the absorption cells was far enough from the hydrogen lamp to prevent heating. Since the absorption of PEPA changes rapidly with wave length (7) the spectral purity must be high. The spectral band width obtained with the Beckman DU was about 17 Å at 240 mμ. Serial dilutions of PEPA gave a straight line when the optical density was plotted as a function of the concentration, showing that the band width of the Beckman DU was sufficiently small.

## RESULTS

NATURE OF THE SYSTEM: As shown in figure 1, a concentration of $5 \times 10^{-3}$ M 2-PGA was necessary to saturate the enzyme. The Michaelis constant ($K_M$) for 2-PGA estimated from the saturation curve is $2.5 \times 10^{-4}$ M. Conversion of 3-PGA to PEPA occurred with an undialyzed extract, but the rate of reaction was less than 10 % of that observed utilizing 2-PGA as the substrate. In the presence of a dialyzed extract 3-PGA was completely ineffective as a substrate for the reaction.

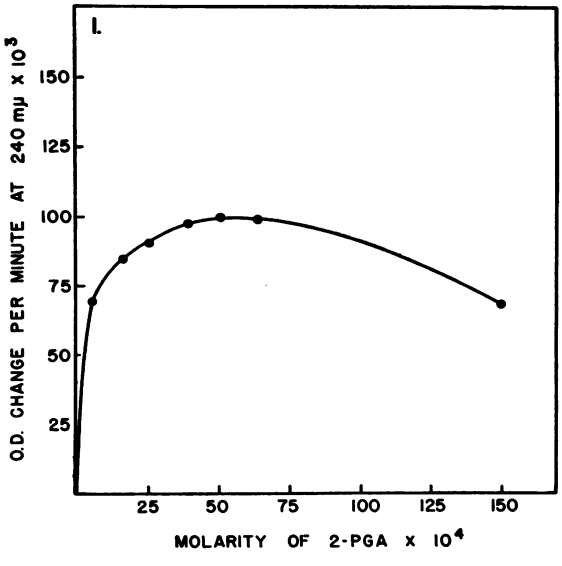
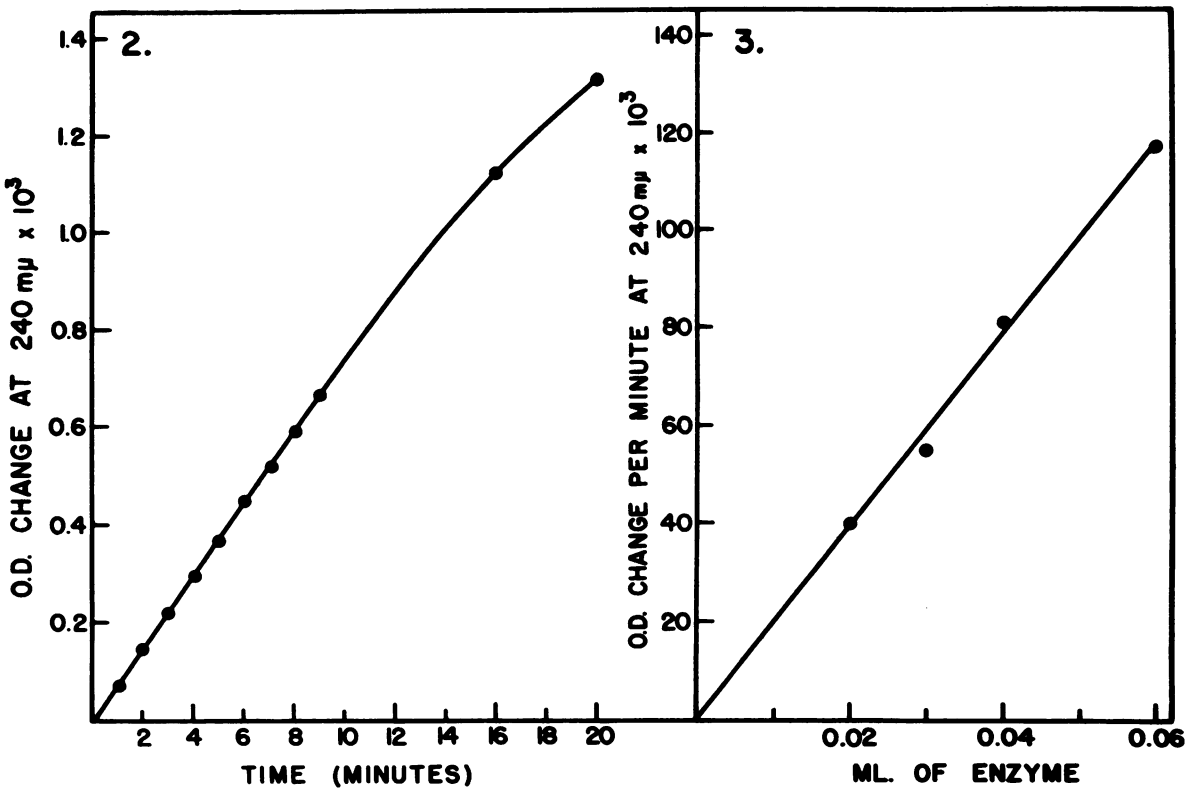


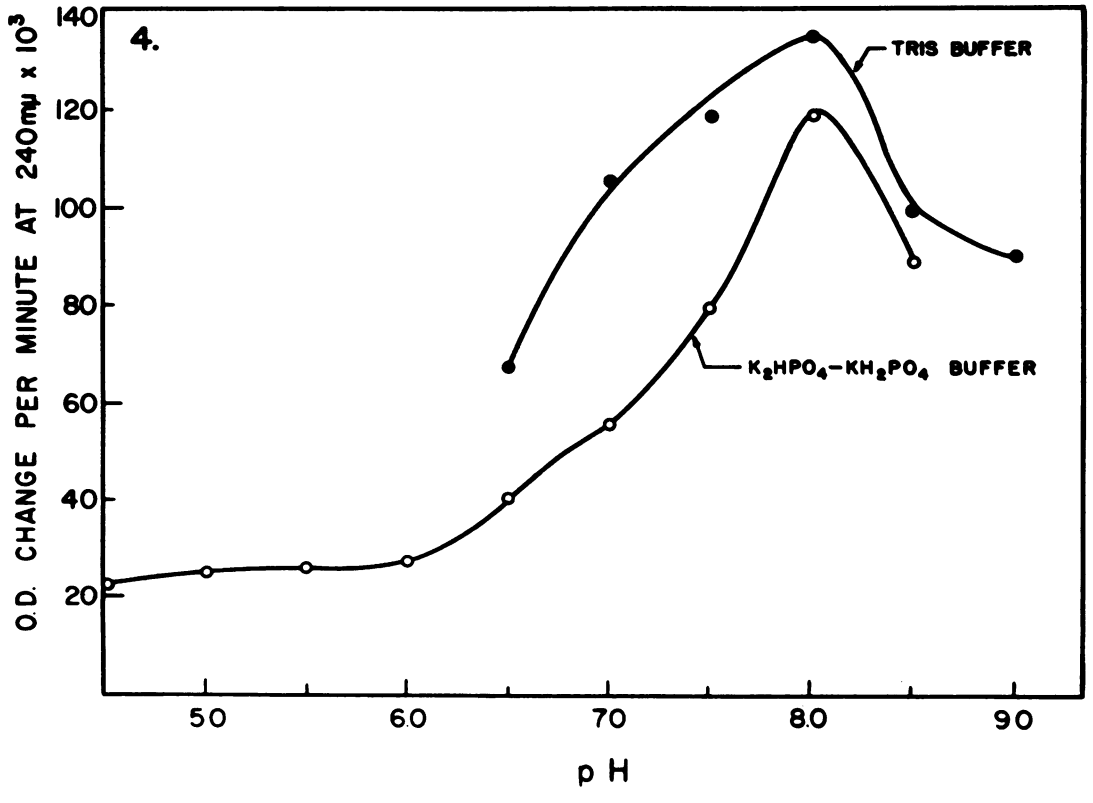

FIG. 1. The rate of enzyme reaction as related to concentration of 2-PGA. The standard assay procedure was used with variation in concentration of 2-PGA as indicated. The extract from pea seed added to each reaction mixture contained 0.13 mg protein.

FIG. 2. Proportionality of enzyme activity with time. The standard assay procedure was used with variation in time as indicated. The enzyme extract added to each reaction mixture contained 0.09 mg protein.

FIG. 3. Proportionality of enzyme activity with enzyme concentration. The standard assay procedure was used with variation in quantity of extract added as indicated. The extract contained 1.0 mg protein per ml.

FIG. 4. Enolase activity as related to pH. The standard assay procedure was used with variation in the pH value as indicated. The enzyme extract added to the reaction mixture contained 0.14 mg protein.



Under conditions of the standard assay the rate of PEPA formation remained essentially linear for the initial 10 minutes as shown in figure 2. Approximately 10 % of the total substrate was hydrolyzed after 10 minutes.

Figure 3 provides evidence that the rate of the reaction was proportional to enzyme concentration. Low amounts of enzyme (0.1 mg protein) were used in the assay medium. PEPA accumulated during the course of the reaction and was not broken down by other enzymes present in the dialyzed extract.

The effect of pH on the activity of enolase from pea seed was studied within the range of 4.5 to 9.0 using phosphate and TRIS buffers. Maximum activity as shown in figure 4 was observed with both buffers at pH 8.0. A sharp decrease in activity occurred at pH values higher or lower than the optimum. TRIS buffer resulted in greater enzyme activity than phosphate buffer throughout the pH range studied.

Effect of Metal Ions on Enzyme Activity: The effect of various divalent cation chlorides on enolase activity is illustrated by the curves in figure 5. In these experiments the standard assay procedure was used with the exception of the variation in divalent cation salt as described. It is evident that $Mn^{++}$ and possibly $Co^{++}$ were more effective in activating enolase at lower concentrations than $Mg^{++}$. Warburg and Christian (14) found $Mn^{++}$ and $Zn^{++}$ to be more effective at low concentrations than $Mg^{++}$ in activation of enolase from yeast. Maximum activity was obtained with $Mg^{++}$, $Mn^{++}$, $Co^{++}$, and $Zn^{++}$ at $10^{-3}$ M, $10^{-4}$ M, $4 \times 10^{-4}$ M and $3 \times 10^{-5}$ M, respectively. The maximum activities measured with the same concentrations of enzyme (maximum activity in the presence of $Mg^{++}$ arbitrarily taken as 1) for $Mg^{++}$, $Mn^{++}$, $Co^{++}$, $Zn^{++}$ and $Ca^{++}$, were 1.00, 0.41, 0.30, 0.07, and 0.00 respectively. In the absence of added salts enzyme activity was approximately 15 % of the maximum attainable with an optimum concentration of $MgCl_2$. Dialysis of the extract for longer periods of time resulted in enzyme activity less than 5 % of the maximum attainable, but complete dependence on salt was not shown. The half saturation concentration for $Mg^{++}$ calculated from figure 5 was $2 \times 10^{-4}$ M.

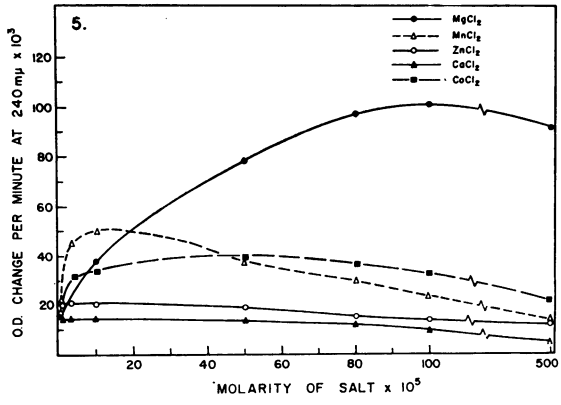



Fig. 5. The activation of enolase by various concentrations of divalent cation salts. The standard assay procedure was used with variation in type and concentration of divalent cation salts as indicated. The enzyme extract added to each reaction mixture contained 0.10 mg protein.

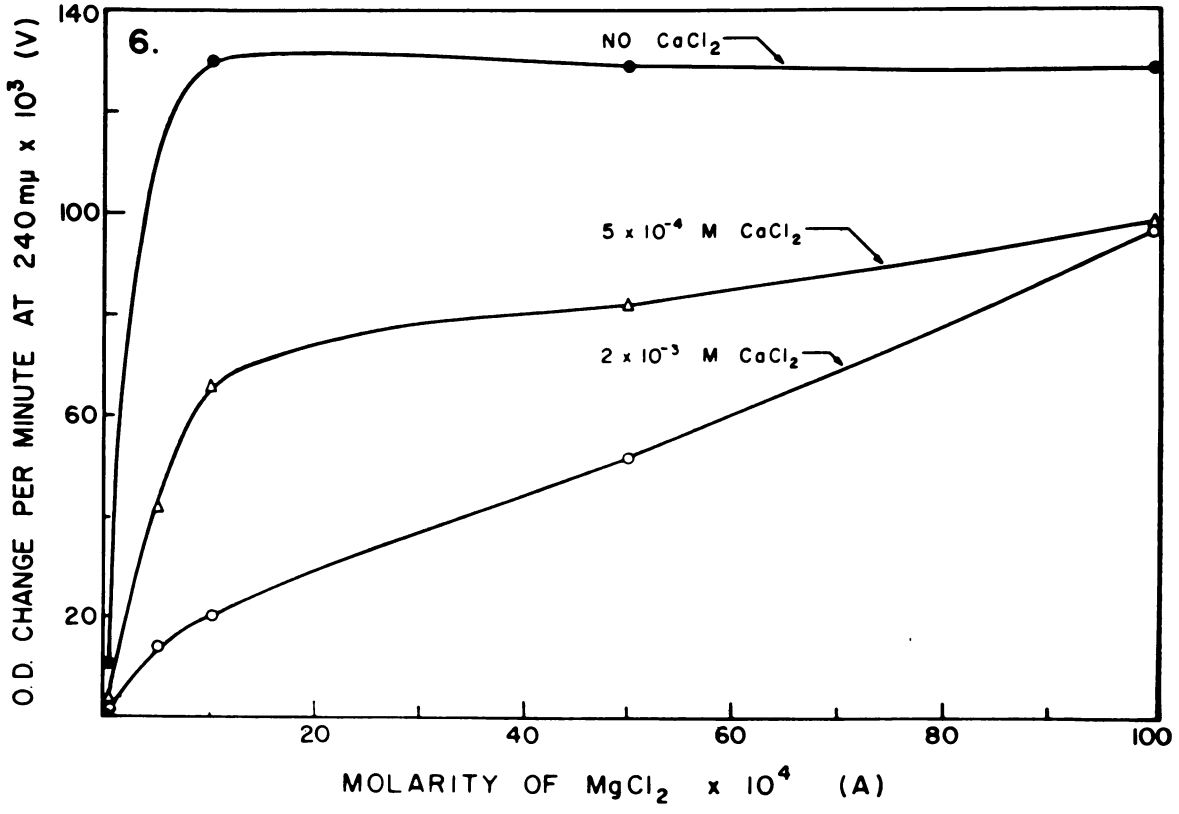
$Ca^{++}$, $K^{+}$, and $Na^{+}$ were completely ineffective as activators for the enolase system. $Ca^{++}$ inhibited the reaction as shown in figure 6. With $2 \times 10^{-3}$ M and $5 \times 10^{-4}$ M $Ca^{++}$, 85 and 50 % inhibition, respectively, was exhibited at $10^{-3}$ M $MgCl_2$. Increasing the $Mg^{++}$ concentration decreased the inhibition manifested by $Ca^{++}$ until the inhibition was almost completely reversed. The curves in figure 7 plotted by the method of Lineweaver and Burk obtained from the data of figure 6 indicate that $Ca^{++}$ is a competitive inhibitor of the enolase system.

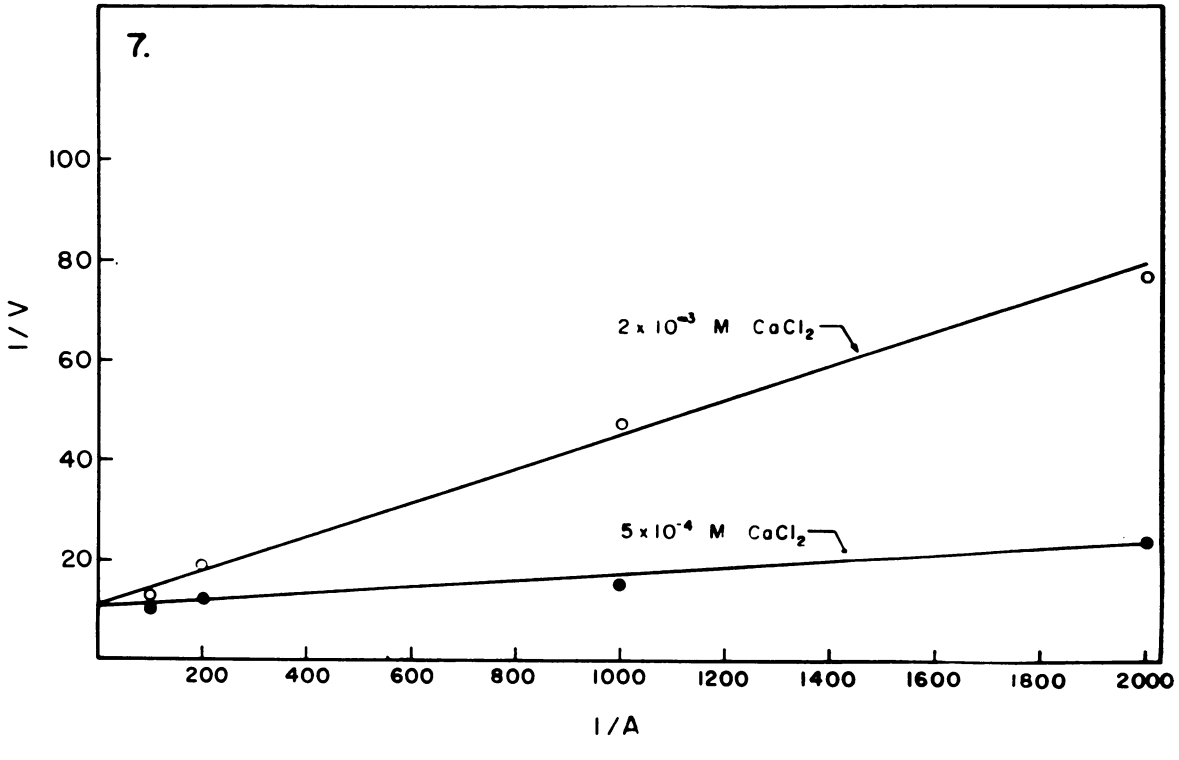
Fluoride Inhibition: The curves in figure 8 show the effect of various fluoride concentrations on enolase activity. The standard assay procedure was used with addition of fluoride as indicated. It was necessary to incubate fluoride with the reaction mixture for one minute before full inhibition developed. Enzyme activity was calculated from the second minute since the activity remained linear for several minutes. The reaction mixture contained less than $10^{-4}$ M endogenous inorganic phosphate. At this phosphate concentration 10 % inhibition was manifested at $5 \times 10^{-3}$ M fluoride and 41 % at $10^{-2}$ M fluoride. Addition of phosphate to the reaction mixtures resulted in a marked increase in inhibition at all fluoride concentrations. At $10^{-3}$ M phosphate 79 and 95 % inhibition was manifested at $5 \times 10^{-3}$ M and $10^{-2}$ M fluoride, respectively; whereas when the reaction mixture contained $5 \times 10^{-3}$ M phosphate, 95 and 100 % inhibition were obtained at the aforementioned fluoride concentrations. Low concentrations of fluoride (5 to 10 ppm) inhibited enolase activity markedly in the presence of $5 \times 10^{-3}$ M phosphate. Addition of phosphate resulted in inhibition of enolase activity in the absence of fluoride. This would be expected since phosphate reduces the effective concentration of $Mg^{++}$ as a cofactor.

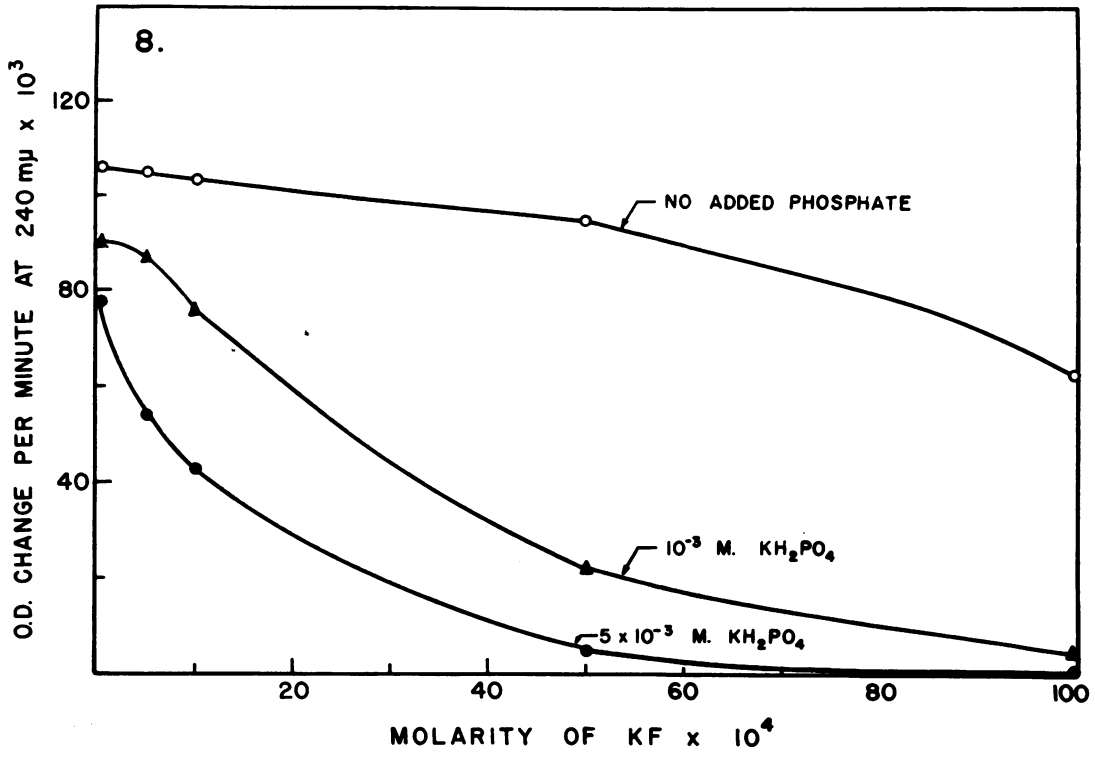
Inhibition of enzyme activity by fluoride is dependent on the concentration of $Mg^{++}$, but increasing the $Mg^{++}$ concentration at fixed levels of fluoride and phosphate does not, as one would suspect, decrease the inhibition. As shown in figure 9 increasing the $Mg^{++}$ concentration with fixed levels of fluoride and phosphate increases the inhibition. At higher $Mg^{++}$ concentrations less fluoride is required to bring about

Fig. 6. The inhibition of enzyme activity by $CaCl_2$ at various concentrations of $MgCl_2$. The standard assay procedure was used with variations in concentration of $MgCl_2$ and $CaCl_2$ as indicated. The extract from pea seed added to each reaction mixture contained 0.12 mg protein.

Fig. 7. The data of figure 6 plotted by the method of Lineweaver and Burk.





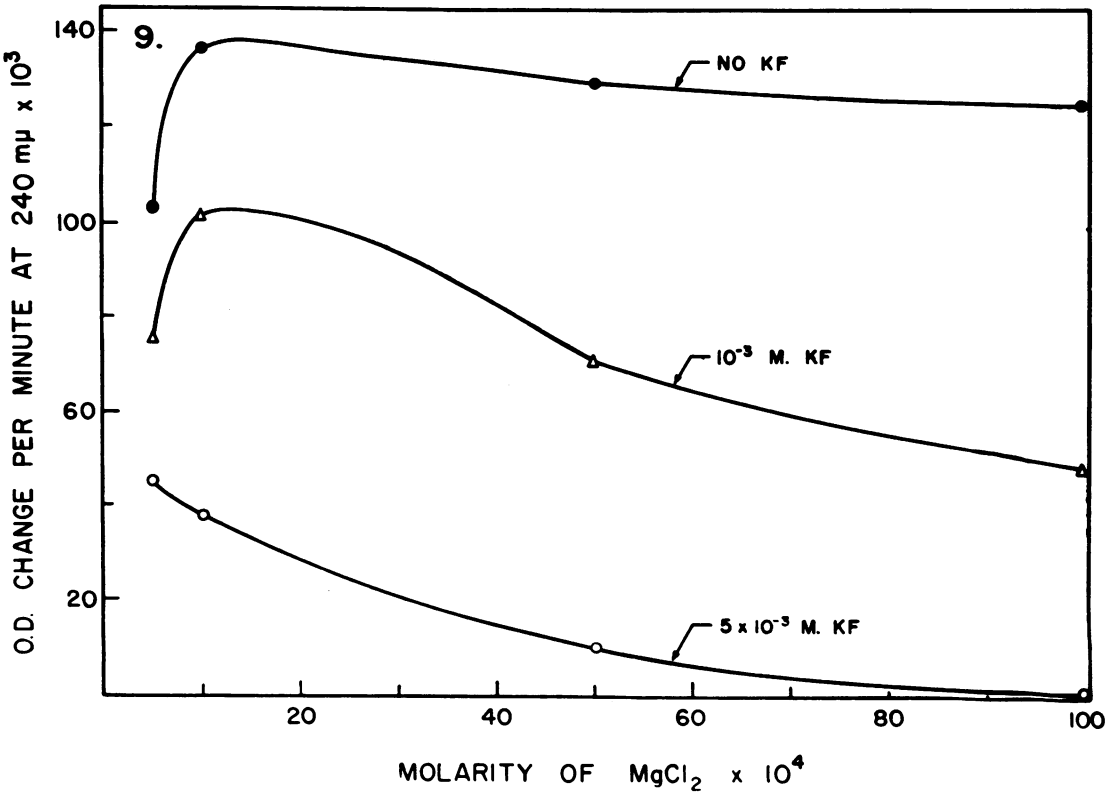



TABLE I

CALCULATION OF CONSTANT (k) = $C_{Mg} \cdot C_{PO_4} \cdot C^2_F \cdot \frac{\text{RESIDUAL ACTIVITY}}{\text{INHIBITED ACTIVITY}}$

| VARIATION | $C_{Mg}$ (M) | $C_{PO_4}$ (M) | $C_F$ (M) | INHIBITED ACTIVITY % | RESIDUAL ACTIVITY % | k ($M^4$) |
|---|---|---|---|---|---|---|
| Phosphate | $10^{-3}$ | $5 \times 10^{-3}$ | $5 \times 10^{-3}$ | 94 | 6 | $8 \times 10^{-12}$ |
| | $10^{-3}$ | $10^{-3}$ | $5 \times 10^{-3}$ | 70 | 30 | $11 \times 10^{-12}$ |
| Fluoride | $10^{-3}$ | $10^{-3}$ | $5 \times 10^{-4}$ | 5 | 95 | $5 \times 10^{-12}$ |
| | $10^{-3}$ | $10^{-3}$ | $10^{-3}$ | 17 | 83 | $5 \times 10^{-12}$ |
| | $10^{-3}$ | $10^{-3}$ | $10^{-2}$ | 94 | 6 | $6 \times 10^{-12}$ |
| Fluoride | $10^{-3}$ | $5 \times 10^{-3}$ | $5 \times 10^{-4}$ | 31 | 69 | $3 \times 10^{-12}$ |
| | $10^{-3}$ | $10^{-3}$ | $10^{-3}$ | 45 | 55 | $6 \times 10^{-12}$ |
| Magnesium | $5 \times 10^{-4}$ | $10^{-3}$ | $10^{-3}$ | 25 | 75 | $2 \times 10^{-12}$ |
| | $10^{-3}$ | $10^{-3}$ | $10^{-3}$ | 26 | 74 | $3 \times 10^{-12}$ |
| | $5 \times 10^{-3}$ | $10^{-3}$ | $10^{-3}$ | 45 | 55 | $6 \times 10^{-12}$ |
| | $10^{-2}$ | $10^{-3}$ | $10^{-3}$ | 54 | 46 | $8 \times 10^{-12}$ |
| Magnesium | $5 \times 10^{-4}$ | $10^{-3}$ | $5 \times 10^{-3}$ | 56 | 44 | $10 \times 10^{-12}$ |
| | $10^{-3}$ | $10^{-3}$ | $5 \times 10^{-3}$ | 72 | 28 | $10 \times 10^{-12}$ |
| | $5 \times 10^{-3}$ | $10^{-3}$ | $5 \times 10^{-3}$ | 92 | 8 | $11 \times 10^{-12}$ |
| | | | | | | Average = $7 \times 10^{-12}$ |

* Data of figures 8 and 9 used to calculate constant (k).

a certain level of inhibition than at lower $Mg^{++}$ concentrations. In the absence of fluoride maximum activity is observed at $10^{-3}$ M $Mg^{++}$ but in the presence of $5 \times 10^{-3}$ M fluoride, maximum activity is found at a lower $Mg^{++}$ concentration.

Warburg and Christian (14) observed with crystalline enolase from yeast that fluoride inhibition resulted from the formation of a magnesium-fluorophosphate complex. In the range of fluoride concentrations from $10^{-2}$ to $10^{-4}$ M the inhibition was correlated with the following relationship: $C_{Mg} \cdot C_{PO_4} \cdot C^2_F \cdot \frac{\text{Residual activity}}{\text{Inhibited activity}} = 3.2 \times 10^{-12}$ $M^4$, where C is the concentration of the indicated ion. Calculations of the data from figures 8 and 9 using this relationship are shown in table I. The calculated constant varied from $2 \times 10^{-12}$ to $11 \times 10^{-12}$ and averaged $7 \times 10^{-12}$. Through the use of this constant and the data represented by the upper curve in figure 8, the phosphate concentration in the medium with no added phosphate was calculated. The value calculated, $4 \times 10^{-5}$ M, agrees closely with the value determined experimentally (3).

A survey of several plant species showed enolase activity to be present in extracts from the leaves of *Pisum sativum*, seed of *Gossypium barbadense*, leaves of *Chenopodium murale*, leaves of *Nicotiana tabacum* and seeds of *Avena sativa*.

## DISCUSSION

The properties of enolase present in an acetone powder of pea seed are similar to those of the crystalline enzyme from yeast. The observed differences are perhaps in part due to the degree of enzyme purification. Divalent cations were activators of the enolase system from higher plants. Magnesium, $Mn^{++}$, $Co^{++}$, and, to a slight extent, $Zn^{++}$ activated this system in the ratio of 1.00 : 0.41 : 0.30 : 0.07, respectively. This compares to the activating capacity of $Mg^{++}$, $Mn^{++}$ and $Zn^{++}$ on crystalline enolase of 1 : 0.425 : 0.419, respectively (7). The effect of $Co^{++}$ on enolase from other sources has apparently not been determined. Maximum activity was obtained with $Mg^{++}$ at $10^{-3}$ M and with the other divalent cations at lower concentrations. The residual activity observed in the absence of added salts was probably due to traces of $Mg^{++}$ present in the reaction mixture. Less than $10^{-5}$ M $Mg^{++}$ would have accounted for the residual activity. In the presence of $Ca^{++}$ the residual activity became essentially zero, indicating the presence of $Mg^{++}$. Addition of a concentration of divalent cations greater than the amount needed for saturation resulted in a decrease in activity. That this is not an ionic phenomenon is indicated by the differences in concentration of the various cations required for above saturation of the enzyme. It has been suggested (7, 8, 9) that the inhibition at high activator concentrations is due to the electro-static

FIG. 8. The inhibition of enolase activity by fluoride at various phosphate concentrations. The standard assay procedure was used with variations in the concentration of KF and $KH_2PO_4$ as indicated. The extract added to each reaction mixture contained 0.08 mg protein.

FIG. 9. Enolase activity as related to $MgCl_2$ and KF concentrations. The standard assay procedure was used with the addition of $10^{-3}$ M phosphate and variations in the concentration of KF and $MgCl_2$ as indicated. The extract added to each reaction mixture contained 0.11 mg protein.

effect of activating ions bound to nonspecific sites. Malmström suggested that the function of magnesium in the enzymatic catalysis is mainly twofold: (a) to furnish one of at least two points of interaction between enzyme and substrate; and (b) to modify the structure of the active site on the enzyme surface. The $K_A$ value calculated for $Mg^{++}$ was $2 \times 10^{-4}$ M. Warburg and Christian (14) observed saturation of enolase with $Mg^{++}$ at $2.8 \times 10^{-3}$ M. Malmström showed $K_A$ values increased with an increase in 2-PGA, phosphate concentration, or pH (9). He observed a $K_A$ value of $2.9 \times 10^{-4}$ M for $Mg^{++}$ in TRIS buffer at pH 7.9.

Fluoride inhibition of the enolase system from pea was found to be dependent on the magnesium, phosphate, and fluoride concentrations. Marked inhibition was manifested at 5 to 10 ppm fluoride in the presence of relatively high concentrations of magnesium and phosphate. The constant calculated according to Warburg and Christian (14), $k = C_{Mg} \cdot C_{PO_4} \cdot C^2_F \cdot \frac{\text{Residual activity}}{\text{Inhibited activity}}$ was found to be about 3 $\times 10^{-12}$ $M^4$. The constant (k) from the data in our experiments was $7 \times 10^{-12}$ $M^4$. Greater purification of the enzyme from pea seed would be necessary to determine the absolute value of k. $Mg^{++}$ serves as an activator for the enolase system and the ion attached to some specific site on the enzyme protein activates the system (14). It seems likely that the magnesium-fluorophosphate complex occupies this same site thus inactivating the enzyme. The inhibition can be reversed by removal of the complex groups through dialysis and addition of $Mg^{++}$ to the system.

There have been many reports of injury to plants that have been exposed to fluoride in the atmosphere or in the soil (15). This injury is manifested by susceptible plant species such as *Chenopodium murale* at concentrations of atmospheric fluoride as low as 0.4 parts per billion. Plant injury may be related to the observed fluoride inhibition of the enolase system. It would be logical to expect that blocking the enolase reaction in plants not containing an alternate pathway would affect metabolism in general. Plants are now being grown in fluoride fumigation chambers and a comparison of the enolase activity between normal and fumigated plants is being made. The results of these observations will be reported later.

## Summary

Experiments were conducted to determine the properties of enolase from higher plants. The concentration of 2-PGA required for saturation of the enzyme from a dialyzed extract of pea seed was determined. After the cation requirements were established evidence was obtained to show that enzyme activity was proportional to both enzyme concentration and reaction time under the specified conditions.

Detailed studies of the system demonstrated a cation requirement that was satisfied by $Mg^{++}$ at $10^{-3}$ M. $Mn^{++}$, $Co^{++}$ and $Zn^{++}$ activated the enzyme reaction, but were much less effective than $Mg^{++}$. $Ca^{++}$ was found to be a competitive inhibitor, reversal of the inhibition being achieved by increasing the $Mg^{++}$ concentration.

Fluoride inhibition of the enolase system in pea seed extracts was dependent on the fluoride, phosphate and magnesium concentration. Using the relationship of Warburg and Christian (14) concerning fluoride inhibition, a constant (k) was calculated and was similar to that observed for crystalline enolase.

Personal communication with Dr. M. D. Thomas, Stanford Research Institute revealed that he and Dr. S. L. Chen, Red Star Yeast Co., had completed experiments on the properties of purified enolase from barley in 1954. This work is now in the process of being submitted for publication.

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