with chloramphenicol (Krenitsky and Fruton, 1966). These observations raise the possibility that the dipeptide ester is hydrolyzed most effectively when it is bound by the dimeric form of the enzyme.

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Mechanistic Studies of Beef Plasma Amine Oxidase*

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ABSTRACT: The initial velocity patterns for the deamination of benzylamine by beef plasma amine oxidase have been determined. The results suggest that the reaction proceeds through a Ping-Pong mechanism in which approximately 1 mole of benzaldehyde is formed anaerobically per mole of enzyme as reported by Reed and Swindell. The product inhibition patterns have been determined at saturating and nonsaturating concentrations of the two variable substrates.

From the results obtained a formal mechanism for the reaction has been proposed and the corresponding rate equation derived.

Beef plasma amine oxidase was initially purified by Tabor *et al.* (1954) and subsequently crystallized by Yamada and Yasunobu (1962a). The latter investigators have shown that this enzyme requires copper for activity (1962b) and in agreement with Tabor *et al.* (1954), that the enzyme contains a prosthetic group with an aldehyde function. Indirect evidence presented by Yamada and Yasunobu (1963a) indicates that the organic prosthetic group may be pyridoxal phosphate which is covalently attached to the enzyme. The molecular weight of the enzyme has been shown to be 170,000 (Achee *et al.*, 1968) and it has been shown that there are two identical subunits which are covalently attached through SS linkages (Achee *et al.*, 1968).

Little is known about the mechanism by which this enzyme catalyzes the oxidation of amines. Enzymes from different sources appear to have different substrate specificities and spectra and there is no evidence to indicate that all the copper–pyridoxal phosphate amine oxidases proceed by the same mechanism. Thus, it is necessary for the time being to consider these amine oxidases from different sources as separate entities. Reed and Swindell (1969) have reported that in the reaction catalyzed by the beef plasma amine oxidase, 1 mole of benzaldehyde is produced anaerobically and that the K_m for oxygen is dependent on the amine concentration. These studies suggest that a Ping-Pong mechanism describes the reaction catalyzed by beef plasma amine oxidase.

Recent advances in the theoretical treatment of multisubstrate–enzyme reactions, due to the pioneering work of Alberty (1953), Boyer (1959), Wong and Hanes (1962), Bloomfield *et al.* (1962), Dalziel (1967), Frieden (1957), and especially Cleland (1963a–c) and Fromm (1967), provide methods for elucidating the formal mechanism by kinetic studies. The present communication reports the results of the kinetic investigation of crystalline beef plasma amine oxidase.

Experimental Section

Materials. Crystalline beef plasma amine oxidase was isolated by a method which has been published previously

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(Yamada and Yasunobu, 1962a). Most chemicals used in the present study were obtained from sources described in previous publications and were of reagent grade. Twice-recrystallized bovine liver catalase and horseradish peroxidase were purchased from Sigma Chemical Co. Ammonium acetate was obtained from Matheson, Coleman & Bell. *o*-Dianisidine, twice recrystallized, was purchased from Mann Research Lab., Inc. [1-^{14}C]Benzylamine (2–5 μCi/μmole) was purchased from the Volk Radiochemical Co. Benzaldehyde was a product of the Eastman Kodak Co. A solution of 70% perchloric acid was obtained from J. T. Baker Chemical Co. A solution of 50% H_2O_2 was purchased from the Fischer Scientific Co. Pure nitrogen (99.95%), 3.0% oxygen–97% nitrogen mixture, and 0.5% oxygen–99.5% nitrogen mixture were purchased from Gaspro Ltd., Honolulu, Hawaii.

Methods

Enzyme Assays and Protein Determination. The spectrophotometric assay (Tabor *et al.*, 1954) in which the formation of benzaldehyde is measured at 250 mμ in a Cary Model 14 automatic recording spectrometer was adopted. In all the experiments, 0.1 M potassium phosphate buffer (pH 7.2) was used and the temperature was 25°. One unit of activity is the amount of enzyme nesessary to cause an absorbancy change of 0.001/min. The protein concentration was determined spectrophotometrically and the $\epsilon_{1\,cm}^{1\%}$ at 280 mμ used was 9.8. All pH determinations were made with a Beckman Research pH meter accurate to ±0.02 pH unit.

Initial reaction velocities were linear during the 5 min required to assay the enzyme. When it was necessary to vary the oxygen concentration, mixtures of N_2–O_2 were prepared in 25-l. carboys by the displacement of H_2O. The reactions in this case were run in Thunberg-type cuvets with two side arms. The benzylamine and buffer were added to the main compartment and the inhibitor and the enzyme (50 units) in the two side arms. The buffer was flushed with the desired gas mixture for several minutes. Then the cuvet was assembled.

Benzaldehyde Inhibition Using Coupled Assay. Initial velocities in the presence of benzaldehyde were determined using a modified glucostat assay (Guidotti *et al.*, 1961; McEwen, 1965a,b) for the determination of hydrogen peroxide. The reaction was coupled to the peroxidase–*o*-dianisidine reaction and velocity measured as the change in absorbance per minute at 460 mμ.

Anaerobic Studies. Anaerobic studies were conducted in a 300-ml flask fitted with two magnetic cups. Oxygen-free nitrogen was obtained by passing the gas through three alkaline pyrogallol traps (0.6 g/15 ml of 10% KOH) and a column of copper beads was heated to 200° and previously reduced with hydrogen gas.

In a typical experiment, 0.1 ml of enzyme solution (8.8×10^{-3} μmole) was pipetted into one cup and 0.2 ml of 70% perchloric acid into the other. The flask was filled with 2.7 ml of 0.33 M phosphate buffer (pH 7.2) containing 5.45×10^{-2} μmole of [^{14}C]benzylamine. The vessel was evacuated and flushed three times before a final flushing of 24 hr. The enzyme was then tipped in and allowed to react for 45 min while maintaining a continuous flow of N_2 gas through the system. The reaction was stopped by adding the perchloric acid which also served to acidify the mixture. Benzaldehyde was extracted with 10 ml of toluene, 1-ml aliquots of which were added to 9 ml of phosphor and counted.

Results

K_m for Benzylamine. Numerous determinations of K_m have been made with benzylamine as the substrate at pH 7.2 and 25° in 0.1 M phosphate buffer and the K_m (app) was 1.4×10^{-3} M in air. It has been previously reported that the activity is dependent on the ionic strength of the buffer (Yamada and Yasunobu, 1962a). Thus, the reported K_m is specifically for the conditions given above. Substrate inhibition was observed at benzylamine concentrations which were higher than 3.4×10^{-3} M.

K_m for O_2. No published data were available on the K_m for O_2 for the beef plasma amine oxidase using benzylamine as the substrate. A $K_m^{O_2}$ of 1.35×10^{-5} M (0.714% O_2) was calculated at pH 7.2 in 0.1 M potassium phosphate buffer at 25° and in the presence of 1.67 mM of benzylamine. It was observed that substrate inhibition occured at oxygen concentrations which were greater than 3×10^{-4} M.

Initial velocity patterns of multireactant systems are indicative of the order of addition of substrate and release of products. The results obtained for beef plasma amine oxidase are presented at Figure 1A,B, using the reciprocal velocity plot (Lineweaver and Burk, 1934). It is evident that parallel lines are observed with respect to both substrates.

Product Inhibition Studies

Cleland (1963a–c) has published methods for using product inhibitors to elucidate enzymatic mechanism. Therefore, the type of inhibition produced by benzaldehyde, NH_3, and H_2O_2 were determined.

Inhibition by Benzaldehyde. The initial reaction velocities were measured at a constant benzylamine concentration (1.67×10^{-3} M) and varying O_2 concentrations in the presence of the product benzaldehyde. The results of the Eadie plot (1952) showed that benzaldehyde was a strictly noncompetitive inhibitor with a K_I value of 1.1×10^{-3} M.

When a similar experiment was conducted in air at various benzylamine concentrations the results shown in Figure 2 were obtained. Benzaldehyde was a strictly noncompetitive inhibitor with a K_I value of 1.45×10^{-3} M.

Inhibition by NH_4OAc. The initial reaction velocities were determined at a constant benzylamine concentration (1.67×10^{-3} M) and varying O_2 concentrations in the presence of NH_4OAc. Ammonium acetate was found to be an uncompetitive inhibitor of O_2 with a K_I value of 0.21 M.

The analogous experiment was performed in air at various benzylamine concentrations. With respect to the amine substrate ammonium acetate was a competitive inhibitor with a K_I value of 0.14 M.

Inhibition by H_2O_2. The experiments were extended to the product H_2O_2. When the oxygen concentration was varied in the presence of H_2O_2 and 1.67×10^{-3} M benzylamine, uncompetitive inhibition was observed. A K_I value of 0.26 M was calculated from the data.

When the benzylamine concentration was varied in the air the results disclosed that H_2O_2 was a competitive inhibitor with respect to benzylamine with a K_I value of 0.15 M.

The concentration of H_2O_2 was 0.075 M and there is a danger that such high concentrations may chemically alter the enzyme. Therefore, control experiments had to be performed. As shown in Figure 3, the inhibition by H_2O_2 was constant during the 5-min interval which corresponds to the length of

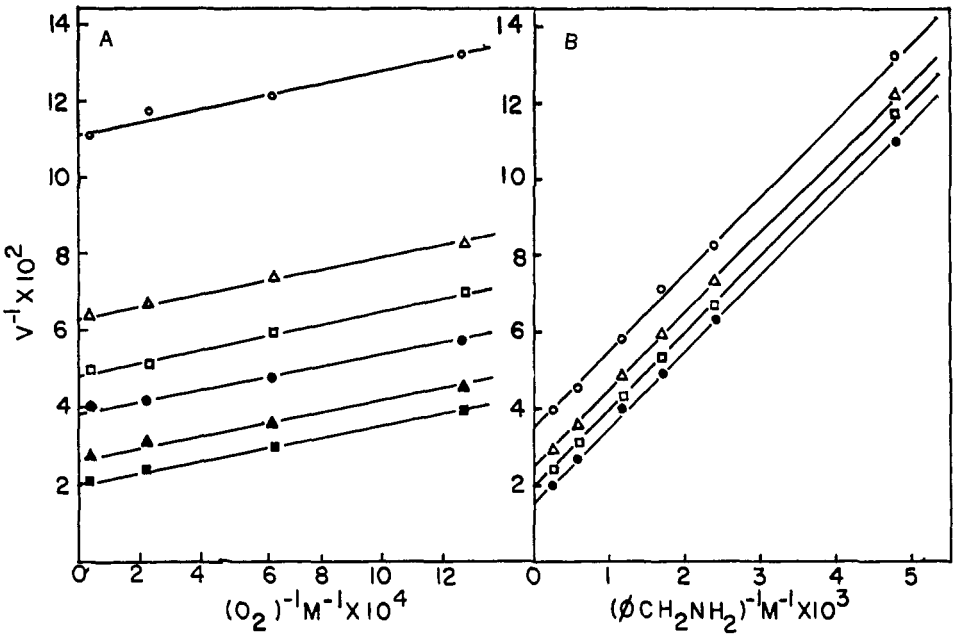

FIGURE 1: Initial velocity pattern for the deamination of benzylamine. (A) The reciprocal velocities are plotted as functions of oxygen concentration of the following fixed levels of benzylamine concentration (○) 0.209 mM, (△) 0.418 mM, (□) 0.584 mM, (●) 0.835 mM, (▲) 1.67 mM, and (■) 3.34 mM. Velocity was measured as the rate of formation of benzaldehyde as described in the Methods section. (B) At fixed levels of oxygen concentration. The initial velocity data from part A have been replotted as functions of benzylamine concentration at the following levels of oxygen (○) 0.5% (7.85×10^{-6} M), (△) 1.0% (1.59×10^{-5} M), (□) 3.0% (4.62×10^{-5} M), and (●) 23% (2.57×10^{-4} M). Velocity was measured as the rate of benzaldehyde formation as described above.

the assay. If there was a time-dependent oxidation of the enzyme by H_2O_2 the inhibition would be expected to increase with time. Addition of catalase, which would destroy the H_2O_2, after the 5-min incubation period of enzyme with H_2O_2 and subsequent assay showed almost complete restoration of enzyme activity to the control value. Furthermore, no oxidation of benzylamine by 0.075 M H_2O_2 was observed.

The K_I values for the products are summarized in Table I under both nonsaturating and saturating concentrations of the substrates.

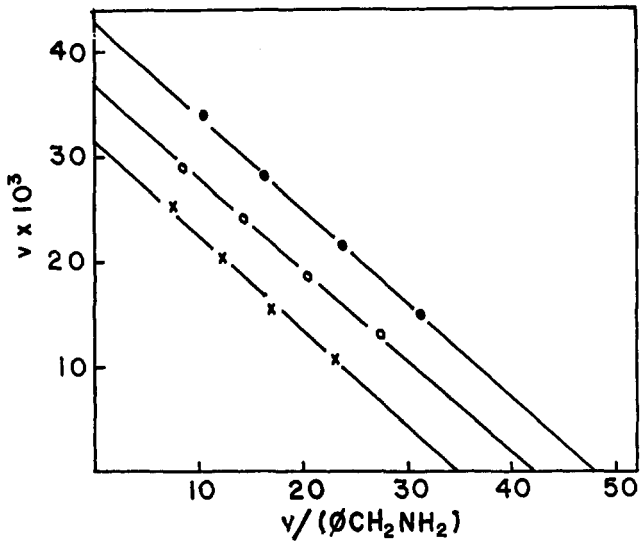

FIGURE 2: Eadie plot of the inhibitory effect of benzaldehyde in air while varying the benzylamine concentration. Noncompetitive inhibition was observed at benzaldehyde concentrations of (●) 0, (○) 3.0 mM, and (×) 4.8 mM. Velocity was measured as the rate of hydrogen peroxide formation using the coupled peroxidase assay described under Methods.

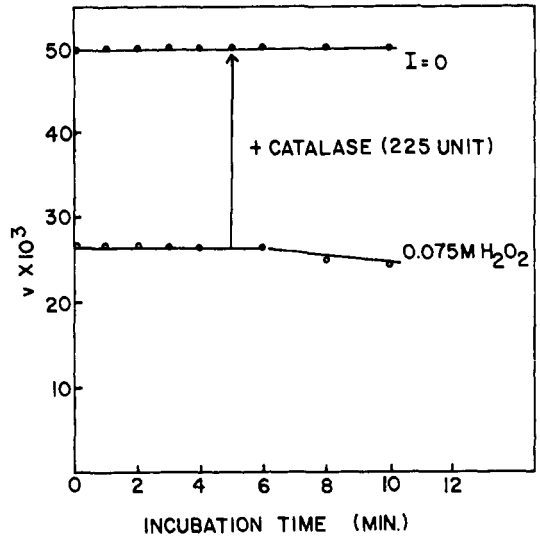

FIGURE 3: Effect of H_2O_2 on the activity of plasma amine oxidase. Fifty units of the enzyme in 2.8 ml of 0.1 M phosphate buffer (pH 7.2) was preincubated with H_2O_2 (final concentration of 0.075 M) for the time intervals shown. Benzylamine (final concentration of 1.67 mM) was added and the enzyme activity was measured. The addition of catalase after 5-min incubation resulted in the restoration of activity to that of the control.

TABLE I: Product Inhibition Pattern of Beef Plasma Amine Oxidase.

Substrate Varied	Product Inhibitor		
	C_6H_5CHO	H_2O_2	NH_4OAc
Benzylamine			
0.5% O_2 (non-saturated)			
Type of inhibition	Noncompetitive	Competitive	Competitive
K_I (M)	2.2×10^{-3}	0.27	0.125
Air (saturated)			
Type of inhibition	Noncompetitive	Competitive	Competitive
K_I (M)	1.45×10^{-3}	0.15	0.14
Oxygen			
1.7 mM benzylamine (nonsaturated)			
Type of inhibition K_I (M)	Noncompetitive	Uncompetitive	Uncompetitive
6.8 mM benzylamine (saturated)	1.1×10^{-3}	0.26	0.21
Type of inhibition	Noncompetitive	Uncompetitive	Uncompetitive
K_I (M)	1.4×10^{-3}	0.27	0.36

Inhibition in the Presence of both H_2O_2 and NH_3. Further evidence pertinent to the detailed formal mechanism was obtained by determining inhibition patterns in the presence of both H_2O_2 and NH_3. An Eadie plot of the results obtained with oxygen as the varied substrate showed strictly noncompetitive inhibition. Figure 4 shows that increasing the NH_3 concentration at a constant level of H_2O_2 makes the inhibition pattern with respect to benzylamine progressively more competitive.

Reaction under Anaerobic Conditions

Production of Benzaldehyde under Anaerobic Conditions. It has been reported that with beef plasma amine oxidase, 1 mole of benzaldehyde is produced when the substrate is added under anaerobic conditions (Reed and Swindell, 1969). When the anaerobic experiments were repeated the results in Table II were obtained. Approximately 1 mole of benzaldehyde was produced per mole of enzyme. The production of benzaldehyde anaerobically is in agreement with the kinetic studies that the reaction catalyzed by beef plasma amine oxidase proceeds by a Ping-Pong mechanism.

Discussion

There are two types of amine oxidase, which can be differentiated most readily on the basis of the prosthetic groups present in the enzyme. The plasma type requires copper and

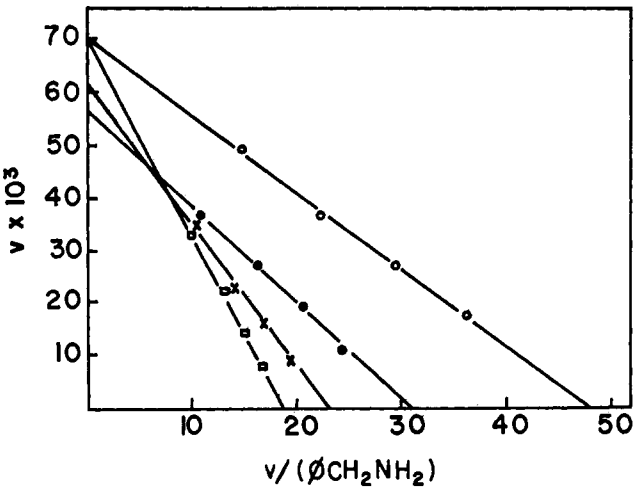

FIGURE 4: Eadie plot of the combined inhibitory effect of hydrogen peroxide and ammonia in air while varying the benzylamine concentration. At a constant H_2O_2 concentration of 0.06 M the inhibition pattern varied from mixed to competitive as the ammonia concentration increased (●) 4.6×10^{-2} M, (×) 6.6×10^{-2} M, and (□) 0.1 M. Velocity was measured as the rate of formation of benzaldehyde as described above, using 5-mm cuvets.

possibly covalently linked pyridoxal phosphate while the mitochondrial enzyme requires covalently attached FAD. However, both enzymes catalyze the same general reaction: $RCH_2NH_2 + O_2 + H_2O \rightleftarrows RC(=O)H + NH_3 + H_2O_2$.

In the present investigation, the mechanism of action of the beef plasma amine oxidase was the subject of study. McEwen (1965a,b; McEwen *et al.*, 1966) has investigated the effect of pH on $K_m^{RNH_2}$ and V_m for the human and rabbit plasma amine oxidases and has shown that there are two types of enzymes. The human enzyme which acts on the un-ionized amine and rabbit type which shows a more complicated pH dependence. In addition, there is a report that a Schiff base intermediate is formed during the reaction (Buffoni, 1966), which can be trapped anaerobically by the addition of sodium borohydride. We (Yamada and Yasunobu, 1963a) have shown earlier that the pink color of the native enzyme is bleached by the anaerobic addition of the substrate. Furthermore the addition of oxygen subsequently restores the pink color. It has also been demonstrated that the copper in the beef plasma amine oxidase probably does not undergo reduction anaerobically (Yamada *et al.*, 1963b). However, Mondovi *et al.* (1969) have recently reported that the copper in the pig kidney histaminase

TABLE II: Anaerobic Production of [^{14}C]Benzaldehyde.[a]

Enzyme (μmoles)	μmoles of C_6H_5CHO Extracted	Turnover
5.95×10^{-3}	6.98×10^{-3}	1.17
5.95×10^{-3}	5.61×10^{-3}	0.94
2.12×10^{-3}	2.17×10^{-3}	1.02
2.12×10^{-3}	2.50×10^{-3}	1.18

[a] See Experimental Section for details of the experiment.

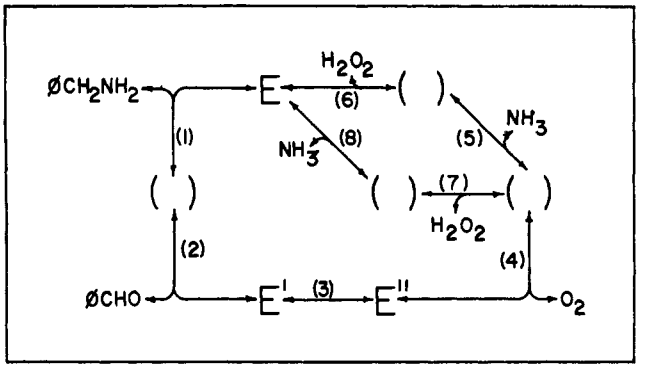

FIGURE 5: Proposed mechanism for reaction catalyzed by beef plasma amine oxidase.

is reduced under aerobic conditions, although it was not settled whether the copper undergoing reduction was functional copper or copper present as impurity. Although the reaction catalyzed by beef plasma amine oxidase is a ternary–ternary reaction and obviously complicated, recent advances in the theoretical treatment of multisubstrate enzymes have made it possible to investigate the formal mechanisms of this enzyme.

The fact that the beef plasma amine oxidase operates by a Ping-Pong mechanism was demonstrated by both kinetic and chemical studies. The initial velocity studies yielded a series of parallel lines with respect to both substrates. Alberty (1953) and Cleland (1963c) have pointed out that this is indicative of a Ping-Pong mechanism. The production of benzaldehyde anaerobically is verification of the double-displacement nature of the reaction. In agreement with the report of Reed and Swindell (1969), 1 mole of benzaldehyde was produced per mole of enzyme in the absence of oxygen.

Now that it appears fairly certain that we must work within a Ping-Pong mechanism we can consider the results of the product inhibition studies. Benzaldehyde is a noncompetitive inhibitor with respect to both the amine substrate and to oxygen. The simple bi–bi Ping-Pong mechanism (Cleland, 1963a) with benzaldehyde forming an abortive binary complex with the unsubstituted enzyme can explain the strictly noncompetitive behavior of benzaldehyde with respect to oxygen. This would be the case if the aldehyde had the same affinity for the unsubstituted, oxidized enzyme as for the reduced, ammonia-substituted enzyme. However this mechanism cannot account for the strictly noncompetitive inhibition of the aldehyde against benzylamine. It clearly requires a mixed-inhibition pattern, *i.e.*, intersection of the inhibited and inhibited plots to the left of the ordinate axis in the Eadie plot. The data in Figure 2 show no tendency toward convergence, thus ruling out this mechanism.

The double-displacement character of the reaction predicts the noncompetitive behavior of the first product, benzaldehyde, toward the amine substrate. The similar pattern observed with respect to oxygen suggests the presence of at least two enzyme–substrate complexes between the step involving the liberation of benzaldehyde and that involving the entry of oxygen as substrate. This amounts to assuming an isomerization step, as commonly found in enzyme mechanisms (Fasella and Hammes, 1967). This additional step prevents competition between benzaldehyde and oxygen for the same enzymic species and would predict the strictly noncompetitive behavior observed.

The product NH_3 and H_2O_2 demonstrate identical inhibition patterns, competitive with respect to benzylamine and uncompetitive with respect to oxygen. Thus, when present separately these products form dead-end complexes with the free enzyme, E (see Figure 5). The uncompetitive behavior toward oxygen arises from the fact that it is necessary to have both products present to make the reactions following the entry of oxygen reversible. This interpretation is confirmed by the results obtained when NH_3 and H_2O_2 were added together as inhibitors; the inhibition was then noncompetitive with respect to oxygen. In this experiment the behavior with respect to benzylamine was also altered, as expected for the formal mechanism given in Figure 5. The pathway through 5 and 6 would yield competitive behavior for H_2O_2, and noncompetitive for NH_3 with respect to the amine; that through steps 7 and 8 would yield the inverse result. Simultaneous operation of the two routes yields the mixed result actually observed (Figure 4). Only when one product is present at overwhelmingly greater concentration is the simple competitive result obtained. These observations constitute good evidence for the random release of the products.

One further point is worthy of mention here. This is the fact that the substrate water is not included in the mechanism. Since the concentration of water cannot be varied over a sufficient range, we cannot postulate the site of its involvement. At any rate, since the concentration of water is 55.5 M, a tremendous excess, we can exclude it in the present study and incorporate it in an apparent rate constant.

The rate equation for the formal mechanism presented in Figure 5 remained to be derived. Utilizing the schematic method of King and Altman (1956) the following expression was obtained for the initial velocity of the reaction where K_3 is the dissociation constant for the isomerization step.

$$\frac{E_0}{v_0} = \frac{k_{-1} + k_{+2}}{k_{+1}k_{+2}}\left[\frac{1}{C_6H_5CH_2NH_2}\right] + \frac{(K_3 + 1)(k_{-4} + k_{+5} + k_{+7})}{K_3k_{+4}(k_{+5} + k_{+7})}\left[\frac{1}{O_2}\right] + \frac{1}{k_{+3}} + \frac{1}{k_{+2}} + \frac{1}{k_{+5} + k_{+7}}\left[\frac{k_{+8}k_{+5} + k_{+7}k_{+6}}{k_{+6}k_{+8}(k_{+5} + k_{+7})}\right]$$

In this slope–intercept form it is evident that for amine substrates where the slope and maximum velocity vary by the same factor, k_{+2} is so small that it dominates V_m. In this instance k_{+2} is also much smaller than k_{-1} so that $K_m^{C_6H_5CH_2NH_2} = K_s^{C_6H_5CH_2NH_2}$, the dissociation constant for the first intermediate.

The formal mechanism proposed here on the basis of chemical studies and initial velocity and product inhibition patterns is consistent with all available data for the beef plasma amine oxidase. This formal mechanism will be tested further by isotope equilibration techniques and individual steps will be studied by use of alternate substrates.

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Chymotrypsin-Catalyzed Phenyl Ester Hydrolysis. Evidence for Electrophilic Assistance on Carbonyl Oxygen*

A. Williams

ABSTRACT: The chymotrypsin-catalyzed hydrolysis of substituted aryl hippurates and *N*-methanesulfonyl-L-phenylalaninates has been measured at pH 6.91, 25°, and 0.1 M ionic concentration. The parameter k_{cat} was essentially constant but k_{cat}/K_m obeyed a Hammett relationship (ρ 0.5 for hippurates, 0.45 for *N*-methanesulfonyl-L-phenylalaninates). The low sensitivity of k_{cat}/K_m to leaving group structure is interpreted as evidence for electrophilic assistance at the *carbonyl oxygen* during the formation of a tetrahedral intermediate. The positive ρ values are reconciled with the negative ones observed in chymotrypsin-catalyzed anilide hydrolysis by a mechanism involving almost complete proton transfer from an imidazolium cation to the departing aniline anion in the transition state for the breakdown of the tetrahedral adduct; proton transfer has not occurred in the corresponding transition state for aryl ester hydrolysis and the rate-determining step in acylation of chymotrypsin by aryl esters is the formation of the tetrahedral intermediate. A mechanism involving protonation of the nitrogen of the anilide prior to tetrahedral adduct formation is demonstrated not to be consistent with the kinetics for chymotrypsin-catalyzed hydrolysis of anilides. Acylation of chymotrypsin by substituted phenyl acetates has a ρ value of +1.8 which could arise from the absence of electrophilic assistance at the carbonyl oxygen owing to the nonspecific esters orientating themselves differently from specific substrates on the enzyme surface.

Few systematic studies have been made on the effects of leaving group structure on reactivity in the acylation of chymotrypsin by esters. Bender and Nakamura (1962) have observed that k_{cat}/K_m for substituted phenyl acetates obeys a Hammett equation, being correlated with σ^- with a ρ value of +1.8. More work has been reported on substituent effects where the acyl group has a specific backbone and an aniline leaving group (Sager and Parks, 1963, 1964; Inagami *et al.*, 1965; Wang and Parker, 1967; Caplow, 1969; Parker and Wang, 1968; Wang, 1968; Inagami *et al.*, 1969). The acylation of chymotrypsin by substituted anilides in general obeys a Hammett relationship with a negative ρ value in contrast to the aryl ester results. The suggestion has been made that acylation by anilides requires electrophilic assistance in the form of a proton transfer to the nitrogen of the anilide from an imidazolium moiety. This catalysis is the microscopic reverse of the general base (imidazole) catalyzed attack of the nucleo-

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