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References

Bray, G. (1960), *Anal. Biochem. 1*, 279.
Eckstein, F. (1968), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 2*, 86.
Eckstein, F. (1970), *J. Amer. Chem. Soc. 92*, 4718.
Eckstein, F., and Gindl, H. (1968), *Chem. Ber. 101*, 1670.
Eckstein, F., Saenger, W., and Suck, D. (1972), *Biochem. Biophys. Res. Commun. 46*, 964.
Griffin, B. E., Jarman, M., Reese, C. B., and Sulston, J. E. (1967), *Tetrahedron 23*, 2301.
Irie, M. (1964), *J. Biochem. (Tokyo) 56*, 495.
Irie, M. (1967), *J. Biochem. (Tokyo) 61*, 550.
Richards, F. M., and Wyckoff, H. W. (1971), *Enzymes 4*, 647.
Rüterjans, H., and Pongs, O. (1971), *Eur. J. Biochem. 18*, 313.
Saenger, W., and Eckstein, F. (1970), *J. Amer. Chem. Soc. 92*, 4712.
Smith, M., Moffatt, J. G., and Khorana, H. G. (1958), *J. Amer. Chem. Soc. 80*, 6204.
Takahashi, K. (1970), *J. Biochem. (Tokyo) 67*, 833.
Takahashi, K. (1971a), *J. Biochem. (Tokyo) 69*, 331.
Takahashi, K. (1971b), *J. Biochem. (Tokyo) 70*, 945.
Takahashi, K., Stein, W. H., and Moore, S. (1967), *J. Biol. Chem. 242*, 4682.
Uchida, T., and Egami, F. (1971), *Enzymes 4*, 205.
Usher, D. A. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 661.
Usher, D. A., Erenrich, E. S., and Eckstein, F. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 115.
Usher, D. A., Richardson, Jr., D. I., and Eckstein, F. (1970), *Nature (London) 228*, 663.
Westheimer, F. H. (1968), *Accounts Chem. Res. 1*, 70.

A Kinetic Investigation of the Interaction of Serine Transhydroxymethylase with Glycine†

Chao-Fu Cheng and John L. Haslam*

ABSTRACT: Five elementary reactions have been observed by the temperature-jump method in the serine transhydroxymethylase–glycine system. Kinetic experiments were carried out over a large glycine concentration range (0.5–100 mM), and the reactions were monitored at three wavelengths 495, 425, and 343 nm in order to determine the concentration and wavelength dependence of each step. From the analysis of the kinetic data a bimolecular and four unimolecular reactions are indicated. In the analysis, all of the reactions were considered as part of a single linear reaction mechanism, and rate constants for two of the possible linear mechanisms have been determined. The steps in the enzyme–glycine interaction are discussed in relationship to the kinetic results and to the proposed intermediates in pyridoxal-P-dependent enzyme reactions.

The temperature-jump method has been used to investigate many enzyme–substrate reactions. See, for example, Hammes (1968) and Gutfreund (1971). Among the enzymes studied by this method are several pyridoxal-P-dependent enzymes (Fasella and Hammes, 1967; Hammes and Haslam, 1968, 1969; and Faeder and Hammes, 1970, 1971). Kinetic investigations with these enzymes are facilitated because of the visible absorptions associated with pyridoxal-P, which absorption can be used as indicators for the enzyme reactions. On the basis of the various peaks in the spectra of these enzymes with their substrates several different intermediate structures have been postulated, such as a Schiff base (Metzler, 1957), geminal diamine (O'Leary, 1971) and quinoid-type structures (Jenkins, 1964). Schirch and Mason (1963) showed that addition of glycine to a solution of serine transhydroxymethylase (L-serine:tetrahydrofolate 5,10-hydroxymethyltransferase, EC 2.1.2.1) resulted in a decrease in the enzyme absorption at 425 nm while producing new peaks at 343 and 495 nm. The peak at 425 nm is still observed even when the enzyme is saturated with glycine. These results indicate the formation of three different enzyme-glycine complexes. Schirch and Diller (1971) concluded that a conformational process may also occur in the enzyme–glycine system based on the results of a temperature-dependence study of the absorption changes at 343 and 425 nm. In the work reported here a kinetic study was done to determine the number and nature of elementary reactions involved in the serine transhydroxymethylase–glycine interaction.

Experimental Section

Serine transhydroxymethylase was prepared from fresh iced rabbit livers by the procedure of Schirch and Gross (1968) with the following modifications: the heat step was performed at 63° in the presence of 0.02 M DL-serine, the pH during the first ammonium sulfate precipitation was adjusted to pH 7 by the addition of 1 M potassium hydroxide, the hydroxylapatite column was eliminated, and the enzyme was crystallized by the addition of ammonium sulfate.

Enzyme concentrations were estimated using an extinction coefficient of 0.95 ml/mg cm at 280 nm (Fujioka, 1969). The

† From the Department of Chemistry, University of Kansas, Lawrence, Kansas 66044. *Received April 17, 1972.* Supported by grants from the Research Corporation and the University of Kansas General Research Fund.

TABLE I: Wavelength Dependence of the Five Reciprocal Relaxation Times.[a]

λ (nm)	$1/\tau_1$	$1/\tau_2$	$1/\tau_3$	$1/\tau_4$	$1/\tau_5$
425	I	D	I	I	N
343	D	N	D	I	N
495	N	N	N	I	D

[a] I and D represent an increase and decrease in absorption and N means not observed at this wavelength.

activity of the enzyme was determined by the procedure described by Schirch and Gross (1968) using DL-allothreonine as substrate. The activity in terms of the serine assay was obtained by multiplying this value by four. In the kinetic studies the enzyme concentration was (1–5) × 10^{-2} mM and the activity based on the serine assay was 8–11.

All experiments were performed at 25° in 0.05 M potassium phosphate buffer (pH 7.3).

The temperature-jump apparatus is similar to the one described by Erman and Hammes (1966) and Faeder (1970) except that it is not set up for stopped-flow work. In our modified instrument a single syringe replaces the two-drive syringes. This instrument produces a 7° temperature change (18–25°).

A Beckman DU and Cary 14 spectrophotometer were used in the enzyme–glycine binding constant determinations and in the enzyme assay procedure.

DL-Allothreonine was purchased from Nutritional Biochemical Co.; pyridoxal-P, DL-serine, yeast alcohol dehydrogenase, and NADH grade III were purchased from Sigma Chemical Co. All other chemicals were standard reagent grade.

Results and Treatment of Data

Examples of some relaxation curves observed in the serine transhydroxymethylase–glycine system are presented in Figure 1. The reciprocal relaxation times ($1/\tau$) were obtained from the oscilloscope traces by plotting on semilog paper the difference between the relaxation curve and its equilibrium value (arbitrary units) against time; the slope of the plot is $-1/\tau$. Many of the graphs showed an initial deviation from linearity indicating that another relaxation process was present. The reciprocal relaxation time for this faster step was obtained by replotting the deviant points using the slope of the slower step as the base line. Table I indicates the wavelength dependence for each of the five relaxation processes observed, and Figures 2–4 show the variations of the measured reciprocal relaxation times with total glycine concentration. In all of these experiments, the glycine concentration (0.5–100 mM) was sufficiently large compared with the enzyme concentration ((1–5) × 10^{-2} mM) so that the sum of the equilibrium concentrations of enzyme and glycine is approximately equal to the total glycine concentration. Over 600 separate kinetic experiments were performed on the system to obtain the kinetic data shown in Figures 2–4 with an average of about ten experimental measurements for each point. Listed in Table II are average values of about three relaxation curves taken on the same solution (Cheng, 1972). The experimental error in these experiments is about 15%.

Figure 2 is a plot of a relaxation process designated as $1/\tau_2$, which process shows a linear dependence with glycine

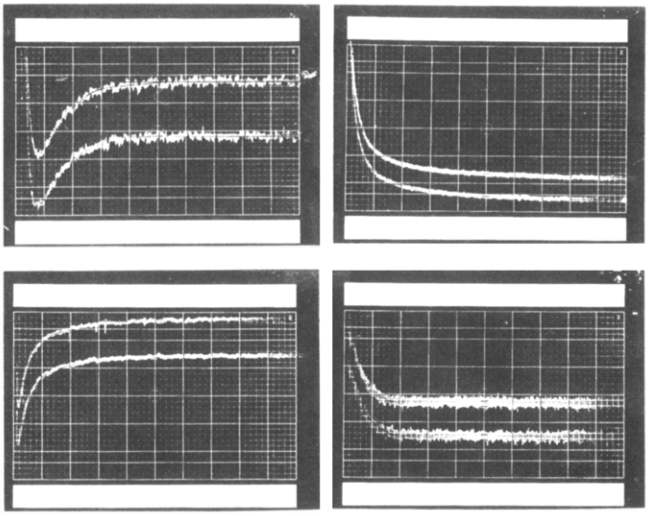


FIGURE 1: Oscilloscope traces of relaxation effects: 0.05 M potassium phosphate buffer (pH 7.3), 25°, enzyme concentration is 2.5 × 10^{-5} M except in the top left picture where it is 3.0 × 10^{-5} M, verticle scale is in arbitrary units of decreasing absorbancy. Top left: horizontal scale is 1 msec per major division, glycine concentration is 7.5 mM, and λ 425 nm. The trace shows two relaxation processes, the shorter is τ_1 and the longer one is τ_2. Bottom left: horizontal scale is 0.5 msec per major division, glycine concentration is 40 mM, and λ 343 nm. The trace shows two relaxation times τ_1 and τ_3. Top right: same as bottom left except λ 425 nm. Bottom right: horizontal scale is 5 msec per major division, glycine concentration is 50 mM, and λ 495 nm. Trace shows τ_4 with some indication of τ_5 at the first.

concentration. This relaxation was observed only at 425 nm. The amplitude for this relaxation process decreased with increasing glycine concentration until it became too small to be observed at approximately 25 mM. Although the glycine concentration range covered for this relaxation process is not as large as for the others, the linearity of the data and the decreasing amplitude indicate a bimolecular reaction (Eigen and de Maeyer, 1963).

Figures 3 and 4 are plots of the four other relaxation processes observed in this system. All of these show an increasing relaxation amplitude with glycine concentration and all are glycine concentration independent in the high concentration

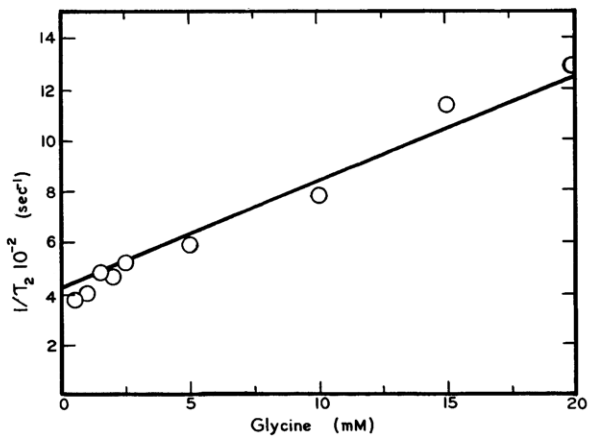


FIGURE 2: Variation of $1/\tau_2$ with glycine concentration. Circles are experimental values obtained at 425 nm. The solid line is a theoretical line based on the mechanism of eq 1 and the rate constants for case II of Table III.

TABLE II: Temperature-Jump Kinetic Data for the Interaction of Serine Transhydroxymethylase with Glycine.[a]

No.	λ (nm)	Glycine (mM)	$1/\tau_1$	$1/\tau_2$	$1/\tau_3$	$1/\tau_4$	$1/\tau_5$	No.	λ (nm)	Glycine (mM)	$1/\tau_1$	$1/\tau_2$	$1/\tau_3$	$1/\tau_4$	$1/\tau_5$
1	425	2		455				60	343	20			2584		
2	425	2		515				61	425	20	7,100		1438		
3	425	2		463				62	425	25	5,227				
4	425	2		476				63	343	25			1897		
5	425	2	8,722					64	425	25				373	
6	425	10	7,962					65	425	50	9,507		1479		
7	425	10	6,950					66	425	50			1850	428	
8	425	10		588				67	425	50			1687	358	
9	425	20	5,564					68	495	50				458	
10	425	20	5,375					69	343	75			1930		
11	425	20	10,645		1604			70	425	75			2000	343	
12	425	5	6,426					71	425	75	7,683		1296		
13	425	15	6,300					72	495	75				279	
14	425	20	6,500					73	343	100			1830		
15	425	25	8,060					74	425	100	7,800		1564		
16	425	50	8,500		2000			75	425	100				435	
17	425	5	7,575					76	425	0.5	5,650				
18	425	5	9,834					77	425	0.5		412			
19	425	5		538				78	495	0.5				188	
20	425	5		535				79	425	1.0	6,433				
21	425	10	7,830					80	425	1.0		424			
22	425	10	8,730					81	492	1.0				203	
23	425	10		646				82	343	1.0				174	
24	343	10				405		83	343	1.0	7,794				
25	425	20	7,633					84	425	2.0	7,103				
26	425	20		1236				85	425	2.0		568			
27	425	20		1337				86	343	2.0	6,906				
28	425	50	9,100		1150			87	343	2.0				310	
29	425	50				413		88	495	2.0				270	
30	425	75	8,550		1530	326		89	495	5.0				260	
31	425	75	7,700		1740			90	425	5.0		627			
32	425	75	6,250		1780	353		91	425	5.0	7,784				
33	425	100			1130			92	343	5.0	7,678				
34	425	100			2300	346	815	93	343	10				236	
35	425	100			1720			94	343	10				266	
36	425	100			1250	394		95	343	10	8,017				
37	425	10	7,251					96	425	10			698		
38	425	10		778				97	425	10	6,970				
39	495	10				205		98	495	10				328	
40	495	50				297		99	495	15				333	
41	425	50	9,250		1178			100	425	15		916			
42	425	50	6,277		1550			101	343	15	7,127				
43	343	100			2160			102	343	15	8,040				
44	495	100				322		103	343	20	7,227				
45	425	100			1700			104	425	20	7,000				
46	425	100	6,350		1529			105	425	10		952			
47	425	0.5	6,705					106	425	10		855			
48	425	0.5		384				107	343	10	6,576				
49	343	0.5				158		108	343	10				327	
50	343	1.0				126		109	495	10				327	
51	425	1.0		419				110	495	20				361	
52	425	1.0	6,793					111	425	20	6,531				
53	495	5.0				103		112	343	20	5,361				
54	425	5.0		588				113	343	30	7,600		1740		
55	425	5.0	8,148					114	343	30	5,683		1254		
56	425	10	5,975					115	425	30	9,688		1980		
57	425	15	5,131					116	425	30	9,183		2150		
58	343	15	5,270					117	425	30	7,045		1144		
59	343	20	8,030		1825			118	495	30				420	

TABLE II (*Continued*)

No.	λ (nm)	Glycine (mM)	$1/\tau_1$	$1/\tau_2$	$1/\tau_3$	$1/\tau_4$	$1/\tau_5$	No.	λ (nm)	Glycine (mM)	$1/\tau_1$	$1/\tau_2$	$1/\tau_3$	$1/\tau_4$	$1/\tau_5$
119	425	40	9,750		1692			150	495	10				260	
120	495	40				454	843	151	425	25	7,432				
121	425	40	9,230		1404			152	425	50	5,958				
122	343	40	9,900		2000			153	495	1.5		480			
123	343	40	8,190		1671			154	425	1.5	6,874				
124	495	40				404		155	425	1.5	7,655				
125	495	40				467	819	156	425	2.0		486			
126	425	50	7,514		1338			157	425	2.0		450			
127	425	50			1098			158	425	2.0	7,546				
128	343	50	6,095		1308			159	425	2.0	6,844				
129	343	100			1788			160	495	2.0				164	
130	425	100			1635			161	425	2.5		626			
131	495	100				420		162	425	2.5		403			
132	425	0.5	4,468					163	425	5.0		809			
133	425	0.5		307				164	425	5.0	7,855				
134	343	0.5				288		165	495	5.0				166	790
135	343	1.0				218		166	425	7.5		1036			
136	425	1.0		326				167	425	10	8,191				
137	425	1.0	4,671					168	425	10		1291			
138	425	1.0		372				169	495	10				180	
139	425	2.0		346				170	343	10	7,547				
140	343	2.0				247		171	425	15	7,503				
141	343	5.0				338		172	425	15	8,000				
142	495	5.0				206		173	425	15	8,130				
143	425	5.0		507				174	495	15				321	867
144	425	5.0		474				175	495	15				285	682
145	425	5.0	6,288					176	425	20	6,839				
146	425	10		608				177	425	20	6,687				
147	425	10		559				178	495	20				301	691
148	425	10	7,027					179	495	25				295	786
149	343	10				378		180	495	25				290	829

[a] The reciprocal relaxation times are averages of about three kinetic experiments at the wavelength and glycine concentration indicated.

region. These characteristics are expected for unimolecular reactions. The fastest relaxation observed is $1/\tau_1$; for this particular step the reaction can be established (from its wavelength and concentration dependence) to be the interconversion of a species absorbing at 425 nm with one absorbing at 343 nm. Another relaxation process, designated as $1/\tau_3$, has the same wavelength dependence as $1/\tau_1$ but is slower and is observed only at glycine concentrations above 10 mM. The wavelength dependence for the particular species involved in this reaction cannot be definitely established.

Two relaxation processes were observed at 495 nm. The slower process ($1/\tau_4$) has the larger amplitude and almost obscures the faster process ($1/\tau_5$) which shows a small but opposite change in absorption at the beginning of the relaxation curves. Unfortunately, $1/\tau_5$ is not observed at either 425 or 343 nm, presumably because of its small amplitude in comparison with those of $1/\tau_1$, $1/\tau_3$, and $1/\tau_4$, all of which are observed at these two wavelengths. The slowest process ($1/\tau_4$) is observed at all three wavelengths as expected. There may be other reactions which were not detected by the temperature-jump method used in this study because the change in absorption for these steps was too small to be detected, or the reactions are not accessible by this method.

The overall binding constant for this system was determined by a titration of the enzyme with glycine following the changes in absorption at 425 and 343 nm. Analysis of the data (Fasella *et al.*, 1966) gave a value of 120 ± 20 M^{-1}. This result can be compared with the value of 169 M^{-1} reported by Schirch and Ropp (1967), in which they measured the binding constant by following absorption changes at 495 nm in solutions of the enzyme with glycine and tetrahydrofolic acid. The reason for the different results is not apparent, but it appears that the two methods give somewhat different results. The overall binding constant is useful in the interpretation of the kinetic results since it places a restraint on the rate constants for a particular mechanism. Acceptable values for the equilibrium constant in respect to a mechanism have been taken to be those within 120 and 169 M^{-1}.

A reaction system of five intermediates represents a large number of possible mechanisms and an even larger number of possibilities exist in order to define the system when the ordering of relaxation times within a particular mechanism is considered. The absence of any concentration dependence for three of the reciprocal relaxation times in the serine transhydroxymethylase–glycine interaction makes this problem more difficult because some concentration dependence for

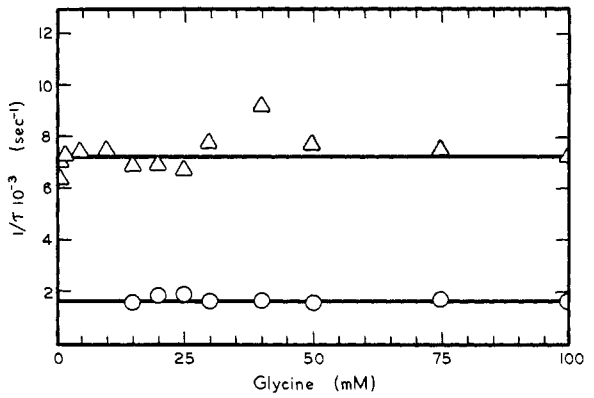


FIGURE 3: Variation of $1/\tau_1$ and $1/\tau_3$ with glycine concentration. The triangles and the circles are the experimental values for $1/\tau_1$ and $1/\tau_3$, respectively. Data are average values at 425 and 343 nm. The solid lines are theoretical lines based on the mechanism of eq 1 and the rate constants for case II of Table III.

TABLE III: Summary of Rate and Equilibrium Constants.

Case	i	k_i (sec^{-1})	k_{-i} (sec^{-1})	K_i	X_i (%)
I	1	4.3×10^4 M^{-1}	3.4×10^2	126 M^{-1}	86.31
	2	4.0×10	6.5×10^2	0.06	5.31
	3	3.9×10^3	2.9×10^3	1.30	7.15
	4	2.2×10^2	1.4×10^3	0.16	1.12
	5	8.7×10	8.7×10^2	0.10	0.11
	1–5			141 M^{-1}[a]	
II	1	4.4×10^4 M^{-1}	3.3×10^2	135 M^{-1}	86.08
	2	4.5×10	3.0×10^2	0.15	12.92
	3	9.0×10	3.3×10^3	0.027	0.34
	4	3.0×10^3	1.7×10^3	1.76	0.61
	5	8.7×10	1.6×10^3	0.056	0.04
	1–5			157 M^{-1}[a]	

[a] Overall equilibrium constant.

the unimolecular steps can be helpful in establishing a mechanism.

If all of the reactions are considered as part of a single reaction scheme, it can be shown that for any mechanism to fit the experimental data the slowest step ($1/\tau_4$) must follow the bimolecular step ($1/\tau_2$). If this were not the case, then $1/\tau_5$, $1/\tau_3$, or $1/\tau_1$, whichever followed the bimolecular step but preceded $1/\tau_4$, would show a concentration dependence as the value of $1/\tau_2$ approached it. This does not occur and helps to eliminate many possible reaction mechanisms. The linear mechanism shown in eq 1 is the simplest to analyze in order to determine whether such a mechanism will fit the experimental data, but even in this case the kinetic data alone cannot distinguish between the ordering of the remaining three relaxation steps.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} X_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} X_3 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} X_4 \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} X_5 \qquad (1)$$

On the basis of the wavelength dependence of these three relaxation processes, their relative values, and the assumption that each intermediate has an absorption at only one of the three wavelengths investigated, X_3 and X_4 can be eliminated as the species absorbing at 495 nm since at least three intermediates in succession each with an absorption at 343 or 425 nm (including one of each) are required to incorporate $1/\tau_1$ and $1/\tau_3$ in reaction 1.

This leaves the possibility of X_2 or X_5 as the 495-nm absorbing species. The mechanism in which X_5 represents the 495-nm species is considered as the most likely linear mechanism based on the kinetic studies with aspartate transaminase as will be discussed later. Two mechanisms which represents the sequence for the last three steps as $1/\tau_1$, $1/\tau_3$, and $1/\tau_5$, and as $1/\tau_3$, $1/\tau_1$, and $1/\tau_5$ are given in Table III as case I and case II, respectively. Although it is difficult to assign relaxation times to specific steps in a coupled system, the assignments given above are based on a comparison of the experimental reciprocal relaxation times and those calculated from the noncoupled equations using the rate constants given in Table III. The sequences given above are those which give the best agreement on this basis.

The procedure for calculating the rate constants for a linear mechanism have been described (Hammes and Haslam, 1969; Haslam, 1972). By the procedure the rate constants and the theoretical reciprocal relaxation times (as a function of substrate concentration) can be calculated, from which results a comparison can be made between the experimental and calculated values based on a given set of rate constants. The calculated values for the reciprocal relaxation times are presented as the solid lines in Figures 2–4 for case II. The agreement between the calculated and experimental results is quite good for all but $1/\tau_2$ and $1/\tau_4$, in which instances the curves could reproduce the data better. The results for case I and case II are the same except that the case I values for $1/\tau_4$ are 14% lower than those of case II. The calculated and experimental results are not sufficiently different to eliminate these mechanisms considering the experimental uncertainty in the data and the difficulty in analyzing all five relaxation processes together as a coupled system.

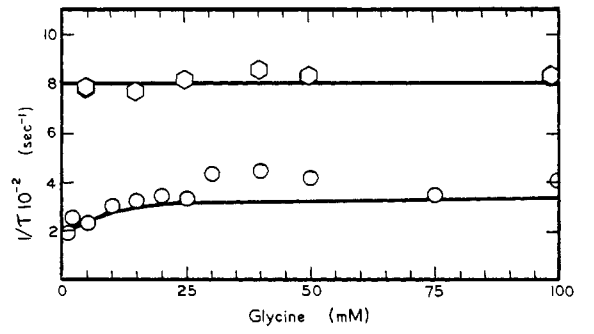


FIGURE 4: Variation of $1/\tau_4$ and $1/\tau_5$ with glycine concentration. The circles and the hexagons are the experimental values for $1/\tau_4$ and $1/\tau_5$, respectively. Data for $1/\tau_4$ are average values at 425, 495, and 343 nm while for $1/\tau_5$ only 495-nm data are represented. The solid lines are theoretical lines based on the mechanism of eq 1 and the rate constants for case II of Table III.

Discussion

On the basis of the present data a unique mechanism cannot be determined, but the first two steps appear to be a bimolecular reaction followed by a slow unimolecular reaction step. The five reactions observed in the interaction of glycine with serine transhydroxymethylase bear some resemblance to the reactions that have been observed with *erythro*-β-hydroxy-

aspartic acid and aspartate transaminase (Hammes and Haslam, 1969). The number of reactions in the serine transhydroxymethylase–glycine interactions is one more than the number observed in the transamination reaction up to the formation of the quinoid-type intermediate. Aspartate transaminase is also a pyridoxal-P-containing enzyme and shows absorptions at 495, 430, 360, and 330 nm with *erythro*-β-hydroxyaspartic acid. On this basis one might expect that several similar structures would be formed in these two enzyme systems. These intermediates may be any or all of the intermediates represented in Figure 5. An interesting difference is observed in the rate of the bimolecular step for these enzymes. It has been estimated that the natural substrates of aspartate transaminase react with the enzyme at rates approaching diffusion-controlled values ($\sim 10^8$ M^{-1} sec^{-1}, Fasella and Hammes, 1967), while the substrate analogs, *erythro*-β-hydroxyaspartic acid and α-methylaspartic acid, have rate constants of about 10^6 and 10^4 M^{-1} sec^{-1}, respectively (Hammes and Haslam, 1968, 1969). The rate constant in the serine transhydroxymethylase–glycine reaction is about 5×10^4 M^{-1} sec^{-1}. This appears to be an unusually low value for a natural substrate and may indicate a substantial steric or energy requirement in this case. Several other possible explanations are: the enzyme may exist in two rapidly interconverting forms with glycine reacting only with a form present in very low concentration. If this were the case, the true rate constant would be equal to the observed rate constant multiplied by the factor of one plus the ratio of inactive to active enzyme. Likewise, if only one form of glycine reacts with the enzyme, say the anion form which at pH 7.3 represents only 0.005 of the total glycine concentration, the value of the bimolecular rate constant would be about 10^7 M^{-1} sec^{-1}, a value similar to those reported for several other enzymes (Hammes, 1968). At present it is not possible to substantiate any of these possibilities.

The last column of Table III lists the calculated percentages of each of the intermediates for case I and II. Both predict that a very large percentage of the enzyme–glycine complexes exist in the form of the first intermediate with much smaller amounts for the remaining four intermediates. The amounts of enzyme–glycine complex for the last intermediate in case I as well as the last three for case II appear to be too small. For example, the extinction coefficient, calculated on the basis of X_5 being the 495-nm species using the percentages of Table III, and the absorption of a glycine-saturated enzyme solution at 495 nm give values unbelievably large. A value of 0.8% would be needed for X_5 to give the value of 118,000 M^{-1} cm^{-1} reported for the 492-nm species in aspartate transaminase (Hammes and Haslam, 1969). These results do call into question the validity of X_5 being the last intermediate in the reaction mechanism, but the results from the kinetic studies on aspartate transaminase indicate that this intermediate is not formed early in the reaction; it was on this basis that the last intermediate instead of the second was chosen as the most likely 495-nm species in the linear mechanism.

Some of the proposed structures involved in pyridoxal-P–enzyme reactions are shown in Figure 5. Schiff base formation between the pyridoxal-P and an ε-amino group of a lysine residue of the enzyme as well as with several amino acid substrates has been established for some enzymes (Fasella, 1967).

These two structures are represented in A and C of Figure 5 in which glycine is shown as the substrate. A possible intermediate occurring between these two structures is the geminal diamine structure B. O'Leary (1971) provides some evidence

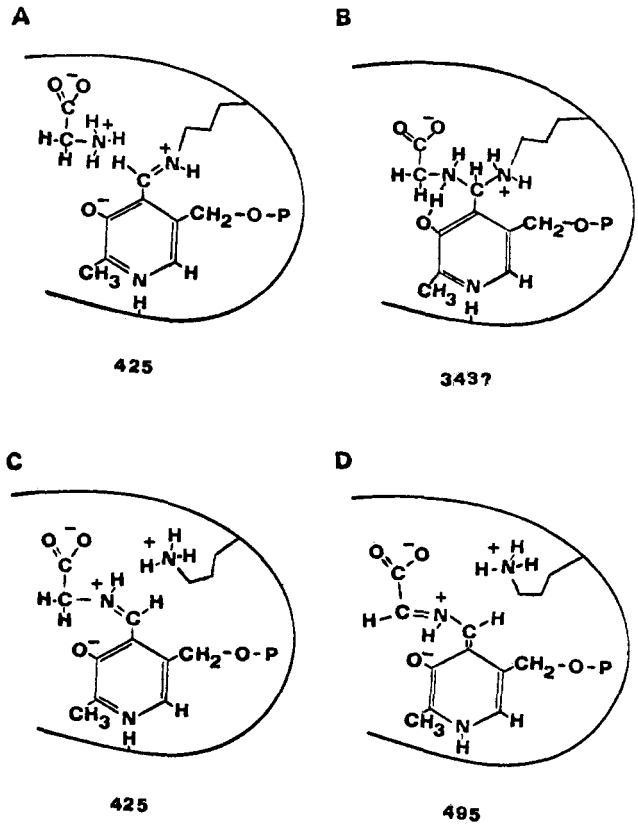


FIGURE 5: Schematic representation of some possible reaction intermediates in the serine transhydroxymethylase–glycine interaction. A. Enzyme in the form of a Schiff base with an ε-amino group of a lysine residue and glycine bound at a possible anion binding site. B. Geminal diamine. C. Schiff base with glycine. D. Quinoid-type structure.

that such a structure may have an absorption around 330 nm. The identification of the 343-nm species in the serine transhydroxymethylase–glycine interaction has not been established but may represent this type of intermediate. The geminal diamine structure has recently been questioned as an intermediate in pyridoxal-P–enzyme reactions. Abbott and Martell (1971) concluded from a nuclear magnetic resonance (nmr) model study that such an intermediate may not occur or if it is formed may not be of importance catalytically in enzyme reactions.

The quinoid-type structure D is characterized by its absorption at wavelengths around 500 nm. It has been shown by Schirch and Jenkins (1964) that the addition of tetrahydrofolic acid to a solution of serine transhydroxymethylase and glycine results in the stabilization of this intermediate indicated by the very large increase in the absorption at 495 nm, and there is also a large increase in the rate of incorporation of tritium into glycine from tritated water. Jordan and Akhtar (1970) have also established that the tritium is lost stereospecifically from [2*S*-^{3}H]glycine to the solvent. It would appear that this intermediate is the most likely candidate to be the active species in the next step in the reactions to form L-serine.

The kinetic results show that five reactions occur in the serine transhydroxymethylase–glycine interaction which means that even if all of the structures shown in Figure 5 occur there is still one intermediate unaccounted for. This may prove to be the conformational change suggested previously by Schirch and Diller (1971). It would be tempting to speculate at this point on a possible sequence of structures

that occur in the reaction but further information is needed to establish a definite mechanism and to identify the reaction intermediates.

References

Abbott, E. H., and Martell, A. E. (1971), *J. Amer. Chem. Soc. 93*, 5852.

Cheng, C. F. (1972), Master's Thesis, University of Kansas.

Eigen, M., and de Maeyer, L. (1963), *in* Technique of Organic Chemistry, Vol. VIII, Part II, Friess, S. L., Lewis, E. S., and Weissberger, A., Ed., New York, N. Y., Interscience.

Erman, J. E., and Hammes, G. G. (1966), *Rev. Sci. Inst. 37*, 746.

Faeder, E. J. (1970), Ph.D. Thesis, Cornell University.

Faeder, E. J., and Hammes, G. G. (1970), *Biochemistry 9*, 4043.

Faeder, E. J., and Hammes, G. G. (1971), *Biochemistry 10*, 1041.

Fasella, P. (1967), *Annu. Rev. Biochem. 36*, 185.

Fasella, P., Giartosio, A., and Hammes, G. G. (1966), *Biochemistry 5*, 197.

Fasella, P., and Hammes, G. G. (1967), *Biochemistry 6*, 1798.

Fujioka, M. (1969), *Biochim. Biophys. Acta 185*, 338.

Gutfreund, H. (1971), *Annu. Rev. Biochem. 40*, 315.

Hammes, G. G. (1968), *Accounts Chem. Res. 1*, 321.

Hammes, G. G., and Haslam, J. L. (1968), *Biochemistry 7*, 1519.

Hammes, G. G., and Haslam, J. L. (1969), *Biochemistry 8*, 1591.

Haslam, J. L. (1972), *J. Phys. Chem. 76*, 366.

Jenkins, W. T. (1964), *J. Biol. Chem. 239*, 1742.

Jordan, P. M., and Akhtar, M. (1970), *Biochem. J. 116*, 277.

Metzler, D. E. (1957), *J. Amer. Chem. Soc. 79*, 485.

O'Leary, M. (1971), *Biochim. Biophys. Acta 242*, 484.

Schirch, L., and Diller, A. (1971), *J. Biol. Chem. 246*, 3961.

Schirch, L., and Gross, T. (1968), *J. Biol. Chem. 243*, 5651.

Schirch, L., and Jenkins, W. T. (1964), *J. Biol. Chem. 239*, 3801.

Schirch, L., and Mason, M. (1963), *J. Biol. Chem. 238*, 1032.

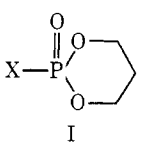
Schirch, L., and Ropp, M. (1967), *Biochemistry 6*, 253.

Inhibition of Cholinesterase by 1,3,2-Dioxaphosphorinane 2-Oxide Derivatives†

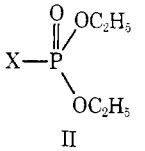
Yacov Ashani, S. L. Snyder, and Irwin B. Wilson*

ABSTRACT: Eel acetylcholinesterase, previously inhibited with 2-chloro-1,3,2-dioxaphosphorinane 2-oxide, was found to reactivate spontaneously with $t_{1/2} \cong 12$ min at pH 7.0, in marked contrast to enzyme inhibited with diethyl phosphoryl derivatives. The spontaneous recovery rate was taken into account in formulating a scheme for the inhibition of the enzyme by the 2-fluoro and 2-*p*-nitrophenoxy derivatives. Inhibition by the chloridate needed a special treatment due to its high hydrolysis rate in aqueous solutions. 2-Fluoro- and 2-*p*-nitrophenoxy-1,3,2-dioxaphosphorinane 2-oxides are poorer inhibitors than the open-chain compounds, whereas the 2-chloro derivative has almost the same activity as *O,O*-diethyl phosphorochloridate. A two-step reaction is suggested for the inhibition of acetylcholinesterase by the ring and open-chain phosphates.

Fukuto and Metcalf (1965) described the preparation and hydrolysis of 1,3,2-dioxaphosphorinane 2-oxides (I) in which X equals Cl, F, and *p*-nitrophenoxy. The anticholinesterase activities of the three compounds were measured



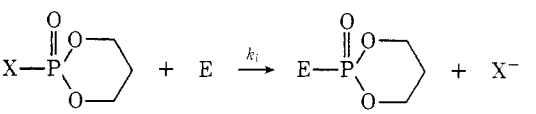
I



II

in terms of I_{50} values using fly head cholinesterase, and these values were compared with the I_{50} value of the open-chain analog *O,O*-diethyl phosphoro-*p*-nitrophenolate (paraoxon) II, X = *p*-nitrophenoxy.

They found that even though the rates of the reactions of the 2-fluoro- and 2-*p*-nitrophenoxy-1,3,2-dioxaphosphorinane 2-oxides with hydroxide ion are comparable to the corresponding open-chain compounds (see also Kahan and Kirby, 1970), the anticholinesterase activities of the cyclic compounds were relatively very poor. Since the reaction of the inhibitor and the enzyme is "formally" a second-order nucleophilic substitution reaction analagous to the reaction of the



inhibitor with hydroxide ion, it seemed interesting to reinvestigate this problem in somewhat greater detail.

We did make a surprising observation. We found that the phosphorylated enzyme (electric eel) (2-enzymo-1,3,2-dioxaphosphorinane 2-oxide) hydrolyzes rather rapidly in water ($t_{1/2} \approx 12$ min) to restore active enzyme in marked contrast to the open-chain *O,O*-diethylphosphoryl-enzyme. This

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