minutes at 50°. The mixture was quickly cooled in ice and filtered to give 7.9 g. of the adduct as a colorless solid, m.p. 81–83°. An additional 4.9 g. (total yield 76%) of product was obtained by concentration of the filtrate *in vacuo*. For analysis, a sample was recrystallized from methanol by cautious heating; m.p. 81–83°.

Anal. Calcd. for $C_{16}H_{18}N_2O_3$: C, 67.1; H, 6.3; N, 9.8. Found: C, 67.0; H, 6.7; N, 9.9.

3,3-Diphenyl-1,2,4-oxadiazolidin-5-one (IIb).—A solution of 5 g. (0.017 mole) of V in 250 cc. of dry methanol was treated with a solution of 1 g. (0.017 mole) of sodium methoxide in 100 cc. of methanol. After having been stored at 25° for 4.5 hours the solution was evaporated to dryness *in vacuo;* the residual solid was dissolved in water and the resulting solution was acidified. The colorless solid was filtered, dried and recrystallized from benzene–ethanol to give 2.8 g. (67%) of the 5-one, m.p. 150–153° dec. An analytical sample, m.p. 156–159° dec., was prepared from another run by recrystallization from ethanol.

Anal. Calcd. for $C_{14}H_{12}N_2O_2$: C, 70.0; H, 5.0; N, 11.7. Found: C, 70.2; H, 5.0; N, 11.4.

3,3-Diphenyl-1,2,4-oxadiazolidin-5-one is sparingly soluble in dilute sodium hydroxide and gives no color change with ferric chloride–pyridine. It shows major bands at 3.14 and 5.73 μ. Admixture of the 5-one and the 3-one produced a marked depression in melting point.

Decomposition of Ethyl [(Hydroxyamino)-diphenylmethyl]-carbamate (V).—A solution of 0.50 g. (0.0017 mole) of V in 50 cc. of methanol was refluxed for 1.5 hours and then evaporated to dryness *in vacuo*. The resulting solid was washed with 1.7 cc. of methanol and filtered. The insoluble portion, 0.2 g. (66%), m.p. 141–142.5°, did not depress the melting point of an authentic sample of benzophenone oxime. Evaporation of the filtrate gave a solid, which, on evaporative distillation *in vacuo*, gave colorless crystals, 0.040 g. (26%), m.p. 47.5–48.5°. This material did not depress the melting point of an authentic sample of ethyl carbamate.

Ethyl (1-phenylpropenyl)-carbamate (VIb) was prepared according to the procedure described by Banfield, *et al.*,[4] for the synthesis of some analogous N-acylketimines. To a solution of the Grignard reagent prepared from 26.4 g. (1.1 g.-atoms) of magnesium, 108 g. (1 mole) of ethyl bromide and 425 cc. of ether there was added, with cooling, 113 g. (1.1 moles) of benzonitrile in 500 cc. of ether. The mixture was refluxed for 1 hour and the supernatant liquid was decanted. A fresh 500-cc. portion of ether was added, the liquid was again decanted and replaced by 500 cc. of fresh ether. A solution of 120 g. (1.1 moles) of ethyl chloroformate in 150 cc. of ether was added with stirring and cooling. The mixture was decomposed carefully with ice and water. The ether layer was separated, washed with dilute sodium bicarbonate solution, then with water and dried. Evaporation to dryness *in vacuo* gave an oily solid which was recrystallized from petroleum ether to give 50 g. (24%) of the product as colorless crystals, m.p. 55–58°. The analytical sample melted at 57.5–58.5°.

Anal. Calcd. for $C_{12}H_{15}NO_2$: C, 70.2; H, 7.3; N, 6.8. Found: C, 70.5; H, 7.6; N, 6.5.

Ethyl (1-phenylpropenyl)-carbamate displays major bands in the infrared at 3.13 and 5.93 μ (shoulder at 5.86 μ).

Acknowledgment.—The authors express their appreciation to Dr. A. C. Osterberg and Mr. C. E. Rauh for the pharmacological data, to Mr. W. Fulmor and associates for spectral determinations, and to Mr. L. Brancone and associates for the microanalyses.

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[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT OF FLORIDA STATE UNIVERSITY]

A Precise Potentiometric Method for Measuring Reaction Rates. Application to the γ-Chymotrypsin-catalyzed Hydrolysis of Methyl Hippurate[1]

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A method is described for following *p*H change with time in a buffered medium in which a reaction is producing acid and for translating that change into reaction rate. The method is used to study the kinetics of the γ-chymotrypsin-catalyzed hydrolysis of methyl hippurate. The dependence of the rate on the substrate concentration conforms to the Michaelis–Menten scheme of enzyme action but the reaction deviates from first order in total enzyme concentration. An explanation for the deviation in terms of the dimerization of the enzyme is offered. The buffer, sodium and potassium chloride and calcium chloride were found to influence the rate in qualitatively different ways. The effect of *p*H was studied over a limited range, and the inactivation of the enzyme also was investigated.

Reactions catalyzed by enzymes are often so complex that their kinetic analysis can be carried out conveniently only in terms of instantaneous reaction rates, dx/dt. At the same time, the direct measurement of dx/dt in open systems[3] may not be feasible because substantial amounts of enzyme might be required. In closed systems, where one measures x as a function of time, accurate derivation is possible only if the experimental points are so dense and so precise that the shape of the rate curve is defined within narrow limits.

The recent commercial availability of highly sensitive *p*H-meters has prompted us to investigate the potentiometric method of rate measurement. For the sample system we chose the γ-chymotrypsin-catalyzed hydrolysis of methyl hippurate.[4] Ester hydrolysis catalyzed by chymotrypsin has proved to be a fruitful model system in the study of enzyme action and a precise knowledge of the kinetics is of considerable current interest.[5] In the present work, a number of kinetic features could be established or confirmed.

Potentiometric Rate Measurement.—A reaction producing acid or base and taking place in a lightly buffered solution may be followed by measuring

(1) Work supported in part by the Office of Naval Research. Reproduction in whole or in part is permitted for any purpose of the United States Government.

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(3) See, for example, H. H. Young and L. P. Hammett, THIS JOURNAL, **72**, 280 (1950); J. Saldick and L. P. Hammett, *ibid.*, **72**, 283 (1950); M. J. Rand and L. P. Hammett, *ibid.*, **72**, 287 (1950).

(4) The α-chymotrypsin-catalyzed hydrolysis of methyl hippurate was investigated by H. T. Huang and C. Niemann, *ibid.*, **74**, 4634 (1952).

(5) N. M. Green and H. Neurath in "The Proteins," Vol. II, Part B, H. Neurath and K. Bailey, eds., Academic Press, Inc., New York, N. Y.

the change in pH by means of suitable electrodes placed in the solution. The unique feature of such a method is that the change in pH per unit of reaction can be varied extensively without extensive alteration of the reaction conditions simply by changing the buffer capacity of the solution. This feature offers the advantage that the sensitivity and precision of the measurement can be held relatively constant over a wide range of concentration of the reactants. In addition, the method is adaptable to continuous recording of the data and even when data are taken manually it is possible to get many closely spaced points, from which the derivative $dp\mathrm{H}/dt$ can be obtained with good accuracy by numerical differentiation.

Relatively little use has been made of the change in pH as a measure of rate.[6] Pertinent recent examples are the hydrolysis of halogenated ketones and of alkyl halides[7] and the measurement of cholinesterase activity.[8]

Kwart and Wilson[7] have described suitable apparatus and have derived equations for calculating rate constants for first-order reactions in unbuffered solutions.

Theory.—If A is a weak acid and B its conjugate base, then

$$K = a_{\mathrm{B}}a_{\mathrm{H}}/a_{\mathrm{A}}, \text{ and } p\mathrm{H}_0 = pK - \log \frac{A}{B} - \log \frac{y_{\mathrm{A}}}{y_{\mathrm{B}}} \quad (1)$$

where a and y represent activity and activity coefficient, respectively, and A and B represent concentrations. If a small amount x of a strong acid is added per liter without changing the volume, the pH becomes

$$p\mathrm{H} = pK - \log \frac{A + x}{B - x} - \log \frac{y'_{\mathrm{A}}}{y'_{\mathrm{B}}} \quad (2)$$

combining equations 1 and 2

$$p\mathrm{H} = p\mathrm{H}_0 - \log\left(1 + \frac{x}{A}\right) + \log\left(1 - \frac{x}{B}\right) + \log q \quad (3)$$

where $q = y_{\mathrm{A}}y'_{\mathrm{B}}/y'_{\mathrm{A}}y_{\mathrm{B}}$.

Equations 1–3 apply to reactions in which one of the products is an acid which is strong compared to the buffer acid, that is, in which K_{A} of the product acid is at least a thousand times K. To follow such a reaction, one can measure pH as a function of the time t and from the data deduce $dp\mathrm{H}/dt$. The amount of product, x, is computed from the pH *via* equation 3[9] and the reaction rate from equation 4

$$dx/dt = (dp\mathrm{H}/dt)/(dp\mathrm{H}/dx) \quad (4)$$

Using equation 3

$$dp\mathrm{H}/dx = -[(A + x)^{-1} + (B - x)^{-1}]/2.303 + \mathrm{d}\log q/dx \quad (5)$$

In practice it is convenient to precalculate $dp\mathrm{H}/dx$ and plot it against pH. For this purpose, it may be preferable to express $dp\mathrm{H}/dx$ as a function of the buffer ratio and the total buffer concentration $C = A + B$, as in equation 6.

$$dp\mathrm{H}/dx = -\{1 + \cosh[\ln(A + x)/(B - x)]\}/1.151C + \mathrm{d}\log q/dx \quad (6)$$

On account of $\mathrm{d}\log q/dx$, $dp\mathrm{H}/dx$ is a function of the ionic strength μ. However, $\mathrm{d}\log q/dx$ is small compared to the other terms and can be evaluated with sufficient accuracy from the interionic attraction theory. At low ionic strength, using the Debye–Hückel limiting law, $\mathrm{d}\log q/dx = S/2\,(\mu_0 + x)^{1/2}$ when A bears unit positive charge, and $\mathrm{d}\log q/dx = -S/2(\mu_0 - x)^{1/2}$ when A is uncharged, where S is the Debye–Hückel limiting slope.

Experimental Part

Materials.—The enzyme γ-chymotrypsin was chosen rather than α-chymotrypsin in order to avoid possible complications due to the conversion of the latter into the β- and γ-forms which is known to occur under similar conditions to those used in the kinetic runs.[10] It was discovered later that the γ-form loses its enzymatic activity more rapidly than the α-form.

A damp filter cake of γ-chymotrypsin was obtained from Worthington Biochemical Sales Co. (lot CD 402). It was crystallized twice from 0.4 saturated ammonium sulfate solution, the crystals were dissolved in 0.001 M hydrochloric acid and exhaustively dialyzed against 0.001 M hydrochloric acid. The resulting solution was stored at 2° and solutions of enzyme for rate runs were made by dilution of this stock solution. Its activity and optical density remained constant over a period of months.

The enzyme concentration was computed from the optical density at 282 mμ in a 1-cm. cell from the equations[11]

$$\text{optical density} = 13.11\ (\text{mg. protein nitrogen/ml.}) = 5.24 \times 10^4\,(E)$$

where (E) is the molar concentration. The second of these equations is based on 16% protein nitrogen and a molecular weight of 25,000.[12]

Methyl hippurate was synthesized by short refluxing of methanol and hippuric acid with sulfuric acid as catalyst. It was crystallized from benzene-cyclohexane and melted at 81.5–84°; literature value, 82–83°.[4] Stock solutions 0.04 M were prepared as needed. This concentration is near the limit of solubility in water at 25°. The stock solutions suffered negligible hydrolysis in one month at room temperature.

A good commerical grade of hippuric acid was recrystallized from water and stored over phosphorus pentoxide.

Buffer-grade tris-hydroxymethylaminomethane (tris) was crystallized twice from methanol and stored over phosphorus pentoxide. The purified amine was titrated with standard hydrochloric acid and assayed 100.0%. Sodium chloride, potassium chloride and calcium chloride were reagent-grade chemicals and were dried before use.

All solutions were made up in deionized water. Potassium acid phthalate from the National Bureau of Standards served as ultimate acidimetric standard.

Apparatus.—The pH-measuring cell was equipped with Beckman #1190-80 glass electrode and #8970-90 calomel electrode. Potentials were measured with a Beckman model GS pH-meter in conjunction with a Rubicon type B potentiometer by a procedure reported elsewhere.[13] With care, pH changes could be measured with a precision of 0.001 unit. Between runs, electrodes were stored in 0.02 M tris buffer of pH 8.2.[14a] After addition of a reagent and swirling, electrode equilibration required 200–300 sec.

(6) R. Livingston, "Investigation of Rates and Mechanisms of Reaction," S. L. Friess and A. Weissberger, eds., Interscience Publishers, New York, N. Y., 1953, p. 66.

(7) E. G. Edwards, D. P. Evans and H. B. Watson, *J. Chem. Soc.*, 1942 (1937); H. Kwart and W. C. Wilson, THIS JOURNAL, **75**, 6147 (1953).

(8) L. E. Tammelin and H. Low, *Acta Chem. Scand.*, **5**, 322 (1951); H. O. Michel, *J. Lab. Clin. Med.*, **34**, 1564 (1949).

(9) If $A \doteq B$, the pH change is nearly linear with x.

(10) M. Kunitz, *J. Gen. Physiol.*, **22**, 207 (1938).

(11) L. W. Cunningham and C. S. Brown, *J. Biol. Chem.*, **221**, 287 (1956).

(12) P. E. Wilcox, J. Kraut, R. D. Wade and H. Neurath, *Biochim. Biophys. Acta*, **24**, 72 (1957), give 25,000 as the most probable molecular weight for chymotrypsinogen.

(13) A. L. Bacarella, E. Grunwald, H. P. Marshall and E. L. Purlee, *J. Org. Chem.*, **20**, 747 (1955).

(14) (a) The buffer concentration refers to the quantity $C = A + B$ as defined for equation 6. (b) The enzyme concentration is in moles/liter.

The reaction vessels were tall 180-ml. electrolytic beakers. A rubber stopper carrying the glass and calomel electrodes, nitrogen inlet tube and an access hole for additions by pipet fitted the beakers snugly. The access hole was plugged with a capillary tube which served as the nitrogen outlet.

The thermostat was of a conventional type. The temperature was adjusted with the aid of a Bureau of Standards thermometer and was maintained at 25.00 ± 0.005°.

Time was measured with a Lab-Chron electric timer reading in seconds. All volumetric apparatus was calibrated.

*p*H Measurements.—If the liquid junction potential is constant

$$p\mathrm{H} = EF/2.303RT + p\mathrm{H}^* \qquad (7)$$

where E is the measured e.m.f. and pH* is a constant for a given set of electrodes at the given temperature. pH* was evaluated by means of Tris buffer solutions of known composition; pH was computed from equation 1. pK for $(HOCH_2)_3CNH_3^+$ was redetermined using a cell with glass and silver–silver chloride electrodes[13] and assuming that ionic activity coefficients are equal to those for HCl at the same ionic strength. For four determinations at ionic strengths ranging from 0.01 to 0.04 the value of pK was 8.077 ± 0.002, in good agreement with 8.076 as reported by Bates and Pinching.[15]

In order to check the accuracy of equation 7 when applied to the kinetic data, a series of measurements was made in which hydrochloric and hippuric acid were added in varying amounts to Tris buffer so as to bracket the pH range employed in the rate measurements. pH changes were computed from the e.m.f. *via* equation 7 and from the solution compositions *via* equation 3. The mean deviation in five separate experiments was 0.002 pH unit, or 1.5% of the mean value of the pH change.

Kinetic Runs.—The total volume of the reaction mixtures was 20 ml. The appropriate volumes of solutions of buffer, of substrate and of water were placed in the reaction vessel, the stopper carrying the electrodes was put in place, the vessel was placed in the thermostat and a slow flow of nitrogen over the solution was started. After 15 min. the desired volume of thermostated enzyme solution was added by pipet and the clock was started simultaneously. Potential and time measurements were recorded at 0.2 mv. intervals.

In order to measure the rate of inactivation of the enzyme, solutions containing buffer and enzyme were kept in the cell at 25.00° for the required length of time. A precalculated amount of substrate solution was then added and the enzymatic activity derived from the rate of change of pH. Volumes were adjusted so that in the measurement of the enzymatic activity the enzyme concentration was about 9×10^{-6} M and the methyl hippurate concentration 0.0200 M. This enzyme concentration is sufficiently low so that the rate is essentially first-order in enzyme (Fig. 1).

Rate Calculations.—pH or e.m.f. was plotted *vs.* t on 50 cm. precision graph paper. The points defined smooth, almost rectilinear curves from which $dp\mathrm{H}/dt$ was obtained by numerical differentiation[16] at 100 sec. intervals. From a statistical analysis of 38 duplicate determinations, the standard precision of a single $dp\mathrm{H}/dt$ determination was found to be 1.8%. dx/dt was computed using equation 4. The buffer capacity of enzyme could be neglected. Allowing 1.5% for possible error resulting from the use of equation 7, the standard error of dx/dt is estimated as 2–3%.

The major limitation of the method is that, due to the time required for electrode equilibration, data for the first 200–300 sec. had to be rejected. Owing to enzyme inactivation during the rate runs, it was desirable to have dx/dt or v (defined in equation 8) at zero time. In the kinetic studies of this inactivation, v_0 was obtained by empirical extrapolation. Plots of v and of v^{-1} *vs.* t were approximately linear and were extrapolated linearly to $t = 0$. Sample values obtained from the v–t and v^{-1}–t plots were: 1.40, 1.47; 1.84, 1.92; 2.31, 2.34; 2.90, 2.91 ($\times 10^{-6}$ M sec.$^{-1}$). The small but consistent discrepancies indicate that some slight but real curvature has been overlooked in the linear extrapolations. By using the mean values, errors arising from this source are minimized. After the kinetics of enzyme inactivation had been studied, all subsequent data were first corrected for inactivation, and v_0 was obtained by extrapolating v^{-1} *vs.* S^{-1} plots to S_0.

(15) R. G. Bates and G. Pinching, *J. Research Natl. Bur. Standards*, **43**, 519 (1949).

(16) W. E. Milne, "Numerical Calculus," Princeton University Press, Princeton, N. J., 1949.

Results

Enzyme Blank.—There was no detectable production of acid by the enzyme in the absence of substrate under the conditions of a kinetic run.

Non-enzymatic Hydrolysis.—The hydroxide ion catalyzed saponification of methyl hippurate proceeded at a rate amounting to a few per cent. of the enzymatic hydrolysis. The second-order rate constant was 1.60 ± 0.06 M^{-1} sec.$^{-1}$ at 25.0°. The velocity of the enzymatic hydrolysis, v, was computed from the total velocity, dx/dt, by means of equation 8 where (S) is the concentration of methyl hippurate.

$$v = dx/dt - 1.60(S)(\mathrm{OH}^-) \qquad (8)$$

Michaelis–Menten Equation.—At constant pH and enzyme concentration, the initial rates closely followed equation 9, conforming to the reaction scheme

$$\mathrm{E} + \mathrm{S} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_3} \mathrm{E} + \mathrm{P}$$

$$v_0 = k_3(E)(S_0)/(K_\mathrm{M} + (S_0)) \qquad (9)$$

(E) is the enzyme concentration and $K_\mathrm{M} = (k_2 + k_3)/k_1$.[17] Sample fits are shown in Table I. This result is significant since at these concentrations the enzyme is an equilibrium mixture of monomer and higher complexes.

TABLE I

TEST OF EQUATION 9 IN THE γ-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF METHYL HIPPURATE. 0.02M TRIS BUFFER AT 25.0°

pH	8.18	8.06	8.06
10^6E (M)	17.7	17.7	25.4
K_M (M)	0.0070	0.0056	0.0056
	$10^6k_3(E)$ (M sec.$^{-1}$) for the following values of S		
0.020	3.96$_5$	4.03$_5$	5.50
.016	3.95$_5$	4.21$_0$	..
.012	(4.13$_0$)	4.14$_0$	..
.010	3.91$_0$	...	..
.008	3.90$_0$	4.11$_5$	..
.006	4.03$_0$	3.96$_5$	5.39
.004	3.94$_0$	4.06$_0$	5.60
Mean	3.95$_0$ ± 0.03$_5$	4.09 ± 0.07	5.50 ± 0.07

Effect of Enzyme Concentration.—At constant pH and substrate concentration, v_0 was not precisely proportional to the enzyme concentration, as illustrated in Fig 1. The data were fitted by an empirical equation of the form

$$v_0 = k(E)(1 - k'(E)) \qquad (10)$$

where k and k' are functions of (S) and pH. Combining equations 9 and 10 and noting that K_M is virtually independent of (E) at constant pH (Table I), one obtains

$$v_0 = k_3^0\,(E)(S)[1 - k'(E)]/[K_\mathrm{M} + (S)] \qquad (11)$$

Numerical values of k_3^0 and k' are[14b]: k_3^0 (sec.$^{-1}$), 0.237$_9$ (pH 8.18), 0.263$_0$ (pH 8.06); k' (M^{-1}), 6540 (pH 8.18), 6920 (pH 8.06).

A plausible explanation of this result may be

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

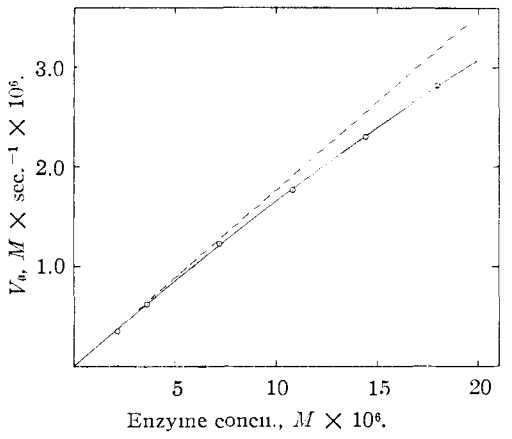

Fig. 1.—Variation of v_0 with (E), 25.00°; $S = 0.0200$ M; pH 8.18. Solid line is a plot of equation 10; $k = 0.176_2$; $k' = 6540$ (M^{-1}). Dashed line is a plot of $v_0 = k(E)$.

found in the known tendency of chymotrypsin to associate in solution.[18,19] Since equation 11 contains no terms of order higher than $(E)^2$, it is sufficient to limit consideration to dimerization. If both monomer and dimer are catalytically active, v_0 is expected to vary according to

$$v_0 = \frac{k_3(M)(S)}{K_M + (S)} + \frac{k'_3(D)(S)}{K'_M + (S)} \qquad (12)$$

where (M) and (D) denote the concentrations of monomer and dimer, and k_3, K_M, k_3' and K_M' are the respective kinetic constants. Introducing the association constant, $K_{assoc.} = (D)/(M)^2 = (D)/(E - 2D)^2$, and neglecting higher-order terms, one obtains $D = K_{assoc.}(E)^2$ and equation 13. Equation

$$v_0 = \frac{k_3(E)(S)}{K_M + (S)} + K_{assoc.}(E)^2(S)\left[\frac{k'_3}{K'_M + (S)} - \frac{2k_3}{K_M + (S)}\right] \qquad (13)$$

13 reduces to the empirical equation 11 if (i) $k_3 = k_3^0$, and (ii) $k'_3 = 0$ or $K'_M = K_M$. The minimum value of $K_{assoc.}$, obtained by setting $k_3 = 0$, is equal to $k'/2$, or approximately 3000 (M^{-1}) in this pH range. This is of a reasonable magnitude. For α-chymotrypsin, Steiner[18b] has reported the value[14b] 1400 (M^{-1}) at pH 6.2 and in acetate buffer of 0.1 M ionic strength from an analysis of light scattering data.

Enzyme Stability.—Although γ-chymotrypsin was stable for several months at pH 3 and 2–3°, it lost its catalytic activity at an appreciable rate at pH 8.2 and 25.0°. The kinetics of inactivation was complex and the complete rate law was not determined. Sample data are shown in Fig 2. Due to the complexity of the inactivation mechanism, it is not possible to assign precise physical significance to the measured residual activity at time t. The inactivation may involve one or several intermediates which are themselves catalysts. In addition, some products may be inhibitory. The residual activity may not, therefore, be assumed proportional to the remaining concentration of the original enzyme. The fractional activity at time t is defined as $\alpha = v_t/v_0$, the velocities being measured under the conditions given in the experimental section, and v_0 being proportional to E_0. $1 - \alpha$ may be expanded in power series

$$1 - \alpha = C_1 t + C_2 t^2 + \ldots \qquad (14)$$

The constants C_1 and C_2 were evaluated for four different initial enzyme concentrations by means of data obtained during the first 15-30 min. The results are listed in Table II. There is a striking decrease in C_1, the initial specific rate of loss of activity, with increasing enzyme concentration. Much of this decrease may be ascribable to self-association of the enzyme, which one might expect to retard the inactivation. Part of the effect may also be due to a greater prominence at higher enzyme concentrations of mechanisms involving intermediates of considerable catalytic activity.

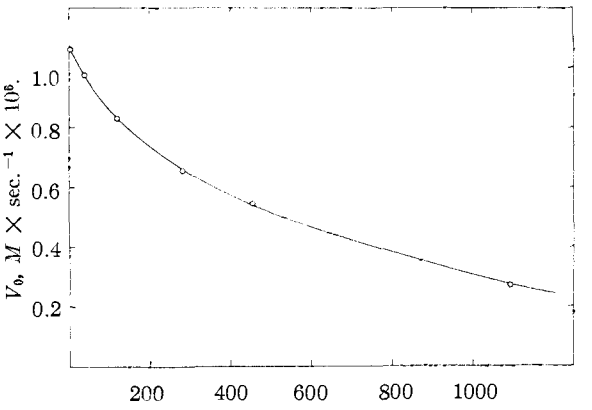

Fig. 2.—Inactivation of 1.44×10^{-4} M γ-chymotrypsin in 0.02 M Tris buffer at pH 8.18, 25.0°. The ordinate is proportional to enzyme activity.

Another way of examining the effect of enzyme concentration is by means of $C_1(E_0)$, which is the actual initial rate of loss of activity. As shown in the last column of Table II, this quantity increases only very slowly at the higher concentrations. This contrasts with the reported second-order inactivation of trypsin.[20]

Table II also lists one set of data for α-chymotrypsin. The rate of inactivation is about one-tenth that for the γ-isomer at the same concentration.

The enzyme inactivation makes itself felt in the presence of substrate in that the observed velocity becomes progressively less than that anticipated from the kinetic constants derived from the v_0 data. Precise analysis of the data is complicated by the changing pH. Assuming the variation of k_3 and k_M with pH to be linear in this narrow range and using data from Table I, values of C_1 in the presence of methyl hippurate were estimated and

(18) (a) G. W. Schwert and S. Kaufman, *J. Biol. Chem.*, **190**, 807 (1951); (b) R. F. Steiner, *Arch. Biochem. Biophys.*, **53**, 457 (1954); (c) V. Massey, W. F. Harrington and B. S. Hartley, *Disc. Faraday Soc.*, **20**, 24 (1955); (d) I. Tinoco, Jr., *Arch. Biochem. Biophys.*, **68**, 367 (1957).

(19) Since this work was completed kinetic evidence for dimerization of α-chymotrypsin has been reported by R. Egan, H. O. Michel, R. Schlueter and B. J. Jandorf, *ibid.*, **66**, 366 (1957). Although our kinetic data do not require that we consider complexes of higher order than dimers, we do not wish to imply that association is limited to dimerization.

(20) M. Kunitz and J. H. Northrup, *J. Gen. Physiol.*, **17**, 591 (1934).

TABLE II

INACTIVATION RATES OF CHYMOTRYPSIN IN 0.02 *M* TRIS BUFFER[14a] AT *p*H 8.18, 25.0°

$10^6E(M)$	10^4C_1 (sec.$^{-1}$)	10^3C_2 (sec.$^{-2}$)	$10^{10}C_1(E_0)$ (*M* sec.$^{-1}$)
	γ-Chymotrypsin		
5.82	3.41	−15.9	20
18.3[a]	2.43	−7.65	44.5
36.7	1.36	−1.95	50
144	0.39	−0.116	56
	α-Chymotrypsin		
8.65	0.28	0.0	2.4

[a] Buffer concentration 0.04 *M*.

are shown in Table III. (The kinetic effect of the products was found to be negligible.) The values of C_1 decrease with increasing (S), indicating that the complexing with the substrate offers some protection against inactivation. However, the values are larger than expected from the data for $(S) = 0$.

TABLE III

INITIAL SPECIFIC RATE OF LOSS OF ACTIVITY OF γ-CHYMOTRYPSIN IN THE PRESENCE OF METHYL HIPPURATE, 25.0°

$(E) = 17.7 \times 10^{-6}$ *M*

(S)	*p*H	10^4C_1 (sec.$^{-1}$)	$K_M/[K_M + (S)]$[a]
0.018	8.11	1.9	0.24
.0145	8.12	2.6	.29
.0067	8.13	3.6	.47
.0032	8.16	3.8	.66
.0000	8.18	2.5	1.00[b]

[a] Fraction of unoccupied sites, assuming $K_M = k_2/k_1$. Based on Table II.

Medium Effects.—The relevant data are shown in Tables IV and V. The rates at *p*H 8.06 are faster than those at *p*H 8.18 partly because of an increase in k_3 and partly because of a decrease in K_M (Table I). Increasing the buffer concentration lowers the rate slightly, the effect of Tris·hippuric acid being somewhat greater than that of Tris·HCl. Addition of sodium chloride to the system 0.02 *M* in buffer results in a progressive increase in rate, fairly abrupt at the low concentrations and more gradual at the higher concentrations of sodium chloride. The effect of potassium chloride is similar but slightly smaller. The observations on the influence of sodium and potassium chloride are consistent with those recently reported by Martin and Niemann in a more detailed study of α- chymotrypsin and methyl hippurate.[21]

The effect of calcium chloride (Table V) is very different from that of sodium chloride. A detectable activation was produced at a concentration of 2×10^{-5} *M* and above about 2×10^{-3} *M* there was no further increase in activity at least up to 0.0085 *M*. This behavior suggests that Ca^{II} is bound relatively tightly to γ-chymotrypsin to form a definite complex having greater catalytic activity than γ-chymotrypsin alone. Assuming the complex to be 1:1, the data were fitted adequately when the association constant, K_{CaE}, was assigned the value 2.6×10^4, as shown in the last column of Table V.

(21) R. B. Martin and C. Niemann, THIS JOURNAL, **79**, 4814 (1957).

TABLE IV

MEDIUM EFFECTS IN THE γ-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF 0.02 *M* METHYL HIPPURATE AT 25.0°

$10^6E(M)$	Buffer[14a]	Other solutes	*p*H$_0$	10^6v_0 (*M* sec.$^{-1}$)
17.7	Tris·HCl, 0.02	.....	8.18	2.94
17.7	Tris·HCl, .02	.....	8.06	3.16
18.4	Tris·HCl, .01	.....	8.13	3.23
18.4	Tris·HCl, .02	.....	8.18	3.03
18.4	Tris·HCl, .04	.....	8.19	2.95
18.4	Tris·HCl, .08	.....	8.21	2.85
17.7	Tris·HCl, .024	Hippuric acid,[a] 0.0018	8.18	2.87
17.7	Tris·HCl, .04	Hippuric acid,[a] 0.0090	8.19	2.67
8.85	Tris·HCl, .02	.....	8.18	1.47
8.85	Tris·HCl, .02	NaCl, 0.00468	8.18	1.52
8.85	Tris·HCl, .02	NaCl, .00923	8.20	1.59
8.85	Tris·HCl, .02	NaCl, .0328	8.22[b]	1.73
8.85	Tris·HCl, .02	NaCl, .0993	8.25[b]	1.87
8.85	Tris·HCl, .02	NaCl, .299	8.25[b]	2.18
8.85	Tris·HCl, .02	NaCl, .700	8.28[b]	2.48
8.85	Tris·HCl, .02	NaCl, 1.00	8.20[b]	2.60
8.85	Tris·HCl, .02	KCl, 0.0166	8.20[b]	1.63
8.85	Tris·HCl, .02	KCl, 0.168	8.28[b]	1.93

[a] Added as Tris·hippuric acid buffer of same composition, B/A, as Tris·HCl buffer. [b] Apparent *p*H. Electrodes not standardized at the same ionic strength.

TABLE V

EFFECT OF CALCIUM CHLORIDE ON THE γ-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF METHYL HIPPURATE AT 25.0° IN 0.02 *M* Tris·HCl BUFFER

$(E) = 8.68 \times 10^{-6}$ *M*

(S) (*M*)	$CaCl_2$ (*M*)	*p*H$_0$	10^6v_0 (*M* sec.$^{-1}$) Obsd.	Calcd.[a]
0.0200	0	8.18	1.464	(1.464)
.0200	2.13×10^{-5}	8.19	1.582	1.585
.0200	4.26×10^{-5}	8.19	1.649	1.649
.0200	12.9×10^{-5}	8.21	1.792	1.746
.0200	42.7×10^{-5}	8.20	1.743	1.805
.0200	85.3×10^{-5}	8.20	1.782	1.820
.0200	0.00215	8.22	1.866	1.827
.0200	.00427	8.22	1.813	1.831
.0160	.00427	8.22	1.88	...
.0120	.00427	8.22	1.68	...
.0080	.00427	8.22	1.45	...
.00399	.00427	8.23	1.27	...
.0200	.00853	8.23	1.826	1.833

[a] Employing $K_{CaE} = 2.6 \times 10^4$ (M^{-1}). See text.

In their study of the chymotrypsin-B catalyzed hydrolysis of phenylalanine ethyl ester at *p*H 6.3, Wu and Laskowski[22] fitted their data with $K_{CaE} = 50$. A much larger value was indicated by their data for α-chymotrypsin.

Kinetic constants were determined for the Ca^{II}–enzyme complex by varying the substrate concentration at a fixed calcium chloride concentration of 0.0043 *M*. The values obtained were $K_M = 0.0032(M)$ and $k_3 = 0.250$ (sec.$^{-1}$), compared with $K_M = 0.0075(M)$ and $k_3 = 0.25$ (sec.$^{-1}$) in the absence of Ca^{II}, extrapolated to the same *p*H and at the same enzyme concentration. It is seen that only K_M is changed and that therefore Ca^{II} in-

(22) F. C. Wu and M. Laskowski, *Biochim. Biophys. Acta*, **19**, 110 (1956).

fluences only the extent of binding of enzyme with substrate, if K_M is accepted as a measure of the binding.[23] The strength of the binding of Ca^{II} to the enzyme suggests chelation, which in turn suggests that Ca^{II} exerts its effect on the kinetics by stabilizing configurations favorable for binding of the substrate.[24,25]

(23) The evidence is summarized by K. J. Laidler, *Disc. Faraday Soc.*, **20**, 83 (1955).

(24) I. M. Klotz in "The Mechanism of Enzyme Action," W. D. McElroy and B. Glass, eds., Johns Hopkins Univ. Press, Baltimore, Md., 1954.

(25) It is possible that addition of Ca^{II} reduces the extent of enzyme–enzyme complexing, thereby raising the rate. However, this would increase k_3 rather than reduce K_M and could not amount to more than a few per cent. at the enzyme concentration employed. Judging by the flatter *p*H *vs.* time curves, Ca^{II} does seem to protect γ-chymotrypsin from inactivation. A similar stabilization by calcium chloride has been reported for α- and B-chymotrypsin.[22]

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Chelating Properties of β-Mercaptopropionic Acid

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The acid dissociation constants of β-mercaptopropionic acid and the stability constants of its chelates with zinc(II) and nickel(II) have been determined potentiometrically by the Calvin–Bjerrum method. The position of zinc(II) in the usual stability sequence of divalent metals has been found to be altered. The chelates of β-mercaptopropionic acid are much less stable than the corresponding chelates of mercaptoacetic acid. This decrease in stability in going from five- to six-membered chelate rings is a general phenomenon which with sulfur present in the chelate ring seems much more pronounced. The reactions of β-mercaptopropionic acid with metal ions have been investigated and the chelates of silver(I), copper(II) and lead(II) have been prepared. The polarographic behavior of β-mercaptopropionic acid has been examined and compared with that of mercaptoacetic acid.

The stability sequence for metal chelates is essentially the same for a large number of chelating agents.[1] In the relatively few cases where the stability of sulfur-containing compounds has been determined, significant alteration of this sequence has been observed. For example, in a comparative study of the chelates of *o*-aminophenol and *o*-aminobenzenethiol,[2] the latter compound was found to give more stable chelates, and the stability order of the divalent metal chelates showed some differences from the usual stability sequence. Also, in the case of mercaptoacetic acid complexes the zinc complex had an anomalously greater stability than the nickel complex.[3] This work on β-mercaptopropionic acid was carried out in order to investigate further the effect of the sulfur atom on the stability of metal chelates.

Experimental

Materials.—β-Mercaptopropionic acid, kindly supplied by Evans Chemetics, N. Y., was found to be 99.85% pure by potentiometric titration with standard sodium hydroxide at 25° in an atmosphere of nitrogen.

In all *p*H measurements, a glass-saturated calomel electrode pair and a model G Beckman *p*H meter, standardized with a potassium acid phthalate buffer at *p*H 4.01 was used.

The compound was found to be quite stable, and aqueous solutions when stored in well stoppered flasks showed no appreciable deterioration for several weeks.

One per cent. aqueous solutions of the common metal ions were prepared from reagent grade salts and used for qualitative tests.

The preparation and standardization of metal perchlorate solutions and of standard sodium hydroxide used in the determination of chelate formation constants have been previously described.[4]

Buffer solutions of ionic strength 0.2 were made with boric acid and sodium hydroxide, disodium hydrogen phosphate and sodium dihydrogen phosphate, acetic acid and sodium acetate, disodium hydrogen phosphate and citric acid, together with appropriate quantities of potassium chloride. All these buffer components were examined polarographically for reducible impurities before use in the polarographic determinations.

Reactions with Metal Ions.—When a 1% aqueous solution of β-mercaptopropionic acid was added to solutions containing various cations, the following observations were made. Nickel(II) formed a deep red color.[5,6] Cobalt(II) gave a deep green color in the presence of ammonia[7]; this solution gradually turned brown on standing due to air oxidation. Iron(III) gave a transient blue color and a white precipitate which was found to be dithiodipropionic acid. Iron(II) gave a blue color which faded very rapidly. Silver(I) and mercury(II) gave white precipitates of the metal complexes soluble in ammonia. Lead(II), copper(II) and bismuth(III) formed pale yellow precipitates which also dissolved in ammonia. Cadmium(II) formed an unstable white precipitate which was soluble in ammonia as well as in excess β-mercaptopropionic acid.

Preparation of β,β′-Dithiodipropionic Acid $HOOC(CH_2)_2SS(CH_2)_2COOH$.—An attempt was made to prepare this compound by the air oxidation of β-mercaptopropionic acid. After passing air through an aqueous solution of the compound for about eight hours no precipitate was obtained. This shows that β-mercaptopropionic acid is very much more stable than thioglycolic acid, which is readily oxidized in air to dithiodiglycolic acid.[8]

On adding a solution of ferric chloride in dilute sulfuric acid to an aqueous solution of β-mercaptopropionic acid, a greenish-blue color was formed and after several minutes a white precipitate was obtained. The addition of the ferric chloride was continued with stirring until an excess of ferric ions was present. The solution was allowed to stand with stirring for about an hour and the precipitate filtered, washed with water, and recrystallized from ethanol; m.p. 151°.

Anal. Calcd. for $C_6H_{10}O_4S_2$: C, 34.27; H, 4.79. Found: C, 34.22; H, 4.55.

Preparation of Chelates.—To aqueous solutions of copper(II), lead(II) and silver(I) nitrates, perchlorates or acetates, an excess of β-mercaptopropionic acid was added with stirring. Precipitates were formed and were allowed to stand

(1) D. P. Mellor and L. E. Maley, *Nature*, **161**, 436 (1948).

(2) R. G. Charles and H. Freiser, THIS JOURNAL, **74**, 1385 (1952).

(3) D. L. Leussing, Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, March, 1958.

(4) H. Freiser, R. G. Charles and W. D. Johnston, THIS JOURNAL, **74**, 1383 (1952).

(5) L. J. Uhlig and H. Freiser, *Anal. Chem.*, **23**, 1014 (1951).

(6) J. B. Lear and M. G. Mellon, *ibid.*, **25**, 1411 (1953).

(7) E. Lyons, *ibid.*, **27**, 1813 (1955).

(8) D. L. Leussing and I. M. Kolthoff, *J. Electrochem. Soc.*, **100**, 334 (1953).