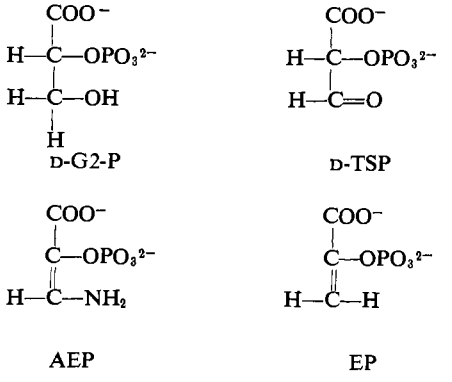
Studies on Two High-Affinity Enolase Inhibitors. Chemical Characterization*

Thomas G. Spring† and Finn Wold‡

ABSTRACT: Two enolase inhibitors, D-tartronate semialdehyde phosphate (TSP, *Biochim. Biophys. Acta 141*, 445 (1967)) and 3-aminoenolpyruvate 2-phosphate (AEP), have been characterized. D-TSP was purified by ion-exchange chromatography and was stable for many months at −20°. In NaOH TSP underwent enolization, a reaction which was facilitated by the presence of Mg^{2+}. The enolate ion of TSP had a strong ultraviolet (uv) absorption at 260 nm (ϵ_{260} 12,000 M^{-1} cm^{-1}), which shifted to 235 nm in the enol (protonated) form. In an experiment designed to verify the enolization, the TSP enolate was reconverted to TSP in the presence of tritiated water. This resulted in the incorporation of tritium at C-2 in TSP, as deduced from the labeling of the borohydride reduction product of TSP, DL-glycerate 2-phosphate-2-*t*. TSP was converted to AEP, an enamine, by incubation in ammonium ion buffers at pH 8–11. AEP also had a strong uv absorbance at 260 nm (ϵ_{260} 8400 M^{-1} cm^{-1}) which shifted to longer wavelength (270 nm) below pH 7.5. AEP was unstable at 25° but was stable for months at −20°. Primary amines (but not secondary or tertiary amines) also gave uv-absorbing enamines from TSP, but these derivatives were not found to be enolase inhibitors. The enamine structure of AEP was deduced from (1) the uv absorption spectrum, (2) the kinetics and pH profile for enamine formation from TSP, and (3) the relative instability of AEP at low pH compared with high pH.

The synthesis of a potent enolase inhibitor, D-tartronate semialdehyde phosphate (TSP[1]), has been described by Hartman and Wold (1967). This compound is an aldehyde analog of the enolase substrate, D-glycerate 2-phosphate (D-G2-P), and was shown to inhibit yeast and rabbit muscle enolases competitively, with a K_i of about 10^{-5} M. One hypothesis put forth to explain the high affinity of TSP for enolase was a suggestion that the aldehyde group of the inhibitor formed an imine (Schiff base) derivative with an amino group at the active site, analogous to reactions known for aldolases (Lai *et al.*, 1965) and decarboxylases (Warren *et al.*, 1966). This hypothesis remains unproved for TSP, however, since Hartman and Wold were unable to demonstrate the presence of an imine by reduction with $NaBH_4$. In order to study the reaction of TSP with enolase more fully, we have purified the inhibitor and studied some of its properties. During the purification, we noticed that incubating TSP in a high concentration of ammonium ion at pH 9 produced a new compound, later identified as 3-aminoenolpyruvate 2-phosphate (AEP). AEP is an enamine analog of the enolase substrate enolpyruvate phosphate (EP), and was found to be an even more potent enolase inhibitor than is TSP, with a K_i (competitive) of about 10^{-7} M. In this paper we report the chemical characterization of TSP and AEP. A discussion of the effects of the two inhibitors on enolases will be presented in a companion paper (Spring and Wold, 1971).



Materials and Methods

All reagents used were of reagent grade quality unless otherwise specified. A commercial source of high purity distilled water was used throughout. D-G2-P was converted to the tricyclohexylammonium salt from the commercially available barium salt (Sigma Chemical Co.) by the method described by Winstead and Wold (1966). TSP was prepared from D-gluconate 2-phosphate according to the method of Hartman and Wold (1967). Quantitative determinations of TSP were most conveniently based on acid-labile phosphate. After heating for 10 min at 100° in 1 or 2 N H_2SO_4, P_i was determined by the method of Fiske and Subbarow (1925) or that of Marsh (1959). Another method, specific for the D isomer of TSP, was based on the quantitative estimation of D-G2-P produced by the reduction of TSP with $NaBH_4$, using enolase to convert D-G2-P to EP which was assayed directly by its ultraviolet (uv) absorbance (Hartman and Wold, 1967). AEP was assayed directly by its strong uv absorption. Enolase assays were carried out according to described methods (Winstead and Wold, 1966). All the spectral studies were also performed with the Cary 15 spectrophotometer. Radioactive counting was done with a Beckman LS-133 liquid scintillation spectrometer using glass vials containing 10 ml of scintillation cocktail (70% toluene, 30% ethanol, and 0.5%

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[1] Abbreviations used are: TSP, D-tartronate semialdehyde phosphate; D-G2-P, D-glycerate 2-phosphate; AEP, 3-aminoenolpyruvate 2-phosphate.

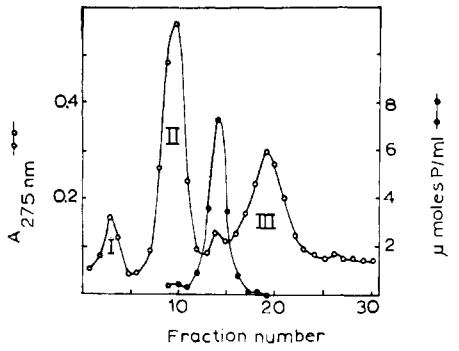


FIGURE 1: Ion-exchange chromatography of TSP. TSP (160 μmoles) was chromatographed on a Dowex 1 column (Bio-Rad resin AG-1-X8), 1.7 × 10 cm. The starting buffer was 0.01 M imidazole·HCl (pH 7.5) and elution was with a 0.0–0.5 M KCl gradient (in buffer). Total elution buffer volume was 150 ml; flow rate, 75 ml/hr, and fraction volume, 5.2 ml. Recovery of TSP (by the G2-P assay) was 123 μmoles (77%).

(w/v) diphenyloxazole). The counting efficiency for ^3H was 30% with this system.

Results

Preparation and Purification of TSP. TSP was prepared from D-gluconate 2-phosphate according to the method of Hartman and Wold (1967). The preparation was completely reproducible, except when solid $NaIO_4$ was used instead of an aqueous $NaIO_4$ solution. Solid $NaIO_4$ does not dissolve well in the cold, buffered solution of gluconate 2-phosphate and under these conditions poor yields of TSP were obtained. When the $NaIO_4$ was added instead as an aqueous solution, no precipitate formed and the yield of TSP after purification was 75–90%. The product was purified by ion-exchange chromatography as shown in Figure 1. TSP eluted as a single peak at about 0.25 M KCl and was well resolved from P_i (not shown) and from three uv-absorbing contaminants, designated I, II, and III. Peak I is probably formaldehyde and peak II is iodate ion, both products of the periodate oxidation.

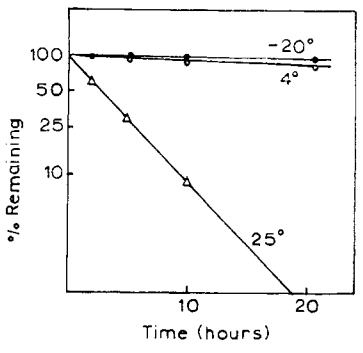


FIGURE 2: Stability of TSP at different temperatures. Solutions of D-TSP (2.2 μmoles/ml, pH 7.5) were incubated at 25, 4, and −20° and aliquots were taken for analysis at 2, 5, 10, and 22 hr. TSP was analyzed by reducing it with a 10-fold molar excess of $NaBH_4$ and assaying for D-G2-P. D-G2-P was assayed by adding a small amount of *E. coli* enolase and measuring equilibrium absorbance of enolpyruvate phosphate (EP) at 240 nm. Authentic D-G2-P was used as a standard.

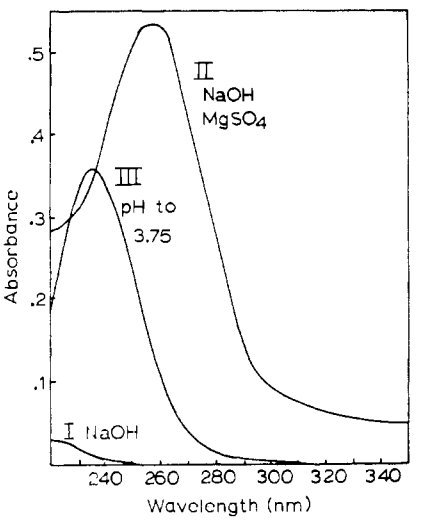


FIGURE 3: Spectral properties of TSP at high pH. TSP (3.5×10^{-5} M) was scanned in a Cary 15 spectrophotometer at 25° under the following conditions: curve I, in 0.01 M NaOH; curve II, in 0.01 M NaOH containing 10^{-3} M $MgSO_4$ (scanned without correcting base line for turbidity); curve III, as in curve II but with HCl added to pH 3.75 and scanned within 1 min (no base-line correction). The blank cuvet contained 0.01 M NaOH for all three curves.

Peak III is an unidentified compound which was observed only in some TSP preparations. It never represented more than 15% of the total phosphate containing material and was often absent entirely. This suggested the possibility that peak III is a partial oxidation product of gluconic acid 2-phosphate, but this has not been explored in depth. Peak III is not an enolase inhibitor and has not been characterized, except to note its strong uv absorption at 275 nm (ϵ_{275} 2×10^4 M^{-1} cm^{-1}, based on phosphate content) which shifts to 250 nm at low pH (p*K* for the transition about 4.1).

Characterization of TSP. The effect of temperature on the stability of TSP is shown in Figure 2. Due to the lability of TSP at room temperature, we were unable to do any chemical characterization of the compound by infrared (ir) or nuclear magnetic resonance (nmr) spectroscopy. The products of TSP decomposition have not been characterized, but likely pathways of destruction are decarboxylation and dephosphorylation.

Stereochemical Purity of TSP. Hartman and Wold (1967) showed that the crude D-TSP present in the reaction mixture after periodate oxidation could be quantitatively reduced with $NaBH_4$ to G2-P with retention of the D configuration at C-2. During the ion-exchange chromatography described here, the TSP also retained the D configuration. This was established by the fact that the assay for total TSP as acid-labile phosphate was identical with the assay for D-TSP after reduction to D-G2-P with $NaBH_4$, and assay with enolase.

Enolization of TSP. In NaOH, TSP was converted to a stable uv-absorbing compound (λ_{max} 260 nm, ϵ_{260} 12,000 M^{-1} cm^{-1}) which was later identified as the enolate ion of TSP. Complete conversion of TSP to its enolate required 10–15 min and a base concentration of at least 1 M NaOH. The presence of Mg^{2+} greatly facilitated the enolization reaction, however, as shown in Figure 3. In 0.01 M NaOH (curve I) TSP did not enolize to any appreciable extent. When 10^{-3} M $MgSO_4$ was added to the 0.01 M NaOH solution of TSP (curve II), two changes were observed: a precipitate of $Mg(OH)_2$ formed and a stable uv peak at about 260 nm appeared very rapidly. The

increase in the base-line absorbance of curve II is due to $Mg(OH)_2$ turbidity, but the 260-nm peak is due to the enolate. Rapid titration of the "Mg^{2+}-induced" enolate to pH 3.75 (curve III) resulted in a shift of the spectrum to 235 nm and a slow disappearance of the uv absorption ($t_{1/2}$ = 7 min). The 235 nm spectrum is thought to be due to the enol (protonated enolate) of TSP, which should be unstable and isomerize back to TSP.

In the presence of Mg^{2+}, the conversion of TSP to its enolate form was essentially complete at or above pH 12. This was established by the fact that higher pH or Mg^{2+} concentrations did not give further increases in the uv absorption of a given sample of TSP. Knowledge of total TSP concentration based on phosphate analysis thus permitted the estimation of the molar extinction coefficient of the Mg·enolate of TSP, and a value of ϵ = 12,000 M^{-1} cm^{-1} at 260 nm was obtained. Below pH 10, Mg^{2+} did not cause enolization. If a solution of TSP enolate (formed in the presence of Mg^{2+}) was titrated from pH 12 to 10, the enolate spectrum disappeared ($t_{1/2}$ = 5 min), and TSP was regenerated.

Attempts to separate the precipitate of $Mg(OH)_2$ from the enolate of TSP were unsuccessful. Apparently the enolate is firmly associated with the precipitate, so firmly that the enolate could be quantitatively collected from the reaction mixture centrifuging out the precipitate containing the excess $Mg(OH)_2$ and all the uv-absorbing enolate.

The identification of the high pH (or high pH + Mg^{2+}) uv-absorbing compound as the enolate seems reasonable in that all its uv properties are consistent with those of model compounds of similar structure (for example, the ascorbic acid–ascorbate system). However, more conclusive evidence was obtained by the demonstration of racemization in the proposed (reversible) process from TSP → enolate → TSP.

Demonstration of the Conversion of D-*TSP to* DL-*TSP through the Proposed Enolate Intermediate.* The Mg·enolate of TSP, associated with $Mg(OH)_2$, was suspended in H_2O-*t* (New England Nuclear) and was acidified with 0.8 equiv of HCl/equiv of NaOH used to produce the enolate. The final pH was above neutrality and small amounts of $Mg(OH)_2$ precipitate remained. The TSP produced in this way from the Mg·enolate was reduced to G2-P-*2-t* by treatment with a 10-fold molar excess of $NaBH_4$ for 2 hr. The reaction mixture was applied at pH 10–11 to a small Dowex 1 (Cl^-) column (1 × 5 cm) and washed with 5–10 ml of water to remove the excess H_2O-*t*. G2-P was then eluted in a narrow band with 0.05 M HCl. Total P and ^3H was determined and an aliquot of the purified G2-P was incubated with enolase under normal assay conditions. The reaction mixture was again fractionated on the Dowex 1 column, and the total amount of ^3H liberated as water as well as ^3H remaining with the G2-P peak was determined. The results given in Table I demonstrate that the compound obtained from the enolate is a DL mixture. Of the total G2-P recovered (as measured by total organic phosphate), only 49% was the D isomer (as measured by the enolase assay for D-G2-P). The position of the incoporated tritium atom in the TSP molecule was demonstrated by showing that 49% of the tritium label as released by treatment of the labeled G2-P with enolase, as would be expected for a tritium on C-2. Since only the D isomer of G2-P is a substrate for enolase (Wold, 1961), the label in the L-G2-P should not be removed by enolase. The conclusion that the remaining 51% of the labeled material was L-G2-P is supported by the fact that all the radioactivity of the original $NaBH_4$ product cochromatographed with authentic G2-P on Dowex 1.

TABLE I: Demonstration of the Racemization of ^3H Incorporation into D-TSP during the Mg^{2+}-Induced Enolization at High pH.[a]

	Organic Phosphate (Total G2-P) (μmoles)	G2-P Susceptible to Enolase (D-G2-P) (μmoles)	Total ^3H Content (cpm)	Fraction of Total G2-P as D-G2-P (%)
1. Starting material	11.4	11.4		100
2. Recovered G2-P after enolization and incorporation of ^3H	8.8	4.3	20,000	49
3. ^3H distribution after enolase treatment of an aliquot from 2			(5,630)	
In water fraction	ND	ND	2,750	49
In G2-P fraction	ND	ND	2,880	

[a] All analyses were carried out after reduction of TSP to G2-P with $NaBH_4$. For experimental detail, see text. The specific radioactivity of the water in the original reaction mixture (2 in the table) was 9 × 10^3 cpm/μatom of H.

Preparation and Characterization of AEP

Equilibrium and Kinetics of the Formation of AEP from TSP. During the purification of TSP, we found that TSP could be converted to a uv-absorbing compound in the presence of ammonium ion at pH 8.5. We have identified this new compound as the enamine, AEP. The amount of AEP produced from a given amount of TSP was dependent on the ammonium concentration (Figure 4a). By means of a double-reciprocal plot (Figure 4b), it was possible to obtain the absorbance of an AEP solution at infinite NH_3 concentration. The molar extinction coefficient of AEP calculated from these data is 8.4 ± 0.3 × 10^3 M^{-1} cm^{-1} (range of two separate determinations).

The rate of enamine formation was dependent on several factors. The initial rate of formation (as measured by the appearance of absorbance at 260 nm) was directly proportional to the concentrations of both TSP and ammonia. The pH was also an important factor, as shown in Figure 5. A maximum in the initial formation rate of AEP occurred at pH 9.5. Temperature also affected the rate of enamine formation, although AEP was normally prepared at 0–4° in order to prevent decomposition of the enamine, which occurred at the higher temperatures. At room temperature, AEP has a lifetime of 1–10 hr, depending on the pH and the ions present, so the aqueous solution of AEP was frozen (−20°) immediately after preparation. With this precaution, AEP could be stored for months with little or no deterioration.

Routinely, AEP was prepared by adding TSP to a solution of 1–3 M NH_4HCO_3 or NH_4Cl at pH 9–10, and incubating for 8 hr at 4°. The product was generally stored at −20° and used without further purification. If required AEP can be separated from the large excess of ammonia by ion-exchange chromatography (see below), but the product is then always contaminated with TSP and other degradation products.

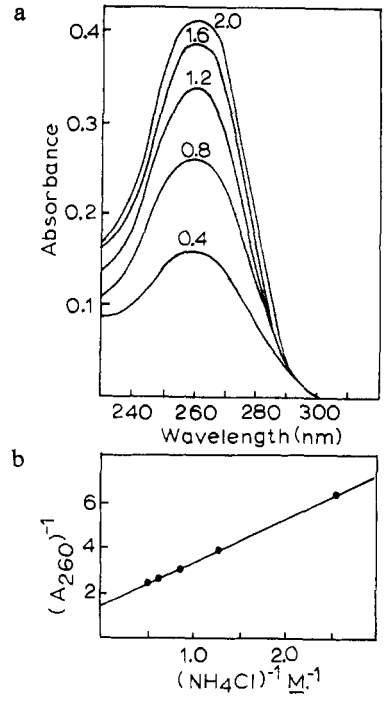


FIGURE 4: Enamine formation as a function of NH_3 concentration. (a) TSP was added to solutions of NH_4Cl of varying molar concentration (indicated on eah curve) and incubated for 12 hr at 4°. Absorption spectra were measured on the Cary 15 spectrophotometer. (b) Double-reciprocal plot of $1/A_{260}$ *vs.* $1/NH_4Cl$ concentration. The 260-nm absorbance at infinite concentration was obtained by extrapolation of the line to the *y* axis.

Ion-Exchange Chromatography of AEP. A solution of AEP in ammonium ion buffer, pH 8–9, was diluted into 0.05 M NaOH to a conductivity of 12 mmhos or less. AEP is reasonably stable even at 20–25° in 0.05 M NaOH, but the purification is best carried out at 4°. A small Dowex 1 column (1 × 10 cm) was equilibrated with 0.05 M NaOH and the AEP was applied

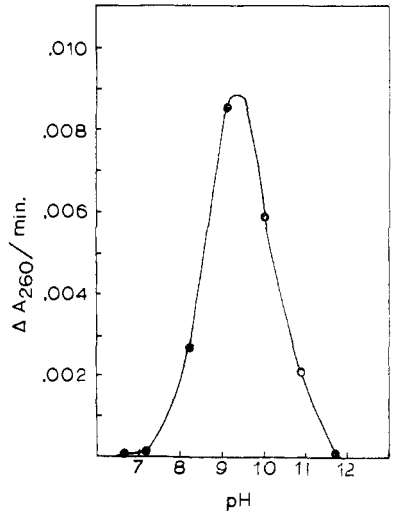


FIGURE 5: Enamine formation at 22° as a function of pH. Solutions of NH_4Cl were adjusted to different pH's with HCl or NaOH and adjusted to a final concentration of 1 M in total $NH_3 + NH_4^+$. TSP was added to give a final concentration of 0.05 M and the initial rate of AEP formation was measured by the increase in absorbance at 260 nm.

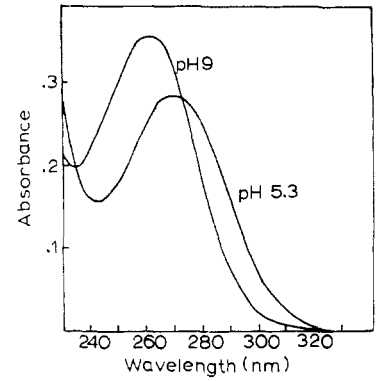


FIGURE 6: Effect of pH on the uv absorption of AEP. The uv absorption spectrum of AEP was measured in dilute NH_4Cl (pH 9) against a water blank. Glacial acetic acid (40 μl) was added to reference and sample cuvets and the spectrum (pH 5.3) was scanned again within 2 min. The pH 5.3 spectrum dropped to blank absorbance within 10–15 min.

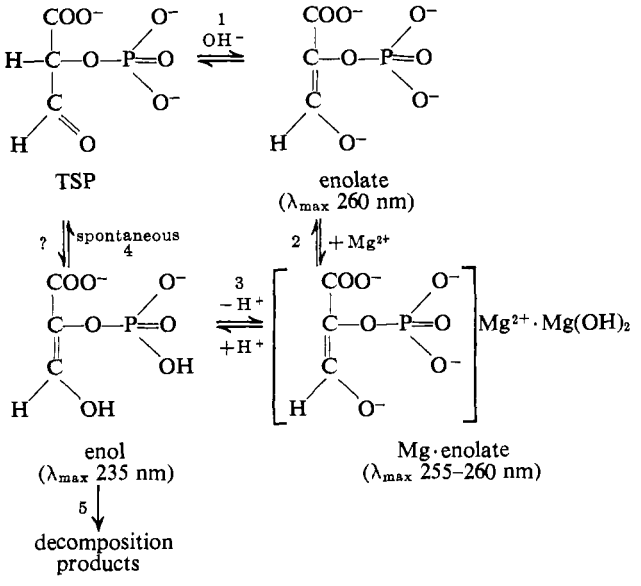
to the column. The column was then washed with 20 ml of 0.05 M NaOH and then 0.3 M KCl in 0.05 M NaOH to remove NH_3 and other contaminants. AEP (and any remaining TSP) was eluted with 0.5 M KCl in 0.05 M NaOH. AEP eluted as a fairly sharp band after 15–20 ml of eluent.

Other Enamines Derived from TSP. If TSP was incubated with a primary amine, rather than ammonia, uv-absorbing derivatives were produced which had properties similar to, yet distinct from, those of AEP. The derivative obtained by incubation with cyclohexylamine, for instance, had a wavelength maximum at 280 nm (at pH 9) and a molar extinction coefficient of 17,000 M^{-1} cm^{-1}. The structure of this enamine was presumably analogous to AEP except for the cyclohexyl group on the enamine nitrogen. The cyclohexyl enamine was not an enolase inhibitor, however (probably due to the bulkiness of the cyclohexyl side chain). Secondary and tertiary amines did not produce uv-absorbing derivatives by this procedure.

The rate of enamine formation was much faster with the primary amines than with ammonia although the rate of decomposition was also greater. At room temperature, glycine or cyclohexylamine produced a maximal enamine absorbance in 1 or 2 min, compared to the 30 min or 3 hr required for the production of AEP, using NH_4Cl and NH_4HCO_3, respectively.

Effect of pH on AEP. Figure 6 shows the effect of pH on the absorption spectrum of AEP. The apparent p*K* for the transition of AEP from its 260-nm-absorbing form (high pH) to its 270-nm-absorbing form (low pH) is about pH 7.5. Presumably the spectral shift reflects the titration of an ionizing group on the AEP molecule, but we do not know whether the phosphate group or the amino group (or both) is primarily involved. A similar shift in the spectrum of the cyclohexyl enamine was also observed (280–295 nm). We also noted that the enamines were very labile at the lower pH, and that the species which absorbed at higher wavelengths were apparently transient intermediates in a reaction which led to disappearance of the enamine spectrum. The effect of pH on the rate of disappearance of the AEP absorption spectrum is shown in Figure 7. It is not possible to interpret the disappearance of AEP at low pH, as we have not been able to identify the product formed. TSP would be the expected hydrolysis product according to the mechanism of enamine hydrolysis proposed by Coward and Bruice (1969), but we

SCHEME I



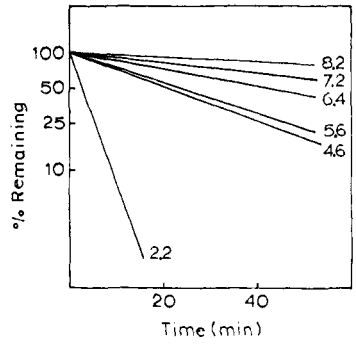


FIGURE 7: Stability of AEP as a function of pH. Aliquots (50 μl) of AEP in 1 M NH_4Cl were diluted into 3 ml of buffer and the disappearance of AEP was measured by the decrease in uv absorbance. HCl was used to adjust the pH of the buffers and the pH values recorded were measured at the conclusion of the reaction. The buffers at pH 2.2, 4.6, and 5.6 were prepared with sodium citrate (0.03–0.07 M); those at pH 6.4 and 7.2 with sodium phosphate (0.05 M); and that at pH 8.2 with Tris (0.025 M).

were not able to recover it from the reaction at pH 4–7. (Side reactions such as decarboxylation and dephosphorylation may be taking place under these conditions.)

Attempts to Derivatize AEP. All attempts to convert AEP to a stable derivative have been unsuccessful. Acetylation with acetic anhydride gave a stable derivative which absorbed at 260 nm but which had no phosphate, and thus was of no use. Similarly, reduction of AEP with $NaBH_4$ at several different pH values never produced the expected amino derivative, isoserine phosphate. This result is perhaps not surprising, since enamines of this type are known to be resistent to borohydride reduction (Tagaki *et al.*, 1968). The procedure of Tanaka and Pigman (1965) for reducing enamines with $NaBH_4$ in the presence of a Pd catalyst was also ineffective in reducing AEP.

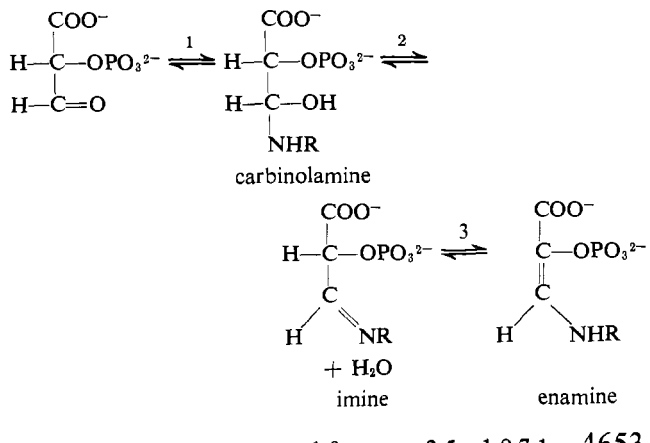
Discussion

Characterization of TSP. Scheme I summarizes the series of reactions which TSP can undergo, and which serve to establish its structure. Reaction 1 is the enolization reaction which can occur in 0.1–1.0 M NaOH. Removal of the C-2 proton of TSP produces a carbanion which is stabilized by resonance with the aldehyde and the carboxylate groups. Similar enolizations are known for β-keto esters and β-diketones (Gould, 1959). Reactions 1 + 2 describe the enolization in the presence of Mg^{2+} and NaOH. In this case, the equilibrium strongly favors the formation of the Mg·enolate complex, possibly because an insoluble or entrapped product is formed. Titration of the $Mg(OH)_2$ with acid would allow reaction 2 to go in the reverse direction, yielding enolate, which could then isomerize back to TSP. Reaction 3 describes the titration of the Mg·enolate to low pH (as in Figure 3, curve III) to give the enol intermediate, which absorbs at a lower wavelength and which can also isomerize rapidly back to TSP (reaction 4). Destruction of the enol may also occur (reaction 5), but the rate of this reaction must be much slower than that of reaction 4, as judged by the reasonably good recoveries of TSP from the enol in short term experiments.

Scheme I also explains the racemization of and incorporation of tritium into TSP upon neutralization of the Mg·enolate complex in H_2O-*t*. The specific activity of the tritiated G2-P (obtained *via* TSP) (Table I) was only 26% of the specific activity of the H_2O-*t*. This apparently low tritium incorporation might be partly the result of a tritium isotope effect. If there is an isotope effect of about 4:1 in favor of hydrogen ion over tritium ion in the protonation step going from enolate to TSP (reaction 1 in Scheme I), then the observed low incorporation would be explained. It must be remembered that the incorporation of tritium into TSP was not done under equilibrium conditions—rather, the Mg·enolate complex was partially titrated with HCl to favor the formation of enolate, and any TSP being produced from the enolate was then immediately reduced with $NaBH_4$.

Characterization of AEP. A reasonable mechanism for the formation of enamines from TSP is shown in Scheme II. In the absence of any well-characterized derivative, the support for Scheme II comes from a consideration of similar reactions in the organic chemistry literature and from the indirect experimental evidence presented under Results. (1) The formation of imines by way of carbinolamine intermediates is a well-established reaction (Cordes and Jencks, 1962), and is analogous to the derivatization of aldehydes with hydroxylamine and semicarbazide to form oximes and semicarbazones, respectively (Jencks, 1959). According to previous work on imine formation, step 1 should require attack by the unprotonated amine or ammonia and step 2 should be subject to

SCHEME II



general acid catalysis. Step 3 involves the isomerization of an imine to an enamine, a reaction which has been described for several different imines of similar structure (Dudek and Holm, 1962; Hay and Caughley, 1965; Hine *et al.*, 1968). The pH optimum in the rate of AEP formation (Figure 5) is consistent with such a rate-limiting attack of NH_3 on TSP at lower pH (reaction 1) and with the rate-limiting conversion of carbinolamine to imine (reaction 2) at higher pH. Similar arguments have been made for pH optima in the rate of formation of oximes and semicarbazones (Jencks, 1959). The fact that the initial rate of enamine formation at pH 9 was proportional to both the RNH_2 concentration and the TSP concentration is also consistent with the mechanism in Scheme II. Furthermore, the stability of AEP at elevated pH, even in the absence of NH_3 (see the purification of AEP in 0.05 M NaOH), can be explained by Scheme II, since the general acid catalyzed hydrolysis of the imine to the aldehyde cannot readily occur at high pH. In addition, the phosphate group of the enamine is fully ionized at high pH, and thus cannot act as a proton donor for any intramolecular general acid catalysis which might accelerate the breakdown of this particular enamine. (2) Under equilibrium conditions, the final concentration of AEP was dependent on the concentration of NH_3, at constant pH and TSP concentration (Figure 4). This observation is also consistent with the reactions shown in Scheme II. (3) The proposed enamine structure is consistent with the uv absorption data (ϵ and λ_{max}) for similar enamine compounds (Glickman and Cope, 1945). The uv data do not indicate whether AEP has a cis or a trans configuration around the double bond. This question remains unanswered at this time. (4) The fact that primary amines and ammonia (but not secondary and tertiary amines) gave enamine-like derivatives from TSP supports Scheme II; the imine intermediate cannot be formed with secondary and tertiary amines. (5) The instability of AEP at low pH (Figure 7) is consistent with the conclusion of Coward and Bruice (1969), who found that at low pH enamines undergo tautomerization to the protonated imine form, which can then hydrolyze to form the parent aldehydes. We were never able to recover TSP from the presumed hydrolysis of AEP, however, and must propose that side reactions which lead to decomposition of AEP (or TSP) take place. The protonated imine tautomer of AEP might well undergo decarboxylation, for instance.

These conclusions regarding the structure and properties of TSP and AEP form the basis for the study of the interaction of the two compounds with the enzyme enolase (Spring and Wold, 1971).

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