Studies of the Reactions of Substituted D,L-*erythro*-β-Phenylserines with Lamb Liver Serine Hydroxymethylase. Effects of Substituents upon the Dealdolization Step†

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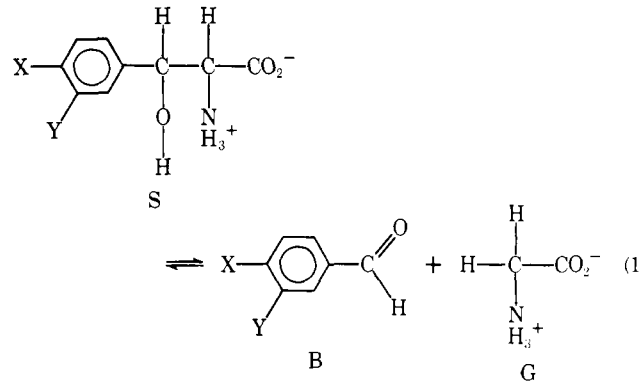
ABSTRACT: Serine hydroxymethylase catalyzes the cleavage of substituted β-phenylserines (S) to form the substituted benzaldehydes (B) and glycine (G). The occurrence in this pathway of a pyridoxal 5′-phosphate (PLP) stabilized quinonoid intermediate with an absorbance maximum at 500 nm (EQ_{500}) has enabled pre-steady-state kinetics studies. The results are consistent with the following minimal kinetic mechanism:

$$E + S \underset{k_{-1}/K_1}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} B + EQ_{500} \xrightarrow{k_3} G + E$$

The rate constants k_2, k_{-2}, and k_3 were evaluated from steady-state kinetic and absorbance measurements and from the dependence of k_{obsd} for the exponential establishment of the steady state with respect to EQ concentration upon the concentrations of S and B. The pre-steady-state rate equation is: $k_{obsd} = k_2\{[S]/([S] + K_1)\} + k_{-2}[B] + k_3$. The Hammett ρ values for the reaction of substituted β-phenylserines with serine hydroxymethylase for k_2 and k_3 are -0.94 ± 0.21, and 0, respectively. The latter ρ value is consistent with a common step in the reaction of all of these substituted β-phenylserines, namely, protonation of the enzyme bound glycine carbanion (quinonoid intermediate). Further support for this assignment is the observation of a solvent kinetic deuterium isotope effect of 6.25 for $k_3(H_2O)/k_3(D_2O)$ consistent with a proton-transfer step. The coincidence in the rates of *p*-hydroxy-*m*-methoxybenzaldehyde appearance (monitored at 370 nm during the serine hydroxymethylase catalyzed cleavage of D,L-*p*-hydroxy-*m*-methoxy-*erythro*-β-phenylserine) with the rate of approach to the steady state with respect to EQ concentration (monitored at 500 nm) indicates that the rate constant k_2 applies to the C_α–C_β bond cleavage step. The ρ value for k_2 indicates significant stabilization in the transition state for the C–C bond cleavage step by electron donating substituents. The ρ value for the transimination sequence, $1/K_1$, of about 0.96 ± 0.25 suggests that complete or almost complete alkoxide formation at the β-OH has already occurred prior to the step involving C_α–C_β bond cleavage. Free formaldehyde has been shown to react with the enzyme bound glycine carbanion in the absence of tetrahydrofolic acid (THF) indicating that activation of formaldehyde by THF is *not* a prerequisite for C_α–C_β bond formation to form serine.

Serine hydroxymethylase (EC 2.1.2.1) purified from mammalian liver catalyzes the reversible cleavage of meta- and para-substituted β-phenylserines to the corresponding benzaldehyde and glycine (eq 1) (Ulevitch and Kallen, 1977a; Schirch and Diller, 1971). Since no previous studies have dealt directly with the pre-steady-state and steady-state kinetics and the mechanism of serine hydroxymethylase catalyzed dealdolization reactions of β-phenylserines, a detailed investigation of these reactions was undertaken.

These studies, utilizing β-phenylserines as substrates, possessed the following advantages: (1) the absorbance of the product, benzaldehyde or substituted benzaldehyde, provides a direct spectrophotometric assay; (2) the chromophoric prosthetic group of serine hydroxymethylase provides the spectrophotometrically detectable enzyme-substrate intermediates such as ES (I) and EQ_{500} (II) which facilitate pre-steady-state kinetic studies (Ulevitch and Kallen, 1977a; Schirch and Jenkins, 1964; Schirch and Diller, 1971); and (3) studies of the enzyme-catalyzed dealdolization of meta- and para-substituted β-phenylserines provide an opportunity for a structure-reactivity correlation. The results of these investigations comprise the present report.



(1)

Experimental Section

Materials. Serine hydroxymethylase (2.3 U/mg) was purified and stored as previously described (Ulevitch and Kallen, 1977a). D,L-*erythro*-β-Phenylserine and D,L-*p*-nitro-*erythro*-β-phenylserine were synthesized according to the

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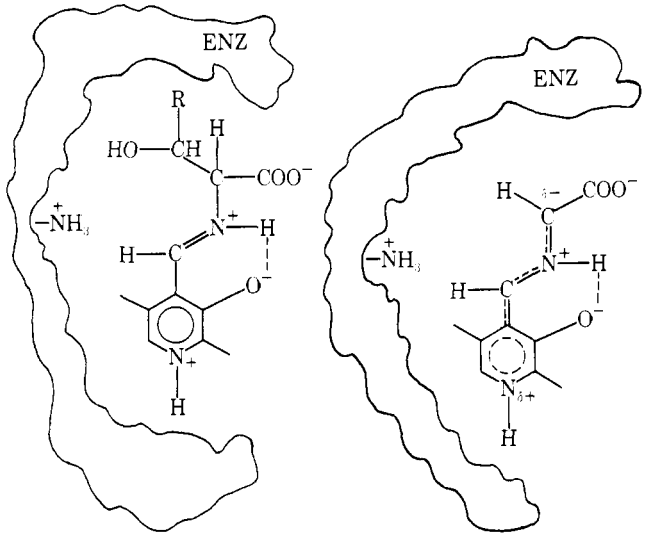
TABLE I: Molar Absorptivities of Meta- and Para-Substituted Benzaldehydes.[a]

Substituents		Wavelength	$\Delta\epsilon^b$	
Para-	Meta	(nm)	(M^{-1} cm^{-1})	Ref
H-	H-	279	1400	*c, d*
HO-	CH_3O-	375	6240[f]	*d*
CH_3O-	H-	310	3000	*d*
Cl-	H-	292	1910	*e, d*
NO_2-	H-	276	3930	*d*
Cl-	Cl-	290	1800	*d*

[a] pH 7.50, 25 °C, ionic strength maintained at 0.1 M with Na_2SO_4. [b] $\Delta\epsilon$ is the (ϵ of the substituted benzaldehyde − ϵ of the parent amino acid). These ϵ values for the substituted benzaldehydes from the literature were in satisfactory agreement with determinations done in this laboratory. [c] Forbes et al. (1958). [d] Ulevitch (1971). [e] Doub and Vandenbelt (1955). [f] $\Delta\epsilon$ = 8140 M^{-1} cm^{-1} at 370 nm, pH 8.00, 25 °C, ionic strength 0.1 M.



ES (I) EQ_{500} (II)

methods of Shaw and Fox (1953) and Holland et al. (1953), respectively, D,L-*p*-hydroxy-*m*-methoxy-*erythro*-β-phenylserine was a gift of Hoffmann-La Roche, and D,L-*p*-methoxy-, D,L-*p*-chloro-, and D,L-3,4-dichloro-*erythro*-β-phenylserine were generously provided by Dr. S. H. Pines (Merck, Sharp and Dohme).

The difference in molar absorptivities between product benzaldehyde and amino acid reactant ($\Delta\epsilon$) utilized to calculate the benzaldehyde concentration in measurements of the initial velocity of β-phenylserine breakdown are contained in Table I. *p*-Hydroxy-*m*-methoxy-, *p*-nitro-, and *p*-methoxybenzaldehydes were of reagent grade and were utilized without further purification. 3,4-Dichlorobenzaldehyde and 4-chlorobenzaldehyde were purified by sublimation under reduced pressure. Benzaldehyde (Aldrich) was purified by vacuum distillation and acetaldehyde was purified by distillation. Other materials of reagent grade were utilized without further purification. Reagent solutions were made fresh daily in deionized water of greater than 5×10^5 ohms cm specific resistance containing 10^{-3} M EDTA.

The source and mode of employment of D_2O have been described (Ulevitch and Kallen, 1977b).

Methods. Initial velocity measurements were obtained and analyzed as previously described (Ulevitch and Kallen, 1977a). The initial rate of *p*-nitrobenzaldehyde production from D,L-*p*-nitro-*erythro*-β-phenylserine was measured at 276 nm in 3.0-mL cells with Pyrocell quartz inserts (pathlength 0.05 cm). Other initial velocity measurements were performed in 1.0-mL quartz cells (pathlength, 1.0 cm) at the wavelengths specified in Table I.

Stopped-flow experiments were performed in a thermostated Durrum-Gibson stopped-flow spectrophotometer equipped with 2-cm lightpath and maintained at 25± 0.1 °C. Transmittance values obtained from oscilloscope traces were converted to ΔA values and analyzed as described elsewhere (Kallen and Jencks, 1966a; Kallen, 1971b) unless otherwise noted.

Stoppered split compartment mixing cells (Pyrocell) with pathlength 0.88 cm after mixing and a Gilford 2000 multiple sample absorbance spectrophotometer with a thermostated cell compartment maintained at 25 ± 0.1 °C were utilized for difference spectra measurements. Kinetic and spectral measurements were performed in 0.05 M Hepes,[1] 0.001 M EDTA, ionic strength 0.1 M with Na_2SO_4. Values of pH measured at the conclusion of kinetic and spectral experiments were within ±0.03 pH unit of the expected value. The p*K* values for the phenolic proton dissociation constants of *p*-hydroxy-*m*-methoxybenzaldehyde and D,L-*p*-hydroxy-*m*-methoxy-*erythro*-β-phenylserine, obtained by spectrophotometric titrations at 375 nm, ionic strength 0.1 M, 25 °C, as described elsewhere (Kallen and Jencks, 1966b; Viale and Kallen, 1971), were 7.22 and 9.55, respectively.

The final concentrations of benzaldehyde and acetaldehyde solutions were determined enzymatically with horse liver alcohol dehydrogenase (Boehringer) and NADH at 340 nm, pH 7.50, 0.05 M Hepes, 0.001 M EDTA, 0.025 M Na_2SO_4, 25 °C.

The estimated error limits for equilibrium and kinetic constants in this study are ±10% unless noted otherwise.

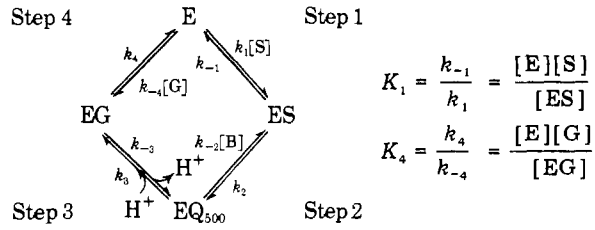
Pre-Steady-State Kinetic Study of Product Release from D,L-p-Hydroxy-m-methoxy-erythro-β-phenylserine. The release of *p*-hydroxy-*m*-methoxybenzaldehyde from D,L-*p*-hydroxy-*m*-methoxy-*erythro*-β-phenylserine was measured by stopped-flow spectrophotometry at 370 nm, 25 °C, pH 8.00. The pseudo-first-order flow rate constant describing the burst of product release (Figure 5A) was calculated from a plot of log {$A_{370,t}$ (from extrapolated steady-state absorbance) − $A_{370,t}$ (from burst absorbance)} vs. time (inset, Figure 5B).

Results

Results from steady-state kinetics, pre-steady-state kinetics, and steady-state absorbance measurements provide experimental data in support of the minimal kinetic scheme for the serine hydroxymethylase catalyzed dealdolization of D,L-

[1] Abbreviations: B, benzaldehyde; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; E_t, total tetrameric enzyme; G, glycine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; PLP, pyridoxal 5'-phosphate; S, substrate; THF, tetrahydrofolic acid; TN, turnover number; NADH, reduced nicotinamide adenine dinucleotide.

SCHEME I



$$K_1 = \frac{k_{-1}}{k_1} = \frac{[E][S]}{[ES]}$$

$$K_4 = \frac{k_4}{k_{-4}} = \frac{[E][G]}{[EG]}$$

erythro-β-phenylserine (Scheme I) where ES and EG are the enzyme bound Schiff bases of pyridoxal 5′-phosphate and β-phenylserines and glycine, respectively, EQ_{500} is the enzyme bound quinonoid intermediate (II) that is responsible for the 500 nm absorption band, and B and G are benzaldehydes and glycine, respectively.

Pre-Steady-State Kinetics for the Approach to Steady State with Respect to EQ Concentration. A new absorbance peak appears at 500 nm when D,L-*erythro*-β-phenylserine is mixed with serine hydroxymethylase (Ulevitch and Kallen, 1977a). The rate of approach to steady state with respect to EQ concentration measured by stopped-flow spectrophotometry is described by an exponential increase in the absorbance at 500 nm (Figure 1A). With the application of the rapid equilibrium assumptions for steps 1 and 4 of Scheme I, in the absence of added glycine and with the irreversibility of step 3 under these conditions, the rate constant, k_{obsd}, for the pre-steady-state appearance of EQ_{500} from β-phenylserine is given by eq 2 where S is a β-phenylserine[2] (see Appendix):

$$k_{obsd} = k_2\{[S]/([S] + K_1)\} + k_{-2}[B] + k_3 \qquad (2)$$

In the absence of added benzaldehyde,[3] since $k_{-2}[B] \ll k_3$, the $k_{-2}[B]$ term is insignificant. From the rectangular hyperbola with a nonzero ordinate intercept for the dependence of k_{obsd} upon L-*erythro*-β-phenylserine concentration, values of K_1, k_2, and k_3 may be calculated (Figure 1B). At $[S] \gg K_1$, eq 2 may be rewritten as eq 3.

$$k_{obsd} = k_2 + k_3 \qquad (3)$$

$$\lim [S] \rightarrow \infty$$

Effect of Carbonyl Compounds on the Pre-Steady-State Kinetics for the Approach to Steady State with Respect to EQ Concentration. In accord with eq 2, the rate constant for the approach to steady state with respect to EQ concentration is linearly dependent upon the carbonyl compound concentration at a fixed β-phenylserine concentration (Figure 2). The slope of the plot of k_{obsd} vs. benzaldehyde concentration yields a value of 4.1×10^4 M^{-1} s^{-1} for the second-order rate constant, k_{-2}. The second-order rate constants, k_{-2}, for formaldehyde and acetaldehyde with respect to the unhydrated aldehyde are 3.6×10^6 M^{-1} s^{-1} and 7×10^4 M^{-1} s^{-1}, respectively. Hydration constants of 2270 (Valenta 1960) and 1 (Lienhard and Jencks, 1966) for formaldehyde and acetaldehyde, respectively

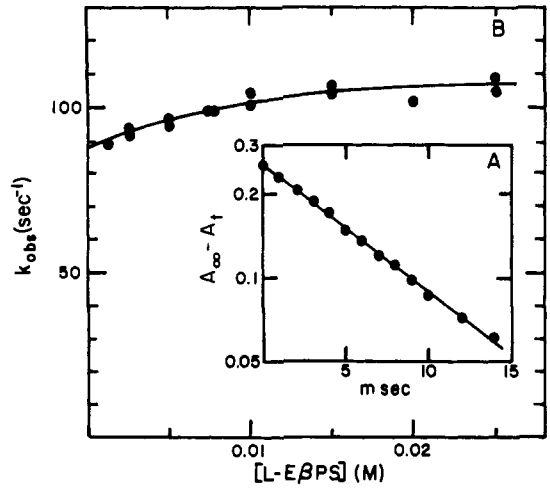


FIGURE 1: (A) Appearance of EQ_{500}; change in absorbance vs. time. Concentration after mixing: serine hydroxymethylase, 14 μM active site concentration, 0.025 M L-*erythro*-β-phenylserine, pH 7.50, 25 °C, ionic strength 0.1 M. A_∞ and A_t are the absorbance values in the steady state and at time (t) after the flow ceased, respectively, $k_{obsd} = 110$ s^{-1}. (B) The dependence of the pseudo-first-order rate constant, k_{obsd}, for the appearance of EQ_{500} on L-*erythro*-β-phenylserine at 25 °C, pH 7.50, ionic strength 0.1 M. Concentration after mixing: serine hydroxymethylase, 2.3 u.mg, 14 μM active site concentration; (●) each experimental point calculated from two superimposable stopped-flow tracings; (—) calculated from eq 2 and $K_1 = 0.009$ M, $k_2 = 28$ s^{-1}, and $k_3 = 85$ s^{-1}.

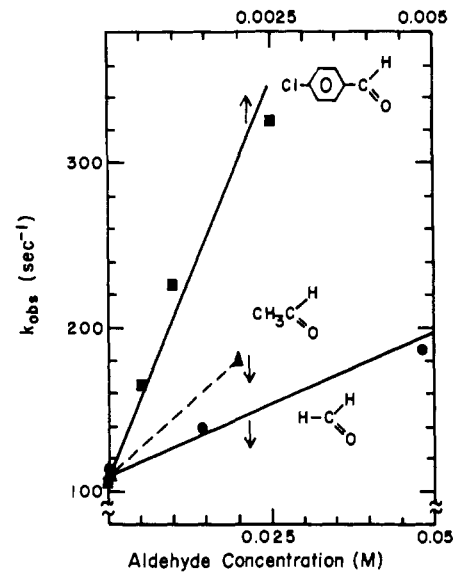


FIGURE 2: The effect of added formaldehyde (●—●), acetaldehyde (▲- - -▲), and benzaldehyde (■—■) upon the pseudo-first-order rate constant for the appearance of EQ_{500}; at 25 °C, pH 7.50, 0.05 M Hepes, 0.001 M EDTA, 0.025 M Na_2SO_4; 0.025 M L-*erythro*-β-phenylserine, 14 μM serine hydroxymethylase, ionic strength 0.1 M. The second-order rate constants for the attack of EQ_{500} on the unhydrated carbonyl compound, k_{-2}, calculated from eq 2 are 3.6×10^6 M^{-1} s^{-1}, 7×10^4 M^{-1} s^{-1}, and 4.1×10^4 M^{-1} s^{-1}, for formaldehyde, and benzaldehyde, respectively. The concentration of aldehyde is total aldehyde (i.e., hydrated plus unhydrated aldehyde); (upper abscissa) benzaldehyde; (lower abscissa) acetaldehyde and formaldehyde.

(K_h = [hydrated aldehyde]/[unhydrated aldehyde]), were used to calculate the unhydrated aldehyde concentrations. The fact that the dependencies of k_{obsd} upon carbonyl compound (e.g., benzaldehyde) concentration are linear indicates that no stable complexes are formed between these carbonyl compounds and serine hydroxymethylase and they are therefore eliminated from kinetic schemes in this paper (cf. Chen and Schirch, 1973b,c).

[2] The magnitude of these constants and the concentration of glycine generated during the time course of the pre-steady-state kinetic experiments are such that there is no significant perturbation of the kinetics due to the accumulation of the product, glycine (i.e., eq 2 applies).

[3] The turnover number of the enzyme at pH 7.50 is 22 s^{-1} per active site and a concentration of 14 μM active sites was used in the stopped-flow experiments measuring the rate of apperance of EQ_{500} from D,L-*erythro*-β-phenylserine. Therefore only 300 μM benzaldehyde could be produced in 1 s (at $[S] \gg K_1$) and since the duration of the stopped-flow experiments was at most 100 ms, 30 μM is the maximum concentration of benzaldehyde present during a stopped-flow experiment. Since k_{-2} is about 10^4 M^{-1} s^{-1} (see below), the assumption that $k_{-2}[B] \ll k_2 + k_3$ holds and the reduction of eq 2 is valid.

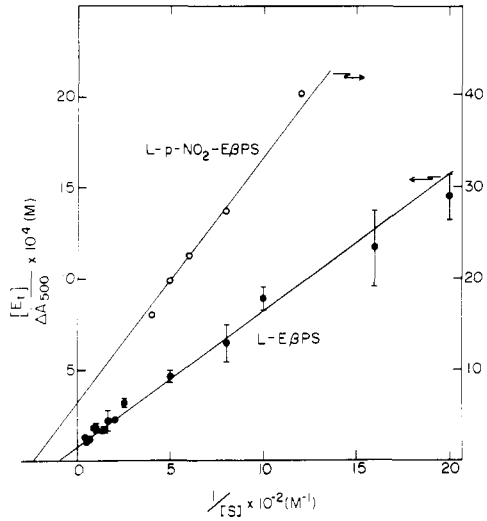


FIGURE 3: Double-reciprocal plot of the change in absorbance at 500 nm against substituted β-phenylserine concentration at pH 7.50, 25 °C, ionic strength 1.0 M. (●) L-*erythro*-β-Phenylserine and serine hydroxymethylase (2.3 U/mg), 14 μM active site concentration. (○) L-*erythro*-β-*p*-Nitrophenylserine and serine hydroxymethylase, 152 μM active site concentration. The solid lines are calculated from eq 5: the values of the constants K_1, k_2, k_3, and ϵ_{500}^{EQ} for the unsubstituted compound are contained in Table II and for the *p*-NO_2 derivative are 4.5×10^{-3} M, 3.5 s^{-1}, 84 s^{-1}, and 4.0×10^4 M^{-1} cm^{-1}, respectively. (Left ordinate) L-*erythro*-β-Phenylserine; (right ordinate) L-*erythro*-β-*p*-nitrophenylserine.

TABLE II: Rate and Dissociation Constants for the Serine Hydroxymethylase Catalyzed Dealdolization of D,L-*erythro*-β-Phenylserine, 25 °C, pH 7.50, Ionic Strength 0.1 M.[a]

Constant	Method of determination	Value
K_1[b] (M)	Steady-state kinetics	0.013
	Steady-state absorbance	0.014
	Pre-steady-state kinetics	0.014
K_4 (M)	Steady-state absorbance and K_i for glycine	0.011
k_2 (s^{-1})	Pre-steady-state kinetics	43 ± 4.1
k_{-2} (M^{-1} s^{-1})	Pre-steady-state kinetics	4.1×10^4
k_3 (s^{-1})	Pre-steady-state kinetics	84 ± 2.4[e]
k_{-3} (s^{-1})	Steady-state absorbance	0.4
Molar absorptivity[c] of EQ_{500}; ϵ_{500}^{EQ} (M^{-1} cm^{-1})	Pre-steady-state kinetics and steady-state absorbance	4.0×10^4 [e]
TN[d] (s^{-1})	Steady-state kinetics	22
	Pre-steady-state kinetics	28 (calcd)
K_m[b] (M)	Steady-state kinetics	0.0095
	Pre-steady-state kinetics	0.0092 (calcd)

[a] Scheme I. [b] Expressed for the concentration of L isomer of *erythro*-β-phenylserine; $K_m = K_1k_3/(k_2 + k_3)$ for calculated value. [c] Molar absorptivity expressed per active site. [d] TN (expressed per active site) $= k_2k_3/(k_2 + k_3)$ for calculated value. [e] Same value obtained for D,L-*p*-NO_2-*erythro*-β-phenylserine.

Steady-State Absorbance Measurements of EQ_{500}. The dependence of the absorbance of EQ_{500} present during steady state, A_{500}, upon the concentration of β-phenylserine is described by a rectangular hyperbola, the equation for which in double-reciprocal form is given by eq 4 based upon Scheme I, where ϵ_{500}^{EQ} is the molar absorptivity of EQ at 500 nm.

$$1/A_{500} = \frac{K_1k_3}{k_2\epsilon_{500}^{EQ}[E_t][S]} + \frac{k_2 + k_3}{k_2\epsilon_{500}^{EQ}[E_t]} \quad (4)$$

Thus, with the values of k_2 and k_3 determined from the pre-steady-state kinetics of EQ_{500} appearance, a known $[E_t]$, and plots of $1/A_{500}$ vs. $1/[S]$ the values for ϵ_{500}^{EQ} and K_1 may be calculated from the ordinate and abscissa intercepts, respectively (Figure 3).

An increase in absorbance at 500 nm of 0.012 is observed after mixing serine hydroxymethylase (62 μM active site concentration, 2.3 U/mg) with 0.3 M glycine. At $[G] \gg K_4$, the proton dissociation constant, K_3 (Scheme I), is given by eq 5.

$$K_3 = k_{-3}/k_3 = \frac{A_{500}/\epsilon_{500}^{EQ}}{[E_t] - A_{500}/\epsilon_{500}^{EQ}} \quad (5)$$

Using values for molar absorptivity of EQ_{500} and k_3 from stopped-flow measurements (Table II) and the absorbance change at 500 nm after the addition of glycine to serine hydroxymethylase, values of K_3 and k_{-3} can be calculated from eq 5. Furthermore, from the inhibition constant of glycine as a competitive inhibitor of D,L-*erythro*-β-phenylserine cleavage catalyzed by serine hydroxymethylase (Ulevitch and Kallen, 1977a) and with values of k_3 and k_{-3} at pH 7.50 (Table II), a value for K_4 may be calculated from eq 6.

$$K_i = \frac{K_4k_3}{(k_3 + k_{-3})} \quad (6)$$

The addition of either glycine or benzaldehyde *decreases* the steady-state absorbance of EQ_{500} obtained upon mixing D,L-*erythro*-β-phenylserine and serine hydroxymethylase. However, the addition of benzaldehyde to a solution of serine hydroxymethylase and glycine has no effect upon the λ_{max} value for the absorbance band at 500 nm.

The visible absorbance and CD spectra of EQ_{500} generated from serine hydroxymethylase and glycine, D,L-*erythro*-β-phenylserine (with or without benzaldehyde), D,L-*threo*-β-phenylserine and the visible spectra of EQ_{500} generated from L-*p*-hydroxy-*m*-methoxy-*erythro*-β-phenylserine or D,L-*p*-nitro-*erythro*-β-phenylserine exhibit identical λ_{max} values of 500 nm.

The Steady-State Kinetics of D,L-erythro-β-Phenylserine Cleavage: The Initial Velocity of Benzaldehyde Production. The steady-state rate equations for the turnover number (TN) and K_m values are given in eq 7 and 8, respectively (Scheme I), derived with the assumption of rapid equilibria for steps 1 and 4 and irreversibility of step 3 (Ulevitch, 1971).

$$TN = k_2k_3/(k_2 + k_3) \quad (7)$$

$$K_m = K_1k_3/(k_2 + k_3) \quad (8)$$

A rectangular hyperbolic dependence of the initial rate of benzaldehyde production upon β-phenylserine concentration was observed, characterized by a TN of 22 s^{-1} per site and K_m (L-*erythro*-β-phenylserine) of 0.0095 M at pH 7.50. The calculated values of TN and K_m based upon eq 7 and 8 and for EQ_{500} appearance (Table II) are 28 s^{-1} and 0.0092 M, respectively, at pH 7.50.

A summary of rate and dissociation constants for Scheme I as applied to D,L-*erythro*-β-phenylserine at pH 7.50 is presented in Table II.

Effects of Meta and Para Substitution on the Rate and Dissociation Constants of the Serine Hydroxymethylase Catalyzed Dealdolization of D,L-erythro-β-Phenylserine. The effects of meta and para substitution on the steady-state and stopped-flow kinetic parameters TN, K_m, K_1, k_2, k_3, and k_{-2} are presented in the form of Hammett $\sigma\rho$ correlations (Hammett, 1970; Ritchie and Sager, 1964).

The dependence of the logarithm of the TN upon σ value is

TABLE III: Rate and Dissociation Constants for the Reactions of Meta- and Para-Substituted D,L-*erythro*-β-Phenylserines with Serine Hydroxymethylase, pH 7.50, 25 °C, Ionic Strength 0.1 M.[a]

Para	Meta	K_m[b] (M)	K_1[b] (M)	TN[c] (s^{-1})	k_2[d] (s^{-1})	k_3[e] (s^{-1})	$\Sigma\sigma$[f]
CH_3O-	H-	0.02 ± 0.005	0.035	31.4 ± 3.6	50		−0.27
HO-	CH_3O-	0.011 ± 0.002	0.0145	20.4 ± 2.4	27		−0.25[f]
H-	H-	0.0095 ± 0.0016	0.014	21.9 ± 1.0	30	82	0.0
Cl-	H-	0.005 ± 0.001	0.006	16.7 ± 1.5	21		0.23
H-	Cl-	0.0019 ± 0.0003	0.002	14.2 ± 0.7	16		0.37
Cl-	Cl-	0.00125 ± 0.0002	0.0025	9.3 ± 0.5	11		0.60[f]
NO_2-	H-	0.003 ± 0.001	0.003	2.3 ± 0.6	2.4	81	0.78

[a] Scheme I. [b] Expressed as the concentration of L isomers. [c] TN expressed per active site; i.e., per mole of PLP bound per mole of enzyme. [d] k_2 has been calculated from the TN and a value of $k_3 = 82\ s^{-1}$; k_3 is independent of meta or para substitution, Figure 4. [e] Determined from the pre-steady-state kinetics of EQ_{500} appearance from D,L-*erythro*-β-phenylserine and D,L-*p*-nitro-*erythro*-β-phenylserine. [f] Hammett (1970). Disubstituted benzenes are satisfactorily correlated by $\Sigma\sigma$.

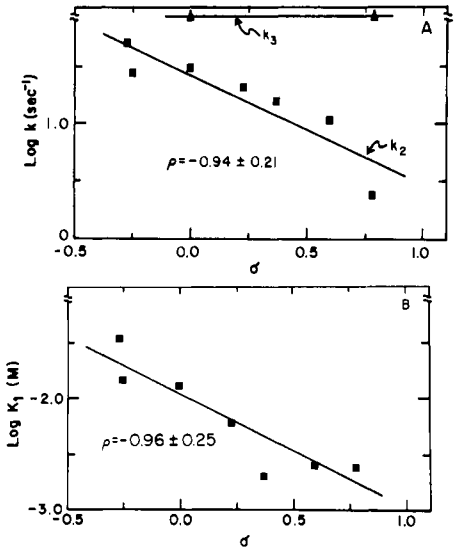


FIGURE 4: (A) The dependence of k_2 and k_3 upon Hammett σ values, pH 7.50, 25 °C, ionic strength 0.1 M, $\rho = -0.91 \pm 0.21$ for k_2 (Scheme I). (B) The dependence of K_1 (Scheme I) on Hammett σ value, pH 7.50, 25 °C, ionic strength 0.1 M, K_1 expressed as the concentration of L-*erythro*-β-phenylserine, $\rho = -0.96 \pm 0.25$.

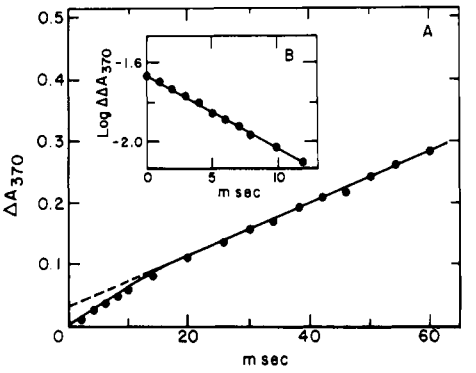


FIGURE 5: (A) The initial transient (burst) during the approach to the steady-state rate of *p*-hydroxy-*m*-methoxybenzaldehyde formation from D,L-*p*-hydroxy-*m*-methoxy-*erythro*-β-phenylserine, pH 8.00, 25 °C. Concentrations before mixing: serine hydroxymethylase (2.3 U/mg) 20 μM active site concentration and 0.02 M D,L-*p*-hydroxy-*m*-methoxy-*erythro*-β-phenylserine. Durrum-Gibson stopped-flow spectrophotometer, 2-cm pathlength. Dashed line (- - -) is the extrapolated steady-state rate of product formation. (B) Determination of the pseudo-first-order rate constant for the initial transient observed during the approach to the steady-state rate of product formation. Data are obtained by subtraction of the absorbance at 370 nm of the burst at any time "*t*" from the absorbance at 370 nm of the steady-state rate of product formation (dashed line (- - -), A).

expected to be nonlinear as a consequence of the composite nature of TN (eq 7). The values of k_3 determined from the pre-steady-state kinetics of the appearance of EQ_{500} (Figure 1) and the absorbance measurements of EQ_{500} in steady state for D,L-*erythro*-β-phenylserine and D,L-*p*-nitro-*erythro*-β-phenylserine are identical within experimental error and, therefore, k_3 is independent of the ring substituent (Figure 4A). Thus, values of k_2 for meta- and para-substituted phenylserines may be calculated from the TN and an average value of k_3 of 82 s^{-1} (Table III); the resultant dependence of log k_2 upon σ value (Figure 4A) is correlated by a ρ value of −0.94 ± 0.21.

The dependence of log K_m upon σ values for substituted phenylserine derivatives is expected to be nonlinear from eq 8. From the values of K_m, k_2, and k_3 obtained as previously described, K_1 may be calculated for each phenylserine derivative, and these values are correlated with a ρ value (Figure 4B) of −0.96 ± 0.25.

Pre-Steady-State Kinetic Study of Product Release from D,L-p-Hydroxy-m-methoxy-erythro-β-phenylserine. The production of *p*-hydroxy-*m*-methoxybenzaldehyde from D,L-*p*-hydroxy-*m*-methoxy-*erythro*-β-phenylserine is characterized by an initial burst followed by the slower steady-state release of product (Figure 5A). A pseudo-first-order rate constant for the burst of product release may be calculated from the plot of log{A_{370} (extrapolated from the steady-state absorbance)$_t$ − A_{370} (from the burst)$_t$} vs. time (inset, Figure 5). The pseudo-first-order rate constants for the burst of aldehyde appearance monitored at 370 nm and EQ_{500} appearance are 120 and 100 s^{-1}, respectively, at pH 8.00.

The steady-state rate of product release after the burst, 2.5 μmol/s per active site of serine hydroxymethylase, in the stopped-flow experiments is in good agreement with the initial rate of product formation, 2.3 μmol/s per active site, measured spectrophotometrically in conventional steady-state experiments.

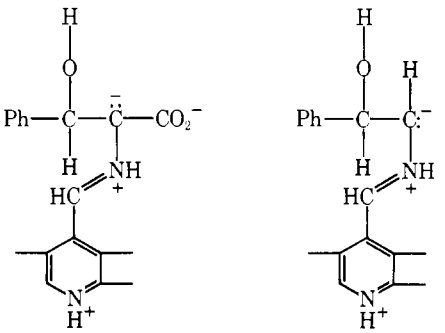
Discussion

Identity of EQ_{500}. Pre-steady-state kinetics, absorbance measurements in the steady state, and steady-state kinetic measurements provide evidence that the minimal kinetic mechanism for the reversible dealdolization of D,L-*erythro*-β-phenylserine is satisfactorily described by Scheme I. The intermediate, EQ_{500}, has been identified as the enzyme-bound quinonoid conjugate base of the Schiff base formed from pyridoxal 5′-phosphate and glycine based upon spectral, kinetic, and chemical evidence which will be discussed in this

section. It is concluded, therefore, that the formation of EQ_{500} occurs by the cleavage of the C_α-C_β bond.

Intermediates absorbing around 500 nm have been observed with several PLP-dependent enzymes including serine hydroxymethylase upon addition of a variety of amino acids (Ulevitch and Kallen, 1977a,b; Morino and Snell, 1967; Jenkins, 1961; Schirch and Jenkins, 1964; Fonda and Johnson, 1970; Martinez-Carrion et al., 1970; Schirch and Diller, 1971; Davis and Metzler, 1972; Miura and Metzler, 1976).

The principal enzyme-bound complexes derived from β-phenylserine and PLP that might be associated with an absorbance band at about 500 nm are the following: (a) the quinonoid intermediate (IV) formed by the decarboxylation of the β-phenylserine-PLP Schiff base; (b) the quinonoid intermediate (III) formed by the loss of an α hydrogen as a proton with or without transamination (cf. Schirch and Jenkins, 1964) from the Schiff base of PLP and D- and/or L-β-phenylserine; and (c) the quinonoid intermediate (II), the conjugate base of the enzyme bound glycine-PLP Schiff base.



III IV

Structure IV can be readily eliminated as the identity of EQ_{500} by the fact that no detectable decarboxylation product, β-phenylethanolamine, can be identified in reaction mixtures following incubation of D,L-*threo*- or D,L-*erythro*-β-phenylserine with serine hydroxymethylase (Ulevitch and Kallen 1977a).

The possibility that enzyme-bound structures such as III may be the EQ_{500} species can be ruled out from: (a) optical rotation experiments (Ulevitch and Kallen, 1977a) which have indicated that the D isomers remain unaltered during the incubation of serine hydroxymethylase with racemic mixtures of *erythro*- or *threo*-β-phenylserines as glycine and benzaldehyde are formed (see also Gilbert, 1954). (b) The possibility that the EQ_{500} species represents an abortive complex formed from the D isomers and serine hydroxymethylase is inconsistent with the observation that the absorbance at 500 nm decreases to zero with time. (c) The failure to detect either β-hydroxyphenylpyruvate or phenylpyruvate as transamination products, by the absence of loss of enzymatic activity (due to pyridoxamine 5′-phosphate formation), and by the lack of ^{1}H NMR evidence for α-H loss from phenylserines to solvent deuterium oxide (Ulevitch and Kallen, 1977a). This last observation is consistent with other studies which show that α-H of serine is retained in the glycine product (Jordon and Akhtar, 1970; Wellner, 1970; Besmer and Arigoni, 1968) and that the cleavage of α-substituted serines is catalyzed by serine hydroxymethylase (Wilson and Snell, 1962; Schirch and Mason, 1963; Fujioka, 1969; Schirch and Diller, 1971). (d) However, the data above do not rule out removal of an α hydrogen (proton) and sequestration at a nonexchangeable site on the enzyme. Therefore, the following observations are crucial to the identification of EQ_{500} as species II (Scheme I) and not as species III formed from the L isomers either on (making little apparent chemical sense, eq 9) or off (eq 10) the catalytic pathway.

$$E + S \rightleftharpoons ES \rightleftharpoons EQ_{500} \rightleftharpoons E + G + B \quad (9)$$

$$E + S \rightleftharpoons ES \rightleftharpoons E + G + B \quad (10)$$
$$\Downarrow\Uparrow$$
$$EQ_{500}$$
$$(III)$$

(1) The pre-steady-state kinetics of substituted β-phenylserines provide ρ values of 0 and -0.94 ± 0.21 for k_3 and k_2, respectively. If EQ_{500} was III then a value of $\rho > 0$ is expected since electron withdrawal should favor α-proton removal. This argument is analogous to one applied in a study of mandelic acid racemase (Hegeman et al., 1970).

(2) The steady-state absorbance at 500 nm achieved in the presence of benzaldehyde is diminished as a consequence of the now significant contribution of k_{-2}[B] in eq 2.

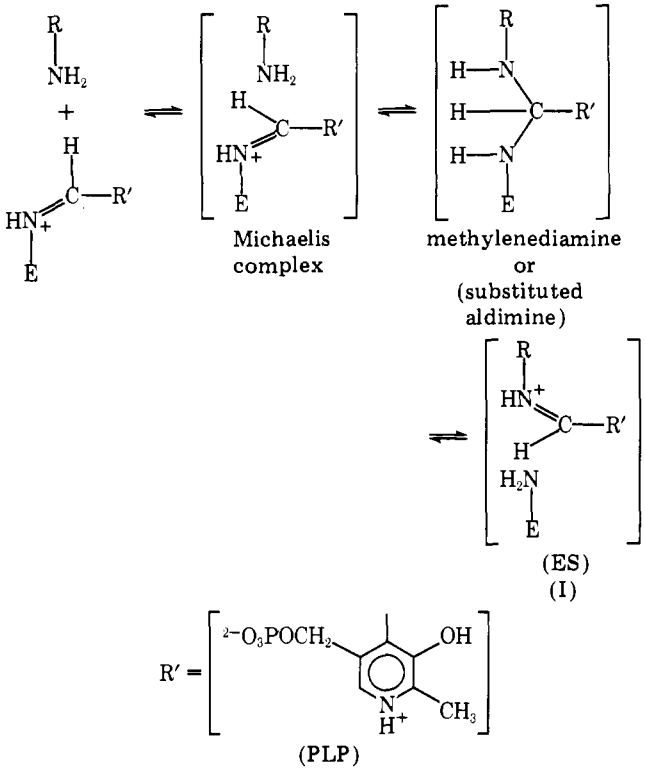
(3) The λ_{max} value for the EQ_{500} species formed from serine hydroxymethylase and all of the substituted β-phenylserines is 500 nm, whereas the λ_{max} values for the quinonoid species derived from serine hydroxymethylase and L-phenylalanine (Ulevitch and Kallen, 1977b) or D-alanine (Schirch and Jenkins, 1964) are 503 and 505 nm, respectively. In addition, the λ_{max} for this absorbance peak is unaltered in the presence of various substituted benzaldehydes. This suggests that the EQ_{500} species is the same for all of the substituted β-phenylserines and, indeed, the ability to form an identical absorbance band at 500 nm upon mixing serine hydroxymethylase and glycine itself provides most straightforward evidence that the identity of EQ_{500} is II.

(4) Compelling evidence for the assignment of the EQ_{500} as species II derives from the pre-steady-state experiments with serine hydroxymethylase and *p*-hydroxy-*m*-methoxy-*erythro*-β-phenylserine, the aldehyde product of which can be readily followed spectrophotometrically. The initial burst of aldehyde appearance is characterized by the same time constant as EQ_{500} appearance during the establishment of the steady state and supports the contention that the EQ_{500} and aldehyde are formed in the same step. A *lag* in product release rather than a *burst* would be observed if EQ_{500} were formed prior to the cleavage of the C_α-C_β bond (eq 9) and in some cases for the mechanism of eq 10 depending upon the magnitude of the kinetic constants. Alternative explanations for the observation of an initial burst (Jencks, 1969) based upon severe product inhibition or the occurrence of substrate induced conformational changes from a more active to a less active form of the enzyme appear unlikely. Thus, for example, studies of the pre-steady-state kinetics of EQ_{500} formation from D,L-*erythro*-β-phenylserine in the presence of either glycine or benzaldehyde indicate no extreme product inhibition.

(5) The pre-steady-state kinetic study of EQ_{500} appearance in deuterium oxide at pH 7.50 reveals kinetic solvent deuterium isotope effects as follows: $k_2(H_2O)/k_2(D_2O) = 0.68$ and $k_3(H_2O)/k_3(D_2O) = 6.25$. The latter isotope effect is similar to that measured for the kinetic solvent deuterium isotope effect for protonation of the enzyme bound quinonoid intermediate formed with serine hydroxymethylase and L-phenylalanine (Ulevitch and Kallen, 1977b) and is consistent with Scheme I in which k_3 is assigned to the protonation of the enzyme bound quinonoid intermediate II.

The Kinetics of the Serine Hydroxymethylase Catalyzed Dealdolization of D,L-erythro-β-Phenylserine. All of the observations regarding the initial velocity of benzaldehyde appearance, the pre-steady kinetics of EQ_{500} appearance, and

SCHEME II: Transimination Sequence for Substrate Binding to Serine Hydroxymethylase.



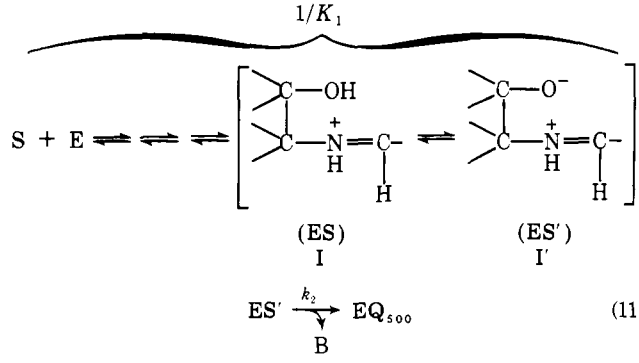
absorbance measurements of EQ_{500} under steady-state conditions are consistent with the minimal kinetic scheme for the serine hydroxymethylase catalyzed dealdolization of D,L-*erythro*-β-phenylserine, Scheme I. The rapid equilibrium steps 1 and 4 almost certainly represent PLP–amino acid Schiff base formation by transimination (see below) sequences (Snell and Jenkins, 1959; Jencks and Cordes, 1963; Tobias and Kallen, 1975) and are associated with spectral changes at 430 nm (Ulevitch and Kallen, 1977a) that are too rapid to measure by stopped-flow techniques as noted earlier in the related case of glutamic-aspartic transaminase (Fasella and Hammes, 1967). Temperature-jump relaxation studies of the reaction with serine hydroxymethylase, which includes the transimination steps, cannot be readily compared with our data due to differences in the source of enzyme and the experimental conditions (Cheng and Haslam, 1972; Schirch, 1975).

The Effect of Carbonyl Compounds on the Rate of Approach to Steady State with Respect to EQ Concentration. Formaldehyde and acetaldehyde both increase the rate of approach to steady state with respect to EQ concentration in stopped-flow experiments with D,L-*erythro*-β-phenylserine and decrease the steady-state absorbance at 500 nm which are both reflections of the decreased concentration of EQ_{500} in the steady state (Ulevitch, 1971). It is reasonable to conclude that these aldehydes react with the quinonoid intermediate as does benzaldehyde to form the corresponding β-hydroxy-α-amino acids. Moreover, the observation that formaldehyde also reacts with the enzyme bound glycine carbanion in the *absence* of added tetrahydrofolic acid (THF) suggests that activation of formaldehyde by THF is not an absolute prerequisite for C_α–C_β bond formation[4] and, indeed, reversible dealdolization of serine in the absence of THF has been observed[5] (Chen and Schirch, 1973b; Akhtar et al., 1975).

Substituent Effects and the Mechanisms of the Dealdolization of D,L-erythro-β-Phenylserine. Kinetic studies of the serine hydroxymethylase catalyzed dealdolization of D,L-*erythro*-β-phenylserine indicate that two steps, described by k_2 and k_3 (Scheme I), are both partially rate-determining (Table II) and the data, kinetic and otherwise (see above discussion), are consistent with the assignment of the rate constants k_2 and k_3 to the cleavage of the C_α–C_β bond and to protonation of the quinonoid intermediate (II), respectively.

The ρ value for the ρσ correlation of $1/K_1$ is 0.96 ± 0.25, where $1/K_1$ represents, in the kinetic mechanism, the formation of enzyme-substrate Schiff base intermediates prior to C_α–C_β bond cleavage: it is assumed that this occurs via a transimination sequence (Scheme II).

A most significant question regards the extent to which proton removal from the substrate β-OH site has occurred during the formation of the enzyme-substrate Schiff base just proximal to the C_α–C_β bond cleavage step (eq 11). Up to this point in the discussion the structure of this intermediate has been depicted possessing a proton on the β-oxygen atom (e.g., I) of the substrate moiety.



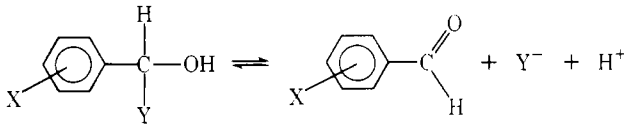
(11)

In view of the similarity of the ρ value for $1/K_1$ with the ρ values of 1.11, 1.01, and 0.70 for the ionization of trifluoroacetophenone hydrates, phenyltrifluoromethylcarbinols (Jaffé, 1953; Stewart and Van der Linden, 1960), and substituted cyanohydrins (Ching, 1977; Ching and Kallen, 1977), respectively, we suggest the possibility that, during the transimination sequence to form the enzyme-substrate Schiff base from free enzyme and substrate in solution, complete or almost complete alkoxide ion formation at the β-OH has already occurred prior to the step involving C_α–C_β bond cleavage. Such an ionization would be clearly aided by electron-withdrawing substituents and is tantamount to a quite substantial decrease in the pK_a value of the β-OH group in the ES complex compared with that for the same group in the substrate in aqueous solution. This formulation (eq 11) contrasts with the situation which appears to occur during the binding of substituted benzyl alcohols to yeast alcohol dehydrogenase, a step which occurs prior to C–H bond cleavage in the oxidation of the alcohols to the corresponding benzaldehydes. From substituent and isotope effect studies it has been concluded that very little alkoxide formation occurs upon binding of the alcohol to the enzyme (Klinman, 1976). It may be that the differences in the leaving groups, a carbanion vs. a hydride ion (if the latter rather than a hydrogen atom is indeed involved in the mechanism of the yeast alcohol dehydrogenase catalyzed oxidation of benzyl alcohols), and/or the involvement of the active site catalytic Zn^{2+} in the latter reactions account for

[4] Studies upon serine hydroxymethylase catalyzed reactions have produced conflicting concepts concerning the role(s) of THF in the reaction mechanisms (Biellmann and Schuber, 1967, 1970; Jordan and Akhtar, 1970; Chen and Schirch, 1973a–c; Kallen and Blanck, 1973; Kallen, 1971a; Akhtar et al., 1975; Jordan et al., 1976).

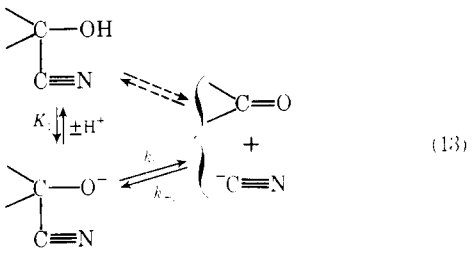
[5] P. S. Tobias and R. G. Kallen, unpublished experiments.

these differences in the mechanism of substituted benzaldehyde formation in these formally quite similar systems (eq 12).



$$(Y = H^{-} \text{ or } {\equiv}C^{-}) \qquad (12)$$

It is noteworthy that no kinetic route for the decomposition of neutral cyanohydrins (dotted arrows, eq 13) has been detected. Rather, complete proton removal (i.e., oxyanion formation) must occur in order that the C-C bond cleavage reaction in cyanohydrin breakdown (solid arrows, eq 13) proceeds with the elimination of CN^-, presumably attributable to the relatively poor leaving ability of CN^- (Ching, 1977; Ching and Kallen, 1977).



(13)

Continuing with the analogy between the β-phenylserine and cyanohydrin cleavage reactions, the ρ value of -0.94 ± 0.21 (or about -0.60 excluding the *p*-nitro derivative[6]) for the k_2 step, C_α-C_β bond cleavage of β-phenylserine catalyzed by serine hydroxymethylase (Scheme I), may be compared with the ρ value of -0.22 for the k_2 step, the C-C bond cleavage of the anionic substituted cyanohydrins in water (Ching, 1977; Ching and Kallen, 1977) (see eq 13), and may be contrasted with the ρ value of 1.28 for the pyridine-catalyzed cleavage of neutral substituted cyanohydrins in 95% ethanol (Baker and Hopkins, 1949; Jaffé, 1953). In view of uncertainties in the dielectric constant to be applied to the active site plus clear differences between the nature of the leaving groups, CN^- vs. the quinonoid intermediate depicted in II, the qualitative similarity in ρ values is consistent with prior β-alkoxide ion development in the serine hydroxymethylase catalyzed pathway (Kallen and Ching, 1975). It would be expected that in a concerted process the substituent effects upon proton removal from the β-OH (aided by electron withdrawal) would oppose those for heterolytic C_α-C_β bond cleavage (aided by electron donation). The enhanced affinity of β-alcohol containing substrates to serine hydroxymethylase compared with β-methylene and β-alkoxy (*O*-methyl) derivatives (Ulevitch and Kallen, 1977a) is consistent with but not proof of β-OH ionization during the transimination (binding) sequence.

The existence of a histidine in the active site peptide of rabbit liver serine hydroxymethylase (Bossa et al., 1976) has led us to the speculation that the proton acceptance by the enzyme from β-alcoholic groups of the serine substrates might involve a charge-relay type of system as apparently occurs in serine proteases (Hunkapiller et al., 1976).

The nature of the proton acceptor(s) is presently unknown, although serine hydroxymethylase in common with other PLP enzymes has an active site lysine which might be employed in this role. It is also unknown currently whether the system employed in proton removal from the β-OH of the serines is the same system involved in the proton addition to EQ_{500} (k_3 in Scheme I).

[6] The *p*-nitro substituent is markedly dipolar and not infrequently deviant in Hammett equation correlations (Kirsch, 1972).

Acknowledgment

The authors appreciate numerous helpful discussions with Drs. David Porter and Richard O. Viale, the many critical contributions of Dr. Wei-Mei Ching and Mrs. Mary Frederick, and the making available of stopped flow equipment by Dr. Harold F. Bright.

Appendix: Derivations of Equations 2 and 4 for Scheme I

Assume steps 1 and 4 are at rapid equilibrium, ES and EG are binary complexes of E with S and G, respectively.

$$[E_t] = [E] + [ES] + [EQ] + [EG]$$

$$K_1 = [E][S]/[ES]$$

$$K_4 = [E][G]/[EG]$$

$$[E] = \frac{[E_t] - [EQ]}{1 + \frac{[S]}{K_1} + \frac{[G]}{K_4}}$$

$$\frac{d[EQ]}{dt} = k_2[ES] - (k_{-2}[B] + k_3)[EQ]$$

$$+ k_{-3}[EG] = \frac{\frac{k_2[S]}{K_1}([E_t] - [EQ])}{1 + \frac{[S]}{K_1} + \frac{[G]}{K_4}}$$

$$- (k_{-2}[B] + k_3)[EQ] + \frac{\frac{k_3[G]}{K_4}([E_t] - [EQ])}{1 + \frac{[S]}{K_1} + \frac{[G]}{K_4}}$$

$$= \underbrace{\frac{(k_2[S]/K_1 + k_{-3}[G]/K_4)[E_t]}{(1 + [S]/K_1 + [G]/K_4)}}_{b}$$

$$- \underbrace{\left[\frac{(k_2[S]/K_1 + k_{-3}[G]/K_4)}{(1 + [S]/K_1 + [G]/K_4} + (k_{-2}[B] + k_3)\right]}_{a}[EQ]$$

$$\frac{d[EQ]}{dt} = b - a[EQ]$$

$$\ln(b - a[EQ]) = -at + C$$

at $t = 0$,

$$[EQ] = 0;\ C = \ln b$$

$$\ln\frac{(b - a[EQ])}{b} = -at$$

$$b - a[EQ] = be^{-at}$$

Upon integration

$$[EQ] = \frac{b}{a}(1 - e^{-at})$$

$$a = k_{obsd} = \frac{k_2[S]/K_1 + k_{-3}[G]/K_4}{1 + [S]/K_1 + [G]/K_4} + k_{-2}[B] + k_3$$

when [G] = 0)pre-steady state)

$$k_{obsd} = k_2\{[S]/(K_1 + [S])\} + k_{-2}[B] + k_3 \qquad (2)$$

when $k_{-3}[G] \ll K_4$ and $k_{-2}[B] \ll k_3$

$$[EQ] = \frac{k_2[E_t]}{k_2 + (K_1/[S] + 1)k_3}$$

$$A_{500} = \frac{[E_t]\epsilon_{500}^{EQ}k_2/(k_2 + k_3)}{\frac{k_3K_1}{(k_2 + k_3)[S]} + 1}$$

$$\frac{1}{A_{500}} = \frac{K_1k_3}{k_2[E_t]\epsilon_{500}^{EQ}[S]} + \frac{k_2 + k_3}{[E_t]\epsilon_{500}^{EQ}k_2} \quad (4)$$

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