cedure B: Pure 1,2-dimethoxyethane (8 g.) was added to 5 g. of finely pulverized cobalt(II) chloride with vigorous shaking. The mixture was allowed to stand overnight and then the blue crystals were collected by filtration. They were thoroughly washed with heptane and dried at reduced pressure (Product B). Procedure C: A mixture of 6 g. of cobalt(II) chloride and 100 g. of 1,2-dimethoxyethane was refluxed for an hour. A dark blue liquid which appeared as the lower layer was separated. This liquid gave a blue solid (Product C) on standing 10 hr. in a vacuum desiccator. All the products were very hygroscopic and turned pink in color in the air. All the handlings described above were made in a desiccated chamber or under nitrogen atmosphere but complete exclusion of moisture was not possible.

None of the products A, B and C is a pure compound. Their typical analytical values (Table IV) indicate that they are mixtures of $Co(C_4H_{10}O_2)_x(H_2O)_yCl_2$ differing in x and y. However, infrared spectra of these products in the range 7–15 μ showed no significant differences. Several products not listed in Table IV obtained by any of the three procedures and differing in carbon content (6–17%) also gave substantially the same spectrum. This means that the ligand, 1,2-dimethoxyethane, assumes the same configuration in all these products. It is possible that the ligand acts as a bidentate group and links two cobalt atoms, because two products whose approximate compositions were, respectively, $Co(C_4H_{10}O_2)_{0.5}Cl_2$ and $Co(C_4H_{10}O_2)Cl_2$ showed the same spectrum.

TABLE IV

ANALYTICAL VALUES FOR THE PRODUCTS

Product	C	H	Co
A	9.65	3.63	29.75
B	16.07	3.73	28.05
C	15.68	4.55	24.33
Calcd. for $CoCl_2 \cdot H_2O$	0	1.36	39.86
$Co(C_4H_{10}O_2)_{0.5}Cl_2$	13.73	2.88	33.70
$Co(C_4H_{10}O_2)_{0.5}Cl_2 \cdot H_2O$	12.45	3.66	30.55
$Co(C_4H_{10}O_2)Cl_2$	21.84	4.58	26.79
$Co(C_4H_{10}O_2)Cl_2 \cdot H_2O$	20.18	5.08	24.77

The infrared spectrum of the liquid intermediate (*Anal.* C, 22.5; H, 6.5; Co, 16.0) obtained in the procedure C had also a strong band at about 850 cm.$^{-1}$ but no band in the range 960–890 cm.$^{-1}$. However, it was somewhat different from that of the solid products especially in the region 1100–970 cm.$^{-1}$. The ligand in this liquid seems still to exist in the *trans* form, but in this case it may act as a unidentate group.

The products were very labile and attempts to purify them by recrystallization failed to give purer products.

Infrared Spectra.—The infrared spectra were obtained with a Perkin–Elmer model 21 spectrophotometer equipped with a sodium chloride prism. Liquid samples were examined as capillary films supported between rock salt plates. Solid samples were examined as Nujol mulls.

[CONTRIBUTION FROM THE NAVAL MEDICAL RESEARCH INSTITUTE, BETHESDA, MARYLAND]

Kinetics of the System α-Chymotrypsin Methyl Hippurate Water Hydroxylamine: the Role of Water in Enzymatic Hydrolysis[1]

BY SIDNEY A. BERNHARD,[2] WILLIAM C. COLES AND JOHN F. NOWELL

RECEIVED JUNE 15, 1959

In view of the characteristic formation of measurable intermediate complexes between enzyme and substrate, it has been of interest to ascertain whether such reactants and complexes equilibrate during the reaction process. Dissociation constants (K_M) can be determined for these complexes. These constants pertain to either a stationary state or a state of quasi-equilibrium. A method has been devised for distinguishing these two possibilities which involves the competition of two reagents for alternate reaction paths. The kinetics of α-chymotrypsin catalyzed hydrolysis and hydroxylaminolysis of methyl hippurate as a function of substrate, hydroxylamine and hydronium ion concentration has been investigated. In the presence of α-chymotrypsin in aqueous solution at the order of tenth-molar hydroxylamine, both hippurate ion and hippuryl hydroxamic acid are formed. The formation of hippuryl hydroxamic acid is accompanied by a diminution in the rate of hippurate formation, although the total rate of disappearance of methyl hippurate is always larger in the presence of hydroxylamine than in its absence. The dissociation constant for methyl hippurate–enzyme complex is independent both of the concentration of hydroxylamine and of the particular reaction path employed in its determination. According to the theory of the above mentioned method, this latter fact constitutes a proof that this dissociation constant is an equilibrium constant, whereas the independence of binding constant on hydroxylamine concentration constitutes a proof that the binding of hydroxylamine (and water by analogy) is independent of the binding of substrate (according to a corollary of the theory). The enzyme-catalyzed hydroxylaminolysis reaction, by its pH dependence, must involve the free base NH_2OH as the principal reactant species in correspondence with the hydrolysis reaction, which involves molecular water. As with all other neutral substrates for this enzyme, the activity depends on the dissociation of a proton (pK ~7) from the enzyme. This dissociation is independent of the nature of the reagent and of both the dissociation of substrate and reagent from the enzyme.

Introduction

Nearly all enzymatic processes involve the interaction of three or more molecules, namely, two or more substrates and an enzyme. In the present paper, we shall consider evidence bearing on the simplification of the complex model which follows from the complete steady-state treatment of a process involving the interaction of three molecular participants for finite times. In another communication[3] the kinetic consequences of the association of these participants for finite times is considered. Often, the chemical kinetics of enzymatic processes have been simplified by neglecting the concentration dependence of one of the substrates, as for example water in hydrolysis reactions. Such omissions, although mathematically valid when the substrate omitted is present in large excess,[4] limit to a large extent the mechanistic

(1) The opinions expressed herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

(2) National Institute of Mental Health, Bethesda, Md.

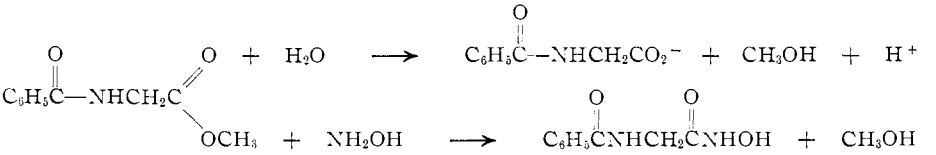
(3) S. A. Bernhard, THIS JOURNAL, in preparation.

(4) More precisely, the omission is valid for either of the following two cases only: (1) All sites for this substrate (W) are occupied. (2) The pathway of complex formation is such that only the terminal complex contains this substrate W, and the terminal complex concentration is infinitesimally small due to extremely rapid reaction (*i.e.*, EW does not exist in appreciable quantities and the formation of ES is rate controlling).

$$W + E + S \rightleftharpoons ES + W \rightleftharpoons ESW \xrightarrow{\text{Very rapid}} E + \text{Products}$$

(S. A. Bernhard, *Discussions Faraday Soc.*, **20**, 267 (1956).)

detail that can be deduced from the kinetic data. Since a large fraction of the data already accumulated regarding enzymatic mechanisms and enzyme–substrate specificity has come from studies of hydrolytic enzymes, a hydrolytic enzyme (α-chymotrypsin) has been selected in the model system. Since the concentration of water cannot be sensibly varied without the possibility of profound alteration to the structure and function of the enzyme, a water analog has been added to the system, *viz.*, hydroxylamine. This analog has the virtue of competing effectively with water at concentrations of hydroxylamine of the order of 0.1 *M*. The substrate selected was methyl hippurate. The α-chymotrypsin catalyzed hydrolysis of this ester already has been studied by Niemann and co-workers[5a,b] and by Lang, Frieden and Grunwald.[5c] The over-all reactions studied here may be represented as



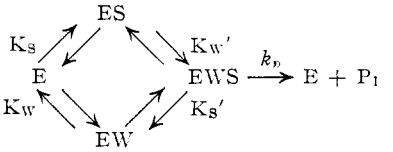
This system has particular advantages due to:

(1) A moderate binding affinity of enzyme for the ester and hence an easily determinable concentration dependence.

(2) Simple analytic procedures for precise estimation of the extent of either reaction.

(3) The possibility of following the reactions under conditions such that the product may be either entirely carboxylate or virtually all hydroxamic acid, without affecting the stability of the enzyme.

One need only consider a general model for a hydrolysis in water, *viz.*



where the step ESW → E + P can include the conversion ESW ⇄ EP_1. The general solution for the rate equation of the above model

$$P_1 = k_p(ESW)$$

i.e., the solution which involves *no assumptions whatsoever*, is

$$\frac{P_1}{k_p E_0} = 1 + \frac{K_S'}{S} + \frac{K_W'}{W} + \frac{K_S K_W'}{SW} + \frac{K_S K_W'}{SW}(k_W + k_S'S)(k_S + k_W'W) + \frac{K_W'}{W} k_S(k_W + k_S'S) + \frac{K_S'}{S} k_W(k_S + k_W'W)$$

$$\frac{}{r_S k_W(k_S + k_W'W) + r_W k_S(k_W + k_S'S)}$$

where the capital K refers to the particular *equilibrium* dissociation constant *e.g.*

$$K_W = \frac{k_{-W}}{k_W}, \text{ and } r_S = \frac{k_{-S}'}{k_p}, \; r_W = \frac{k_{-W}'}{k_p}$$

Even a cursory inspection of the rate equation reveals that the predicted functional dependence of rate on S concentration is different depending on whether the general model given above or the model which omits consideration of W is assumed. The two models are of course identical for an equilibrium situation ($r_S, r_W \to \infty$). If $W >>> K_W'$ then the *form* of the rate equation given above is the same as that given by the Michaelis–Menten relationship but this relationship can certainly not be assumed.

(5) (a) H. T. Huang and C. Niemann, THIS JOURNAL, **74**, 4634 (1952); (b) T. H. Applewhite, H. Waite and C. Niemann, *ibid.*, **80**, 1457 (1958); (c) J. H. Lang, E. Frieden and E. Grunwald, *ibid.*, **80**, 4923 (1958).

(4) The ease of preparation or ready availability of all reagents as highly purified crystalline materials.

The reactions were studied as a function of varying hydroxylamine concentration and of varying methyl hippurate concentration under conditions such that [Methyl hippurate] >> [Enzyme] << [Hydroxylamine] << (H_2O).

Experimental

I. **Reagents. Methyl Hippurate.**—Hippuric acid (Eastman Kodak Co.) was recrystallized from water and dried *in vacuo*. A 1.0 *M* solution of the acid in absolute methanol was refluxed with 25 g. of methanol extracted dry Amberlite IR-120 resin per mole of hippuric acid. When the titer of acid solution fell below 0.05 *M*, refluxing was discontinued and approximately 90% of the methanol was removed. The solution was cooled to room temperature and the ester precipitated by the addition of an amount of water equal to the methanol removed. The mixture was brought to about *p*H 7 with 1 *M* NaOH with vigorous and prolonged stirring. The precipitate was twice recrystallized (in long needles) from 70° H_2O and dried *in vacuo*. Saponification equivalent (by basic hydrolysis at 80°), 196; (theoretical, 194); m.p. 83°.

Hippuryl Hydroxamic Acid.—To 2.0 g. of methyl hippurate were added in succession 10 ml. of 5.0 *M* hydroxylamine hydrochloride and 10 ml. of 5.5 *M* NaOH. The mixture was stirred until all of the ester disappeared and then brought to *p*H 6.5 with concd. HCl. The resultant precipitate was recrystallized twice from boiling water and dried *in vacuo*. Equivalent weight (by titration with standard base) 195 g. (theoretical 195).

Hydroxylamine Hydrochloride.—Fisher Certified Reagent Grade material was recrystallized from boiling methanol and dried *in vacuo*. Equivalent weight, 69.5 g. (by titration with standard base); (theoretical 69.5).

α-Chymotrypsin.—A Worthington Co. recrystallized salt-free preparation (batch No. 5675) was stored at 5° and used without further purification in all experiments.

Monochloroacetic acid (Fisher Reagent Grade) and $FeCl_3 \cdot 6H_2O$ (Mallinckrodt Reagent Grade) were not further purified. Acid and base standards (approximately 0.1 *M* HCl and 0.1 *M* CO_2-free NaOH) were prepared by the usual procedures.

II. **Analysis for Hydroxamic Acid.**—The basis of the analytical procedure is the formation of a purple Fe^{+3}–hydroxamic acid complex. Previous procedures for quantitative estimation of hydroxamic acid on this basis were found not to meet the precision requirements of these experiments. The complicating features of the color test being due primarily to (1) the rapid fading of the color with time, (2) the sensitivity of color intensity to *p*H and (3) the evolution of gaseous side products causing bubbles in the color tube. More details concerning the principles of the present method will be presented elsewhere[6]; the method developed is as follows:

Reagents.—(1) 4 *M* monochloroacetic acid (MCA); (2) HCl at a concentration of 3 × $[NH_2OH]$ (± 2%) in the reaction mixture; (3) $FeCl_3 \cdot 6\,H_2O$, 5 g./100 ml. in 0.05 *M* HCl.

Method.—Equal volumes of MCA and HCl are mixed prior to the kinetic run and 2 ml. of the mixture transferred to each of a set of test tubes. Three ml. aliquots of the reaction mixture are transferred to each tube and the time of addition recorded; the reaction being stopped instantaneously at the resultant *p*H (about 2.3). The tubes may be stored at room temperature for several hours without any detectable change. The colored iron–hydroxamic acid complex is developed by the addition of 2 ml. of the ferric reagent, and the color intensity (against a water blank) is measured 5 minutes after the addition of the ferric reagent.

(6) S. A. Bernhard and J. F. Nowell, in preparation.

A small drift (about 0.003 optical density unit) occurs during the first few minutes. A color blank consisting of the reaction solvent and test reagents is read against water in a similar manner. A Beckman Model DU Spectrophotometer at a wave length of 520 mμ was used for all measurements reported here. As an added precaution, the concentration–optical density dependence of purified samples of the hydroxamic acid in the same reaction solvents was determined. Excellent agreement with Beers' law was invariably found and the extinction co-efficient was insensitive to ionic strength.

Hydroxylamine Test for Esters.—Essentially this same method was found to be an excellent quantitative test for esters. To 1 ml. of an adequately diluted ester sample were added in succession 1 ml. of 4 *M* hydroxylamine hydrochloride, 1 ml. of 5 *M* NaOH and 2 ml. of a solution 2.5 *M* in HCl and 2 *M* in MCA. The color reaction with $FeCl_3$ is then run as above.

III. **Hydrolysis Measurements.**—The hydrolysis of methyl hippurate was followed by potentiometric titration at *p*H 8.00 as described previously.[7] At higher concentrations of substrate an appreciable drop in *p*H with time was noted in the absence of enzyme. An apparatus for eliminating atmospheric CO_2 was designed. CO_2-free stock solutions were used and were continuously bubbled with CO_2-free nitrogen (which had previously been equilibrated with water at the ionic strength and temperature of the solvent). The rate of hydroxyl ion catalysis of the hydrolysis of methyl hippurate determined in the CO_2-free atmosphere at *p*H 8 was appreciable, the velocity was found to be first order in substrate and OH^- concentration (see results below).

IV. **Preparation of Reaction Mixtures.**—Both the hydrolysis and the hydroxylaminolysis reactions were set up in the CO_2-free apparatus described above, and the *p*H was maintained constant (to a *precision* of ± 0.001 *p*H) by *manual* addition of base *via* a microburet. Concentrations of methyl hippurate varied between 2×10^{-2} and 5×10^{-4} *M*. In the hydrolysis experiments, the NH_2OH concentration was varied between zero and 0.10 *M*, whereas in hydroxylaminolysis experiments this concentration was usually in the range 0.05–0.25 *M*. The ionic strength was generally 0.25 *M*, the *p*H 8.00 and the temperature was always 25.00 ± 0.02°. A number of experiments at different *p*H and ionic strengths and at higher [NH_2OH] were run in addition. To 40 ml. of substrates was added 1–2 ml. of an enzyme solution containing 1–2 mg. enzyme/ml. In this range of enzyme concentration the velocity (corrected for blank reaction) is strictly proportional to enzyme concentration. Enzyme catalyzed reactions were measured over periods of from 5–25 minutes. Enzyme solutions were checked daily by following the hydrolysis of 40 ml. of 0.01 *M* methyl hippurate in 0.25 *M* KCl. It was found that such enzyme solutions could be prepared by direct weighing of crystalline α-chymotrypsin to yield activities reproducible to ± 1%. Solutions could be stored for periods of as long as one week without any measurable change in activity in this air-conditioned sunlight-free laboratory.

The "uncatalyzed" rates of hydroxylaminolysis in the same solvent were determined in the absence of added buffer (such as "Tris"). The rate of hydroxylaminolysis was found to be first order in NH_2OH and first order in substrate. These uncatalyzed rates were determined in the same apparatus as all other rates described above. Enzyme catalyzed rates were properly corrected for these uncatalyzed rates of hydroxylaminolysis and hydrolysis.

Hydroxylamine was prepared from the hydrochloride *immediately before each run* by mixing equal volumes of 1.000 *M* $NH_3OH^+Cl^-$ and 1.00 *M* KOH and adjusting to *p*H 8.00. Reaction mixtures were prepared by mixing *x* ml. of the above solution with 20 − *x* ml. of 0.50 *M* KCl and 20 ml. of aqueous methyl hippurate solution.

V. **Precision Measure.**—Under the conditions of these experiments the limiting concentration was always that of methyl hippurate. In the experiment at lowest methyl hippurate concentration the total initial amount of ester was 2×10^{-2} meq. Titrant could be delivered with the microburet to ± 1×10^{-5} meq. Hence, over the first 5% of the total extent of reaction, the rate of hydrolysis could be measured to ± 2% under the most unfavorable condition and to ± 1% usually. The rates of hydrolysis were virtually linear under those conditions. The hydroxamic acid test (see above) was precise ± 5×10^{-6} *M* hydroxamic acid. The kinetic runs of lowest ester concentration hence required measurement of a greater extent of total reaction. The initial rate, however, could be derived from measurements over this extent of reaction (≤ 20% reaction) by successive approximation assuming a Michaelis–Menten type equation and a crude numerical value for the constant (K_m) estimated from the higher methyl hippurate concentration data. By this method, linear plots for determining initial velocity were obtained readily for the lowest concentration of methyl hippurate in the hydroxylaminolysis reaction. At higher concentrations of methyl hippurate the hydroxylaminolysis as well as the hydrolysis could be followed in the range of linear concentration *versus* time range. Precision of the hydroxylaminolysis rate determinations were to ± 1% in all but the lowest concentration experiments. The precision measure by least square analysis of the Michaelis–Menten constants determined was ± 2% for hydrolysis and ± 3–4% for hydroxylaminolysis. Reproducibility of these constants in separate sets of runs was to ± 5% for both reactions. At no time did subtraction of the uncatalyzed rates significantly affect the precision measure of the experiments.

Ionization constants were determined with a Beckman Model G *p*H meter to ± 0.02 *p*H unit. In all cases the meter was standardized with a buffer within 0.5 *p*H unit of the *pK* of the particular acid. The total precision measure of the *pK* was ± 0.04 *pH* unit.

Results

Variation of Substrate Concentrations.—The effect of NH_2OH on both the rate of hydrolysis and of hydroxylaminolysis of methyl hippurate was studied at *p*H 8.00 and 25°. In Figs. 1 and 2 the effect of NH_2OH on the two enzymic reactions is shown. The rate of hydrolysis (v_W) decreased with increasing concentration of NH_2OH while (over this same concentration range) the rate of hydroxylaminolysis (v_N) increased. At all concentrations of NH_2OH shown in the figures it was found that

$$v_W + v_N > v_W^0$$

(where v_W^0 is the rate of hydrolysis under conditions identical except that [NH_2OH] = 0). The enzyme thus appears not to be inhibited by these concentrations of NH_2OH. It should be noted that the rates in both curves approach a saturation level with respect to NH_2OH. Following the theoretical treatment[3] (see Discussion), the velocities v_N and v_W were measured as a function of varying methyl hippurate concentrations at fixed [NH_2OH], and as a function of varying [NH_2OH] at fixed concentrations of methyl hippurate. The results are summarized in Figs. 1 and 2 and Table I.

Since both reactions proceed to a measurable extent at *p*H 8.0 in the absence of enzyme, it was necessary to determine the "uncatalyzed" reaction rates under all the conditions encountered in the various experiments. A detailed account of these experiments will be published later. The conclusions regarding the non-enzymatic rate can be summarized as:

(1) The velocities of both hydrolysis and hydroxylaminolysis are first order in methyl hippurate.

(2) The velocities of both reactions are first order in hydroxyl ion concentration over the range of *p*H 8–9.

(3) The rate of hydroxylaminolysis as a function of hydroxylamine concentration over the range *p*H 6–10 can be expressed by the equation

$$v = a[NH_2OH] + b[NH_2O^-]$$

where $b > 10^6 a$.

(7) G. W. Schwert and M. A. Eisenberg, *J. Biol. Chem.*, **179**, 665 (1949).

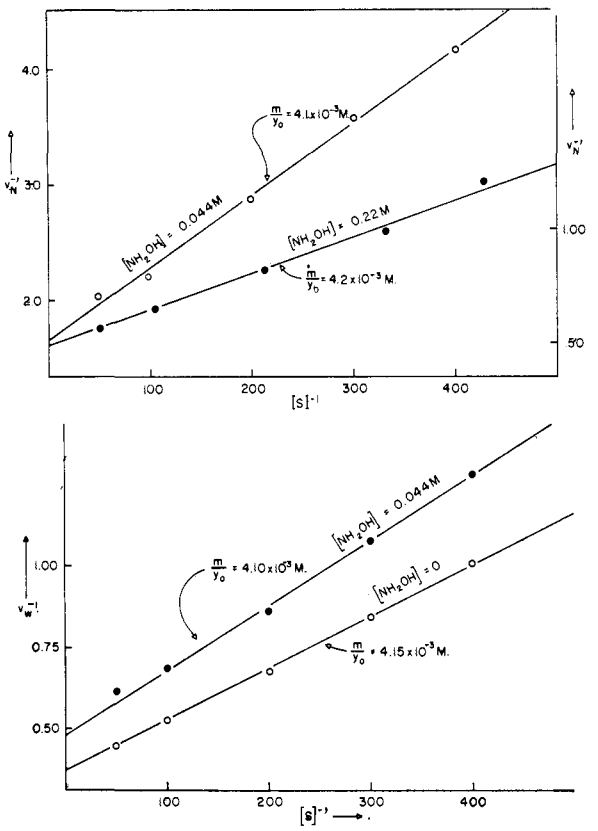


Fig. 1.—Reciprocal plots for the rates of hydroxylaminolysis: (1) O, left hand ordinate; ●, right hand ordinate, and hydrolysis (1) of methyl hippurate, as a function of methyl hippurate concentration; *p*H 8.00, 0.25 *M* KCl. Velocities in arbitrary units; v_N and v_W ($S \rightarrow \infty$) are given in Table I.

(4) The rate of hydrolysis is *increased* by the addition of hydroxylamine at *p*H 7–8.

(5) Both reactions are ionic strength dependent and can be expressed by the velocity equation $\ln v = \ln v_0 + k\mu^{1/2}$, where v_0 is the rate at zero ionic strength and μ is the ionic strength.

TABLE I

α-CHYMOTRYPSIN CATALYZED HYDROLYSIS AND HYDROXYLAMINOLYSIS OF METHYL HIPPURATE

25°, *p*H 8.00 and 0.25 *M* KCl. Chymotrypsin concentrations 1.4×10^{-6} *M*.

$[NH_2OH]$, *M*	$V_{max}{}^{N}$ [b]	$V_{max}{}^{W}$ [b]	$K_M{}^{N}$, $M \times 10^3$	$K_M{}^{W}$, $M \times 10^3$
0.020	...	0.262	..	4.20
.044	0.210	.232	4.10	4.10
.050	.240	.229	4.30	4.25
.100	.435	.191	4.25	4.10
.220	.670	...	4.20	..
.250	.720	...	4.15	..
.500[a]	.960	...	4.20	..
.0	.00	.273	..	4.15

[a] In 0.5 *M* KCl. [b] In units of sec.$^{-1}$, assuming a mol. wt. of 2.4×10^4 for α-chymotrypsin.

At *p*H 8.00 and $\mu = 0.25$ *M*, the specific rates of hydrolysis (in the absence of NH_2OH) and hydroxylaminolysis are $k_W = 2.81 \times 10^{-6}$ sec.$^{-1}$ and $k_N = 2.48 \times 10^{-5}$ M^{-1} sec.$^{-1}$ respectively, where $v_W = k_W[S]$ and $v_N = k_N[S]\,[NH_2OH]$.

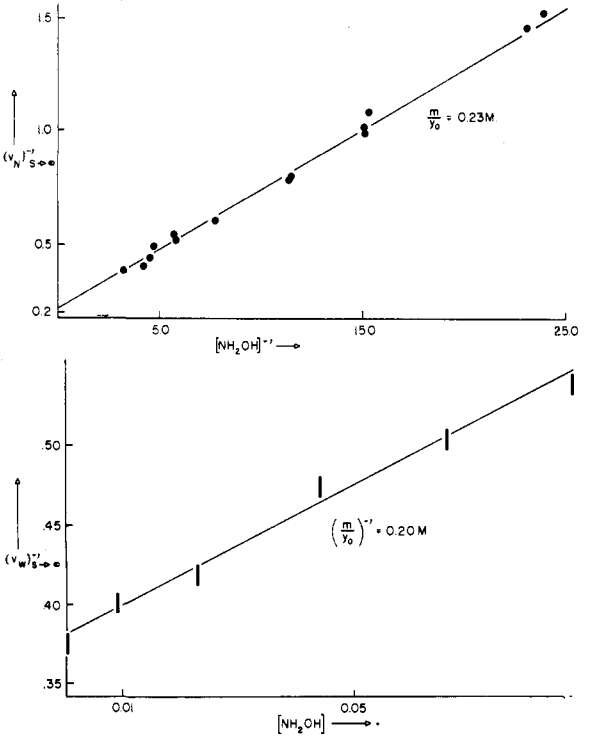


Fig. 2.—Reciprocal plots for the limiting rates of hydroxylaminolysis (2a) and hydrolysis (2b) of methyl hippurate, as a function of hydroxylamine concentration; *p*H 8.00, 0.25 *M* KCl. Absolute velocities at $N \rightarrow \infty$ and $N = 0$ are given in text.

Effect of *p*H.—In order to determine the effect of *p*H on the active site of the enzyme, it was first necessary to study the equilibria between hydrogen ion and the reactants and products in the system, *viz.*

$$\text{hippuryl-OH} \rightleftharpoons \text{hippuryl-O}^- + \text{H}^+$$

$$\text{hippuryl NHOH} \rightleftharpoons \text{hippuryl NHO}^- + \text{H}^+$$

$$\text{NH}_3\text{OH}^+ \rightleftharpoons \text{NH}_2\text{OH} + \text{H}^+$$

Titration studies of the individual compounds in 0.25 *M* KCl at 25° were therefore carried out. The concentrations of acids employed in these studies were representative of the actual concentrations measured in rate experiments. The pK_a's of *hippuric acid, hippuryl hydroxamic acid* and NH_3OH^+ were calculated to be *3.45 ± 0.05, 8.95 ± 0.02,* and *6.08 ± 0.02,* respectively, under these conditions. The association of hippurate ion with hydrogen ion hence could be neglected in rate experiments. The dissociation of the hydroxamic acid, however, had to be accounted for in measurements of the hydrolysis rate since the method of measurement of the extent of hydrolysis was dependent on the liberation of hydrogen ions during the course of the reaction. The total reaction scheme at constant *p*H is represented in Fig. 3. [Y] is the total concentration of hydroxamic acid as determined by the hydroxamic acid test, $X + Y(\alpha + \beta)$ is the total hydrogen ion concentration liberated as determined by titration and α and β at fixed *p*H are given by the equations

$$\alpha = [H^+]/(K_\alpha' + [H^+]) = [H^+]/(9.2 \times 10^{-7} + [H^+])$$

$$\beta = K'/(K\beta' + [H^+]) = 1.17 \times 10^{-9}/(1.17 \times 10^{-9} + [H^+])$$

The rate of formation of hippurate was determined from the rates of hydrogen ion and hydroxamic acid production by the relationship

$$dx/dt = d[H^+]/dt - (\alpha + \beta)\frac{dy}{dt}$$

In order to obtain sufficient sensitivity in the determination of dx/dt, it was necessary to minimize the coefficients $(\alpha + \beta)$ by confining the pH range of experiments. Fortunately the pH range of high enzymatic activity (pH 7.0–8.0) coincided with low values for $\alpha + \beta$ (< 0.1).

The possibility of the two reverse reactions

$$RCO_2^- + NH_2OH \rightleftharpoons RCONHOH + OH^-$$

could be excluded since the velocity of either of these reactions was found to be very slow even at concentrations comparable to the original methyl hippurate. The dissociation constants of both these products from the enzyme were found to be an order of magnitude larger than that for the ester. Since a maximum of 10% of the total extent of reaction was followed, inhibition by-product could be ignored.

The previously noted proton inhibition of chymotrypsin catalyzed reactions was found to occur in hydroxylaminolysis as well. In this latter case it is important to differentiate between proton binding to substrate (NH_2OH or NH_2O^-) and enzyme. Since the pH region of enzymatic activity is near the pK_a of NH_3OH^+ and far removed from the pK_a of NH_2OH, it was possible to establish the reactive charge species in catalysis. This can be deduced by inspection of Table II. Between pH 7.5 and 8.0, V_{max}^N changes by only a factor of 1.1 whereas the ratio $\frac{[NH_2O^-]}{[NH_2OH]}$ changes by a factor of 3.2. V_{max}^W changes by the factor of 1.1 as well indicating that in this pH range proton binding to enzyme is the major inhibition determining factor. In the lower pH region, however, V_{max}^N falls more precipitously with pH than does V_{max}^W indicating proton binding inhibition of the substrate as well as of the enzyme. If NH_2OH were the only reactive species in hydroxylaminolysis, then V_{max}^N under these conditions would be given by

$$V_{max}^N = k_P^N E_0 \left\{\frac{N}{N + K_N(1 + H/K'_\alpha)}\right\}\frac{K_H}{(K_H + H)}$$

where K_α' is the dissociation constant of hydroxylammonium ion, K_H is the enzyme-H^+ dissociation constant, N is the total concentration of acidic and basic species of hydroxylamine, and E_0 is the total concentration of enzyme sites. If the hydrolysis and hydroxylaminolysis reactions are both dependent upon the same enzyme–H^+ dissociation, the qualities $V_{max}^N\{1 + (K_N/N)(1 + H/K_\alpha')\}$ and V_{max}^W should have the same pH dependencies. This is borne out by the results in Table III. Since at 0.25 M total hydroxylamine concentration the ratio K_N/N is equal to 0.9, the possibilities could be adequately distinguished. From these data K_H could be determined for both the hydrolysis and hydroxylaminolysis reactions from the slope of the linear plots of $(V_{max}^W)^{-1}$ or $[V_{max}^N\{1 + (K_N/N)(1 + H/K_\alpha')\}]^{-1}$ *vs.* $[H^+]$, K_H for both reactions was the same, namely, $1.0 \pm 0.1 \times 10^{-7}$ M, a value substantially the same as that determined for other reactions.

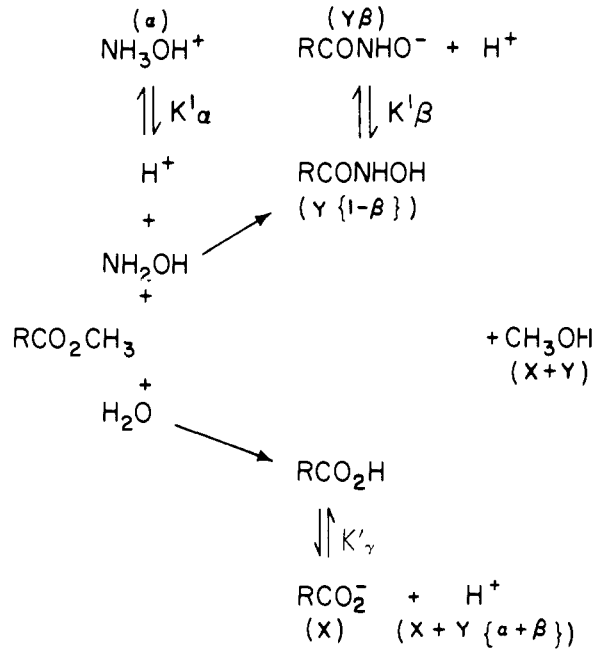


Fig. 3.—Competing reaction scheme (see text).

TABLE II

pH DEPENDENCE OF THE α-CHYMOTRYPSIN CATALYZED HYDROLYSIS AND HYDROXYLAMINOLYSIS OF METHYL HIPPURATE

pH	$K_M^{N\,a}$ (M × 10^3)	$K_M^{W\,b}$ (M × 10^3)	$V_{max}^{N\,a}$ (sec.$^{-1}$) ^{c}	$V_{max}^{W\,b}$ (sec.$^{-1}$) ^{c}	$V_{max}^N\{1 + (K_N/N)(1 + H/K_\alpha')\}$ (sec.$^{-1}$)
8.00	4.15	4.15	0.72	0.27	1.36
7.50	4.2	4.3	.67	.25	1.28
7.00	4.4	4.2	.35	.145	0.70
6.50	4.3	4.1	.165	.073	.37
6.00	4.5	4.2	.043	.025	.13

[a] In 0.25 M KCl and 0.25 M total concentration of hydroxylamine and hydroxylammonium. [b] In 0.25 M KCl only. [c] Assuming a mol. wt. of 2.4×10^4 for α-chymotrypsin.

From these data and Figs. 3 and 4 these conclusions can be drawn:

(1) Hydrogen ion is a true non-competitive inhibitor of both the α-chymotrypsin catalyzed hydrolysis and hydroxylaminolysis of methyl hippurate, *i.e., the binding of substrate and hydrogen ion to the enzyme are independent of each other.* As has been pointed out previously (see Discussion) this condition is both a necessary and a sufficient condition for quasi-equilibrium.

(2) The apparent dissociation constant of enzyme–H^+ inhibited complex is the same in the presence of high concentrations of NH_2OH and in its absence.

Equilibria Studies.— Since conditions could be specified such that either product (carboxylate or hydroxamate) would predominate, it was possible to study the equilibrium between hydroxamate and carboxylate as a function of hydroxylamine concentration. Total concentration of products was established by determination of hydroxa-

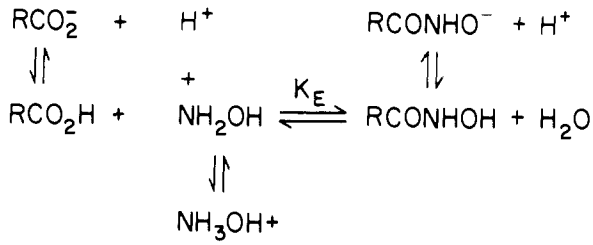


Fig. 4.—Equilibria among products (see text).

mate after periods of time sufficiently long such that the alkaline hydroxylamine test for both esters and hydroxamates gave quantitative agreement with the direct test for hydroxamate alone. The equilibrium constant for hydrolysis of esters

$$K = [RCO_2CH_3][CH_3OH]/[RCO_2H][H_2O]$$

is near unity[8,9]; hence in dilute aqueous solution at *p*H 8 the concentration of ester remaining at equilibrium would be expected to be trivially low.

The constant at fixed *p*H, K_N', defined as

$$K_N' = \frac{K_N}{[H_2O]} = \frac{[RCONHO^-] + [RCONH_2OH]}{[RCO_2^-][NH_2OH]} = \frac{x}{(1-x)[N]}$$

has been determined. To calculate the thermodynamically significant constants, we define the scheme in Fig. 4, hence

$$\frac{[N]}{H_2O} K_E = \frac{x}{(1-x)} \frac{(K_\alpha' + H)(K_\alpha' + H)}{K_\alpha'(K_\beta' + H)}$$

where [N] is the sum of activities of charged and uncharged species of hydroxylamine, and $x/(1-x)$ is the ratio of activities of total hydroxamate to total carboxylate. Assuming unit activity coefficients throughout, $K_E \sim 7.5 \times 10^6$ *M*.

It was of interest to compare at a fixed hydrogen ion concentration, the values of three parameters, *viz.*, (1) the pseudo equilibrium constant, K_N', (2) the "binding constant" of NH_2OH to the enzyme determined from both the rates of hydrolysis and hydroxylaminolysis (as defined below), and (3) the "competition" constant defined as

$$R = V_{N,S}/V_{W,S}$$

where $V_{W,S}$ is the maximum velocity of hydrolysis at infinite substrate concentration in the absence of NH_2OH and $V_{N,S}$ the corresponding maximum velocity of hydroxylaminolysis at infinite substrate and hydroxylamine concentrations. Table III summarizes the pertinent results.

Discussion

The use of hydroxylamine as a water analog in this system stems from the naive assumption that a mechanism in which k_3 (in the Michaelis–Menten—Briggs–Haldane model[10,11]) can be varied under conditions of the steady state, will lead to information concerning the nature of the parameter K_M, *viz.*

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3(N)} E + P \quad K_M = \frac{k_2 + k_3(N)}{k_1} \quad (1)$$

$$v_N = k_3(N)\, E_0[S]/(K_M + [S]) \quad (2)$$

(8) L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Co., New York, N. Y., 1940, p. 213.

(9) (a) G. E. K. Branch and D. S. McKittrick, THIS JOURNAL, **45**, 321 (1923). (b) Prof. M. L. Bender has found a value of ~5 for esters of this type (to be published.)

TABLE III

COMPETITION BETWEEN WATER AND HYDROXYLAMINE FOR ENZYME, SUBSTRATE AND ENZYME-SUBSTRATE COMPLEX

*p*H 8.00, 0.25 *M* KCl, 25°

Type of experiment	Constant	Value
Equilibrium	$(K_N')^{-1}$	0.18 *M*
Competitive binding	K_N	0.22 *M*
Velocity at substrate saturation	V_N/V_W	5.0

The naiveté of such a model is immediately obvious in general, in that the binding of the second reactant (N) to the enzyme has been ignored, and in this specific case, in that the competition with water (W) (both for binding site and reaction) has been neglected. Nevertheless, by working under the assumptions involved in derivation of equations 1 and 2 and carrying out the rate *vs.* concentration experiments dictated by these equations, the empirically derived parameter $K_M(N)$ is indistinguishable from the similarly determined parameter $K_M(W)$ using another rate (the hydrolysis rate) and a different second reactant (water). It is evident, therefore, that the parameter K_M is in this case independent of the second reactant. Since the so-called "turnover rate" is critically dependent on the concentration of second reactant, K_M *cannot be a function of* k_3 *in this system.*

At this point it is well to restate the pertinent facts concerning the system methyl hippurate–α-chymotrypsin–NH_2OH–H_2O, *viz.*: (a) The Michaelis–Menten constant (K_M), as determined from Lineweaver and Burk plots, is invariant to [NH_2OH] and to the particular reaction velocity employed in the determination. (b) The rate of hydroxylaminolysis is dependent on [NH_2OH] and approaches a limiting maximum velocity at high concentrations of NH_2OH and methyl hippurate. (c) The hydrolysis of methyl hippurate is noncompetitively inhibited by NH_2OH. The apparent inhibition constant, as determined by the method of Lineweaver and Burk,[12] is the same as the apparent Michaelis–Menten constant (K_M^N) as determined from the variation of v_N with [NH_2OH]. (d) The limiting rates of hydrolysis V_W([S] → ∞ [NH_2OH] = 0) and hydroxylaminolysis V_N· ([S] → ∞, [NH_2OH] → ∞) are of the same order of magnitude although not equal to each other. (e) The direct rates of interconversion of $P_N \rightleftarrows P_W$ are small relative to the over-all rates S → P_N, S → P_W.

It might appear that the independence of K_M on hydroxylamine concentration constitutes a proof that K_M is an equilibrium constant. However, this is not a necessary condition. Consider the general model for competitive binding and reaction shown in Fig. 5. If equilibrium does not obtain, the rate of a particular reaction is governed by both the equilibrium binding constants (K_j^i with respect to both ESN and ESW) and by the rate at which these complexes decompose. Specifically, these rates of reaction are given by equations 3 and 4, where N and W are the maintained concentrations of the two reactants. Q_N and Q_W in equations 3 and 4 may be considered

(10) L. Michaelis and M. L. Menten, *Biochem. Z.*, **49**, 333 (1913).

(11) G. E. Briggs and J. B. S. Haldane, *Biochem. J.*, **19**, 338 (1925).

(12) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

$$v_N = k_P^N E_0 / \left[\left\{ 1 + \frac{W}{K_N^S} + \frac{N}{K_N^S} + \frac{K_S}{S}\left(1 + \frac{W}{K_W} + \frac{N}{K_N}\right)\right\}\frac{K_N^S}{N} + Q_N \right] \quad (3)$$

$$v_W = k_P^W E_0 / \left[\left\{ 1 + \frac{W}{K_W^S} + \frac{N}{K_N^S} + \frac{K_S}{S}\left(1 + \frac{W}{K_W} + \frac{N}{K_N}\right)\right\}\frac{K_W^S}{W} + Q_W \right] \quad (4)$$

as perturbation terms which determine the extent to which the concentration of the corresponding triple complex is altered from its equilibrium value due to the decomposition rates (k_P^N and k_P^W).[13] The terms to the left of Q in equations 3 and 4 are equal to $E_0/[ESN]$ and $E_0/[ESW]$, respectively, under conditions such that k_P^N/k_{-i}^j and k_P^W/k_{-i}^j are negligible, *i.e.*, at quasi-equilibrium. These equations may be rewritten

$$v_N = k_P^N[ESN]; \; v_W = k_P^W[ESW] \quad (5)$$

$$[ESN] = [ESN]_{eq} \Big/ \left\{1 + Q_N \frac{[ESN]_{eq}}{E_0}\right\}$$

$$[ESW] = [ESW]_{eq} \Big/ \left\{1 + Q_W \frac{[ESW]_{eq}}{E_0}\right\}$$

The exact expressions for the Q's have been considered elsewhere.[3] These expressions, although highly complex (they contain all the parameters and variables of the model shown in Fig. 8), have some predictable properties. The two Q's are not equal, nor do they exhibit the same dependence on the concentration of a particular reagent. Each Q is directly proportional to the value of the corresponding k_P, *i.e.*

$$Q_N = k_P^N \Phi(N,W,S)$$
$$Q_N = k_P^W \Phi(N,W,S)$$

At quasi-equilibrium the Q's vanish and by equations 3–5 we have

$$\frac{v_N^{-1} k_P^N E_0 N}{K_N^S} = 1 + \frac{W}{K_W^S} + \frac{N}{K_N^S} + \frac{K_S}{S}\left\{1 + \frac{W}{K_W} + \frac{N}{K_N}\right\} = \frac{v_W^{-1} k_P^W E_0 W}{K_W^S} \quad (6)$$

which predicts a linear relationship between the inverse of either velocity and the inverse of the substrate concentration (when all reagent concentrations are maintained) in correspondence with the usual Michaelis–Menten model[10] of enzyme kinetics. By carrying out the analysis of the velocity–concentration relationship predicted by this latter model one obtains from equation 6 the two parameters K_M^W and K_M^N defined as

$$\frac{d(1/v_N)}{d(1/S)} \Big/ \left(\frac{1}{V_{max}^N}\right) = K_S \frac{\left(1 + \frac{W}{K_W} + \frac{N}{K_N}\right)}{\left(1 + \frac{W}{K_W^S} + \frac{N}{K_N^S}\right)} = K_M^N \quad (7)$$

$$= \frac{d(1/v_W)}{(1/S)} \Big/ \left(\frac{1}{V_{max}^W}\right) = K_M^W$$

where V_{max} = limit $v_{(S \to \infty)}$

From equation 7 we conclude that the independence of K_M^N on hydroxylamine concentration *is not* a necessary condition for the maintenance of quasi-equilibrium but that *the equality of* K_M^N *and* K_M^W *(at fixed concentrations of N and W) is a necessary condition.* Since Q_N and Q_W of equations 3–5 are not equal, the condition $K_M^N = K_M^W$ is a sufficient condition for the maintenance of quasi-equilibrium as well.[14]

(13) J. Z. Hearon, S. A. Bernhard, D. J. Botts, S. L. Friess and M. F. Morales, in "The Enzymes," Academic Press, Inc., New York, N. Y., 1959, p. 130.

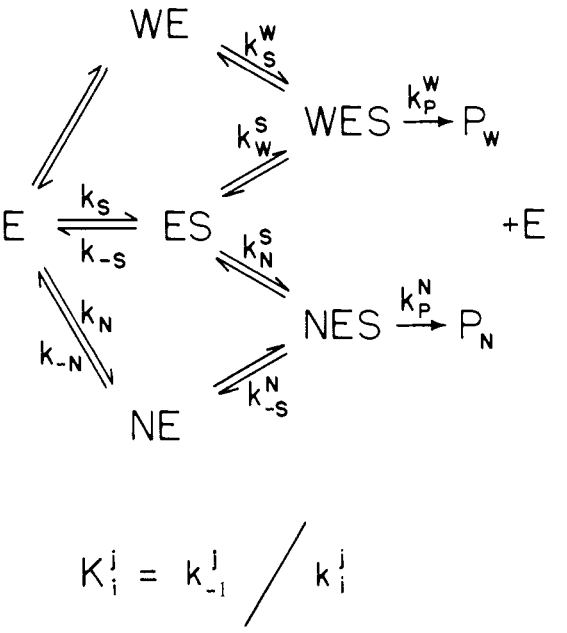


Fig. 5.—Competing reaction model: each process has a forward (k_i) and reverse (k_{-i}) step. Components which are enzyme bound at both sides of the double arrows are noted by superscripts; components bound only at the right hand side of the double arrows are shown as subscripts.

On the basis of the above arguments we conclude for the system α-chymotrypsin-water-hydroxylamine-methyl hippurate, *the concentration of the intermediate enzyme–substrate–reactant complexes are essentially the same as those which would obtain under equilibrium conditions.*

We note, in addition, that the equilibrium constants $K_M^N = K_M^W$ are independent of hydroxylamine concentration. Only two alternatives can possibly satisfy this condition (see equation 7): (a) the ratios N/K_N and N/K_N^S are both very small, or (b) $K_N = K_N^S$; $K_W = K_W^S$. These alternatives can be resolved by considering the effect of hydroxylamine on the extrapolated velocity at infinite substrate concentration, as derived from equation 6.

Since saturation in N is observed (Fig. 2), it follows that the ratio N/K_N^S is either greater than or of the order of unity in most of the hydroxylaminolysis reactions reported here. Since these

$$V_N = \frac{k_P^N E_0 N}{K_N^S(1 + W/K_W^S)}; \; \frac{N}{K_N^S} \ll 1 \quad (8a)$$

$$V_N \longrightarrow \infty \text{ as } N \longrightarrow \infty$$

$$V_N = \frac{k_P^N E_0}{\frac{K_N^S}{N}(1 + W/K_W^S) + 1} \quad (8b)$$

$$\therefore V_N \longrightarrow k_P^N \text{ as } N \longrightarrow \infty$$

reactions as well as the hydrolysis reactions have been studied over a wide range of hydroxylamine

(14) If quasi-equilibrium is not maintained, the expression for v^{-1} (equations 3 and 4) is not necessarily linear in S^{-1}, N^{-1} and W^{-1} as has been emphasized by Botts,[15] and this fact could, in principle, be used as a criterion for quasi-equilibrium. However, the linear terms (in S^{-1}, N^{-1}, and W^{-1}) and the concentration independent terms in Q may predominate and hence obscure such conclusions. Even in systems where v^{-1} is linear in S^{-1}, N^{-1} and W^{-1}, however, if quasi-equilibrium is not maintained and k_P^N is not equal to k_P^W, K_M^N cannot be equal to K_M^W.[15]

(15) D. J. Botts, *Trans. Faraday Soc.*, **54**, 593 (1958).

concentrations, we conclude that K_N^S is equal to K_N, and hence from the model (Fig. 5) the equilibrium relationships which must hold are

$$K_S^W = K_S^N = K_S; K_W = K_W^S$$

In this system, therefore, binding of reagent and substrate are strictly non-competitive. Binding of the two reagents, hydroxylamine and water, is strictly competitive since the inhibition constant for hydroxylamine inhibition of hydrolysis is identical to the binding constant of hydroxylamine for hydroxylaminolysis.

The fact that binding of substrate and reagent are strictly non-competitive is, as has been derived rigorously by Botts and Morales,[16,17] a necessary and sufficient condition for quasi-equilibrium. In simpler terms, if the triple complex (ESN) decomposes at a significant rate relative to the rate of desorption of either species (the non-equilibrium case), then the concentration of either species must affect the binding of the other even though the two are bound non-competitively.

The pseudo binding constant for hydroxylamine to the enzyme, *viz.*: $K_N(1 + W/K_W)$ cannot be resolved into its constituent parameters due to the impossibility of sensibly varying the water concentration. The similarity of the numerical value of the "pseudo" constant to the equilibrium constant K' (see results) is suggestive of a similar role for hydroxylamine in both processes.

$$K' = \frac{([RCO_2H] + [RCO_2^-])[NH_2OH]}{[RCONHOH] + [RCONHO^-]};$$

$$K_N\left(1 + \frac{W}{K_W}\right) = \frac{[EW][NH_2OH]}{[EN]}$$

It should be borne in mind however, that the assumption $1 + W/K_W \sim W/K_W$ cannot be tested by these methods. Furthermore, at *p*H 8.0, K' can be approximated by

$$K' \sim \frac{[RCO_2^-][NH_2OH]}{[RCONHOH]}$$

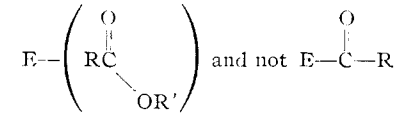
If the above two processes were analogous, contrary to the results, the two rates $k_P^N[ESN]$ and $k_P^W[ESW]$ should have different *p*H dependencies. If as has been shown, the principal active reagent species in hydroxylaminolysis is NH_2OH, then by analogy with K', the hydrolysis should involve OH^- rather than water. The *p*H independent hydrolysis rate between *p*H 7.8 and 8.4 is not in accord with this conclusion.

The maximum rate of hydroxylaminolysis, k_P^N, is five times the maximum observable rate of hydrolysis in the absence of hydroxylamine, $k_P^W/(1 + K_W/W)$. This value is somewhat surprising. If strong binding of water to the site occurs, $k_P^N \sim 5k_P^W$. Since hydroxylamine is a much stronger nucleophilic reagent than water, this relatively small difference in rate would be indicative of an activation process involving *primarily* only the substrate and enzyme. If on the other hand, K_W/W is much greater than unity (*i.e.*, most of the enzyme sites do not contain bound water and $k_P^N \lesssim k_P^W$) the treatment of hydrolytic enzyme–substrate data according to the Michaelis–Menten model (the usual procedure) cannot be valid *unless quasi-equilibrium is maintained.*

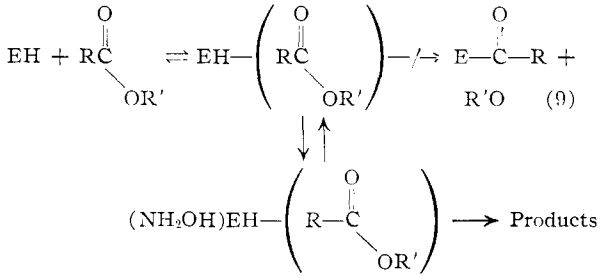
(16) D. J. Botts and M. F. Morales, *Trans. Faraday Soc.*, **49**, 696 (1953).

(17) M. F. Morales, THIS JOURNAL, **77**, 4169 (1955).

Since in this system quasi-equilibrium is maintained, it is clear that the intermediate enzyme substrate complex has the structure



i.e., the virtual irreversibility of the acyl enzyme intermediate excludes it as the metastable species "ES." Moreover, since "ES" reversibly complexes with hydroxylamine, the triple complex ESN must still preserve the aliphatic ester linkage



The existence of this triple complex of enzyme, substrate and attacking reagent is strongly indicative of the absence of acylated enzyme intermediates as a rate influencing species in this system. Since the acyl bond is moderately stable in this substrate (methyl hippurate), it is not surprising that an attack of both enzyme and reagent is required for reaction. The peptide bond is, of course, even more stable and hence the same double (and possibly concerted) attack by enzyme and reagent would be anticipated. When the acyl bond is labile, as for example with acyl derivatives exhibiting "general base catalysis" such as acyl anhydrides and nitrophenyl esters, direct acylation of the basic catalyst (in this case the enzyme) may occur.[18] It should be pointed out that acylation of α-chymotrypsin has been demonstrated *only* with such labile acyl derivatives.

From the *p*H dependence of the reaction velocity presented here and elsewhere, it is evident that a proton dissociation from the enzymatic site (with dissociation constant approximately equal to 10^{-7} *M*) is necessary for catalytic activity. The binding of both this substrate and these reagents (water or hydroxylamine) is independent of this dissociation. Any chemically detailed model of proteolytic activity and specificity will have to account for at least these three independent binding phenomena. It is difficult to conceive of a mechanism involving only one serine and one histidine residue of a protein (the two chemical groups which have thus far been implicated in proteolytic enzyme action) which can account for these three essential binding phenomena, even in view of the large amount of freedom in speculation which our present ignorance of protein configuration allows.

Acknowledgments.—The authors are indebted to Drs. J. Durell, S. Friess and J. Z. Hearon for many valuable discussions and especially to Drs. M. F. Morales and D. J. Botts for their critical analysis of the theoretical conclusions.

(18) M. L. Bender and B. W. Turnquest, *ibid.*, **79**, 1656 (1957).