reaction on the enzyme surface, either glycinamide or methanol would be required to occupy a site normally occupied by the water molecule in the hydrolysis reaction. Because of the specificity exhibited by the enzyme the latter requirement appears too stringent and would not likely be fulfilled. A more reasonable explanation would be that a relatively stable acyl–enzyme intermediate is formed which is capable of reacting with nonbonded water, methanol or glycinamide molecules. One can explain the concurrent methanolysis and hydrolysis reactions by a competition of the nucleophiles, water and methanol for the acyl–enzyme intermediate. From the results presented here, it appears that methanol is a better nucleophile than water. Presumably in the transamination and transpeptidation reactions,[19] the same kind of competition exists. The hypothesis that the hydrolysis, methanolysis, transamination and transpeptidation reactions occur *via* an acyl–enzyme intermediate suggests that the distinction between the hydrolysis and transfer properties of enzymes may be overdrawn.

The evidence presented in this investigation in conjunction with that of studies mentioned above and with investigations of *p*H[20,21] and of diisopropylfluorophosphate inactivation[22] is consistent with a double displacement mechanism for the hydrolytic action of α-chymotrypsin. A similar mechanism has been described by Wilson[23] for the action of acetylcholinesterase. In contrast to the mechanism of Wilson, it is suggested that the enzyme–substrate complex, defined by the Michaelis–Menten constant, consists of adsorbed substrate on the enzyme in which the ester linkage has not been altered by the enzyme; that is, binding involves those parts of the molecule other than the ester group. We would then interpret Wilson's intermediates as steps in the activation process, involved in the rate constant, k_3. These intermediates, all short-lived, include the product of addition of a nucleophilic agent to the carbonyl carbon atom (a tetrahedral intermediate), the acyl–enzyme intermediate and the product of addition of a molecule of water to the acyl–enzyme intermediate (a second tetrahedral intermediate). The instability of the two tetrahedral intermediates is indicated by analogy with such compounds in non-enzymatic hydrolysis.[14] The instability of the acyl–enzyme intermediate is indicated by analogy with the instability of acetylimidazole.[24] Reversible formation of an enzyme–substrate complex, which is indicated in several α-chymotrypsin systems,[7] would not be possible with such unstable intermediates[14] but must rather involve an adsorbed substrate with an intact ester linkage. While this double displacement mechanism involving an acyl–enzyme intermediate is consistent with all presently known facts, it clearly must await further definitive proof.

Acknowledgment.—The authors acknowledge valuable discussions with Dr. D. E. Koshland, Jr. The mass spectrometer on A. E. C. contract At(11-1)-92 was made available through the courtesy of Dr. H. Taube. A generous gift of α-chymotrypsin from Armour and Co. is gratefully acknowledged.

(20) K. J. Laidler, *Disc. Faraday Soc.*, **20**, 83 (1955).

(21) B. Hammond and H. Gutfreund, *Biochem. J.*, **61**, 187 (1955).

(22) E. F. Jansen, M. D. F. Nutting, R. Jang and A. K. Balls, *J. Biol. Chem.*, **179**, 189 (1949).

(23) I. B. Wilson, in "Mechanism of Enzyme Action," edited by McElroy and Glass, Johns Hopkins Univ. Press. Baltimore, Md., 1954, p. 653.

(24) Reference 23, p. 585. Unpublished results of this Laboratory.

CHICAGO, ILLINOIS

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, ILLINOIS INSTITUTE OF TECHNOLOGY]

The Kinetics of the α-Chymotrypsin-catalyzed Oxygen Exchange of Carboxylic Acids[1]

BY MYRON L. BENDER AND KENNETH C. KEMP[2]

RECEIVED JULY 26, 1956

A study was made of the kinetics of the α-chymotrypsin-catalyzed oxygen exchange between the solvent, water, and the following acids which were labeled with oxygen-18 in the carboxyl group: benzoyl-L-phenylalanine, acetyl-L-tryptophan, benzoyl-D-phenylalanine and β-phenylpropionic acid. It was found that the first two acids underwent oxygen exchange with the solvent, whereas the last two did not. A symmetrical mechanism is proposed for the oxygen exchange. The values of K_O, the Michaelis constant of the oxygen exchange, and k', the composite rate constant of this reaction, were determined from simultaneous solution of equations of the type: $2.30 \log(x_o - 0.204)/(x - 0.204) = k'(E)_o t/(K_O + (S))$. Assuming negligible kinetic isotope effects, it is demonstrated that K_O is equal to k_2/k_1, an equilibrium constant. The values of K_O determined for the oxygen exchange of benzoyl-L-phenylalanine and of acetyl-L-tryptophan are in good agreement with the values of K_I determined for these substances when used as competitive (product) inhibitors. The equivalence of K_O and K_I suggests that K_I for product inhibition is an equilibrium constant in these cases. The similarity of enzymatic oxygen exchange and enzymatic hydrolysis with respect to mechanism and kinetics is pointed out. This similarity suggests that in the determination of the kinetics of hydrolytic reactions, the Michaelis constants may be approximately equilibrium constants and the k_3's may be composite rate constants. This conclusion, in conjunction with other data, suggests that the Michaelis constants for α-chymotrypsin–amide systems are equilibrium constants.

Introduction

Many of the carboxylic acids which are products of hydrolysis of α-chymotrypsin-catalyzed reactions are competitive inhibitors of this enzyme.[3] That is, they compete with the substrate for the active site on the enzyme. However, it has been shown that α-chymotrypsin catalyzes the exchange of oxygen atoms between the solvent and the car-

(1) This investigation was aided by research grant G-3787 of the National Institutes of Health, U. S. Public Health Services. Paper IV in the series: The Mechanism of Action of Hydrolytic Enzymes.

(2) From the Ph.D. thesis of K.C.K.

(3) H. Neurath and G. Schwert, *Chem. Revs.*, **46**, 69 (1950).

boxylic acid group of these acids.[4] They, therefore, may be regarded as substrates as well as inhibitors of this enzyme.

From a study of the competitive inhibition of carboxylic acids in enzymatic hydrolyses, it is possible to evaluate inhibition constants which are a measure of the affinity of the enzyme for the inhibitor. These equilibrium constants, K_I, have been evaluated for a large number of carboxylic acids with respect to the enzyme, α-chymotrypsin.[5] One of the recurring problems with respect to the determination of kinetic data in enzymatic systems is the decision as to whether the Michaelis constant, K_M, is an equilibrium constant and, therefore, whether it is a true measure of the affinity of an enzyme for a substrate.[6] A study is presented here of the kinetics of the α-chymotrypsin-catalyzed oxygen of carboxylic acids for which inhibition constants already have been evaluated. From such a study it should be possible to compare the Michaelis constants and inhibition constants of identical systems.

A similar study has been reported recently by Vaslow[7] who compared the equilibrium binding constant, K_E, determined by the equilibrium dialysis technique, with the Michaelis constant for the oxygen exchange reaction using the system chymotrypsin–N-acetyl-3,5-dibromo-L-tyrosine. In the present communication are reported the kinetics of the α-chymotrypsin-catalyzed oxygen exchange of the following acids which were labeled in the carboxyl group with oxygen-18: benzoyl-L-phenylalanine, acetyl-L-tryptophan, benzoyl-D-phenylalanine and β-phenylpropionic acid.

Experimental

Materials.—α-Chymotrypsin was an Armour and Co. product which was used without recrystallization. The enzyme concentrations were determined by measuring the optical densities at 282 mμ in a Beckman DU spectrophotometer. A standard calibration curve was obtained by measuring the nitrogen concentration by means of a micro-Kjeldahl procedure.[8] β-Phenylpropionic acid-O^{18} was prepared by the equilibration of unlabeled acid with H_2O^{18} in the presence of 0.1 *N* hydrochloric acid at reflux temperature for 92 hours. L-Phenylalanine was labeled by the same method and was converted to benzoyl-L-phenylalanine-*carboxyl*-O^{18} by benzoylation according to the method of Carter and Stevens.[9] Benzoyl-D-phenylalanine-*carboxyl*-O^{18} was prepared in a manner identical with that described for the L-enantiomorph. L-Tryptophan was converted to L-tryptophan-O^{18} according to the equilibration technique with H_2O^{18}. The latter was converted to acetyl-L-tryptophan-*carboxyl*-O^{18} according to the procedure of du Vigneaud and Sealock.[10]

Kinetics.—A typical procedure followed in studying the α-chymotrypsin-catalyzed oxygen exchange of the carboxylic acids is described. To 0.2664 g. of benzoyl-L-phenylalanine-*carboxyl*.-O^{18} in a 25-ml. volumetric flask, 5.26 ml. of 0.188 *N* NaOH (an equivalent amount), 5.0 ml. of 0.1 *M* phosphate buffer of *p*H 7.8 and 4.0 ml. of redistilled water were added. The solution was thermostated at 25.04 ± 0.01°. Ten ml. of enzyme solution which also was thermostated was pipetted into the flask and the volume of the solution was diluted to the mark with distilled water. The *p*H of the resulting solution was 7.7.

At appropriate time intervals, 3-ml. samples were removed; the reaction was stopped by destruction of the enzyme by boiling the solution for one minute. The solution was cooled, centrifuged, decanted and then acidified with 1 *N* HCl to precipitate the benzoyl-L-phenylalanine. The precipitate was washed by decantation several times and recrystallized from water. The purity of the recovered acid was checked by its melting point. The recovered acid was analyzed for its oxygen-18 content mass spectrometrically.

Oxygen-18 Analysis.—Analysis of oxygen-18 in the carboxylic acids was accomplished by converting the oxygen atoms of the labeled compounds to carbon dioxide by a method described by Doering and Dorfmann[11] and by Steyermark,[12] and then analyzing the carbon dioxide in a Consolidated-Nier model 21-201 isotope-ratio mass spectrometer. The procedure involves the pyrolysis of the oxygen-containing compound to carbon monoxide over carbon at 1120° in a stream of pre-purified nitrogen and the subsequent oxidation of the carbon monoxide to carbon dioxide by iodine pentoxide at 114°. The yield of the pyrolysis reaction was determined by measurement of the carbon dioxide produced in a constant volume manometer.

In the micro determination of oxygen by the Schutze–Untersaucher[13] method, upon which this method of oxygen-18 analysis is based, it is necessary to apply a correction for a blank, which is due to a reaction of the carbon packing with the combustion tube (silica) and quartz wool. The presence of a blank dilutes the enriched carbon dioxide sample with carbon dioxide of normal composition. The magnitude of this blank was measured during every set of pyrolyses and the dilution caused by the blank was taken into account in the calculation of the atom fraction of oxygen-18 from the mass spectrometric data.

The following equations[14] were used to calculate the atom fraction O^{18} where

n = mm. blank
y = mm. pyrolysis sample − n
R_u = mass spectrometer reading for the 46/44 ratio of unknown CO_2
R_s = mass spectrometer reading for the 46/44 ratio of standard CO_2

Atom fraction O^{18} in the carboxyl group of an acid in which the only oxygen atoms present are those of the carboxyl group equals

$$\frac{R_u/R_s - 0.5000 + 0.4990n/y((R_u/R_s) - 1)}{(R_u/R_s) + 244.10}$$

Atom fraction O^{18} in the carboxyl group of an acid which contains three oxygen atoms per molecule, only the carboxyl group being labeled, equals

$$\frac{2.998(R_u/R_s) - 1.9980 + 0.9980\ n/y((R_u/R_s) - 1)}{2(R_u/R_s) + 488.21}$$

A series of twelve samples of the same labeled compound (benzamide-O^{18}) resulted in oxygen-18 analyses with an average deviation of 0.4%. We consider this the precision that we can obtain with this method.

Results and Discussion

The oxygen exchange reaction may be represented by eq. 1

$$RCOO^{18}H + H_2O \rightleftharpoons RCOOH + H_2O^{18} \qquad (1)$$

The enzyme-catalyzed, as well as the acid-catalyzed, oxygen exchange of carboxylic acids is reversible; from consideration of the principle of microscopic reversibility and in the absence of isotope effects, the reaction path of this exchange reaction must

(4) D. Sprinson and D. Rittenberg, *Nature*, **167**, 484 (1951); D. Doherty and F. Vaslow, THIS JOURNAL, **74**, 931 (1952).

(5) R. Foster, H. Shine and C. Niemann, *ibid.*, **77**, 2378 (1955).

(6) S. Bernhard, *ibid.*, **77**, 1973 (1955).

(7) F. Vaslow, *Biochim. et Biophys. Acta*, **16**, 601 (1955); *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **30** [4], 45 (1956).

(8) A. Hiller, J. Plazin and D. D. Van Slyke, *J. Biol. Chem.*, **176**, 1401 (1948). A methylene blue–methyl red indicator was used for titration instead of alizarin red.

(9) H. Carter and C. Stevens, *ibid.*, **138**, 627 (1941).

(10) V. du Vigneaud and R. Sealock, *ibid.*, **96**, 511 (1932).

(11) W. E. Doering and E. Dorfmann, THIS JOURNAL, **75**, 5595 (1953).

(12) A. Steyermark, "Quantitative Organic Microanalysis," Blakeston and Co., Philadelphia, Pa., 1951, p. 208.

(13) M. Schutze, *Z. Anal. Chem.*, **118**, 241 (1939); J. Untersaucher, *Ber.*, **73B**, 391 (1940).

(14) The derivation of these equations may be found in the Ph.D. thesis of K.C.K., Illinois Institute of Technology, 1956.

then be symmetrical. Consequently, the enzyme-catalyzed exchange should be formulated in terms of a symmetrical scheme such as

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EP \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E + P \qquad (2)$$

where ES represents a complex of enzyme and $RCOO^{18}H$ and EP represents the identical but isotopically distinguishable complex of enzyme and RCOOH. The general rate expression for eq. 2 is[15]

$$-\frac{d(S)}{dt} = \frac{[k_1k_3k_5(S) - k_2k_4k_6(P)](E)_o}{k_2k_4 + k_2k_5 + k_3k_5 + k_1(S)(k_3 + k_4 + k_5) + k_6(P)(k_2 + k_3 + k_4)} \qquad (3)$$

In the case of the oxygen exchange reaction, the symmetry dictates the following relationships: $k_1 = k_6$ and k_2 and k_5; k_3 and k_4 may be replaced by $k_3'(H_2O^{16})$ and $k_3'(H_2O^{18})$, respectively (assuming no kinetic isotope effects).

McKay[16] has shown that the rate of exchange of a virtual reaction (a reaction at equilibrium except for the exchange reaction itself) obeys first-order kinetics, regardless of the mechanism involved. The application of the McKay equation to the oxygen exchange reaction represented by eq. 2 utilizing the symmetry condition $k_2 = k_5$ leads to the expression (4)[17]

$$2.30 \log \frac{(x_o - 0.204)}{(x - 0.204)} = \frac{k'(E)_o t}{(k_2/k_1) + (S)} \qquad (4)$$

where $k' = k_2k_3'/(k_2 + k_3')$ and x refers to the concentration of labeled acid; (k_2/k_1) will be referred to as K_O, the "Michaelis constant" of the oxygen exchange reaction. This equation is similar to one reported by Vaslow.[7] Equation 4 requires that a plot of the logarithm of the atom % O^{18} excess *versus* time be linear and that the slope be equal to $k'(E)_o/2.3(K_O + (S))$. K_O and k' can then be determined from the slopes by solving simultaneous equations. The graphical results for benzoyl-L-phenylalanine and acetyl-L-tryptophan are presented in Figs. 1 and 2, respectively. The values of K_O and k' calculated from the slopes of the lines in Figs. 1 and 2 together with the inhibition constants of these compounds which have been evaluated previously by independent competitive inhibition studies are given in Table I.

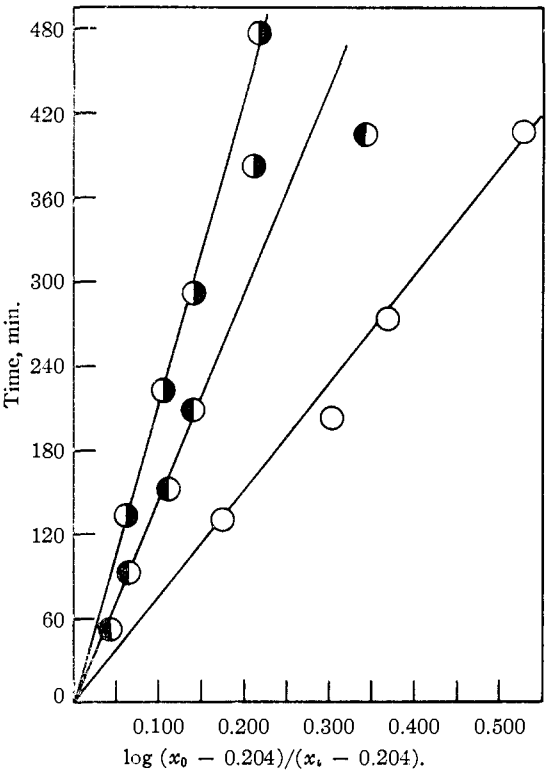

Fig. 1.—α-Chymotrypsin-catalyzed oxygen exchange of acetyl-L-tryptophan-*carboxyl*-O^{18} at *p*H 7.9 and 25.04° (0.024 *M* tris-(hydroxymethyl)-aminomethane buffer): ○ (A) = 4.03×10^{-2} *M*, (E) = 0.291 mg. N/ml.; ◑, (A) 6.91×10^{-2} *M*, (E) = 0.177 mg. N/ml.; ◐, (A) = 5.35×10^{-2} *M*, (E) = 0.208 mg. N/ml.

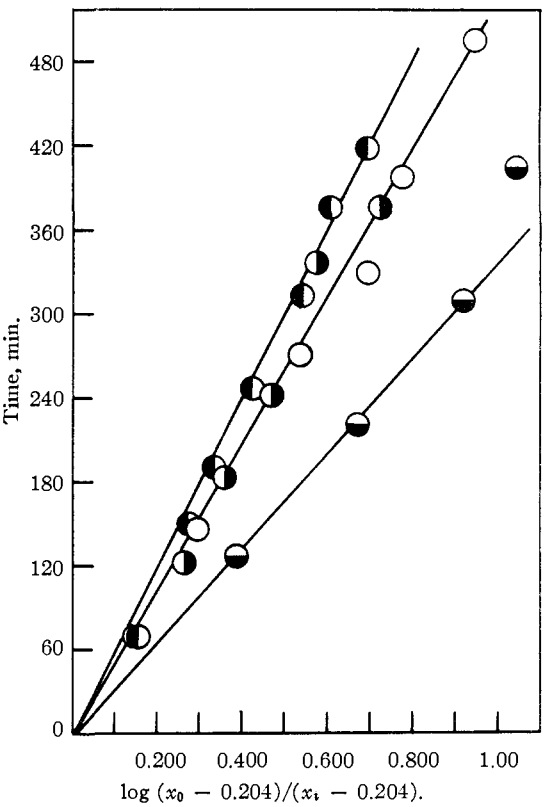

Fig. 2.—α-Chymotrypsin-catalyzed oxygen exchange of benzoyl-L-phenylalanine-*carboxyl*-O^{18} at *p*H 7.8 and 25.04° (0.02 *M* phosphate buffer): ○, (A) = 3.97×10^{-2} *M*, (E) = 0.300 mg. N/ml.; ◐, (A) = 4.37×10^{-2} *M*, (E) = 0.285 mg. N/ml.; ◑, (A) = 3.51×10^{-2} *M*, (E) = 0.291 mg. N/ml.; ◒, (A) = 1.78×10^{-2} *M*, (E) = 0.305 mg. N/ml.

The Michaelis constant for the oxygen exchange of benzoyl-L-phenylalanine, K_O, is, within experimental error, identical with its inhibition dissociation constant. In the case of acetyl-L-tryptophan,

(15) J. B. S. Haldane, "Enzymes," Longmans, Green and Co., London, 1930; R. M. Bock and R. A. Alberty, THIS JOURNAL, **75**, 1921 (1953).

(16) H. A. C. McKay, *Nature*, **142**, 997 (1938).

(17) See the Ph.D. thesis of K.C.K., Illinois Institute of Technology, 1956, for the derivation of this equation. The authors gratefully acknowledge a personal communication of Prof. R. Alberty in which this result is obtained by the direct integration of eq. 3.

TABLE I

KINETICS OF OXYGEN EXCHANGE OF BENZOYL-L-PHENYLALANINE AND ACETYL-L-TRYPTOPHAN[c]

Acid	K_O, 10^2 M	K_I, 10^2 M	k', 10^3 m./l. min. mg. N/ml.
Benzoyl-L-phenylalanine[a]	4.0	2.9[18]	1.21
	2.5		0.99
	1.7		.79
	2.6		1.00
	Av. 2.7 ± 0.7		Av. 1.00 ± 0.11
Acetyl-L-tryptophan[b]	0.38	1.0 ±	0.45
	.57	0.2[5]	.46
	.24		.44
	Av. 0.40 ± 0.12		Av. 0.45 ± 0.01

[a] Phosphate buffer. [b] THAM buffer. [c] Aqueous solution; 25.04 ± 0.01°.

K_O is less than K_I. In view of the inherent experimental errors and the simultaneous solution of K_O, its value must be regarded as less accurate than K_I. It is seen that the oxygen exchange experiments yield values of an equilibrium constant for the association of carboxylic acid and enzyme, k_2/k_1, equal to those obtained by competitive inhibition studies. The complex rate constant, k', will reduce to k_3' if $k_2 >> k_3$.

The question of whether K_I determined from experiments involving product inhibition is necessarily an equilibrium constant is pertinent to the present discussion. Product inhibition may be formulated in the following simplified manner.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P \qquad (5)$$

In the oxygen exchange case, K_P is an equilibrium constant since $k_2 = k_2'(H_2O^{18})$ and $k_3 = k_2'(H_2O^{16})$ ((H_2O^{18}) and (H_2O^{16}) are mole fractions), and therefore, $K_P = K_S = k_2'/k_1$. But in the general case of product inhibition, $K_P = (k_2 + k_3)/k_4$; that is, the inhibition constant in a case involving product inhibition is not necessarily an equilibrium constant but rather a collection of rate constants, resembling the Michaelis constant. It is generally assumed that for product inhibition in hydrolytic reactions, the reverse reaction is so slow that the inhibition constant is an equilibrium constant. In the present case an equilibrium constant, determined unequivocally by means of the oxygen exchange reaction, has been equated to a product inhibition constant in two cases. This result leads directly to the conclusion that product inhibition constants in these cases are equilibrium constants.

An interesting experiment performed by Bernhard[6] consisted of the determination of the Michaelis constant of a poor substrate of α-chymotrypsin and the determination of the inhibition constant of this compound in the presence of an efficient substrate of α-chymotrypsin. The equivalence of the Michaelis and inhibition constants in this instance was presented as evidence of the equilibrium nature of both the Michaelis constant and the inhibition constant. This equivalence might also be interpreted as the equivalence of the same rate constants by the use of an argument somewhat analogous to the one stated above.

(18) S. Kaufman and H. Neurath, *J. Biol. Chem.*, **181**, 623 (1949).

Since it was deemed necessary to postulate a symmetrical mechanism for the enzyme-catalyzed exchange reaction, it appears possible that such a scheme may also be required for enzyme-catalyzed hydrolytic reactions because of similarities in the mechanisms of these two reactions.[19] The hydrolysis of an ester would then be formulated as

$$E + Ester \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\cdot Ester \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\cdot Acid \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E + Acid \qquad (6)$$

It is assumed in 6 as well as in 2 that the binding of the co-substrate (water or alcohol) to the enzyme can be neglected. In this scheme, the following simplifying assumptions will be made: $k_1 = k_6$ and $k_2 = k_5$; k_3 can be shown to be much greater than k_4 since $k_3 = k_3'(H_2O)$ and $k_4 = k_4'(ROH)$ and since $k_3' = k_4'$ and $H_2O >> ROH$. The first two assumptions are a consequence of the fact that the binding of an ester (or an amide) and its corresponding acid to the enzyme should be similar since the major structural element responsible for the binding will be identical. One can illustrate this hypothesis by the comparison of the Michaelis constants and inhibition constants of a series of substrates of α-chymotrypsin shown in Table II.

TABLE II

MICHAELIS AND INHIBITION CONSTANTS OF A SERIES OF SUBSTRATES OF α-CHYMOTRYPSIN[a]

	Benzoyl-L-phenylalanine, 10^2 M	Acetyl-L-tryptophan, 10^2 M	β-Phenylpropionic acid, 10^2 M
K_M of ester	0.46[20]	...	0.39[20]
K_M of amide	0.35[3]	0.50[20]	.62[3]
K_I of acid	2.7[18]	1.00[5]	.55[22]
K_O of acid	2.9[21]	0.40	...

[a] These values do not correspond to constant ionic strength.

The rough equivalence of these constants indicates that all may be equilibrium constants since the oxygen exchange constants are equilibrium constants. This approximate equivalence further indicates that k_1/k_2 may equal k_6/k_5. The most straightforward explanation of such an equation, in the light of the chemistry involved here, is that $k_1 = k_6$ and $k_2 = k_5$ as assumed before. If the assumptions outlined above are made, eq. 3 then reduces to eq. 7 for the initial phase of a hydrolytic reaction (when $P = 0$).

$$-d(S)/dt = k'(E)_0(S)/((k_2/k_1) + (S)) \qquad (7)$$

where

$$k' = k_2k_3/(k_2 + k_3)$$

Equation 7 is essentially of the same form as that for the oxygen exchange reaction (eq. 4) and suggests that the Michaelis constants determined in certain hydrolytic reactions may be equilibrium constants, as are those determined in the oxygen exchange reaction. It further suggests that the k_3 determined in certain hydrolytic reactions is a composite rate constant as given above. This anal-

(19) I. Roberts and H. C. Urey, THIS JOURNAL, **61**, 2584 (1939); M. L. Bender, R. R. Stone and R. S. Dewey, *ibid.*, **78**, 319 (1956).
(20) J. E. Snoke and H. Neurath, *Arch. Biochem.*, **21**, 351 (1949).
(21) R. Foster and C. Niemann, THIS JOURNAL, **77**, 1886 (1955).
(22) H. Neurath and J. A. Gladner, *J. Biol. Chem.*, **188**, 407 (1951).

ysis may have interesting consequences when applied to the known kinetic data of hydrolytic reactions.

α-Chymotrypsin did not catalyze oxygen exchange between benzoyl-D-phenylalanine or β-phenylpropionic acid and the solvent. The results of the exchange experiments are given in Table III. The fact that benzoyl-D-phenylalanine does not undergo oxygen exchange in the presence of α-chymotrypsin while the corresponding L-enantiomorph is a substrate, is another example of the stereospecificity requirements of the enzyme. It might be anticipated that β-phenylpropionic acid would participate in an oxygen exchange reaction since the corresponding ester is a substrate of α-chymotrypsin.[20] However, the ester is a poor substrate and the amide is not a substrate at all. Since the oxygen exchange is considerably slower than ester hydrolysis, it is possible that the former is undetectably slow.[23]

TABLE III

OXYGEN EXCHANGE OF β-PHENYLPROPIONIC ACID AND BENZOYL-D-PHENYLALANINE[d]

Acid	Time, hr.	Atoms % O^{18}
β-Phenylpropionic acid[a]	0	1.30
	3.0	1.25
	3.0	1.26[c]
	5.0	1.26
	6.0	1.28[c]
	24.0	1.26
Benzoyl-D-phenylalanine[b]	0	0.817
	4.8	.813
	6.3	.815
	8.6	.812
	10.1	.812

[a] Acid concn. = 1.3×10^{-2} *M*; enzyme concn. = 1.5 mg. N/ml., *p*H 7.8. [b] Acid concn. = 4.2×10^{-2} *M*; enzyme concn. = 2 mg. N/ml., *p*H 7.7. [c] Blank runs in the absence of enzyme. [d] 25.04°.

The Michaelis constants, K_O, and the rate constants, k', found for the oxygen exchange of benzoyl-L-phenylalanine and acetyl-L-tryptophan are of the same order of magnitude as the constants of the hydrolysis of the corresponding amides.[3,21] Vaslow noted the same similarity with respect to acetyl-3,5-dibromotyrosine and its corresponding amide.[7] This similarity suggests that the Michaelis constants of the amides may be regarded as equilibrium constants. This suggestion has also been made by Huang and Niemann[24] from a consideration of the ratios of Michaelis constants and inhibition constants of enantiomorphic pairs, by Shine and Niemann[25] from a consideration of the effect of ionic strength on the kinetic constants and by Bernhard[6] from the consideration mentioned above of the Michaelis and inhibition constants of a poor substrate.

Acknowledgment.—The authors acknowledge valuable discussions with Drs. R. A. Alberty, R. M. Bock and F. Vaslow. The mass spectrometer on A. E. C. Contract At(11-1)-92 was made available through the courtesy of Dr. H. Taube. A generous gift of α-chymotrypsin from Armour and Co. is gratefully acknowledged.

(23) Personal communication from Dr. R. M. Bock indicates that benzoyl-D-phenylalanine methyl ester may be a substrate (although a poor one) of α-chymotrypsin. In this case the failure to observe oxygen exchange with the D-acid would again be due to the extreme slowness of the reaction.

(24) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 1541 (1951).

(25) H. Shine and C. Niemann, *ibid.*, **77**, 4275 (1955).

CHICAGO, ILLINOIS

[CONTRIBUTION FROM THE LILLY RESEARCH LABORATORIES]

Hygromycin. III. Structure Studies

BY ROBERT L. MANN AND D. O. WOOLF

RECEIVED JUNE 20, 1956

Hygromycin, an antibiotic with a wide antibacterial spectrum, has been degraded to three principal units: 3,4-dihydroxy-α-methylcinnamic acid, an inosamine and 5-keto-6-deoxy-D-arabohexose. The hexose is attached by a glycosidic linkage at the 4-hydroxy position of the 3,4-dihydroxy-α-methylcinnamic acid. The inosamine is linked to the acid as a carboxamide.

Among the metabolic products of the actinomycete *Streptomyces hygroscopicus* (Jensen) Waksman and Henrici is hygromycin,[1,2] which has a relatively broad spectrum of activity against gram-positive and gram-negative bacteria. This paper is concerned with the elucidation of the structure of the antibiotic.

Hygromycin has been isolated as an amorphous substance of the composition $C_{23}H_{29}NO_{12}$,[3] a formula that has been confirmed by analysis of a crystalline 2,4-dinitrophenylhydrazone. Titration of the antibiotic gave a pK_a' of 8.9 in water and 10.7 in 66% dimethylformamide, indicating an acidic group.[4] A positive reaction with the Folin–Ciocalteu reagent and the pK_a' value suggested that the acid function may be phenolic. This was also indicated by the strong absorption of hygromycin in the ultraviolet at 214 and 272 mμ in acid; the intensity and position of the maxima varied with the *p*H of the solution.

Reactivity of the antibiotic with carbonyl reagents, its capacity for reducing Fehling and Benedict solutions and the strong infrared absorption at 5.84 μ indicated the presence of an aldehyde or ketone group. At least one C-methyl was demon-

(1) R. C. Pittenger, R. N. Wolfe, M. M. Hoehn, Phoebe Nelms Marks, W. A. Daily and J. M. McGuire, *Antibiotics and Chemotherapy*, **3**, 1268 (1953).

(2) R. L. Mann, R. M. Gale and F. R. Van Abeele, *ibid.*, **3**, 1279 (1953).

(3) The formula for hygromycin was previously reported to be $C_{25}H_{33}NO_{13}$.[2]

(4) T. V. Parke and W. W. Davis, *Anal. Chem.*, **26**, 642 (1954).