ation of the relative locations of the points in, for instance, Figs. 1 and 4 shows that those in the region of the fluidity–conductivity plot which are interpreted as falling in a region of changing complex formation correspond rather well with those in the conductivity–concentration plot which display the minimum–maximum effect. By induction, therefore, it appears that the effects noted in the first three figures are probably due more to complex formation than to viscosity alone.

A completely quantitative development of these ideas probably will result in confirmation of the complexing hypothesis. However, the correlation must await the accumulation of further information, both on the exact nature and strength of whatever complexing does occur, and also on further conductance studies in the form of the investigation of systems containing mixed solutes involving a common ion. Such conductance studies are now in progress and will be reported in a subsequent communication.

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Kinetics of Carboxypeptidase Action. I. Effect of Various Extrinsic Factors on Kinetic Parameters[1]

BY RUFUS LUMRY,[2] EMIL L. SMITH AND RUBY R. GLANTZ

Investigation of the kinetics of crystalline pancreatic carboxypeptidase has shown that there is a marked effect of ionic strength on the activity of this enzyme. Normal rate parameters are obtained with carbobenzoxyglycyltryptophan and carbobenzoxyglycylphenylalanine only at substrate concentrations of 0.02 M and below; above this range there is strong substrate inhibition. With the corresponding leucine substrate, inhibition does not occur. Orthophosphate has a small effect as a competitive inhibitor of this enzyme. The effect of temperature on the rate constants for carboxypeptidase has been measured and values for the activation parameters are presented. Evidence is presented for interpreting K_m (the Michaelis constant) as a ratio of the dynamic constants of the consecutive reactions rather than as a thermodynamic equilibrium constant. The effect of pH and D_2O are considered in terms of the mechanism of this enzyme and the role of water and its component ions in the hydrolytic process.

I. Introduction

It has generally been found that the kinetic scheme suggested by Henri[3] but developed by Michaelis and Menten[4] and later by Briggs and Haldane[5] holds for the steady state velocities of most enzyme systems. The usual formulation of this scheme is

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_0} P + E$$

where E is enzyme, S is substrate and P the products of the reaction. The velocity is described as

$$\frac{d[P]}{dt} = \frac{k_0 e[S]}{[S] + K_m} \qquad (1)$$

where $K_m = (k_{-1} + k_0)/k_1$ and e is the total concentration of enzyme, always small with respect to the substrate concentration.[5]

Evaluation of velocity–substrate relationships under different conditions provides potential data for the elucidation of enzymic mechanisms, especially for enzymes where it is possible to compare different substrates. Although it has been customary to measure K_m, it has not been possible to interpret such data because of a lack of knowledge as to the meaning of K_m which as k_{-1}/k_1 may have thermodynamic significance or as k_0/k_1 may be a ratio of possibly unrelated specific rate constants. In recent years there have been a few integrated kinetic studies, for example, those of Laidler and co-workers.[6] Unequivocal interpretation of K_m has been secured only where it has been possible to make direct estimates of the kinetic constants which are part of K_m, as in the work on catalase and peroxidase.[7] It is a purpose of this paper to describe experiments which lead to a highly probable interpretation of K_m for carboxypeptidase. The technique depends on the retardation of reactions involving water in the presence of deuterium oxide and a critical study of the temperature coefficients of catalysis velocity. With an interpretation of K_m, it is possible to obtain values for the rate constants for the reactions involving formation of the intermediate compound ES and its decomposition to products.

Carboxypeptidase was selected because a large body of data for this enzyme already exists.[8,9] In our work, it was found that many of our data were not in accord with earlier kinetic studies by Neurath and his co-workers.[8] Control experiments demonstrated that the lack of agreement is probably due to various combinations of three factors: (1) Although carboxypeptidase is inhibited by some component of phosphate buffer,[10] this has been interpreted[11] as due to a decrease in the rate of product–enzyme dissociation. However, there is also a weaker effect of phosphate which can be interpreted as competitive inhibition. (2) The

(1) This investigation was aided by research grants from the National Institutes of Health, United States Public Health Service.

(2) Merck Fellow in the Natural Sciences.

(3) V. Henri, "Lois generales de l'Action des Diastases," A. Hermann & Cie, Paris, 1903.

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

(5) J. B. S. Haldane, "Enzymes," Longmans Green and Co., London, 1930.

(6) (a) K. J. Laidler and J. P. Hoare, THIS JOURNAL, **71**, 2699 (1949). (b) E. J. Casey and K. J. Laidler, *ibid.*, **72**, 2159 (1950).

(7) B. Chance, *J. Biol. Chem.*, **151**, 553 (1943); *Acta Chem. Scand.*, **1**, 236 (1947).

(8) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 96 (1950).

(9) E. L. Smith, *Advances in Enzymol.*, **12**, 191 (1951).

(10) E. L. Smith and H. T. Hanson, *J. Biol. Chem.*, **179**, 803 (1949).

(11) H. Neurath and G. de Maria, *ibid.*, **186**, 653 (1950).

reactivity of carboxypeptidase depends strongly on ionic strength up to values of about 0.3 *M*. (3) With carbenzoxyglycyl-L-tryptophan and carbobenzoxyglycyl-L-phenylalanine as substrates, the reactivity of carboxypeptidase is decreased by the substrate at concentrations above 0.02 *M*.

II. Materials and Methods

The experiments were generally performed in 5-ml. volumetric flasks in which all components of the reaction mixture except the enzyme were equilibrated in a water-bath regulated to ±0.01°. The enzyme was then added, the solution thoroughly mixed and samples withdrawn for analysis. When the ninhydrin method was used, the enzyme concentration was adjusted so that 6 or 7 aliquots could be removed at 5-minute intervals before more than a few per cent. of substrate was hydrolyzed. At substrate concentrations of 0.01 *M* and lower, it was necessary to correct observed velocities to initial values by a method of successive approximations. A straight line fit of the first few points of the product–time curve represents a chord of the true fit. At low substrate concentrations the average velocity over the length of the chord was readily converted to the velocity at initial substrate concentration by use of equation (1). The corrections were seldom greater than 10% of the original velocity.

For the most part, the liberated free amino acid was estimated by the colorimetric ninhydrin procedure of Moore and Stein.[12] Aliquots of the reaction mixture were added to 2 ml. of ninhydrin reagent at *p*H 6; this immediately stopped the enzymic reaction. This mixture was heated in a boiling water-bath for 20 minutes. (At the altitude of this Laboratory, water boils at 94°.) The color produced was stable for more than 24 hours and gave an average deviation of ±2% in replicate samples, and it was generally possible to reproduce reaction velocities on supposedly identical samples to ±3%. Color intensity was determined by using a Coleman Junior spectrophotometer at 570 mμ after dilution of the boiled ninhydrin mixture with 10 ml. of a mixture of equal volumes of *n*-propanol and water. The procedure was calibrated with standards which contained the reaction components of the digest plus fixed amounts of reaction products. Unsplit substrates occasionally gave some color but did not change the calibration. Buffers, enzyme, acylated amino acid, inhibitors, ammonia and inorganic salts did not affect the standard color development.

Veronal buffers have a large temperature coefficient in contrast to most other common buffers. Corrections for temperature variation were made in compiling data. The *p*H of the reaction mixture was determined before and after each experiment. Except in the range above *p*H 8.2 the veronal concentrations given kept the fluctuation to less than 0.15 *p*H unit; above *p*H 8.0 double strength veronal was usually employed.

Crystalline carboxypeptidase[13] was prepared from frozen beef pancreas and recrystallized in excess of 4 times by the procedure of Neurath, *et al.*[14] The final crystallization was performed as previously described.[15] All preparations used were electrophoretically and ultracentrifugally homogeneous. The activity on a nitrogen basis was in the range repeatedly described for this enzyme.[8,9] Stock solutions were prepared about once every two months by saturating a 1.0 *M* NaCl solution buffered at *p*H 7.5 with veronal. Dilutions were made as needed since the very dilute solutions were stable for only a few days. However, the activity of these solutions was checked by nitrogen determinations only occasionally, enzymic activity being itself the best measure of the effective concentration of enzyme as required for evaluation of absolute reaction rate constants. A standard unit was defined and an activity determination performed with each experiment. This determination was made at 25° with 0.02 *M* carbobenzoxyglycyl-L-tryptophan at an ionic strength of 0.5 *M* and at *p*H 7.5 in veronal buffer. These standards were correlated with preparations of known nitrogen content. Concentrations of enzyme were calculated on the basis that carboxypeptidase contains 14.3% nitrogen[13] and that the molecular weight per enzymic center is 34,000.[15] It has tentatively been assumed that there is but one enzymic center per molecule of protein.

We are indebted to Dr. E. E. Hays and Dr. J. B. Lesh of the Armour Laboratories for some samples of carboxypeptidase which were used in this work for comparative purposes.

Carbobenzoxyglycyl-L-tryptophan (CGT) was prepared as already described.[16] Occasional preparations could not be readily recrystallized from the solvents previously used or gave high blanks with the ninhydrin reagent. The following purification procedure gave satisfactory products. The crude product was taken up in chloroform and washed several times with dilute HCl. The chloroform solution was dried, filtered clear and allowed to evaporate slowly at room temperature. Crystals were deposited which give satisfactory physical constants and which give a negligible blank with the ninhydrin reagent.

Carbobenzoxyglycyl-DL-phenylalanine (CGP) was prepared as previously described.[14] The D-isomer is not hydrolyzed nor does it inhibit the enzyme.[14]

Carbobenzoxyglycyl-L-leucine (CGL) was prepared as already described.[17] In our hands, the carbobenzoxy dipeptide ester was isolated as a crystalline intermediate product.[18] The melting point of carbobenzoxyglycyl-L-leucine has been given as 142–143°[17] and as 102–103°[11]; several of our preparations gave 102–103°.

We gratefully acknowledge the coöperation of Dr. W. J. Polglase and Mr. D. Spackman in preparing some of the substrate preparations used in this investigation.

Commercial samples of ninhydrin have frequently required recrystallization from hot water to eliminate colored impurities.

The heavy water (99.8+% D_2O) was obtained from the Stuart Oxygen Co., San Francisco, California.

As a check on the ninhydrin method, many of our findings were checked by titration of liberated carboxyl groups with alcoholic KOH.[19]

III. Results

1. **Ionic Strength and Reactivity of Carboxypeptidase.**—It was soon found that inconsistent results were obtained from reaction mixtures containing low concentrations of ionized substances. The variation in relative velocity is shown in Fig. 1 for several concentrations of CGT where the ionic strength was changed by adding KCl. Other constitu-

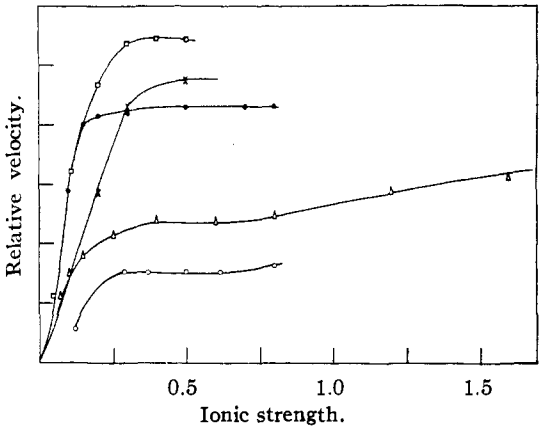

Fig. 1.—Relative velocities for the hydrolysis of carbobenzoxyglycyl-L-tryptophan by carboxypeptidase as a function of ionic strength: □, 0.020 *M* at 25°; ×, 0.025 *M* at 25°; △, ●, 0.005 *M* at 25°; ○, 0.015 *M* at 5°. The vertical positions of the different curves are not related. Each curve is the result of an isolated experiment.

(12) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

(13) M. L. Anson, *J. Gen. Physiol.*, **20**, 663 (1937).

(14) H. Neurath, E. Elkins and S. Kaufman, *J. Biol. Chem.*, **170**, 221 (1947).

(15) E. L. Smith, D. M. Brown and H. T. Hanson, *ibid.*, **180**, 33 (1949).

(16) E. L. Smith, *ibid.*, **175**, 39 (1948).

(17) M. A. Stahmann, J. S. Fruton and M. Bergmann, *ibid.*, **164**, 753 (1946).

(18) E. L. Smith and N. B. Slonim, *ibid.*, **176**, 835 (1948).

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

ents of the reaction mixture are the same. In Table I are given the data on the action of carboxypeptidase on CGT in media where the ionic strength was constant but the species of added ion changed. In comparison with the magnitude of the change produced by increasing ionic strength, the variation in velocity with LiCl, NaCl and KCl is insignificant. However, there are slight differences which indicate the following relative efficiencies for anions at an ionic strength of 0.5 *M* where the added salt contributes 80% of the total ionic strength: $Cl > Br > NO_3 > SO_4$.

TABLE I

INFLUENCE OF IONIC STRENGTH ON CARBOXYPEPTIDASE[a]

Each bracket indicates a single experiment at 25° with 0.02 *M* CGT

Principal salt	Ionic strength	Relative velocity
KCl	0.10	19
	.20	23
	.25	23
NaCl	.40	25
	.80	25
LiCl	.20	23
	.40	25
	.80	26
KCl	.50	17.0
KBr	.50	16.0
KNO_3	.50	15.0
K_2SO_4	.50	14.0

[a] Veronal buffer at *p*H 7.5.

Data for CGP and CGL indicate a similar dependence on ionic strength for these substrates. As an additional check an entire substrate concentration–velocity plot for CGT was secured at 25° with $MgSO_4$ instead of KCl to bring the ionic strength to 0.5 *M*. The k_0 value of a control containing KCl was identical. The K_m value in $MgSO_4$ was 0.0044 *M* as compared to the average value of 0.0052 *M* for a number of runs in KCl. There may be a slight effect of $MgSO_4$ but the statistical reliability of one run suggest that there are no differences when compared to the measurements where KCl or NaCl supply the primary ionic components of the reaction mixture. Since the same rate parameters are obtained at the same total ionic strength, it must be concluded that the effect of added salts on carboxypeptidase action is through ionic strength.

Figure 1 actually shows two regions of ionic strength in which the velocity dependence is considerable. In the region from about 0.3 to 0.9 *M* the velocity of hydrolysis of CGT is independent of ionic strength, and identical rate parameters were obtained at various ionic strengths in the plateau range. However, it may be noted that the substrate concentration has some influence on the ionic strength necessary to reach the flat portion of the curve. It was the usual procedure in investigating variables other than ionic strength to hold the ionic strength constant at 0.5 *M*. No study of effect of ionic strength on carboxypeptidase activity has been reported. It has been stated[20] that there is no effect of ionic strength up to 0.25 *M* on the hydrolysis of an ester substrate where substrate inhibition occurs.

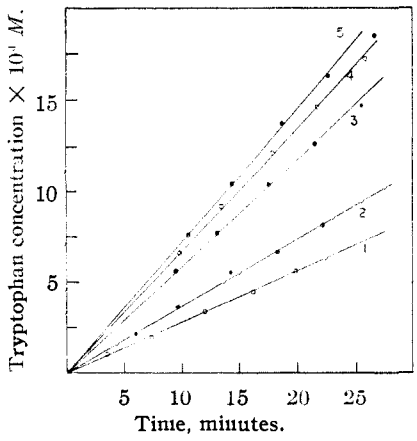

Fig. 2.—The production of L-tryptophan by carboxypeptidase as a function of time at various initial concentrations of carbobenzoxyglycyl-L-tryptophan: 1, 0.0025 *M*; 2, 0.005 *M*; 3, 0.010 *M*; 4, 0.015 *M*; 5, 0.020 *M*. Conditions are 25°; ionic strength = 0.5 *M* in KCl–veronal at *p*H 7.5.

2. **Velocity–Substrate Relationships.**—Carboxypeptidase yields "normal" hydrolysis of CGT and CGP if the concentrations of these substances are less than 0.025 *M* in veronal buffer with salt sufficient to give an ionic strength of at least 0.3 *M*. Under these conditions velocities corresponding to a number of initial substrate concentrations were measured. A typical experiment is shown in Fig. 2.

The substrate concentrations [S] were then divided by corrected velocities (v)[21] and their ratio plotted against substrate concentration as suggested by Lineweaver and Burk.[22] The resulting figure is a straight line as required by the rate law which may be written in the form

$$\frac{[S]}{v} = \frac{[S]}{k_0 e} + \frac{K_m}{k_0 e} \qquad (2)$$

Figure 3 shows the data of Fig. 2 treated in this way. In Table II are presented the averages for K_m and k_0 values calculated by the method of least squares from data of this type. k_1 values are also given in this table; they were calculated on the basis that for these systems $K_m = k_0/k_1$, an assumption which will be discussed below.

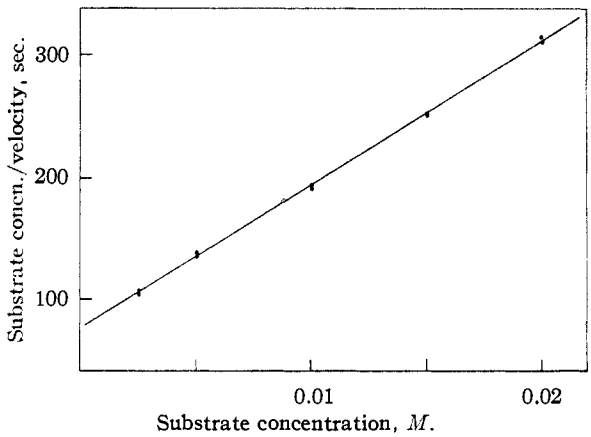

Fig. 3.—A plot of initial substrate concentration [S] divided by velocity *versus* initial substrate concentration for the hydrolysis of carbobenzoxyglycyl-L-tryptophan. The data are taken from Fig. 2.

The precision of the ninhydrin technique sets the lower limit of permissible substrate concentration at 0.001 *M*. Substrate inhibition sets the upper limit at 0.020 *M* for both CGT and CGP. This limited range serves to restrict the precision of K_m and k_0 determinations and makes repeated duplication necessary. CGL is free of inhibiting influence up to at least 0.075 *M* so that rate parameters for this substrate were obtained in the range 0.002 to 0.075 *M*.[23]

Values for the absolute rate constant (k_0) for CGP are in approximate agreement with those reported by Neurath's group. On the other hand, K_m values found by us for both CGP and CGL are very much lower. Neurath[8,11] gives 0.035 and 0.09 *M* for CGP and CGL, respectively. Consideration of equation (1) will show that actual maximum velocities of hydrolysis observed in this Laboratory must have been higher than those found earlier.

(20) J. E. Snoke and H. Neurath, *J. Biol. Chem.*, **181**, 789 (1949).

(21) Velocities were corrected by the procedure discussed in the section on Materials and Methods.

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

(23) The larger the range of substrate concentrations used in determining rate constants the more certain the interpretations and the fewer experiments required to establish good accuracy. A tenfold range of substrate concentrations would appear to be about minimal for quantitative kinetic studies.

TABLE II

RATE PARAMETERS FOR CARBOXYPEPTIDASE

Substrate	Temp., °C.	No. of runs	pH[e]	Ionic strength,[f] M	Substrate concentration M	K_m M	k_1,[a] liter × 10^{-4} mole-sec.$^{-1}$	k_0,[b] sec.$^{-1}$
Carbobenzoxy-	25.0	10[c,d]	7.5	0.30 to 0.89	0.001 to 0.020	0.0051 ± 0.0004[h]	1.77 ± 0.14[h]	89
glycyl-L-trypto-	15.0	4	7.5	0.50	.002 to .020	.0052 ± .0008	0.98 ± .16	50
phan	5.4	6	7.5	.50	.002 to .020	.0050 ± .0005	0.55 ± .05	27
Carbobenzoxy-	25.0	5	7.5	.50	.002 to .020	.0065 ± .0005	2.78 ± .20	181
glycyl-L-phenyl-	15.0	3	7.5	.50	.002 to .020	.0067 ± .0004	1.56 ± .09	104
alanine[g]	5.0	4	7.4	.50	.002 to .020	.0062 ± .0002	0.94 ± .03	57
Carbobenzoxygly-	25.0	3	7.5	.50	.0013 to .075	.027 ± .002	0.393 ± .023	106 ± 3
cyl-L-leucine	5.0	3	7.5	.50	.002 to .075	.036 ± .004	0.104 ± .011	37.5 ± 0.6

[a] Based on $K_m = k_0/k_1$. [b] Activity of H_2O taken as unity. [c] One run with commercial carboxypeptidase (Armour and Company). [d] In one run $MgSO_4$ replaced KCl as added salt. [e] 0.007 *M* veronal buffer. [f] KCl used as inert salt. [g] Equivalent D-isomer of substrate always present. [h] Error equals ±1 standard deviation.

TABLE III

ACTIVATION PARAMETERS FOR CARBOXYPEPTIDASE

(Per mole of active site per liter)

*p*H 7.5; ionic strength = 0.50 (KCl); temp. range 5 to 25°

Substrate	$\Delta F_1^{\ddagger}$,[a] kcal.	$\Delta H_1^{\ddagger}$, kcal.	$\Delta S_1^{\ddagger}$, e.u.	$\Delta F_0^{\ddagger}$,[b] kcal.	$\Delta H_0^{\ddagger}$,[e] kcal.	$\Delta S_0^{\ddagger}$, e.u.
Carbobenzoxyglycyl-L-tryptophan	11.7 ± 0.1[c]	9.3 ± 1.3[d]	−8.5 ± 4.2	14.9	9.3	−19.8
Carbobenzoxyglycyl-L-phenyl-alanine	11.5 ± 0.4	9.0 ± 0.9	−8.5 ± 4.2	14.5	9.0	−18.5
Carbobenzoxyglycyl-L-leucine	12.6 ± 0.1	10.4 ± 1.6	−7.5 ± 5.6	14.8 ± 0.2[c]	8.0 ± 0.4[d]	−20.0 ± 1.8

[a] Standard state taken as one mole per liter of reactants at *p*H 7.5. [b] Standard state taken as at *p*H 7.5 with H_2O at unit activity. [c] Error is ±1 standard deviation. [d] Error calculated by coupling rate constant value plus one standard deviation at one temperature with rate constant minus one standard deviation at the other temperature. Where more than two temperatures were involved the smallest error was taken. [e] $\Delta H_1^{\ddagger}$ = experimental activation energy − RT.

3. Effect of Temperature.—Free energies ($\Delta F^{\ddagger}$), heats ($\Delta H^{\ddagger}$) and entropies ($\Delta S^{\ddagger}$) of activation calculated from the data of Table II are presented in Table III. These quantities[24] are defined by the following expression for the absolute rate constant k

$$k = \frac{\kappa T}{h} e^{-\frac{\Delta F^{\ddagger}}{RT}} = \frac{\kappa T}{h} e^{-\frac{(\Delta H^{\ddagger} - T\Delta S^{\ddagger})}{RT}} \quad (3)$$

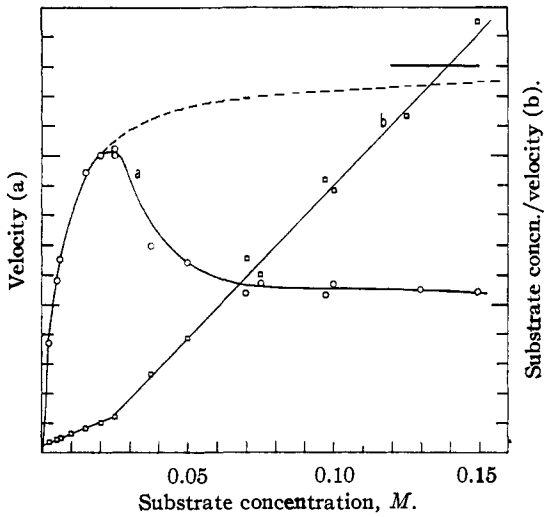

Fig. 4 (a).—Velocity as a function of initial concentration of carbobenzoxyglycyl-L-tryptophan at 5°, *p*H 7.5 and 0.04 *M* phosphate buffer. The dashed line represents the relationship in the absence of inhibition by substrate. The solid segment at top right is the maximum velocity reached at infinite substrate concentration in the absence of inhibitor. (b).—The data of Fig. 4a plotted after the manner of Lineweaver and Burk (*cf.* Fig. 3).

(24) S. Glasstone, K. J. Laidler and H. Eyring, "The Theory of Rate Processes," McGraw-Hill Book Company, Inc., New York, N. Y., 1941.

where κ (kappa) is the Boltzmann constant, h is Planck's constant, R is the gas constant and T is the absolute temperature. The transmission coefficient has been assumed to be unity.

Velocities were determined at three temperatures and the fit to an Arrhenius plot was better than the validity of the averaged rate constants. Except for CGL, values for k_0 were determined in separate experiments in which the rate of hydrolysis at 0.02 *M* substrate was determined simultaneously at the several temperatures. k_0 is calculated from these values once K_m is known as a function of temperature. It is seen in Table II that K_m values for CGT and CGP are not dependent on temperature.

Thermal studies were also made in media containing 0.04 *M* phosphate buffer. A decided temperature coefficient exists for the apparent K_m under these circumstances (*cf.* Neurath and Elkins-Kaufman).[25] The data are presented later in an Arrhenius plot (see Fig. 5). The interpretation is discussed below.

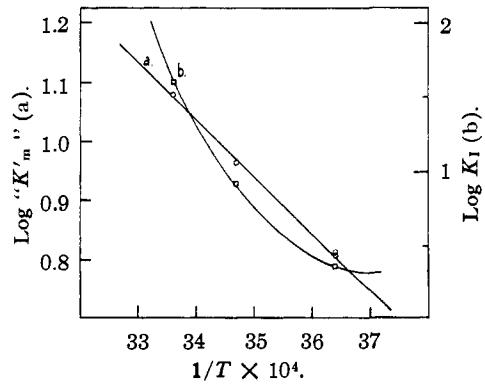

Fig. 5.—Arrhenius plot of "K'_m" values measured in phosphate buffer: (a) "K'_m" values as measured; (b) K_I values calculated from "K'_m" data on the basis of competitive inhibition.

(25) H. Neurath and E. Elkins-Kaufman, *Federation Proc.*, **8**, 232 (1949).

4. **Effect of Phosphate on Reactivity.**—Many earlier investigations of the kinetics of carboxypeptidase were performed in media containing phosphate. Smith and Hanson[10] subsequently demonstrated that this buffer inhibits carboxypeptidase. Neurath and de Maria[11] have defended the use of phosphate buffer with carboxypeptidase for measurements made in a brief time after mixing the enzyme and the buffer. They have demonstrated apparently equivalent reaction rates in phosphate and in veronal during a short period. In this work relatively high concentrations of CGP were involved. With the lower concentrations of CGP and CGT used in our experiments, phosphate was found to have a pronounced inhibitory action which increased as the substrate concentration was lowered. Results with phosphate were somewhat less precise than with veronal largely because of the erratic behavior of determinations at 0.005 *M* substrate. However, a typical reciprocal plot shows excellent linear precision over the short region of substrate concentrations available (Fig. 4). Decreasing velocity at high substrate concentration is observed as with veronal (see section III-7) and is denoted by the second straight segment, that with a higher slope. In phosphate-containing media a temperature coefficient is found to exist for K_m. That for k_0 is nearly identical ($\Delta H^{\ddagger} = 9.6 \pm 0.4$ kcal. per mole of site) with the value measured in veronal for this substrate (CGT). Apparent values for K_m (designated K'_m) are given in Table IV. The value for CGP obtained under the conditions used by Neurath, *et al.*[8] (with NaCl substituted for LiCl) is in agreement with that previously observed. The other listings are average values for CGT and because of ionic strength considerations, only the first is completely dependable. It will be noted that k_0 values are 7% higher in veronal than in phosphate buffers. Consequently at substrate concentrations higher than about 0.03 *M* relatively higher precision would be required to note any inhibition by phosphate. The earlier workers did not observe any such inhibition probably because their substrate concentrations lay in the higher range above 0.03 *M*. It would not be expected, therefore, that the type of inhibition discussed here entered to produce the much higher value of K_m observed by these workers for CGP. However, we have not investigated the phosphate inhibition of the system carboxypeptidase-CGP and the effect may be larger at higher concentrations for this substrate as indicated by the first listing of Table IV. Furthermore, we have not investigated the net effect produced in reaction mixtures containing inhibiting concentrations of substrate (see III-7) as well as phosphate and having ionic strengths in a critical range as already indicated (section III-1). These conditions were customary in Neurath's experiments and may produce a net artifact which would explain high K_m values.[26]

TABLE IV

ACTION OF CARBOXYPEPTIDASE IN PHOSPHATE BUFFER
0.04 molar in PO_4, *p*H 7.5

Substrate	Temp., °C.	K'_m *M*	$\frac{k_0(PO_4)}{k_0(control)}$	Ionic strength
CGP	25	0.033	..	0.2 *M* up, varying with substrate concn. (0.20 to 0.225 *M*)
CGT	25	.0095	0.93	0.4 to 0.5 *M*
CGT	25	.011	..	0.2 *M* up, varying with substrate concn. (0.20 to 0.225 *M*)
CGT	15	.009	..	
CGT	5	.006	..	

K_m values for CGT in 0.04 *M* in phosphate buffer are nearly twice the values found in veronal buffer.

5. **The Effect of Deuterium Oxide on Kinetics.**—It has frequently been found that D_2O alters the rate of chemical reactions in which water enters as a reactant.[27,28] This effect, attributable to a greater participation of the zero-point energy of the molecule in the normal rather than in the activated state, may serve to alter the rate of a reaction as much as five or six-fold. A common state of affairs for hydrolytic reactions is a 20 to 50% decrease in rate. However, the concentration of water will not enter kinetic considerations if the step involving the addition of a water molecule cannot be made rate limiting in the reaction scheme. To secure information on the participation of water as a cosubstrate for carboxypeptidase and also to aid in elucidating the nature of K_m, the hydrolysis of CGT by carboxypeptidase was studied at a very low H_2O concentration. Veronal buffer, salt and substrate were prepared in D_2O and diluted to final concentration with the same isotopic form of water. The enzyme was added in H_2O and was the sole source of this kind of water.

Preliminary experiments in which ionic strength was not controlled are not listed in Table V because of their erratic nature. However, they showed that K_m and k_0 changed by the same factor when different concentrations of heavy water were employed. *p*H was poorly controlled but there was some relationship between rate decrease in D_2O and *p*D (*p*H analog for deuterium-ion activities). A further series of experiments is given in Table V. Up to *p*D 8.3, the agreement of the ratios of the values of the rate parameters in D_2O and H_2O lies within the precision of the method. Furthermore, the influence of D_2O is small at *p*D 7.5 and causes a decrease in K_m and k_0 of about 25% to *p*D 8.3. The agreement in each case is not attributable to a single error in the slope of the [S]/*v versus* [S] plot since such an error would enter once in evaluating k_0 and twice in evaluating K_m as can be seen by consideration of the form of the relation (equation 2). Identical ratios would not be expected in such a case. This effect of D_2O on the velocity of CGT hydrolysis leads to two conclusions: (1) At certain *p*H values the velocity of hydrolysis of CGT by carboxypeptidase depends on the properties of water. (2) Between *p*D 7.5 and 8.3, K_m is affected to exactly the same extent as k_0. The simplest hypothesis that explains the fact that $(k_{-1} + k_0)/k_1$ changes in the same way as k_0 is that k_{-1} is negligible compared with k_0, and k_1 is independent of the properties of water below *p*D 8.3. Above this value K_m falls rapidly in D_2O whereas the relative value of k_0 begins to increase.

TABLE V

EFFECT OF D_2O ON THE PARAMETERS OF CARBOXYPEPTIDASE

The concentration of D_2O varied between 94 and 99.5%. Experiments were performed in veronal buffer at an ionic strength of 0.5 *M* at 25.0°

*p*D[a]	K_m (D_2O), *M*	$\frac{K_m(D_2O)}{K_m(H_2O)}$	$\frac{k_0(D_2O)}{k_0(H_2O)}$
7.7	0.0049	0.94	0.94
7.9	.0044	.88	.85
7.9	.0042	.81	.86
8.0	.0038	.73	.76
8.3	.0039	.75	.78
8.4	.0036	.52	.89
8.4	.0029	.38	.80
8.7	.0040	.45	1.00

[a] Glass electrode observation corrected by +0.4 *p*H unit.

The absolute values of deuterium-ion concentrations in the heavy water experiments are not known. A glass electrode was used for relative determinations but it does not appear that the influence of D_2O on the glass electrode has been reported. It has not been possible to make direct comparisons of glass electrode values with values of *p*D measured with a gaseous deuterium electrode. However, the glass electrode is probably very nearly independent of the isomer of water involved. This follows from the work of Dole[29] which suggests that the slow step is not diffusion of water cations through the glass membrane but rather the displacement of a water molecule held to the outer glass surface by ion-dipole interactions with cations of the glass.

(26) There is another difficulty in evaluating the causes of the differences in K_m values determined in the two laboratories. Part of the explanation undoubtedly resides in the differences in ionic strength which have been used. Experiments in which *p*H 7.5 phosphate buffer at 0.04 *M* and LiCl at 0.1 *M* have been used together[8,11,14,20,25] present some ambiguity in view of the sparing solubility of lithium phosphate indicated in the standard tables. In fact, when the above solutions are mixed, there is a slow precipitation. In the presence of substrate and enzyme, supersaturated solutions are formed but it is not easy to assess the degree of ionization or the amount of ionized phosphate under these conditions.

(27) K. F. Bonhoeffer, *Ergeb. Enzymforsch.*, **6**, 47 (1937).

(28) V. K. La Mer, *Chem. Revs.*, **19**, 363 (1936).

(29) M. Dole, "The Glass Electrode," John Wiley and Sons, Inc., New York, N. Y., 1941.

A proton is apparently carried from bulk water into the water film as H_3O^+. Size and charge will not depend on the nature of the form of hydrogen used. Furthermore, since water rather than the individual hydrogen ion is involved, mass effects will be inappreciable. As a result it is reasonable to assume that the glass electrode value for hydrogen-ion activity measured in solutions also containing deuterium-ion will give approximately the correct total activity of deuterium-ion plus hydrogen-ion. The glass electrode potential then according to our interpretation of Dole's results is essentially a double junction potential with properties independent of the isomer of hydrogen involved in forming the potential. This has been verified by a simple experiment in which stoichiometrically identical acetate buffers were prepared in 99.8% D_2O and in H_2O. The former yielded a glass electrode "pH" of 4.77 and the latter 4.67. La Mer and Chittum[30] give the acidic dissociation constant for deuterium acetate as 0.58×10^{-5} compared to 1.84×10^{-5} for acetic acid. Consequently

$$\frac{[H^+]}{[D^+]} = 3.2 \left\{ \frac{\gamma_{Ac(H_2O)}\gamma_{HAc}}{\gamma_{Ac(D_2O)}\gamma_{DAc}} \right\}$$

The activity coefficients may be taken as equal in the two solvents; then

$$p\mathrm{D} - p\mathrm{H} = \log 3.2 = 0.5$$

Since the observed difference pD − pH is 0.1 the glass electrode yields a higher value of $[D^+]$ than actually exists in the solution by 0.4 pH unit. This correction factor of +0.4 pH unit has been used throughout in tabulating pD values. The correction factor may be slightly high for media containing appreciable H_2O. It is not likely to be very sensitive to absolute pH in an intermediate range. Though the actual concentrations of water cations differed by no more than 2.5 times in the two types of water, factors involved in determining the rate of any one of the component enzyme reactions which are dependent on hydrogen or hydroxyl ion may have markedly different values in high deuterium-ion concentration because of the changes in ionization constants for dissociable acid or base groups when the heavier isotope of hydrogen is employed.

6. **Effect of pH.**—According to Neurath and co-workers,[8] both K_m and k_0 are strongly dependent on pH. They find a minimum in both rate parameters in the region of pH 7.5 when CGP is employed as substrate. Despite the minimum in K_m and k_0 at this pH, the fact that they enter as a ratio allows a maximum velocity of reaction at this hydrogen-ion activity. Above this value both rate parameters rise to a maximum at about pH 8.3 and then fall rapidly at higher pH values. The maximal values of the two constants are more than twice the values at pH 7.5.

We have reinvestigated the effect of pH on K_m and k_0 for the action of carboxypeptidase on CGP and CGT at high ionic strength and in the range of substrate concentration where inhibition does not occur (Table VI). It is apparent that the influence of pH on K_m and k_0 is far smaller than that described earlier though the broad maximum still includes pH 7.5. In any event the rate parameters are so slightly modified as to make a quantitative consideration useless. The range of pH values investigated is smaller than that previously studied since it was not thought wise to use buffers other than veronal.

The data of Table VI for CGT were obtained over a wide range of substrate concentration. Each listing represents the determination of a complete substrate–velocity curve. It was because the magnitude of the observed effect of pH was so small and is in contrast to the findings of other workers that the two experiments of Table VII (given in separate columns) were performed at constant substrate concentration (0.02 M) and varying pH. The data do not show a significant variation in velocity of hydrolysis as a function of pH.

This was confirmed by the results of two other experiments with CGP (Table VI) at two substrate concentrations. The use of only two points in calculating K_m leads to some unreliability. Nevertheless, there is small monotonic increase of K_m from pH 7.5 to pH 8.3; below pH 8.3, k_0 is unaffected. At pH 8.5, both k_0 and K_m appear to decrease slightly which is in slight contrast to the behavior of CGT but in qualitative agreement with the findings of Neurath.[8]

(30) V. K. La Mer and J. P. Chittum THIS JOURNAL, **58**, 1642 (1936).

TABLE VI

EFFECT OF pH ON K_m AND k_0 FOR CARBOXYPEPTIDASE

Substrate	No. of determinations	pH	K_m M	$k_{0\epsilon}$ at pH / $k_{0\epsilon}$ at pH 7.5
CGT[a]	1	7.35	0.0055	1.01
	1	7.50	.0052	1.00
	1	7.7	.0059	1.03
	1	7.9	.0052	1.04
	2	8.2	.0061	1.06
	1	8.4	.0076	1.05
CGP[a,b]	1	7.6	.0064	1.0
	2	7.8	.0078	1.0
	1	8.0	.0078	1.1
	1	8.3	.0080	1.0
	2	8.5	.0060	0.8

[a] Measurements in veronal buffer at 0.5 M ionic strength and at 25°. [b] Paired velocity determinations at 2 substrate concentrations, 0.0075 M and 0.020 M.

TABLE VII

EFFECT OF pH ON HYDROLYSIS OF CGT[a]

pH	Relative velocity	Average	pH	Relative velocity	Average
7.55	154		7.65	145	
	149		7.8	136	
	148	150	8.0	143	
8.0	157			135	139
	154		8.1	140	
	156	156	8.3	141	
8.6	140			144	143
	142		8.6	141	
	139	140		134	138

[a] Data of 2 experiments at 25° with a substrate concentration of 0.02 M in veronal buffer at an ionic strength of 0.5 M.

A single determination of K_m and k_0 at pH 8.2 for CGL showed a slight drop in the first parameter from 0.027 to 0.023 M. k_0 was increased slightly to 106% of its value at pH 7.5 agreeing with the observations on CGT. The change of K_m, on the other hand, was in a direction opposite to that observed with CGT indicating an increase in k_1 for CGL at pH 8.2.

To check further the effect of pH on CGT, two full curves were run at 5° and pH 8.2. It is interesting to note (Table VIII) that k_0 is unchanged but that k_1 is increased as a result of a decreasing activation energy though the entropy change in forming the activated complex becomes more negative.

TABLE VIII

KINETIC CONSTANTS AND PARAMETERS AT pH 8.2 FOR HYDROLYSIS OF CARBOBENZOXYGLYCYL-L-TRYPTOPHAN[a]

k_0 (25.0°) = 94 sec.$^{-1}$
K_m (25.0°) = 0.0061 M
k_1 (25.0°)[b] = 1.54×10^4
k_1 (5.0°) = 25 sec.$^{-1}$
K_m (5.0°) = 0.0041 M
k_1 (5.0°)[b] = 0.61×10^4 liter mole.$^{-1}$ sec.$^{-1}$
$\Delta H_0^{\ddagger}$ = +10.3 kcal. per mole of active center
$\Delta F_0^{\ddagger}$ = +15.0 kcal. per mole of active center
$\Delta S_0^{\ddagger}$ = −16 e.u. per mole of active center
$\Delta H_1^{\ddagger}$ = +6.8 kcal. per mole of active center
$\Delta F_1^{\ddagger}$ = +12.7 kcal. per mole of active center
$\Delta S_1^{\ddagger}$ = −20 e.u. per mole of active center

[a] Veronal buffer; ionic strength = 0.5 M. [b] Calculated on the basis that $K_m = k_0/k_1$.

7. Decrease in Velocity at High Substrate Concentrations.—Figure 6 is a substrate–velocity plot for the hydrolysis of CGT by carboxypeptidase measured over a wide range of concentrations. The curve is the best fit of the data from

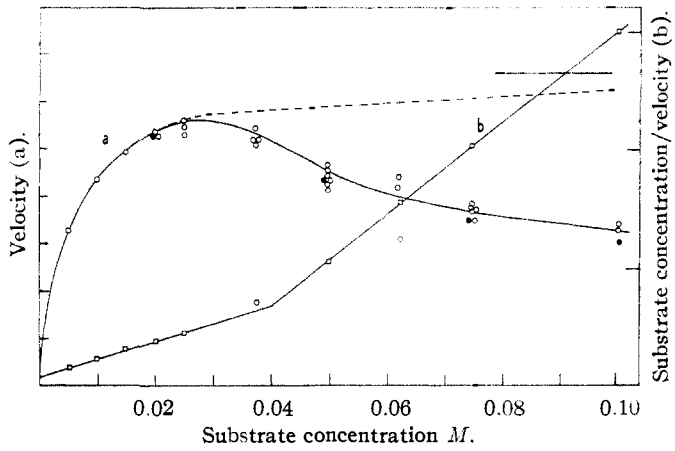

Fig. 6 (a).—Velocity as a function of initial carbobenzoxyglycyl-L-tryptophan concentration under "standard" conditions (veronal buffer; *cf.* text). The dotted line represents uninhibited velocities and the solid segment at the upper right represents the maximum uninhibited velocity: O, determined with the use of the ninhydrin technique; ●, determined by using the titrimetric procedures. (b).—[S]/v *versus* [S]. The data were calculated from points on the curve of Fig. 6a.

4 experiments at 25° in veronal buffer at *p*H 7.5 and at an ionic strength of 0.5 M. The dotted line is the uninhibited velocity as calculated from the velocity of the general control point at 0.02 M substrate with the K_m value of 0.0052 M. The curve clearly indicates inhibition at substrate concentrations of 0.025 M and higher.

That the effect is due to some interaction of enzyme and substrate other than the normal interaction of catalysis and is not due to substrate-substrate interaction is shown by Fig. 7 in which the electrical conductivity of solutions of CGT is plotted as a function of the square root of the concentrations. Other conductivity experiments have shown that there is a common ion effect with 0.5 M NaCl since the partial conductivity of CGT is uninfluenced by the added salt.

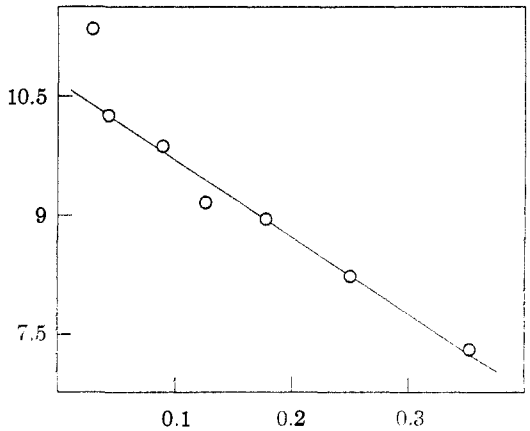

Fig. 7.—Conductivity of solutions of the potassium salt of carbobenzoxyglycyl-L-tryptophan as a function of the square root of ionic strength at approximately *p*H 7.5.

The extent of inhibition at a given substrate concentration is slightly affected by temperature and by the presence of phosphate (*cf.* Fig. 4). The final extent of inhibition at 0.15 M substrate concentration is approximately the same. D_2O and indole-3-acetic acid (a characteristic non-competitive inhibitor) do not alter the shape of the substrate–velocity curve nor the extent of inhibition. Methanol does not alter the shape but raises slightly the substrate concentration required to produce inhibition. Furthermore, the inhibition portion of the plot is essentially unchanged if CGT is replaced by a DL mixture of the isomers of CGP (Fig. 8). That inhibition is not due to the carbobenzoxyglycyl portion of the molecule or the liberated L-amino acid is shown by the fact that no inhibition was obtained when CGL was employed as the substrate (Fig. 9). It is apparent that substrate inhibition depends on the character of the terminal amino acid residue. Since CGP and CGT appear to be nearly identical substrates for carboxypeptidase (Tables II and III), it is not surprising to find that they inhibit in almost the same quantitative manner.

It may be noted that Snoke and Neurath[20] found substrate inhibition of carboxypeptidase with benzoylglycyl-β-phenyllactic acid but not with CGP. As an additional check on the reliability of our findings the kinetics were reinvestigated with the titration technique. As shown in Fig. 6 the results were identical. Furthermore, similar results were obtained with several different preparations of carboxypeptidase, including a commercial preparation. Finally, we have convinced ourselves that the substrate inhibition is not due to an artifact by a systematic check of the compounds, procedures, calibrations and manipulations which have been used.

IV. Discussion

1. Interpretation of K_m.—In order to secure maximum information from studies of reaction rate *versus* substrate concentration it is necessary to know whether the Michaelis constant applies to an equilibrium or a stationary-state formation of the intermediate compound of enzyme and substrate. There is no *a priori* reason

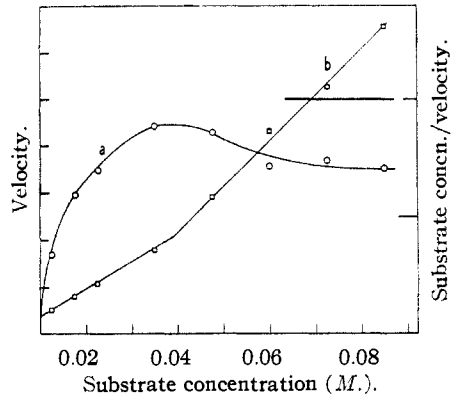

Fig. 8 (a).—Velocity of hydrolysis of carbobenzoxyglycyl-L-phenylalanine as a function of the initial substrate concentration. The solid segment at the upper right is the maximum uninhibited velocity; "standard" conditions at 25°. (b).—The data of Fig. 8a plotted after the manner of Lineweaver and Burk (*cf.* Fig. 3).

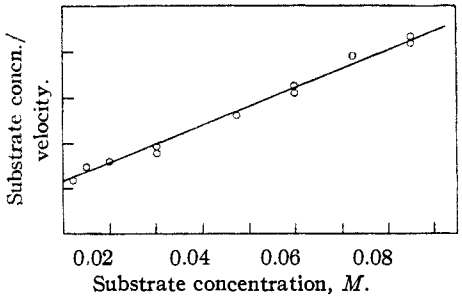

Fig. 9.—[S]/v *versus* [S]: the substrate is carbobenzoxyglycyl-L-leucine; "standard" conditions at 25°.

why either situation should be preferred. Chance[7] has proved that stationary-state conditions generally apply for peroxidase ($K_m = k_0/k_1$) by direct measurement of the changes in concentration of intermediate compounds. Goldstein[31] has indicated a similar situation for a hydrolytic enzyme, cholinesterase, using non-steady-state kinetics. Neither method has been convenient for use with carboxypeptidase so that the interpretation of K_m must be inferred from the results of experiments in which K_m and k_0 are varied by changing experimental conditions.

Three lines of evidence support the interpretation of K_m for carboxypeptidase as equal to k_0/k_1: (I). The data in Table V show that D_2O has an identical and significant effect on K_m and k_0 below *p*D 8.3. These results are reasonably interpreted to mean that $K_m = k_0/k_1$ and that k_1 is independent of the type of water used below *p*D 8.3. The precision of the data enforce this analysis but it is certainly not unique.

(II) Certain modified substrates produce similar changes in K_m and k_0 when compared to values of these constants for CGP. For example, with carbobenzoxyglycyl-*o*-fluorophenylalanine, K_m and k_0 are increased to the same degree, whereas with carbobenzoxyglycyl-*p*-fluorophenylalanine, K_m and k_0 are decreased to the same extent.[32] Again the results are most easily interpreted on the basis that $K_m = k_0/k_1$ and that k_0 alone is altered by the substitution in the phenyl ring.

(III) Hydrocinnamic ion, which possesses two of the total of three points of interaction of CGP with the enzyme, is a strictly competitive inhibitor for carboxypeptidase.[3,9,33] The free energy and heat changes in forming a mole of enzyme-inhibitor compound are -5 and -7 kcal., respectively. The absence of a temperature dependence for K_m when CGP is the substrate would mean a zero heat change in forming a mole of intermediate compound and a free energy change of -3 kcal., if $K_m = k_{-1}/k_1$. A similar interpretation for CGL would yield a positive heat change and a negative free energy change. Although there need be no relationship between thermodynamic constants for substrates and inhibitors, somewhat better agreement might be expected. The disparity is removed if K_m is the stationary-state constant for when the activation energies for k_0 and k_1 are nearly the same, the heat change associated with K_m will be small ($\Delta H_m = \Delta H_0^{\ddagger} - \Delta H_1^{\ddagger}$). By itself this argument carries little weight. In conjunction with arguments I and II it provides some additional support for the dynamic interpretation of K_m. The total of arguments must be said to indicate rather than prove the interpretation but further development of II has shown that considerable confidence may be put in the analysis.

Similar arguments may be used with existing data to give tentative interpretations of K_m for a number of enzymes. For example, carbonic anhydrase must form its intermediate compound in equilibrium. Any other interpretation of Kiese's data[34] leads to a large negative activation energy. Similarly, the intermediate compound for the hydrolysis of sucrose by invertase is probably formed in equilibrium since k_0 varies by a factor of 20 in varying the *p*H from 4 to 8[35] whereas K_m does not change at all (*cf.* Kuhn).[36] These results though certainly only indicative suggest that no general interpretation of K_m will be found and that the nature of the Michaelis constant must be determined independently for each enzyme and indeed in many cases for each substrate as well.

Casey and Laidler[6b] have given kinetic data for the urease–urea system and for two substrates of pepsin. The assumption is made throughout that K_m is the reciprocal of the association equilibrium constant for enzyme-substrate compound formation. It seems at least as reasonable to interpret K_m as a dynamic constant. This eliminates the need for considering the large positive entropy changes calculated for the formation of ES compound. For the three systems the heats of formation of ES calculated on the assumption of an equilibrium formation of the compound are positive, negative free energy changes resulting from positive entropy changes of the order of 15 to 20 e.u. per mole of active center. Casey and Laidler have pointed out the possible significance of positive entropy changes of this magnitude in terms of major alterations in the protein. Their interpretation may indeed be correct but the significance of large entropy changes must be established by experiments leading to a description of K_m for the systems involved.

The description of K_m as a dynamic constant composed of k_0/k_1 has been used to calculate k_1 for three substrates of carboxypeptidase, CGP, CGT and CGL (Tables II and III). It is apparent that most activation parameters are small. This is true for the entropy of activation in forming ES suggesting that no unusual or remarkable rearrangements of any of the involved molecular species occurs. Relatively larger negative entropies of activation have been found for the slow step in the sequence of reactions leading to bond rupture.

2. Ionic Strength.—Since enzymes function in aqueous solution and, in many cases, act by means of ionic mechanisms, it may be expected that the rate of certain steps will vary when the ionic strength is altered. In general, our understanding of enzyme-catalyzed reactions has not progressed to the point where such studies have great usefulness and very few have been made. The reactivity of urease has recently been shown to be dependent on ionic strength[37] and, undoubtedly, many enzymes will be found in this category.

For carboxypeptidase, the effect of low ionic strength is considerably greater than would be expected from the Brønsted equation relating reaction velocity to ionic strength. This effect is also larger than would be predicted by the simple

(31) A. Goldstein, *J. Gen. Physiol.*, **27**, 529 (1944).

(32) E. L. Smith, W. J. Polglase, R. Lumry, R. R. Glantz, M. D. Armstrong, and J. D. Lewis, to be published.

(33) (a) E. L. Smith, R. Lumry and W. J. Polglase, *J. Phys. Colloid Chem.*, **55**, 125 (1951); (b) R. Lumry, W. J. Polglase, E. L. Smith, R. R. Glantz and S. L. Wendelboe, to be published.

(34) M. Kiese, *Biochem. Z.*, **307**, 400 (1941).

(35) L. Michaelis and M. Rothstein, *ibid.*, **110**, 217 (1920).

(36) R. Kuhn, *Z. physiol. Chem.*, **125**, 28 (1923).

(37) W. R. Pasterczyk and H. J. McDonald, Abstracts, Chicago Meeting, A. C. S., September, 1950, 7C.

Debye–Hückel theory for a single charged catalytic site unless the protein has a high negative valence at the point of interaction with substrate, the substrate being always negatively charged under the conditions of these experiments. If we exclude the very improbable close aggregation of several monovalent negative protein groups, there are no groups in proteins which would yield multiple valences. A reasonable explanation for the effect is that as the ionic strength is increased, the activities of a number of groups on the enzyme are altered by means of Debye–Hückel effects. The individual changes may be quite small but their total effect is to stabilize a more reactive configuration of the protein.

3. **Phosphate Inhibition.**—In contrast to the slowly-occurring inhibition observed in phosphate-containing media by earlier workers, the weak but rapidly-occurring inhibition noted in this work has not previously been reported. It is not of the non-competitive type nor does the Arrhenius plot of K_I calculated on the basis of competitive inhibition yield a straight line (Fig. 5). When the observed "K_m," which is a function of the concentration of phosphate buffer, is plotted directly on an Arrhenius plot (Fig. 5), a straight line results. The effect of phosphate can, therefore, be interpreted as modifying the rate constants probably by reducing k_1 through modification of configuration or by elimination of some group at or near the catalytic site which affects the rate.

4. **Effect of Water and Its Ions.**—It has been found that neither variation in *p*H nor the type of water produce large changes in rate parameters below *p*H or *p*D 8.3. Above this value, k_1 especially changes rapidly with hydrogen-ion concentration. Data are insufficient to treat the higher range. In the lower range it appears that the slow step in the products-forming groups of reactions (k_0) is changing from a water-independent to a water-dependent reaction. The shift is probably incomplete in the range available. The entropy of activation for the slow-steps of k_0 is increased from −20 to −16 e.u. (see Table VIII) in the shift. k_1 is negligibly altered in value but the contributions of entropy and heat factors to its free energy of activation are markedly changed. $\Delta S_1^{\ddagger}$ decreases rapidly over the range of *p*H values from −8.5 to −20 e.u. at *p*H 8.3. This is paralleled by a change of 2.5 kcal. in the heat of activation. The slow-step in the group of reactions leading to the formation of the intermediate compound consequently appears also to change with *p*H.

Since the entropy of activation at the higher *p*H is similar to that associated with k_0, it is possible that some of the processes originally included in the k_0 group of reactions are transferred to the k_1 group. Evidence from phenylalanine substrates which have been substituted in the phenyl ring[32] suggests that at *p*H 7.5 the ES compound is formed with only two of the three (or perhaps four) binding points of the substrate molecule. The slow-step in the zero-order group of reactions appears to involve the subsequent binding of phenyl ring to protein. It can be proposed that at higher *p*H values the ES compound is actually formed using three points of binding. The subsequent reaction in which a molecule of water adds across the strained peptide bond then becomes the slow-step in the k_0 group of reactions. In this way the slow-step of the zero-order group is shifted to the first-order group of reactions thus accounting for the observed changes in entropy of activation.

Variations in relative proportions of deuterium oxide and water at high concentrations of the former have no pronounced effect on rate constants. On the other hand the equilibrium constants for the formation of HDO and D^+ are such that the relative concentrations of hydrogen and deuterium ions change very markedly with slight alterations in H_2O concentration at high D_2O. From this it is suggested that the effect of deuterium oxide is not mediated through its ions. Furthermore, the slow rate with which specific rate constants change with hydrogen-ion concentration indicate that the ions of water do not directly enter in any of the slow steps. The validity of this conclusion will rest on a knowledge of the mechanism by which changes in *p*H produce shifts in slow-steps. The extent of dissociation of neither substrate nor products is much altered by the change from *p*H 7.5 to 8.3 since all dissociation constants are well over on the side of complete dissociation. Apparently the shift in slow-step is caused in some way by altering the concentrations or reactivities of groups on the protein. The data do not justify a further consideration of the mechanism at this time.

5. **Substrate Inhibition.**—Inhibition of an enzyme by its own substrates is a well known phenomenon. In some cases the effect has been attributed to a decrease in the concentration of water. For invertase the addition of inert substances decreases the water concentration and the rate of the reaction.[38] In our experiments, the addition of 5% glucose did not influence the substrate inhibition of carboxypeptidase nor alter the velocity of catalysis. Substrate inhibition of this enzyme is, therefore, not of this type.

In another case, it has been proposed that urea inhibits urease because a postulated site for water-binding at the point of catalysis is covered by a urea molecule.[6a] Such a mechanism might apply here. However, any explanation for substrate inhibition of carboxypeptidase must explain the novel feature that only approximately half the enzyme activity can be eliminated by even the highest possible concentration of substrate. This partial inhibition suggests a dimerization in which the reactivity of one molecule of enzyme is eliminated or a selective inhibition of one of each pair of nearly identical catalytic sites. The latter case may be rather improbable since catalytic efficiency must then be nearly identical at each site yet only one site can be inhibited. However, there is evidence that dimerization does not occur. Ultracentrifuge experiments in the presence of a high concentration of substrate do not indicate the presence of other than monomeric protein molecules. Furthermore, a study of the dependence of inhibition on enzyme concentration shows a strictly

(38) J. M. Nelson and M. P. Schubert, THIS JOURNAL, **50**, 2188 (1928); J. M. Nelson, *Chem. Revs.*, **12**, 1 (1933).

linear first-order relationship (Fig. 10). It would appear, therefore, that inhibition by substrate is a function of the unit catalytic site. It is also found that the extent of inhibition by substrate for CGT and apparently also for CGP is proportional to the fourth power of the substrate concentration. This conclusion is derived from the following argument.

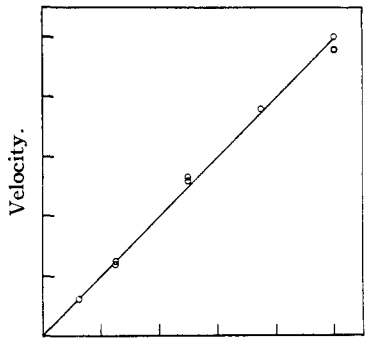

Fig. 10.—Relative velocity as a function of carboxypeptidase concentration for the hydrolysis of 0.05 *M* carbobenzoxyglycyl-L-tryptophan. The absence of deviations from a straight-line fit of the data demonstrate a first-order dependence of the substrate inhibition reaction on enzyme concentration; "standard" conditions at 25°.

If there are two types of enzymic sites with identical reactivities but differing susceptibilities for substrate inhibition, we may assume $[E]_T = [e] + [e']$ where $[E]_T$ = total enzyme concentration, and e and e′ are the two types of sites. Let $[e] = [e'] = [E]_T/2$. In analogy with the reaction scheme given in section I

$$e + S \xrightarrow{k_1} eS \xrightarrow{k_0} P + e$$

$$e' + S \xrightarrow{k_1} e'S \xrightarrow{k_0} P + e'$$

$$e' + nS \rightleftarrows e'S_n$$

$$K_I = \frac{[e'S_n]}{[e'][S]_n}$$

where the symbols have their usual meanings and $e'S_n$ is inactive catalytically.

$$[E]_{effective} = e + \frac{e'}{1 + K_I[S]^n} = \frac{[E]_T}{2}\left(1 + \frac{1}{1 + K_I[S]^n}\right)$$

$$v_I = \left(\frac{dP}{dt}\right)_I = \frac{k_0[E]_T[S]}{2}\left(\frac{2 + K_I[S]^n}{1 + K_I[S]^n}\right) \quad (4)$$

Since K_m is known from the data of Table II, the full uninhibited substrate-velocity curve can be plotted as has been done in the dashed curves of Figs. 4a, 6a and 8a. The proper rate equation in the absence of inhibitor is

$$v = \left(\frac{dP}{dt}\right) = \frac{k_0[E]_T[S]}{[S] + K_m} \quad (5)$$

hence

$$\frac{v_I}{v} = \frac{1 + \frac{[S]^n K_I}{2}}{1 + [S]^n K_I} \quad (6)$$

for graphical representation it is more convenient to use

$$2\frac{v_I}{v} - 1 = \frac{1}{1 + K_I[S]^n} \quad (7)$$

The ratio v_I/v can be obtained from the solid and the dashed curves of Figs. 4a and 6a, the solid line being the original fitting by eye of the experimental points. If now, in the usual manner, $\left(2\frac{v_I}{v} - 1\right)$ is plotted against log [S], Fig. 11 results. The solid curve of this figure was calculated from the right side of the equation (7) with $K_I = 1.35 \times 10^5$ molar^{-1} and $n = 4$. The experimental points were taken from the data of Fig. 6a. The agreement of experiment and theory is very good and is not destroyed by a determination of v_I/v from the most extreme deviations of the experimental points of Fig. 7a although these points show considerable scatter at any given substrate concentration. n is very clearly 4 and not 3 or 5. Therefore, the solid curve of Fig. 11 is identical with a plot of equation (7). It must be concluded that 4 molecules of substrate are required to inhibit one enzymic center with an over-all inhibition (association) constant of 1.35×10^5 molar^{-1}.

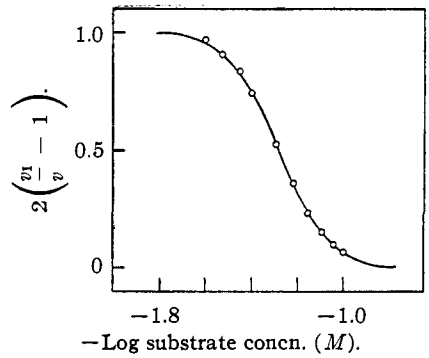

Fig. 11.—The curve is a plot of the function $1/(1 + K_I[S]^4)$ *versus* log [S]. The points are measured values of the function $\left(2\frac{v_I}{v} - 1\right)$ *versus* log [S] as given by equation (7) for carbobenzoxyglycyl-L-tryptophan at 25°; $K_I = 1.35 \times 10^5$ liter^4 mole^{-4}. The data are taken from Fig. 6a.

At 5° the course of substrate inhibition by CGT is identical in 0.04 *M* phosphate and in 0.007 *M* veronal. Within the available precision the data also yield a fourth-order dependence on substrate concentration, but the data are too few to exclude completely a fifth-order dependence. At 5° the velocity at maximal inhibition was taken as 0.4 of the uninhibited velocity instead of 0.5 as at 25°. With this modification an application of the previously described treatment produces a symmetrical mass-law curve with the substrate dependence mentioned above. The requirement that total extent of inhibition increases with lowered temperature throws some doubt on this method of interpretation. However, if we assume a fourth-order dependence, the association constants for inhibition by the substrate per mole of enzymic site are at 5°, 5.7×10^5 and, at 25°, 1.4×10^5. The heat of association is calculated to be −7.5 kcal. per mole of enzymic center or 1.9 kcal. per mole of substrate. Since the free energy change in the reaction at 25° is −7 kcal. per mole of site, the entropy change is insignificant.

As an inhibitor, CGL is considerably poorer than CGT or CGP. The value of k_1 is also considerably lower than corresponding values for CGT and CGP

though the k_0 values are all of the same general magnitude. The latter observations can be correlated with a weaker bonding between enzyme and substrate and the consequent reduction in the rate constant k_1. The polarizability of the conjugated ring structures in the terminal amino acids of CGT and CGP is much greater than that of the saturated residue of leucine in that position. The result is a reduction of van der Waals interaction between the R group and the centers of such interaction on the protein. Evidence has already been presented that the interaction of the side chain of a substrate or an inhibitor depends on van der Waals interaction.[33a,39,40] The ability of a substrate to inhibit carboxypeptidase is also apparently determined by the nature of the side chain at R.

Though inhibition by substrate may be interpreted as non-competitive with itself, the details of its action are not clear. The reaction scheme used in this derivation is, of course, not uniquely related to the conclusions. For instance, the same order in reactants results from the assumption of a mechanism in which 4 molecules of substrate reduce the value of k_0 for each center to one-half its maximum value. There is no additional evidence which favors either mechanism. For present purposes it is important to note that substrate inhibition does not occur in the range of substrate concentration used for determination of k_0 and K_m values. At [S] = 0.02 M, the highest concentration used for such determinations, 1% of the enzyme is inhibited by substrate. At [S] = 0.025 M, the percentage rises to 2.5% and the rise is extremely rapid at higher [S]. This rapid change is seen in a [S]/v *versus* [S] plot of the data of Figs. 4b, 6b, 8b. One straight line changes into another nearly straight line in a very small region of substrate concentrations. The straight line at low [S] is given by the uninhibited velocity expression, which is also the limiting case of the inhibited velocity expression (8) for low [S]. The second straight line no longer has a simple significance because of the complex dependence of slope on substrate concentration

$$\frac{S}{v} = \frac{[S] + K_m}{\frac{k_0 e}{2}\left((1 + \frac{1}{1 + [S]\,4K)_1}\right)} \qquad (8)$$

(39) E. L. Smith, *Federation Proc.*, **8**, 581 (1949).

(40) E. L. Smith and R. Lumry, *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 168 (1950).

However, if measurements were not made in the region free of inhibition, the second straight line might be mistaken for a correct description of the Michaelis–Menten scheme. The intercept and hence the value of K_m calculated in this manner would be very small, and in many cases negative, hence meaningless. A number of carboxylic acids have been found to inhibit carboxypeptidase[8,9,33a,39,40] Two of these, indoleacetic and hydrocinnamic, increase the solubility of the protein in aqueous solution at low ionic strength[41] at concentrations greater than 0.01 M. The effect appears to be non-specific and may be due to a detergent-like action of the inhibitor in shielding hydrophobic surfaces of the protein from water. General interaction of organic acids with proteins is now well-known. Luck and co-workers[42] have found a larger thermal stabilizing influence of short chain fatty acids on human serum albumin. In shielding the hydrophobic groups from water, inhibitors may cover essential protein groups at the site of catalysis or may stabilize a different configuration of the protein with lower intrinsic reactivity. Either mechanism could give the type of results observed in the substrate inhibition of carboxypeptidase. It is probable that substrate acts at four points of the protein (per enzymic site) to increase the solubility of the protein in water solution and to make stable a modified configuration of the protein with less catalytic ability than that of the "normal" form (which occurs in the absence of high concentrations of polar–nonpolar organic ions).

Studies to be presented[41] provide additional evidence that compounds like hydrocinnamic acid as well as inorganic ions like pyrophosphate can produce profound changes in the electrophoretic behavior of the protein with high order dependencies on the concentration of inhibiting agent. It is not as yet certain how these effects are related to that of substrate–enzyme interaction. However, the similarity between types and concentrations of substrate molecules and inhibitors makes a close relationship seem likely.

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(41) E. L. Smith, D. M. Brown and R. Lumry, unpublished observations.

(42) P. D. Boyer, G. A. Ballou and J. M. Luck, *J. Biol. Chem.*, **162**, 199 (1946); F. G. Lunt, G. A. Ballou, J. M. Luck and R. G. Rice, *ibid.*, **162**, 181 (1946).