# Studies on Two High-Affinity Enolase Inhibitors. Reaction with Enolases*

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ABSTRACT: Enolases (from rabbit muscle, salmon muscle, yeast, and *Escherichia coli*) are strongly inhibited by two substrate analogs, D-tartronate semialdehyde phosphate (TSP) and 3-aminoenolpyruvate phosphate (AEP). With both inhibitors, the enolases form stable complexes with characteristic ultraviolet (uv) difference spectra (TSP–enolase: maximum at 285 nm; AEP–enolase: maximum at 295 nm). The difference spectra are sufficiently intense (molar extinction coefficients about $10^4$ $M^{-1}$ $cm^{-1}$) to allow direct spectrophotometric titration of the active sites in the enzymes. The titration of all four enolases demonstrates that there are two equivalent binding sites per mole of enzyme. The spectrophotometrically determined dissociation constants for the EI complexes are in good agreement with the catalytically determined $K_I$ values (about $10^{-5}$ M for TSP and about $10^{-7}$ M for AEP). These results together with the published data on the subunit structure of enolases establish that enolase activity in widely different biological species is uniformly expressed by enzymes which are made up of two identical or very similar subunits and which contain two active sites per native dimer.

The glycolytic enzyme enolase has been purified from a large number of biological sources, including rabbit muscle (Holt and Wold, 1961), yeast (Westhead and McLain, 1964), trout muscle (Cory and Wold, 1966), human muscle (Baranowski *et al.*, 1968), coho and chum salmon muscle (Ruth *et al.*, 1970), and recently, *Escherichia coli* (Spring and Wold, 1971a). All the enolases studied so far have similar physical and catalytic properties, such as a divalent cation requirement ($Mg^{2+}$, $Mn^{2+}$, or $Zn^{2+}$) for activity, inhibition by fluoride + phosphate (Warburg and Christian, 1942), molecular weights in the 80,000–100,000 range (including yeast enolase, see Mann *et al.*, 1970), and a dimeric subunit structure (Winstead and Wold, 1964, 1965; Brewer and Weber, 1968; Gawronski and Westhead, 1969; Brewer *et al.*, 1970; Ruth *et al.*, 1970; Cardenas and Wold, 1968; Spring and Wold, 1971a). Rabbit muscle enolase has also been shown to have two binding sites for a substrate analog, glycolate phosphate, by a gel filtration method (Cardenas and Wold, 1968), thus suggesting a model for enolase in which the native enzyme dimer contains two active sites.

We have recently found that two enolase inhibitors, D-tartronate semialdehyde phosphate (TSP)[1] (Hartman and Wold, 1967) and 3-aminoenolpyruvate phosphate (AEP), form complexes with strong and characteristic absorption spectra when they interact with enolases. This finding has permitted us to determine the number of analog binding sites in different enolases by direct spectrophotometric titration. Some of the properties of the two analogs were presented in the accompanying paper (Spring and Wold, 1971b). This paper presents the data on the interaction of the two inhibitors with enolases and a discussion of the results as they relate to the structure and function of enolases and the mechanism of the enolase reaction.

## Materials and Methods

*Inhibitors.* D-TSP and AEP were prepared as described previously (Spring and Wold, 1971b). TSP was stored frozen after purification on a Dowex 1 column and was thawed immediately prior to use. AEP was prepared in 1 M $NH_4Cl$ (pH 9.5) from TSP and frozen without purification. The AEP stock solution thus contained some contaminating TSP, but the TSP but did not interfere with these experiments.

*Enzymes and Enzyme Assays.* Pyruvate kinase (rabbit skeletal muscle, type I) was purchased from Sigma Chemical Co. and assayed by the method of Pon and Bondar (1967) using tricyclohexylammonium enolpyruvate phosphate (Sigma Chemical Co.) and $Na_2ADP$ (California Biochemical Corp.) as substrates.

Yeast enolase A, prepared by the method of Westhead and McLain (1964), was a gift of Dr. Paul Hargrave. Crystalline rabbit muscle enolase was prepared by the method of Winstead and Wold (1966). Coho salmon enolase was a crystalline sample prepared in 1967 by the method of Ruth *et al.* (1970). *E. coli* enolase was prepared by the method of Spring and Wold (1971a). All enolase samples were of high specific activity and were homogeneous by disc gel electrophoresis. Enolase assays were performed by the spectrophotometric method of Warburg and Christian (1942), using tricyclohexylammonium glycerate 2-phosphate (D-G2-P) as substrate (prepared from the barium salt (Sigma Chemical Co.) by the method of Winstead and Wold (1966)). The assay buffer contained 0.2 M KCl, $10^{-3}$ M $MgSO_4$, $10^{-5}$ M EDTA, and either 0.05 M Tris-HCl (pH 8.0) (*E. coli* and yeast enolase) or 0.05 M imidazole-HCl (pH 7.0) (rabbit and coho muscle enolase). In the assays for TSP or AEP as inhibitors, the enzyme was preincubated with the inhibitor in the assay system for 2 min prior to initiation of the enzymatic reaction by the addition of substrate.

*Spectrophotometric Titrations of Enolase.* Four different enolases (rabbit, coho, yeast, and *E. coli*) were titrated with TSP and with AEP in solutions containing 0.2 M KCl–$10^{-3}$ M $MgSO_4$–$10^{-5}$ M EDTA, and buffered with either 0.05 M imidazole at pH 7 (coho and rabbit enolase) or with 0.05 M Tris at pH 8 (yeast and *E. coli* enolase). Enzyme concentration was 0.3–0.8 mg/ml and stock TSP and AEP solutions were

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[1] Abbreviations used are: TSP, D-tartronate semialdehyde phosphate; AEP, 3-aminoenolpyruvate phosphate.

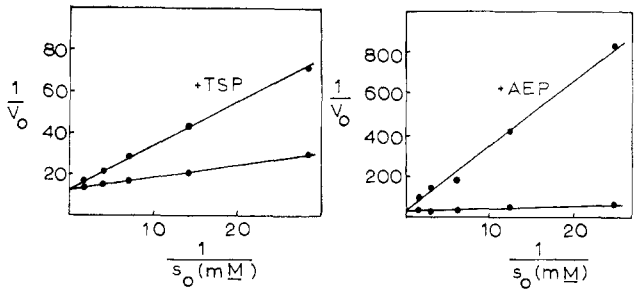



FIGURE 1: Lineweaver–Burk plots of the substrate kinetics of rabbit muscle enolase in the absence and presence of TSP ($9.5 \times 10^{-6}$ M) and AEP ($4.75 \times 10^{-7}$ M). The assays were performed as described in the Methods, at an enzyme concentration of approximately $10^{-9}$ M.

in the millimolar concentration range. The enzyme concentrations were calculated from the 280-nm absorbance, using published values for the $\epsilon_{280}^{0.1\%}$ of each enzyme (rabbit, 0.9; coho, 0.74; yeast, 0.9; and *E. coli*, 0.57) (see Wold, 1971). Microliter amounts of TSP or AEP were added to 3 ml of the enzyme solution in a quartz cuvet and the absorption spectrum from 350 to 230 nm was measured in the Cary 15 recording spectrophotometer immediately after each addition. The reference cuvet contained solvent only, and no additions were made to it. All volume changes were negligible. The spectrophotometer was thermostatted at 25° throughout the titration. A titration requiring 10–20 separate additions of inhibitor took less than 1 hr.

## Results

*Inhibition of Enolase with TSP and AEP.* Typical results for the effect of TSP and AEP on the enzymatic activity are shown in Figure 1 for rabbit muscle enolase. The Lineweaver–Burk plots indicate that both TSP and AEP are competitive inhibitors of enolase. AEP is considerably more effective as an inhibitor than is TSP, the $K_I$ for AEP being about 100 times smaller than the $K_I$ for TSP.

*Rates of Formation and Dissociation of the EI Complexes.* The rate of association of AEP with rabbit muscle enolase

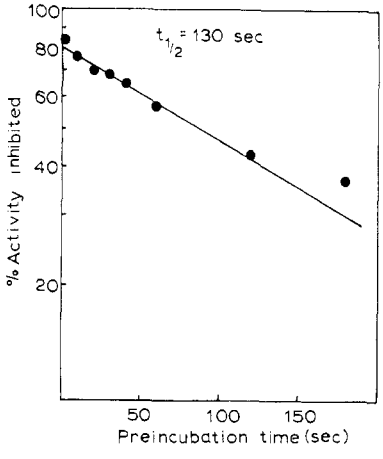



FIGURE 2: Dissociation of the enolase·AEP complex. A rabbit muscle enolase solution ($4.9 \times 10^{-6}$ M in subunits) was titrated with a twofold molar excess of AEP. Aliquots of this solution were diluted 1:1000 into the assay buffer for the enolase assay and preincubated for different lengths of time prior to the initiation of the assay with G2-P. The amount of active enzyme was determined by the initial velocity of the enzymatic assay.

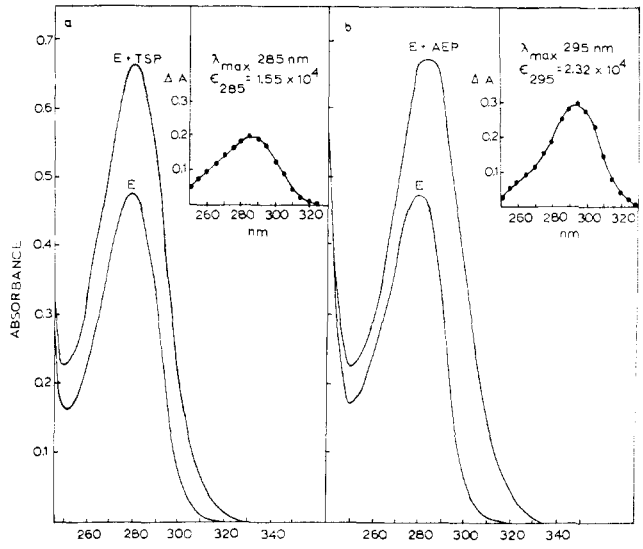



FIGURE 3: Difference spectra of the E·TSP and E·AEP complexes for rabbit muscle enolase. (a) The spectra of the enzyme at $6.5 \times 10^{-6}$ M without inhibitor (E) and in the presence of $5 \times 10^{-5}$ M TSP (E + TSP). TSP alone at this concentration has no significant uv absorption in the range studied. The difference spectrum ([E + TSP] − E) is shown in the upper right-hand corner. The molar difference extinction coefficient ($\epsilon_{285}$) was calculated assuming that the molarity of the 285 nm chromophore was equal to the concentration of enolase subunits present ($2 \times 6.5 \times 10^{-6}$ M). (b) The spectra of the enzyme at $6.5 \times 10^{-6}$ M without inhibitor (E) and in the presence of $1.9 \times 10^{-5}$ M AEP (E + AEP). The difference spectrum and the calculation of $\epsilon_{295}$ are analogous to part a.

at pH 7.0 was slow enough to be estimated directly in the enolase assay. When enolase was added to the reaction mixture containing $\sim 10^{-7}$ M AEP and $10^{-3}$ M D-G2-P (final enzyme concentration $\sim 10^{-9}$ M in subunits), there was a lag of about 2 min before the AEP inhibition of enolase became significant, suggesting that the association of enolase with AEP at those concentrations was slow relative to the association with substrate. A rough estimate of the bimolecular rate constant ($k_1$) for

$$\text{AEP} + \text{E} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{E·AEP (E = enolase)}$$

is $2 \times 10^4$ $M^{-1}$ $sec^{-1}$. The rate of dissociation ($k_{-1}$) for the E·AEP complex was also a slow reaction, and could be estimated by diluting the E·AEP complex into assay buffer and measuring the rate of recovery of enzyme activity (Figure 2). Under these conditions, the dissociation of the E·AEP complex to give active enzyme was first order with respect to the complex, and the calculated first-order rate constant ($k_{-1}$) was $5.3 \times 10^{-3}$ $sec^{-1}$. The ratio $K = k_{-1}/k_{+1}$ ($\sim 3 \times 10^{-7}$ M) gives an independent measure of the dissociation constant ($K_d$) for the E·AEP complex.

Estimation of the association and dissociation rate constants for the reaction of E with TSP was not possible by the above methods, since the reactions were much too fast to permit direct observation.

*Spectrophotometric Titrations of Enolases.* Characteristic uv difference spectra for the formation of enzyme–inhibitor complexes are given in Figure 3. The maxima (285 nm for TSP and 295 nm for AEP) were identical for all four enolases, and the difference spectra are sufficiently intense ($\epsilon_M \cong 10^4$ $M^{-1}$ $cm^{-1}$) so that spectrophotometric titrations could be per-

TABLE I: Data on Difference Spectra Produced in Titrations of Enolases with TSP and EAP.

| Enolase Source | Subunit[a] Concn (M × $10^5$) | Observed[b] $\Delta A$ (max) | Calcd $\epsilon$[c] | Titration End Point[d] |
|---|---|---|---|---|
| Rabbit | 1.29 | 0.20 | 15,500 | ~1 |
| Coho | 1.28 | 0.17 | 13,300 | ~1 |
| Yeast | 1.24 | 0.16 | 12,900 | ~1 |
| *E. coli* | 1.71 | 0.24 | 14,000 | ~1 |
| Rabbit | 1.29 | 0.300 | 23,200 | 0.98 |
| Coho | 1.16 | 0.205 | 17,700 | 0.97 |
| Yeast | 1.22 | 0.255 | 21,000 | 1.00 |
| *E. coli* | 1.71 | 0.305 | 17,800 | 1.06 |

[a] Concentration of enolase subunits calculated assuming two subunits per native enzyme and native molecular weights of 82,000 (rabbit), 100,000 (Coho), 88,000 (yeast), and 90,000 (*E. coli*). Enzyme concentration (milligrams per milliliter) determined from $A_{280}$ using published $E_{280}^{0.1\%}$ values. [b] $\Delta A$ (max) refers to the maximal absorbance changes (at the end point of the titration) observed in the TSP ($\lambda_{max}$ 285 nm; top set of data) and EAP ($\lambda_{max}$ 295 nm; bottom set of data) titrations, respectively. [c] Molar extinction coefficients at $\lambda_{max}$ for the difference spectra $\epsilon = \Delta A(\text{max})/\text{enzyme subunit concentration}$ (M), assuming that the difference spectrum is due to the presence of one chromophoric group per enzyme subunit. [d] Obtained by extrapolation or direct observation of the stoichiometry of binding at high concentration of inhibitor (Figure 4); moles of inhibitor per mole of subunit.

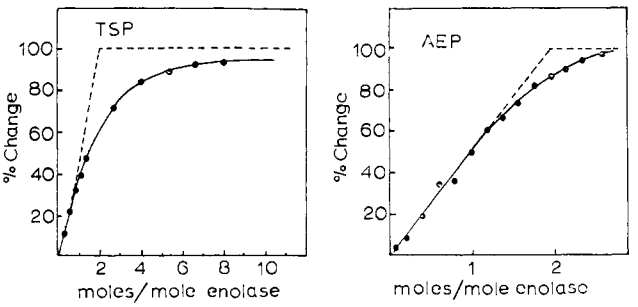



FIGURE 4: Spectrophotometric titration of rabbit muscle enolase with TSP and AEP. Both titration curves (solid lines) represent plots of the per cent change in the respective difference spectra produced by the addition of inhibitor to the enzyme, as described in Methods. The enolase concentration was 6.5 × $10^{-6}$ M. The dashed curves represent theoretical titration curves, assuming irreversible binding of 2 moles of inhibitor/mole of enolase.

formed. Typical titration curves are shown in Figure 4, and the titration data for all four enolases are summarized in Table I. In all cases the end point of the spectrophotometric titration (either extrapolated or observed directly, see Figure 4) corresponded to 2 moles of inhibitor bound per mole of enzyme, or 1 mole of inhibitor bound per enzyme subunit. The molar extinction coefficients for the inhibitor–subunit complexes in Table I are calculated from the known molecular weights of the four enzymes and suggest that the nature of the chromophore formed by the different enzymes with TSP is very similar and also that those formed with AEP are very similar.

*Are the Analog Binding Sites and the Catalytic (Substrate Binding) Sites Identical*? This question, of obvious relevance to the conclusion that the enolases contain two active sites, can be explored by many different, albeit indirect approaches. One of these is to compare the affinity between enzyme and inhibitor measured by direct binding ($K_d$) and by kinetic analysis ($K_I$). If the two constants have very similar values, the two methods must measure the same process. The data in Table II summarize the $K_d$ values from the titration experiments and also give a partial comparison to corresponding $K_I$ values. Because of the high affinity between AEP and enolase, the $K_d$ values can only be estimated as maximum values, and the comparison for AEP is therefore not very precise. However, the $K_I$ values obtained for rabbit and *E. coli* enolase agree reasonably well with the corresponding $K_d$ values. For the E·TSP complexes, however, $K_d$ can be determined quite accurately and the good agreement with the corresponding $K_I$ values for the rabbit, yeast, and *E. coli* enzymes in this case strongly suggests that binding and kinetics measure the same interaction.

An extension of this approach is to compare $K_m$ values for individual enolases with the corresponding $K_d$ values for TSP and AEP (see Table II). The quantitative agreement is far from perfect but the general pattern of relative substrate affinity as measured by $K_m$, coho enolase > rabbit enolase > yeast enolase = *E. coli* enolase, is completely consistent with the relative analog affinities measured by $K_d$.

Another approach is to evaluate the effect of $Mg^{2+}$ on the interaction of enolase with TSP and AEP. It is well established that in the absence of $Mg^{2+}$ (the elimination of $Mg^{2+}$ is most conveniently accomplished by the addition of an excess of a chelating agent, such as EDTA) all enolases are completely inactive. A similar absolute requirement for $Mg^{2+}$ in the binding of TSP and AEP to enolase can readily be demonstrated. Thus, when rabbit enolase was first treated with an excess of EDTA and then with either TSP or AEP, the characteristic uv difference spectra were totally absent. Similarly, the addition of EDTA to a solution of E·TSP or E·AEP caused rapid dissociation of the complexes, as judged by the disappearance of the uv absorption. The uv spectra which resulted from the treatment of the EI complexes with EDTA suggested that the

TABLE II: Comparison of Kinetic Data and Spectrophotometric Titration Data for the Binding of Substrate and Inhibitors of Enolases.[a]

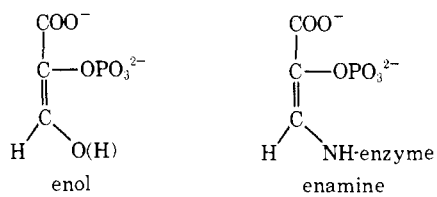
| Enolase Source | $K_m$ | TSP $K_I$[b] | TSP $K_d$[c] | EAP $K_I$[b] | EAP $K_d$[c] |
|---|---|---|---|---|---|
| Rabbit | 45 | 4.1 | 2.5 | 0.02 | 0.1 |
| Coho | 40 | | 1.6 | | 0.04 |
| Yeast | 100 | 14[d] | 15 | | 0.5 |
| *E. coli* | 100 | 19 | 15 | 0.09 | 0.5 |

[a] All $K$ values expressed in micromolar concentration. [b] Competitive inhibition constant derived from kinetic measurements (as in Figure 1). [c] Calculated dissociation constants for EI complex as determined from the spectrophotometric titrations (Figure 4): $K_d = (E)(I)_{free}/(EI)$, where E and EI was determined from the titration curve. [d] Hartman and Wold (1967).

complexes dissociated directly into free enzyme + TSP and free enzyme + AEP, respectively.

A final point related to the question of identity of the two processes is based on the following argument. Each analog binds to the enolases with an affinity considerably greater than that of the substrate. Thus, in addition to the normal substrate binding site, there is some feature of the enolase active site that "matches" TSP and AEP uniquely. If this argument is correct, the strong binding of TSP and AEP should be specific for enolase, and other enzymes for which the two inhibitors are good substrate analogs should not be affected at the low inhibitor concentrations at which enolase is inhibited. Only one enzyme has been tested, namely pyruvate kinase. This enzyme was selected because of the structural similarity of TSP and especially AEP to enolpyruvate phosphate. At concentrations of TSP and AEP which strongly inhibited enolase activity, no inhibition of pyruvate kinase was observed. Thus TSP and AEP appear to be specific inhibitors of enolases, not merely for any enzyme which uses enolpyruvate phosphate as a substrate. We assume that TSP and AEP may inhibit pyruvate kinase at higher inhibitor concentrations (greater than $10^{-4}$ M) but this has not been examined, due to the difficulty of preparing concentrated solutions of TSP and due to interference by AEP in the spectrophotometric assay for pyruvate kinase.

*Some Properties and Possible Structures of the Enzyme–Inhibitor Complexes.* Although neither TSP nor AEP formed irreversible derivatives with enolases, the inhibitors were greatly stabilized by their combination with the enzyme at 25° and at pH 7–8 (the half-lives of the uv spectra characteristic of the complexes were of the order of 10–20 hr at 25°). At reduced temperatures (4°) the $t_{1/2}$ of the uv spectra were of the order of several days. It appears that there is little or no breakdown of the inhibitors in the EI complexes themselves, and that the slow decay is due to spontaneous decomposition of free inhibitor which is in equilibrium with the corresponding EI complex, and which is less stable ($t_{1/2}$ at 25° and pH 7 is about 7 hr for TSP and 1 hr for AEP; Spring and Wold, 1971b). We have made several attempts to stabilize and identify the two enzyme–inhibitor complexes. Hartman and Wold (1967) suggested the possibility that the aldehyde group of TSP might react with an amino group of enolase to form a Schiff base derivative. They tested this hypothesis by treating the E·TSP complex with $NaBH_4$ in an attempt to reduce the Schiff base formation by this approach. Based on the 285-nm difference spectrum produced by titrating enolases with TSP, the Schiff base hypothesis now appears untenable, since a Schiff base of TSP would not be expected to absorb strongly in the 285-nm region. A more likely structure for the E·TSP complex would be an enol or possibly an enzyme bound enamine, since model compounds of similar structure show strong absorption in this region (Spring and Wold, 1971b). We have attempted to




test these two hypotheses by incubating TSP with rabbit muscle enolase in the presence of $H_2O$-*t* and then looking for incorporation of tritium into the C-2 position of TSP. A positive finding would show that the C-2 proton of TSP had been labilized during the formation of the E·TSP complex, and would constitute direct evidence in favor of either one of the two suggested structures. Only insignificant tritium incorporation was found in these experiments. This negative result is inconclusive, however, since lack of incorporation might be due to kinetic factors which prevented the C-2 proton of TSP from exchanging rapidly enough with the solvent. It is worth noting that the interaction of TSP with enolase never racemized the D-TSP.

To test the hypothesis that either TSP or AEP might form a covalent enamine derivative with enolase, we denatured the E·TSP and the E·AEP complexes rapidly by dilution into 8 M urea. We hoped that a covalently bound enamine would not be destroyed by this procedure, but that it would remain attached to the denatured enzyme and would be detectable spectrophotometrically. However, the uv spectra of the urea-denatured EI complexes showed that instantaneous dissociation of the E·TSP and E·AEP complexes occurred, yielding free E + TSP and E + AEP, respectively. (Dilution of the EI complexes into buffer containing no urea caused no dissociation in the control experiment.) Again, the negative results do not lead to any conclusion as to the chemical nature of the complexes.

Attempts to reduce the enamine intermediates (postulated for the E·TSP and the E·AEP complexes) to stable amine derivatives with $NaBH_4$ have also been unsuccessful. However, since reduction of enamines with $NaBH_4$ is known to be difficult, and since AEP itself could not be reduced in this way (Spring and Wold, 1971b), a negative result here is not surprising.

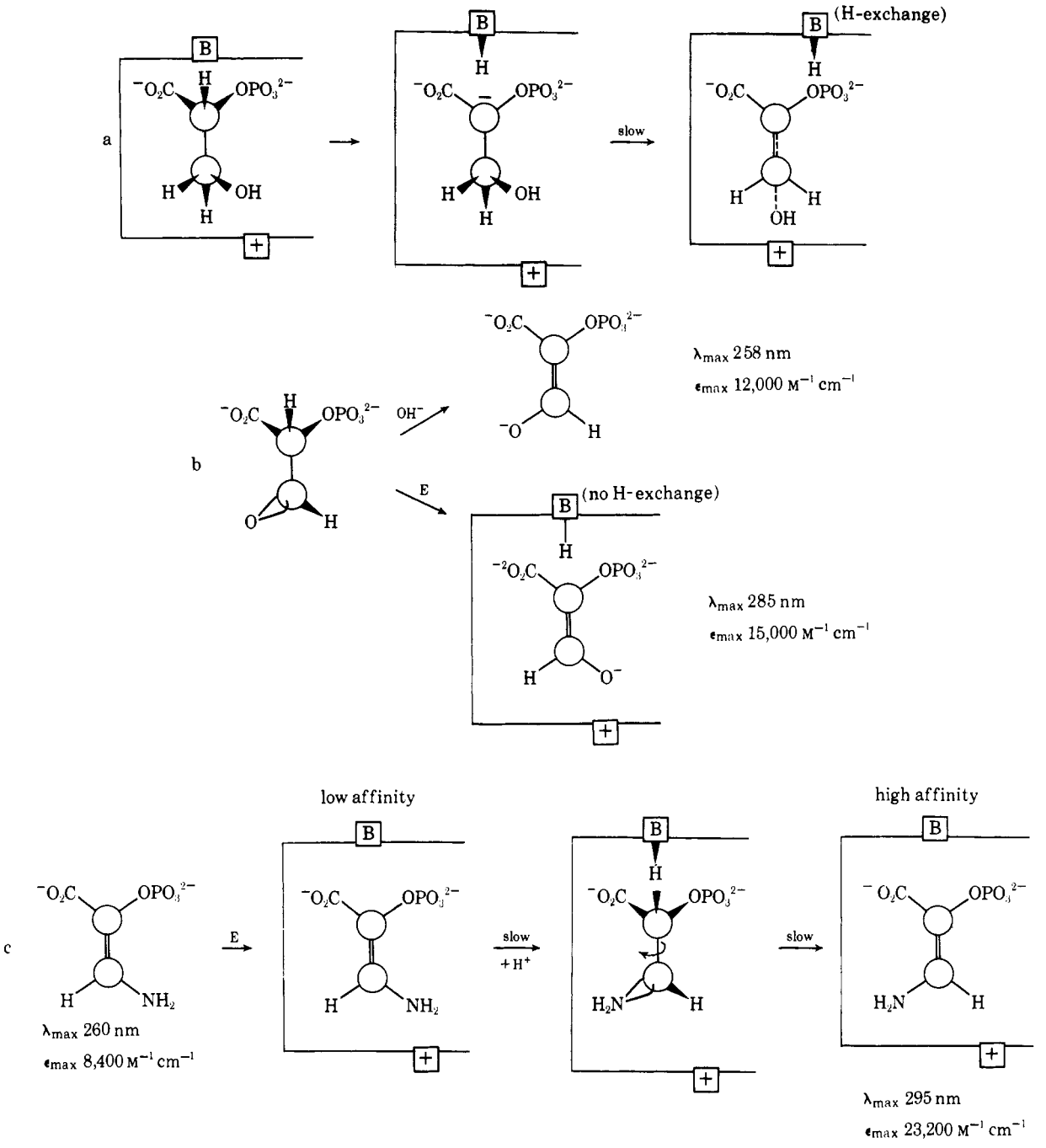
## Discussion

The main conclusion from the results reported here is that the four different enolases all contain 2 moles of analog binding sites per mole of active enzyme. This conclusion is consistent with the earlier data on the binding of glycolate phosphate (Cardenas and Wold, 1968) and glycidol phosphate (Rose and O'Connell, 1969) to rabbit muscle enolase which also demonstrated two binding sites. Within the sensitivity of the titrimetric assays used it can also be concluded that the two sites are equivalent and do not interact. This conclusion is consistent with the fact that all enolases studied to date have been found to give normal, hyperbolic substrate kinetics (Hanlon and Westhead, 1969; Holt and Wold, 1961; Ruth *et al.*, 1970; Spring and Wold, 1971a). If one accepts the arguments presented in the Results section that the analog binding sites are identical with the catalytic sites, then the full conclusion of this work is that the four phylogenetically different enolases from *E. coli*, yeast, coho salmon, and rabbit contain two noninteracting active sties. It is now well established that all these four enolases are dimers consisting of two identical or very similar subunits (for a review of enolase structure and function, see Wold, 1971). Thus we arrive at an apparently universal model for enolases as dimers with two active sites per active dimer molecule. (A recent report on enolase from a thermophilic bacterium suggests that higher molecular weight aggregates may also have to be considered for the native enolase structure (Cronlund and Stellwagen, 1971).)

In addition to the conclusion about the number of binding sites in enolases, we would also like to draw some more tentative conclusions as to the nature of the enolase sites and the mechanism of the reaction catalyzed by different enolases.

From the very similar spectral properties exhibited by the four enolase·TSP complexes and also by the four enolase·

SCHEME I




AEP complexes, we would like to strengthen the argument that the catalytic sites of enolases are very similar throughout the biosphere. At the same time, the small differences in $K_d$ obtained in this work also enforce the significance of the minor differences observed previously for $K_m$ and $K_I$ values as well as for pH optima of different enolases (Cardenas and Wold, 1971; Wold, 1971). Thus, while the active sites of different enolases appear to be very similar, the small but consistent differences in the affinity of the various enzymes for both substrate and substrate analogs suggest that some changes have taken place in the active-site components during biochemical evolution.

Finally, the possible relevance of our data to the enolase mechanism should be considered. Scheme I summarizes some of the data and presents the current picture of the dehydration mechanism together with hypothetical reaction schemes for the interaction of the two inhibitors with enolase. The first reaction (a) in this scheme summarizes the most recent data on the enolase mechanism obtained by Dinovo and Boyer (1971) by the study of isotope effects on the enolase reaction. It was concluded from these studies that the first step of the dehydration reaction catalyzed by enolase is the fast, base-catalyzed extraction of the C-2 proton, and that the rate-determining step is the $sp^3$–$sp^2$ transition of the resulting carbanion intermediate. Part of the argument in favor of this mechanism was based on the rapid, enolase-catalyzed exchange of the C-2 proton of the substrate with the solvent. It is interesting to note that the observation that D-2,3-dihydroxybutyrate 2-

phosphate (Wold and Ballou, 1957) and D-erythronate 2-phosphate (Wold and Barker, 1964) are inert as substrates for enolase may well be consistent with the proposed rate-limiting step. In these cases, the rotation of the C-2–C-3 bond may be prevented in the enzyme–analog complex by the bulky substituents on C-3.

The second reaction (b) in the scheme rationalizes the reactions of TSP within the framework of this mechanism. The proton extraction accomplished by 1 M base in the conversion of TSP to enolate in the model reaction (Spring and Wold, 1971b) is accomplished by the base in the active site of enolase, in agreement with the proposed dehydration reaction, and the resulting carbanion is immediately stabilized as the enolate–enzyme complex. The proposed active site acidic group, for which there is no direct evidence, is included to facilitate the elimination of OH in the normal dehydration reaction, and could in the case of the TSP stabilize the enolate–enzyme complex. This would explain the high affinity of enolase for TSP and also predict that only one geometrical isomer of the enolate would be formed. We further predict that the spectral shift to longer wavelength in the enzyme–enolate as compared to the high pH enolate reflects the different geometry of the two otherwise identical compounds. The main aspect of our data which does not support this proposal is our failure to demonstrate a C-2 proton exchange with the solvent in the presence of the enzyme, wherease such exchange could be readily demonstrated in the base treatment of TSP (Spring and Wold, 1971b) and also in the enzyme-catalyzed dehydration of the substrate (Dinovo and Boyer, 1971). Perhaps the strong interaction of the TSP enolate with the active site of enolase leads to a conformation in which the C-2 proton has no access to solvent (compare a and b in Scheme I).

The third reaction (c) in Scheme I presents a possible structure of the AEP·enolase complex, attempting to account for the spectral properties as well as the unique feature of this reaction, namely the low rate of formation of the E·AEP complex. It is again proposed that only one geometrical isomer of the enamine can form a stable complex with enolase, and that there is an obligatory transition from one (preferred) configuration in solution to the opposite configuration in the complex through a Schiff base intermediate protonated by the enzyme (in the E·BH form). This transition, in analogy to the proposed dehydration–hydration mechanism (a), could represent the slow step in the formation of the E·AEP complex. Again the different geometry of the enzyme-bound enamine and the free compound is proposed to be the basis for both the spectral shift to longer wavelength and the almost threefold increase in the extinction coefficient. This simple scheme incorporating the transition state analog concept proposed by Wolfenden (1969, 1970) does not explain the very great stability of the E·AEP complex, and it may well be that a covalent enzyme–enamine derivative will provide a better model. We were, however, unable to provide any evidence for a covalent derivative.

Although reactions b and c in the scheme are only hypothetical, they may lend themselves to experimental tests, and the scheme thus has predictive value. A study of the pH effect on the formation of the high-affinity AEP complex should for example provide a preliminary test for the proposed mechanism in c, and if a method can be found which will distinguish between the proposed geometrical isomers, the fundamental concept of this model could be critically examined. Even without unequivocal data on the nature of the complexes, however, these inhibitors with their unique spectral properties should provide excellent experimental tools for the study of other aspects of enolase catalysis. The role of metal ions in the enolase reaction may, for example, be studied through the quantitative effect of metal ions on the formation of the enzyme–inhibitor complexes.

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