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The Kinetics of the α-Chymotrypsin-catalyzed Hydrolysis and Methanolysis of Acetyl-L-phenylalanine Methyl Ester. Evidence for the Specific Binding of Water on the Enzyme Surface[1]

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RECEIVED JULY 29, 1959

The kinetics of the α-chymotrypsin-catalyzed hydrolysis and methanolysis of acetyl-L-phenylalanine methyl-C^{14} ester have been measured in aqueous solutions containing 0.25 and 0.50 *M* methanol. The hydrolysis was followed by titration at a constant *p*H. The methanolysis was followed by determining the change during the hydrolytic reaction in the specific activity of the labeled ester in the presence of an excess of unlabeled methanol. It has been shown that methanol is a non-competitive inhibitor of the hydrolysis reaction and further, that methanolysis of the ester occurs according to Michaelis–Menten kinetics for an isotopic exchange reaction. The equivalence of the classical Michaelis constant for hydrolysis and for methanolysis, at fixed concentrations of methanol and water, has demonstrated the existence of quasi-equilibrium in this system. The kinetic results can not be interpreted in terms other than the independent binding of both the ester and water (or methanol) on the enzyme surface. *Assuming binding of water or methanol on the enzyme surface*, it is calculated that the rate constant of methanolysis is 8.3 times that of hydrolysis. This value compares favorably with solvolytic reactions of N-acetylimidazole in which methanolysis/hydrolysis ratios of 4.3 to 6.1 were found. The specific binding of methanol or water on the enzyme surface in addition to binding of the ester substrate must be considered in any mechanism of α-chymotrypsin action.

Introduction

In a previous paper, the kinetics of base-catalyzed hydrolysis and alcoholysis of esters in aqueous alcohol solutions was investigated by means of an isotopic tracer technique.[3] The present investigation is concerned with a study of the α-chymotrypsin-catalyzed hydrolysis and alcoholysis of an ester in order to specify the role of water in enzymatic hydrolysis and to compare enzymatic and non-enzymatic hydrolysis. Water may act as a true substrate for chymotrypsin and other hydrolytic enzymes; that is, a binding site may exist for water on the enzyme surface. However, the impossibility of varying the water concentration, without varying the solvent appreciably, prohibits the use of the usual kinetic evidence for the determination of binding and requires the use of a model substance to simulate the role of water in enzymatic hydrolysis. The model substance chosen was methanol since it is structurally and chemically similar to water. The nucleophilic reactivities of these two compounds are similar, as will be shown later, and thus the methanolic analogy to hydrolysis (methanolysis) should be a good model for the study of the water reaction. It is the concern of this investigation to determine whether water reacts directly from solution or is bound on the enzyme surface in a hydrolytic reaction.

The idea of using a "water analog" to solve this type of problem was first enunciated by Koshland.[4] He reasoned that if the enzyme myosin can catalyze the reaction between phosphates and water, one should expect a similar catalysis of the reaction between phosphates and methanol, in water as the solvent, unless specific binding of water on the enzymatic surface was involved. Methanol-C^{14} was utilized in these experiments. Methanolysis was detected by analyzing for methyl phosphate using the isotope dilution method. Little methyl phosphate was found. It was concluded that water is much more reactive than methanol in this enzymatic reaction, in direct contrast to the non-enzymatic reaction. The conclusion was, therefore, reached that myosin contains a specific site for water on its surface.

Hydroxylamine has been used as a "water analog" in investigations of α-chymotrypsin-catalyzed reactions of a number of esters.[5,6]

In the present investigation methanol has been used as a "water analog." The effect of methanol, at relatively low concentrations in water, on the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-phenylalanine methyl ester was studied. If methanol, acting as a "water analog," is capable of competing with water in the enzymatic reaction, then the effect of added methanol on the rate of the hydrolysis reaction will be inhibitory only if there is a specific site for water on the enzyme surface (assuming no kinetic solvent effect at low methanol concentrations). This conclusion arises because the methanolysis of a methyl ester leads to no net change in the species present and because of the assumption that methanol will be occupying the enzymatic site normally occupied by water. In this regard, it is interesting to note that various workers[7–10] have noted inhibition of α-chymotrypsin-catalyzed hydrolysis by methanol. However, in all cases, the methanol concentrations used in these studies were greater than two molar. For example, the influence of methanol on the kinetics of the α-chymotrypsin-hydrocinnamic ester system at concentrations of methanol from 15 to 25% was treated as a dielectric constant effect,[8] as well may be the case in the other experiments cited. A

(1) This research was supported by Grant H-2416 of the National Institutes of Health.

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(3) M. L. Bender and W. A. Glasson, THIS JOURNAL, **81**, 1590 (1959).

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(8) M. L. Bernard and K. J. Laidler, *ibid.*, **74**, 6099 (1952).

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(10) S. Kaufman and H. Neurath, *ibid.*, **180**, 181 (1949).

recent investigation has utilized methanol concentrations which approach the low values used in the present work.[11] In that study, the marked inhibition of the hydrolysis reaction brought about by small amounts of methanol (similar to that found in the present study), is too great to be explained as a solvent effect, and was postulated to be due to a specific interaction between the enzyme and the methanol.

If inhibition occurs, it is necessary to prove that such an inhibition is not caused by non-specific effects; *i.e.*, solvent effects, structural change in the enzyme, etc. To show that the inhibition is indeed specific, it is necessary to show that methanol reacts with the ester analogously to water. By labeling the ester with carbon-14 in the alcohol moiety, this can be demonstrated as a decrease in the specific activity of the ester as a function of the time of contact with the active enzyme solution. An indication that α-chymotrypsin does catalyze an alcoholysis reaction can be seen in the enhanced rate of reaction of *p*-nitrophenyl acetate in the presence of various alcohols.[12]

In the study of the α-chymotrypsin-catalyzed hydrolysis and methanolysis of acetyl-L-phenylalanine methyl ester in methanol–water solutions, it was hoped that (1) definitive evidence could be obtained concerning possible binding of water on the enzyme surface and (2) the kinetic consequences of the ternary-complex mechanism involving the substrate and the cosubstrate (water or methanol) could be verified (see Mathematical Appendix).

Experimental

Materials.—The α-chymotrypsin used was an Armour and Company recrystallized, salt-free preparation. Water used in all enzyme experiments was distilled from alkaline permanganate solution in glass apparatus. Eastman Kodak (White Label) methanol was used without further purification. Sodium hydroxide solutions were standardized against potassium acid phthalate using phenolphthalein as indicator. Hydrochloric acid solutions were standardized against standard sodium hydroxide solutions. A stock solution of tris-(hydroxymethyl)-aminomethane buffer (*p*H 7.80) was prepared by dissolving 9.08 g. (0.07 mole) of the amine in 200 ml. of water and adding 6 *M* hydrochloric acid until the *p*H fell to 7.80; the resulting solution was diluted to 250 ml. with water.

Acetyl-L-phenylalanine methyl ester and acetyl-L-phenylalanine methyl-C^{14} ester have been described previously.[3] N-Acetylimidazole was a gift from Prof. T. C. Bruice. The sample was recrystallized from acetic anhydride, washed with ether and dried in a vacuum desiccator, m.p. 99–100°.

Kinetics of Enzyme-catalyzed Hydrolysis.—The kinetics of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-phenylalanine methyl ester were measured in pure water, and in one, and two volume % methanol–water solutions. The method of titration to a constant *p*H[13] was used to determine the amount of acid liberated as a function of time. The *p*H was measured with a Beckman Model G *p*H meter equipped with external electrodes. The reactions were run in a 250 ml. beaker which was half-submerged in a constant temperature bath (25.2 ± 0.1°). The solution was stirred with a glass stirrer (motor driven) and nitrogen (Matheson, oil pumped) was bubbled through the solution slowly to prevent carbon dioxide from affecting the *p*H. A 1.0 ml. microburet (Manostat Company) was used which has a mechanical counter allowing the volume delivered to be read to (0.0001 ± 0.00001) ml. directly. The tip of the buret was beneath the surface of the liquid throughout the course of a run. A typical run will be described. 10.0 ml. of a solution 0.0268 *M* in acetyl-L-phenylalanine methyl ester, in water, 1.0 ml. of a solution 4.0 *M* in sodium chloride, 5.0 ml. of a buffer solution of tris-(hydroxymethyl)-aminomethane-hydrochloric acid (0.0149 *M*), and 80.0 ml. of water were added to the reaction vessel. The stirring was then initiated and the bubbler turned on. After the system had reached equilibrium, the *p*H was adjusted to 7.88 by adding small volumes of 0.4730 *M* sodium hydroxide solution from the microburet. At this point, 2.0 ml. of a solution $1.89 \times 10^{-6} M$ in α-chymotrypsin, in water, were added and the timer started. After the *p*H had fallen to 7.80, 0.0100 ml. of 0.4730 *M* sodium hydroxide solution was added. When the *p*H had again fallen to 7.80, the time was recorded and another 0.0100 ml. of base was added. This procedure was repeated until the reaction was 98% complete. All solutions were thermostatted at 25.2° prior to addition to the reaction vessel.

The initial concentration of the ester was determined in two ways: (1) by total alkaline hydrolysis of an aliquot of the stock solution of the ester followed by addition of excess standard hydrochloric acid and titration of the excess acid with standard alkali using phenolphthalein as the indicator and (2) by total enzymatic hydrolysis[13] using the data of a given kinetic run. In some runs, good agreement was obtained between the two methods; whereas, in other runs, the agreement was very poor. This point will be discussed later.

The enzyme concentrations, in units of mg. of protein nitrogen per ml., were determined spectrophotometrically by measurement of the optical density at 282 mμ and comparison with a curve,[14] calibrated over the range, 0 to 0.060 mg. N/ml. The relationship can be expressed as $O.D._{282} = 12.5$ mg. N/ml. Conversion of this unit to moles/l. was accomplished by assuming the nitrogen content of the (undried) enzyme to be 14.0% and taking the mol. wt. to be 22,500.[15] This nitrogen content was observed during the course of this investigation and is in agreement with the value given by Spencer and Sturtevant,[16] 14.2%.

Kinetics of Enzyme-catalyzed Alcoholysis.—The kinetics of the enzyme-catalyzed methanolysis of acetyl-L-phenylalanine methyl C^{14}-ester were determined in one and two volume % methanol–water solutions. For the most part, conditions used in the methanolysis experiments were the same as those used in the hydrolysis experiments except for the use of a higher concentration of buffer (to keep the *p*H constant). A typical run will be described. 20.0 ml. of a solution 0.0181 *M* in acetyl-L-phenylalanine methyl-C^{14} ester, 5.0 ml. of tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer (0.300 *M* in amine, *p*H 7.80), 5.0 ml. of a solution 3.76 *M* in sodium chloride, 5.0 ml. of methanol, and 460 ml. of water were added to a 500 ml. volumetric flask and the contents thermostatted at 25.2 ±0.1° (all solutions were thermostatted at this temperature prior to addition). After thermal equilibrium had been attained, 1.0 ml. of a solution $2.20 \times 10^{-5} M$ in α-chymotrypsin was added, the timer started, and the solution was diluted to the mark with water. At the appropriate time, the reaction was quenched by pouring the reaction mixture into 5.0 ml. of a solution of trichloroacetic acid in water (1.09 g./ml.)(that this treatment was sufficient to inactivate the enzyme was demonstrated by performing a typical hydrolysis run, adding the requisite amount of trichloroacetic acid solution, adjusting the *p*H back to 7.80, and noting no change in the *p*H as a function of time). The time was noted and five drops of an alcoholic solution of brom thymol blue added. Saturated sodium bicarbonate solution was then added until the color changed from yellow to blue-green (*p*H 6.0–7.6), and the resulting solution was then repeatedly extracted with methylene chloride. The isolation, purification and radioassay of the samples have been described previously.[3]

Solvolysis of N-Acetylimidazole.—In order to find the relative reactivities of methanol and water, in a case where

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(13) G. W. Schwert, H. Neurath, S. Kaufman and J. Snoke, *ibid.*, **172**, 221 (1948).

(14) K. C. Kemp, Ph.D. Thesis, Illinois Institute of Technology, 1956.

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

(16) T. Spencer and J. Sturtevant, THIS JOURNAL, **81**, 1874 (1959).

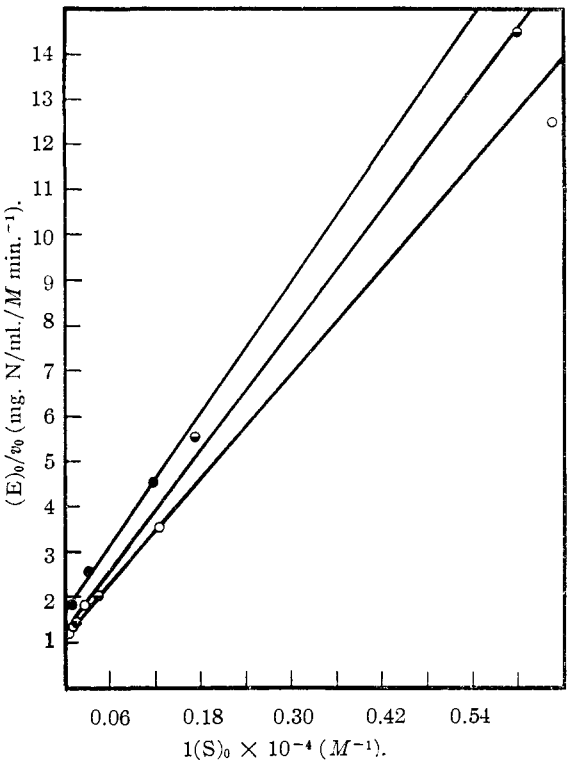

Fig. 1.—$(E)_0/v_0$ as a function of $1/(S)_0$ for the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-phenylalanine methyl ester in methanol–water mixtures, at 25.2 ± 0.1°: ○, 0.000; ◒, 0.251 and ●, 0.497 M methanol.

it is known that these species react directly from solution, the relative rates of methanolysis and hydrolysis of N-acetylimidazole, a model for the acyl-enzyme, in methanol–water mixtures was determined. The general method consisted of adding N-acetylimidazole to methanol–water mixtures, buffered at pH 7, and analyzing for total ester in the solution after the solvolysis had gone to completion. Runs were performed in eight and twenty volume % methanol. The analytical method for the determination of total ester content followed that of Hestrin.[17]

Results

Kinetics of Enzyme-catalyzed Hydrolysis.—The results of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-phenylalanine methyl ester in water, one and two volume % methanol–water solutions are given in Table I, and are graphically presented (from a least squares analysis of the data) in the form of Lineweaver–Burk plots,[5] in Fig. 1.

Various workers,[7-11] have studied the effect of methanol on the rate of α-chymotrypsin-catalyzed hydrolysis reactions. The results obtained in this investigation are in qualitative agreement with the data of the other investigators in that there is a rate depression on addition of methanol to the solvent system. The primary difference between this investigation and the above mentioned studies lies in the fact that the concentrations of methanol used are much lower in the present investigation than in all but one previous investigation.

Kinetics of Enzyme-catalyzed Methanolysis.—The results of the α-chymotrypsin-catalyzed methanolysis of acetyl-L-phenylalanine methyl ester in one and two volume % methanol–water solutions

(17) S. Hestrin, *J. Biol. Chem.*, **180**, 249 (1949).

TABLE I

THE KINETICS OF THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF ACETYL-L-PHENYLALANINE METHYL ESTER IN WATER AND METHANOL–WATER MIXTURES[a]

Volume % alcohol	Ester × 10^4,[b] M	Enzyme × 10^8,[c] M	v_0 × 10^6,[d] M/min.
0.0	137	3.80	100
.0	68.3	3.80	90.1
.0	27.3	3.80	66.2
.0	7.96	3.80	33.7
.0	1.55	3.80	10.8
1.0	67.7	4.35	97.4
1.0	21.5	4.35	68.6
1.0	5.77	4.35	25.0
1.0	1.67	4.35	9.66
1.0	1.29	4.35	6.37
2.0	105	9.28	157
2.0	30.3	9.28	114
2.0	8.42	9.28	64.2
2.0	2.04	9.28	15.1
2.0	1.24	9.28	7.23

[a] All runs were carried out at 25.2±0.1°. The ionic strength was maintained at 0.042 M through addition of aqueous sodium chloride solution. The pH was 7.80. [b] The values used are from total alkaline hydrolysis. In a few instances, the disagreement between the values obtained from calculation of the kinetic data and those obtained from the total alkaline hydrolysis of aliquots of the ester stock solution occurred. This is believed to be due to the autocatalytic hydrolysis of the ester in the stock solution. The main reason for this conclusion lies in the fact that deviations were not apparent until more than one week had elapsed after preparation of the stock solution. [c] The concentration of the enzyme was calculated as described in the experimental section. [d] Initial velocities were determined from the limiting slopes (at zero time) of plots of ml. of base used *versus* time.

are given in Table II and are graphically presented from a least squares analysis of the data in the form of Lineweaver–Burk plots in Fig. 2.

It can be seen that the initial velocity of methanolysis increases with increasing initial concentration of ester and with increasing methanol concentration (for comparable initial ester concentrations). The implications of these observations will be discussed later.

The Solvolysis of N-Acetylimidazole.—The results of the competitive hydrolysis and methanolysis of N-acetylimidazole in methanol–water mixtures are given in Table III.

The values of k_m/k_w (relative rates of methanolysis) were calculated from the equation

$$x_e/x_a = (k_m/k_w)(\mathrm{MeOH}/\mathrm{H_2O}) \qquad (1)$$

where x_e and x_a are the moles of ester and acid formed per 10 ml. of solution.

The values of k_m/k_w at eight and twenty volume % methanol are significantly different. A possible explanation for this difference can be found in a kinetic solvent effect in these systems. With regard to this point, it is of interest that in the ethanolysis and hydrolysis of acetic anhydride at 20°[18] the ratio of the rate constants for alcoholysis and hydrolysis are 1.18 and 1.06 in eighty and forty weight % water in ethanol solutions. The solvent effect would probably be more pronounced

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TABLE II

THE KINETICS OF THE α-CHYMOTRYPSIN-CATALYZED METHANOLYSIS OF ACETYL-L-PHENYLALANINE METHYL ESTER IN VARIOUS METHANOL-WATER MIXTURES[a]

Volume % alcohol	Ester × 10⁴,[b] M	Enzyme × 10⁸,[c] M	r_t × 10⁶[d]	Time, min.	v_0^* × 10⁶,[e] M/min.
1.0	145	1.57	9.08	3.88	
1.0	145	1.57	8.98	7.95	
1.0	145	1.57	8.98	11.90	22.5
1.0	145	1.57	8.92	15.95	
1.0	145	1.57	9.02	19.90	
1.0	6.76	4.41	8.09	4.80	
1.0	6.76	4.41	6.65	9.50	19.0
1.0	6.76	4.41	6.16	14.50	
1.0	3.38	4.41	8.08	4.65	
1.0	3.38	4.41	7.25	7.90	9.26
1.0	3.38	4.41	6.28	11.78	
1.0	1.69	4.41	7.78	4.75	
1.0	1.69	4.41	7.18	8.04	5.66
1.0	1.69	4.41	6.31	11.90	
2.0	145	1.57	9.08	4.90	
2.0	145	1.57	8.86	9.95	48.2
2.0	145	1.57	8.76	14.80	
2.0	7.04	2.09	8.30	4.90	
2.0	7.04	2.09	7.67	9.95	14.2
2.0	7.04	2.09	7.02	14.85	
2.0	3.52	2.09	8.04	4.95	
2.0	3.52	2.09	7.37	9.70	10.8
2.0	3.52	2.09	6.68	15.00	
2.0	1.69	4.41	7.62	3.84	
2.0	1.69	4.41	6.45	7.75	7.80
2.0	1.69	4.41	5.44	11.70	

[a] All runs were carried out at 25.2±0.1° and *p*H 7.80. The ionic strength was maintained at 0.042 *M* except for those runs with an initial ester concentration of 145 × 10^{-4} *M*, in which the ionic strength was 0.060 *M*. [b] The initial ester concentrations were determined by total alkaline hydrolysis of aliquots of the ester stock solutions. The ester stock solutions were used within two days of preparation. [c] Calculated as described previously. [d] The values of r_t are the absolute mole fractions of C^{14}-ester in the given sample. [e] Initial velocities were determined from the limiting slopes (at zero time) of plots of r_t *vs.* time. The values of the limiting slopes were calculated by expressing r_t as a second order polynomial in the time, *i.e.*, $r_t = a + bt + ct^2$, where *a* is equal to the initial value of r_t (9.20 × 10^{-6}) and *b* is the desired limiting slope. Using least-squares analysis, the best value of *b* was obtained. It is readily shown[19] that $v_0^* = (dr_t/dt)_{t=0}\,(S)_0/r$, and thus the initial velocity can be evaluated from a knowledge of the initial concentration of ester and the previously described limiting slope. It is on the basis of this equation that the values of v_0^* were calculated.

TABLE III

THE HYDROLYSIS AND METHANOLYSIS OF N-ACETYLIMIDAZOLE IN METHANOL-WATER MIXTURES[a]

Volume % alcohol	N-acetylimidazole (μmoles/10 ml.)	x_e[b] (μmoles/10 ml.)	x_a[c] (μmoles/10 ml.)	k_m'/k_w[d]
8.0	115.3	21.8	93.5	6.08
20	110.8	43.0	67.8	4.26

[a] The runs were carried out at 25.2 ±0.1°, *p*H 7.00, and an ionic strength of 0.024 *M*. [b] Determined as described previously. [c] Calculated as difference between total N-acetylimidazole and ester found. [d] Calculated from equation 1.

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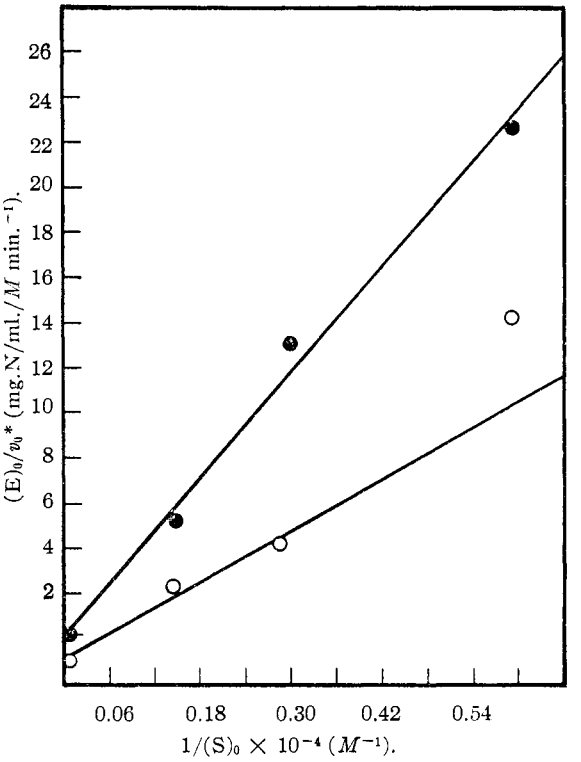

Fig. 2.—$(E)_0/v_0^*$ as a function of $1/(S)_0$ for the α-chymotrypsin-catalyzed methanolysis of acetyl-L-phenylalanine methyl ester in methanol-water mixtures, at 25.2 ± 0.1°: ●, 0.251 and ○, 0.497 *M* methanol.

with the less reactive N-acetylimidazole compared to acetic anhydride.

Discussion

In Table IV the values of K_m and k_3 calculated from Figs. 1 and 2 are tabulated. While the accuracy of K_m and k_3 are not all that might be desired, there is no question of the conformity of the kinetic data to that of the Michaelis–Menten formulation. These results constitute the second kinetic investigation of a hydrolytic enzyme in which a co-substrate other than water has been successfully carried out, the other being the kinetics of the simultaneous hydrolysis and hydroxylaminolysis of an ester.[5,6]

TABLE IV

THE MICHAELIS PARAMETERS FOR THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS AND METHANOLYSIS OF ACETYL-L-PHENYLALANINE METHYL ESTER IN WATER AND METHANOL-WATER MIXTURES[a]

	Hydrolysis		Methanolysis	
Methanol	K_m × 10³,[b] M	V,[b] sec.$^{-1}$	K_m × 10³,[b] M	V,[b] sec.$^{-1}$
0.00	1.80	48.2		
.25	1.84	43.1	1.79	24.2
.50	1.45	31.1	1.60	45.4

[a] All kinetic measurements were carried out at *p*H 7.80 and 25.2°. [b] These values are estimated to be accurate to ±25%.

As illustrated in equations 10 and 11, a necessary and sufficient condition for quasi-equilibrium is that K_m(methanolysis) = K_m(hydrolysis) at a given concentration of methanol and water. That

quasi-equilibrium obtained in this system is illustrated by Table IV. In 0.25 M methanol in water, K_m(hydrolysis) = 1.84 and K_m(methanolysis) is 1.79×10^{-3} M; and in 0.50 M methanol in water, the K_m(hydrolysis) = 1.45 and K_m(methanolysis) = 1.60×10^{-3} M. Thus in both instances K_m(hydrolysis) = K_m(methanolysis) to within ten per cent. which is well within the experimental uncertainty of 25%. Within the experimental error, the data constitutes evidence for the existence of quasi-equilibrium in this system. It is interesting to note that Gutfreund[20] has asserted that $k_2 \gg k_3$ (in the classical Michaelis sense) in the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-phenylalanine ethyl ester. This condition is tantamount to a state of quasi-equilibrium for the reaction considered.

Specific binding of reactant water at the catalytic center of fumarase,[21] myosin,[7] and α-chymotrypsin[3] has been inferred from experimental data. The following evidence obtained in this investigation is in agreement with the results of Bernhard,[3] for α-chymotrypsin. (1) The hydrolysis of acetyl-L-phenylalanine methyl ester, catalyzed by α-chymotrypsin, is inhibited by methanol, at low concentrations, in a non-competitive manner with respect to the substrate (ester). This conclusion is based on the independence of K_m (hydrolysis) with respect to the concentration of methanol, within the region of methanol concentrations used (although K_m is obviously not independent of methanol over all methanol concentrations.[7-10] This inhibition is indicative of methanol competing with water for the co-substrate-site since methanolysis gives no net change in the species concentration. (2) α-Chymotrypsin catalyzes the methanolysis of the ester at low concentrations of methanol. This fact supports the use of methanol as an analog for water, in that methanol apparently reacts in the same way as water. (3) The value of k_5, as obtained from equation 8 is 290 sec.$^{-1}$; whereas, the value of $k_3/(1 + K_2/(W))$, as obtained from equation 7 (reaction in pure water) is 48.2 sec.$^{-1}$. The ratio of these rate constants is 8.3.

If water and methanol react from solutions then the limiting velocities of hydrolysis and methanolysis are given by

$$V(\text{hydrolysis}) = k_3(W) \quad (2)$$

$$V(\text{methanolysis}) = k_5(A) \quad (3)$$

since water and methanol are in great excess over the substrate (ester). This situation is analogous to a pseudo-first order reaction. Thus, the ratio of the limiting rates of methanolysis to hydrolysis is given by

$$V(\text{methanolysis})/V(\text{hydrolysis}) = k_5/k_3\,(A)/(W) \quad (4)$$

Rearrangement of equation 4 allows calculation of the ratio k_5/k_3. This ratio was found to be 123 and 160 in 0.25 and 0.50 M methanol, respectively.

The two alternatives, *i.e.*, reaction from a specific site or from solution yield markedly different values for the relative reactivity of methanol to water in the α-chymotrypsin-catalyzed reactions of the substrate used in this study. As a means of choosing between these two alternatives, the relative reactivity of methanol to water, toward a model for the enzyme-substrate complex, was measured. The model chosen was N-acetylimidazole because of the implication of the imidazole group of histidine in the catalytic action of α-chymotrypsin and the acyl-enzyme theory. The relative reactivities, in eight and twenty volume % methanol–water solution, were found to be 4.3 and 6.1, respectively. It is apparent that the relative reactivity of methanol to water toward the model is very similar to that calculated from the ternary-complex mechanism and vastly different from that calculated on the basis of reaction of the nucleophiles from solution. On the basis of the above observations, it is necessary to conclude that there is specific binding of water (and methanol) on the surface and near the catalytic center of α-chymotrypsin.

The use of methanol as a water analog is felt to be superior to the use of hydroxylamine as employed in Bernhard's study[6] since it has been demonstrated, in non-enzymatic studies, that hydroxylamine is a very special nucleophile, in that it reacts as a bifunctional catalyst in the hydroxylaminolysis of esters, in neutral solution.[22] The mode of action of hydroxylamine in enzymatic reactions is not known. For this reason, the conclusions obtained in this methanolysis study may be less open to criticism than those obtained in the hydroxylaminolysis study.

It is interesting to note the similar values of the relative reactivities of methanol and water toward acetyl-L-phenylalanine methyl ester and the relative reactivity of hydroxylamine and water toward methyl hippurate.[8]

$$k_5\,(\text{methanolysis})/k_3\,(\text{hydrolysis}) = 8.3$$

$$k_5\,(\text{hydroxylaminolysis})/k_3\,(\text{hydrolysis}) = 5$$

Further, the binding constants for methanol[18] and hydroxylamine[6] on the enzyme surface are

$$K_4\,(\text{methanol}) \leqq 2.7\ M$$

$$K_4\,(\text{hydroxylamine}) = 0.25\ M$$

These values are directly comparable since quasi-equilibrium has been demonstrated in both investigations. The obvious interpretation of these two sets of values is that any structural or electronic differences between methanol and hydroxylamine are primarily operative in the binding step rather than in the subsequent decomposition step.

A Comparison between Base- and Enzyme-catalyzed Hydrolysis and Alcoholysis of Esters.—As mentioned earlier, it was the intention of this study to compare enzymatic and non-enzymatic hydrolysis with the hope that further knowledge concerning the catalytic action of α-chymotrypsin, would result therefrom. α-Chymotrypsin catalysis has been compared to acid and base catalysis in the hydrolysis of a series of α-substituted ethyl-β-phenylpropionates.[23] It was observed, that in all cases, the enzymatic reaction was faster and in the case of benzoyl-L-phenylalanine ethyl ester, the rate increase was extremely large. The latter

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(21) T. C. Farrar, H. S. Gutowsky, R. A. Alberty and W. G. Miller, THIS JOURNAL, **79**, 3978 (1957).

(22) W. P. Jencks, *ibid.*, **80**, 4585 (1958).

(23) M. L. Bender and B. W. Turnquest, *ibid.*, **77**, 4271 (1955).

fact is not surprising since this particular ester is a specific substrate of α-chymotrypsin. The data of this study are indicative of the fact that no direct comparison can be made between enzymatic and non-enzymatic hydrolysis. Bernhard[24] has studied the rates of acetylcholinesterase- and hydroxide ion-catalyzed hydrolysis of ethyl acetate and acetylcholine. He found that the ratio of the rates for the two substrates, for enzymatic and non-enzymatic catalysis, were the same for the two types of catalysis and concluded that the activation step for both must be similar.

Table V lists the appropriate ratios of the kinetic constants for the α-chymotrypsin-and base-catalyzed hydrolysis and alcoholysis of acetyl-L-phenylalanine methyl ester, in water as the solvent. k_{H_2O} and k_{ROH} refer to the maximum rates of hydrolysis and alcoholysis, respectively, and correspond to $k_3/(1 + K_2/(W))$ and k_5 of equations 7 and 8, respectively. It should be noted that the ratios k_{H_2O}/k_{OH} and k_{ROH}/k_{OR} are comparisons of first order rate constants to second order rate constants. These ratios can be thought of as the concentrations of hydroxide ion or alkoxide ion necessary to achieve the first order rate given by k_{H_2O} or k_{ROH}.

TABLE V

A COMPARISON BETWEEN α-CHYMOTRYPSIN- AND BASE-CATALYZED[a] HYDROLYSIS AND METHANOLYSIS OF ACETYL-L-PHENYLALANINE METHYL ESTER[a,b]

k_{H_2O}/k_{OH} (enzyme/base) M	k_{ROH}/k_{OR} (enzyme/base) M	k_{ROH}/k_{H_2O} (enzyme) M	k_{OR}/k_{OH} (base)
22	110	8.3	1.6

[a] In water, at 25°. [b] k_{OH} and k_{OR} were obtained by extrapolation of the experimental data to pure water as solvent.

Column 1 of Table V adds to the already overwhelming evidence pointing to greater efficiency of an enzymatic hydrolysis compared to the corresponding non-enzymatic hydrolysis. Column 2 gives evidence for a generalization, of the above concept, to solvolyses of carboxylic acid derivatives. This evidence has value in that the role of water in enzymatic hydrolyses is not unique but rather water is acting as a member of a general group of nucleophilic reagents.

Columns 3 and 4 illustrate the importance of the hydrogen-bonding ability of the reactant molecule. Methanol is more reactive than ethanol in enzymatic reactions, while ethoxide ion is more reactive than methoxide ion in base-catalyzed reactions. In enzymatic hydrolysis and alcoholysis, the alcohol, bound on the surface of the enzyme, is polarized in relation to its acidity. Thus, the relative reactivity is proportional to the acidity of the alcohol in the enzymatic reaction. The relative reactivity of the alcohol is inversely proportional to the acidity in base-catalyzed alcoholysis, *i.e.*, proportional to the basicity and to a limited extent, the nucleophilicity.[3]

Mathematical Appendix[24]

Kinetic Analysis of Ternary Complex Mechanisms.—The simple Michaelis–Menten approach to the kinetics of enzymatic hydrolysis does not explicitly include the role of water in the reaction. Enzymatic hydrolysis has subsequently been treated kinetically assuming that water acts as a true cosubstrate. The kinetic expressions obtained from these two treatments are experimentally indistinguishable[6] due to the impossibility of varying the water concentration without incurring extraneous effects (solvent effects, etc.).

The kinetics of the following systems must be considered for present purposes: (a) Ester–water–methanol–α-chymotrypsin (hydrolysis is followed) and (b) ester-C^{14}–water–methanol–α-chymotrypsin (methanolysis is followed). System (a) is described by the equations

$$\begin{array}{ll} (1)\ E + S \rightleftarrows ES & (4')\ ES + A \rightleftarrows ESA \\ (2)\ E + W \rightleftarrows EW & (1')\ EW + S \rightleftarrows ESW \\ (4)\ E + A \rightleftarrows EA & (1'')\ EA + S \rightleftarrows ESA \\ (2')\ ES + W \rightleftarrows ESW & (3)\ ESW \longrightarrow \text{products} \end{array} \quad (5)$$

and system (b) is described by the equations

$$\begin{array}{ll} (1)\ E + S^* \rightleftarrows ES^* & (4')\ ES^* + A \rightleftarrows ES^*A \\ (2)\ E + W \rightleftarrows EW & (1')\ EW + S^* \rightleftarrows ES^*W \\ (4)\ E + A \rightleftarrows EA & (1'')\ EA + S^* \rightleftarrows ES^*A \\ (2')\ ES^* + W \rightleftarrows ES^*W & (5)\ ES^*A \rightleftarrows E + S + A^* \end{array} \quad (6)$$

where E, S*, S, W, and A refer to the enzyme, labeled and unlabeled ester, water and alcohol, respectively. The kinetics of systems (a) and (b) have been described by Bernhard.[6] The expressions for the initial rates of hydrolysis (v_0) and methanolysis (v_0^*) corresponding to systems (a) and (b), respectively, are given by equations 7 and 8 in the Lineweaver–Burk form

$$(E)_0/v_0 = 1/k_3[(1 + (W)/K_2' + (A)/K_4') + K_1/(S)_0(1 + (W)/K_2 + (A)/K_4)K_2'/(W) + Q_W] \quad (7)$$

$$(E)_0/v_0^* = 1/k_5[(1 + (W)/K_2' + (A)/K_4' + K_1/(S)_0(1 + (W)/K_2 + (A)/K_4)K_4'/(A) + Q_A] \quad (8)$$

assuming the fifth equation of (6) is irreversible (this assumption is valid in the present work where the methanolysis kinetics were followed at low conversions), where the equilibrium constants, K_i, are written as dissociation constants and Q_w and Q_A are the non-equilibrium terms for hydrolysis and methanolysis, respectively. The Q's may be considered as perturbation terms which determine the extent to which the concentrations of the corresponding ternary complexes are altered from their equilibrium values due to the decomposition rates (k_3 and k_5). Q_w and Q_A are equal to zero when k_3 is much less than k_{-1} and k_{-2}', and k_5' is much less than k_{-4}' and k_{-1}'', respectively. These conditions correspond to a state of quasi-equilibrium, *i.e.*, all the complexes are in rapid equilibrium, as opposed to the stationary state exemplified by Q not equal to zero.

The exact expressions for Q_w and Q_A have been obtained by Bernhard.[6] These expressions, although highly complex, have some useful properties, *i.e.*, Q_w is not equal to Q_A and these Q's do not show the same dependence on the concentration of a particular reagent. As noted above, at quasi-equilibrium, the Q's vanish yielding

$$v_0^{*-1}k_5(E)_0(A)/K_4' = (1 + (W)/K_2' + (A)/K_4') + K_1(S)_0[1 + (W)/K_2 + (A)/K_4] = v^{-1}k_3(E)_0(W)/K_2' \quad (9)$$

(24) S. A. Bernhard, THIS JOURNAL, **77**, 1966 (1955).

As in the usual Michaelis–Menten equation, equation 9 predicts a linear relationship between the inverse of the initial velocity (hydrolysis or methanolysis) and the inverse of the substrate concentration, $(S)_0$. By carrying out the analysis of the velocity-concentration relationship predicted by equation 9, two apparent Michaelis constants can be defined as

$$d(1/v_0^*)/d(1/(S)_0)/(1/v_0^*)_{(S)_0=\infty} = K_5(1 + (W)/K_2 + (A)/K_4)/(1 + (W)/K_2' + (A)/K_4') = K_m \text{ (methanolysis)} \quad (10)$$

$$d(1/v_0)/d(1/(S)_0/(1/v_0)_{(S)_0=\infty} = K_1(1 + (W)/K_2 + (A)/K_4)/(1 + (W)/K_2' + (A)/K_4') = K_m \text{ (hydrolysis)} \quad (11)$$

Comparison of equations 10 and 11 indicates that K_m(methanolysis) is equal to K_m(hydrolysis) *at fixed concentrations of methanol and water.* Since Q_A and Q_w, of equations 7 and 8, respectively, are not equal, the condition K_m (hydrolysis) = K_m (methanolysis) is a necessary and sufficient condition for the maintenance of quasi-equilibrium.[6]

Acknowledgment.—The authors gratefully acknowledge valuable discussions with Dr. S. A. Bernhard.

[CONTRIBUTION NO. 2508 FROM THE DIVISION OF CHEMISTRY AND CHEMICAL ENGINEERING, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA]

Further N-Terminal Sequences in Human Hemoglobins A, S and F by Edman's Phenylthiohydantoin Method

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RECEIVED JANUARY 4, 1960

By means of the Edman phenylthiohydantoin method, the N-terminal sequences in the peptide chains of several human hemoglobins have been found to be as follows: α^A and α^F, val-leu-ser-pro-ala-aspNH_2-; β^A, val-his-leu-thr-pro-glu-; β^S, val-his-leu-thr-pro-val-; and γ^F, gly-his-phe.

Introduction

By Sanger's DNP-method, normal adult human hemoglobin (hemoglobin A) has been shown to contain four N-terminal valyl residues and two kinds of polypeptide chains. The two chains termed α chains terminate in val-leu[1] and the two termed β chains in val-his-leu.[1b,2] Human fetal hemoglobin (hemoglobin F) also contains two chains N-terminal in val-leu (α chains), and, in addition, two chains N-terminal in glycine (γ chains).[3] The α^A, α^S and α^F chains are identical.[4–6] (The superscripts denote the hemoglobin that was the source of the peptide chain.) All attempts to extend the above N-terminal sequences by careful partial hydrolysis of the DNP-proteins were unsuccessful because of the lability of the adjacent peptide bonds. Only a step-wise degradation procedure such as the Edman method[7] seemed capable of extending the N-terminal sequences. When the Edman method as modified by Fraenkel-Conrat[8] was applied to the several hemoglobins the above sequences were verified and extended.

Experimental

Preparation of Hemoglobin Solutions.—Solutions of hemoglobin from the blood of normal adults and sickle cell anemics and from the umbilical cord blood of new-born infants were prepared as described by Clegg and Schroeder[9] with the exception that immediately prior to use, the hemoglobin was dialyzed against water instead of developer. For most experiments with hemoglobin A, solutions so prepared were used without chromatographic purification to remove minor components.[9,10] However, the main component in sickle-cell hemoglobin and Zone F_{II}[10] were isolated chromatographically and used as hemoglobins S and F, respectively. Solutions with concentrations of about 10 mg./ml. were used. The results were unaltered when globin replaced hemoglobin in the degradation procedure.

Preparation of Single Polypeptide Chains of Hemoglobin.—Ingram[11] has successfully applied the method of Wilson and Smith[12] for the separation of the peptide chains of horse globin to the separation of the chains of human globin. The isolation of the chains was carried out in these Laboratories without alteration of procedure. The purity of the α chains was 95–100% and that of the β^A, β^S and γ^F chains about 75%.

Paper Strips.—The Fraenkel-Conrat modification[8] of the Edman procedure was used with only minor modifications. Strips of Whatman No. 1 filter paper (1 × 7 cm.) in which a small hole had been punched near one end were used as carriers for hemoglobin and the peptide chains of hemoglobin. Each strip would absorb about 0.08 ml. of solution without difficulty. The strips carrying the isolated peptide chains were oven dried at 90° for 5 minutes prior to the first treatment with phenyl isothiocyanate.

Formation of PTC-Protein and Subsequent Degradation.—Essentially, the only modification of these steps in the procedure[8] lay in the method of exposing the strips to reagents. The strips were not placed in beakers[8] or laid in petri dishes.[8] Rather, racks of glass rods with glass hooks were used. On the glass hooks, the strips were suspended above the reagents by means of the small hole in each strip. Suspension in this way permitted good access by the reagent to both sides of the strip. The time of exposure to acid during the degradation was varied from step to step as experience suggested; the time at each step is given in the results below.

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