α-N-Carbethoxy-DL-tyrosine Ethyl Ester.—To a solution of 100 g. of DL-tyrosine ethyl ester in 500 ml. of pyridine, maintained at 5–10°, was added dropwise and with stirring over a period of 1 hr., 58 g. of ethyl chlorocarbonate. The pyridine was removed *in vacuo*, the resulting sirup dissolved in 300 ml. of chloroform, the solution extracted with cold 1 *N* aqueous hydrochloric acid, the chloroform phase dried and the solvent removed *in vacuo* to give 91 g. of colorless prisms, m.p. 87.2–90.3°.

α-N-Carbethoxy-D-tyrosine Ethyl Ester.—α-Chymotrypsin, 75 mg., was added to a stirred solution of 75 g. of α-N-carbethoxy-DL-tyrosine ethyl ester in 500 ml. of methanol and 1400 ml. of water, maintained at 30–35° and containing sufficient phenol red indicator to follow visibly the *p*H of the reaction mixture. As the asymmetric hydrolysis proceeded, 5.46 g. of sodium hydroxide in 336 ml. of water was added at a rate sufficient to maintain the *p*H of the reaction mixture at *p*H 7–8. After 4 hr. the reaction was essentially complete and the volume of the solution was reduced by *ca.* 400 ml. by evaporation *in vacuo*. The residue was adjusted to *p*H 8.5 and extracted with four 200-ml. portions of chloroform. Evaporation of the chloroform phase gave a sirup which could not be crystallized.

α-N-Carbethoxy-D-tyrosinamide.—A sirupy preparation of α-N-carbethoxy-D-tyrosine ethyl ester obtained from the enzymatic resolution of 55 g. of α-N-carbethoxy-DL-tyrosine ethyl ester was dissolved in 150 ml. of absolute methanol and the solution saturated with anhydrous ammonia at 0–5°. The reaction mixture was allowed to stand at room temperature for 2 days, the excess ammonia and solvents removed by evaporation *in vacuo* and the residue allowed to crystallize. Recrystallization of the crude product from water gave 7.6 g. of α-N-carbethoxy-D-tyrosinamide, colorless needles, 156.6–158.1°, $[\alpha]^{25}D$ −19.4° (*c* 5% in methanol).

Anal. Calcd. for $C_{12}H_{16}O_4N_2$ (252): C, 57.1; H, 6.4; N, 11.1. Found: C, 57.4; H, 6.2; N, 11.1.

Enzyme Solutions.—Aqueous stock solutions of crystalline α-chymotrypsin were prepared daily and were kept at 4° between the intervals in which they were brought to 25° prior to introduction into the reaction mixtures.

Buffer Solutions.—THAM was recrystallized three times from distilled water prior to the preparation of stock solutions which were adjusted to the desired *p*H by titration with aqueous hydrochloric acid.

Formaldehyde.—Merck reagent grade formaldehyde (36–38% in water) was adjusted to *p*H 8 prior to its use in the formol titrations.

Ferric Chloride Solution.—The ferric chloride solution used for the spectrophotometric determination of α-N-acetyl-L-tyrosinhydroxamide was prepared as follows: 54.0 g. of reagent grade ferric chloride hexahydrate was dissolved in 500 ml. of water and 16.3 ml. of concd. hydrochloric acid. The solution was then made up to 1000 ml. with absolute methanol. This solution was turbid when first prepared but became clear after filtration through a Whatman No. 1 paper and standing for two days.

Enzyme Experiments.—All enzymatic hydrolyses were conducted at 25.0° in aqueous solutions containing a THAM–HCl buffer and in the manner described by Huang and Niemann.[9] The titrimetric method of analysis used in all experiments with α-N-carbethoxy-L-tyrosinamide was identical with that described by Huang and Niemann[9] and earlier by Iselin and Niemann.[36] The experiments involving the use of α-N-acetyl-L-tyrosinhydroxamide as a specific substrate were followed by a modification of the procedure of Hogness and Niemann.[28] In the present experiments, the absorbance, at any time *t*, of unhydrolyzed α-N-acetyl-L-tyrosinhydroxamide, as its ferric chloride complex, was determined at 515 mμ with a Beckman Model B Spectrophotometer. From the reaction mixtures at *p*H 7.6 and 0.27 *M* in the THAM component of the THAM–HCl buffer 1.0-ml. aliquots were withdrawn at pre-selected time intervals and delivered into 10-ml. G. S. volumetric flasks containing 1.0 ml. of the ferric chloride solution and 7.5 ml. of absolute methanol. The solutions were adjusted to 10.0 ml. with absolute methanol, mixed and the absorbance determined with the spectrophotometer set at 100% transmission for a blank, prepared in the same manner as above, but containing instead of 10 ml. of reaction mixture, 1.0 ml. of a solution consisting of enzyme, buffer and water. In the case of those experiments with $[S]_0 = 15$ to 40×10^{-3} *M* it was necessary to increase the dilution of the colored ferric complex in order to facilitate measurement. This was achieved by employing 25-ml. G. S. volumetric flasks and delivering the 1.0-ml. aliquot of the reaction mixture into 1.0 ml. of the ferric chloride solution and 22.5 ml. of absolute methanol and then making up to volume with absolute methanol.

Determination of *p*H-Optimum.—The experiments, which led to the data summarized in Fig. 1, were conducted in aqueous solutions at 25° and 0.02 *M* in the THAM component of a THAM–HCl buffer with [E] = 0.266 mg. protein-nitrogen per ml. of Armour preparation no. 10705, $[S]_0 = 10 \times 10^{-3}$ *M* and *t*, time of reaction, of 20 min.

(36) B. M. Iselin and C. Niemann, *J. Biol. Chem.*, **182**, 821 (1950).

PASADENA, CALIFORNIA

[CONTRIBUTION NO. 2263 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Effect of Various Salts on the α-Chymotrypsin-catalyzed Hydrolysis of Two Acylated α-Amino Acid Esters[1]

BY R. BRUCE MARTIN AND CARL NIEMANN[2]

RECEIVED OCTOBER 4, 1957

A detailed analysis has been made of the effect of sodium and calcium chlorides on the rate of the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate and of sodium, calcium and magnesium chlorides on the rate of the comparable reaction involving acetyl-L-valine methyl ester. Less extensive studies with seven additional salts also are reported.

It has been shown[3] that in the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate, in aqueous solutions at 25° and *p*H 7.90 ± 0.01 in the presence of sodium chloride, at concentrations of sodium chloride greater than 1 *M* the value of K_S'[4] is essentially constant but as the concentration of sodium chloride is decreased below 1 *M*, the value of K_S' begins to increase, slowly and then rapidly, and as the system approaches zero ionic strength the value of K_S' tends to become large. While the value of k_3' generally decreases with decreasing concention of sodium chloride, at concentrations below 1 *M* the value of k_3' decreases more rapidly than at concentrations above 1 *M* and as the system approaches zero ionic strength, the value of k_3' appears to become small. In this investigation the above studies have been extended to include ex-

(1) Supported in part by a grant from the National Institutes of Health, U. S. Public Health Service.

(2) To whom inquiries regarding this article should be sent.

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

(4) The symbols K_S' and k'_3 are used herein with reference to the dependence of the rate upon the initial specific substrate concentration and refer to apparent constants which may be composite in nature. However, for the purposes of this communication it is unimportant whether the constants are simple or complex.

amination of the effect of varying concentrations of sodium and calcium chlorides on the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate in aqueous solutions at 25.0° and *p*H 7.90 ± 0.01,[5] of varying concentrations of sodium, magnesium and calcium chlorides on the comparable hydrolysis of acetyl-L-valine methyl ester[6] and the relative effect of 1 *M* concentrations of lithium chloride, potassium chloride, sodium chloride, sodium bromide, sodium iodide, sodium nitrate, sodium acetate, magnesium chloride, calcium chloride and tetramethylammonium chloride and 0.5 and 1 *M* sodium sulfate on the values of K_S' and k_3' of the latter specific substrate.

The basic data relative to the effect of varying concentrations of sodium and calcium chlorides on the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate and of sodium, magnesium and calcium chlorides on the comparable reaction of acetyl-L-valine methyl ester are summarized in Table I. It should be noted that at each salt concentration the kinetics with respect to dependency upon the initial specific substrate concentration are described by equation 1 and in every case values of K_S' and k_3' were obtained by a least squares fit to the relation $([S_0][E]/v_0) = (K_S'/k_3') + ([S]_0/k_3')$.[7]

$$v_0 = d[P]/dt = k_3'[E][S]/(K_S' + [S]) \qquad (1)$$

The agreement between the experimentally determined values of v_0, evaluated by the empirical orthogonal polynomial procedure of Booman and Niemann[8,9] and those expected on the basis of a linear relationship between $[S]_0/v_0$ and $[S]_0$[7] is illustrated by representative data presented in Fig. 1.

Exploratory experiments involving prior incubation of the enzyme or the specific substrate with either sodium or calcium chloride produced no change in the rate of reaction, which otherwise was observed after adding an essentially salt free enzyme solution to the remainder of the initial reaction system. Therefore, it may be concluded that all components of the initial reaction system were in equilibrium with each other. At no time was any evidence obtained to suggest that the presence or absence of any of the salts employed in this study led to an irreversible process, *e.g.*, classical denaturation and total inactivation of the enzyme.[10]

The data given in Table I were examined with respect to possible linear relationships between log K_S' and log k_3' and ionic strength or its square root. In no case was such a relationship observed, and it appears reasonable to conclude that the data summarized in Table I cannot be explained in terms of a simple ionic strength effect.

TABLE I

DEPENDENCE OF K_S' AND k_3' UPON ADDED SALT[a]

Molarity	Molality	Activity	K_S'[b]	k_3'[c,d]
Methyl hippurate–sodium chloride[e]				
0.020	0.020	0.017	7.6 ± 0.4	2.9
.080	.080	.063	5.3 ± .4	3.3
.100	.100	.078	5.1 ± .3	3.4
.150	.151	.114	3.7 ± .3	3.5
.20	.201	.148	3.5 ± .2	3.6
.50	.504	.343	2.9 ± .2	4.2
1.00	1.02	.670	2.8 ± .1	4.5
1.50	1.55	1.01	2.6 ± .1	4.7
2.00	2.08	1.39	2.8 ± .1	4.9
2.70	2.86	2.01	2.7 ± .1	5.2
Methyl hippurate–calcium chloride[f]				
0.20	0.201	0.095	4.5 ± 0.2	4.9
.40	.404	.181	4.0 ± .2	5.2
.60	.609	.276	3.1 ± .1	5.3
.90	.919	.448	2.4 ± .1	5.5
1.20	1.24	.678	2.2 ± .1	6.2
1.60	1.67	1.12	1.8 ± .1	6.3
Acetyl-L-valine methyl ester–sodium chloride[g]				
0.10	0.100	0.078	108 ± 9	2.2
.30	.302	.214	81 ± 4	2.3
.50	.504	.343	73 ± 3	2.4
.80	.812	.537	52 ± 3	2.5
1.00	1.02	.670	44 ± 2	2.6
1.25	1.28	.838	39 ± 2	2.6
1.50	1.55	1.01	35 ± 2	2.7
2.00	2.08	1.39	25 ± 1	2.7
2.70	2.86	2.01	17 ± 1	2.8
Acetyl-L-valine methyl ester–calcium chloride[h]				
0.20	0.201	0.095	87 ± 6	3.0
.40	.404	.181	73 ± 4	3.6
.60	.609	.276	62 ± 3	3.8
.80	.816	.385	53 ± 2	3.9
1.0	1.03	.518	51 ± 2	4.2
1.5	1.56	.988	49 ± 2	5.5
2.0	2.11	1.81	46 ± 2	5.9
Acetyl-L-valine methyl ester–magnesium chloride[i]				
0.10	0.101	0.054	84 ± 7	2.2
.30	.312	.149	48 ± 3	2.1
.50	.534	.258	36 ± 3	2.1
.75	.829	.439	32 ± 2	2.3
1.00	1.15	.705	28 ± 2	2.3
1.50	1.86	1.78	28 ± 2	3.0

[a] In aqueous solutions at 25.0° and *p*H 7.90 ± 0.01. [b] In units of 10^{-3} *M*. [c] In units of 10^{-3} *M*/min./mg. protein-nitrogen per ml. [d] ±0.1. [e] Values of K_S' and k_3' based upon 8 to 11 experiments with $[S]_0$ varied from 5 to 30 × 10^{-3} *M* and [E] from 0.035 to 0.15 mg. protein-nitrogen per ml. [f] Values of K_S' and k_3' based upon 9 to 10 experiments with $[S]_0$ varied from 5 to 30 × 10^{-3} *M* and [E] from 0.035 to 0.15 mg. protein-nitrogen per ml. [g] Values of K_S' and k_3' based upon 7 to 12 experiments with $[S]_0$ varied from 10 to 60 × 10^{-3} *M* and [E] from 0.050 − 0.15 mg. protein-nitrogen per ml. [h] Values of K_S' and k_3' based upon 8 to 10 experiments with $[S]_0$ varied from 10 to 55 × 10^{-3} *M* and [E] from 0.045 to 0.15 mg. protein-nitrogen per ml. [i] Values of K_S' and k_3' based upon 7 to 9 experiments with $[S]_0$ varied from 10 to 50 × 10^{-3} *M* and [E] from 0.050 to 0.15 mg. protein-nitrogen per ml.

Alternatively, let us consider the reactions represented by equations 2 to 7 inclusive.

There are two general methods that may be used for a solution of the situation represented by equa-

(5) T. H. Applewhite, R. B. Martin and C. Niemann, THIS JOURNAL, **80**, 1457 (1958).

(6) T. H. Applewhite, H. Waite and C. Niemann, *ibid.*, **80**, 1465 (1957).

(7) H. Lineweaver and D. Burk, *ibid.*, **56**, 658 (1934).

(8) K. A. Booman and C. Niemann, *ibid.*, **78**, 3642 (1956).

(9) In those cases where the recorder traces of extent of reaction *vs.* time were linear in the interval from 0 to 8 min., v_0 was evaluated directly from the slopes of the traces.

(10) There are two salts, *i.e.*, sodium perchlorate and sodium thiocyanate, that may not fall in this category. Because their use resulted in enzyme blanks[8] an order of magnitude greater than those otherwise observed and because the rates observed in their presence were erratic no attempt was made to use them in this study.

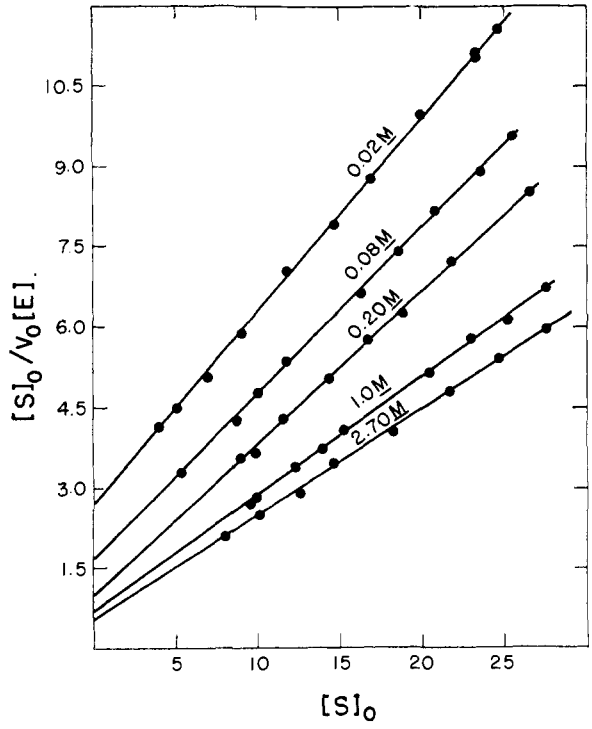

Fig. 1.—α-Chymotrypsin–methyl hippurate–sodium chloride: $[S]_0/v_0[E]$ in units of min./mg. protein-nitrogen per ml., $[S]_0$ in units of 10^{-3} M; M molarity of sodium chloride.

tions 2 to 6 inclusive, *i.e.*, a steady-state treatment where $K_1 = (k_{-1} + k_6)/k_1$, $K_2 = k_{-2}/k_2$, $K_3 = (k_{-3} + k_5)/k_3$ and $K_4 = (k_{-4} + k_5)/k_4$ and the less exact equilibrium solution where $K_1 = k_{-1}/k_1$,

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \quad (2)$$

$$E + M \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E' \quad (3)$$

$$ES + M \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E'S \quad (4)$$

$$E' + S \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E'S \quad (5)$$

$$E'S \xrightarrow{k_5} E' + P \quad (6)$$

$$ES \xrightarrow{k_6} E + P \quad (7)$$

$K_2 = k_{-2}/k_2$, $K_3 = k_{-3}/k_3$ and $K_4 = k_{-4}/k_4$. It has been shown by Segal, Kachmar and Boyer[11] that the equilibrium solution of equations 2 to 6 inclusive leads to equation 8, where in this case [M] is the mean ion activity of the added salt, v_0 the ini-

$$[S]_0/v_0 = ([S]_0/V)(1 + (K_3/[M])) + (1/V)(K_4 + (K_1K_3/[M])) \quad (8)$$

tial velocity and V the maximum limiting velocity at high values of $[S]_0$ and [M]. Because of the free energy conditions of a cyclic process $K_1K_3 = K_2K_4$.

It will be seen that equation 8 predicts that plots of $[S]_0/v_0$ *vs.* $[S]_0$ at constant [M] and $[M]/v_0$ *vs.* [M] at constant $[S]_0$ will be linear. The complete steady-state solution of equations 2 to 6 inclusive[12] is extremely complex and predicts that plots of $[S]_0/v_0$ *vs.* $[S]_0$ and $[M]/v_0$ *vs.* [M] will not be linear. While plots of $[M]/v_0$ *vs.* [M] based upon data summarized in Table I are not linear for the higher values of [M] those of $[S]_0/v_0$ *vs.* $[S]_0$ are. Since both are linear at lower values of [M] and because the equation resulting from the complete steady-state solution of reactions 2 to 6 inclusive[12] is essentially intractable for the problem at hand, the equilibrium procedure leading to equation 8 was employed in this study.

For a reaction represented by equation 9 we may,

$$E + S \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} ES \xrightarrow{k_3'} E + P \quad (9)$$

under certain conditions[13] write equation 10, where $V' = k_3'[E]$. Thus, for the case under considera-

$$([S]_0/v'_0) = ([S]_0/V') + (K_S'/V') \quad (10)$$

tion the slope $1/V' = 1/V(1 + (K_3'/[M]))$ and $[M]/k_3' = ([M]/k_5) + (K_3/k_5)$. Therefore, a plot of $[M]/k_3'$ *vs.* [M] should give a straight line of slope $1/k_5$ and intercept K_3/k_5. Similarly, from the relation $K_S' = V'(K_S'/V')$, *i.e.*, the product of V' and the intercept K_S'/V', it follows that $K_S' = V'\{1/V(K_4 + (K_1K_3/[M]))\}$ and $([M]K_S'/k_3') = ([M]K_4/k_5) + (K_1K_3/k_5)$. Thus, a plot of $([M]\cdot K_S'/k_3')$ *vs.* [M] should be linear with a slope of K_4/k_5 and intercept of K_1K_3/k_5.

It was noted previously, *vide post*, that plots of $[S]_0/v_0$ *vs.* $[S]_0$ were linear. The extent to which plots of $[M]/k_3'$ *vs.* [M] and $([M]Ks/k_3')$ *vs.* [M] were also linear is illustrated by the representative example given in Fig. 2. In general, the plots of

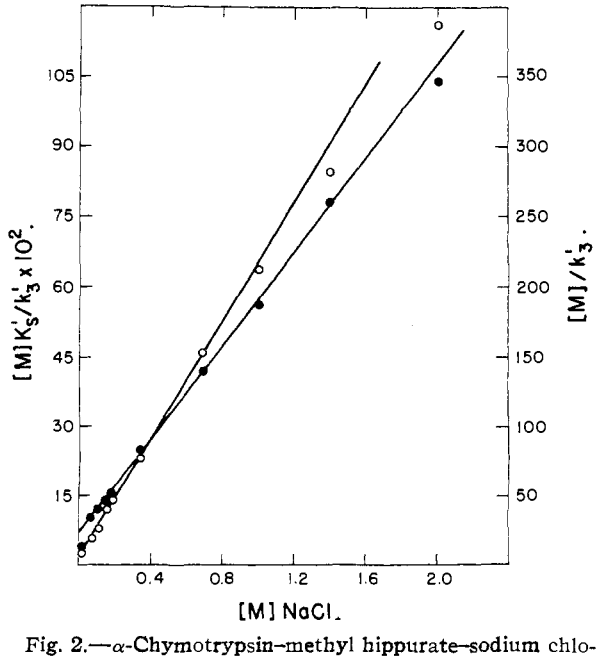

Fig. 2.—α-Chymotrypsin–methyl hippurate–sodium chloride. Closed circles, $[M]K_s'/k_3' \times 10^2$; open circles, $[M]/k_3'$; [M] mean ion activity of sodium chloride.

(11) H. L. Segal, J. F. Kachmar and P. D. Boyer, *Enzymologia*, **15**, 187 (1952).

(12) L. L. Ingraham and B. Makower, *J. Phys. Chem.*, **58**, 266 (1954).

(13) R. J. Foster and C. Niemann, THIS JOURNAL, **77**, 1886 (1955).

$([M]K_S'/k_3')$ *vs.* [M] were linear over the range of salt concentrations studied but occasionally tended to depart from linearity at the higher salt concentrations, *cf.*, Fig. 2. This was most noticeable with acetyl-L-valine methyl ester and sodium chloride at concentrations above 1 *M*. The plots of $[M]/k_3'$ *vs.* [M] generally were of the character of the example given in Fig. 2. The observed departure from linearity at high salt concentrations may be due to a superimposed ionic strength effect which might tend to cancel out in plots involving K_S'.

A subjective estimate of the slopes and intercepts of the $[M]/k_3'$ *vs.* [M] and $([M]K_S'/k_3')$ *vs.* [M] plots led to the values of K_1, K_2, K_3, K_4 and k_5 summarized in Table II.

TABLE II

VALUES OF K_1, K_2, K_3, K_4 AND k_5[a]

Specific substrate	Salt	K_1[b]	K_2[b]	K_3[b]	K_4[b]	k_5[c]
Methyl hippurate	NaCl	1.2	14	2.8	0.25	4.8
Methyl hippurate	$CaCl_2$	1.5	20	2.5	0.19	6.1
Acetyl-L-valine methyl ester	NaCl	19	20	3.8	3.6	2.6
Acetyl-L-valine methyl ester	$CaCl_2$	19	21	4.1	3.7	4.4
Acetyl-L-valine methyl ester	$MgCl_2$	18	21	2.6	2.2	2.3

[a] Based upon equations 2 to 8 inclusive and the corresponding definitions. [b] In units of 10^{-2} *M*. [c] In units of 10^{-3} *M*/min./mg. protein-nitrogen per ml.

The constant K_1 should be independent of the nature of the added salt and dependent only upon the nature of the specific substrate. It is also the limiting value of K_S' that should be observed in the absence of any added salt if it were experimentally possible to extend observations made at finite concentrations to zero salt concentration. It will be seen that the values of K_1 given in Table II are essentially independent of the nature of the added salt and are in reasonable agreement with the requirement that they be dependent only upon the nature of the specific substrate.

The constant K_2 should be independent of the nature of the specific substrate and may be dependent only on the nature of the added salt. The values of K_2 summarized in Table II appear to be in fair agreement with the first requirement since the value of K_2 for methyl hippurate–sodium chloride while lower than the rest is of the same order of magnitude as the others. The apparent lack of dependence of K_2 upon the nature of the added salt, *cf.*, Table II, suggests that the nature of the added salt may not be as important as the resultant mean ion activity associated with its presence. It is this result along with other considerations, *vide ante*, that led us to equation 3 rather than one of the form $E + M \rightleftarrows EM$.

There is little that can be said with reference to the constant K_3 other than that the values given in Table II suggest that this constant may be dependent upon the nature of the specific substrate and in some cases also may be dependent upon the nature of the added salt.

The constant K_4 is the limiting value of K_S' at high salt concentrations. In view of the fact that with both K_3 and K_4 the values observed in the presence of magnesium chloride appear to be low relative to those observed in the presence of either sodium or calcium chloride, it should be noted that at *p*H 7.90 magnesium chloride possesses considerable buffering capacity which could lead to erroneous values of v_0.[14] Whether this situation is or is not responsible for the results summarized in Table II is not known.

It has been shown by Segal, Kachmar and Boyer[11] that several cases other than the one considered above will give rate equations of the form required for a reasonable description of the experimental data. Two of these cases are the steady-state solutions of equations 2, 4 and 6 and of equations 3, 5 and 6. However, these latter two cases would allow no deviation from linearity as was actually observed in some of the $([M]K_S'/k_3')$ *vs.* [M] plots. The equilibrium treatment of equations 2 to 6 inclusive may be considered to be the limiting case. The deviations from linearity noted above could be ascribed to the necessity of introducing the steady-state solution which, as noted previously, is very complex. In all of the alternative cases the interpretation of the evaluated constants was less satisfactory than that arising from the equilibrium solution of equations 2 to 6 inclusive and which was used to obtain the values given in Table II.

The cases considered so far are examples of so-called essential activation,[11] *i.e.*, the presence of a salt is necessary for the complete reaction and the reaction represented by equation 7 does not occur. In the $([M]K_S'/k_3')$ *vs.* [M] plots the point corresponding to the very lowest value of [M] was often low as may be seen in Fig. 2. This anomaly could arise from a slow decomposition of ES, assuming that reaction 7 occurs. However, the evaluation of this rate constant, *i.e.*, k_6, would be very difficult.

The inclusion of equation 7 into the over-all representation requires consideration of several cases of so-called non-essential activation.[11] Both the equilibrium and steady-state solutions of equations 2, 4, 6 and 7 give linear $[S]_0/v_0$ *vs.* $[S]_0$ plots and non-linear $[M]/v_0$ *vs.* [M] plots.[11] The latter condition may appear to be satisfied in the present study since the $([M]K_S'/k_3')$ *vs.* [M] and $[M]/k_3'$ *vs.* [M] plots are not always linear particularly at high values of [M]. However, in the limit where $k_6K_2 \ll k_5[M]$ only the steady-state treatment would be compatible with the experimental observations and in this case the reciprocal of the slope of the $[M]/k_2'$ *vs.* [M] plot would be k_1 which appears to be a less satisfactory interpretation than the one leading to k_5 and used above.

A more acceptable interpretation of the experimental results, and one also involving non-essential activation,[11] is given by an equilibrium treatment of equations 2 to 7 inclusive,[11] which predicts a linear $[S]_0/v_0$ *vs.* $[S]_0$ plot and a non-linear $[M]/v_0$ *vs.* [M] plot as has been observed in this study for the higher values of [M]. When equation 7 becomes insignificant this case reduces to that considered in detail previously. The steady-state treatment of equations 2 to 7 inclusive requires that both the $[S]_0/v_0$ *vs.* $[S]_0$ and $[M]/v_0$ *vs.* [M] plots be non-linear.[11]

While it has not been possible to establish unequivocally one particular mechanism, it appears

(14) C. F. Jacobsen, J. Leonis, K. Linderstrom-Lang and M. O. Heson, "Methods of Biochemical Analysis," Vol. 4, Interscience Publishers, New York, N. Y., 1957, p. 190.

that a representation based upon equations 2 to 7 inclusive affords the most satisfactory interpretation and that the equilibrium solution for this case is adequate. In the absence of procedures suitable for the evaluation of k_6, the equilibrium solution of the representation based upon equations 2 to 6 inclusive may be taken as the best available but admittedly approximate description of the influence of several added salts on the α-chymotrypsin-catalyzed hydrolysis of several representative ester type neutral specific substrates.

In a previous communication[3] the dependence of K_S' and k_3', for methyl hippurate, upon added sodium chloride was represented by two hyperbolic curves. The curve for K_S' may be described by the equation $[M](K_S'/k_3' - K_4/k_5) = K_1K_3/k_5$ and as indicated previously K_4 is the limiting value of K_S' at high salt concentrations. The value of K_4 given in Table II, *i.e.*, 2.5×10^{-3} *M* is in good agreement with the value of 2.7×10^{-3} *M* observed at 2.70 *M* sodium chloride,[3] *cf.*, Table I. From the relation $k_3' = k_5[M]/([M] + K_3)$ it follows that the curve given for k_3 has the form $[M](k_5 - k_3') = k_3'K_3$ with the limiting value of k_3' at high values of [M] being k_5. The value of k_5 given in Table II, *i.e.*, 4.8×10^{-3} *M*/min./mg. protein–nitrogen per ml. is in reasonable agreement with the value of 5.19, in the same units, observed at 2.70 *M* sodium chloride,[3] *cf.*, Table I.

It has been observed previously that in the α-chymotrypsin-catalyzed hydrolysis of chloroacetyl-L-tyrosinamide in aqueous solutions 0.05 to 1.50 *M* in sodium chloride,[15] of acetyl-L-tyrosinamide in aqueous solutions 0.10 to 0.40 *M* in sodium phosphate[16] and of α-N-nicotinyl-L-tyrosinhydrazide in aqueous solutions 0.01 to 0.90 *M* in the amine component of a THAM–HCl buffer[17] the value of K_S' was constant within the limits of experimental error whereas the value of k_3' increased with increasing concentration of the added salt which in two of the three cases was also functioning as a buffer.

For the equilibrium solution of the representations involving equations 2 to 6 inclusive and 2 to 7 inclusive and which were considered above, we may write $K_S' = (K_4[M] + K_2)/([M] + K_3)$. For K_S' to be constant as [M] varies at least one of the following alternatives must be obeyed. First, $K_3 = K_2$ and $K_S' = K_4 = K_1$; second, $[M] >> K_3$ and K_2 and hence $K_S' = K_4$; and third, K_3 and $K_2 >> [M]$ and therefore $K_S' = K_1$.

Some choice may be made from amongst the above three alternatives if other considerations are introduced. The second alternative, *i.e.*, $[M] >> K_3$ and K_2 would predict, for the equilibrium solution of equations 2 to 6 inclusive and 2 to 7 inclusive that k_3' would not vary with [M]. Since this is not observed the second alternative may be excluded.

The first alternative, *i.e.*, $K_3 = K_2$, predicts that the $[M]/k_3'$ *vs.* [M] plot employed earlier would be linear for the equilibrium solution of equations 2 to 6 inclusive. For the equilibrium solution of equations 2 to 7 inclusive the situation would be more complex since both k_5 and k_6 would have to be considered.

The third alternative, *i.e.*, K_3 and $K_2 >> [M]$ predicts that k_3' would not vary with [M] for the equilibrium solution of equations 2 to 7 inclusive, but for the equilibrium solution of equations 2 to 6 inclusive the prediction would be the same as that for the first alternative, *i.e.*, a plot of $[M]/k_3'$ *vs.* [M] would be linear. While it is not possible to decide between the first and third alternatives, it is difficult to understand why K_3 and $K_2 >> [M]$ for two amide and one hydrazide type of specific substrates and this is not so for two ester type of specific substrates, *cf.*, Table II.

In the preceding discussion care was taken to use the term added salt rather than to ascribe the effects noted to a particular cation. The reason for this precaution may be seen from the data given in Table III where values of K_S' and k_3', obtained for the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-valine methyl ester in aqueous solutions at 25.0° and *p*H 7.90 ± 0.01 and 1.0 *M* in a number of different salts, have been summarized.

TABLE III

VALUES OF K_S' AND k_3' FOR ACETYL-L-VALINE METHYL ESTER IN 1.00 *M* SOLUTIONS OF VARIOUS SALTS[a]

Salt	Activity	K'_S[e]	k_3'[f,g]
Lithium chloride		52 ± 2	2.3
Sodium chloride	0.670	44 ± 2	2.4
Sodium bromide	.705	52 ± 2	2.3
Sodium iodide		60 ± 3	2.0
Sodium nitrate	.566	49 ± 2	2.1
Sodium acetate		53 ± 3	2.7
Sodium sulfate[c]	.135	38 ± 1	2.5
Sodium sulfate[d]	.206	17 ± 1	2.7
Potassium chloride	.623	48 ± 2	2.3
Tetramethylammonium chloride[b]		90 ± 6	2.2
Calcium chloride	.518	51 ± 2	4.2
Magnesium chloride	.705	28 ± 2	2.3

[a] In water at 25.0° and *p*H 7.90 ± 0.01 unless otherwise stated. [b] Reaction mixture titrated with tetramethylammonium hydroxide, tetramethylammonium and hydronium ion only cations in reaction system. [c] 0.50 *M* sodium sulfate. [d] 1.00 *M* sodium sulfate. [e] In units of 10^{-3} *M*. [f] In units of 10^{-3} *M*/min./mg. protein-nitrogen per ml. [g] ±0.1.

The data for sodium, calcium and magnesium chlorides, which were taken from Table I and which are approaching the limiting values of K_4 and k_5 given in Table II emphasize the behavior of magnesium chloride in causing a diminution in the value of K_S', or K_4, with no significant change in the value of k_3', or k_5, relative to the values observed in the presence of sodium chloride. Since a similar behavior is noted with respect to sodium sulfate, *cf.*, Table III, it is not surprising that Jandorf[18] found that the addition of magnesium sulfate caused a marked increase in the rate of hydrolysis of acetyl-L-tyrosine ethyl ester which now appears to be due to the effectiveness of both magnesium and sulfate ion in depressing the values of K_S', or K_4, and thereby increasing the rate. In contrast, the effect produced by calcium chloride appears to be due almost entirely to an enhancement in the value of k_3',

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or k_5, with little or no effect on the value of K_S', or K_4. It is unfortunate that the insolubility of calcium sulfate does permit observations of the expected increase in rate arising from an increase in k_5 and a decrease in K_4.

While it is tempting to speculate about the results obtained with lithium, sodium, potassium and tetramethylammonium chlorides and with sodium chloride, bromide, iodide, nitrate and acetate, *cf.*, Table III, it is clear that such speculation must be deferred until comparisons can be made of systems possessing uniform mean ion activities. Such systems and those involving binary mixtures of constant mean ion activity are being investigated.

It is appropriate that some reference be made to the possible nature of the species E'. It is known that α-chymotrypsin may associate to form a dimer at high ionic strengths or a polymer at low ionic strengths.[19-27] In view of the fact that all studies were conducted at *p*H 7.90 ± 0.01 and at enzyme concentrations in the range from 0.035 to 0.15 mg. protein–nitrogen per ml., it is unlikely that a significant amount of dimer was present and we can exclude the possibility that in the transformation $E + M \rightleftarrows E'$, E is the dimer and E' the monomer. The absence of any information relative to the catalytic properties of α-chymotrypsin polymer does not permit us to be as positive about rejecting E as α-chymotrypsin polymer. However, since the experiments with the exception of two points in the case of methyl hippurate–sodium chloride were conducted at values of $M > 0.10$, it does not appear likely that the reaction $E + M \rightleftarrows E'$ can be interpreted as a transformation of polymer to monomer. The two remaining alternatives are that E' is actually EM or that E and E' are different forms of monomeric α-chymotrypsin. The lack of dependence of K_2 upon the nature of the salt suggests that EM is not involved but that in proceeding from an environment of low mean ion activity the conformation of monomeric E is altered to produce new conformational species E' that are more effective catalysts than is E.

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Experimental

The general procedure has been described previously.[5] The two specific substrates were prepared as before.[5,6] All experiments were performed in chemically unbuffered solutions using a *p*H-Stat which poised the reaction systems at *p*H 7.90 ± 0.01 at 25.0°. The enzyme preparation was Armour no. 234. The concentration of enzyme was varied from 0.035 to 0.15 mg. protein–nitrogen per ml., the lower concentrations of enzyme being employed at the higher salt concentration and *vice versa*. This was done to limit possible consequences of dimerization at the higher salt concentrations. Other pertinent details are given in Table I. The upper limits used for calcium chloride and magnesium chloride were determined by the precipitation of enzyme at higher concentrations. Both the calcium and magnesium chlorides were reagent samples stated to contain 99% calcium or magnesium chloride. It was necessary to use mean ion activities in order to interpret the data meaningfully. The values of the activity coefficients of sodium and potassium chloride were interpolated from those given by Stokes and Levien.[28] The activity coefficients of calcium and magnesium chlorides and of sodium sulfate were interpolated from those reported by Stokes.[29] Those of sodium bromide and sodium nitrate were interpolated from the data given by Harned and Owen.[30] The observed initial velocities were corrected for an enzyme blank, evaluated for each salt concentration, and for a blank arising from the hydrolysis of the specific substrate in the absence of enzyme, which also was evaluated for each salt concentration.

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Studies on Polypeptides. X. The Synthesis of a Pentapeptide Corresponding to an Amino Acid Sequence Present in Corticotropin and in the Melanocyte Stimulating Hormones[1]

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A synthesis of the pentapeptide histidylphenylalanylarginyltryptophylglycine (L,L,L,L) is described. The synthetic product behaved as a single component when analyzed by paper chromatography in two different solvent systems. The behavior of the pentapeptide toward leucine aminopeptidase and trypsin, respectively, was investigated. The former enzyme converted the peptide into an equimolar mixture of the constituent amino acids, and the latter cleaved the arginine-tryptophan bond with the formation of histidylphenylalanylarginine and tryptophylglycine. These results provided conclusive evidence for the stereochemical homogeneity of the synthetic product.

In connection with our studies of synthetic polypeptides corresponding to amino acid sequences which are common to corticotropin and to the

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(2) The name of this author was misspelled in communication IX of this series (see ref. 6).