[CONTRIBUTION NO. 2239 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The α-Chymotrypsin-catalyzed Hydrolysis of Methyl Hippurate in Aqueous Solutions at 25° and *p*H 7.9, its Inhibition by Indole and its Dependence upon Added Non-aqueous Solvents[1]

BY THOMAS H. APPLEWHITE, R. BRUCE MARTIN AND CARL NIEMANN[2]

RECEIVED AUGUST 19, 1957

The kinetics of the initial stages of the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate in aqueous solutions at 25° and *p*H 7.90 and 0.02 *M* in sodium chloride have been determined. The inhibition of the above reaction by indole has been investigated and a previous interpretation of this latter reaction has been confirmed. A study of the effect of added non-aqueous solvents upon the kinetics of the first reaction has shown that with methanol, *t*-butyl alcohol, acetonitrile, dimethylformamide, dimethylacetamide and formaldehyde the value of k_3' decreases and that of K_S' increases with increasing concentration of the non-aqueous component. With acetone, methyl ethyl ketone and dioxane the value of K_S' has a similar dependency but the value of k_3' remains constant. A possible interpretation of the effects observed in aqueous-acetone and aqueous-dioxane has been proposed.

The kinetics of the initial stages of the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate in aqueous solutions at 25° and *p*H 7.9 ± 0.1 were first determined by Huang and Niemann[3] for systems 0.02 *M* in the THAM[4] component of a THAM–HCl buffer. It was concluded[3] that the dependence of the initial rate upon the initial specific substrate concentration was described by equation 1 when $K_S'^{5} = 8.5$ and $k_3'^{5} = 2.2$. In a subsequent communication[6] the primary data obtained by the above investigators[3] were re-evaluated by procedures more objective than those used previously and values of $K_S' = 6.5 \pm 0.5$ and $k_3' = 2.2 \pm 0.2$ were obtained.

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

With the development of the so-called *p*H-Stat[7,8] it became possible to examine the above basic reaction system in a more convenient manner and in the absence of conventional buffers. Since the system α-chymotrypsin–methyl hippurate, possesses a reactivity comparable to several systems involving an amide type of specific substrate,[6] can be examined in aqueous as well as in aqueous–non-aqueous media and provides an example of a system containing a bifunctional specific substrate,[3] which can be employed in studies involving the ternary interaction of the enzyme with a monofunctional competitive inhibitor,[9] it was decided to investigate the system α-chymotrypsin–methyl hippurate in aqueous solutions at 25° and *p*H 7.90 ± 0.01 and 0.02 *M* in sodium chloride in order to establish a point of reference for subsequent studies.

Six series of experiments were performed and the results obtained in each are summarized in Table I. The first series was exploratory in nature and led to values of K_S' and k_3', obtained from a $1/v_0$ *vs.* $1/[S]_0$ plot,[10] which were used to establish optimal experimental conditions for subsequent evaluations.

In the second series of experiments the separate evaluation of the primary data by the empirical orthogonal polynomial procedure of Booman and Niemann[11,12] and by the procedure of the same authors[13] based upon definite integration of equation 1 gave values of v_0 which demonstrated that the variability arising from a difference in evaluation procedures is comparable with that associated with duplicate experiments evaluated by either one of the two procedures, *cf.*, Table II. The agreement between the two sets of values of K_S' and k_3' not only supports the choice of equation 1 as the correct rate equation in the second of the two evaluation procedures[11,13] but also establishes the validity of this equation for the evaluation of K_S' and k_3'. However, the agreement noted does not imply that values of K_S' and k_3' so obtained are necessarily correct since the presence of a systematic error would remain undetected.[11]

In both the second and third series of experiments specific substrate stock solutions adjusted to *p*H 7.90 ± 0.01 were employed. However, the order of their use in the third series was the reverse of that of the second. The fact that the value of K_S' obtained from the third series was significantly lower than that obtained from the second, suggested that a systematic error was to be found in a non-enzyme catalyzed hydrolysis of the specific substrate prior to its introduction into the reaction mixture. Therefore, the non-enzyme catalyzed hydrolysis of methyl hippurate in aqueous solutions at 25° and *p*H 7.90 ± 0.01 was examined at values of $[S]_0 = 8$ and 16×10^{-3} *M* and a value of $k = 1 \times 10^{-4}$ *M*/min. was obtained. From the relation $[S]_{0\ cor} = [S]_0\, e^{10^{-4}\ \text{min.}^{-1}\ t}$, with t being the elapsed time between the preparation of a given stock solution and its use, a set of corrected values of $[S]_0$ for the second and third series was computed and used for a separate evaluation of K_S' and k_3'.

When any one of the three plots[10,14] based upon equation 1 are employed for the evaluation of K_S and k_3, it can be shown that $K_S^* < K_S$ and $k_3^* <$

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

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

(3) H. T. Huang and C. Niemann, THIS JOURNAL, **74**, 4634 (1952).

(4) Tris-(hydroxymethyl)-aminomethane.

(5) All values of K_S' given in this communication are in units of 10^{-3} *M* and all values of k_3' in units of 10^{-3} *M*/min./mg. protein-nitrogen per ml.

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

(7) C. F. Jacobsen and J. Leonis, *Compt. rend. trav. lab. Carlsberg, Ser. Chim.*, **27**, 333 (1951).

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

(12) R. T. Birge, *Rev. Mod. Phys.*, **19**, 298 (1947).

(13) K. A. Booman and C. Niemann, THIS JOURNAL, **77**, 5733 (1955).

(14) G. S. Eadie, *J. Biol. Chem.*, **146**, 85 (1942).

TABLE I

VALUES OF K_S' AND k_3' FOR METHYL HIPPURATE[a]

Series	No. expts.	[E][b]	$[S]_0$[b]	t[d]	t[e]	K_S'[f]	k_3'[g]
1	17	0.140	4–24[h]	1–2	1	7.7[i]	3.3[i]
2	12	.150	4–24[h,j]	0–8	1	8.3 ± 0.4[k,l]	3.37 ± 0.08[k,l]
2a	..	...	..	..	..	8.5 ± 0.2[m,l]	3.37 ± 0.04[m,l]
3	12	.150	4–24[h,n]	0–24	3	6.86 ± 0.16[k,l]	3.13 ± 0.03[k,l]
2,3	..	...	..	..	..	6.8[k,o,p]	3.2[k,o,p]
4	12	.150	4–24[h]	0–16	2	6.57 ± 0.45[k,l]	3.04 ± 0.09[k,l]
5	12	.150	8–20[q]	0–24	3	6.61 ± 0.86[k,l]	2.90 ± 0.16[k,l]
6	11	.150	4–24[h]	0–8	1	7.55 ± 0.30[k,l,r]	2.91 ± 0.05[k,l,r]

[a] For the system involving α-chymotrypsin in aqueous solutions at 25° and *p*H 7.90 ± 0.01 and 0.02 *M* in sodium chloride. [b] In units of mg. protein-nitrogen per ml. [c] In units of 10^{-3} *M*. [d] Total time of observation in min. [e] Uniform time interval between observations in min. [f] In units of 10^{-3} *M*. [g] In units of 10^{-3} *M*/min./mg. protein-nitrogen per ml. [h] Six *ca.* equally spaced concentrations within this interval. [i] Obtained from a subjective estimate of the slope and intercept of a $1/v_0$ *vs.* $1/[S]_0$ plot.[10] [j] Specific substrate stock solutions adjusted to *p*H 7.90 ± 0.01 and allowed to stand for periods of from 0.5 to 11.25 hr. prior to use in the order from the highest to the lowest concentration. [k] v_0 evaluated by the orthogonal polynomial procedure.[11] [l] K_S' and k_3' evaluated by a least squares fit to the equation $([S]_0[E]/v_0) = (K_S'/k_3') + ([S]_0/k_3')$.[10] [m] v_0 evaluated by procedure based upon definite integration of equation 1.[13] [n] Same as *j* except stock solutions used in the order from the lowest to the highest concentration. [o] $[S]_0$ corrected for hydrolysis prior to initiation of enzyme catalyzed reaction. [p] Obtained from a subjective estimate of the slope and intercept of a $[S]_0/v_0$ *vs.* $[S]_0$ plot.[10] [q] Four equally spaced concentrations within this interval. [r] Extent of reaction corrected for an enzyme blank.

k_3, when K_S^* and k_3^* are the values of the constants corrected for a non-enzyme catalyzed hydrolysis of the specific substrate proceeding simultaneously with the enzyme catalyzed reaction and when K_S and k_3 are the values not so corrected. It also can be shown that $K_S^{**} < K_S$ and $k_3^{**} = k_3$, when K_S^{**} and k_3^{**} are the values that have been corrected for a non-enzyme catalyzed hydrolysis of the specific substrate occurring prior to the initiation of the enzyme catalyzed reaction. The experimental conditions that were employed in the second series would tend to maximize an error arising from a non-enzymatic hydrolysis of the specific substrate occurring prior to the initiation of the enzyme catalyzed reaction and those in the third series would tend to minimize such an effect. Since the values of $K_S' = 8.3 \pm 0.4$ and $k_3' = 3.37 \pm 0.08$ obtained from the second series, the values of $K_S' = 6.86 \pm 0.16$ and $k_3' = 3.13 \pm 0.03$ from the third series and the values of $K_S' = 6.8$ and $k_3' = 3.2$ from the corrected data of the second and third series are in the order expected, it may be concluded that the difference noted in the values of K_S' and k_3' from the second and third series is due to a non-enzyme catalyzed hydrolysis of the specific substrate prior to the initiation of the enzyme catalyzed reaction.

TABLE II

COMPARISON OF VALUES OF INITIAL VELOCITIES OBTAINED BY TWO DIFFERENT EVALUATION PROCEDURES

$[S]_0$[a]	P_m[b]	v_0[c]	v_0[d]	$[S]_0/v_0$[e]	$[S]_0/v_0'$[e,f]	v_0'[g]
4	3	1.63	0.02	24.54	24.85	1.61
4	3	1.69	.02	23.67	24.50	1.63
8	3	2.51	.03	31.87	32.15	2.49
8	3	2.52	.05	31.75	32.80	2.44
12	3	2.99	.01	40.13	40.70	2.95
12	3	2.98	.04	40.27	40.70	2.95
16	3	3.29	.06	48.63	48.70	3.28
16	3	3.23	.02	50.47	49.40	3.24
20	3	3.64	.02	54.95	56.00	3.57
20	3	3.50	.03	57.14	57.50	3.48
24	3	3.85	.05	62.34	63.45	3.78
24	3	3.76	.03	63.83	64.35	3.73

[a] In units of 10^{-3} *M*. [b] Order of polynomial employed in evaluation. [c] In units of 10^{-4} *M*/min. and obtained by orthogonal polynomial procedure.[11] [d] ± variability in units of 10^{-4} *M*/min. and obtained by orthogonal polynomial procedure.[11] [e] In units of min. [f] Obtained from definite integration procedure. [g] In units of 10^{-4} *M*/min. and calculated from *f*.

In order to verify the above conclusion and to obtain more accurate values of K_S' and k_3', a fourth series of experiments was conducted under conditions where the specific substrate stock solutions were prepared not more than 30 minutes prior to their use and were adjusted to *p*H 7.90 ± 0.01 immediately before the initiation of the enzyme catalyzed reactions. The values of K_S' and k_3' so obtained are given in Table I. Approximately six months later a fifth series conducted under similar conditions gave the next to last set of values of K_S' and k_3' presented in Table I.

The agreement between the values of K_S' and k_3' obtained from the fourth and fifth series and between these values and those obtained from the second and third series after correction for the non-enzyme catalyzed hydrolysis of the specific substrate occurring prior to the initiation of the enzyme catalyzed reaction leaves no doubt that the systematic error arising from the non-enzyme catalyzed hydrolysis of the specific substrate in the stock solutions maintained at 25° and *p*H 7.9 ± 0.01 can be eliminated through the use of the experimental procedure employed in the fourth and fifth series of experiments. Furthermore, with the knowledge that $k = 1 \times 10^{-4}$ *M*/min. for the non-enzyme catalyzed hydrolysis of methyl hippurate in aqueous solutions at 25° and *p*H 7.9 ± 0.01, it can be concluded that the values of K_S' and k_3' obtained from the fourth and fifth series are substantially free of systematic error arising from the non-enzyme catalyzed hydrolysis of the specific substrate during the course of the enzyme-catalyzed reaction.

A further uncertainty with respect to the accuracy of the above values of K_S' and k_3' resides in a so-called enzyme blank which in effect places another enzyme catalyzed reaction in competition with the enzyme catalyzed hydrolysis of the added specific substrate. No evidence for the existence

of such a blank was obtained in the earlier studies of Huang and Niemann.[3] Therefore, the values of $K_S' = 6.5 \pm 0.5$ and $k_3' = 2.2 \pm 0.2$, which are the revised values for a system 0.02 M in the THAM components of a THAM–HCl buffer,[3,6] were not corrected for an enzyme blank. When these latter values are compared with the values of $K_S' = 6.57 \pm 0.45$ and 6.61 ± 0.86 and $k_3' = 3.04 \pm 0.09$ and 2.90 ± 0.16 obtained in this study for a system 0.02 M in sodium chloride and which also were not corrected for an enzyme blank it is seen that the two sets of K_S' values are in good agreement but that the value of k_3' for the system 0.02 M in the THAM component of a THAM–HCl buffer is substantially lower than the k_3' value for the nearly comparable system 0.02 M in sodium chloride.

With the availability of a pH-Stat[8] it became possible to examine a system containing only α-chymotrypsin in aqueous solutions at 25° and pH 7.90 ± 0.01 and 0.02 M in sodium chloride. It was found that such a system led to the formation of acidic reaction products in modest but significant amounts. Since it has been observed that the preceding reaction possesses many of the characteristics of an α-chymotrypsin catalyzed hydrolysis,[15] it appears that the existence of such a reaction in the α-chymotrypsin-catalyzed hydrolysis of an added specific substrate, when followed by means of a pH-Stat, creates a situation, either real or apparent, where two substrates, *i.e.*, the separately added specific substrate and that associated with the enzyme preparation are in competition for the same enzyme. An analysis of this situation has shown that $K_S^+ > K_S$ and $k_3^+ < k_3$ when K_S^+ and k_3^+ are the values of the constants corrected for an enzyme blank by simply subtracting the extent of reaction observed with enzyme preparation alone from the extent of reaction observed in the presence of the separately added specific substrate and when K_S and k_3 are the values not so corrected. Since the nature of the substrate associated with the enzyme preparation is unknown, the validity of the above correction is questionable. In order to determine the magnitude of such a correction with respect to its effect upon values of K_S' and k_3', a sixth series of experiments were performed to give the last set of values of K_S' and k_3' presented in Table I. When the values of K_S' and k'_3 corrected for an enzyme blank are compared with the mean values of K_S' and k_3' not so corrected, it is seen that the values of k_3' are relatively insensitive to the correction but that the values of K_S' differ significantly and in the direction anticipated. At the present time it cannot be determined which of the values of K_S' is the more accurate. Until the substrate present in the enzyme preparation can be characterized and the nature of its reaction described, it is necessary to recognize the existence of two sets of values for K_S' and k_3', *i.e.*, $K_S' = 6.60 \pm 0.30$ and $k_3' = 2.97 \pm 0.12$ derived from data not subjected to correction for an enzyme blank and $K_S' = 7.55 \pm 0.30$ and $k_3' = 2.91 \pm 0.05$ derived from data that have been corrected.

The remaining concern with respect to the reliability of the above values of K's and k_3' relates to the magnitude of values of E_S' and S_S', the possibility of product inhibition in a classical sense and the maintenance of the constancy of reaction parameters that do not appear in the stoichiometric equation. If it is assumed as before[6] that the molecular weight of monomeric α-chymotrypsin is 22,000, its nitrogen content is 16.0% and that all of the enzyme is present in the reaction system as the monomer then, in the experiments leading to the final values of K_S' and k_3', [E] = 4.26 to 4.40 $\times 10^{-5}$ M, $E_S' = 0.58$ to 0.64×10^{-2} and $S_S' = 0.53$ to 3.64. Since all of these values are well within the limits demanded of a ± 5% experimental error, it may be concluded that all assumptions inherent in the evaluation procedures have been satisfied.[6] As all initial velocities were evaluated by an empirical procedure, which by definition is independent of an assumed rate equation,[11] it may be concluded that any classical competitive inhibition of the reaction by its reaction products would have no significant influence upon the values obtained for the initial velocities, and in turn upon the values of K_S' and k_3', particularly since it has been found that the value of K_I' for hippuric acid in aqueous solutions at 25° and pH 7.9 ± 0.01 and 0.02 M in sodium chloride is *ca.* 16×10^{-3} M.[16] Finally, with the temperature and pH being controlled within adequate limits, the only remaining factors requiring comment are those that are characteristic of the pH-Stat, *i.e.*, the change in volume of the reaction system caused by the addition of standard base to the system during the course of reaction, the attendant change in the molarity of the enzyme and specific substrate and the change in the ionic strength and species of the reaction system. It will be seen from the account given in the Experimental section that the errors produced by these factors are of little or no significance.

Inhibition of the α-Chymotrypsin-catalyzed Hydrolysis of Methyl Hippurate by Indole.—The availability of a set of relatively precise values of K_S' and k_3' for the system α-chymotrypsin–methyl hippurate in aqueous solutions at 25° and pH 7.90 ± 0.01 and 0.02 M in sodium chloride suggested the desirability of examining the system α-chymotrypsin–methyl hippurate–indole under the same conditions since the earlier analysis, which was conducted in a system 0.02 M in the THAM component of a THAM–HCl buffer,[9] was based upon values of constants that since have been revised.[6,17] It was hoped that with the pH-Stat a more precise evaluation could be performed and the basic nature of the system more firmly established.

The system α-chymotrypsin–methyl hippurate–indole may be regarded as the prototype of a number of systems which may involve, at least in part, the simultaneous interaction of the enzyme with a bifunctional specific substrate and a monofunctional competitive inhibitor to give an intermediate ternary complex ESI capable of yielding reaction products. It is important that the factual basis of the prototype system be well founded. Such systems are important not only because of their theo-

(15) Unpublished observations of T. H. Applewhite, R. B. Martin and T. Gordon.

(16) Unpublished experiments of R. B. Martin.

(17) R. J. Foster and C. Niemann, THIS JOURNAL, **77**, 3370 (1955).

TABLE III

α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF METHYL HIPPURATE IN THE PRESENCE OF INDOLE[a]

$[I] \times 10^3$	0.00	0.55	1.10	2.20
$1/V'' \times 10^{-3}$ [b,c]	2.23 ± 0.03	2.37 ± 0.03	2.50 ± 0.04	2.90 ± 0.04
$V'' = k_3''[E] \times 10^3$	0.448 ± .006	0.422 ± .005	0.401 ± .006	0.345 ± .005
$k_3'' \times 10^3$	2.91 ± .05	2.73 ± .05	2.60 ± .05	2.24 ± .05
K_S''/V'' [b,d]	16.9 ± .7	23.3 ± .8	28.2 ± 1.0	36.8 ± 1.2
$(K_S'/V')(1 + [I]/K_I)$ [e]	16.9 ± .7	29 ± 3	40 ± 6	63 ± 11
$K_S'' \times 10^3$	7.55 ± .3	9.9 ± 0.3	11.3 ± 0.4	12.3 ± 0.4
$K_S'(1 + [I]/K_I)$ [e]	7.55 ± .3	12.8 ± .3	18 ± 3	28 ± 5
$rK_S/C\sigma \times 10^{-3}$	....	0.407	0.385	0.328
$C\sigma \times 10^6$	....	13.9	14.0	13.5

[a] In aqueous solutions at 25.0° and *p*H 7.90 ± 0.01 and 0.02 *M* in sodium chloride with [E] = 0.154 mg. protein-nitrogen per ml., 7 to 8 values of $[S]_0$ between the limits of 8 to 25 × 10^{-3} *M*, total time of observation from 0 to 8 min. with a uniform interval of 1 min. between observations, extent of reaction corrected for an enzyme blank and initial velocities computed by the orthogonal polymial procedure.[11] [b] Obtained by a least squares fit to the family of lines described by the relation $1/v_0 = 1/V'' + ((K_S''/V'')/[S]_0)$, where $V'' = k_3''[E]$. [c] Value of intercept. [d] Value of slope. [e] Based upon $K_S' = 7.55 \pm 0.30$, $k_3' = 2.91 \pm 0.05$ and $K_I = 0.8 \pm 0.2 \times 10^{-3}$ *M*.[17]

retical implications but also because of their use as probes for the exploration of the topography of the catalytically active site of the enzyme.

The experimental conditions employed in the present study are summarized in Table III. It will be seen from Fig. 1 that the same general features observed by Huang and Niemann[9] for the system 0.02 *M* in the THAM component of a THAM–HCl buffer have now been observed for the comparable system 0.02 *M* in sodium chloride.

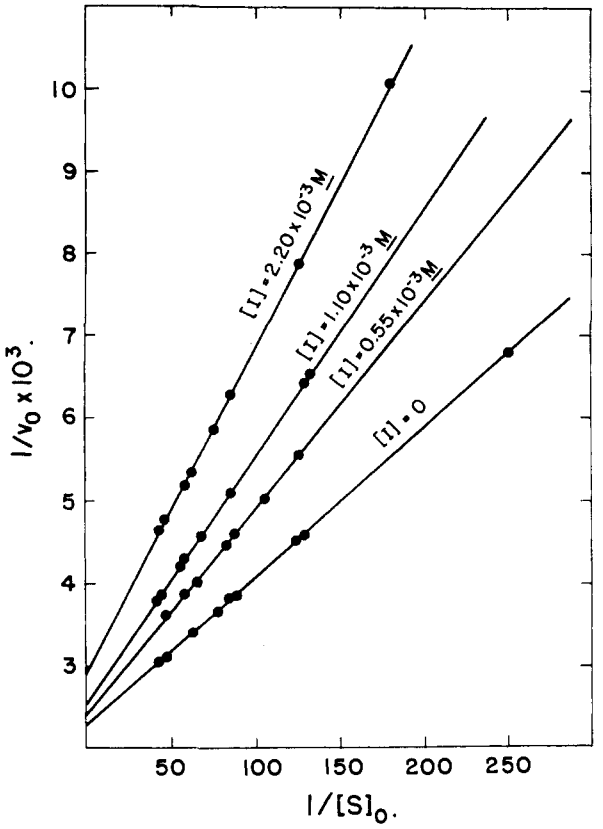

Fig. 1.—Inhibition of α-chymotrypsin-catalyzed hydrolysis of methyl hippurate by indole.

Inspection of Fig. 1 and consideration of the values of $1/V''$, K_S''/V'', K_S'', k_3'', $(K_S'/V')(1 + [I]/K_I)$ and $K_S'(1 + [I]/K_I)$ given in Table III confirms the earlier conclusion of Huang and Niemann[9] that the kinetics of inhibition of the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate by indole cannot be reconciled with those of a simple competitive system.

The quantities $rK_S/C\sigma$ and $C\sigma$ were evaluated on the basis of equation 2 which is linear with respect to $1/v_0$ and $1/[S]_0$ but in which both the slope

$$1/v_0 = K_S'/V'(1 + [I]/K_I)(1/(1 + rK_S[I]/C\sigma))\ 1/[S]_0 + (1/(1 + rK_S[I]/C\sigma))\ (1/V' + (K_S'/V')([I]/C\sigma)) \quad (2)$$

and intercept are dependent upon [I],[9,18] *cf.*, Fig. 1. As noted previously[9] when $r = 0$, *i.e.*, when the ternary complex ESI does not give rise to reaction products, the slope of equation 2 is reduced to $K_S'/V'(1 + [I]/K_I)$ and the intercept to $1/V'(1 + K_S'[I]/C\sigma)$. The fact that K_S''/V'' is again found = $K_S'/V'(1 + [I]/K_I)$ confirms the conclusion reached earlier[9] that the values of K_S''/V'' observed for the system α-chymotrypsin–methyl hippurate–indole are not compatible with the supposition that a ternary complex is formed but does not undergo further reaction, *i.e.*, the types of reversible inhibition that do not include a ternary complex capable of yielding reaction products are excluded.

The agreement of the three values of $rK_S/C\sigma$ and of $C\sigma$, *cf.*, Table III, may be considered satisfactory when it is realized that values of $rK_S/C\sigma$ are very sensitive to errors in values of K_S''/V''. Thus, within the limits of experimental error, the results of the present investigations are in agreement with and confirm those obtained previously.[9] It is also evident that further examination of the system α-chymotrypsin–methyl hippurate–indole cannot lead to a more rigorous test of equation 2 and of the argument upon which it is based[9] because the limited solubility of indole makes it impossible to examine this system over a sufficiently wide range of values of [I], which is required if a lack of dependence of $rK_S/C\sigma$ and of $C\sigma$ upon [I] is to be established beyond all doubt.

The mean values of $rK_S/C\sigma = 0.37$[22] and $C\sigma =$

(18) Equations comparable if not equivalent to equation 2 have been derived by Segal, Kachmar and Boyer[19] and by Botts and Moralles[20,21].

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

(20) J. Botts and M. Morales, *Trans. Faraday Soc.*, **49**, 696 (1953).

(21) M. F. Morales, THIS JOURNAL, **77**, 4169 (1955).

(22) All values used in the discussion which follows are based upon units of 10^{-3} *M* for K_S and K_I.

13.8 obtained in this study leads to a value of rK_S = 5.11 which may be compared to the value of 5.36 obtained previously[9] from values of $rK_S/C\sigma$ = 0.55 and $C\sigma$ = 9.75. From the former value of rK_S and a value of K_I = 0.8 it may be inferred[9] that $0.44 < K_SK_I/C\sigma < 1$, $7.55 < K_S < 17.3$ and $0.30 < r < 0.68$. The previous values[9] are $0.61 < K_SK_I/C\sigma < 1$, $8.5 < K_S < 13.9$, $0.39 < r < 0.64$. The lower limit of 0.44 may be interpreted[9] to mean that the over-all combining ability of the binary complexes must compare favorably with that of the free enzyme but may be somewhat less than that inferred from the lower limit of 0.61 obtained previously.[9] From the limiting values of K_S given above, *i.e.*, $7.75 < K_S < 17.3$, it may be estimated that $13.4 \leq K\sigma \leq \infty$ and from the lower limit, that $V' \leq V \leq 2.29\ V'$ from which it follows[9] that $2.91 \leq k_3 \leq 6.66$, and that $0.3\ k_3 \leq k_{3\cdot1} \leq 0.7$. From these latter values, it follows that the rate of hydrolysis of ESI is *ca.* one-half that of ES.[9]

The Effect of Aqueous–Non-aqueous Solvent Systems on the α-Chymotrypsin-catalyzed Hydrolysis of Methyl Hippurate.—Aqueous–non-aqueous solvent systems have been employed in studies involving the reaction of α-chymotrypsin with specific substrates possessing limited solubility in water. However, relatively few attempts have been made to determine the consequences of varying the concentration or nature of the non-aqueous component with all other factors being kept constant.

Kaufman, Neurath and Schwert[23] reported that the so-called proteolytic coefficients determined for the hydrolysis of glycyl-L-tyrosinamide and benzoyl-L-tyrosine ethyl ester in aqueous methanol decreased logarithmically with increasing concentration of methanol. However, these results are difficult to interpret because of the ambiguity inherent in the use of proteolytic coefficients and in the former case because of the known facile transformation of dipeptide amides to the corresponding diketopiperazines and ammonia.[24,25]

Kaufman and Neurath[26] studied the hydrolysis of acetyl-L-tyrosinamide at three concentrations of methanol, up to 17 wt. % and found k_3 to be constant and K_S to increase with increasing methanol concentration. Simple competitive inhibition was excluded and a plot of $1/K_S$ *vs.* M methanol was found to be linear.

Barnard and Laidler[27] examined the hydrolysis of methyl hydrocinnamate at three concentrations of methanol, up to 15 wt. %, and it may be inferred from their results that k_3 decreases and K_S increases with increasing methanol concentration.

A change in the nature or composition of the solvent system would be expected to have a profound influence upon a number of reaction parameters. Therefore, in order to achieve some degree of simplicity it was decided to limit interest to those systems where the only variables were the initial specific substrate concentration and the nature or composition of the solvent system and where either K_S or k_3 would be the dependent variable.

A series of experiments with α-chymotrypsin and methyl hippurate in aqueous–non-aqueous solvents at 25° and *p*H 7.90 ± 0.01 and 0.02 M in sodium chloride were conducted under conditions similar to those described in the first section of this communication. Values of v_0 were obtained by the orthogonal polynomial procedure,[11] from primary data that had been corrected for an enzyme blank and values of K_S' and k_3' by a least squares fit of values of $[S]_0/v_0$ and $[S]_0$ to the equation $([S]_0[E]/v_0) = (K_S'/k_3') + ([S]_0/k_3')$.[10]

It will be seen from the data summarized in Table IV that for systems containing methanol, *t*-butyl alcohol, acetonitrile, dimethylformamide, dimethylacetamide and formaldehyde k_3' decreased and K_S' increased with increasing concentration of the non-aqueous component. However, for systems containing acetone, methyl ethyl ketone and dioxane k_3' remained constant while K_S' increased with increasing concentration of the non-aqueous component. Since our interest was limited to the case of a single dependent variable no further attention was given to the first group of solvents. The experiments with formaldehyde were prompted by the observation of Bernhard[28] that the rate of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-phenylalanine ethyl ester was reduced materially

TABLE IV

DEPENDENCE OF VALUES OF K_S' AND k_3 FOR THE SYSTEM α-CHYMOTRYPSIN–METHYL HIPPURATE AT 25° AND *p*H 7.9 UPON THE NATURE OR COMPOSITION OF THE SOLVENT[a]

Non-aqueous component	Vol. %	M	K_S'[b,c]	k_3'[c,d]
None	..	..	7.55 ± 0.3	2.91 ± 0.05
Methanol	5.0	1.24	17 ± 4	0.76 ± .08
	10.0	2.49	20 ± 4	0.44 ± .04
t-Butyl alcohol	10.0	..	13 ± 1	2.26 ± .07
Acetone	5.03	0.69	12 ± 1	2.9 ± .1
	10.1	1.38	21 ± 2	2.8 ± .2
	15.2	2.08	28 ± 3	2.8 ± .2
	20.4	2.79	40 ± 7	2.7 ± .3
Methyl ethyl ketone	10	..	24 ± 2	2.8 ± .2
Dioxane	0.4	0.047	9.1 ± 0.5	2.8 ± .1
	5.0	0.59	25 ± 4	2.8 ± .2
	10.0	1.18	50 ± 6	2.8 ± .2
	15.0	1.78	92 ± 9	2.7 ± .2
Acetonitrile	10.0	..	19 ± 3	2.5 ± .2
Dimethyl-formamide	8.0	..	13 ± 1	2.7 ± .1
	16.0	..	18 ± 2	2.1 ± .1
Dimethyl-acetamide	10.0	..	21 ± 2	2.6 ± .1
	20.0	..	50 ± 8	2.3 ± .2
Formaldehyde	1.8[e]	..	9 ± 1	1.00 ± .05
	5.4[e]	..	19 ± 5	0.8 ± .1

[a] Reaction system 0.02 M in sodium chloride with [E] = 0.150 and 0.155 mg. protein-nitrogen per ml., 8 to 10 values of $[S]_0$ between the limits of 6 to 26 × 10^{-3} M, total time of observation from 0 to 8 min. with a uniform interval of 1 min. between observations and with the extent of reaction corrected for an enzyme blank. [b] In units of 10^{-3} M. [c] Initial velocities determined by the orthogonal polynomial procedure[11] and K_S' and k_3' evaluated by a least squares fit to the equation $([S]_0[E]/v_0) = (K_S'/k_3') + ([S]_0/k_3')$. [d] In units of 10^{-3} M/min./mg. protein-nitrogen per ml. [e] w./v. %.

(23) S. Kaufman, H. Neurath and G. W. Schwert, *J. Biol. Chem.*, **177**, 793 (1949).

(24) H. T. Huang and C. Niemann, THIS JOURNAL, **72**, 921 (1950).

(25) L. Meriwether and F. H. Westheimer, *ibid.*, **78**, 5119 (1956).

(26) S. Kaufman and H. Neurath, *J. Biol. Chem.*, **180**, 181 (1949).

(27) M. L. Barnard and K. J. Laidler, THIS JOURNAL, **74**, 6099 (1952).

(28) S. A. Bernhard, *Biochem. J.*, **59**, 506 (1955).

TABLE V

DEPENDENCE OF VALUES OF K_S' UPON CONCENTRATION OF NON-AQUEOUS COMPONENT

Vol. %	M	K_S'[a]	Solubility[b] H_2O	Solubility[b] H_2O–non-aq.	R_S[c]	K'/R_S[a]	$K_S'/1.2R_S$[a]	K_S'/R_S^2[a]	$K_S'/(1.2R_S)^2$[a]
0	0	7.6	0.0375	....	..	7.6	7.6	7.6	7.6
				Acetone					
5.03	0.69	12.2	....	0.0528	1.41	8.7	7.3	..	..
10.1	1.38	20.6	....	.0802	2.14	9.6	8.0	..	..
15.2	2.08	28.1	....	.1150	3.07	9.2	7.7	..	..
20.4	2.79	40.4	....	.1605	4.28	9.4	7.8	..	..
				Dioxane					
5.0	0.59	25.0	....	0.0551	1.47	17.0	..	11.6	8.1
10.0	1.18	50.4	....	.0827	2.21	22.8	..	10.3	7.2
15.0	1.78	92.3	....	.1113	2.97	31.1	..	10.5	7.3

[a] In units of 10^{-3} M. [b] G. per 5.0 ml. of solution 0.02 M in sodium chloride at 25°. [c] Ratio of solubility in aqueous–non-aqueous system relative to aqueous system.

by the addition of 2 w./v. % of formaldehyde to the reaction mixture but that the residual activity appeared to remain constant throughout the experiment. This observation has been confirmed and extended with a second specific substrate under conditions where the reaction with formaldehyde resulted in the initial release of *ca.* 95 and 120 equivalents of acid per mole of enzyme.

The limited solubility of methyl ethyl ketone in water prevented further studies with this solvent. The upper limits of 2.79 M acetone and 1.78 M dioxane were determined by the sluggish electrode response observed at higher concentrations.

The systems α-chymotrypsin–acetyl-L-tyrosinamide – aqueous methanol,[26] α - chymotrypsin–methyl hippurate–aqueous acetone and α-chymotrypsin–methyl hippurate–aqueous dioxane are similar in that in all three the value of k_3, or k_3', is independent of, and K_S, or K_S', increases with increasing concentration of the non-aqueous component of the solvent system. Furthermore, in all three it is clear that the non-aqueous component does not function as a simple competitive inhibitor since in every case it can be shown that the value of K_1 decreases with increasing concentration of the non-aqueous component. However, the first system differs from the other two in that only with the first is there an apparent linear relationship between $1/K_S$ and the molarity of the non-aqueous component.

Since there was no reason to search for a purely empirical relationship between K_S' and the molarity of the non-aqueous component present in the two systems involving methyl hippurate attention was directed to possible relationships of theoretical significance. Plots of log $1/K_S'$ *vs.* $1/D$, log $1/K_S'$ *vs.* $1/D^2$ and log $1/K_S'$ *vs.* $(D-1)(2D+1)$ were constructed but in no case was a linear relationship obtained. While it cannot be concluded that the gross dielectric constant of the reaction system is without influence upon values of K_S' no simple dependency was observed nor was it expected.

The fact that k_3' remained constant while K_S' increased with increasing concentration of the non-aqueous component coupled with the knowledge that methyl hippurate is more soluble in acetone or dioxane than it is in water suggested a treatment reminiscent of one used by Dimroth[29,30] in his interpretation of the influence of the nature of the solvent upon the equilibrium constants of certain desmotropes.

In principle it was assumed that the aqueous–non-aqueous reaction system was microscopically inhomogeneous and that the value of K_S' observed in such a system was composed of two partition coefficients, one describing the partition of the specific substrate between the enzyme and water and equal to the value of K_S' observed in the aqueous system, and the other of the specific substrate between the aqueous and non-aqueous components. The ratio of the solubility of methyl hippurate in the particular solvent system associated with a given value of K_S' to its solubility in the aqueous system, *i.e.*, R_S, was assumed to be equal to the second partition coefficient.

It will be seen from the data given in Table V that for aqueous acetone the values of the quotient K_{S_R}'/R_S are reasonably constant but give a mean value of 9.2 which is *ca.* 20% greater than the value of 7.6 observed for the aqueous system. Therefore, if it is assumed that the ratio R_S is not equal to but is proportional to the second partition coefficient and that the proportionality constant is 1.2, it is seen, *cf.*, Table V, that the values of $K_{S_{H_2O}}' = K_{S_R}'/1.2R_S$ are in agreement with the value of K_S' determined for the aqueous system.

For aqueous dioxane the values of K_{S_R}'/R_S are not constant but increase with increasing concentration of dioxane. However, values of K_{S_R}'/R_S^2 are essentially constant and the values of $K_{S_{H_2O}}' = K_{S_R}'/(1.2R_S)^2$ are in reasonable agreement with the directly determined value. The use of $1.2R_S$ for aqueous acetone and $(1.2R_S)^2$ for aqueous dioxane may appear capricious. However, in the absence of knowledge relative to the behavior of methyl hippurate in aqueous acetone and aqueous dioxane solutions, it is not an unreasonable practice. It is hoped that future investigations will determine whether the relationships $K_{S_{H_2O}}' = K_{S_R}'/1.2R_S$ for aqueous acetone systems and $K_{S_{H_2O}}' = K_{S_R}'/(1.2R_S)^2$ for aqueous dioxane systems, which incidentally are the most satisfactory empirical relationships we have found, are fortuitous or have a rational basis.

Experimental[31,32]

General Procedures.—An extensive set of exploratory

(29) O. Dimroth, *Ann.*, **399**, 91 (1913).

(30) L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill, New York, N. Y., 1940, p. 89.

(31) All melting points are corrected.

(32) Microanalyses by Dr. A. Elek.

experiments[33] led to the following general procedures. The precautions characteristic of quantitative operations were observed throughout including the use of carbon dioxide-free distilled water.

Standard Sodium Hydroxide Solutions.—These were prepared as directed by Swift,[34] stored in polyethylene bottles with siphons and soda lime protection and were individually standardized against Merck "Primary Standard" potassium acid phthalate.

Methyl Hippurate.—Matheson hippuric acid (0.1 mole) was esterified by reaction with methanol and thionyl chloride. The crude product (92%) was purified by three recrystallizations from benzene and one from isopropyl ether to give long, colorless needles, m.p. 82.0–83.0°; yield 35%.

Anal. Calcd. for $C_{10}H_{11}O_3N$ (193): C, 62.2; H, 5.7; N, 7.3. Found: C, 62.5; H, 5.6; N, 7.0.

A second preparation of this compound employing 0.1 mole of once recrystallized Matheson hippuric acid gave a much purer product. Only one recrystallization from isopropyl ether was necessary to obtain an analytical sample, m.p. 82.8–84.1°; lit.[8] m.p. 82–83°.

Anal. Calcd. for $C_{10}H_{11}O_3$ (193): C, 62.2; H, 5.7; N, 7.3. Found: C, 62.4; H, 5.7; N, 7.1.

Specific Substrate Solutions.—The required amounts of specific substrate necessary to provide suitable concentrations in the individual stock solutions were accurately weighed into G. S. Pyrex volumetric flasks. About 20 minutes before the start of a run the sample was dissolved in nearly the total volume of carbon dioxide-free water by warming if necessary. The flask was then thermostated at 25 ± 0.1°.

Enzyme Solutions.—These were prepared just prior to use by weighing the required amount of enzyme directly into a tared G. S. Pyrex volumetric flask containing *ca.* 1 ml. of carbon dioxide-free water. The solution was slowly diluted to the mark, gently inverted *ca.* five times and thermostated. If a delay in use of the enzyme was anticipated, the solutions were stored at 4°. Only enough enzyme stock solution was prepared for nine to twelve runs, and as these could be carried out in less than 3 hr., refrigeration of the enzyme solution was usually unnecessary.

Operation and Calibration of the *p*H-Stat.—This instrument, a modification of the design of Neilands and Cannon,[8] was built by M. D. Cannon, International Instruments Co., Canyon, California. The essential features for use in constant *p*H titrations are: an "Agla" micrometer syringe driven by a variable speed, reversible motor controlled by a moving coil galvanometer (Contact Meter Relay) with manually adjustable fixed contacts that permit the choice of the desired *p*H; a Leeds and Northrup Model 7664-41 A.C.-operated *p*H meter, mounted in its original case, with input from a Beckman No. 4990-29 glass electrode and No. 5970-29 calomel reference electrode and output across the coil of the Contact Meter Relay; a Leeds and Northrup Speedomax Type G Recorder whose pen response was electrically controlled by a bridge circuit in which one leg of the bridge was geared directly to the syringe drive motor; and, allied circuitry allowing the operator to zero the pen and calibrate the pen response for delivery and tracking, start, stop and reverse the syringe drive unit, control the syringe drive motor speed and select the desired scale setting to obtain various pen responses for a certain volume of titrant delivered.

The following procedure was employed to prepare the instrument for use. The main power switch was turned on at least a day prior to use. The battery was inserted and the recorder drive activated 15 minutes before calibration. Buffer (*p*H 7.00) was placed in a clean dry reaction vessel, *vide post*, and the *p*H meter standardized. The recorder was manually standardized and then visually zeroed by bringing the pen upscale to the first division. With the scale switch at position 5 the micrometer head was advanced exactly ten revolutions by placing the drive switch in the forward position. This corresponds to a delivery of 0.100 ml. of titrant and should move the pen exactly four inches from its zero position. If this was not the case the pen position was corrected with the compensate potentiometer. Switching the scale selector to position 10 should move the pen exactly two inches toward the zero position. If this response was not obtained, the position of the pen was corrected with the adjust potentiometer. The scale selector was then switched back and forth between positions 5 and 10 with the corresponding adjustments at each position until the correct pen tracking was achieved. Following calibration the pen was returned to zero by placing the drive switch in reverse and allowing the instrument to run to the reverse limit. The pen response was held to within 0.5% by a recalibration after each four runs.

A final step in the calibration was a check of the Contact Meter Relay (CMR). This was carried out by locking the zero knob on the *p*H meter and moving the *p*H indicator needle upscale by means of the standardization potentiometer. With the CMR switch in the low position and S_7 at position A the indicating pointer of the CMR will operate ("kick" upscale) whenever the *p*H, simulated manually in this case, drops below the pre-selected value. After the desired setting was achieved, it was seldom necessary to readjust this portion of the instrument, a daily check being sufficient.

Titration Cell.—In the preliminary experiments[33] a cell similar to that of Neilands and Cannon[9] was employed. It became apparent that cleaning and drying the cell between each experiment reduced the speed and, hence, the utility of the system. Accordingly, a new type of cell was designed. This cell combined the temperature control features of the above cell with an inner container that could be rapidly removed and replaced with a clean, dry vessel. With standardization in mind, the outer jacket was designed to hold 50-ml. Pyrex beakers with the lip cut off to provide a cell of *ca.* 40-ml. total capacity. The fit between the jacket and the beakers was as close as possible and a few drops of water were added to increase contact area and aid heat transfer. With this arrangement and the full output of a Precision constant temperature bath at 25.0 ± 0.1° passing through the jacket it was found that the temperature within the cell, checked with a calibrated, precision thermometer, agreed exactly with the temperature of the constant temperature bath. However, it also was noted that the equilibration time was approximately 10 minutes for a five degree temperature differential. Therefore, all solutions introduced in this cell were maintained at 25.0 ± 0.1° until they were transferred.

The cell was covered with a Lucite plate with a circular groove to provide alignment of the cell and the cover. Holes in the cover were provided to allow the introduction of the pair of Beckman electrodes, a nitrogen inlet tube, a small glass propeller *ca.* 1 cm. in diameter driven by a stirring motor, the hypodermic needle from the "Agla" syringe and the reactants. The cover and all of the components were clamped firmly in position and the cell was supported in correct alignment by an air operated, elevating table.

The stirrer, of low pitch to reduce cavitation, was designed to move the solution in a manner that reduced introduction of gas into the solution and at the same time directed the flow of liquid against the glass electrode. The glass electrode and the hypodermic needle were positioned dimetrically opposite to one another across the stirrer. This system was very satisfactory, and no effect on the rates was observed except when the stirring speed was very low, giving rise to poor mixing, or excessively fast, causing foaming of the solution.

The reaction system was maintained free of carbon dioxide by introducing dry nitrogen, previously passed through a bed of potassium hydroxide pellets and a distilled water bubbler, near the top of the cell. In this way, air was swept out and evaporation errors minimized. No change in *p*H could be observed when the nitrogen flow system was in operation, but when the flow was turned off the system would titrate rapidly the carbon dioxide taken up by the slightly alkaline solutions.

Enzyme *p*H-Adjustment System.—Enzyme solutions could not be maintained near *p*H 7.9 for extended periods without loss of activity. In addition, the problems of correct zero time required a constant knowledge of the *p*H of the enzyme solution prior to its introduction into the titration cell. For these reasons a separate titration system was devised for the adjustment of the enzyme stock solution to the required *p*H. A Lucite cover plate with holes positioning a pair of electrodes as above, a nitrogen inlet tube, a

(33) T. H. Applewhite, Ph.D. Thesis, Calif. Inst. Tech., Pasadena, Calif., 1957.

(34) E. H. Swift, "A System of Chemical Analysis," Prentice-Hall, Inc., New York, N. Y., 1939, pp. 99–100.

small glass propeller driven by a stirring motor and a hypodermic needle from a syringe held in a micrometer measuring device[35] was used in conjunction with a 10-ml. beaker and a separate Leeds and Northrup *p*H meter. This system allowed the adjustment of small samples of enzyme stock solution to the required *p*H, and, in addition, permitted continuous observation of this *p*H.

The Procedure for a Typical Kinetic Run.—The specific substrate stock solution prepared as above was diluted to the mark, gently inverted 10–12 times and the required volume, *i.e.*, 4–5 ml., pipetted into the titration cell. Sufficient 0.2 *M* aqueous sodium chloride was introduced to give a final concentration of 0.02 *M*. Water, if necessary, was added to bring the total volume to within 1 ml. of the final volume of 10 ml. This solution was then adjusted to *ca.* *p*H 7.95 by the addition of standard sodium hydroxide solution from the previously filled and positioned "Agla" syringe. With all of the controls in the operating position, *ca.* 2.5 ml. of the enzyme stock solution was rapidly adjusted to *ca.* *p*H 8 by the addition of very small amounts of 0.85 *N* sodium hydroxide solution. A weight-calibrated syringe in a two-stop holder[36] was quickly rinsed twice with *ca.* 0.25-ml. portions of the enzyme solution. The *p*H of each system was continuously observed during this period and as each approached the operating *p*H, *i.e.*, 7.9, the syringe was quickly filled and the 1-ml. enzyme aliquot introduced beneath the surface of the solution in the titration cell. With this technique the titration started within 0–5 seconds after the enzyme was introduced. The syringe-drive speed control was then adjusted to maintain the addition of standard base at a rate essentially that required by the reaction. This practice avoids the possibility of the apparatus falling behind the reaction.[8] The reaction was then allowed to proceed for the required time (8–24 minutes) with occasional readjustment of the drive speed control as necessary. When the run was completed, the zero button on the *p*H meter was locked in, the drive switch reversed,[37] the reaction mixture removed and discarded, the electrodes and stirrer rinsed and dried, the "Agla" syringe refilled, and when a clean, dry cell was introduced the cycle could be repeated.

In all of the studies described in this communication the consequences of dilution of the reaction mixture by the added standard aqueous sodium hydroxide solution were ignored. Consideration of a hypothetical reaction system with $[S]_0 = 5 \times 10^{-3}$ *M*, [E] = 0.15 mg. protein-nitrogen per ml. and [NaCl] = 0.02 *M* in an initial volume of 10 ml. will show that 50% reaction requires the addition of 0.500 ml., the total volume of the "Agla" syringe, of 0.05 *N* sodium hydroxide. In this case the 5% dilution causes a 5% lowering of $[S]_t$ and $[E]_t$ below their respective values if no dilution is assumed. At the same time the titration of the liberated carboxylic acid with the standard base increases the ionic strength by 7%. The former effect will tend to cause a diminution in rate whereas the latter will cause an increase in rate[38] and even in the extreme case the errors will be partially compensated. With the proper choice of the normality of the standard base, the above error is further minimized and in the present studies this practice resulted in a dilution of the reaction mixture of less than 3.5%. When it is realized that all data were reduced by an empirical evaluation procedure[11] to arrive at the initial situation, the effects produced by a dilution of the above magnitude are without significance relative to the other experimental errors.

Interpretation of Recorder Traces.—The chart paper is graduated in tenths of inches in both dimensions and the chart speed may be varied to move the paper either one-half or one inch per minute. These facts coupled with the calibration mentioned previously allow the time and volume data to be taken directly from the charts. The volume information may be transformed directly into milliequivalents per milliliter by multiplication by a constant times the base concentration, or, as in these studies, may be employed to calculate initial velocities in terms of volume. The latter values can then be converted to milliequivalents per milliliter per min. Initially the volume data were estimated to $\pm 0.5 \times 10^{-3}$ ml. and rounded off to the nearest 1×10^{-3} ml. Later, improved techniques and increased sensitivity of the instrument permitted an estimate of the volume data, when necessary, to $\pm 0.2 \times 10^{-3}$ ml. A chart speed of one-half inch per minute was generally employed in these studies, and the time readings could be estimated to less than ±3 seconds.

Experimental Conditions.—Many of the essential details of the individual experiments are given in Tables I to V inclusive. Of the experiments summarized in Table I, the first series were conducted with Armour preparation lot no. 00592, the second, third, fourth and fifth with lot no. 90492 and the sixth with lot no. 283. All experiments involving inhibition by indole or the use of aqueous–nonaqueous solvent systems were performed with the latter preparation. An Eastman preparation of indole was recrystallized from ethanol.

Acknowledgment.—The authors wish to acknowledge their indebtedness to Mr. Hal Waite for his valuable assistance during the course of this investigation.

PASADENA, CALIFORNIA

(35) P. A. Shaffer, Jr., P. S. Farrington and C. Niemann, *Anal. Chem.*, **19**, 492 (1947).

(36) A. Krogh, *Ind. Eng. Chem., Anal. Ed.*, **7**, 130 (1935).

(37) In addition to the other features mentioned this instrument is provided with forward and reverse limit switches which automatically turn off all auxiliary circuits at either extreme of the syringe travel.

(38) H. J. Shine and C. Niemann, THIS JOURNAL, **77**, 4275 (1955).