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REACTION OF TRIS WITH ALDEHYDES

EFFECT OF TRIS ON REACTIONS CATALYZED BY HOMOSERINE DEHYDROGENASE AND GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

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Summary

Tris buffer was observed to produce an apparent inhibition of the homoserine dehydrogenase (EC 1.1.1.3)-catalyzed reduction of aspartic β-semialdehyde and an apparent inhibition of the glyceraldehyde phosphate dehydrogenase (EC 1.2.1.9)-catalyzed oxidation of glyceraldehyde 3-phosphate. In each case, the apparent inhibition was found to be due to a lowering of the substrate concentration as a result of a reversible reaction between the free base form of Tris and the substrate, an aldehyde. The product of the reaction was tentatively identified as an imine on the basis of its spectral properties. The inhibition of these two enzymatic reactions by Tris was employed to investigate the kinetics of the reaction of Tris with their substrates. Assuming that these aldehydes exist entirely as the free aldehyde in aqueous solution, equilibrium constants of $369 \pm 12\ M^{-1}$ and $68 \pm 1.5\ M^{-1}$ were determined at 25°C for the reaction of the free base form of Tris with glyceraldehyde 3-phosphate and aspartic β-semialdehyde, respectively. Correcting for the existence of the hydrated form of glyceraldehyde 3-phosphate in aqueous solution, an equilibrium constant of $1.1 \cdot 10^4\ M^{-1}$ was obtained for the reaction of this aldehyde with the free base form of Tris. Forward and reverse direction rate constants for the reaction of Tris with glyceraldehyde 3-phosphate were determined at pH 7.45 and pH 8.5, and both were found to be pH-dependent.

Introduction

In 1961 Mahler discussed a number of ways in which Tris and other amine buffers might participate in or interfere with enzyme-catalyzed reactions [1]. Among the modes of participation considered were: (a) Tris as a possible sub-

strate for the enzyme, (b) compound formation between Tris and substrates containing carbonyl groups, (c) chelate formation between Tris and metal ions, (d) Tris-catalyzed chemical transformations of the substrate, and (e) interactions of Tris with the enzyme or coenzyme. We have recently encountered two examples of apparent inhibition of enzymatic reactions by Tris which appear to be solely the result of compound formation between Tris and the carbonyl-containing substrates.

One of the reactions catalyzed by the aspartokinase I-homoserine dehydrogenase I (EC 1.1.1.3) complex of *Escherichi coli* K 12 can be represented by Eqn. 1.

$$\text{aspartic } \beta\text{-semialdehyde} + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{homoserine} + \text{NADP}^+ \quad (1)$$

We have previously reported the results of kinetic studies of this reaction in the reverse direction in the presence of Tris buffer [2,3]. Tris buffer has also been used in active enzyme-centrifugation studies employing the forward reaction assay [4,5]. In this communication we report the results of our investigation of the effects of Tris on the enzymatic catalysis of the forward reaction as represented by Eqn. 1. In addition, we report the results of our investigation of the effects of Tris on the glyceraldehyde-3-phosphate dehydrogenase catalysis of the reaction represented by Eqn. 2.

$$\text{glyceraldehyde 3-phosphate} + \text{NAD}^+ + \text{H}_2\text{O} \xrightarrow[\text{Arsenate}]{} \text{3-phosphoglycerate} + \text{NADH} + \text{H}^+ \quad (2)$$

Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.9) was selected for study because the analysis of the data is simplified somewhat by the irreversibility of the reaction in the presence of arsenate. Evidence for imine formation between the aldehyde substrates and Tris as well as the equilibrium constants and rate constants for the reaction of these aldehydes with Tris are presented herein.

Experimental Procedure

Materials

Tris base and Tris · HCl were obtained from Schwartz-Mann and NAD^+, NADPH, dithiothreitol, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase and the barium salt of DL-glyceraldehyde-3-phosphate diethylacetal were obtained from Sigma. The DL-glyceraldehyde 3-phosphate was prepared from the diethylacetal barium salt by treatment with Dowex-50 Hydrogen Form Resin as described by Sigma. Aspartic β-semialdehyde was prepared by the method of Black and Wright [6] and the aspartokinase I-homoserine dehydrogenase I complex was isolated and purified from *E. coli* K12 (λ) as previously described [2,7].

Enzyme assays

All assays were carried out in cuvettes with a 1-cm light path employing reaction mixtures with a final volume of 3 ml. The initial velocities of the enzyme-

catalyzed reactions were measured by following the appearance of NADH or the disappearance of NADPH spectrophotometrically at 340 nm with a Gilford spectrophotometer equipped with a Sargent SRL recorder and a cell compartment thermostatically maintained at 25°C. All reaction mixtures were allowed to equilibrate for at least 10 min in the thermostatically temperature-controlled cell compartment before the reaction was initiated by the addition of enzyme or substrate. Experiments were carried out in duplicate, and the average percent deviation of the individual velocity determinations from the mean was less than 2.5%. The pH of each reaction mixture was measured with a Radiometer pH Meter 26. The forward reaction (aspartic β-semialdehyde → homoserine) assay mixture for homoserine dehydrogenase contained 0.25 M potassium phosphate, pH 7.6, 0.11 mM NADPH, 23.9 μM L-aspartate β-semialdehyde, and either 0, 33 mM, 67 mM, or 133 mM Tris, pH 7.6. The assay mixture for glyceraldehyde-3-phosphate dehydrogenase contained 26 mM sodium arsenate, 3.3 mM dithiothreitol, 0.25 mM NAD^+, 53 μM D-glyceraldehyde 3-phosphate, and either 13 mM sodium pyrophosphate buffer, pH 8.5, or 9 mM sodium pyrophosphate/26.7 mM Tris buffer, pH 8.5, or 6.5 mM sodium pyrophosphate/43.4 mM Tris buffer, pH 8.5, or 86.7 mM Tris buffer, pH 8.5, or 6.5 mM sodium pyrophosphate/43.3 mM Tris buffer, pH 7.45, or 3 mM sodium pyrophosphate/66.7 mM Tris buffer, pH 7.45, or 86.7 mM Tris buffer, pH 7.45.

Kinetic procedure and analysis

The reaction of an aldehyde with a primary amine may be represented by Eqn. 3 [8].

$$S + T \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} I + H_2O \tag{3}$$

Where S, T, C, and I represent the aldehyde, amine, carbinolamine, and imine, respectively. Eqn. 3 simplifies to Eqn. 4 if, under the conditions employed, the concentration of C is negligible compared to [S], [T], and [I].

$$S + T \underset{k_r}{\overset{k_f}{\rightleftharpoons}} I \tag{4}$$

In the presence of a large excess of the amine, [T] ≫ [S], the reaction of Eqn. 4 in the forward direction is pseudo-first-order, i.e., $v_f = k'_f$ [S] where $k'_f = k_f$ [T]. Thus, under pseudo-first-order conditions, the rate is described by Eqn. 5.

$$\ln\left(\frac{[S]_0 - [S]_e}{[S]_t - [S]_e}\right) = (k'_f + k_r)t = k_{obs}t \tag{5}$$

where $[S]_0$, $[S]_t$, and $[S]_e$ represent the concentration of the aldehyde initially, at time t, and at equilibrium, respectively [9]. The equilibrium constant for the reaction is given in Eqn. 6.

$$K = \frac{[I]_e}{[S]_e[T]_e} = \frac{[S]_0 - [S]_e}{[S]_e[T]_e} = \frac{K_{app}}{[T]_e} \tag{6}$$

where $K_{app} = ([S]_0 - [S]_e)/[S]_e = k'_f/k_r$ and $[T]_e$ is the equilibrium concentra-

tion of the amine species, i.e. protonated or free base, involved in the equilibrium.

An equation similar in form to Eqn. 5 would also describe the rate of the reaction represented by Eqn. 3 if the rate of formation of the carbinolamine were rate-limiting and the carbinolamine and imine were in rapid equilibrium. However, in this case, k_r would be $k_{-1}/(K + 1)$ where $K = k_2/k_{-2}$.

In the present studies, $[S]_0$, $[S]_t$, and $[S]_e$ were determined from initial velocity measurements in enzymatic assays employing the Michaelis-Menten equation, $[S] = vK_m/(V - v)$. Thus,

$$\ln\left(\frac{[S]_0 - [S]_e}{[S]_t - [S]_e}\right) = \ln\left[\frac{\left(\frac{v_0}{V - v_0}\right) - \left(\frac{v_e}{V - v_e}\right)}{\left(\frac{v_t}{V - v_t}\right) - \left(\frac{v_e}{V - v_e}\right)}\right] \quad \text{and} \quad K_{app} = \frac{\left(\frac{v_0}{V - v_0}\right) - \left(\frac{v_e}{V - v_e}\right)}{\left(\frac{v_e}{V - v_e}\right)}$$

where v_0 represents the initial velocity measured in the absence of the amine, Tris, or in the presence of Tris without preincubation of the Tris and aldehyde together, v_t represents the initial velocity measured after preincubation of a solution of the aldehyde and Tris in the absence of enzyme for the specified time t, v_e represents the initial velocity obtained after preincubation of a solution of the aldehyde and Tris for approximately 10 half-lives in the absence of enzyme, and V represents the maximum velocity for the enzyme-catalyzed reaction. The values of K_m and V for each enzyme and at each experimental condition employed were determined in independent experiments. The aldehyde concentrations to be determined were always less than the K_m of the enzyme used in their measurement. Linear regression analyses and analyses by the method of Wilkinson [10] were performed on a Hewlett-Packard 9100A calculator.

Difference spectra

The difference spectra were obtained between 350 and 210 nm in a Cary 118 spectrophotometer with the range set at 0—0.02 absorbance units full-scale and the scanning speed set at 0.5 nm/s employing split-compartment cells with a total light path of 0.88 cm. The DL-glyceraldehyde 3-phosphate was prepared as described in Materials and then treated with acid-washed Norite and filtered through a Millipore filter to remove ultraviolet-absorbing contaminants resulting from the Dowex-50 treatment. The final concentration of D-glyceraldehyde 3-phosphate was determined enzymatically using glyceraldehyde phosphate dehydrogenase. The difference spectra were obtained in 0.43 M potassium phosphate buffer, pH 7.6. The 5.3 mM DL-glyceraldehyde 3-phosphate and 143 mM Tris, pH 7.6, were present in separate compartments in the reference cell and in the same compartment in the sample cell.

Results

Effect of Tris on reactions catalyzed by glyceraldehyde-3-phosphate dehydrogenase and homoserine dehydrogenase

The effect of Tris on the rate of the reaction catalyzed by glyceraldehyde-

3-phosphate dehydrogenase is depicted in Fig. 1. The reactions were initiated by addition of glyceraldehyde 3-phosphate to the enzyme-containing assay mixtures with and without Tris; consequently, there was no preincubation of the Tris with the aldehyde. The salient features of Fig. 1 are (a) the initial velocities measured after the 15-s mixing period are virtually identical in the absence and presence of Tris, (b) the velocity falls off more rapidly in the early time period when Tris is present, and (c) the total amounts of NADH produced after 4 h is, within experimental error, independent of the Tris concentration. These data suggest that Tris competes with the enzyme for the free aldehyde, that formation of the Tris-aldehyde complex is not an extremely rapid reaction, and that either the formation of the Tris-aldehyde complex is a readily reversible process or that the Tris-aldehyde complex can serve as a poor substrate for the enzyme. In view of the specificity of enzymes, the latter possibility would appear unlikely. A similar effect of Tris on the homoserine dehydrogenase-catalyzed reduction of aspartic semialdehyde by NADPH was observed.

Additional support for a time-dependent formation of a Tris-aldehyde complex via a reversible reaction is presented in Table I. In these experiments, the aldehyde and Tris were preincubated together for specified times in the absence of enzyme, after which the free aldehyde concentration remaining was determined by enzymatic assay. The data clearly indicate a time-dependent reduction of free aldehyde concentration via a reaction which must be reversible since the final amount of NADH formed, a measure of the total potentially free aldehyde present, is identical regardless of the actual free aldehyde present initially as determined by the initial velocity measurements.

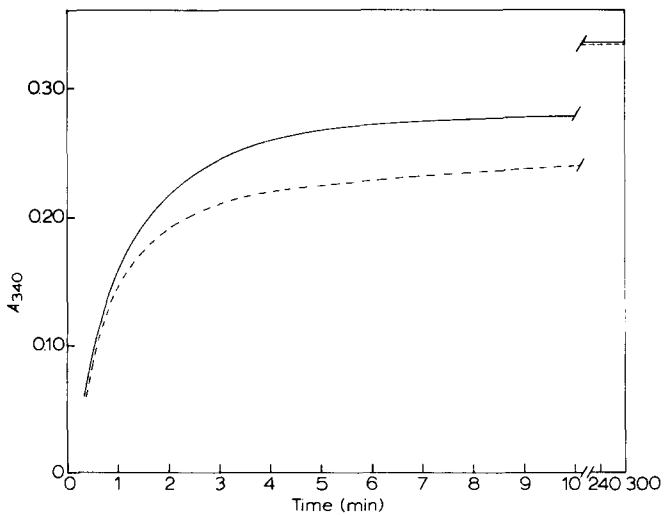

Fig. 1. Effect of Tris (pH 7.45) on the reaction catalyzed by glyceraldehyde phosphate dehydrogenase. The solid curve (———) represents the time course for formation of NADH when 25 μl of 12.9 mM DL-glyceraldehyde 3-phosphate is added to 2.975 ml assay solution containing 13 mM sodium pyrophosphate (pH 7.45), 26 mM sodium arsenate, 3.3 mM dithiothreitol, 0.25 mM NAD^+, and glyceraldehyde phosphate dehydrogenase. The dashed curve (- - - - -) represents the time course for formation of NADH when 25 μl of 12.9 mM DL-glyceraldehyde 3-phosphate is added to 2.975 ml assay solution identical to that above except that it contains 86.7 mM Tris (pH 7.45) instead of 13 mM sodium pyrophosphate. The initial 15 s is the mixing time.

TABLE I

EFFECT OF PREINCUBATION OF GLYCERALDEHYDE 3-PHOSPHATE WITH TRIS (pH 7.45) AT 25°C ON THE INITIAL VELOCITY OF THE GLYCERALDEHYDE PHOSPHATE DEHYDROGENASE-CATALYZED OXIDATION OF THE ALDEHYDE

2.975 ml solution containing 6.5 mM sodium pyrophosphate (pH 7.45), 43.3 mM Tris (pH 7.45), 26 mM sodium arsenate, 3.3 mM dithiothreitol, 0.25 mM NAD^+, and 106 μM DL-glyceraldehyde 3-phosphate was preincubated at 25°C in a cuvette for the specified time before the enzymatic reaction was initiated by the addition of 25 μl of a glyceraldehyde phosphate dehydrogenase solution. The final ΔA_{340} was measured 4—5 h after addition of the enzyme.

Preincubation time (min)	Initial Velocity (ΔA340/min)	Inhibition (%)	Final ΔA_{340}
0	0.198	0	0.331
2	0.154	22	0.332
4	0.127	36	0.331
90	0.058	71	0.327

Determination of equilibrium and apparent rate constants for the reaction of Tris with glyceraldehyde 3-phosphate and aspartic semialdehyde

In the presence of a large excess of Tris, the approach of the reaction of Tris with glyceraldehyde 3-phosphate to an equilibrium position at pH 7.45 and 8.5 is consistent with that of a reversible reaction which is first-order or pseudo-first-order in either direction (Fig. 2). Similar plots were obtained when the reaction of Tris with aspartate semialdehyde was investigated at pH 7.6 in the presence of a large excess of Tris. The values of k'_f and k_r, the apparent first-order rate constants for the formation and hydrolysis of the aldehyde-Tris complex, were calculated from the relationships $k'_f + k_r = k_{obs}$ and $K_{app} = k'_f/k_r$ (see Eqns. 5 and 6).

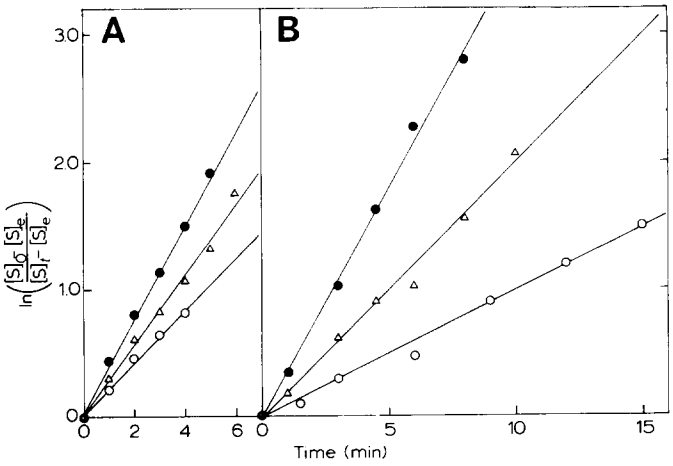

Fig. 2. First-order plots for the approach to equilibrium of the reaction of glyceraldehyde 3-phosphate with Tris at 25°C in the presence of a large excess of Tris. The values of $[S]_0$, $[S]_e$, and $[S]_t$ were determined enzymatically as described in the text. A: At pH 7.45, 107 μM DL-glyceraldehyde 3-phosphate, and either 86.7 mM Tris (●), 66.7 mM Tris (△), or 43.3 mM Tris (○). B: At pH 8.5, 107 μM DL-glyceraldehyde 3-phosphate, and either 86.7 mM Tris (●), 43.3 mM Tris (△), or 26.7 mM Tris (○).

In order to calculate the apparent second-order rate constant, k_f, for the reaction of the aldehyde S, with Tris, T, to form product I (see Eqn. 4), identification of the amine species, i.e. protonated or free base, involved in the reaction was essential. This was accomplished by determining the apparent equilibrium constant, K_{app}, for the reaction of glyceraldehyde 3-phosphate with Tris at pH 7.45, 8.3, and 8.5 via Eqn. 6. As can be seen in Fig. 3, a plot of K_{app} vs. the concentration of the free base or unprotonated species of Tris is linear (correlation coefficient = 0.978), indicating that it is the free base species of Tris involved in the reaction. The free base concentrations were calculated employing a pK_a of 8.07 for Tris [11]. From the data of Fig. 3, an equilibrium constant, K, of 369 M^{-1} can be calculated for the reaction of glyceraldehyde 3-phosphate with Tris. Also plotted in Fig. 3 are the data for the aspartic semialdehyde-Tris reaction (correlation coefficient = 0.996), from which an equilibrium constant, K, of 68 M^{-1} can be calculated.

The apparent second-order rate constant, k_f, was obtained graphically by

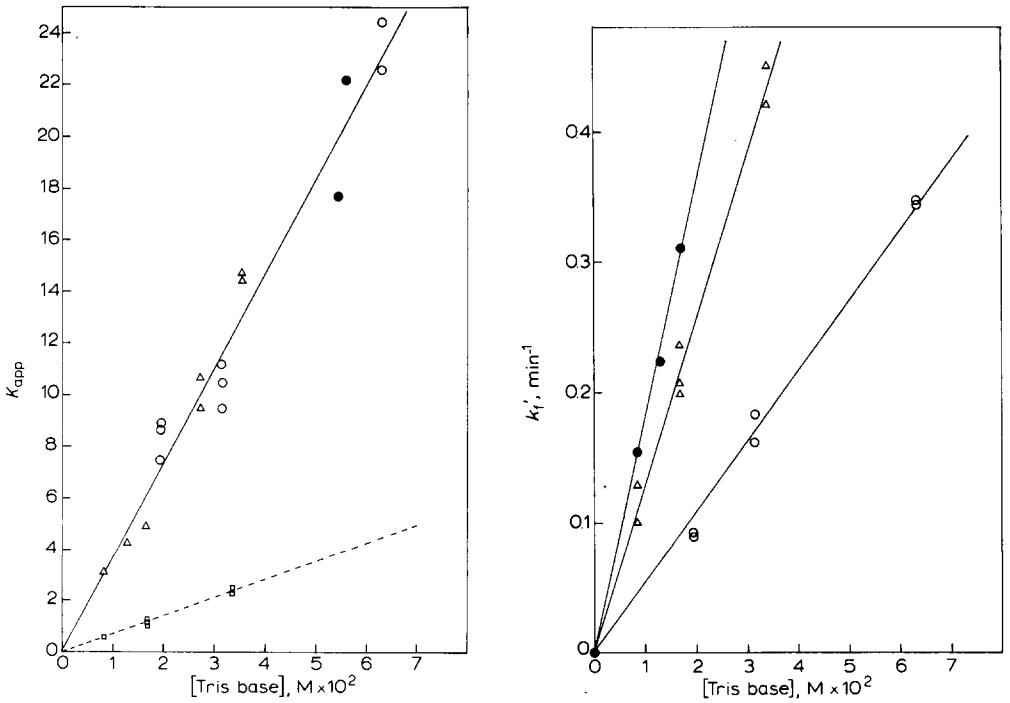

Fig. 3. Plots of K_{app} vs. the concentration of the free base form of Tris. The solid line (———) was determined from a linear regression analysis of the data for the reaction of Tris with glyceraldehyde 3-phosphate at pH 7.45 (△), pH 8.3 (●), and pH 8.5 (○). Correlation coefficient = 0.978. The dashed line (- - - - - -) was determined from a linear regression analysis of the data for the reaction of Tris with aspartic semialdehyde at pH 7.6 (□). Correlation coefficient = 0.996.

Fig. 4. Plots of the pseudo-first-order rate constant, k_f', vs. the concentration of the free base form of Tris. The lines were determined from linear regression analyses of the data for the reaction of Tris with glyceraldehyde 3-phosphate at pH 7.45 (●) and at pH 8.5 (○), and for the reaction with aspartic semialdehyde at pH 7.6 (△). The correlaction coefficients were 0.998, 0.998, and 0.996, respectively.

plotting k_f' against the concentration of the free base form of Tris. These plots are shown in Fig. 4 and the k_f values obtained are summarized in Table II, from which it can be seen that k_f displays a significant pH-dependence. The values for the apparent first-order rate constant, k_r, calculated from the relationship $k_r = k_f'/K_{app}$, are presented in Fig. 5, from which it can be seen that k_r does not show a significant or consistent dependence on the total concentration of Tris. The mean values of k_r under each set of experimental conditions are presented in Table II. Comparison of the k_r values for the Tris · glyceraldehyde 3-phosphate complex at pH 8.5 and pH 7.45 clearly indicates a significant pH-dependence, with k_r being four times greater at the lower pH.

Spectroscopic evidence for imine formation

A difference spectrum for glyceraldehyde 3-phosphate vs. glyceraldehyde 3-phosphate plus Tris at pH 7.6 is presented in Fig. 6. Inspection of this plot reveals that the ultraviolet absorption spectrum of glyceraldehyde 3-phosphate is markedly altered by Tris in that the carbonyl absorbance band of the aldehyde at approx. 285 nm decreases in the presence of Tris and a new absorbance band at approx. 243 nm appears. Somewhat similar spectral changes have been observed in the formation of furfuraldoxime and other oximes [12]; thus, these observed spectral changes would appear to be consistent with the conversion of $\rangle C = O$ to $\rangle C = N-$, or imine formation. Furthermore, the time course for the disappearance of the absorbance band at 285 nm or the appearance of the absorbance band at 243 nm is consistent with the kinetics of the inhibition of the glyceraldehyde-3-phosphate dehydrogenase reaction by Tris. From the results of the enzyme inhibition studies, a $t_{1/2}$ of approximately 1.2 min would be expected for the disappearance of free aldehyde under the experimental conditions employed in obtaining the difference spectra. Since scan 2 of Fig. 6 was started 1.5 min after mixing the Tris and aldehyde, approximately three half-lives had elapsed before scan 2 reached 285 nm. Scan 3, on the other hand, was obtained after approximately 24 half-lives had elapsed. Furthermore, since the rate of appearance of the 243 nm absorbance band appears to be comparable to the rate of disappearance of the 285 nm absorbance band, there is no evidence in these difference spectra for the transient accumulation of significant concentrations of a carbinolamine intermediate such as was observed in furfuraldoxime formation [12].

TABLE II

EQUILIBRIUM AND RATE CONSTANTS FOR THE REACTION OF ALDEHYDES WITH TRIS AT 25°C

Aldehyde	pH	$K \pm$ S.E. * (M^{-1})	k_f ** ($M^{-1} \cdot min^{-1}$)	k_r *** (min^{-1})
Glyceraldehyde-3-*P*	7.45	369 ± 12 (17)	18.2	0.056
Glyceraldehyde-3-*P*	8.5	369 ± 12 (17)	5.5	0.014
Aspartic semialdehyde	7.6	68 ± 1.5 (8)	12.8	0.19

* Determined from data of Fig. 3.

** Determined from data of Fig. 4.

*** Mean of the values for k_r calculated from the relationship $k_r = k_f'/K_{app}$.

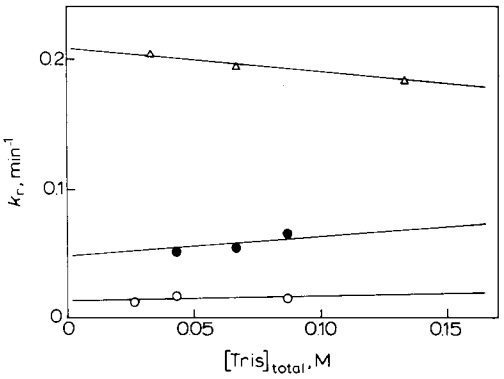

Fig. 5. Plots of the mean apparent first-order rate constants, k_r, vs. the total concentration of Tris for the Tris-glyceraldehyde-3-phosphate reaction at pH 7.45 (●) and pH 8.5 (○) and for the Tris aspartate semialdehyde reaction at pH 7.6 (△).

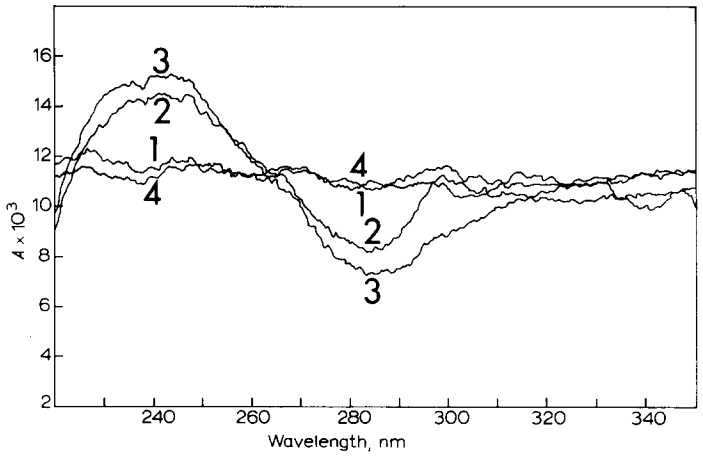

Fig. 6. Difference spectra for glyceraldehyde 3-phosphate vs. glyceraldehyde 3-phosphate plus Tris. Scan 1: 6.2 mM DL-glyceraldehyde 3-phosphate in 0.5 M potassium phosphate buffer (pH 7.6) vs. 6.2 mM DL-glyceraldehyde 3-phosphate in 0.5 M potassium phosphate buffer (pH 7.6). Scan 2: 5.3 mM DL-glyceraldehyde 3-phosphate in 0.43 M potassium phosphate buffer (pH 7.6) vs. 5.3 mM DL-glyceraldehyde 3-phosphate in 0.43 M potassium phosphate/143 mM Tris buffer (pH 7.6). Scan initiated 1.5 min after mixing the aldehyde and Tris; scanning speed = 0.5 nm/s. Scan 3: Repeat of scan 2, 27.2 min after mixing the aldehyde and Tris. Scan 4: air vs. air.

Discussion

The results of this investigation indicate that the apparent inhibition of glyceraldehyde-3-phosphate dehydrogenase and homoserine dehydrogenase produced by Tris is the direct result of the lowering of the free substrate concentration due to a readily reversible reaction between Tris and the aldehyde substrates of the enzymes. The spectral properties of the final product of the reaction between Tris and glyceraldehyde 3-phosphate are consistent with those of an imine. The observation that the appearance of the 243 nm absorbance band, tentatively assigned to $\equiv C = N-$, closely parallels the disappear-

ance of the carbonyl absorption band at 285 nm suggests that dehydration of the carbinolamine to form the imine is not rate-limiting.

The kinetic analysis performed in this study employed the simplifying assumption that the aldehyde exists exclusively as the free aldehyde in aqueous solution. Trentham, McMurray and Pogson [13] have demonstrated that glyceraldehyde 3-phosphate in aqueous solution exists in equilibrium with its hydrated form, a geminal diol, and that the free aldehyde is the active chemical state of D-glyceraldehyde 3-phosphate in its reaction with glyceraldehyde-3-phosphate dehydrogenase.

$$\begin{array}{l} CH(OH)_2 \\ | \\ CHOH \\ | \\ CH_2OPO_3^{2-} \end{array} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \begin{array}{l} CHO \\ | \\ CHOH \\ | \\ CH_2OPO_3^{2-} \end{array} + H_2O.$$

Furthermore, they have reported that the molar ratio of the geminal diol to the free aldehyde is 29 : 1 at 20°C. If the dehydration of the geminal diol to form free aldehyde is rapid compared to the rate of reaction of Tris with the free aldehyde, the expression for K_{app} for the reaction of the aldehyde with Tris is

$$K_{app} = \frac{(K_H + 1)([S]_0 - [S]_e)}{[S]_e}$$

where K_H = [aldehyde hydrate]/[aldehyde].
The rate of the reaction of Tris with the aldehyde is described by

$$\ln\left(\frac{[S]_0 - [S]_e}{[S]_t - [S]_e}\right) = \left(\frac{k_f'}{K_H + 1} + k_r\right) t = k_{obs} t$$

Hence, the values of K_{app}, K, k_f' and k_f reported in Table II and Figs. 3 and 4 for the reaction of glyceraldehyde 3-phosphate with Tris can be corrected for the existence of the equilibrium between the aldehyde and its hydrate by multiplying each of these constants by the factor (K_H + 1). The value of k_r is not altered by the existence of the equilibrium between the aldehyde and its hydrate. Employing K_H = 29, the value reported at 20°C [13], the corrected values for K and k_f for the glyceraldehyde 3-phosphate reaction with Tris would be $K = 1.1 \cdot 10^4\ M^{-1}$, $k_f = 546\ M^{-1} \cdot min^{-1}$ at pH 7.45 and $k_f = 165\ M^{-1} \cdot min^{-1}$ at pH 8.5. From the corrected k_f' values, the value of $k_{+1} = 8.7 \cdot 10^{-2} \cdot sec^{-1}$ reported for the conversion of the aldehyde hydrate to free aldehyde at 20°C [13], and the equilibrium concentrations of the aldehyde and aldehyde hydrate, it can be calculated that the rate of conversion of the hydrate to free aldehyde was at least 15—56 times greater than the rate of reaction of the free aldehyde with Tris in these experiments. Thus, the assumption that the dehydration of the aldehyde hydrate is rapid relative to the rate of the reaction of Tris with the free aldehyde appears valid. A similar correction for the values reported in Table II for the reaction of Tris with aspartate semialdehyde cannot be made because the extent of hydration of this aldehyde in aqueous solution is not known.

Segal and Boyer [14] have reported that DL-glyceraldehyde 3-phosphate and

DL-glyceraldehyde have half-lives of 10—20 min in 0.01 M Tris, pH 8.4. This half-life range observed by Segal and Boyer is entirely consistent with our results since we would calculate from our kinetic data a half-life of approximately 13 min for glyceraldehyde 3-phosphate in 0.01 M Tris at pH 8.5.

The assay procedure recommended by Sigma Chemical Co. for the determination of glyceraldehyde 3-phosphate concentrations employs glyceraldehyde-3-phosphate dehydrogenase, arsenate, and 0.1 M Tris, pH 8.5. In spite of the reaction of Tris with aldehydes, this assay procedure gives valid results because the reaction between Tris and the aldehyde is readily reversible while the enzyme-catalyzed reaction is irreversible in the presence of arsenate. Thus, the only effect of Tris in this particular assay is to increase the length of time required for the enzyme-catalyzed reaction to reach completion.

Tris buffers have also been employed in both the forward and reverse reaction assays of homoserine dehydrogenase activity as represented by Eqn. 1 [2—5]. The position of equilibrium of the reaction as written in Eqn. 1 lies far to the right [15], i.e.

$$K = \frac{[\text{homoserine}]\,[\text{NADP}^+]}{[\text{aspartate semialdehyde}]\,[\text{NADPH}]\,[\text{H}^+]} = 1.3 \cdot 10^{11}\,\text{M}^{-1}.$$

Thus, in initial velocity studies in the reverse reaction assay, Tris buffer is an excellent buffer to employ since it combines with the aldehyde product formed, thereby serving as an aldehyde trap and delaying the onset of the forward reaction. On the other hand, in the forward reaction assay, Tris buffer, particularly if preincubated with the aldehyde substrate, will lead to erroneous K_m values for the aldehyde as well as reduce the length of time during which appearance of product is approximately linear with time. In the forward reaction active enzyme-centrifugation studies of homoserine dehydrogenase carried out in the presence of Tris buffer [4,5], the Tris and aspartic semialdehyde were, in essence, preincubated together before contacting the enzyme. Calculations based on an equilibrium constant of 68 M^{-1} (Table II) indicate that the concentration of free aspartic semialdehyde present in these active enzyme-centrifugation studies would have been reduced by approximately 40% due to the reaction with Tris. This reduction should have no effect on the results of these studies provided the concentration of free aldehyde substrate remained saturating at all times. In fact, the presence of the product of the Tris-aldehyde reaction could prove advantageous in such experiments by acting as a buffer against changes in the free aldehyde concentration due to utilization by the enzyme.

Thus, although the use of Tris and other amine buffers can lead to complications when used in enzymatic assays employing carbonyl containing substrates, their use can also be advantageous, particularly if the equilibrium and rate constants for their reaction with the carbonyl compounds of interest are known. Not only can they serve as a trap for products containing carbonyl groups, but also they can serve as a buffer of free carbonyl group concentration in those circumstances when it is desirable to have a high concentration of potentially free carbonyl groups while maintaining a lower and relatively constant concentration of free carbonyl groups.

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