ocimene. Two of these, α- and β-pyronene, were previously known to occur in the *allo*-ocimene pyrolysate. Two other known terpenes, α-terpinene and dipentene, and three known aromatic compounds, *m*-xylene, *m*-ethyltoluene, and hemimellitene, were also found. In addition, five new hydrocarbons have been found and their structures determined. These are 1,3-dimethyl-1-ethyl-3,5-cyclohexadiene, 1,2,3,5-tetramethyl-2,4-cyclohexadiene, 1,2,3,5-tetramethyl-1,3-cyclohexadiene, 1,1-dimethyl-3-ethyl-3,5-cyclohexadiene and 1,1-dimethyl-3-ethyl-2,4-cyclohexadiene.

The structures were determined by reaction of the cyclohexadienes with the dimethyl ester of acetylenedicarboxylic acid and by dehydrogenation, disproportionation, or dealkanation to give aromatic compounds.

In addition to the new cyclohexadienes, several other new compounds were prepared. These include the naphthoquinone adducts of 1,3-dimethyl-1-ethyl-3,5-cyclohexadiene, of 1,2,3,5-tetramethyl-2,4-cyclohexadiene, of 1,2,3,5-tetramethyl-1,3-cyclohexadiene, and of α-terpinene. Also, the phthalic anhydride homologs, 3,4,5-trimethylphthalic anhydride, 3,4,6-trimethylphthalic anhydride, 3-ethylphthalic anhydride, and 4-ethylphthalic anhydride, their dimethyl esters (except for the dimethyl ester of 4-ethylphthalic acid) and the free acids were obtained. In addition, the dimethyl esters of 3-methylphthalic acid and of 3,4-dimethylphthalic acid have been crystallized and their melting points determined.

NEW ORLEANS, LA. RECEIVED NOVEMBER 25, 1949

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, THE CATHOLIC UNIVERSITY OF AMERICA]

The Molecular Kinetics of Pepsin-Catalyzed Reactions[1]

BY E. J. CASEY AND K. J. LAIDLER

Introduction

Considerable importance attaches to the question of the heats and entropies of reaction and activation associated with reactions catalyzed by enzymes, since the results provide valuable evidence with regard to the detailed mechanisms of these reactions. Very little relevant information exists: about the only systematic studies which enable both heats and entropies of activation to be determined are those of J. A. V. Butler[1a] on reactions catalyzed by trypsin and chymotrypsin, those of H. Neurath, *et al.*,[2] on peptidase and esterase activities of chymotrypsin, and the work in this Laboratory on the urease-catalyzed hydrolysis of urea.[3,4] In the present paper are presented data on the pepsin-catalyzed hydrolysis of two synthetic dipeptides, namely, carbobenzoxy-l-glutamyl-l-tyrosine and its ethyl ester. We have carried out a more detailed investigation than was done by Butler, and are suggesting a new interpretation of his data which brings them into line with those we have obtained.

The results on most of the hydrolytic enzymes may be analyzed in terms of the simple Michaelis–Menten law[5] which may be written in the form

$$v = k_0 K c_e c_s/(1 + K c_s) \qquad (1)$$

Here v is the rate of reaction, c_e and c_s are the molar concentrations of enzyme and substrate, respectively, K is the equilibrium constant for the *formation* of the enzyme–substrate complex (*i. e.*, the reciprocal of the Michaelis constant, K_m) and k_0 is the specific rate constant for the decomposition of the complex. The equilibrium constant K may be written as

$$K = e^{-\Delta F/RT} = e^{-\Delta S/R}\, e^{-\Delta H/RT} \qquad (2)$$

where ΔF, ΔS and ΔH are the increases in free energy, entropy and heat associated with the formation of the enzyme-substrate complex, R is the gas constant and T the absolute temperature. In the units of liter mole^{-1}, K is invariably greater than unity; ΔF is therefore a negative quantity.

According to Eyring's theory of absolute reaction rates,[6] the rate constant k_0 may be expressed as

$$k_0 = (kT/h)e^{-\Delta F^*/RT} \qquad (3)$$

$$= (kT/h)e^{\Delta S_0^*/R} e^{-\Delta H_0^*/RT} \qquad (4)$$

Combining equations (2), (3), (4) with (1), it may be seen that at low substrate concentrations (when $Kc_s \ll 1$), the rate is equal to

$$v = k_0 K c_e c_s \qquad (5)$$

$$= c_e c_s (kT/h) e^{-(\Delta F_0^* + \Delta F)/RT} \qquad (6)$$

$$= c_e c_s (kT/h)\, e^{(\Delta S_0^* + \Delta S)/R} e^{-(\Delta H_0^* + \Delta H)/RT} \qquad (7)$$

At high substrate concentrations, on the other hand, when $Kc_s \gg 1$

$$v = k_0 c_e \qquad (8)$$

$$= c_e (kT/h) e^{-\Delta F_0^*/RT} \qquad (9)$$

$$= c_e (kT/h) e^{\Delta S_0^*/R} e^{-\Delta H_0^*/RT} \qquad (10)$$

(1) Abstracted from part of a dissertation submitted by E. J. Casey to The Catholic University of America in partial fulfillment of the requirements for the degree Doctor of Philosophy in Chemistry, July, 1949.

(1a) J. A. V. Butler, THIS JOURNAL, **63**, 2971 (1941).

(2) S. Kaufman, H. Neurath and G. W. Schwert, *J. Biol. Chem.*, **177**, 793 (1949); *cf.* H. Neurath and E. Elkins-Kaufman, *Federation Proc.*, **8**, 232 (1949).

(3) K. J. Laidler and J. P. Hoare, THIS JOURNAL, **71**, 2699 (1949).

(4) K. J. Laidler and J. P. Hoare, *ibid.*, in the press.

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

(6) H. Eyring, *J. Chem. Phys.*, **3**, 107 (1935); *cf.* W. F. K. Wynne-Jones and H. Eyring, *ibid.*, **3**, 492 (1935); S. Glasstone, K. J. Laidler and H. Eyring, "The Theory of Rate Processes," McGraw-Hill Book Co., Inc., New York, N. Y., 1941.

It is therefore clear that by measuring rates at low and at high concentrations of substrate, and over a range of temperature at each concentration, it is possible to obtain values for ΔF_0^* and ΔF, and for the corresponding heats and entropies. This has been done for the two substrates in the present investigation; in Butler's work only one concentration was used with each substrate.

Bergmann[7] has shown that pepsin is specific to certain types of structures, and that, among other similar dipeptides, carbobenzoxy-l-glutamyl-l-tyrosine is hydrolyzed at the peptide linkage. This dipeptide and its ethyl ester then were chosen as substrates for this investigation; the ethyl ester would show the effect of substitution of the tyrosyl carboxyl group at a point just two atoms removed from the peptide linkage under consideration.

Experimental Method and Results

General Kinetic Procedure.—The work was carried out at *p*H 4.0, maintained by acetate buffer when necessary; this *p*H was chosen because Bergmann and Zervas found it to be the optimum for the action of pepsin on carbobenzoxy-l-glutamyl-l-tyrosine. The enzyme solution was prepared using Armour crystalline pepsin, which was examined by micro-Kjeldahl analysis and found to contain 14% total nitrogen by weight; a 1% solution was used containing 1.4 mg. protein nitrogen per ml., this being equivalent to 759 absolute activity units[8] (the number of milliequivalents of tyrosine produced per ml.). The technique used for measuring rates was to mix 50 ml. of buffered dipeptide solution with 10 ml. of a solution of enzyme made up in dilute acid to *p*H 4.0. The volumes were measured at 0° for uniformity, and the solutions were heated separately to the bath temperature before the run was started. All temperatures used were well below the critical range of temperature where enzyme inactivation becomes appreciable. Immediately after mixing, and at successive times afterward, slightly more than 5 ml. was removed from the reaction vessel and cooled in ice to diminish the rate of reaction to a negligible figure. Exactly 5 ml. of this mixture was mixed with neutral formaldehyde solution, and titrated potentiometrically in the manner to be described. In order to conserve substrate, some of the runs were carried out using a semimicro technique. The concentration of enzyme in all cases in the reaction mixture was calculated to be 4.70×10^{-5} mole per liter, assuming pure enzyme with a molecular weight of 35,500.

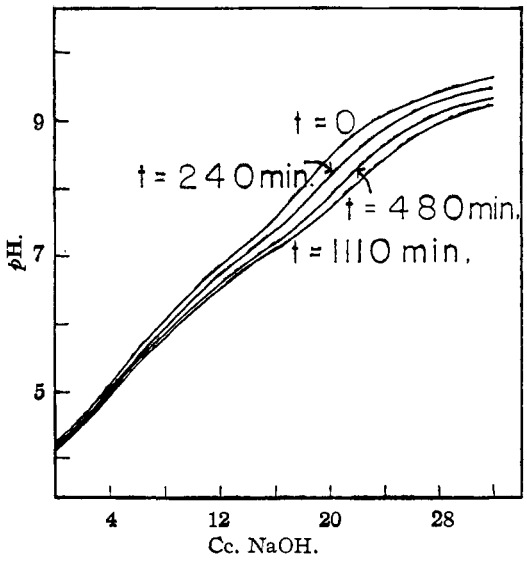

Fig. 1.—Titration curves for a solution containing 8.35×10^{-4} *M* carbobenzoxy-l-glutamyl-l-tyrosine ethyl ester and buffer, corresponding to various stages of hydrolysis.

The Potentiometric Formol Titration.—Several methods have been used to follow the hydrolysis of the peptide linkage. Bergmann and Zervas[7] used the van Slyke amino-nitrogen method, and Zamecnik and Stevenson[9] have recently employed a method which depends upon the liberation of carbon dioxide from free tyrosine by decarboxylase, the rate being determined manometrically. Titration methods have been used by a number of investigators, including Butler,[1] Grassmann and Heyde,[10] Bergmann, *et al.*,[11] and Neurath and his co-workers.[12] However, the presence of the buffer greatly reduces the gradient of *p*H *vs.* base at the neutralization point, and so lowers the accuracy of the method when colorimetric indicators are used. We prefer the potentiometric formol titration method as being more convenient than the van Slyke and manometric methods, and more accurate than the indicator formol titration method.

The technique involved the use of a glass electrode and a standard Beckman *p*H meter. Several titrations were run for the system containing enzyme, buffer and dipeptide, and it was found that the *p*H corresponding to maximum gradient of the *p*H *vs.* base curves did not vary appreciably over the course of the hydrolysis. Figure 1 is the plot of a typical set of data for the pepsin-catalyzed hydrolysis of a solution containing 0.000835 *M* carbobenzoxy-l-glutamyl-l-tyrosine-ethyl-ester and buffer, corresponding to various stages of the hydrolysis. It is seen that the point of maximum gradient is *p*H 8, and it was to this *p*H that the titrations were carried out.

The general procedure was as follows. The 5.00-ml. reaction mixture, to which 0.5 ml. of formaldehyde solution had been added, was placed in a 50-ml. beaker; the electrodes were inserted, and the titration carried out. On the blank, that is the solution removed at zero time, a full titration curve was run, and from that the *p*H of maximum gradient determined; subsequent samples were titrated up to this *p*H of maximum gradient. The difference between the titer value at time *t* and that of the blank is a measure of the number of carboxyl groups liberated in the hydrolysis. Plots were made of moles-of-base-liberated *vs.* time, and initial rates determined from initial slopes.

The base used was 10^{-3} molar, and was protected from atmospheric carbon dioxide. It was delivered to the beaker from a semi-micro buret of total volume 5.00 ml. graduated in hundredths of a ml.

Hydrolysis of Carbobenzoxy-l-glutamyl-l-tyrosine-ethyl Ester.—The dipeptide was synthesized according to

(7) J. S. Fruton and M. Bergmann, *J. Biol. Chem.*, **127**, 627 (1939); *cf.* also M. Bergman and L. Zervas, *Z. physiol. Chem.*, **224**, 11 (1934); M. Bergmann, *Advances in Enzymology*, **2**, 49 (1942); C. R. Harington and R. V. Pitt Rivers, *Biochem. J.*, **38**, 717 (1944).

(8) M. L. Anson and A. E. Mirsky, *J. Gen. Physiol.*, **13**, 121 (1929).

(9) P. C. Zamecnik and M. L. Stevenson, *J. Biol. Chem.*, **169**, 349 (1947).

(10) W. Grassmann and W. Heyde, *Z. physiol. Chem.*, **183**, 32 (1929).

(11) K. Hoffmann and M. Bergmann, *J. Biol. Chem.*, **134**, 225 (1940); M. Bergmann and J. S. Fruton, *ibid.*, **145**, 247 (1942); M. A. Stahmann, J. S. Fruton and M. Bergmann, *ibid.*, **164**, 753 (1946); G. W. Irving, J. S. Fruton and M. Bergmann, *ibid.*, **138**, 231 (1941); J. S. Fruton, G. W. Irving and M. Bergmann, *ibid.*, **141**, 763 (1941); J. S. Fruton and M. Bergmann, *ibid.*, **145**, 253 (1942).

(12) G. W. Schwert, H. Neurath and S. Kaufmann, *ibid.*, **172**, 221 (1948); J. E. Snoke and H. Neurath, *Arch. Biochem.*, **21**, 351 (1949).

the method of Bergmann and Zervas.[6] The ethyl ester hydrochloride of tyrosine was prepared by condensing tyrosine with absolute alcohol, dry hydrogen chloride being bubbled in as catalyst. The hydrochloride group was removed by means of potassium carbonate to give tyrosine ethyl ester. Condensation of benzyl alcohol with phosgene gave carbobenzoxy (cbz) chloride, which was condensed with 1-glutamic acid to give cbz-1-glutamic acid. The inner anhydride of cbz-1-glutamic acid was prepared by the dehydrating action of acetic anhydride. Then cbz-1-glutamic acid anhydride and tyrosineëthyl ester coupled to give the required dipeptide. Recrystallization from alcohol and from warm dilute sodium hydroxide solution gave prisms of pure cbz-1-glutamyl-1-tyrosine-ethyl ester, which melted at 175.5°, in good agreement with Bergmann's value of 176°.

This dipeptide is very insoluble in water. In order to get it into solution at *p*H 4.0, a weighed amount was dissolved in a small amount of dilute sodium hydroxide and warmed slightly (to less than 40°) for two minutes. Then dilute hydrochloric acid was added slowly until the solution had reached *p*H 4.0. Distilled water was then added to bring the volume up to that required. By this method it was possible to obtain reproducible solutions of the dipeptide up to 0.01 *M* which were stable in the cold. It was found that with this dipeptide no acetate buffer was needed in the reaction mixture; the *p*H remained constant during the hydrolysis.

The rate of hydrolysis was measured at 38.0° at a series of concentrations. Figure 2 shows typical data at three concentrations of substrate. The initial rates were determined, and the Michaelis curve so derived is shown in Fig. 3 (upper curve, a). From this curve the Michaelis constant, K_m, and its reciprocal, K, were determined, as well as the initial and limiting specific rate constants.

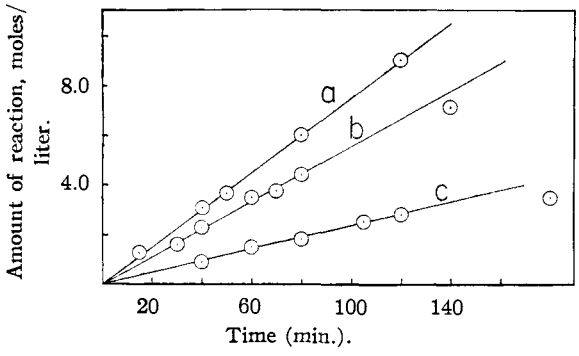

Fig. 2.—Curves showing amount of reaction *vs.* time for three concentrations of substrate at 38°, using 1% pepsin solution. The substrate concentrations are: (a) 0.00830 *M*, (b) 0.00250 *M*, (c) 0.00050 *M*.

The activation energy and entropy were also determined at low and at intermediate concentrations, by measuring the rates at the four temperatures, 23.8, 31.6, 38.0 and 47.2°. Unfortunately it was not possible to get into solution a sufficiently high concentration to determine directly the energy and entropy of activation in the region of limiting rate. However, approximate values of ΔH and ΔS were derived in the following manner. Figure 4 shows Arrhenius plots for extremely low (line a) and intermediate (line b) concentrations of substrate. The experimental

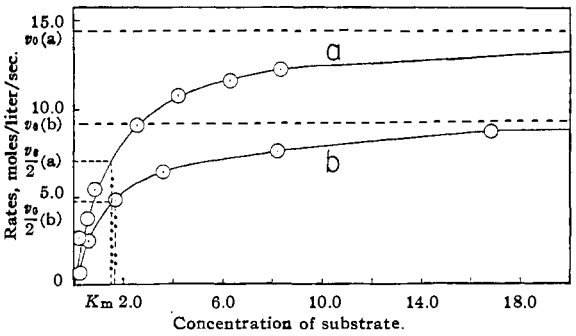

Fig. 3.—Initial rate *vs.* concentration of substrate at 38.0°: (a) carbobenzoxy-1-glutamyl-1-tyrosineëthyl ester, (b) carbobenzoxy-1-glutamyl-1-tyrosine.

activation energy is seen to decrease with increasing substrate concentration, which implies a positive ΔH, *i. e.*, a negative heat of formation for the intermediate complex. At 38.0° the rate *vs.* concentration data are quite complete; at the other temperatures there are two points only available. For these other three temperatures the Michaelis curves were plotted and the values of K and k_0 calculated at each temperature. A plot of log K *vs.* $1/T$ is included in Fig. 4 (line c), and gives ΔH directly; ΔS was then calculated from this information.

The rate equation at very low concentration of substrate ($Kc_s \ll 1$ in eq. (1)) reduces to $v = k_0 K c_s c_e$. It was assumed that the experimental activation energy for the low substrate concentration investigated really applies to $k_0 c_e K$, and is thus (*cf.* eq. (7)) equal to $\Delta H_0^* + \Delta H$, which we call ΔH^*. From ΔH^* and the known rate constant, ΔS^* ($= \Delta S_0^* + \Delta S$) can then be calculated using eq. (7). The values of ΔH_0^* and ΔS_0^* are then readily obtained. The results in this manner are summarized in Table I, and are represented schematically in Fig. 5. The work at low concentration of substrate, corresponding to ΔF^*, ΔH^* and ΔS^*, is quite accurate; the other values given must be considered only as approximate.

TABLE I

SUMMARY OF VALUES OBTAINED IN THE HYDROLYSIS OF CARBOBENZOXY-1-GLUTAMYL-1-TYROSINEËTHYL ESTER

Temp., °C.	$k_0c_e \times 10^6$, moles liter^{-1} sec.$^{-1}$	$K \times 10^2$, liter mole^{-1}	$k_0Kc_e \times 10^4$, sec.$^{-1}$	$k_0K \times 10^4$, liter mole^{-1} sec.$^{-1}$
23.8	2.66	5.27	0.14	0.30
31.6	6.67	5.60	0.37	0.79
38.0	14.4	6.66	0.96	2.04
47.2	43.3	7.04	3.03	6.45

$\Delta F^* = 18.1 \pm 1.5$ kcal./mole; $\Delta H^* = 23.1 \pm 1.2$ kcal./mole; $\Delta S^* = 16.1 \pm 0.8$ cal./mole

$\Delta F = -4.0$ kcal./mole; $\Delta H = 2.4$ kcal./mole; $\Delta S = 20.6$ cal./mole

$\Delta F_0^* = 22.1$ kcal./mole; $\Delta H_0^* = 20.7$ kcal./mole; $\Delta S_0^* = -4.5$ cal./mole

Hydrolysis of Carbobenzoxy-1-glutamyl-1-tyrosine.—Carbobenzoxy-1-glutamyl-1-tyrosine ethyl ester was hydrolyzed to cbz-1-glutamyl-1-tyrosine by heating with a twofold excess of 0.01 *M* sodium hydroxide at 120° for

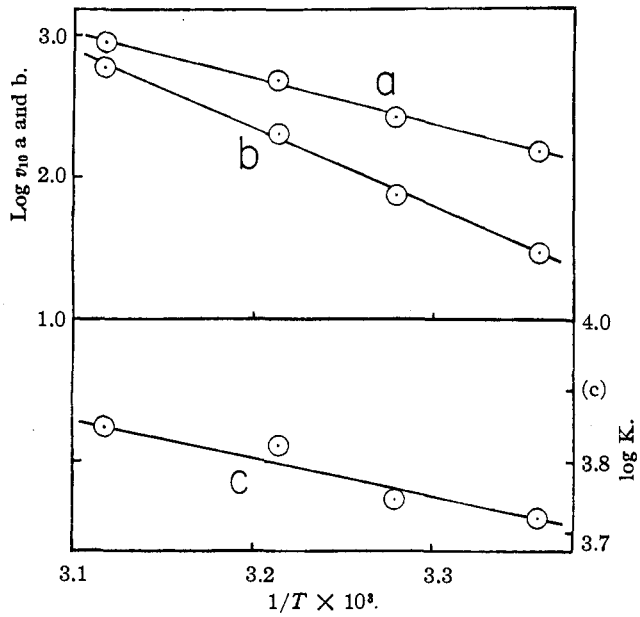

Fig. 4.—(a) and (b) plots of log (rate) *vs.* $1/T$ for low (a) and intermediate (b) concentrations of substrate. (c) Plot of log K *vs.* $1/T$.

twelve minutes. The free acid was recrystallized from warm water, and when pure melted at 186° in good agreement with Bergmann's value (185°) for the melting point of this dipeptide. Cbz-l-glutamyl-l-tyrosine is readily soluble in acid at *p*H 4.0. Since the hydrolysis in the unbuffered reaction mixture in this case is accompanied by a slight *p*H change, and since the rate of the reaction is very sensitive to *p*H,[6] 1.0 ml. of 0.1 *N* acetate buffer was added per 50 ml. of dipeptide solution at high concentrations of dipeptide.

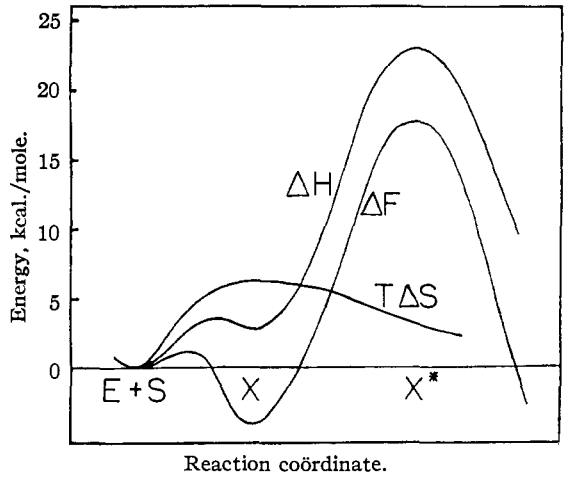

Fig. 5.—Schematic representation of the variations in *F*, *H* and *TS* during the course of hydrolysis of the ethyl ester.

The rate of hydrolysis was studied as a function of concentration of substrate at 38.0°; the results are plotted in Fig. 3 (lower curve, b). The values of K and of initial and limiting rates were determined from the data, and also the specific rate constants for low (k_0Kc_e) and for high (k_0c_e) concentrations of substrate. In addition, the energy of activation was determined corresponding to low and high concentrations of substrate; the Arrhenius plots are given in Fig. 6. From the rate constants and temperature coefficients at high and at low concentrations the values of ΔF, ΔS, ΔH, ΔF_0^*, ΔH_0^*, ΔF^*, ΔS^* and ΔH^* were calculated, and are included in Table II. Figure 7 is a schematic representation of the results. Since no approximations are involved, it is probable that these results on carbobenzoxy-l-glutamyl-l-tyrosine are quite accurate.

General Discussion

It is of interest to compare the results obtained in the present investigation with those obtained for the trypsin,[1] chymotrypsin[1] and urease[4] systems. The values of ΔH, ΔH_0^* and ΔH^*, and the corresponding entropy changes are summarized in Table III.

In the case of the urease and pepsin reactions, the data are complete, the rates and temperature coefficients having been determined over a range of substrate concentrations. With the trypsin and chymotrypsin reactions, however, only a single concentration was used, and some discussion of the assignment of values is necessary.[13] In the cases of the three

TABLE II

SUMMARY OF VALUES OBTAINED IN THE HYDROLYSIS OF CARBOBENZOXY-l-GLUTAMYL-l-TYROSINE

Temp., °C.	$k_0c_e \times 10^5$, moles liter^{-1} sec.$^{-1}$	$K \times 01^{-2}$, liter mole^{-1}	$k_0Kc_e \times 10^5$, sec.$^{-1}$	$k_0K \times 10^4$, liter mole^{-1} sec.$^{-1}$
31.6	5.08	5.32	2.70	1.13
38.0	9.18	5.80	5.40	1.23
47.4	18.4	6.80	12.5	1.34
$\Delta F^* = 15.8 \pm 1.0$ kcal./mole		$\Delta H^* = 20.2 \pm 0.3$ kcal./mole		$\Delta S^* = 4.6 \pm 0.4$ cal./mole
$\Delta F = -4.6 \pm 2.0$ kcal./mole		$\Delta H = 3.0 \pm 0.6$ kcal./mole		$\Delta S = 24.4 \pm 0.8$ cal./mole
$\Delta F_0^* = 20.4 \pm 1.0$ kcal./mole		$\Delta H_0^* = 17.2 \pm 0.3$ kcal./mole		$\Delta S_0^* = -19.8 \pm 0.4$ cal./mole

substrates other than chymotrypsinogen, the molarity of substrate used by Butler was very much greater than 10^{-3} mole per liter, which is the order of magnitude of the Michaelis constant for systems of this type: thus a value of 2.1×10^{-3} mole per liter has been given recently[14] for the action of trypsin on benzoylargininеamide, and for this system Butler used a concentration of 0.36 mole per liter. It follows that for these three reactions the measured heat and entropy of activation correspond to ΔH_0^* and ΔS_0^* (*cf.* eq. (10)). However the concentrations for the substrates

(13) It has recently been pointed out by S. Kaufman, H. Neurath and G. W. Schwert, *J. Biol. Chem.*, **177**, 793 (1949), that Butler apparently uses 5.7×10^{13} instead of 5.7×10^{12} for the value of kT/h at ordinary temperatures. If this is so, Butler's entropy values should all be increased by about 4 entropy units.

(14) K. M. Harmon and C. Niemann, *J. Biol. Chem.*, **178**, 743 (1949).

TABLE III

SUMMARY OF AVAILABLE KINETIC INFORMATION ON ENZYME-CATALYZED HYDROLYSIS

Enzyme	Substrate	ΔH	ΔS	ΔH_0^*	ΔS_0^*	ΔH^*	ΔS^*
Trypsin	Benzoyl-l-arginineamide	..	..	14.9	− 6.2	..	..
Trypsin	Chymotrypsinogen	..	..	....	.....	16.3	8.5
Trypsin	Sturin	..	..	(11.8)	(− 4.7)	..	..
Chymotrypsin	Benzoyltyrosylglycyl amide	..	..	(10.5)	(−17.8)	..	..
Urease	Urea	3.3	13.3	9.25	− 7.5	12.5	7.5
Pepsin	Cbz-l-glutamyl-l-tyrosine ethyl ester	2.4	20.6	20.7	− 4.5	23.1	16.1
Pepsin	Cbz-l-glutamyl-l-tyrosine	3.0	24.4	17.2	−19.8	20.2	4.6

sturin and benzoyl-l-tyrosylglycylamide do not exceed the Michaelis constant by as large a factor as that for the benzoyl-l-arginineamide, and the assignment of the values in this way is therefore not so accurate; this has been indicated by including the figures in parentheses.

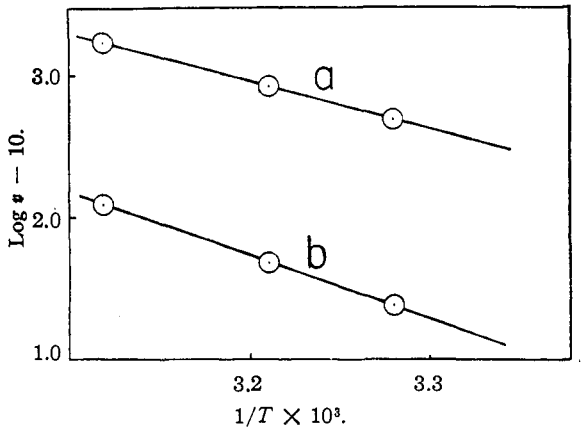

Fig. 6.—Arrhenius plots for the hydrolysis of carbobenzoxy-l-glutamyl-l-tyrosine, at two substrate concentrations: (a) high, (b) low.

In the case of the action of trypsin on chymotrypsinogen, on the other hand, the values must be regarded as corresponding to ΔH^* and ΔS^*, since the molar concentration of the protein substrate must have been extremely low because of the size of the molecule.

The values in Table III may be seen to show certain well-marked regularities which were not evident on the basis of the assumption, which was generally made previously, that Butler's values all represented ΔH^* and ΔS^*. The following points are to be noted in particular: (1) The entropies of activation ΔS^* are all positive; these are the entropy changes involved in going from the separated enzyme and substrate molecules to the activated state. (2) The entropies of reaction ΔS are all positive; these are the entropies of formation of the complex from the reactants. (3) The entropies of activation ΔS_0^* are all negative; these are the entropies involved in going from the complex to the activated state.

A positive entropy of formation of the complex, that is, a positive ΔS, was first discovered for the urease system,[3] and was originally thought to be unique. However, the above analysis suggests that it may not be uncommon. Complex formation normally is associated with a decrease in entropy, and it is suggested that the present results may be due to one of two factors: the adsorption of the dipeptide molecule must be accompanied by the elimination of several water molecules from the surface of the enzyme, the total entropy of desorption of water molecules being greater than the entropy of adsorption of the dipeptide molecule; or alternatively the enzyme molecule itself may change its configuration in forming the stable complex with the substrate. Such changes might consist of an opening-out of the enzyme structure, an unwinding, perhaps, of the same general character as is supposed to occur in protein denaturation.[15] This opening of the enzyme molecule would account for the positive entropy of formation of the complex, and leads immediately to a negative entropy change as the complex goes through the state of activation and thence to complete reaction, the enzyme then being restored to its original shape and rendered able to catalyze another hydrolysis.

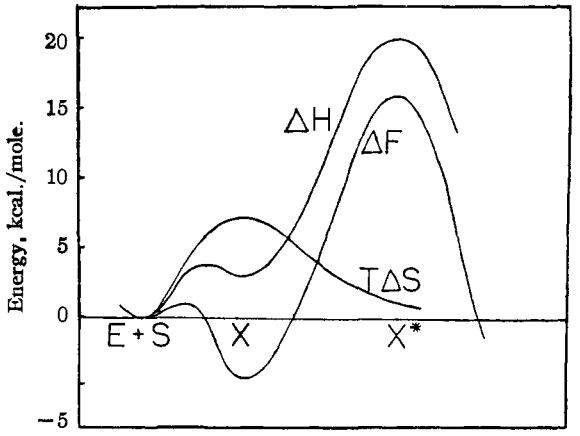

Fig. 7.—Schematic representation of the variations in F, H and TS during the course of hydrolysis of carbobenzoxy-l-glutamyl-l-tyrosine.

The positive values of ΔS^* found in these systems are to be compared with the values found

(15) J. D. Bernal and D. Crowfoot, *Nature*, **133**, 794 (1934); *cf.* H. Eyring and A. E. Stearn, *Chem. Revs.*, **25**, 253 (1939); A. E. Stearn, *Ergb. Enzymforsch.*, **1**, 1 (1938); I. Sizer, *Adv. in Enzymol.*, **3**, 35 (1943).

in non-enzymatic hydrolyses: these are usually about −15 cal./deg./mole (*cf.* Table VII of ref. 4). In view of this, and of the fact that it is highly probable that the big substrate molecule is attached to the enzyme molecule at not more than three or four places[7] (thus necessitating the removal of not more than a few water molecules), the proposal of structural change within the enzyme molecule on complex formation seems to be the more plausible.

Because of the scarcity of data, any discussion along these lines must necessarily be considered as extremely tentative, subject to revision in the light of further experimental evidence. However, the idea of a structural change in the enzyme molecule is attractive when it is remembered that in biological systems enzymes appear to be responsible for the conversion of chemical energy into mechanical work.

Thanks are due to Drs. Rufus Lumry and E. L. Smith for suggestions relative to this paper.

Summary

1. The pepsin-catalyzed hydrolyses of carbobenzoxy-l-glutamyl-l-tyrosine and of its ethyl ester have been investigated over a range of temperature and of substrate concentrations.

2. The results have been analyzed on the basis of a simple model involving complex formation between substrate and enzyme. Values have been calculated for the heat of formation of this complex, ΔH, of the heat of activation for its decomposition, ΔH_0^*, and of the heat of activation in going from the reactants to the activated complex, ΔH^*. The corresponding entropy values (ΔS, ΔS_0^* and ΔS^*) have also been obtained. It is found that ΔS is positive for both systems, that ΔS_0^* is negative, and that ΔS^* is positive.

3. These results are found to fall into line with those for the urea–urease system, and also with those for the reactions investigated by J. A. V. Butler, on the basis of a new interpretation of Butler's results. All of these investigations are discussed tentatively in the light of the proposal that a structural change occurs in the enzyme molecule during the process of complex formation.

WASHINGTON, D. C. RECEIVED JULY 25, 1949

[CONTRIBUTED FROM THE VENABLE CHEMICAL LABORATORY OF THE UNIVERSITY OF NORTH CAROLINA]

Electromotive Force Studies in Dilute Solutions of Hydrochloric Acid in Aqueous Ethylene Glycol at 25°

BY H. D. CROCKFORD, SAMUEL B. KNIGHT AND HELEN A. STATON

Knight, Masi and Roesel[1] determined the activity coefficients of hydrochloric acid in 10, 20, 40 and 60% by weight aqueous ethylene glycol solutions by electromotive force measurements at 25°. From their data they calculated the values of the ion size parameter for hydrochloric acid in these solutions and noted the fact that these values were not satisfactory inasmuch as they were greater than 4.3 Å. in the two higher concentrations and lower than this value in the two smaller concentrations.

The purpose of the work presented in this paper was to expand the electromotive force measurements to the 5, 15 and 30 weight per cent. solutions and from the data to determine the value of the ion size parameter for the purpose of clearing up the lack of constancy found by Knight, Masi and Roesel[1] in their values.

For this purpose the same cell used by Knight Masi and Roesel[1] was employed.

H_2 | HCl(m), Ethylene glycol (x) H_2O (y) | AgCl–Ag

Electromotive force measurements were made with 5, 15 and 30 weight per cent. of the glycol and over an acid range extending to 0.01 m. The standard potentials of the cell at the various ethylene glycol concentrations, the ion size parameter, and the mean activity coefficients of hydrochloric acid in the various mixtures were calculated from the data obtained.

Experimental

The methods of purification of the chemicals, the preparation of the electrodes, and the experimental procedures were the same as those used by Knight, Masi and Roesel.[1]

Density measurements were made on all solutions at 25°. The vapor pressures used were from the data of Trimble and Potts[2] and the dielectric constant values are from Akerlof.[3]

The electromotive force values reported are the average of at least three checks agreeing within ±0.05 mv. Such checks were easy to obtain. All values are corrected to 1 atm. of hydrogen.

Calculations

Calculations were made by use of the equation

$$E' = E + 0.1183 \log m - \frac{0.1183A\sqrt{c}}{1 + aB\sqrt{c}} - 0.1183 \log (1 + 0.002mM_{xy}) = E^0_m + f(m) \quad (1)$$

in which E is the observed electromotive force corrected to 1 atm. of hydrogen, m is the molality. A and B are the Debye–Hückel constants, a is the ion size parameter, c is the concentration of the hydrochloric acid in moles per liter, and M_{xy} is the mean molecular weight of the solvent. The values of A, B, M_{xy}, along with the values

(1) S. B. Knight, J. F. Masi and D. Roesel, THIS JOURNAL, **68**, 661 (1946).

(2) H. M. Trimble and W. Potts, *Ind. Eng. Chem.*, **27**, 66 (1935).

(3) G. Akerlof, THIS JOURNAL, **54**, 4125 (1932).