I. THE KINETICS OF THE ALPHA-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-HEXAHYDROPHENYLALANINAMIDE

II. THE INFLUENCE OF pH ON THE KINETIC CONSTANTS FOR ALPHA - CHYMOTRYPSIN CATALYZED REACTIONS INVOLVING SELECTED SUBSTRATES AND INHIBITORS

III. GENETIC STUDIES IN NEUROSPORA CRASSA

Thesis by

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ABSTRACT

The kinetic constants for the alpha-chymotrypsin-catalyzed hydrolysis of acetyl-L-hexahydrophenylalaninamide have been determined at 25° and pH 7.9. This study indicates that aromaticity in the amino acid side chain is neither essential nor particularly helpful to the action of alpha-chymotrypsin. Some computational procedures are discussed.

The kinetic constants for the alpha-chymotrypsin-catalyzed hydrolyses of L-tyrosinhydroxamide and acetyl-L-tyrosinhydroxamide have been determined at 25° at several pH's. The inhibition constants of variously ionizing inhibitors have also been determined at several pH's. The observations are generally consistent with formulations based on the existence of differently protonated forms of the enzyme and enzyme-substrate complexes.

Potentiometric titrations of some hydroxamic acids reveal that the pK_A values for these acids range from 9.0 to 9.5, and that these acids are much weaker than previously believed.

Some of the conditions necessary for the good expression of the phenotype dotted in Neurospora crassa have been determined. The linkage and centromere distance of the gene controlling tyrosinase thermostability in Neurospora crassa have been determined by genetic studies.

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PART I. THE KINETICS OF THE ALPHA-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-HEXAHYDROPHENYLALANINAMIDE

[Reprinted from the Journal of the American Chemical Society, 75, 4687 (1953).]

[Contribution No. 1793 from the Gates and Crellin Laboratories of Chemistry, California Institute of Technology]

The Kinetics of the α-Chymotrypsin Catalyzed Hydrolysis of Acetyl-L-hexahydrophenylalaninamide in Aqueous Solutions at 25° and *p*H 7.9[1]

By Robert R. Jennings[2] and Carl Niemann[3]

Received April 10, 1953

On the basis of the respective K_S and k_3 values of acetyl-L-phenylalaninamide and of acetyl-L-hexahydrophenylalaninamide it has been concluded that the affinity of α-chymotrypsin for the former specific substrate is no greater and is probably less than for the latter. In the course of this study a rational procedure has been developed for the determination of the initial velocities of certain enzyme-catalyzed reactions and the scope and limitations of an alternative method for the determination of the K_S and k_3 values of comparable enzymatic systems in which the initial velocities need not be determined has been examined.

In view of the tendency to associate α-chymotrypsin activity with specific substrates possessing aromatic side chains,[4-9] and to consider that the aromatic character of these side chains is, in some way, important for α-chymotrypsin activity[7-9] it was thought worthwhile to compare the behavior of a specific substrate such as acetyl-L-phenylalaninamide[10] with that of its non-aromatic analog, *i.e.*, acetyl-L-hexahydrophenylalaninamide. A preliminary investigation revealed that the latter compound was hydrolyzed in the presence of α-chymotrypsin and that the α-chymotrypsin-catalyzed hydrolysis of this specific substrate at 25° in aqueous solutions 0.02 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer appeared to be optimal, with respect to the *p*H of the above reaction medium, in the region of *p*H 7.9 ± 0.1, *cf.* Fig. 1. Since this behavior was identical with that observed for the comparable reaction with acetyl-L-phenylalaninamide,[10] the above conditions were selected for a more detailed study of the kinetics of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-hexahydrophenylalaninamide.

It was noted previously[10] that with a specific substrate such as acetyl-L-phenylalaninamide operational difficulties are aggravated because of the relatively slow rate of hydrolysis of the specific substrate. Anticipating comparable difficulties with acetyl-L-hexahydrophenylalaninamide, it was decided to consider, in a general way, the use of improved techniques for the evaluation of the kinetic constants rather than to study another pair of specific substrates wherein the behavior of the non-aromatic member of the pair could be complicated by the possible presence of several stereoisomers.

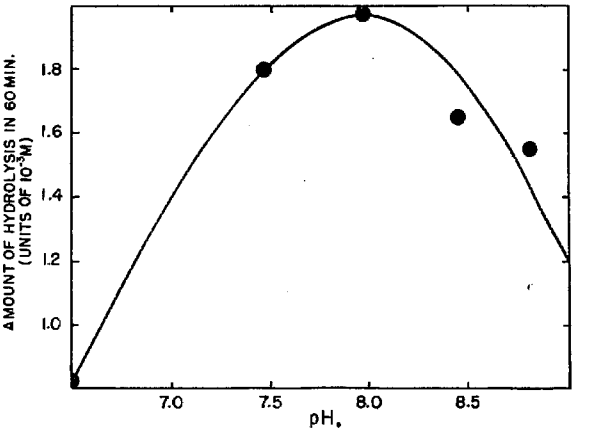

Fig. 1.—*p*H–activity relationship of the system α-chymotrypsin–acetyl-L-hexahydrophenylalaninamide in aqueous solutions at 25° and 0.02 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

For the system

$$E_f + S_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_f + P_{1f} + P_{2f} \ldots \quad (1)$$

where $K_S = (k_2 + k_3)/k_1$, and when zone A conditions[11-13] are satisfied, the kinetic constants K_S and

(1) Supported in part by a grant from Eli Lilly and Co.
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(4) M. Bergmann and J. S. Fruton, *Advances in Enzymology*, **1**, 63 (1941).
(5) J. S. Fruton in D. E. Green "Currents in Biochemical Research," Interscience Publishers, Inc., New York, N. Y., 1946, p. 123, *et seq.*
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k_3 may be evaluated from the corresponding rate equation, *i.e.*, equation 2 where $v = -d[S]/dt$ and $V = k_3[E]$, by rearrangement of this equation to equations 3, 4 or 5 which lend themselves to the evaluation of K_S and k_3 by virtue of the linear plots of $1/v_0$ *vs.* $1/[S]_0$, or $[S]_0/v_0$ *vs.* $[S]_0$, or v_0 *vs.* $v_0/[S]_0$, respectively.[14-18] The plot suggested by Veibel[19] and by Pigman,[20,21] *i.e.*, $1/k'$ *vs.* $[S]$, where k' is the observed "first order" constant is simply a variant of that based on equation 4.

$$v = V[S]/(K_S + [S]) \quad (2)$$

$$1/v = (K_S/V)(1/[S]) + 1/V \quad (3)$$

$$[S]/v = (1/V)[S] + (K_S/V) \quad (4)$$

$$v = -(v/[S])K_S + V \quad (5)$$

While the plot of v_0 *vs.* $v_0/[S]_0$ may possess certain advantages[17,18] it must be emphasized that none of the above plots in themselves provides an adequate solution of the basic operational difficulty of arriving at a reasonable procedure for the estimation of the initial velocities at any given specific substrate concentration particularly when the extrapolation has to be made from a curve constructed from a limited number of points.

There appear to be two general solutions of the problem of estimating initial velocities with a reasonable degree of precision, *viz.*, one, to employ, whenever possible, specific substrates and procedures that will permit continuous observation of the systems under investigation so that the extrapolations to zero time are minimized and thus operationally become relatively unambiguous; and two, for those cases where the above procedures cannot be applied, to devise and use a rational procedure, based upon successive approximations, for determining the initial velocities rather than to depend upon the usual practice of attempting to construct, by visual means, tangents to curves based upon a limited number of experimental points. There is, of course, a third solution[22] which is reminiscent of the procedure of Guggenheim[23,24] in that it avoids the problem of estimating initial velocities by the use of a procedure which does not require them. In this communication we shall give examples of the two latter procedures as applied in an investigation of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-hexahydrophenylalaninamide.

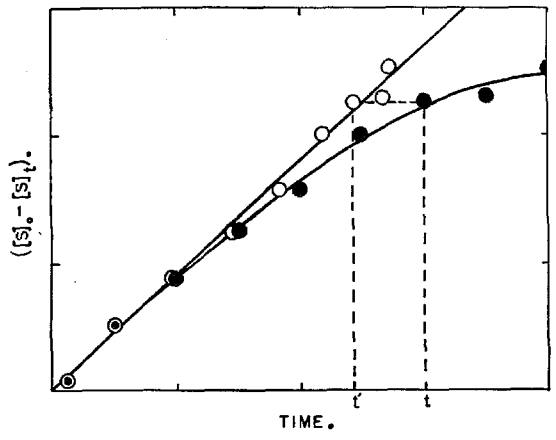

Fig. 2.—Hypothetical zero-order plot: solid circles, observed values; open circles, corresponding values if initial velocity were maintained. Curve through solid circles best fit to observed values; line through open circles the initial velocity.

For the reaction given in equation 1 and when zone A conditions are satisfied[11-13] it is to be expected from the integrated rate equation 6

$$k_3[E]t = K_S \ln [S]_0/[S]_t + ([S]_0 - [S]_t) \quad (6)$$

that a set of conditions exist wherein a plot of $([S]_0 - [S]_t)$, or of $\ln [S]_0/[S]_t$, *vs.* t will give a straight line within the limits of experimental error. However, in practice, the limitations imposed by the solubility of the specific substrate and the sensitivity of the analytical method employed may prevent the attainment of the desired set of conditions and instead of a linear plot a curve will be obtained in both cases.

For the "zero order" case a plot of $([S]_0 - [S]_t)$ *vs.* t will give a curve similar to, but perhaps not as exaggerated as, that depicted in Fig. 2. It will be seen from Fig. 2 that the initial slope of this curve, corresponding to the initial velocity v_0, will be determined by the parameters $([S]_0 - [S]_t)$ and t'. For v_0 we may write equation 7 and upon substitution obtain equation 8. Rearrangement of equation 6 leads to equation 9 and from equations 8 and 9 we may obtain equation 10 where the zero order correction factor $f_0 = t'/t$ and the fraction of the total amount of specific substrate reacting in time t, $h_0 = ([S]_0 - [S]_t)/[S]_0$. In practice it has been found convenient to plot the parameters f_0, h_0 and $K_S/[S]_0$ as a family of curves, as in Fig. 3, and to thus determine f_0 as a function of h_0 for predetermined values of $K_S/[S]_0$.

$$v_0 = k_3[E][S]_0/(K_S + [S]_0) = ([S]_0 - [S]_t)/t' \quad (7)$$

$$t' = ([S]_0 - [S]_t)\{(K_S + [S]_0)/k_3[E][S]_0\} \quad (8)$$

$$t = \{K_S \ln [S]_0/[S]_t + ([S]_0 - [S]_t)\}/k_3[E] \quad (9)$$

$$f_0 = h_0(K_S/[S]_0 + 1)/\{(K_S/[S]_0)\ln(1/(1 - h_0)) + h_0\} \quad (10)$$

A similar correction factor f_1 can also be employed to determine the initial velocities from first order, *i.e.*, from $\ln [S]_0/[S]_t$ *vs.* t, plots as may be seen from equation 11 where $f_1 = t'/t$ and $h_1 = \ln [S]_0/[S]_t$.

$$f_1 = h_1(K_S/[S]_0)/\{(K_S/[S]_0)h_1 + 1 - [S]_t/[S]_0\} \quad (11)$$

As before it has been found convenient to evaluate f_1 graphically from a plot such as that given in Fig. 4 in which f_1 is determined as a function of h_1 for predetermined values of $K_S/[S]_0$.

In the application of the above procedure to the determination of initial velocities, the experimental

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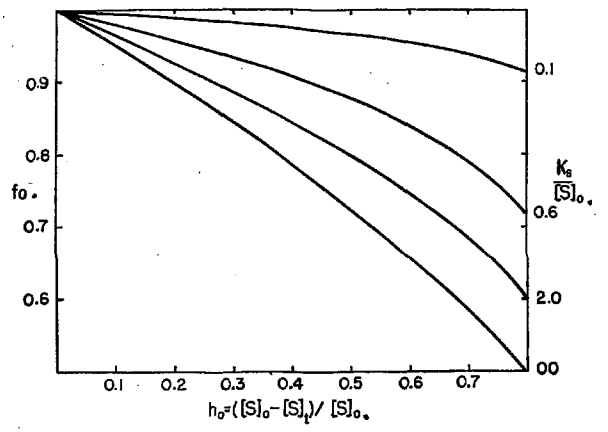

Fig. 3.—Graphical evaluation of the correction factor f_0 for zero-order plots.

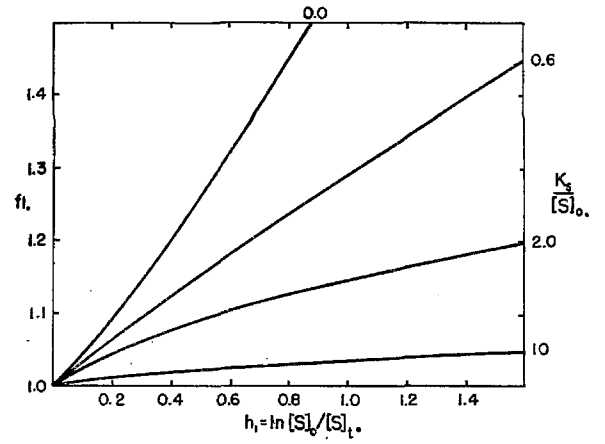

Fig. 4.—Graphical evaluation of the correction factor f_1 for first order plots.

data are first presented in the form of both ($[S_0] - [S]_t$) *vs.* t and ln $[S]_0/[S]_t$ *vs.* t plots. From these plots the initial velocities are estimated by visual means[25] and an approximate value of K_S determined by any of the plots based upon equation 3. This approximate value of K_S may then be used to determine the appropriate correction factors, *i.e.*, f_0 and f_1, and the initial velocities determined from the now linear corrected ($[S]_0 - [S]_t$) *vs.* t and ln $[S]_0/[S]_t$ *vs.* t plots may be used in turn to determine a more precise value of K_S. If the corrected ($[S]_0 - [S]_t$) *vs.* t and the ln $[S]_0/[S]_t$ *vs.* t plots are not linear and the second K_S value differs substantially from the first, the approximation process is repeated until satisfactory results are obtained.[26]

The above procedure was not used extensively in the present study because of the desire to gain experience with the procedure of Walker and Schmidt.[22] However, an example of the approximation method for the determination of initial velocities using data obtained in the present investigation is given in Figs. 5 and 6 and in Table I.[27] It is noteworthy that the initial velocity determined from each plot was 0.040 μM./min.

(25) It can usually be determined by inspection which type of plot will permit the most satisfactory extrapolation. Since only approximate values are needed at this point extrapolation based upon only one type of plot will ordinarily be satisfactory.

(26) Since the factors f_0 and f_1 are not profoundly influenced by modest changes in the value of K_S, a second approximation is rarely required.

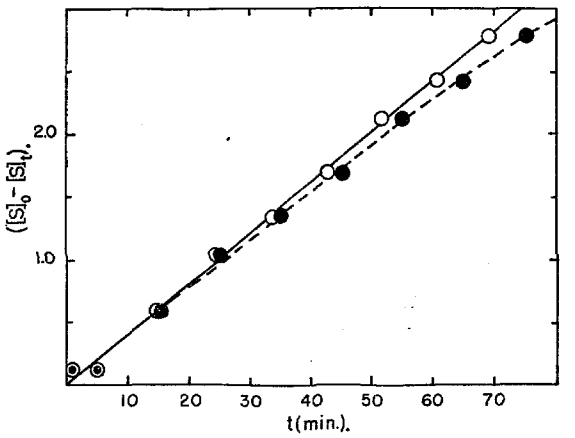

Fig. 5.—Zero-order plot of set of data obtained in experiment no. 33 and summarized in Table I: solid circles, observed values; dotted curve, best fit to observed values; open circles, corrected values; solid line, least squares fit to corrected values.

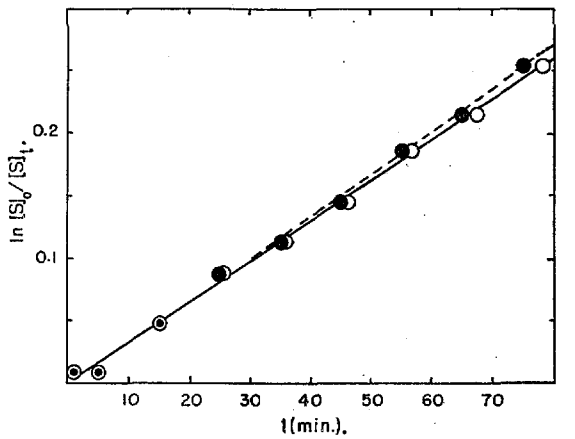

Fig. 6.—First-order plot of set of data obtained in experiment no. 33 and summarized in Table I: solid circles, observed values; dotted curve, best fit to observed values; open circles, corrected values; solid line, least squares fit to corrected values.

In the development of the procedure of Walker and Schmidt[22] it was assumed that the case depicted by equations 12, 13 and 14 was of sufficient general interest to be used as an example. When $K_S = (k_2 + k_3)/k_1$, $K_{P1} = k_5/k_4$ and $K_I = k_7/k_6$, and

$$E_f + S_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_f + P_{1f} + P_{2f} \quad \ldots \quad (12)$$

$$E_f + P_{1f} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EP_1 \quad (13)$$

$$E_f + I_f \underset{k_7}{\overset{k_6}{\rightleftharpoons}} EI \quad (14)$$

when zone A conditions[11-13] are satisfied, the inte-

(27) Further examples of this procedure will be given in subsequent communications from this Laboratory including its application to the recalculation of the kinetic constants of all systems which we have studied previously.

TABLE I

DETERMINATION OF INITIAL VELOCITY FROM ZERO AND FIRST ORDER PLOTS OF DATA OBTAINED IN EXPERIMENT NO. 33[a]

t, min.	$([S]_0 - [S]_t) \times 10^3$ M	$([S]_0 - [S]_t)/[S]_0$	f_0	t_0', min.	$\ln [S]_0/[S]_t$	f_1	t_1', min.
0	0.00	~0	1.000	0.0	0.000	1.000	0.0
1	.12	~1	1.000	1.0	.009	1.000	1.0
5	.12	1	1.000	5.0	.009	1.000	5.0
15	.58	6	0.980	14.7	.048	1.010	15.1
25	1.04	8	.972	24.2	.087	1.015	25.4
35	1.34	11	.960	33.6	.113	1.020	35.7
45	1.69	14	.950	42.8	.145	1.025	46.1
55	2.12	17	.940	51.7	.186	1.030	56.6
65	2.42	19	.933	60.6	.215	1.035	67.3
75	2.79	22	.920	69.0	.253	1.040	78.0

[a] [E] = 0.208 mg. protein-nitrogen/ml., $[S]_0$ = 12.41 $\times 10^{-3}$ M, $K_S/[S]_0 = 2$, f_0 and f_1 from plots given in Figs. 3 and 4, $v_0 = 0.040$ μM./min. from plots given in Figs. 5 and 6.

grated rate equation can be rearranged to give equation 15

$$k_3[E] = K_S(1 + [I]/K_I + [S]_0/K_{P_I})(\ln [S]_0/[S]_t)/t + (1 - K_S/K_{P_I})([S]_0 - [S]_t)/t \quad (15)$$

Thus when [I] = 0 and K_{P_I} is so large that its influence is negligible, it follows from equation 15 that a plot of $([S]_0 - [S]_t)/t$ *vs.* $(\ln [S]_0/[S]_t)/t$ will give a straight line whose slope will be equal to $-K_S$ and whose intercept will be equal to $k_3[E] = V$.[22,28] If [I] is finite, K_{P_I} is again very large and K_S is a known quantity from previous experiments in which [I] = 0 it follows from equation 15 that a plot of $([S]_0 - [S]_t)/t$ *vs.* K_S $(\ln [S]_0/[S]_t)/t$ will give a straight line whose slope will be equal to $-(1 + [I]/K_I)$ and whose intercept will be equal to k_3 [E] = V if the inhibition is competitive.

When K_{P_I} is small and the extent of reaction is appreciable it is to be expected that the procedure of Walker and Schmidt[22] will not lead to results as accurate as those obtained by a method based upon the determination of initial velocities unless some attempt is made to evaluate K_{P_I}. This may be done either by evaluating P_1 as a competitive inhibitor in a system containing another specific substrate whose value of K_S is known and where the value of K_{P_I} for the latter system is negligibly large, or by a method of successive approximations. As an example of the latter procedure an approximate value of K_S is first determined and then the reaction is studied in the presence of an added quantity of P_1 so as to obtain an approximate value of K_{P_I}. This approximate value of K_{P_I} can be applied to obtain a more accurate value of K_S which in turn can be used to evaluate a more accurate value of K_{P_I} and the process repeated until satisfactory values of both K_S and K_{P_I} are obtained. When $K_{P_I} > K_S$ the correct values will be approached quite rapidly since in this case even an approximate value of K_{P_I} will give a reasonably accurate value of K_S.

If the above precautions are heeded, the Walker and Schmidt procedure[22] enjoys an advantage over methods based upon the determination of initial velocities in that it is frequently possible to determine more accurately the amount of reaction occurring in a given time interval than it is to make the measurements necessary for the determination of initial velocities. However, it does suffer from the disadvantage that any change in [E] will cause far more serious errors than in any method based upon initial velocities. Therefore some assurance must be provided that during the time interval used no change in [E] occurs.

The α-chymotrypsin-catalyzed hydrolysis of acetyl-L-hexahydrophenylalaninamide was observed in two different ways. In the first, the extent of reaction at various times was determined by withdrawing and titrating a single aliquot, and in the second, several aliquots were withdrawn and titrated at the start of a given experiment and a second set withdrawn and titrated after a preselected time interval which was usually 60 minutes. Since it was desired to present all of the data in the form of a Walker–Schmidt plot[22] the data obtained by the first procedure were first presented in the form of a $([S]_0 - [S]_t)$ *vs.* t plot, the experimental points then corrected with the aid of the factor f_0 as described earlier, the best straight line drawn through the corrected points, and then by an "uncorrecting" process the amount of hydrolysis occurring in 60 minutes was determined by using the corrected linear plot to reconstruct the portion of the experimental curve in the vicinity of 60 minutes. The plot given in Fig. 5 and the data summarized in Table I is a representative example of this latter operation.

Sixty separate experiments were conducted and of this number the results of nine, *i.e.*, experiments no. 19–23, 36, 43, 44 and 46, were so obviously aberrant that they were not considered further. A second set of experiments, *i.e.*, no. 1, 9, 18, 25 and 40 also gave results which were sufficiently erratic as to justify their exclusion although in this instance the variations observed were not markedly greater than those expected on the basis of the probable experimental error. Experiments no. 53–60 were conducted with an enzyme preparation of slightly lesser activity than that used for the remainder of the experiments and for this reason will be considered separately.

Since it was anticipated from previous studies[10,29] that the K_{P_I} value of acetyl-L-hexahydrophenylalanine was of the order of 0.1 M it was concluded that in the case at hand K_S and k_3 could be evaluated by a plot of $([S]_0 - [S]_t)/t$ *vs.* $(\ln [S]_0/[S]_t)/t$ as K_{P_I} was sufficiently large to be ignored. The results of the remaining 38 experiments are summarized in Table II and in Fig. 7 where a least squares analysis with the quantity $(\ln [S]_0/[S]_t)/60$ as the so-called errorless parameter gave a value of $K_S = 26 \pm 3 \times 10^{-3}$ M and a value of $k_3 = 0.61 \times 10^{-3}$ mole/min./mg. protein-nitrogen/ml. With the quantity $([S]_0 - [S]_t)/60$ as the errorless parameter, a value of $K_S = 29 \pm 2 \times 10^{-3}$ M and $k_3 = 0.64 \times 10^{-3}$ mole/min./mg. protein-nitrogen/ml. was obtained. The data from experiments no. 53–60, *cf.* Table III and Fig. 8, gave, on the basis

(28) Although Walker and Schmidt[22] indicate that equal time intervals should be used there appears to be no compelling reason for this precaution in this particular case.

(29) D. W. Thomas, R. V. MacAllister and C. Niemann, THIS JOURNAL, **73**, 1548 (1951).

TABLE II

SUMMARY OF EXPERIMENTS USED FOR PLOT GIVEN IN FIG. 7[a]

Expt. no.	$[S]_0$ $\times 10^3$ M	$([S]_0 - [S]_{60})/60$ $\times 10^5$ mole/min.	$(\ln [S]_0/[S]_{60})/60$ $\times 10^3$/min.
2	10.00	3.37	3.74
3	10.00	3.08	3.38
11	11.00	3.65	3.69
16	12.53	3.92	3.46
33	12.41	3.77	3.34
39	12.41	3.77	3.34
48[b]	12.54	3.68	3.24
50	12.59	4.03	3.56
51[b]	12.50	3.67	3.23
52[b]	12.50	3.70	3.26
15	12.88	3.75	3.20
28	14.39	4.35	3.34
29	14.53	4.45	3.40
30	14.34	4.07	3.11
8	15.00	4.30	3.14
17	15.03	4.10	2.98
12	16.67	4.47	2.90
14	16.67	4.73	3.11
4[b]	20.00	4.97	2.72
24	20.14	4.97	2.72
32	20.00	5.32	2.90
35	20.03	5.22	2.83
38	20.00	5.15	2.80
13	22.22	5.77	2.82
6	25.00	5.66	2.44
27	25.47	6.03	2.55
5	30.00	6.50	2.32
7	35.00	6.92	2.09
10	40.00	7.76	2.06
26	40.00	7.50	1.99
31	40.10	7.51	2.00
34	40.00	7.53	2.01
37	40.10	7.68	2.03
41[b]	40.00	7.25	1.91
42[b,c]	40.10	7.20	1.89
45[b]	40.00	7.78	2.07
47[b]	40.00	7.87	2.09
49[b]	40.00	7.87	2.09

[a] Performed at 25° and *p*H 7.9 in aqueous solutions 0.02 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer, [E] = 0.208 mg. protein-nitrogen/ml. unless otherwise noted. [b] Experiments in which observations were limited to $t = 0$ and $t = 60$, for all others, value at $t = 60$ determined as described in text. [c] [E] = 0.104 mg. protein-nitrogen/ml.

TABLE III

SUMMARY OF EXPERIMENTS USED FOR PLOT GIVEN IN FIG. 8[a]

Expt. no.[b]	$[S]_0$ $\times 10^3$ M	$([S]_0 - [S]_{60})/60$ $\times 10^5$ mole/min.	$(\ln [S]_0/[S]_{60})/60$ $\times 10^3$/min.
53	12.54	3.44	2.99
56	12.54	3.74	3.28
58[c]	12.54	3.45	3.01
60[c]	25.00	5.63	2.54
54	39.90	6.92	1.83
55	40.00	7.17	1.89
57	40.05	7.22	1.90
59[c]	40.00	7.37	1.95

[a] Performed at 25° and *p*H 7.9 in aqueous solutions 0.02 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer, [E] = 0.196 mg. protein-nitrogen/ml. unless otherwise noted. [b] In all experiments observations limited to $t = 0$ and $t = 60$; [c] [E] = 0.098 mg. protein-nitrogen/ml.

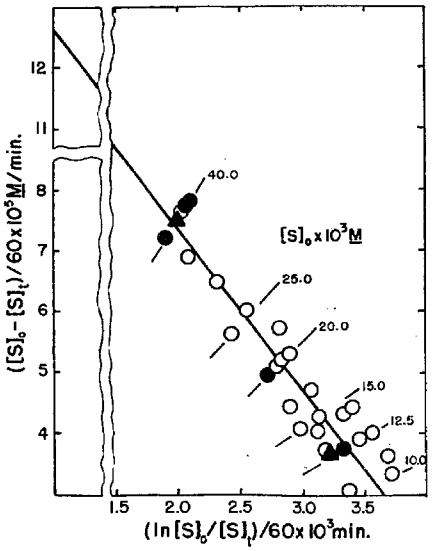

Fig. 7.—Walker–Schmidt plot of the results of the 38 experiments summarized in Table II: open circles, results of single experiments; solid circles, mean of duplicate experiments; solid triangles, mean of triplicate experiments. Line is a least squares fit.

of a least squares treatment with $(\ln [S]_0/[S]_t)/60$ as the errorless parameter a value of $K_S = 30 \pm 3 \times 10^{-3}$ *M* and a value of $k_3 = 0.67 \times 10^{-3}$ mole/min./mg. protein-nitrogen/ml. However, since the lesser activity of the enzyme preparation used in these experiments was probably due to exposure to the preparation to the atmosphere for a prolonged period the kinetic constants obtained from experiments no. 53–60 should not be given as much weight as those obtained from the more extended series described above.

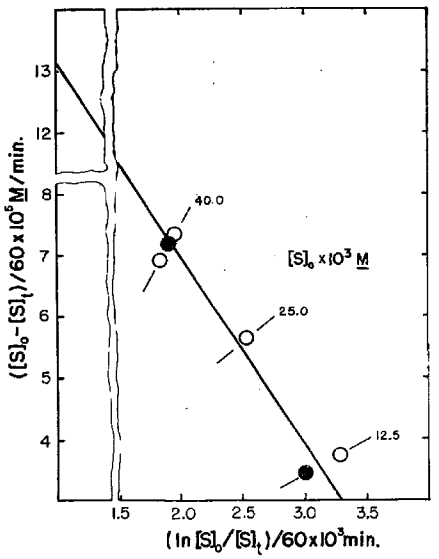

Fig. 8.—Walker–Schmidt plot of the results of the 8 experiments summarized in Table III: open circles, results of single experiments; solid circles, mean of duplicate experiments. Line is a least squares fit.

From a comparison of the K_S and k_3 values of acetyl-L-phenylalaninamide, *i.e.*, $34 \pm 5 \times 10^{-3}$ *M* and 0.7×10^{-3} mole/min./mg. protein-nitrogen/

ml.,[10] and of acetyl-L-hexahydrophenylalaninamide, *i.e.*, $27 \pm 4 \times 10^{-3}$ *M* and 0.6×10^{-3} mole/min./mg. protein-nitrogen/ml., it can be seen that the replacement of a benzyl group by a hexahydrobenzyl group has relatively little effect upon either the K_S or k_3 values, at least at 25° and *p*H 7.9. Since there is reason to interpret the K_S values of these two specific substrates in terms of the corresponding enzyme–substrate dissociation constants, *i.e.*, the k_2/k_1 values,[9,13,29–31] it appears that the π-electrons present in an aromatic side chain are not involved in the combination process. The possible greater affinity of the active site for the corresponding hydroaromatic side chain may be due to the slightly greater effective mass of the latter if, as it seems likely that, van der Waals forces are the principal forces involved in the combination of uncharged specific substrates and competitive inhibitors with the active site of the enzyme.

Experimental[32,33]

N-Acetyl-L-hexahydrophenylalanine.[34]—A solution of 12.6 g. of acetyl-L-phenylalanine in 75 ml. of glacial acetic acid was hydrogenated over platinic oxide at 40 p.s.i. of hydrogen at 25°, the reaction mixture filtered, the filtrate largely freed of solvent, the residue triturated with water, the colorless crystalline solid collected and dried to give 11.8 g. of product, m.p. 182–183°. Recrystallization from water gave a product, m.p. 188–189°, $[\alpha]^{25}D$ −5.5° (*c* 8.2% in ethanol). A portion of the product, m.p. 182–183°, was hydrolyzed with aqueous hydrochloric acid, the hydrolysate neutralized and treated with *p*-toluenesulfonyl chloride and aqueous sodium hydroxide to give *p*-toluenesulfamido-L-hexahydrophenylalanine, m.p. 162–163°, lit.[35] m.p. 160.5°.

Acetyl-L-hexahydrophenylalaninamide.—A solution of 11.8 g. of acetyl-L-hexahydrophenylalanine, m.p. 182–183°, in 80 ml. of absolute ethanol was saturated at 0° with dry hydrogen chloride, the reaction mixture allowed to stand at 25° for 3 days, and then evaporated *in vacuo* to a thick sirup. The sirup was dissolved in 200 ml. of methanol, the solution saturated at 0° with anhydrous ammonia, the reaction mixture allowed to stand at 25° for one week, then evaporated to dryness, the solid residue extracted with chloroform and the residual solid recrystallized twice from water to give 5.6 g. of the desired amide, colorless needles, m.p. 156–157°, $[\alpha]^{25}D$ −16.5 ± 0.7° (*c* 3.8% in ethanol). Recrystallization of this product from ethyl acetate and then from water gave a product of identical m.p.

Anal. Calcd. for $C_{11}H_{20}O_2N_2$ (212.3): C, 62.2; H, 9.5; N, 13.2. Found: C, 62.0; H, 9.6; N, 13.6.

Acetyl-DL-hexahydrophenylalaninamide, m.p. 205–207°, was prepared in an analogous manner from either acetyl-DL-phenylalanine or acetyl-DL-tyrosine except in the latter instance ethanol was used in lieu of glacial acetic acid in the hydrogenation and hydrogenolysis of acetyl-DL-tyrosine.

Enzyme Experiments.—The general technique was identical with that described previously[13] and in every instance a formol titration[13] was used to follow the course of the reaction. All measurements were made at 25° in solutions 0.02 *M* with respect to the amine component of a tris-(hydroxymethyl)-amino methane–hydrochloric acid buffer and the enzyme preparation was Armour lot no. 90402. The enzyme preparation used in experiments no. 53–60 was a sample of the same lot no. which was lost for a period and when recovered was found to possess a diminished activity corresponding to a lesser amount of protein-nitrogen. It will be noted that in all experiments the relative concentrations of E and S were such as to permit the attainment of zone A conditions[11–13] and that there is no question as to the stability of α-chymotrypsin in aqueous solutions at 25° for the periods required in this investigation.[13,36]

(30) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 3223 (1951).

(31) H. T. Huang, R. V. MacAllister, D. W. Thomas and C. Niemann, *ibid.*, **73**, 3231 (1951).

(32) All melting points are corrected.

(33) Microanalyses by Dr. A. Elek.

(34) D. Shemin and R. M. Herbst, *ibid.*, **61**, 2471 (1939).

(35) P. Karrer and W. Kehl, *Helv. Chim. Acta*, **13**, 50 (1930).

(36) D. S. Hogness and C. Niemann, *ibid.*, **75**, 884 (1953).

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SUPPLEMENTARY EXPERIMENTAL SECTION

The requirements of brevity in publication do not permit the inclusion of bulky tables such as those presenting the primary experimental data from which the results given in the text were extracted. However, it seemed advisable to offer these data in an organized form in some accessible place, hence this supplementary section.

By way of brief comment, it must be confessed that the experimental results obtained are not of a high order of precision. Furthermore, the amount of data which has been rejected may seem excessively large. Nevertheless, the text gives the best interpretation which could be placed on the experiments, and it may be considered to be reliable within the specified limits of accuracy.

TABLE IV

Primary Data For Experiments Presented in Table II and Figure 7 of the Publication

Expt. No.	$[S]_o$ x 10^3 [M]	$[E]_o$ mg. protein N/ml.	Time [Min]	Titer [ml]	Interpolated Titers 0 Min.	Interpolated Titers t Min.	Normality of Base
2	10.00	0.208	2	1.505	1.508	1.710	0.0100
			13	1.555		t = 60	
			25½	1.600			
			35	1.645			
			45	1.657			
			60	1.710			
3	10.00	0.208	2	1.450	1.469	1.654	0.0100
			10	1.500		t = 60	
			20	1.540			
			30	1.570			
			40	1.595			
			50	1.630			
			60	1.650			
11	11.00	0.208	2	1.375	1.380	1.599	0.0100
			10	1.425		t = 60	
			20	1.440			
			30	1.496			
			40	1.541			
			50	1.573			
			60	1.593			
			75	1.647			
			90	1.676			
16	12.53	0.208	2	1.411	1.428	1.664	0.0100
			10	1.470		t = 60	
			30	1.545			
			38	1.583			
			45	1.607			
			52	1.642			
			60	1.660			
			70	1.702			

Expt. No.	$[S]_o$ x 10^3 [M]	$[E]_o$ mg. protein N/ml.	Time [Min]	Titer [ml.]	Interpolated Titers [ml.] 0 min.	t min.	Normality of Base
33	12.41	0.208	1	1.308	1.298	1.520	0.01023
			5	1.309		t = 60	
			15	1.353			
			25	1.400			
			35	1.428			
			45	1.463			
			55	1.504			
			65	1.534			
			75	1.571			
39	12.41	0.208	1	1.374	1.374	1.600	0.01022
			5½	1.400		t = 60	
			15	1.438			
			25	1.468			
			35	1.500			
			45	1.530			
			55	1.575			
			60	1.595			
			75	1.650			
48[a]	12.54	0.416	1	1.697	1.726	1.945	0.01008
			1½	1.740		t = 30	
			2	1.730			
			2½	1.750			
			60	2.130			
			60½	2.140			
			61	2.143			
			61½	2.117			
50[a]	12.59	0.416	1	1.660	1.715	1.959	0.01008
			5	1.750		t = 30	
			15	1.847			
			25	1.932			
			35	1.990			
			40	2.035			
			50	2.098			
			60	2.150			
			60½	2.122			

Expt. No.	$[S]_o$ x 10^3 [M]	$[E]_o$ mg. protein N/ml.	Time [Min]	Titer [ml.]	Interpolated Titers [ml.] 0 Min.	t Min.	Normality of Base
51[a]	12.50	0.416	1	1.698	1.698	1.916	0.01008
			1½	1.720		t = 30	
			2	1.710			
			2½	1.714			
			60	2.110			
			60½	2.100			
			61	2.104			
			61½	2.116			
52[a]	12.50	0.416	1	1.720	1.715	1.936	0.01008
			1½	1.735		t = 30	
			2	1.735			
			2½	1.730			
			60	2.120			
			60½	2.136			
			61	2.127			
			61½	2.127			
15	12.88	0.208	2	1.413	1.430	1.654	0.01000
			10	1.469		t = 60	
			20	1.503			
			30	1.553			
			40	1.583			
			50	1.617			
			60	1.653			
28	14.39	0.208	5	1.378	1.351	1.606	0.01023
			10	1.410		t = 60	
			20	1.449			
			30	1.493			
			40	1.528			
			50	1.563			
			60	1.613			
			70	1.660			
			80	1.687			
29	14.53	0.208	1	1.381	1.388	1.650	0.01023
			5	1.413		t = 60	
			10	1.441			
			15	1.450			
			20	1.471			
			30	1.521			
			40	1.567			
			50	1.613			
			60	1.644			

Expt. No.	$[S]_o \times 10^3$ [M]	$[E]_o$ mg. protein N/ml.	Time [Min]	Titer [ml.]	Interpolated Titers [ml.] 0 Min.	t Min.	Normality of Base
30	14.34	0.208	1	1.399	1.390	1.630	0.01023
			5	1.410		t = 60	
			10	1.430			
			15	1.450			
			20½	1.480			
			30	1.510			
			40	1.551			
			50	1.600			
			60	1.623			
8	15.00	0.208	2	1.405	1.396	1.654	0.01000
			10	1.425		t = 60	
			20	1.490			
			30	1.530			
			40	1.570			
			50	1.610			
			60	1.665			
			75	1.720			
			90	1.790			
17	15.03	0.208	2	1.425	1.432	1.678	0.01000
			10	1.471		t = 60	
			20	1.520			
			30	1.560			
			40	1.593			
			50	1.651			
			59	1.677			
			60	1.677			
12	16.67	0.208	2	1.37	1.379	1.647	0.0100
			10	1.425		t = 60	
			20	1.491			
			30	1.515			
			36	1.566			
			45	1.595			
			60	1.646			
			75	1.700			
			90	1.775			
14	16.67	0.208	2	1.394	1.408	1.692	0.0100
			6	1.435		t = 60	
			10	1.463			
			20	1.507			
			30	1.566			
			41	1.613			
			50	1.639			
			60	1.691			

Expt. No.	$[S]_o \times 10^3$ [M]	$[E]_o$ mg. protein N/ml.	Time [Min]	Titer [ml.]	Interpolated Titers [ml.] 0 Min.	t Min.	Normality of Base
4	20.0	0.208	2	1.520	1.519	1.818	0.01000
			10	1.570		t = 60	
			20	1.625			
			30	1.670			
			40	1.735			
			50	1.770			
			60	1.810			
24	20.14	0.208	5	1.400	1.371	1.668	0.01000
			6	1.400		t = 60	
			15	1.456			
			30	1.530			
			31	1.513			
			45	1.604			
			46	1.592			
			60	1.672			
			61	1.672			
32	20.00	0.208	6	1.320	1.293	1.606	0.01023
			15	1.380		t = 60	
			25	1.426			
			35	1.480			
			45	1.535			
			55	1.574			
			65	1.630			
35	20.03	0.208	1	1.317	1.312	1.618	0.01023
			5	1.335		t = 60	
			15	1.400			
			25	1.450			
			35	1.491			
			40	1.526			
			45	1.550			
			55	1.593			
			65	1.637			

Expt. No.	$[S]_o$ x 10^3 [M]	$[E]_o$ mg. protein N/ml.	Time [Min]	Titer [ml]	Interpolated Titers [ml.] 0 Min.	t Min.	Normality of Base
38	20.00	0.208	1	1.347	1.374	1.677	0.01022
			5	1.399		t = 60	
			15	1.454			
			25	1.514			
			35	1.556			
			$45\frac{1}{2}$	1.625			
			55	1.661			
			65	1.700			
			75	1.740			
13	22.22	0.208	2	1.583	1.570	1.916	0.01000
			5	1.596		t = 60	
			10	1.638			
			20	1.721			
			30	1.754			
			40	1.795			
			50	1.869			
			60	1.917			
6	25.00	0.208	2	1.460	1.485	1.824	0.01000
			10	1.530		t = 60	
			20	1.605			
			30	1.660			
			45	1.740			
			60	1.825			
			75	1.905			
			90	1.980			
			121	2.150			
27	25.47	0.208	5	1.400	1.375	1.728	0.01023
			10	1.440		t = 60	
			15	1.477			
			20	1.493			
			30	1.565			
			40	1.624			
			50	1.675			
			60	1.720			

Expt. No.	$[S]_o$ x 10^3 [M]	$[E]_o$ mg. protein N/ml.	Time [Min]	Titer [ml.]	Interpolated Titers [ml.] 0 Min.	t Min.	Normality of Base
5	30.00	0.208	2	1.530	1.540	1.930	0.01000
			10	1.655		t = 60	
			20	1.675			
			30	1.730			
			40	1.805			
			50	1.880			
			60	1.930			
7	35.00	0.208	2	1.430	1.416	1.831	0.01000
			11	1.495		t = 60	
			20	1.560			
			30	1.645			
			40	1.695			
			50	1.775			
			60	1.830			
			75	1.925			
10	40.00	0.208	2	1.370	1.365	1.831	0.01000
			10	1.440		t = 60	
			20	1.530			
			30	1.600			
			40	1.690			
			50	1.750			
			60	1.830			
			75	1.940			
			105	2.120			
26	40.00	0.208	5	1.408	1.381	1.821	0.01023
			10	1.457		t = 60	
			15	1.500			
			20	1.537			
			30	1.604			
			40	1.695			
			50	1.742			
			60	1.823			
31	40.10	0.208	1	1.388	1.372	1.813	0.01023
			5	1.410		t = 60	
			15	1.490			
			25	1.558			
			35	1.630			
			45	1.703			
			55	1.779			
			65	1.850			
			75	1.899			

Expt. No.	$[S]_o$ x 10^3 [M]	$[E]_o$ mg. protein N/ml.	Time [Min]	Titer [ml.]	Interpolated Titers [ml.] 0 Min.	t Min.	Normality of Base
34	40.00	0.208	1	1.314	1.311	1.752	0.01023
			5	1.350		t = 60	
			15	1.427			
			25	1.503			
			35	1.581			
			45	1.663			
			55	1.720			
			65	1.787			
			75	1.860			
37	40.10	0.208	1	1.345	1.370	1.820	0.01022
			5	1.428		t = 60	
			15	1.483			
			25	1.559			
			35	1.645			
			45	1.715			
			55	1.794			
			65	1.907			
41	40.00	0.208	1	1.384	1.378	1.803	0.01022
			1½	1.390		t = 60	
			2	1.396			
			2½	1.405			
			60	1.800			
			60½	1.826			
			61	1.810			
			61½	1.807			
42[a]	40.10	0.104	1	1.265	1.253	1.676	0.01022
			1½	1.254		t = 120	
			2	1.268			
			2½	1.250			
			60	1.470			
			61	1.470			
			61½	1.479			
			62	1.488			
45	40.00	0.208	1	1.490	1.455	1.917	0.01008
			1½	1.470		t = 60	
			2	1.470			
			2½	1.467			
			60	1.890			
			60½	1.930			
			61	1.930			
			61½	1.950			

Expt. No.	$[S]_o$ x 10^3 [M]	$[E]_o$ mg. protein N/ml	Time [Min]	Titer [ml.]	Interpolated Titers [ml.] 0 Min.	t Min.	Normality of Base
47	40.00	0.208	1	1.444	1.440	1.907	0.01008
			1½	1.453		t 60	
			2	1.454			
			2½	1.460			
			60	1.900			
			60½	1.913			
			61	1.920			
			61½	1.920			
49	40.00	0.208	1	1.423	1.414	1.883	0.01008
			1½	1.433		t 60	
			2	1.420			
			2½	1.437			
			60	1.887			
			60½	1.887			
			61	1.893			
			61½	1.883			

TABLE V

Primary Data For Experiments Presented in Table III

and Figure 8 of the Publication

Expt. No.	$[S]_o$ x 10^3 [M]	$[E]_o$ mg. protein N/ml.	Time [Min]	Titer [ml.]	Interpolated Titers [ml.] 0 Min.	Interpolated Titers [ml.] t Min.	Normality of Base
53[a]	12.54	0.196	1	1.293	1.295	1.487	0.01075
			1½	1.300		t = 60	
			2	1.302			
			2½	1.310			
			120	1.624			
			120½	1.656			
			121	1.660			
			121½	1.665			
56[a]	12.54	0.196	1	1.296	1.283	1.492	0.01075
			1½	1.296		t = 60	
			2	1.291			
			2½	1.296			
			120	1.673			
			120½	1.664			
			121	1.677			
			121½	1.675			
58[a]	12.54	0.098	2½	1.480	1.472	1.681	0.00989
			3	1.475		t = 120	
			3½	1.482			
			4	1.504			
			120	1.680			
			120½	1.685			
			121½	1.685			
			122	1.695			
60[a]	25.00	0.098	1	1.475	1.463	1.805	0.00989
			1½	1.463		t = 120	
			2	1.474			
			2½	1.470			
			90	1.720			
			90½	1.716			
			91	1.730			
			91½	1.724			

Expt. No.	$[S]_o$ x 10^3 [M]	$[E]_o$ mg. protein N/ml	Time [Min]	Titer [ml.]	Interpolated Titers [ml.] 0 Min.	t Min.	Normality of Base
54	39.90	0.196	$1\frac{1}{2}$	1.308	1.302	1.688	0.01075
			2	1.323		t = 60	
			3	1.322			
			4	1.333			
			60	1.690			
			$60\frac{1}{2}$	1.692			
			61	1.693			
			$61\frac{1}{2}$	1.712			
55	40.00	0.196	1	1.290	1.284	1.684	0.01075
			$1\frac{1}{2}$	1.293		t = 60	
			2	1.295			
			$2\frac{1}{2}$	1.305			
			60	1.667			
			$60\frac{1}{2}$	1.688			
			61	1.692			
			$61\frac{1}{2}$	1.692			
59[a]	40.00	0.098	1	1.482	1.477	1.924	0.0989
			$1\frac{1}{2}$	1.488		t = 120	
			2	1.484			
			$2\frac{1}{2}$	1.485			
			60	1.705			
			$60\frac{1}{2}$	1.700			
			61	1.704			
			62	1.713			

TABLE VI

Primary Data For The pH Optimum Determination Presented in Figure 1 of the Publication

The substrate concentration was 10 x 10^{-3} M and the enzyme concentration 0.208 mg. protein nitrogen/ml. for each experiment.

Expt. No.	pH	Time [Min]	Titer [ml.]	Interpolated Titers [ml.] 0 Min.	t Min.	Normality of Base	Amt. of Hydrolysis in 60 min. x 10^{3} [M]
1	6.50	1	2.110	2.111	2.193	0.0100	0.82
		2	2.116				
		60	2.200				
		61	2.187				
2	7.50	1	1.781	1.780	1.960	0.0100	1.80
		2	1.788				
		60	1.953				
		61	1.970				
3	8.43	1	0.801	0.800	0.965	0.0100	1.65
		2	0.805				
		60	0.960				
		61	0.972				
4	8.80	1	0.495	0.488	0.644	0.0100	1.56
		2	0.500				
		60	0.647				
		61	0.651				
5	7.90	1	1.320	1.318	1.505	0.0100	1.87
		2	1.323				
		60	1.495				
		61	1.515				

TABLE VII

Primary Data For Experiments Rejected as Aberrent

Expt. No.	$[S]_o$ x 10^3 [M]	$[E]_o$ mg. protein N/ml.	Time [Min]	Titer [ml.]	Normality of Base
1	10.00	0.208	1	1.39	0.01000
			10	1.44	
			15	1.46	
			30	1.52	
			45	1.57	
			60	1.61	
9	12.50	0.208	2	1.39	0.01000
			10	1.44	
			20	1.47	
			30	1.54	
			40	1.56	
			50	1.61	
			60	1.64	
			75	1.68	
18	12.92	0.208	2	1.437	0.01000
			9½	1.464	
			10	1.470	
			20	1.483	
			29½	1.520	
			40	1.599	
			50	1.637	
			63	1.693	
19	9.92	0.208	5	1.557	0.01000
			6	1.573	
			15	1.614	
			16	1.610	
			30	1.657	
			31	1.667	
			45	1.722	
			46	1.716	

Expt. No.	$[S]_o$ x 10^3 [M]	$[E]_o$ mg. protein N/ml.	Time [Min]	Titer [ml.]	Normality of Base
20	11.46	0.208	$5\frac{1}{2}$	1.313	0.01000
			6	1.338	
			15	1.347	
			16	1.390	
			30	1.443	
			45	1.497	
			68	1.593	
			80	1.648	
			$91\frac{1}{2}$	1.680	
21	12.79	0.208	5	1.360	0.01000
			6	1.370	
			15	1.403	
			16	1.423	
			30	1.476	
			31	1.483	
			45	1.526	
			60	1.574	
			61	1.603	
22	13.92	0.208	5	1.323	0.01000
			6	1.327	
			15	1.383	
			30	1.460	
			31	1.467	
			45	1.526	
			60	1.576	
			75	1.648	
			90	1.752	
23	16.80	0.208	5	1.373	0.01000
			6	1.380	
			15	1.437	
			30	1.516	
			31	1.513	
			45	1.584	
			46	1.580	
			60	1.657	
			61	1.660	

Expt. No.	$[S]_o$ x 10^3 [M]	$[E]_o$ mg. protein N/ml.	Time [Min]	Titer [ml.]	Normality of Base
25	20.14	0.208	5	1.400	0.01000
			6	1.400	
			15	1.456	
			30	1.530	
			31	1.513	
			45	1.604	
			46	1.592	
			60	1.672	
			61	1.672	
36	12.5*	0.208	1	1.317	0.01023
			5	1.357	
			17	1.416	
			25	1.455	
			35	1.498	
			45	1.536	
			55	1.560	
			65	1.600	
40	12.35	0.208	1	1.350	0.01022
			5	1.375	
			22	1.445	
			40	1.505	
			60	1.593	
			80	1.665	
			100	1.730	
			121	1.780	
			140	1.823	
43	40.00	0.416	Not computed		
44	12.50	0.416	1	1.610	0.01008
			1½	1.627	
			2	1.660	
			2½	1.690	
			60	2.120	
			60½	2.135	
			61	2.150	
			61½	2.160	

* Approximate

Expt. No.	$[S]_o$ x 10^3 [M]	$[E]_o$ mg. protein N/ml.	Time [Min]	Titer [ml.]	Normality of Base
46	12.43	0.416	1	1.680	0.01008
			5	1.730	
			15	1.830	
			25	1.923	
			35	1.970	
			45	2.000	
			55	2.285	
			65	2.125	
			75	2.227	

PART II

THE EFFECT OF pH ON THE KINETIC CONSTANTS FOR THE ALPHA-CHYMOTRYPSIN-CATALYZED HYDROLYSES OF L-TYROSINHYDROXAMIDE AND ACETYL-L-TYROSINHYDROXAMIDE; THE EFFECT OF pH ON THE INHIBITION BY SELECTED COMPOUNDS OF THE ALPHA-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINHYDROXAMIDE.

INTRODUCTION

One of the most common observations made with respect to enzyme-catalyzed reactions is that the reaction appears to proceed most readily at some one pH. This pH optimum phenomenon does not appear in all enzyme-catalyzed reactions, but it does seem to be present in a majority of such reaction systems; and, because of its widespread occurrence, its explanation constitutes one of the major problems in quantitative enzymology. Naturally the pH optimum effect has been the subject of rather extensive discussion (for recent reviews see 1 - 7), but rather less experimentation. No extended studies of the effect of pH on reactions catalyzed by proteolytic enzymes have been carried out, possibly because of the lack of suitable analytical techniques.

The derivation of equations intended to give quantitative explanation to the pH optimum phenomenon usually has taken the form of an extension of the original mechanism for enzyme-catalyzed reactions proposed by Michaelis and Menten (8). Ordinarily the derivation of these equations is carried out with the assumption that, of all of the reactions involved in the various schemes, only the breakdown of the ultimate enzyme-substrate complex constitutes a rate determining step. The more rigorous steady-state solution has been applied to such reaction schemes by Alberty (4), who states that the forms of the resulting equations are the same as those derived on the basis of the equilibrium assumptions. The only difference lies in the interpretation of the constant which corresponds to the Michaelis - Menten constant in simpler formulations (cf. 8). This problem has been considered in these simpler formulations first by Haldane (9) and, subsequently, by many others. These equations describing the influence

of pH on kinetic reactions have been fitted to experimental data in some instances (cf. examples in 3, 5, and 7); however, predictions based on the application of the values of the constants thus obtained to other reactions involving the same enzyme have ordinarily not been made, chiefly because only one substrate is known for most of the enzymes so investigated.

The enzyme alpha-chymotrypsin seems particularly well suited to the study of the pH optimum effect. Its desirable properties for the study of enzyme reactions in general have been discussed previously (10, 11). This enzyme has a large number of recognized substrates, many of which have already been subjected to considerable study. Also, many competitive inhibitors (cf. 7) of the reactions catalyzed by this enzyme have been discovered and studied. The large number of substrates and inhibitors which have been uncovered in these past investigations make this enzyme a particularly fruitful one for the exploration of the pH optimum effect.

The substrates employed in the present investigation, L-tyrosinhydroxamide and acetyl-L-tyrosinhydroxamide, were selected because of an excellent colorimetric analytical technique which has been developed for hydroxamides (12, 13, 14). Both of these substrates possess ionizable groups. This is an advantage in one respect: it is certainly desirable to obtain information regarding the effect of pH on the hydrolysis of substrates possessing such ionizable groups, and a disadvantage in another respect: for the initial investigation of the effect of pH on the enzyme-catalyzed reaction, it would perhaps be preferable to avoid the complexity introduced by such ionizing groups and to study a neutral substrate. Although such neutral substrates now appear to be available for study (15), the analytical techniques

for these substrates had not been sufficiently well refined at the time the experimentation on the pH optimum behavior of alpha-chymotrypsin was started. Previous investigators have determined the pH optimum for acetyl-L-tyrosinhydroxamide and L-tyrosinhydroxamide and some tentative conclusions were advanced regarding the effects observed (14, 16). The kinetic constants were also determined for each of these substrates at its optimum pH.

These experiments were undertaken, then, with the object of determining the influence of hydrogen ion concentration on the kinetic course of the hydrolysis of the above substrates, both in the absence and presence of competitive inhibitors of various ionizing types. Through observation of the behavior of these constants with changing pH, it was hoped that a mechanism fitting the experimental data might be proposed which would allow some conclusions to be drawn regarding various kinetically important ionizing groups on the enzyme.

CALCULATIONS AND EXPERIMENTAL RESULTS

Some general considerations. The determination of the kinetic constants for an enzyme-catalyzed reaction ordinarily requires that a series of kinetic studies be made with the enzyme concentration held at a single value and the initial substrate concentration for each experiment varied over a fairly wide range. If the influence of pH on these kinetic constants is to be studied, then, under ideal conditions, a set of kinetic experiments would be made at each of several pH's, the constants would be evaluated for each pH, and, finally, some conclusions regarding the effect of pH on the kinetic constants of the reaction might be reached. While this ideal might be attainable in some cases, it was found necessary to effect some compromises in the present series of experiments.

First of all, the progress of the hydrolysis reaction causes the liberation of a carboxylic acid and hydroxylamine. Since hydroxylamine is a very weak base, the net effect is the release of acid into the solution. When the substrate concentration is rather large, the amount of hydrolysis which is required to give satisfactory information regarding the course of the reaction may cause rather large amounts of acid to be liberated. The pH might be maintained at a constant value in spite of these increasing amounts of acidity either by the continuous addition of alkali to the reaction mixture (in conjunction with a pH measuring device) or by the use of large amounts of buffer. The dilution of the reaction mixture which would be caused by the addition of alkali, the necessity for standardizing the pH measuring device several times during the interval of time required for the experiment, and the complexity of the experimental set-up combine to make the first

alternative rather unattractive. The second alternative also offers some difficulties in that the concentrations of buffer required to effect complete buffering with large amount of hydrolysis would have to be very large. It was felt that such large concentrations of buffer, because of the effects of ionic strength and the alteration of the activity of the water, would not be very desirable. Accordingly, a compromise solution of this problem was attempted; namely, a certain amount of change in the pH of the reaction mixture was accepted, the extent of this change was measured or estimated in various ways (for which see the following experimental section), and allowance for this change was made in the interpretation of the results.

Kinetic studies on L-tyrosinhydroxamide. It was decided that the study of the effects of pH on the kinetic constants of the alpha-chymotrypsin-catalyzed hydrolysis of this substrate might well be carried out by the methods of Walker and Schmidt (1). This method, which is reviewed and applied in the first section of this thesis, involves the determination of the amount of hydrolysis which occurs within a fixed time interval, here chosen as sixty minutes. It seemed most reasonable to select the median pH, ie. the pH halfway between the starting and concluding pH's, as the one characteristic of the run. The assumption is, then, that the effect of the changing pH will be thus averaged out, and that the result of the run will be the same as that which would be obtained for a hypothetical run made at the median pH alone. For the small changes in pH observed, the approximation seems well justified.

The experimental quantities which must be obtained for use in the Walker - Schmidt method of plotting are $(1/t)([S]_o - [S]_t)$ and

$(1/t) \ln [S]_o/[S]_t$, where t = time, $[S]_o$ = initial substrate concentration, and $[S]_t$ = substrate concentration remaining after time t. Evidently, if $[S]_o$ and t are known quantities, a knowledge of the value of one of these functions allows the value of the other function to be computed. According to Beer's Law

$[S]_t = k\underline{e}_t$, where $\underline{e}_t$ is the extinction observed in any colorimetric method designed to measure $[S]_t$. Similarly

$[S]_o = k\underline{e}_o$.

Now, since the value of k seems to vary slightly with a number of experimental variables (but not within a given run), the use of a quantity which does not involve k seems desirable. Such a quantity is $(1/t) \ln [S]_o/[S]_t = (1/t) \ln \underline{e}_o/\underline{e}_t$. As a consequence, $(1/t) \ln \underline{e}_o/\underline{e}_t$ was used as a basis for the computations involved in these experiments. Actually the variations noted in k were very small, and gross errors in measuring out the substrate for a run or in dilution could be detected very easily by comparing the $\underline{e}_o$ value for a particular run with the $\underline{e}_o$ value which might reasonably be expected on the basis of Beer's Law behavior.

The first step, then, in carrying out a kinetic run was to make observations of the extinction values at various times during the course of the reaction. Sometimes these observations were made at intervals fairly well distributed throughout the time of the reaction; sometimes they were grouped at the beginning of the run and at sixty minutes, the time used for the Walker - Schmidt plot. Next the natural logarithms of the extinction values thus obtained were plotted against

elapsed time, and the values of ln $\underline{e}_o$ and ln $\underline{e}_t$ were obtained by very short interpolations. The value of (1/60) $\ln[S]_o/[S]_t$ = (1/60) ln $\underline{e}_o/\underline{e}_t$ was then calculated. For runs made with a single substrate concentration, but at various pH's, a plot of (1/60) $\ln[S]_o/[S]_t$ vs. pH is a smooth curve. The values of this quantity obtained at various pH's and initial substrate concentrations are summarized in Table VIII and presented as plots of (1/60) $\ln[S]_o/[S]_t$ vs. pH for constant substrate concentrations in Figures 9 through 13.

In order to get values for the kinetic constants which are characteristic of a particular pH, it is necessary to extrapolate the experimental values of (1/60) $\ln[S]_o/[S]_t$, which are obtained at many pH's, to a single pH. The plots of (1/60) $\ln[S]_o/[S]_t$ vs. pH were employed for this purpose. Rather than trying to fit a fair curve through the experimental points and then using points taken from the curve as a basis for computation, the curve was merely used as a guide to determine the method of extrapolation. The extrapolation procedure was made easier by the fact that both the acidic and basic branches of the curve appeared to be nearly linear. For example, take the treatment of Expt. No. 41. The value of (1/60) $\ln[S]_o/[S]_t$ determined from this run was found to be 13.4 and the median pH was found to be 6.47. The point corresponding to these values is indicated by the arrow in Figure 9. A linear extrapolation, indicated by the dotted line parallel to the fair curve, gives values of (1/60) $\ln[S]_o/[S]_t$ of 13.8 x 10^{-3} min.$^{-1}$ at pH 6.50 and 10.5 x 10^{-3} min.$^{-1}$ at pH 6.25. Near the optimum pH, the change of the value of (1/60) $\ln[S]_o/[S]_t$ with pH is much slower, and no change in the value of (1/60) $\ln[S]_o/[S]_t$ results from short range extrapolations. (Extrapolations made over

the regions where the curve changes shape rapidly appear to be rather hazardous since the exact shape of the curve is not well enough defined by the experimental data; such extrapolations were not used.) Obviously, the extrapolation procedure will yield the most accurate values if it is employed over short intervals only. However, the best determinations of the kinetic constants result when a large amount of information is available. The following procedure was established with these factors in mind: 1) All of the runs below pH 6.25 were extrapolated to pH 6.0 (Table IX), 2) All of the runs between pH 6.0 and 6.5 were extrapolated to pH 6.25 (Table X), 3) All of the runs between pH 6.25 and 6.75 were extrapolated to pH 6.5 (Table XI), 4) All of the runs near the optimum were extrapolated to pH 7.0 (Table XII), 5) All of the runs between pH 7.25 and 7.75 were extrapolated to pH 7.55 (Table XIII), 6) All of the runs between pH 7.50 and 8.00 were extrapolated to pH 7.75 (Table XIV), 7) All of the runs at pH's greater than 7.75 were extrapolated to pH 7.95 (Table XV). Note that this overlapping causes most runs to appear at least twice in the computations. No extrapolation is greater than about 2.5 - 3.0 pH units, and most extrapolations are shorter than this. At a single pH, then, several values for the quantity $(1/60)\ \ln[S]_o/[S]_t$ were obtained for each substrate concentration. These values were averaged, and the average deviation was calculated. From the average value of $(1/60)\ \ln[S]_o/[S]_t$ obtained for a given substrate concentration, the corresponding value for $(1/60)([S]_o - [S]_t)$ was computed, and these averages were presented in the form of a Walker - Schmidt plot. The average deviations of observed values of $(1/60)\ \ln[S]_o/[S]_t$ were also indicated on the plot; these errors are the most significant ones. The best straight line was drawn through the points, and the

values for K_s (11) and V_{max} (11) estimated from the slope and intercept respectively. Next, using the average deviations in the values of (1/60) $\ln[S]_o/[S]_t$ as a guide, lines were drawn to estimate reasonable extremes of interpretation of the data. The Walker - Schmidt plots of the data (extrapolated to the various pH's as indicated) are shown in Figures 14 through 20, and the values of the kinetic constants obtained from these plots are summarized in Table XVI and presented graphically in Figures 21 and 22.

A function which will prove to be important in later discussions is K_s/V_{max}. As it happens, this function can be obtained from runs made at low substrate concentrations if V_{max} is known even very approximately. Only a rough interpolation in order to obtain values for V_{max} at different pH's from Figure 22 is required. Naturally, accuracy is somewhat improved by the fact that changes in pH during the course of runs started with low initial substrate concentrations are usually negligible; consequently errors are not introduced in the process of trying to assign a characteristic pH to the run. The integrated rate equation for enzymatic reactions (see Part I of this thesis) has, in the absence of inhibition or other influences by the products of the reaction, the form

$$V_{max}\, t = K_s \ln[S]_o/[S]_t + [S]_o - [S]_t. \qquad (1)$$

This can be rearranged to

$$K_s/V_{max} = t/(\ln[S]_o/[S]_t)\,[1 - ([S]_o - [S]_t)/t\, V_{max}]. \qquad (2)$$

Examination shows that $([S]_o - [S]_t)/t\, V_{max}$ is usually much smaller than 1 if $[S]_o$ is small and V_{max} is fairly large. Runs made at

3×10^{-3} M and 5×10^{-3} M substrate concentration are employed for the computation of K_s/V_{max} for this substrate. The results of these computations are summarized in Table XVII and are presented as log K_s/V_{max} vs. pH in Figure 23.

Runs 69, 70, 92, 93, and 94 were used to study the effects of ionic strength and the effect of calcium ion in particular. The results of these runs are summarized in Table XVIII.

Not all of the runs in the numerical sequence were utilized in the above calculations. In a few cases where high substrate concentrations were used at high pH's, the substrate precipitated out of the solution before the run was completed. This was the case with runs 18, 19, and 87. One series of runs was made with an enzyme preparation later demonstrated to have a low activity (runs 51 - 56). A few runs appeared to be aberrant for no recognizable reason, but were adjudged to be in sufficient conflict with the bulk of the data that they were summarily rejected (runs 43, 44, 85, and 97). The same stock solution of enzyme was used for runs 43 and 44; it seems likely that an error may have been made in the preparation of this solution.

Kinetic studies on acetyl-L-tyrosinhydroxamide. Partly because of uncertainty regarding the inhibitory effects of the split products resulting from the hydrolysis of this compound, the initial velocities of the hydrolysis reaction were employed for the determinations of K_s and V_{max}. It seems reasonable, therefore, to use the pH at the start of a given run as the pH corresponding to the value for the initial velocity (v_o) obtained from the run. These initial pH's were

determined by the methods outlined in the experimental section. The ionizations of the very weakly acidic groups of this substrate are almost entirely suppressed at lower pH's. Consequently a series of runs could be made at a single pH using different initial substrate concentrations, and the kinetic constants could be calculated for this pH without resorting to the extrapolation methods required for L-tyrosinhydroxamide. Attempts were made to start all runs of a series at the same pH, even when the ionization of the substrate became significant. Although complete success was not achieved, the extrapolations required were very short.

For the estimation of the initial velocities of the reaction, it seemed reasonable to use the method of successive approximations described in Part I of this thesis. However, it soon became evident that one of the split products of the hydrolysis reaction, acetyl-L-tyrosine, was inhibiting very strongly at low pH's. A correction factor taking this additional complication into account may be derived very easily. The integrated rate equation which takes into account both inhibition by one of the products of the reaction and inhibition by an added inhibitor has the form

$$V_{max} \quad t = K_s(1 + [S]_o/K_p + [I]/K_I) \ln [S]_o/[S]_t + (1 - K_s/K_p)([S]_o - [S]_t), \tag{3}$$

where K_I refers to the dissociation constant for the enzyme-inhibitor complex,

$$K_I = [E][I]/[EI], \tag{4}$$

and K_p refers to the dissociation constant for the enzyme-product complex,

$$K_p = [E][P]/[EP]. \tag{5}$$

The equation of the line which expresses the initial slope of a first order plot, ie., a plot of $\ln[S]_t$ vs. time, is given by

$$\ln[S]_o/[S]_t = t\, V_{max}/\, K_s(1 + [I]/K_I) + [S]_o \tag{6}$$

From (3) and (6) one may obtain

$$f_1 = t/t$$

$$= \frac{(K_s/[S]_o)(1 + [I]/K_I) + 1}{(K_s/[S]_o)(1 + [I]/K_I)\ln[S]_o/[S]_t + h + (K_s/K_p)(\ln[S]_o/[S]_t - h)}$$

$$\times \ln[S]_o/[S]_t \tag{7}$$

where $h = ([S]_o - [S]_t)/[S]_o$ = % hydrolysis in time t. Evidently a similar equation can be derived for corrections to a zero order plot. (A plot of $[S]_t$ vs. t).

$$f_o = \frac{(K_s/[S]_o)(1+ [I]/K_I) + 1}{(K_s/[S]_o)(1+ [I]/K_I)\ln[S]_o/[S]_t + h + (K_s/K_p)(\ln[S]_o/[S]_t - h)}$$

$$\times h. \tag{8}$$

It would, of course, be desirable to apply these correction factors to both zero and first order plots and to check the initial velocities thus obtained for consistency. Because of the large amount of computation which this would entail in the present case, only the first order plots were employed. Since the correction factors are smaller for

the first order plots than for the zero order plots, the estimates of the values of the factors need not be as accurate for first order plots as for zero order plots.

The process of obtaining the initial velocities was then as follows: 1) From a set of runs made at a given pH, rough estimates of the initial velocities were made visually. 2) From these estimates, an approximate value for K_s was obtained. 3) A similar procedure was followed in the case of runs made at the same pH, but containing added quantities of acetyl-tyrosine. An approximate value for $K_s(1 + [P]/K_p)$ was obtained, where [P] = the concentration of added tyrosine. 4) From these approximate values, a value for K_p was obtained. 5) The approximate values for K_p and K_s were used to obtain values for f_1. The plots were then corrected, and better estimates of K_s and K_p were obtained from the more accurate values for the initial velocities obtained from these corrected points. 6) The more accurate values for K_p and K_s were then used to obtain new values for f_1, and the process was repeated if significant differences from the previous f_1 values were noted. In practice, the first estimate was usually good enough. By this procedure, the only influence on the kinetic course of the reaction which could not be quantitatively estimated was that caused by the changing value of the pH during the course of the run; this change in pH was usually small.

In the final stages of calculation, when the initial velocities had been estimated as well as possible, the data were presented as a plot of $[S]_o/v_o$ vs. $[S]_o$ (cf. 17). In this plot, the slope of the straight line through the experimental points is equal to V_{max}, and the intercept of the line on the vertical axis is equal to K_s/V_{max}. K_s and V_{max} were calculated from plots of this type for a number of pH's.

There is actually no particularly cogent reason for employing this method of plotting in preference to a number of other available methods for representing the data. Indeed, the method conceals the actual independent variation of the kinetic constants. However, the quantity K_s/V_{max} is a particularly important one in the inhibition studies and in the evaluation of some of the effects of hydrogen ion, and it was deemed advisable to obtain this as a single estimate from a graph rather than as a composite of two separate estimates. Also the quantity $[S]_o/v_o$ is readily obtained from the first order plots which are well suited to the colorimetric method.

Those runs which were made with an inhibitor added were treated in an exactly analogous manner insofar as the estimation of the initial velocities was concerned.

In Table XIX the velocities of all of the uninhibited runs made at pH 6.2 are presented, and the $[S]_o/v_o$ vs. $[S]_o$ plot of these runs is shown in Figure 24. It may be noted that at this pH the hydroxamide does not ionize; hence all runs start at the same pH if the same salt: acid ratio in the buffer is used throughout. Also at this pH the buffering action of the cacodylic acid system is very good, and the amounts of hydrolysis are usually fairly small. As a consequence, the pH changes very little over the course of a run. A number of inhibitors were also added to runs made at this pH, a constant amount of each inhibitor being added to a number of runs of different initial substrate concentrations. It was assumed that the inhibition was competitive in each case (cf. 7), and the inhibition constants were evaluated from Lineweaver - Burk plots (18) of the data. The initial velocities of these runs are presented in Tables XX through XXII and the Lineweaver - Burk plots are shown in Figure 25. Cacodylic acid buffer was used for

all of the experiments at pH 6.20.

A series of runs was made at pH 6.60, also in cacodylic acid buffer. Treatment of these runs was the same as for those at pH 6.20. The results are summarized in Table XXIII and presented graphically in Figure 26. Some difficulty was experienced in interpreting the runs made at high substrate concentration, presumably because of the effect of changing pH.

Results of runs made in cacodylic acid at pH 6.95 are presented in Table XXIV and Figure 27.

Four runs were made at pH 7.12 using a phosphate buffer system 0.3f in phosphorous. This buffer required a special solution for the colorimetric reaction, and the colored solutions showed some tendency to fade. In spite of a greater concentration of ions, and in spite of some uncertainty as to the exact effect of phosphate on the hydrolysis reaction, the runs do not seem out of line with others made at about this pH. The results of these runs are presented in Table XXV and Figure 28.

An attempt to use a tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer system at a pH of about 7.2 was not wholly successful. This pH represents about the limit of effectiveness of this buffer, and the pH change during runs made where a large amount of hydrolysis occurred was very large. As a consequence, the four runs made under these circumstances are of somewhat doubtful value. They are presented in Table XXVI and Figure 29.

One of the buffer systems investigated in an attempt to find an effective buffer for pH's near 7.0 which did not display unfavorable side effects was hydroxylamine-hydroxylamine hydrochloride. Four

runs were made with this buffer system with the buffer 0.3f in the amine. Unfortunately, the hydroxylamine has an effect on the velocity of the hydrolysis reaction, which eliminates it from consideration as a buffer for general use at this pH. However, the runs made in the presence of hydroxylamine are of interest because of the fact that hydroxylamine is one of the products of the hydrolysis reaction. The actual data for these runs are rather poor, partly because of slow fading of the color produced by the colorimetric reaction. Results of these runs are presented in Table XXVII and Figure 30. The initial pH for all runs was 7.15. Note that, since the buffer mixture was prepared by neutralization of $NH_2OH \cdot HCl$, the reaction mixture also contains NaCl.

The buffer system consisting of ethylenediamine-monohydrochloride and ethylenediamine-dihydrochloride is suitable for use at pH's in the vicinity of 7.0. It has the disadvantage of introducing a larger number of ions into the solution than previously used buffers. Four runs were made with this buffer at pH 7.3 with the buffer 0.3f in the amine component. These runs are presented in Table XXVIII and Figure 31. The change in pH during runs made at high initial substrate concentrations was still fairly large.

At pH 7.6, the reported optimum pH for acetyl-L-tyrosinhydroxamide, five runs were made. Tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer 0.3f in the amine was used to hold the pH. The conditions for these experiments were identical to those employed by other investigators (14, 19) except for the concentration of the enzyme and the lot number of the enzyme preparation. The results of these runs are summarized in Table XXIX and presented graphically in Figure 32.

Calcium ion has been reported to cause an increase in the rate of hydrolysis of peptide linkages (10). Four runs were made at pH 7.60 under conditions identical to those employed for the runs discussed in the preceding paragraph except that calcium ion was added to the extent of 0.04 M (as $CaCl_2$). The results of these runs are presented in Table XXX and Figure 33.

Four runs were made at pH 7.60 with 55.4×10^{-3} M L-tyrosinamide as an inhibitor. This concentration of tyrosinamide proved to be very strongly inhibitory. Competitive inhibition was assumed, and the data were presented in a Lineweaver - Burk plot (Figure 34). The results of the individual runs are summarized in Table XXXI.

A series of five runs made at pH 7.70 is presented in Table XXXII and Figure 35. No inhibitors were added.

In the case of four runs made at pH 7.88, it was noted that for each substrate concentration the velocity observed was low in comparison with its near neighbors on a velocity - pH curve. This is reflected as well in a low value for the V_{max} obtained from these runs. The K_s, however, seems to agree with neighboring values quite well. These effects are probably to be attributed to a low activity in the enzyme stock solution used for all of these runs. Thus the K_s value appears to be trustworthy while V_{max} obtained from these runs may be lower than the correct value. The cause of the decreased activity of this particular enzyme solution is not clear. The results of these runs are presented in Table XXXIII and Figure 36.

The usual method employed for the evaluation of competitive inhibition constants requires the determination of the initial velocities of a number of runs in which the inhibitor concentration is held

constant while the initial substrate concentration is varied. This was the method employed for the evaluation of most of the inhibition constants in this paper. However, it can be shown that it is equally as possible to determine an inhibition constant from a series of runs in which the initial substrate concentration is held constant while the inhibitor concentration is varied. The expression for the initial velocity of an enzyme catalyzed reaction in the presence of a competitive inhibitor is

$$v_o = \frac{V_{max}[S]_o}{K_s(1 + [I]/K_I) + [S]_o} \quad . \qquad (9)$$

While this equation can be rearranged in many ways to suggest possible plots for runs made in this way, one of the more useful forms is

$$[I]v_oK_s/(K_s + [S]_o) = V_{max}[S]_oK_I/(K_s + [S]_o) - K_Iv_o \ , \qquad (10)$$

where v_o = initial velocity, $[I]$ = inhibitor concentration, and the other symbols have their usual meanings. If the quantity on the left-hand side of the equation is plotted against v_o, a straight line will be obtained, the slope of which is equal to $-K_I$, the intercept on the vertical axis equal to $V_{max}[S]_oK_I/(K_s + [S]_o)$, and the intercept on the horizontal axis equal to the velocity of an uninhibited run.

The case under consideration does not offer an ideal illustration of this method of determining inhibition constants. In the first place, the compound acting as an inhibitor, acetyl-L-tyrosinamide, is actually a substrate in its own right. Since it is hydrolyzed at a much slower rate than acetyl-L-tyrosinhydroxamide (ratio about 10 to 1), it would seem that, at least as a first approximation, the

hydrolysis of the acetyl-L-tyrosinamide might be neglected, and the compound considered to act entirely as an inhibitor. A quantitative discussion of the validity of this assumption might well be deferred. Secondly, the run made without inhibitor added shows a velocity much higher than velocities of other uninhibited runs for the same substrate concentration, higher even than those made at the pH optimum. This is probably an indication of a mistake in weighing out enzyme for the preparation of the stock solution. In theory, at least, this should have no effect on the value obtained for the "inhibition" constant for the acetyl-L-tyrosinamide. The data are offered principally for illustrative purposes, and not for the reliability of the value of the constant which is obtained. The velocities and other computed quantities are presented in Table XXXIV and the plot described above is shown in Figure 37. It might be noted that the value of an inhibition constant obtained by this method is remarkably insensitive to errors in the value of K_s which is used to compute the necessary quantities for the plot.

The above method offers the advantage of allowing the substrate concentration to be selected so as to obtain optimal conditions for making experimental observations. In the case described above, the substrate concentration used for the experiments is small enough that the changes in pH during the course of a run are negligible, but large enough to give optimum conditions for the analytical method used in following the hydrolysis reaction.

At pH's greater than about 7.9, the ionization of the hydroxamic acid grouping begins to become important, and the attainment of the same initial pH for a group of runs becomes more difficult. A series of runs was made in the vicinity of pH 8.20 and extrapolated to that

pH in a manner similar to that employed for L-tyrosinhydroxamide. The extrapolations for acetyl-L-tyrosinhydroxamide were made over much smaller distances, however. These runs near pH 8.2 are summarized in Table XXXV and Figure 38. Both observed and extrapolated values are given in the table.

This same problem was encountered in a somewhat more aggravated form at about pH 8.6. The extrapolations were never longer than 0.1 pH unit, however. Some erratic behavior of the points was noted for the runs made at this pH. Runs 30 - 38 probably give the best indication of the correct values for the points as these runs were made in two groups of four within a space of a few days. Agreement between these runs seems to be fairly good, and they seem to fit into the pH spectrum, which includes some runs made at about this same time at higher pH's. In view of these facts, it seems very likely that these runs represent the situation at this pH fairly satisfactorily. At the same time, it should be kept in mind that relatively small errors in the estimated velocities can cause large errors in the final constants when the value of K_s becomes fairly large.

The balance of the runs made near pH 8.6 were made in isolated instances as controls for inhibition studies. These, in general, appear to have higher velocities than the runs in the 30 - 38 sequence. The magnitude of this increase appears to be about 8% for the lower concentrations and about 4% for the larger substrate concentrations. Run 96^b and its accompanying inhibitor runs (made with the same enzyme stock solution) have low velocities when compared to other runs, including run 99^a and the runs made with it which contain the same amount of inhibitor as 96^b. Run 99^a is in agreement with some of the other runs made with the same initial substrate concentration. The

following procedure was adopted: 1) Runs 30 - 38 were considered separately; 2) The remainder of the runs were considered without runs 30 - 38; 3) All of the runs were considered jointly. Although the values obtained from 1) are probably the most reliable, 2) and 3) serve to indicate the possible extent of error. Evidently a confirmation of the results obtained at this pH is desirable. The results of these runs are summarized in Table XXXVI and Figures 39a, 39b, and 39c.

Because of the spread in values observed at pH 8.60, it was decided to evaluate inhibition constants using a control run employing the same enzyme stock solution. If competitive inhibition is assumed, a value for the inhibition constant can be obtained by a comparison of a single run to the control. In order to do this, a value for K_s must be assumed; this, however, need not be particularly accurate. If symbols subscripted "o" indicate initial quantities corresponding to the control run, and if symbols subscripted "oi" refer to similar quantities for the inhibited run, then the following relationships apply

$$v_o = V_{max}[S]_o/(K_s + [S]_o) \tag{11}$$

$$v_{oi} = \frac{V_{max}[S]_{oi}}{K_s(1 + [I]/K_I) + [S]_{oi}} \quad . \tag{12}$$

Since the control and the inhibited run are both made with the same enzyme activity, V_{max} is the same in (11) and (12). The ratio of the velocities is then

$$\frac{v_o}{v_{oi}} = \frac{[S]_o}{[S]_{oi}} \frac{K_s(1 + [I]/K_I) + [S]_{oi}}{K_s + [S]_o} \quad . \tag{13}$$

Evidently a value for K_I can be obtained from this relationship. This method was successfully employed for acetyl-D-tryptophanamide (Table XXXVII) and acetyl-L-tyrosinamide (Table XXXVIII). It might be indicated again that acetyl-L-tyrosinamide is a substrate in its own right, and that this may mean that the apparent inhibition constant which is obtained by the above method may not be a pure inhibition constant. An attempt was made to obtain a value for acetyl-L-tyrosine, but the amount of inhibition was too small to be quantitatively evaluated.

A number of runs were made in the general vicinity of pH 9.0 (Table XXXIX). While these were not accurate enough to permit values of K_s and V_{max} to be obtained at this pH, they were of considerable use in determining curves for extrapolation purposes. These runs also yielded some values of K_s/V_{max} on the basis of some simple assumptions.

The values of K_s and V_{max} determined at the various pH's are summarized in Table XL together with some other values reported in the literature. The values are presented graphically in Figures 40 and 41.

Equation (11) can be rearranged to form

$$K_s/V_{max} = [S]_o/v_o - [S]_o/V_{max} \quad . \qquad (14)$$

This equation is, of course, the basis of the method of plotting which has been used for the kinetic studies of acetyl-L-tyrosinhydroxamide. One valuable property of this expression is that, for small values of $[S]_o$ and rather large values for V_{max}, the term $[S]_o/V_{max}$ may be quite small compared to $[S]_o/v_o$. This means that accurate values for K_s/V_{max} can be obtained from runs made at low substrate concentrations.

Additional practical advantages in the present case result from the fact that the change in pH is negligible over the course of a hydrolysis experiment made with a low substrate concentration. Since the quantity K_s/V_{max} appears to be an important one for subsequent discussion, it has been calculated from runs made at low concentrations of acetyl-L-tyrosinhydroxamide. With the reasonable assumption that V_{max} at pH's greater than 8.6 can be obtained fairly well by an extension of the curve in Figure 41, values for K_s/V_{max} have been calculated for some runs at about pH 9.0. A rather large error in this extrapolation would cause only small errors in K_s/V_{max}. The results of these calculations are presented in Table XLI and Figure 42. The logarithms of K_s/V_{max} rather than K_s/V_{max} itself are plotted in Figure 42 in order to give a reasonable spread to the values.

From equation (12) it is evident that a value for K_I can be obtained from a single run if values of K_s and V_{max} are known. In order to obtain a more accurate idea of the behavior of the inhibition constants of acetyl-L-tyrosine and L-tyrosinamide with respect to pH, a series of runs at a low substrate concentration were made, and values for K_s and V_{max} taken from Figures 40 - 42 were used to calculate the constants. Table XLII and Table XLIII summarize the data for acetyl-L-tyrosine and L-tyrosinamide respectively. The variation in the inhibition constants with pH is shown graphically in Figures 43 and 44.

The behavior of the constants of some of the other inhibitors with respect to pH is indicated by the data presented in Tables XLIV and XLV.

DISCUSSION OF RESULTS

A. The effect of ionic strength, calcium ion, and hydroxylamine on the hydrolytic reactions.

From the data which are given in Table XVIII, an increase in ionic strength of about 50% does not appear to cause any change in the velocity of the enzymatic hydrolysis of L-tyrosinhydroxamide, at least not at pH's up to 7.0. Calcium ion does seem to have an accelerating effect on the reaction. Magnesium ion may have a small effect; however, this was observed only at pH 7.0, and the limits of experimental error do not permit any rigorous conclusions to be drawn. It might be noted that the range of ionic strengths explored here is greater than that resulting from the varying ionic strengths of the buffer.

In the study of the hydrolysis of acetyl-L-tyrosinhydroxamide, some of the buffer systems employed possessed higher ionic strengths than the others. Thus the KH_2PO_4 - K_2HPO_4 buffer system and the ethylenediamine-monchydrochloride-dihydrochloride systems had considerably higher ionic strengths than either the cacodylic acid or tris-(hydroxymethyl)-aminomethane buffer systems for a corresponding pH. Thus at pH 7.0 the two latter buffer systems, as used in the present studies, would have ionic strengths of roughly 0.27 M, while the ionic strength of the ethylenediamine system (pK_{A_2} = 7.0) is about 0.6. A similar increase holds for the phosphate buffer. However, examination of Figures 40 - 42 fails to disclose any effect of this increased ionic strength. It should perhaps be emphasized that the effect of ionic strength may become more important at higher pH's;

evidence which indicates that this may be so is currently being obtained (22). Other substrates than acetyl-L-tyrosinhydroxamide are being used for these studies.

H. Neurath has reported a rather profound effect of calcium ion on the activity of chymotrypsin (10). It was thought that it might be of interest to investigate the influence of calcium ion on the hydrolysis of the hydroxamides. Calcium ion did cause an increase in the rate of hydrolysis of L-tyrosinhydroxamide at pH 6.0 and 7.0. Controls indicated that this effect was not due to ionic strength. The kinetic constants for the enzyme-catalyzed hydrolysis of acetyl-L-tyrosinamide were determined in the presence of 0.04f $CaCl_2$ at pH 7.6. This series of experiments was uncontrolled, ie. the part of the increased activity due merely to ionic strength was not determined. One might suppose, from the evidence that the higher ionic strength in the ethylenediamine buffer system used at pH 7.3 caused no observable increase in activity, that the ionic strength effect at pH 7.6 might also be small. The constants obtained in the presence of calcium ion were $K_s = 40 \times 10^{-3}$ M, and $V_{max} = 1.61 \times 10^{-3}$ M/min. as compared to $K_s = 47 \times 10^{-3}$ M and $V_{max} = 1.41 \times 10^{-3}$ M/min. for the reaction in the absence of calcium ion. It is not possible to determine within the limits of experimental error whether or not the value of K_s is affected. It is, however, possible to say that V_{max} is definitely increased by the presence of calcium ion. According to the steady state treatment of the reaction sequence

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} \text{products},$$

K_s is equal to $\frac{k_2 + k_3}{k_1}$ (9). $V_{max} = k_3[E]_o$, where $[E]_o$ is the total enzyme concentration. Evidence has been accumulating that $k_2 \gg k_3$ in reactions catalyzed by alpha-chymotrypsin (23). In the case of acetyl-L-tyrosinhydroxamide, which has the largest k_3 value of any substrate so far studied, the increase in V_{max} (and hence of k_3) of about 15% certainly does not cause any increase in the value of K_s, and, therefore, it might be supposed that $k_3 \ll k_2$. Better controlled experiments involving larger amounts of activation should be performed in order that a more positive indication of this effect may be obtained.

When hydroxylamine was employed as a buffer, it inhibited the enzymatic hydrolysis of acetyl-L-tyrosinhydroxamide. This inhibitory action of the hydroxylamine seemed to be exerted mainly on V_{max}. Since the enzyme stock solution was prepared with the 0.3f hydroxylamine buffer and then used for runs over a period of several hours, it might be expected that, if the inhibition were due to irreversible destruction of the enzyme, the effect on the values of the individual velocities and upon K_s would have been more profound than that observed. Evidence indicates the possibility that the same sort of statement might be made for hydroxylamine as for calcium ion, but the present experimental evidence is insufficient. Probably the most important result of these experiments with hydroxylamine is that hydroxylamine, in the quantities liberated in the hydrolysis of the hydroxamide substrates by chymotrypsin, has a negligible effect on the kinetic reaction. The largest quantity of hydroxylamine liberated in a hydrolysis experiment was of the order of 0.025f, while 0.3f hydroxylamine caused a decrease in velocity of only about 10%. Incidentally, a short experiment

demonstrated that the inhibitory effect of hydroxylamine was not due to the existence of an appreciable back reaction.

B. Agreement of results with those of previous investigators.

The alpha-chymotrypsin-catalyzed hydrolysis of L-tyrosinhydroxamide has been studied at pH 6.9 - 7.0 using the initial velocities of the reactions for the evaluation of the kinetic constants (16). If the split products of the reaction caused any inhibition of the enzyme, the constants obtained in this way would, at least in theory, differ from constants obtained by the method of Walker and Schmidt, since the quantities used as a basis for computation in the latter method are determined from an advanced stage in the reaction. From the first method: $K_s = 41 \pm 2 \times 10^{-3}$ M and $k_3 = 3.6 \pm 0.2 \times 10^{-3}$ M/min. From the second method: $K_s = 40 \pm 4 \times 10^{-3}$ M and $k_3 = 3.6 \pm 0.3 \times 10^{-3}$ M/min. That agreement should be fairly good is hardly surprising since some of the experiments used for the Walker - Schmidt method were also used for the method based on initial velocities. However, the results do indicate that there are no serious effects from the split products, at least at pH 7.0. It is assumed that an equally fortunate situation exists at other pH's.

Values for the kinetic constants for the alpha-chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide obtained in these experiments may be compared to those obtained by other investigators at pH 7.6. Present experiments: $K_s = 47 \pm 3 \times 10^{-3}$ M; $k_3 = 35 \pm 2 \times 10^{-3}$ M/mg. protein nitrogen/ml./min. Foster and Niemann (19): $K_s = 42 \pm 2 \times 10^{-3}$ M; $k_3 = 34 \pm 2 \times 10^{-3}$. Hogness as corrected by Foster (20): $K_s = 45 \pm 5 \times 10^{-3}$ M; $k_3 = 32 \pm 3 \times 10^{-3}$. Agreement

appears to be fairly good. It might be remarked that Foster used the same lot of the Armour preparation of alpha-chymotrypsin as that used in the present experiments, while Hogness used a different lot.

Foster and Niemann (19) obtained a value of $90 \pm 10 \times 10^{-3}$ M for the inhibition constant of acetyl-L-tyrosine at pH 7.6. This compares well with a value of 87×10^{-3} M obtained in this series of investigations. The curve for K_I vs. pH for acetyl-L-tyrosine (Figure 43) also seems to be within the limits of error of $115 \pm 15 \times 10^{-3}$ M for the K_I of acetyl-L-tyrosine at pH 7.9 as reported by Thomas et al (21).

The value of 41×10^{-3} M determined as the apparent inhibition constant for acetyl-L-tyrosinamide at pH 7.9 does not agree particularly well with a value of 32×10^{-3} M for K_s determined by Thomas et al (21) as recalculated by Foster (20). The value of 41×10^{-3} also appears to be high compared to other K_s values which have been reported (cf. 21) and recently determined in these laboratories (24).

C. Acid constants of hydroxamic acids.

Recent determinations of the acid constants of the hydroxamic acid groups in L-tyrosinhydroxamide (16), and acetyl-L-tyrosinhydroxamide (14) by potentiometric methods have thrown into question the value of $pK_A = 7.55$ for acethydroxamide. This value for acethydroxamide was determined in 1910 by a conductimetric technique (25). The acid constant for acethydroxamide was consequently redetermined potentiometrically and found to be about 9.4. This is in agreement with the values obtained from the amino acid hydroxamides. Some glycinhydroxamide was also prepared and its acid constants determined; however, because of a somewhat low decomposition point (but a fairly good neutral equivalent) the values of the acid constants for this compound may be

slightly questionable. The acid constants for hydroxamides are summarized in Table XLVI. It seems fairly clear that a revision in the older value, still accepted in the most recent review (26), is called for. A pK_A of about 9.0 - 9.5 appears to be characteristic for hydroxamic acids.

D. The effect of pH on the hydrolysis reactions catalyzed by alpha-chymotrypsin.

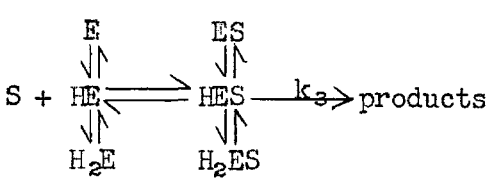
The effect of pH on the hydrolysis of L-tyrosinhydroxamide has been discussed briefly on the basis of the pH optimum curve alone (16). Acetyl-L-tyrosinhydroxamide has been considered in somewhat more detail, also on the basis of its pH optimum curve, and a calculation based upon a comparison with the optimum curve of acetyl-L-tyrosinamide and the ionization constants of the hydroxamic acid was apparently successful (14). A discussion of the effect of pH on the hydrolysis of this substrate has appeared in a recent article (7). It is unfortunate that, in the treatment which appears here, the wrong form of the substrate (the completely unprotonated one) has been selected as the one upon which the enzyme acts. It is interesting that, in spite of this mistaken starting point, the qualitative conclusions which are reached are the right ones.

The effect of pH on enzyme-catalyzed reactions is usually attributed to the existence of several protonated forms of the enzyme, and equations have been derived from this standpoint in several instances (1, 2, 3, 4, 5, 7, others). In the usual case, only three different protonated species related by two acid constants have been found necessary to describe the pH phenomenon for most systems. The range of application of this idea has recently been discussed (7).

In addition, it has been found that, in all of the cases so far described, it is sufficient to postulate that only one protonated form of the enzyme is catalytically active (7). In the deriving of expressions relating pH and the kinetic behavior of enzymatic reactions, almost any desired degree of complexity may be attained (5). The problem is one of finding a simple, reasonable explanation for experimental results, and not one of considering all of the various possibilities for the effect of pH on enzymatic reactions.

It is customary in making these derivations to assume that the rate of reaction of the active enzyme-substrate complex to give products is much slower than the other reactions in the series. As stated in the introduction, the steady state solution appears only to yield the information the K_s is not necessarily a true equilibrium constant. The derivations which follow are consequently based on the customary assumptions.

We may start by considering the following system, assuming that zone A conditions (27) hold. The resulting expressions are the same as those obtained by other investigators (3, 4). This system would be a likely one for a substrate whose charge state is of no kinetic consequence.



$$\text{Rate} = v = k_3(HES) \tag{15}$$

$$[HE]h/[H_2E] = K_a \tag{16}$$

$$[E]h/[HE] = K_b \tag{17}$$

$$[HES]h/[H_2ES] = K_c \qquad (18)$$

$$[ES]h/[HES] = K_d \qquad (19)$$

$$[HE][S]/[HES] = K_m \qquad (20)$$

The total enzyme concentration is given by

$$[E]_o = [E] + [HE] + [H_2E] + [ES] + [HES] + [H_2ES] \; . \qquad (21)$$

By substitution of the equilibrium equations in (21):

$$[E]_o/[HES] = (K_m/[S])(h/K_a + 1 + K_b/h) + h/K_c + 1 + K_d/h \; . \qquad (22)$$

When (22) is solved explicitly for [HES], and substitution made into (15),

$$v = \frac{k_3[E]_o[S]}{K_m(h/K_a + 1 + K_b/h) + [S](h/K_c + 1 + K_d/h)} \; . \qquad (23)$$

It can be seen that a reaction proceeding according to (23) will still follow Michaelis - Menten kinetics, ie., obey a rate law of the form

$$v = V_{max}[S]/([S] + K_s) \; . \qquad (24)$$

From (23), the observed constants, K_s and V_{max}, would be expected to vary with pH according to

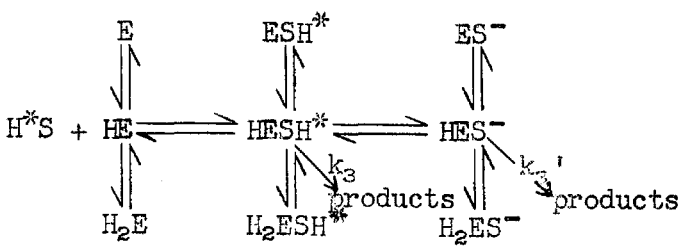
$$K_s = K_m \frac{(h/K_a + 1 + K_b/h)}{(h/K_c + 1 + K_d/h)} \qquad (25)$$

$$V_{max} = \frac{k_3[E]_o}{h/K_c + 1 + K_d/h} \qquad (26)$$

$$K_s/V_{max} = (K_m/k_3[E]_o)(h/K_a + 1 + K_b/h) \quad . \qquad (27)$$

It might be remarked that for a specific case some of the equilibrium constants may equal zero or infinity. One sees from these equations that it is not necessarily true that either of the kinetic constants is pH independent. These equations have been employed by several investigators with more or less success and with more or less modification (cf. 3, 5, and 7). The greatest success so far obtained has been in the case of fumarase investigated by Alberty (4). A number of good quantitative relationships seem to be forthcoming.

The above situation may be rendered rather more complex by the existence of variously charged species of substrate. Some of the effects of substrate ionization have been treated by other investigators (3, 4, 5, 7). It is with some of these more complex cases that we are presently concerned. The case of acetyl-L-tyrosinhydroxamide will be considered first. In the pH range studied, the ionization of the phenolic hydroxyl group is not too important, and the effect of this ionization is uncertain, hence it will be neglected as a first approximation. It seems best to examine a rather general case and then to specialize it in the light of the experimental results. In a manner analogous to that employed above we may write the reaction sequence



If we simplify by assuming that the ionization of the substrate hydrogen in the enzyme-substrate complexes is the same for all of the complexes and equal to the ionization constant of the hydroxamic acid by itself, we may write the following equations:

$$\text{Rate} = k_3[HESH^*] + k_3'[HES^-] \ . \tag{28}$$

All of the equilibrium constants represented by equations 16 through 20 apply, and in addition

$$[S]h/[HS] = [ES^-]h/[ESH^*] = [HES^-]h/[HESH^*] = [H_2ES^-]h/[H_2ESH^*] = K' \tag{29}$$

apply approximately. The total enzyme concentration is

$$[E]_o = [E] + [HE] + [H_2E] + [ESH^*] + [HESH^*] + [H_2ESH^*] + [ES^-] + [HES^-] + [H_2ES^-] \ . \tag{30}$$

(28) in light of (29) can be re-expressed

$$v = (k_3 + k_3'K'/h)[HESH^*] \ . \tag{31}$$

The total substrate concentration may be expressed by

$$[S]_t = [HS] + [S^-] = [HS]/(1+K'/h) = [S^-]/(1+H/K') \ . \tag{32}$$

When the equilibrium expressions are substituted into (30), one obtains

$$[E]_o/(HESH^*) = (K_m/[S]_t)(1+K'/h)(h/K_a+1+K_b/h) + (1+K'/h)(h/K_c+1+K_d/h) \ . \tag{33}$$

Substituting for [HESH*] in (31) gives

$$v = \frac{(k_3 + k_3'K'/h)[E]_o[S]_t}{K_m(1+K'/h)(h/K_a + 1 + K_b/h) + (1+K'/h)(h/K_c + 1 + K_d/h)} \quad (34)$$

$$V_{max} = \frac{(k_3 + k_3'K'/h)[E]_o}{(1+K'/h)(h/K_c + 1 + K_d/h)} \quad (35)$$

$$K_s = K_m \frac{h/K_a + 1 + K_d/h}{K_c/h + 1 + h/K_d} \quad (36)$$

$$K_s/V_{max} = (K_m/[E]_o)\frac{(1+K'/h)(h/K_a + 1 + K_b/h)}{k_3 + k_3'K'/h} \quad (37)$$

Consider first the behavior which has been observed for V_{max}. Figure 41 seems to indicate a fairly rapid rise on the acid branch with a virtual pH independence (or something approaching it) on the basic side. We might note also that the behavior of acetyl-L-tyrosine with respect to pH (Figure 42) seems to indicate that negatively charged groups are much less tightly combined with the enzyme with increasing pH. An adequate explanation of these observed effects can be obtained if 1) the unionized form of the substrate does not interact with ES, and 2) the ionized form of the substrate interacts only with H_2E. This probably represents an oversimplification of the actual state of affairs; it seems likely that there is at least some affinity between hydroxamate ions and the enzyme species HE. It does not seem worthwhile to increase the complexity of the resulting equations by introducing terms for this interaction, which is very likely quantitatively small over the pH range studied.

As a consequence of these assumptions, equation (31) becomes

$$v = k_3[HESH^*], \tag{38}$$

equation (30) becomes

$$[E]_o = [E] + [HE] + [H_2E] + [HESH] + [H_2ESH] + [H_2ES^-] , \tag{39}$$

equation (33) is then

$$\begin{aligned}[E]_o/[HESH^*] &= (K_m/[S]_t)(1+ K'/h)(h/K_a + 1 + K_b/h) \\ &\quad + 1 + h/K_c(1+ K'/h) \\ &= (K_m/[S]_t)(1+ K'/h)(h/K_a + 1 + K_b/h) \\ &\quad + (1+ K'/K_c) + h/K_c \ .\end{aligned} \tag{40}$$

From equations (40) and (38)

$$v = \frac{k_3[E]_o[S]_t}{K_m(1 + K'/h)(h/K_a + 1 + K_b/h) + (1+ K'/K_c) + h/K_c} \ . \tag{41}$$

From this equation

$$V_{max} = \frac{k_3[E]_o}{(1+ K'/K_c) + h/K_c} \tag{42}$$

$$K_s = K_m(1+ K'/h)\frac{(h/K_a + 1 + K_b/h)}{(1 + K'/K_c) + h/K_c} \tag{43}$$

$$K_s/V_{max} = (K_m/k_3[E]_o)(1+ K'/h)(K_a/h + 1 + h/K_b) \ . \tag{44}$$

Equation (42) predicts that the value of V_{max} will rise to a constant value. By inverting one obtains

$$1/V_{max} = (1+ K'/K_c)/k_3[E]_o + h/k_3[E]_oK_c \ . \tag{45}$$

Thus if $1/V_{max}$ is plotted against h, a straight line should be obtained. The equation may also be rearranged to

$$V_{max} = k_3[E]_o/(1+K'/K_c) - V_{max}h/K_c(1+K'/K_c) \quad . \qquad (46)$$

This form shows that a plot of V_{max} vs. $V_{max}h$ is also a straight line. This form seems better for purposes of quantitative evaluation than (45). Table XLVII and Figure 45 present the actual data obtained experimentally. The slope of the line, equal to

$$-1/K_c(1+K'/K_c) \quad , \qquad (47)$$

was found to be 4.2×10^{-7}. If $K' = 10^{-9}$, the constant determined for acetyl-L-tyrosinhydroxamide, the value of K_c is 4.2×10^{-7}. Note that the value obtained for K_c is not affected much by K'. The straight line relationship lies within the limits of experimental error of the individual points. This is perhaps more evident in Figure 46 which is merely a re-plotting of Figure 41 with the curve derived from Figure 45. This representation fits the data within the limits of error of the experiments. The apparent falling off of the velocity on the basic branch of the curve might easily be due to decreasing ionic strength of the buffer solution with increasing pH. Experiments should be performed with the ionic strength held constant in order that a better idea of the behavior of V_{max} with pH may be obtained, particularly at higher pH's.

In equation (44) can be seen a particularly useful relationship. This may be rearranged to

$$(K_s/V_{max})(1+K'/h) = (Km/k_3[E]_o)(h/K_a + 1 + K_b/h) \quad . \qquad (48)$$

The left-hand side of this equation contains experimentally observed quantities while the right-hand side contains acid constants which are typical of the enzyme alone and which should apply in any instance regardless of the substrate being studied. This would not necessarily be true of K_c. The right-hand side of the equation has its pH minimum at

$$h_o = K_a K_b \tag{49}$$

and may be represented by

$$(K_s/V_{max})(1 + K'/h) = (1/k_3[E]_o) + (K_b/K_a/k_3[E]_o) \times (h_o/h + h/h_o) \tag{50}$$

$$= (1/k_3[E]_o) + (K_b/K_a/k_3[E]_o)\, f(pH) \text{ (Ref. 7).}$$

Evidently a plot of the left side of the equation against f(pH) will yield a straight line from which K_a and K_b can be calculated. The correct value for the minimum pH is best obtained by trying different values for h_o until the best straight line is obtained. The value for K_a obtained by this method should be fairly good; K_b suffers from the possible effect of ionic strength at higher pH's mentioned above, from errors in K', from interaction of hydroxamate ion with HE, and from some uncertainty regarding the effect of the ionization of the phenolic group of tyrosine on the kinetic reaction. This latter effect would be particularly important at pH 9.0. The quantities used in the final calculation are summarized in Table XLVIII and Figure 47. The values obtained are $K_a = 1.4 \times 10^{-7}$ and $K_b = 2.9 \times 10^{-9}$. The curve obtained by plotting

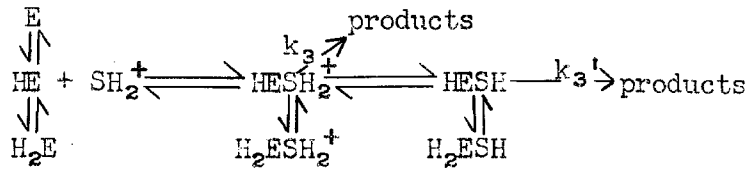
$\log(1+K'/h)(h/K_a+1+K_b/h)$ vs. pH

is shown superimposed on the experimental data in Figure 48. Note that by employing logarithms it is not necessary to determine $K_m/k_3[E]_o$, and the shapes of the curves can be compared directly.

The value of $K_a = 1.4 \times 10^{-7}$ ($pK_A = 6.85$) is in agreement with the value of pK = 6.8 obtained recently by direct titration of alpha-chymotrypsin (28). This acid group is not found in chymotrypsinogen, and it was therefore presumed to be kinetically important. These investigators proposed that this ionization might be that of the basic group of histidine ($pK_A = 7.0$).

This same curve fitting has been applied to the experimental data for K_s in Figure 49. Again logarithms have been used. Although the fit of this curve appears somewhat rough, it agrees within the limits of experimental error. The use of logarithms causes differences in the smaller values to be magnified.

When the behavior of V_{max} for L-tyrosinhydroxamide is examined in the light of the foregoing, it seems evident that the state of protonation of the amino group is important kinetically. Furthermore, it appears that protonated and unprotonated molecules both are combining with the enzyme, since only by combination of both forms can the behavior of V_{max} be influenced by the status of the amino group. The behavior of V_{max} seems to indicate that the protonation of the amino group has an activating influence for the enzymatic hydrolysis. It may be true that the unprotonated form is completely inactive and acts merely as an inhibitor; however, it seems likely that it is also split, albeit more slowly than the protonated form. The reaction scheme which expresses this idea is shown below.



$k_3 > k_3'$

The ionization of the enzyme hydrogen in the enzyme-substrate complexes may be affected by the presence of the positive charge on the substrate, hence we cannot assume that both of these dissociations have the same value for K_c. This also means that in the enzyme-substrate complex the acid constant for the amino group of the substrate is affected by the protonation state of the enzyme. Consider the following equilibria:

$$[HE]h/[H_2E] = K_a \tag{51}$$

$$[E]h/[HE] = K_b \tag{52}$$

$$[HE][SH_2]/[HESH_2] = K_m \tag{53}$$

$$[HESH_2^+]h/[H_2ESH_2^+] = K_c \tag{54}$$

$$[HESH]h/[H_2ESH] = K_c' \tag{55}$$

$$[SH]h/[SH_2^+] = K_o' \tag{56}$$

$$[S^-]h/[SH] = K_o'' \tag{57}$$

$$[HESH]h/[HESH_2^+] = K_1' \tag{58}$$

$$[H_2ESH]h/[H_2ESH^+] = K_2' \tag{59}$$

K_1', K_2', K_c and K_c' are not independent of one another; any one of these constants can be expressed in terms of the other three.

The expression for the total enzyme concentration is then

$$[E]_o = [E] + [HE] + [H_2E] + [HESH_2^+] + [H_2ESH_2^+] + [HESH] + [H_2ESH] . \tag{60}$$

$$\text{Rate} = k_3[HESH_2^+] + k_3'[HESH] = k_3[HESH_2^+](1 + rK_1'/h), \tag{61}$$

where $r = k_3'/k_3$.

The total substrate concentration may be expressed by

$$[S]_t = [S^-] + [SH] + [SH_2^+] = [SH_2^+](1 + K_o'/h + K_o'K_o''/h^2). \tag{62}$$

Substitution of the equilibrium constants into (60) gives

$$[E]_o/[HESH_2^+] = (K_m/[S]_t)(1 + K_o'/h + K_o'K_o''/h^2)(h/K_a + 1 + K_b/h) + 1 + K_2'/K_c + h/K_c + K_1'/h . \tag{63}$$

Equation (63) might also be expressed in other ways. From (63) and (61)

$$v = \frac{k_3[E]_o[S]_t(1 + rK_1'/h)}{K_m(1 + K_o'/h + K_o'K_o''/h^2)(h/K_a + 1 + K_b/h) + 1 + K_2'/K_c + h/K_c + K_1'/h}, \tag{64}$$

from whence

$$K_s = K_m\frac{(1 + K_o'/h + K_o'K_o''/h^2)(h/K_a + 1 + K_b/h)}{1 + K_2'/K_c + h/K_c + K_1'/h} \tag{65}$$

$$V_{max} = \frac{k_3[E]_o(1 + rK_1'/h)}{1 + K_2'/K_c + h/K_c + K_1'/h} \quad , \tag{66}$$

$$K_s/V_{max} = (K_m/k_3[E]_o)\frac{(h/K_a + 1 + K_b/h)}{1 + rK_1'/h} \quad . \tag{67}$$

Consider first equation (67). If we assume that r = 0, that is, HESH is not susceptible to hydrolysis, the use of the values of K_a and K_b from the kinetic studies on acetyl-L-tyrosinhydroxamide gives the solid curve in Figure 50. This curve successfully predicts the behavior of the data on the acid side of the minimum, and it also predicts the correct position for the minimum within the limits of experimental error. The discrepancy on the alkaline side may be easily explained by some susceptibility to hydrolysis of the species represented by HESH. The dotted curve represents the behavior obtained for a value of rK_1' of 1×10^{-8}. If K_1' is assumed to be the same as K_o' (this will certainly be nearly true), r = 0.1. This means that protonation of the substrate causes an increase in the rate of hydrolysis of the enzyme-substrate complex of approximately ten-fold, which is certainly a reasonable figure.

Equation (66) can be written in the form

$$\begin{aligned}(1/V_{max})(1 \quad rK_1'/h) &= (1/k_3[E]_o)(1 + K_2'/K_c + h/K_c + K_1'/h) \\ &= (1/k_3[E]_o)(1 + K_2'/K_c) \\ &\quad + (K_1'/K_c/k_3[E]_o)(h/h_o + h_o/h) \quad ,\end{aligned} \tag{68}$$

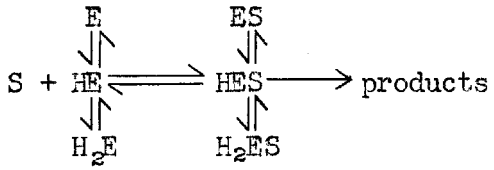
where $h_o = K_cK_1'$ = pH at the optimum for V_{max}. The left-hand side

of the equation consists of known quantities if we accept rK_1' as previously determined. A plot of the left-hand side of the equation against $f(pH) = h_o/h + h/h_o$ will yield a straight line (Figure 51). The slope of this line was found to be 2.38×10^2, the intercept was found to be 9.5×10^2, and $h_o = 1.12 \times 10^{-7}$ ($ph_o = 6.95$). The number of constants in (68) is one more than the number of constants which can be obtained from the straight line. It is interesting, therefore, to assign either to K_1' or K_2' the constant for the free substrate, 1×10^{-7}. If $K_1' = 1 \times 10^{-7}$, $K_2' = 3.22 \times 10^{-7}$, $K_c = 1.26 \times 10^{-7}$, $K_c' = 3.91 \times 10^{-8}$, and $k_3[E]_o = 3.74 \times 10^{-3}$. If $K_2' = 1 \times 10^{-7}$, $K_1' = 3.64 \times 10^{-8}$, $K_c = 3.46 \times 10^{-7}$, $K_c' = 1.26 \times 10^{-7}$, and $k_3[E]_o = 1.36 \times 10^{-3}$. These values are very sensitive to slight changes in the slope and intercept of the line, and are, in reality, not particularly significant. The fact that $K_c > K_c'$ and $K_2' > K_1'$ would be anticipated from the influence of charges resulting from protonation of the enzyme or substrate; protonation of the enzyme should make the ionization of substrate hydrogens more facile, and vice-versa. The order of magnitude of these changes is probably about right for charge interactions of this type. The value of K_c in the second set of data is fairly close to the value of K_c for acetyl-L-tyrosinhydroxamide (4.2×10^{-7}), but this is probably fortuitous. The use of the second set of constants ($K_2' = 10^{-7}$) gives the curve in Figure 52 when substituted into equation (66). The curve agrees with the data within experimental error, and might even give a somewhat closed fit if the line in Figure 51 were drawn a little differently. If K_1' has the value of 3.64×10^{-8}, obtained by assuming $K_2' = 1 \times 10^{-7}$, then $r = 0.27$, still a reasonable value.

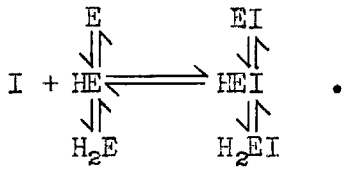
The second set of constants substituted into (65), along with other constants previously determined, should predict the shape of the curve for K_s. It is convenient to plot this logarithmically so that K_m becomes an additive constant. The curve thus obtained is shown superimposed on the experimental data in Figure 53. Agreement of the curve with the experimental points is within the limits of error.

E. The effect of pH on the inhibition constants of competitive inhibitors of hydrolytic reactions catalyzed by alpha-chymotrypsin.

In view of the large amount of information which has been obtained for competitive inhibitors of hydrolysis reactions catalyzed by chymotrypsin, it is important to consider what the influence of pH on the observed inhibition constants might be. The effect of pH on inhibition constants has been formulated by Alberty (4), among others, who treated only the case for the interaction of neutral inhibitors and substrates. He considered the following reaction scheme:



and



If

$$[HE]h/[H_2E] = K_a \tag{69}$$

$$[E]h/[HE] = K_b \tag{70}$$

$$[HES]h/[H_2ES] = K_c \tag{71}$$

$$[ES]h/[H_2ES] = K_d \tag{72}$$

$$[HEI]h/[H_2EI] = K_e \tag{73}$$

$$[EI]h/[HEI] = K_f \tag{74}$$

$$[HE][S]/[HES] = K_m \tag{75}$$

$$[HE][I]/[HEI] = K_{HEI} \tag{76}$$

and if K_I is the observed inhibition constant for the competitive inhibitor,

$$K_I = K_{HEI}\frac{1 + h/K_a + K_b/h}{1 + h/K_e + K_f/h} . \tag{77}$$

The evidence for the case of alpha-chymotrypsin seems to indicate that substrates, and, presumably, inhibitors do not combine with E. Thus for the case of the inhibition of the alpha-chymotrypsin-catalyzed hydrolysis of a neutral substrate by a neutral inhibitor,

$$K_I = K_{HEI}\frac{1 + h/K_a + K_b/h}{1 + h/K_e} . \tag{78}$$

In general, then, K_I becomes a function of pH. However, the value of K_I will be the same for a given pH regarless of the neutral substrate used in the evaluation since K_a and K_b are characteristic of the enzyme alone, and K_e and K_{HEI} are characteristic only of the enzyme-inhibitor

complexes, and comparisons of K_I values at a single pH are valid. When the equation for the influence of hydrogen ion on K_s is written,

$$K_s = K_m \frac{1 + h/K_a + K_b/h}{1 + h/K_c} \quad , \qquad (79)$$

it can be seen that comparisons of the observed values of K_s and K_I are equivalent to comparisons of K_m and K_{HEI} if either 1) $K_e = K_c$ or 2) the pH is high. Since the values of K_c so far obtained seem to be greater than 10^{-7}, and since K_e is probably nearly the same as K_c for most inhibitors, the chances are very good that such comparisons will be valid at pH's of about 7.9 where most of these comparisons have been made.

It can be shown that for the case of acetyl-L-tyrosinhydroxamide the expression for inhibition by neutral inhibitors is the same as (78).

In the case of acetyl-D-tryptophanamide, the values obtained are: pH 6.2, $K_I = 2.0$; pH 6.9, $K_I = 2.0$; pH 7.9, $K_I = 2.7$; pH 8.6, $K_I = 2.4$. The drop from 2.7 to 2.4 is not significant experimentally. The equation (78) gives a qualitative explanation for the rise in K_I, but predicts a much higher value at pH 8.6. The value of 2.4 is the product of some rather poor data and perhaps should be rechecked. The value at 6.2 might be higher or that at 6.9 lower to suit the theoretical equation better.

For the case of acetyl-L-tyrosine, it appears that combination of this molecule takes place chiefly with the species H_2E, and not with HE. In past formulations, the dissociation constants for the enzyme substrate complex have always been stated in terms of the species HE. In the case of acetyl-L-tyrosine, it is preferable to discuss the

observed behavior in terms of a dissociation constant for the species H_2EI:

$$[I][H_2E]/[H_2EI] = K_{H_2EI} \quad . \tag{80}$$

If it is assumed that acetyl-L-tyrosine does not interact at all with HE,

$$\begin{aligned} K_I &= K_{H_2EI}K_a/h(h/K_a + 1 + K_b/h) \\ &= K_{H_2EI}(1 + K_a/h + K_aK_b/h^2) \quad . \end{aligned} \tag{81}$$

A plot of the experimental observations of K_I against 1/h should produce a parabola. The curve which is actually obtained is not, however, concave upward, and some interaction of acetyl-L-tyrosine with HE seems to be indicated. The behavior of the curve before this interaction becomes very significant and before K_b becomes of importance should follow

$$K_I = K_{H_2EI}(1 + K_a/h) \quad . \tag{82}$$

Figure 54 shows values observed up to pH 7.6 plotted against 1/h. Actually this pH is perhaps a little too advanced for rigorous consideration, but the effects of K_b and of the interaction of the acetyl-L-tyrosine with HE are in opposite directions, and these points may be reasonably accurate by virtue of the counterbalancing effects. The value for K_a obtained from the straight line is 1.4×10^{-7}. This agrees well with the value obtained from the kinetic study of the hydrolysis of acetyl-L-tyrosinhydroxamide. These two values for K_a actually represent completely independent determinations.

This behavior of acetyl-L-tyrosine with pH was anticipated from the results of experiments of R. J. Foster, who noted an increase in the K_I value of the acetyl-D-and-L-tryptophans and indoyl-3-propionic acid in going from pH 6.9 to pH 7.9 (13), and from experiments using equilibrium dialysis methods with radioactive acetyl-L-3,5-dibromotyrosine (33).

The manner of change of the inhibition constant for L-tyrosinamide is rather unexpected in comparison with the variation of K_s noted for L-tyrosinhydroxamide. A decrease in K_I, similar to that observed for L-tyrosinhydroxamide, was noted by Foster for D-tryptophanamide in going from pH 6.9 to pH 7.9 (13). The equation relating the change in K_I to hydrogen ion concentration is very similar in form to the equation for K_s for L-tyrosinhydroxamide:

$$K_I = K_{EI}\frac{(1+K_o'/h)(h/K_a+1+K_b/h)}{1+K_1'/h+h/K_e+K_2'/K_e} \qquad (83)$$

$$K_{EI} = [IH^+][HE]/[HEIH^+] \quad . \qquad (84)$$

K_o' is the acid constant for the protonated amino group of the substrate; K_1' and K_2' are quantities analogous to those for the proposed reaction scheme for the substrate. Although K_o' for this compound has not been determined, it would be expected to be of the same order of magnitude as the acid constants for the alpha-amino group of other amino acid amides. pK_A for this group in glycinamide is 7.9. Although it is conceivable that equation (83) could describe the behavior of the inhibition constant for L-tyrosinamide, the magnitudes of the constants which would be required would probably

be quite different from those employed in the description of the behavior of the related substrate. The behavior of the inhibition constant is based on a rather slim amount of data, yet some doubt is cast on the validity of some of the proposed reaction schemes. Of course, the behavior of K_I for L-tyrosinamide may very well not be strictly analogous to the behavior of K_s for L-tyrosinhydroxamide; a better inhibitor for comparison purposed would be D-tyrosinhydroxamide. (A determination of the K_I value of the latter compound has been made at pH 6.9, and the value found was 40×10^{-3} M (16). This value is unusually high for an inhibition constant of a D-inhibitor in comparison to the K_s value for the L-substrate.) One not too unlikely possibility is that amino acid hydroxamides exist as zwitter ions. In this case the pK_A of 9.2 would refer to the amino group (compare to 9.6 in glycine), and the value of 7.0 would refer to the hydroxamic acid group (compare 4.8 for the carboxyl group in acetic acid with about 2.5 for this group in glycine). In the event that this situation should exist, the interpretation of the effect of pH on the hydrolysis of L-tyrosinhydroxamide would necessarily be different, although the form of the equation relating velocity and the various equilibrium constants would still be similar to the equation proposed. Most likely, the K_s/V_{max} equation would have the same form and the same values of the constants as proposed in the preceding discussion, even though the ionization constants for the groups were interchanged.

In the case of inhibition by acetyl-L-tyrosinamide, the variation with pH of the value of the apparent K_I is about that which would be predicted on the basis of equation (78). Using the values of K_a, K_b, and $K_c = K_e$ obtained from the studies on acetyl-L-tyrosinhydroxamide

together with a value of 32 x 10^{-3} M for K_s = "K_I" of acetyl-L-tyrosinamide (see Table XLV) at pH 7.9, a value for K_I of 55 x 10^{-3} M would be indicated for pH 6.2, and a value of 54 x 10^{-3} M at pH 8.6. If the value of 40 x 10^{-3} M determined in the present series of experiments at pH 7.83 is accepted as correct for K_I, then values of 72 x 10^{-3} M at pH 6.2 and 71 x 10^{-3} M at 8.6 would be predicted. These values may be compared to the observed ones of 67 x 10^{-3} M at pH 6.2 and 68 x 10^{-3} M at pH 8.6. The manner of variation of this constant with pH seems to be adequately explained by equation (78) if the assumption is made that K_e for the "inhibitor" is equal to K_c for the hydroxamide. The observed values for "K_I" are likely to be high because of hydrolysis of the acetyl-L-tyrosinamide itself. The agreement obtained is probably within the limits of the experimental error. This small amount of data would not seem to justify a more extended discussion of the quantitative aspects of hydrogen ion influence on the simultaneous hydrolysis of two substrates.

EXPERIMENTAL SECTION

A. The preparation and properties of compounds.

Acetyl-L-tyrosine. This compound has been prepared by many investigators (cf. 29); however, some comments might well be added to the general fund of information. Since acetyl-L-tyrosine is fairly soluble in H_2O, it may well be advantageous to keep the volume of the acetylation reaction mixture small. One experiment, not carried through for optimum yield, may be illustrative: 25 gm. L-tyrosine was added to a solution of 13 gm. NaOH pellets in slightly less than 50 ml. of H_2O. This solution was cooled in ice, and 23 ml. of acetic anhydride was added over a period of an hour with frequent vigorous agitation. The solution was tested frequently to insure basic conditions, and portions of a concentrated NaOH solution were added as needed. The solution was placed overnight in the refrigerator. The next day the solution was acidified while tested with a pH meter. It was noted that no precipitate was formed at the isoelectric point of tyrosine. The pH was ultimately brought to 0.7, and the resulting solution reduced to a thick syrup in vacuo. Some toluene was added and then removed in vacuo in an effort to eliminate some of the traces of acetic acid and water by azeotropic distillation; this did not seem to be particularly successful. The syrup was then extracted with two portions of acetone, and the extracts were filtered free of the salt which precipitated out. (Extraction with alcohol at this stage may lead to varying amounts of esterification in subsequent steps.) The acetone was removed in vacuo. A small quantity of water was added to the resulting syrup, and this solution was somewhat reduced in volume by distilling in vacuo in order

to remove traces of acetone. Water was then added until the volume was about 50 ml., the solution was heavily seeded, and the mixture was placed in the refrigerator. Crystallization was aided by occasional stirring during the two days necessary to get reasonable amounts of material. (It may be noted that acetyl-L-tyrosine is very soluble in hot water and may be recrystallized from very small volumes. Furthermore, crystallization from larger volumes is slow and incomplete.) The crystals were collected and thoroughly dried in vacuo over P_2O_5. In this experiment, 13 gms. of material, M. P. 152-153° (lit. 152-154 (29)), was obtained. Probably a larger yield would have resulted from the re-treatment of filtrates.

Acetyl-L-tyrosine ethyl ester. This compound has also been prepared on numerous occasions (cf. 21). The standard technique of dissolving the acetylated acid in absolute ethanol, followed by saturation of the chilled solution with gaseous HCl was used with good success. The following observations might be useful: 1) After the solution saturated with HCl had been allowed to stand overnight, the alcohol and HCl were removed in vacuo, and the residual syrup was dissolved in water. If crystallization does not occur spontaneously, neutralization of the solution with solid $NaHCO_3$ may be required; 2) The solid material resulting from this treatment and from subsequent recrystallizations was found to have a low melting point unless very thorough drying methods were used. (Evidently a semi-stable hydrate melting at about 70° C is formed. Hard crystals of this hydrate resulting from vacuum desiccation of the solid over $CaCl_2$ will actually become very gummy when subjected to more stringent drying conditions. This gummy material will again become hard and exhibit the melting point of

acetyl-L-tyrosine ethyl ester if dried further.) Several days in vacuo over P_2O_5 were necessary for complete drying. Alternatively the hydrate was dried by dissolving it in ethanol and benzene and reducing the volume of the solution by boiling at atmospheric pressure. Water distilled off in the azeotrope. The remaining solvent was stripped off in vacuo and the resulting syrup induced to crystallize by stirring it into ligroin and scratching with a glass rod.

Acetyl-L-tyrosinhydroxamide. The preparation of this compound has been described by Hogness (14 . The method given here may be used alternatively. 10.5 Gms. Na metal was dissolved in about 160 ml. of methanol. About 80% of this solution was added to a nearly saturated solution of 24.4 gms. of hydroxylamine hydrochloride, and the resulting salt was filtered off. The solution was chilled, and 22 gms. of acetyl-L-tyrosine ethyl ester was added. Then the remaining 20% of the sodium methoxide solution was added, and the resulting reaction mixture was allowed to stand two days in the refrigerator. The solid which resulted, the sodium salt of the hydroxamide, was collected by filtration, washed with methanol and dried. The product weighed 20.6 gms. and melted at 185-186° with extensive decomposition. (The difficult part of this preparation lies in the conversion of this sodium salt to the free acid. The reason for this difficulty is unknown. The following procedure is somewhat analogous to some employed for the purification of other simpler hydroxamides.) The sodium salt was dissolved in about 300 ml. of H_2O, and a saturated solution of $CuSO_4$ was added dropwise with stirring. A green, gelatinous precipitate of the copper salt of the hydroxamic acid formed upon addition of the $CuSO_4$. This salt appears to have some base incorporated in it, and 2N NaOH is added alternatively with the $CuSO_4$ solution until a test of a small

amount of centrifugate with $FeCl_3$ solution gave no red color, thus indicating complete precipitation. It is not certain whether or not the solution should be prevented from becoming acid during the precipitation. The use of a pH meter in this operation might be advantageous. The large volumes of copper salt were centrifuged and collected, then twice washed with H_2O and centrifuged. The damp Cu salt was then placed in methanol and the resulting mixture saturated with H_2S while stirring. (When the Cu salt is placed in the methanol, quite a lot of heat may be evolved, and the character of the precipitate may change. This is particularly true if the volume of methanol is small. This effect is probably undesirable, and no doubt can be avoided by adding the salt in small portions to the methanol while stirring and passing in H_2S continuously. Methanol was used for this purpose in preference to water because the CuS precipitate coagulates more readily in methanol, and the methanol is more easily removed than water from the hydroxamide.) To the mixture of CuS and methanol solution was added a considerable amount of decolorizing charcoal to help speed the coagulation of the CuS and to remove traces of color from the solution. The charcoal and CuS were filtered off, and the methanol was completely removed by stripping in vacuo. The resulting solid was dried and found to weigh 16.6 gms. (The removal of the methanol in one other case resulted in a syrup which crystallized upon continued exposure to vacuum.) The solid was placed in a very small amount of warm isopropanol -- caution is required since the solubility of the hydroxamide is very high -- and more isopropanol was added in portions with continuous warming. When the solid was largely dissolved, the residues were removed by rapid filtration. The solution, now brown in color, was cooled quickly to prevent further

darkening. Decolorizing charcoal was found to be relatively ineffective in removing this color. The solution was seeded heavily and placed in the refrigerator. Crystallization was very slow, and the crystallizing solution was allowed to remain in the cold for four days. Occasional stirring seemed to help promote crystallization. The crystals were then collected on a small Hirsh filtration funnel and washed 2 times with small portions of ice-cold isopropanol. This procedure proved to be quite effective in removing traces of color from the precipitate. 6.5 Gms. of white solid was obtained, M. P. 143-144° with extensive decomposition (lit. 144°), $[\alpha]_D^{25}$ = + 38.7° (lit. 38.4°(14)), c = 5% in H_2O. No effort was made to increase the yield by reworking filtrates, although this would probably have been effective. The necessary recrystallization always seemed to result in rather considerable losses, yet a better solvent was not found in spite of a fairly extensive search. There were some indications that mixed solvents of higher alcohols (eg. benzyl alcohol) and toluene might prove satisfactory.

Acethydroxamide. (30) 20 Gms. of hydroxylamine hydrochloride was dissolved in a previously chilled solution of 25 gms. NaOH pellets in 100 ml. H_2O. To this solution, which was chilled in ice, was added 29 ml. of ethyl acetate in several small portions. This solution was allowed to come to room temperature, and concentrated aqueous $CuSO_4$ and NaOH were added alternately until the green Cu salt of the hydroxamide no longer was formed. The resulting green precipitate was washed twice with water with centrifuging between washings, then pressed between paper towels to remove excess water. The solid was suspended in methanol and treated with H_2S. The solids were filtered off, retreated with H_2S in methanol, and the resulting mixture filtered. The filtrates

were combined and reduced to a syrup *in vacuo*. The resulting syrup was further dried by dissolving it in ethyl alcohol–benzene mixture and stripping. This syrup was extracted with three 100 ml. portions of ethyl acetate and the extract was cooled in ice. The solid which resulted was collected by filtration, washed with ligroin, and dried *in vacuo*. It weighed 6.0 gms., and melted at 87–89°. When a concentrated acetone solution containing 5.3 gms. of this solid was treated with ligroin, a precipitate was formed. This precipitate, after twofold washing with ligroin and drying, weighed 4.6 gms. and melted at 88–90° (lit. 90° (30)).

Glycinhydroxamide. (21) To 70 ml. of 1.11 N sodium methoxide was added 4 gms. of hydroxylamine hydrochloride, and the resulting salt was filtered off. Similarly 7.75 gms. of glycine ethyl ester hydrochloride was added to 50 ml. of 1.11 N sodium methoxide, and the resulting salt was removed by filtration. The resulting solutions of glycine ethyl ester and hydroxylamine–sodium methoxide were mixed together and allowed to stand for 3 days. Some white solid separated from the solution. Without separating the solid, the methanol was removed *in vacuo*, giving still more solid. All of the solid was dissolved in hot water and allowed to crystallize. The crystals were collected, washed with cold water, and then dessicated over $CaCl_2$ *in vacuo* for 4 days. The product thus obtained weighed 2.9 gms. and decomposed at 135° with extensive bubbling and the appearance of a white solid. Recrystallization twice from H_2O–methanol did not seem to raise the decomposition point, which has been reported as 140° (31) and 142–143° (32). The neutral equivalent, determined in part B of this section, was found to be 91 ± 2 (calculated 90.0).

B. Titrations of acethydroxamide and glycinhydroxamide.

Titration of acethydroxamide. 0.1294 Gms. of acethydroxamide was placed in a beaker and titrated with 0.112N NaOH. The course of the titration was followed with a Beckman pH meter. A plot of this titration is given in Figure 55. The pH at the theoretical equivalence point is 9.4. The neutral equivalent corresponding to the endpoint estimated from the data is 77 (theoretical 75). This solution was then back titrated with HCl (Fig. 56). The pH at the calculated $\frac{1}{2}$ equivalence point was again found to be 9.4, and the neutral equivalent corresponding to the difference in the two end points estimated from the data was determined to be 73. The pK_A of this acid thus appears to be about 9.4, and the neutral equivalents obtained from the titrations indicate satisfactory purity of the material.

Titration of glycinhydroxamide. 0.450 Gms. of glycinhydroxamide was dissolved in water and quantitatively diluted to 50 ml. A 10 ml. aliquot of this was titrated with 0.100 HCl (Fig. 57) and the resulting solution was back titrated with 0.112N base (Fig. 58). Next a 10 ml. aliquot was titrated with base alone (Fig. 59). The neutral equivalent from the HCl titration alone was found to be 93, and the pH at both the theoretical and experimental $\frac{1}{2}$ equivalence points was 7.4. From the back titration with NaOH, the molecular weight was estimated to be 90. The pH at the $\frac{1}{2}$ equivalence point was 7.4, and the pH at the 1.5 equivalence point was 9.4. A neutral equivalent of 89 was obtained from the titration with base alone, and the pH at the $\frac{1}{2}$ equivalence point was found to be 9.4. Comparison of the neutral equivalent values determined above with the theoretical value (90.1) seemed to

indicate satisfactory purity of the glycinhydroxamide. Assuming that all of the ionization at the $\frac{1}{2}$ equivalence point is due to the amino group, the pK_A is found to be 7.4. If it is assumed that the amino group is completely unprotonated at the 1.5 equivalence point, the pK_A of the hydroxamic acid group is 9.4.

C. The kinetic studies on the alpha-chymotrypsin-catalyzed hydrolysis of L-tyrosinhydroxamide.

The L-tyrosinhydroxamide used for this experiment was the same as that used by Foster (13) in his work with this compound. The reactions were all carried out in a thermostatically regulated bath maintained at $25.0 \pm 0.1^{\circ}$.

An Armour preparation of bovine alpha-chymotrypsin (lot number 90402) was employed for these experiments. Since the molecular weight of alpha-chymotrypsin is not accurately known, and since commercial preparations contain some amounts of inorganic salt, it has been customary to express enzyme concentrations in terms of milligrams of protein nitrogen per milliliter, the assumption being that all of such nitrogen is associated with active enzyme. The concentration used in these experiments was 0.208 milligrams of protein nitrogen per milliliter. The enzyme solutions were freshly prepared each day and kept cold except when brought to 25° for use.

Both cacodylic acid - sodium cacodylate and tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer systems were used to maintain nearly constant pH in the reaction solutions. The concentration of the organic component of the buffer was 0.10 formal in all cases. While, in general, the cacodylic acid system was used for the lower pH region,

and the amine buffer was employed at higher pH's, both buffers were used in the median pH region in order that any influence of the nature of the buffer on the kinetic course of a reaction might be observed. Results indicate that there is no difference between the two buffers insofar as their effect on the hydrolysis reaction is concerned (Fig. 9-13).

The analytical method which was used to follow the reaction was essentially the same as that developed by previous investigators (12, 13, 14). The basis of the method is the well known fact that hydroxamic acids give an intense red color with $FeCl_3$ solutions. Studies by previous investigators have established that the color development under standardized conditions follows Beer's Law. Foster, who has made fairly exhaustive studies on the use of the colorimetric technique with L-tyrosinhydroxamide, found that the color development was very good in methanol solutions 0.02f in $FeCl_3$ and HCl. (The colored complex formed when a hydroxamic acid is added to this solution is very stable, and the observed extinction does not change for periods as long as 16 hours). Foster noted that many added substances affected the intensity of the color; in addition, casual observation seemed to indicate some small variations which were dependent upon the age of the stock preparation of $FeCl_3$, the particular stock preparation used, and so forth. These minor variations were seldom greater than 5%. Furthermore, Beer's Law was always obeyed. It was observed that both cacodylic acid and water caused diminished color intensities, but did not affect Beer's Law behavior.

An actual kinetic experiment might be described as follows: First, the correct amount of substrate is either weighed into a 10 milliliter

volumetric flask or added in the form of a measured volume of a freshly prepared stock solution. Next, the appropriate buffer is prepared by mixing a 1f solution of free amine with a 1f solution of its salt in proportions designed to give about the desired pH (cacodylic acid buffers are similarly prepared), and 1 ml. of the mixed buffer is added to the flask containing the substrate. Water is then added until the volume is about 8.5 milliliters, and the substrate is dissolved. With high substrate concentrations, heating and agitating the flask may be necessary to effect solution. This does not cause any hydrolysis of the substrate, since the zero time extinction value may be observed to fall on the Beer's Law curve constructed from observations on unheated solutions. This solution is placed in the thermostatically controlled bath. A stock solution of the enzyme is also placed in the bath together with a dropper bottle of distilled water, which is to be used for diluting the reaction mixture to volume. A number of volumetric flasks are partially filled with methanol and the appropriate amount of a stock solution to give a solution 0.02f in $FeCl_3$ and 0.02f in HCl upon dilution. The range of intensities of color resulting from the various substrate concentrations employed in these experiments is too large to allow the same dilution for color development to be used for all experiments, hence either the size of the flasks used for the development of the color or the size of the aliquot may vary from experiment to experiment. In order to prepare an appropriate blank for use in making the colorimetric determinations, 1 milliliter of the buffer mixture prepared above and 1 milliliter of the enzyme solution are placed in a 10 milliliter volumetric flask and diluted to volume with water. An appropriate aliquot of this solution is placed in one

of the flasks containing the methanol - $FeCl_3$ - HCl solution, and this is diluted to volume with methanol to give the solution to be used as a blank. (Sometimes the enzyme may be omitted from the blank, as it has no effect on the extinction of either the blank or the ferric hydroxamate solutions.) The blank is placed in a Beckman Model B spectrophotometer, and its transmission is determined against an air path; this is done routinely so that any errors in the preparation of the blank or other solutions can be detected immediately. At zero time, one milliliter of the stock solution of the enzyme is placed in the reaction flask, which is immediately diluted to volume and mixed thoroughly by inverting repeatedly. Aliquots of the reaction mixture are then withdrawn at various times and placed in the flasks containing the $FeCl_3$ etc., which are immediately diluted to volume with methanol and mixed. These colored solutions are placed in a cell with a 1 cm. light path, and their extinctions are determined in the spectrophotometer at a wave length of 505 millimicrons (13). At the conclusion of the run, the remaining reaction mixture is placed in a Beckman pH meter and the final pH is determined. The meter is carefully adjusted beforehand and standardized at 25° with a buffer solution of a known pH. The error in determining the pH is probably about 0.02 - 0.03 pH units. In the case of some few runs, a sample of the reaction mixture is withdrawn near the start of the run, and its pH is determined; by this means an idea of the change in pH during the course of the reaction may be obtained. This rough estimate of the change of pH per unit of substrate hydrolyzed can be applied to other runs where only the final pH has been determined, and the median pH, which is used in calculation, can then be estimated with sufficient accuracy. Plots of the change in pH

per 10^{-3} M of substrate hydrolyzed vs. pH have been made for cacodylic acid and tris-(hydroxymethyl)-aminomethane buffers and are shown in Figure 60. (It might be noted that an accuracy of ± 30% is adequate.)

The data which have been obtained from experiments with L-tyrosinhydroxamide are presented in Table XLIX.

D. The kinetic studies on the alpha-chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide.

The acetyl-L-tyrosinhydroxamide used for these experiments was obtained from several sources (cf. part A this section), but was always checked for purity using both specific rotation and the comparison of the extinction value obtained in the colorimetric test (as described below) with that of an authentic sample. The reactions were all carried out in a thermostatically controlled bath at 25°.

An Armour preparation of alpha-chymotrypsin (lot 10705) was used for all experiments, and the same precautions were observed for the enzyme stock solutions used in these experiments as for the solutions employed for the kinetic studies on L-tyrosinhydroxamide. The enzyme concentration used for all of these experiments was 0.040 mg. protein nitrogen per milliliter.

The following table indicates the buffers used for these experiments.

A	B	Concentration A (formal)
Tris-(hydroxymethyl)-aminomethane	HCl	0.3
Cacodylic acid	NaOH	0.3
Phosphoric acid	KOH	0.3
Ethylenediamine	HCl	0.3
Hydroxylamine	HCl	0.3

Tris-(hydroxymethyl)-aminomethane and cacodylic acid buffer systems

were used for the most part. Results appear to indicate that, of the buffers listed above, only hydroxylamine-hydrochloric acid buffer seems to influence the kinetic course of the reaction.

The analytical method, essentially that used by Hogness for this substrate (14), has been described in sufficient detail in part C of this section. The wave length of light employed for these studies was 515 millimicrons. Beer's Law behavior was observed regardless of the buffer system employed. It may be noted that the solubility of acetyl-L-tyrosinhydroxamide in water is such that heat was never required to effect solution. Also, the ferric chloride stock solution employed for many of these experiments was 0.5f in HCl. This was particularly necessary for those experiments which were performed at high pH's. This increase in the amount of HCl did not seem to cause any appreciable change in the observed extinction values. A special reagent was required for use with the phosphate buffer, and was prepared as follows: 54 gms. $FeCl_3 \cdot 6H_2O$ was dissolved in water and 200 ml. of concentrated hydrochloric acid was added. This solution was diluted to 500 ml. with water, and then to 1 liter with methanol. This stock solution was employed at a 1:10 dilution for the color reaction. Some fading of the color was noted with time; however, the extent of this fading probably was not sufficient to cause errors large enough to affect the experimental results.

For low pH's, the starting pH for each run was found to be the same as that for the buffer alone. However, when the pH of the reaction mixture was larger than about 8.0, the effect of ionization of the hydroxamic acid grouping became noticeable. It was found possible to estimate the starting pH's of these runs if acid constants of

1×10^{-9} for the hydroxamic acid and 7×10^{-9} for tris-(hydroxymethyl)-aminomethane-hydrochloride were assumed. The results of calculations based on these assumed constants were verified by experiment. An example of such an experiment is given in Table L.

When charged inhibitors were added to the solution, the pH was adjusted to the desired pH by placing a solution of substrate, inhibitor, and the correct amount of the unionized species of buffer in a Beckman pH meter and adding NaOH or HCl until the desired pH was reached. The 8 ml. of solution resulting from this operation was then transferred quantitatively to a 10 ml. volumetric flask; extinction values indicated that this operation was conducted without mishap. It was assumed that dilution from 8 to 10 ml. would not significantly affect the pH, and this is borne out by observation of the final pH of runs at low substrate concentration where the change in pH resulting from hydrolysis is negligible.

The runs made with acetyl-L-tyrosinhydroxamide are presented in Table LI.

SYMBOLS AND CONVENTIONS

In writing protonated forms of enzyme–substrate or enzyme inhibitor complexes, the protons are placed adjacent to the member of the complex which possesses the ionizing group. Thus H_2ESH indicates a complex in which two protons are combined with basic groups on the enzyme, and one proton is combined with a basic group on the substrate. Since the exact charge state of the enzyme is uncertain, charges are indicated only for substrate or inhibitor molecules. For example

$$H_2ESH \rightleftharpoons H_2ES^- + H^+$$

would be used to indicate the loss of a proton from a carboxyl group of the substrate portion of the complex. Protonated and charged forms of free inhibitor or substrate molecules are similarly indicated. E, HE, H_2E refer to various kinetically important protonated states of the free enzyme molecule.

Symbols used in the text are summarized below.

[ ] Brackets are used to denote concentration.

E Enzyme molecule.

$[E]_0$ Total enzyme concentration [free and combined] in a reaction system.

f_1 Correction factor for first order plots of kinetic data [equation 7, p. 37].

f_0 Correction factor for zero order plots of kinetic data [equation 8, p. 37].

h Hydrogen ion concentration or percent hydrolysis.

h_0 The optimum hydrogen ion concentration for a particular enzyme–substrate system.

I Inhibitor molecule.

k_1 Reaction rate constant [p. 50 – 51].

k_2 Reaction rate constant [p. 50 – 51].

k_3 Reaction rate constant [p. 50 – 51].

k_3' Reaction rate constant [p. 58].

K' Acid constant of substrate molecule [equation 29, p. 58].

K_0' Acid constant of substrate molecule [equation 56, p. 64].

K_0'' Acid constant of substrate molecule [equation 57, p. 64].

K_1' Acid constant of substrate when combined with enzyme [equation 58, p. 64].

K_2' Acid constant of substrate when combined with enzyme [equation 59, p. 64].

K_a Acid constant of free enzyme [equation 16, p. 55].

K_b Acid constant of free enzyme [equation 17, p. 56].

K_c Acid constant of enzyme when combined with substrate [equation 18, p. 56].

K_c' Acid constant of enzyme when combined with substrate [equation 55, p. 64].

K_d Acid constant of enzyme when combined with substrate [equation 19, p. 56].

K_e Acid constant of enzyme when combined with inhibitor [equation 73, p. 69].

K_f Acid constant of enzyme when combined with inhibitor [equation 74, p. 69].

K_{HEI} Dissociation constant of enzyme–inhibitor complex [equation 76, p. 69].

K_{H_2EI} Dissociation constant of enzyme–inhibitor complex [equation 80, p. 71].

K_I Observed dissociation constant for an enzyme–inhibitor complex [equation 4, p. 36].

K_m Dissociation constant for an enzyme-substrate complex [equation 20, p. 56; equation 53, p. 64; equation 75, p. 69].

K_p Dissociation constant for an enzyme-split product complex [equation 5, p. 37].

K_s Observed Michaelis – Menten constant.

P Inhibiting split product molecule.

r Ratio between rate constants of two competing reactions [equation 61, p. 65].

S Substrate molecule.

$[S]_o$ Initial substrate concentration for a kinetic experiment.

$[S]_t$ Substrate concentration in a kinetic experiment after elapsed time t.

t Time.

v Velocity of reaction.

v_o Initial velocity of reaction.

V_{max} The limiting maximum velocity for a given enzyme concentration.

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Figure 9. Plot of $(1/60)\ln[S]_o/[S]_t$ vs. pH for substrate concentrations of 2.00, 3.00, and 5.00 x 10^{-3} M.

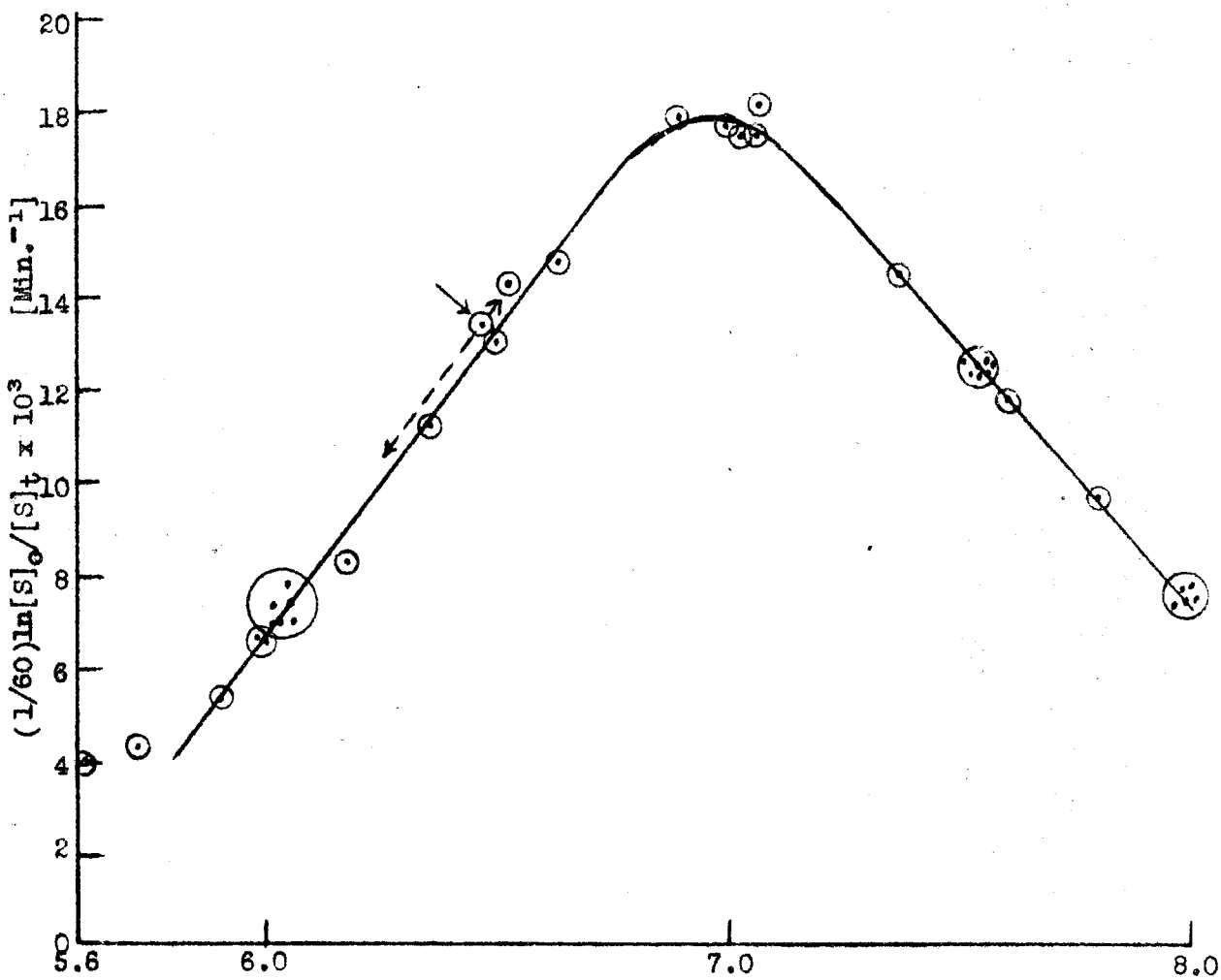


Figure 10. Plot of $(1/60)\ln[S]_o/[S]_t$ vs. pH for substrate concentration of 10.0×10^{-3} M.

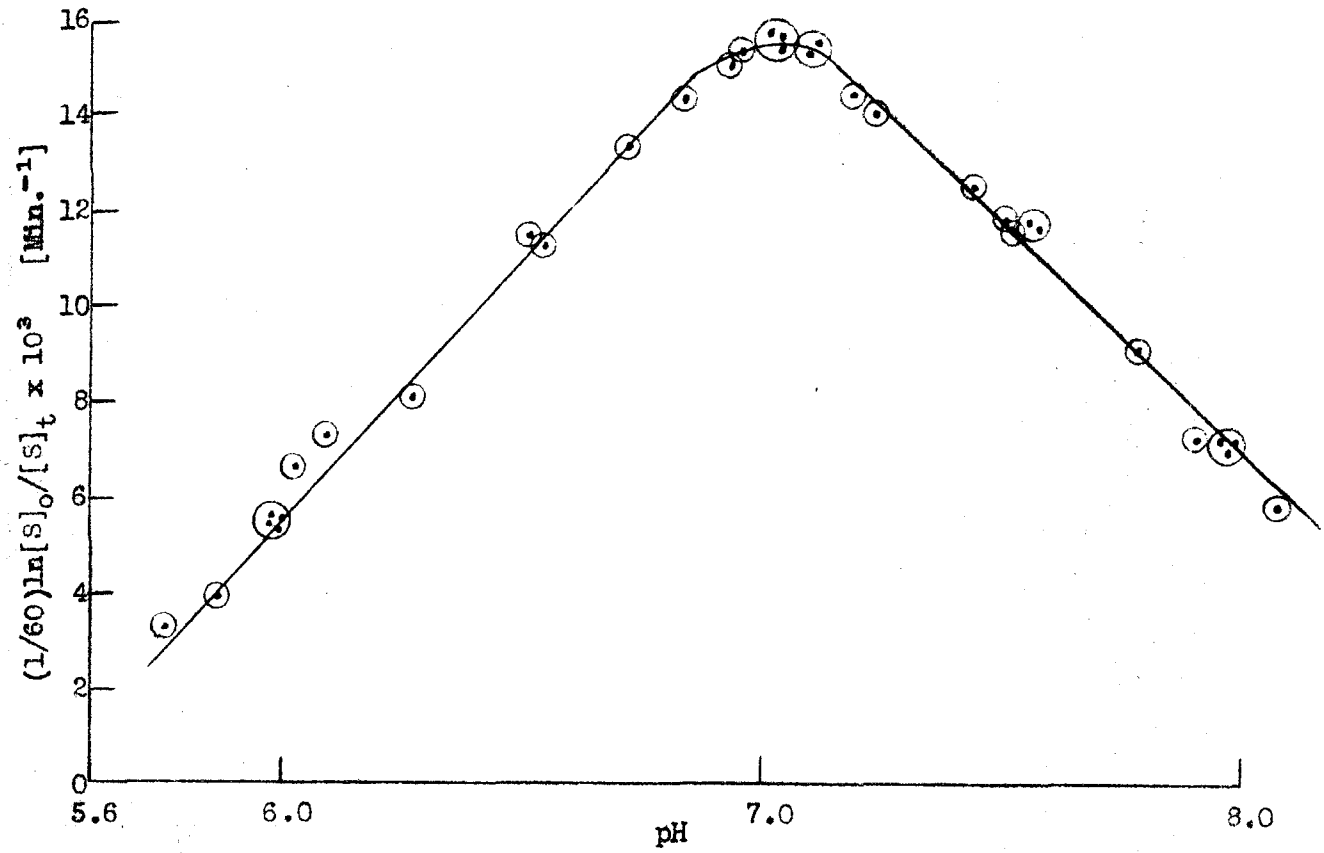


Figure 11. Plot of $(1/60)\ln[S]_o/[S]_t$ vs. pH for substrate concentration of 20.0×10^{-3} M.

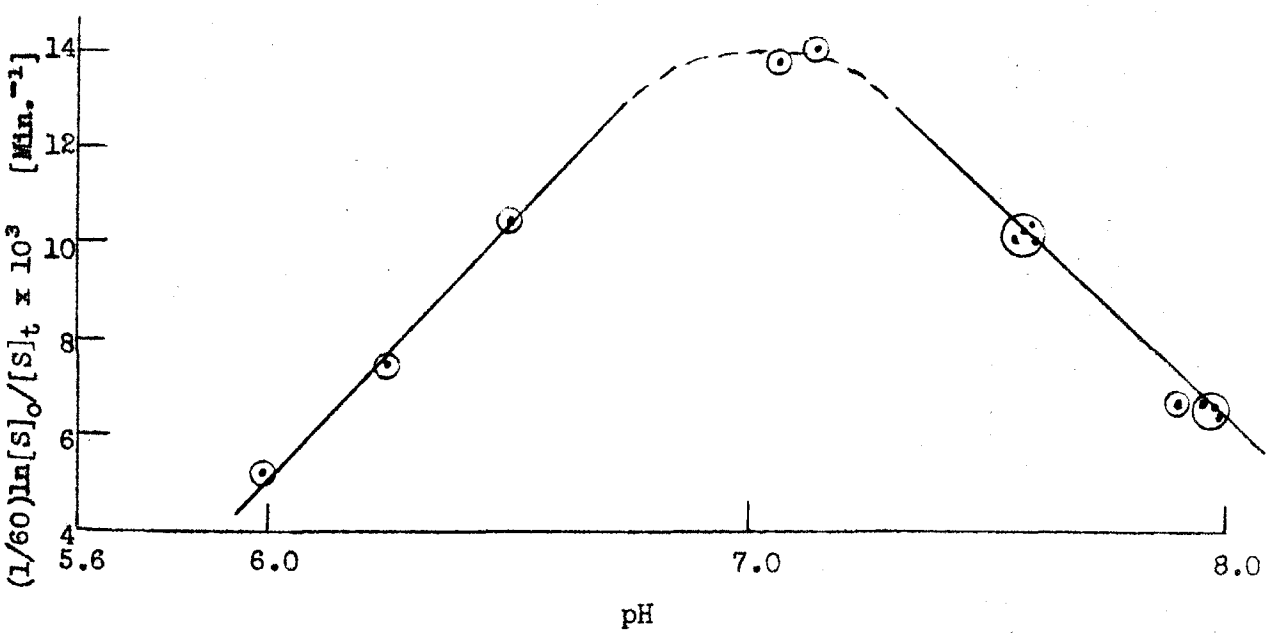


Figure 12. Plot of $(1/60)\ln[S]_o/[S]_t$ vs. pH for substrate concentration of 30.0×10^{-3} M.

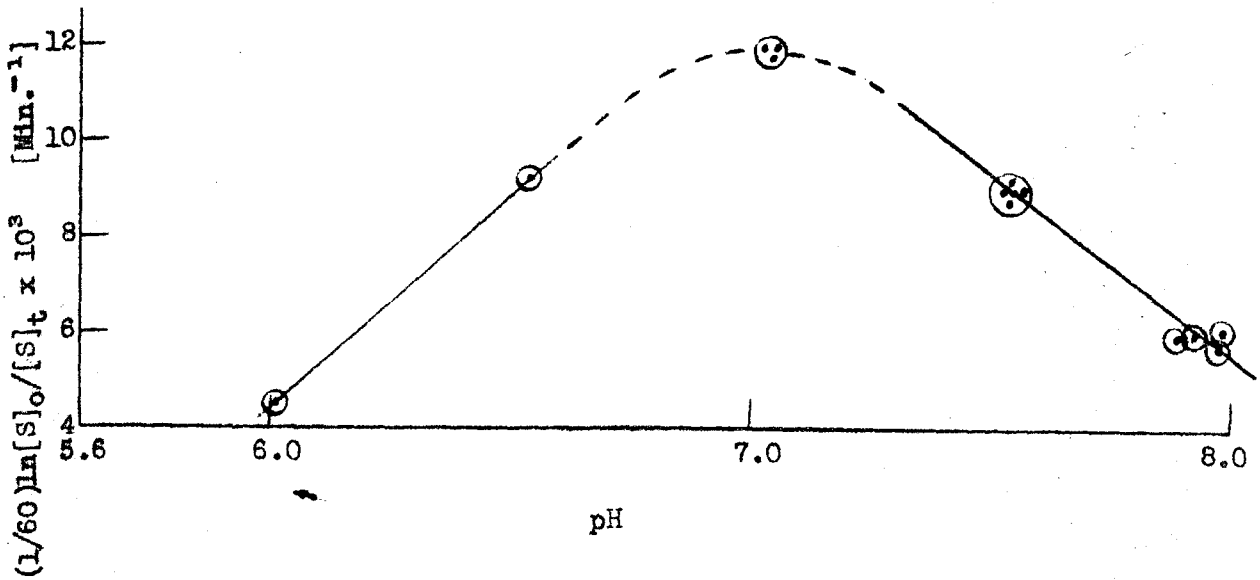


Figure 13. Plot of $(1/60)\ln[S]_o/[S]_t$ vs. pH for substrate concentration of 40.0×10^{-3} M.

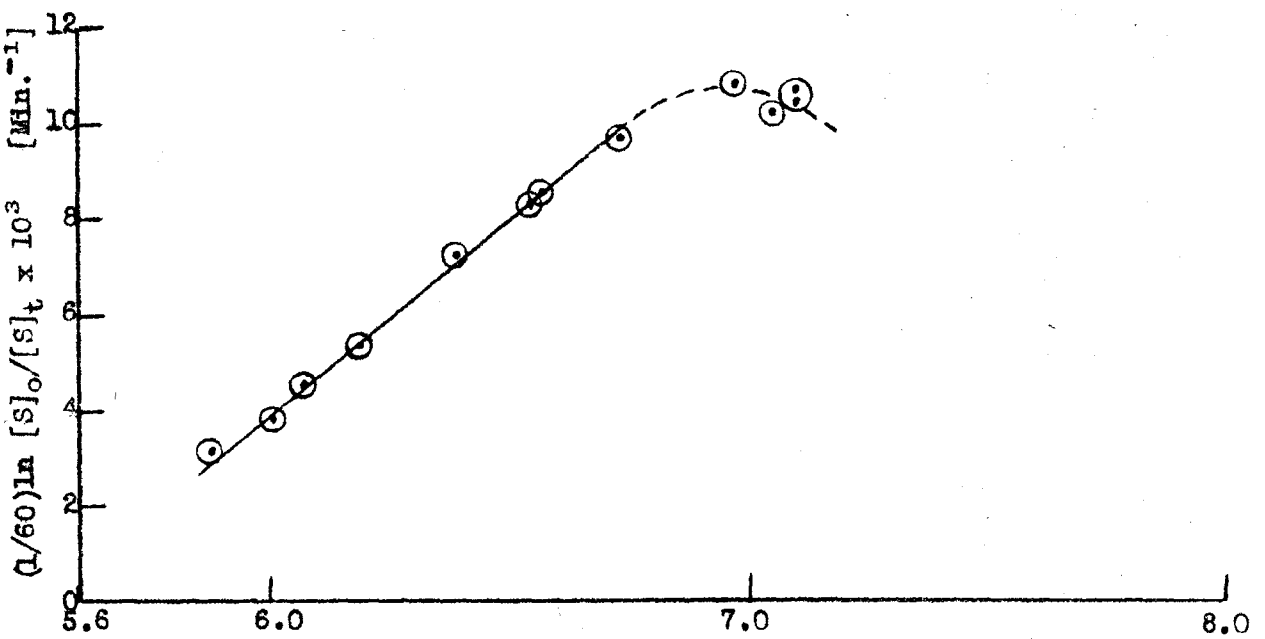


Figure 14. Walker Schmidt plot of data extrapolated to pH 6.0. Heavy line is best estimate of the straight line through the points. Arrows indicate the average deviation in the value of $(1/60)\ln[S]_o/[S]_t$ where this could be determined. The dotted lines indicate reasonable limits of error in the data.

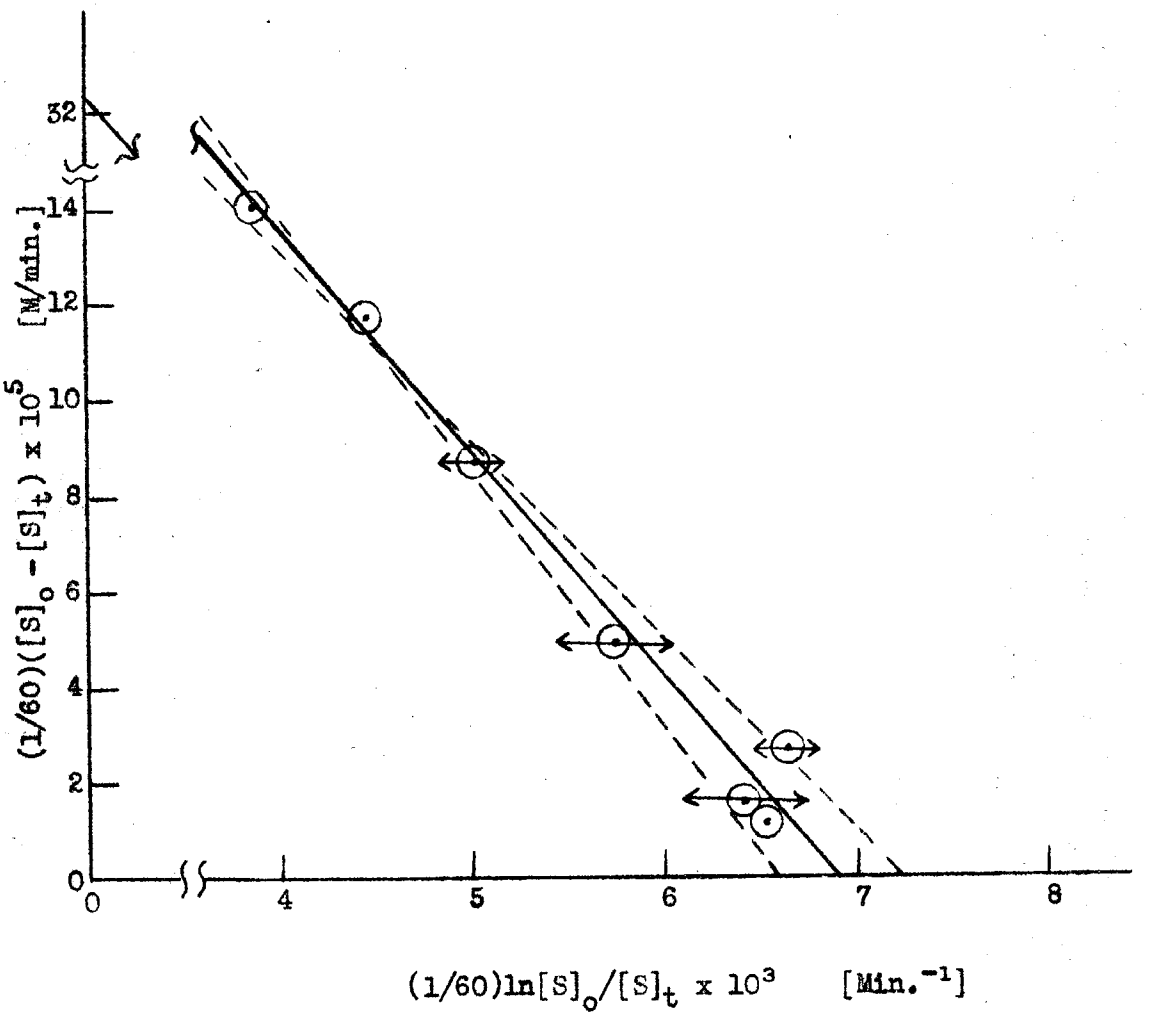


Figure 15. Walker-Schmidt plot of data extrapolated to pH 6.25. Heavy line is best estimate of the straight line through the points. Arrows indicate the average deviation in the value of $(1/60)\ln[S]_o/[S]_t$ where this could be determined. The dotted lines indicate reasonable limits of error in the data.

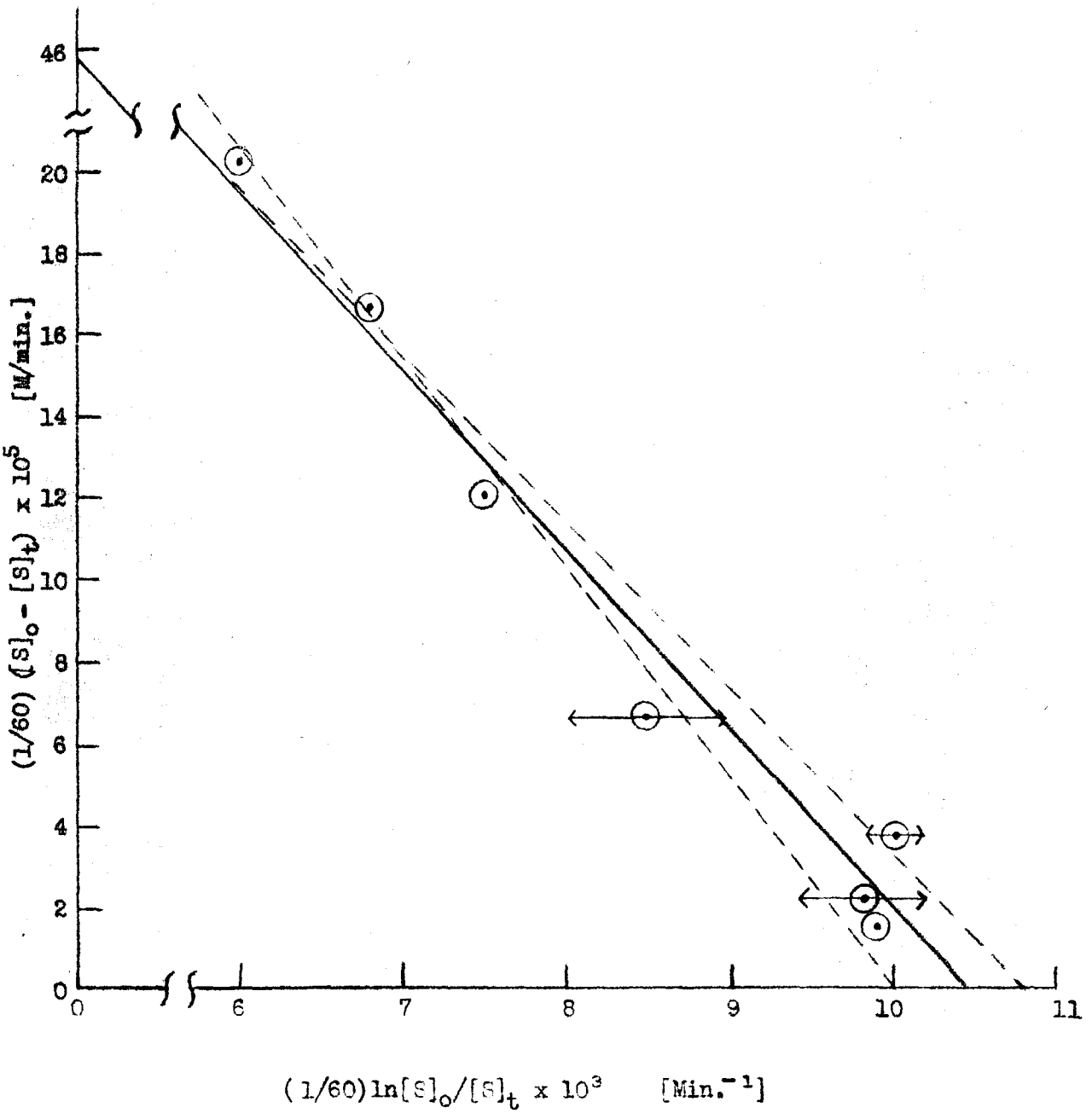


Figure 16. Walker-Schmidt plot of data extrapolated to pH 6.50. Heavy line is best estimate of the straight line through the points. Arrows indicate the average deviation in the value of $(1/60)\ln[S]_o/[S]_t$ where this could be determined. The dotted lines indicate reasonable limits of error in the data.

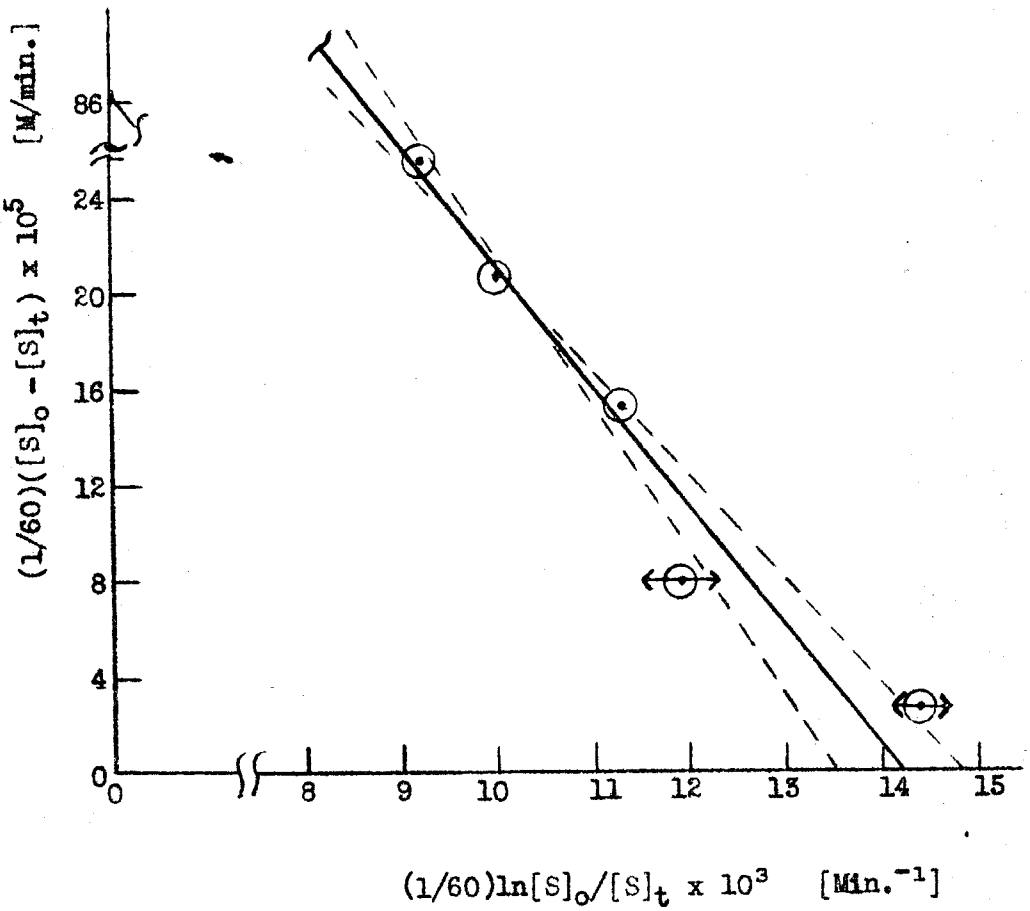


Figure 17. Walker-Schmidt plot of data extrapolated to pH 7.00. Heavy line is best estimate of the straight line through the points. Arrows indicate the average deviation in the value of $(1/60)\ln[S]_o/[S]_t$ where this could be determined. The dotted lines indicate reasonable limits of error in the data.

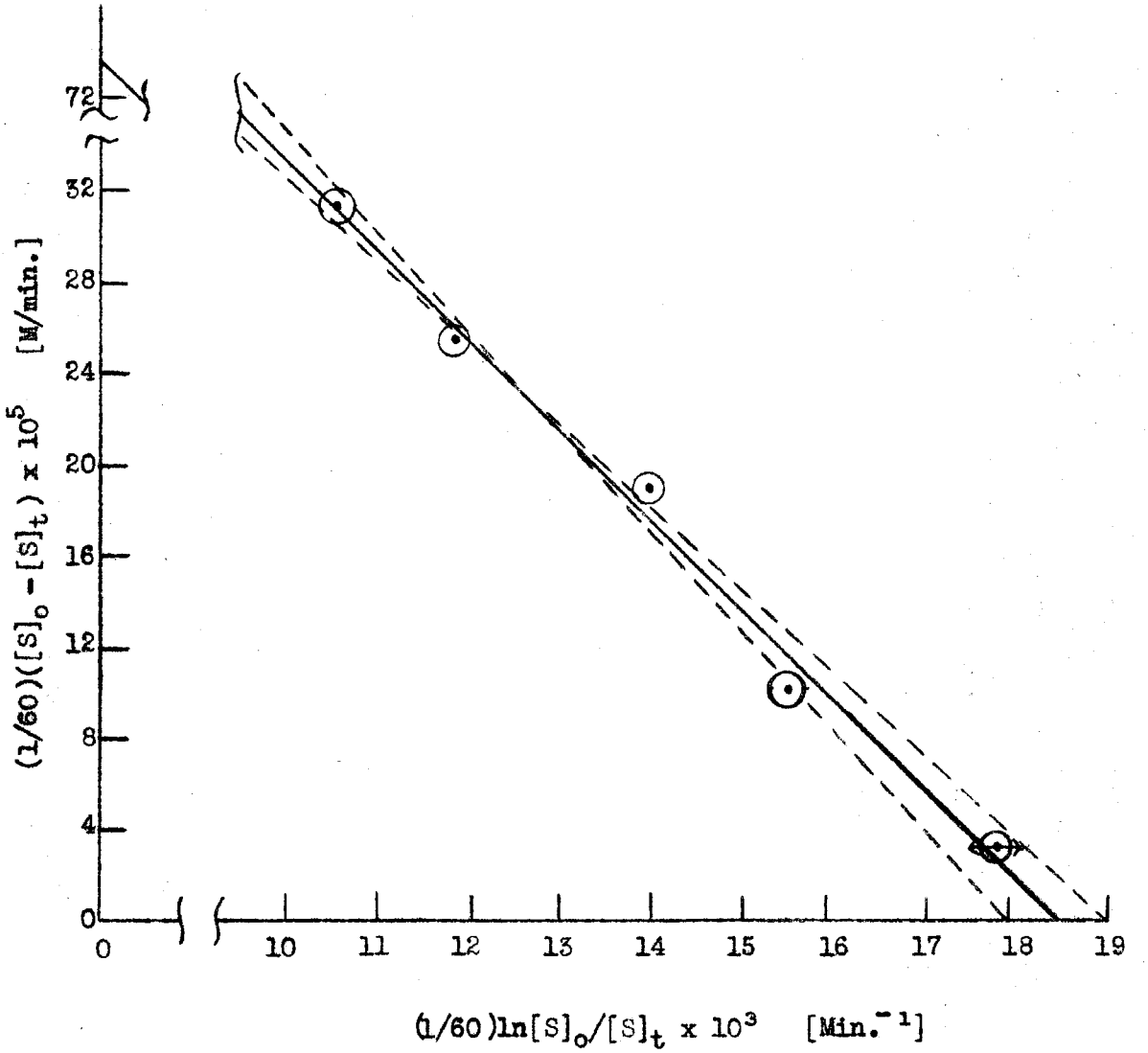


Figure 18. Walker-Schmidt plot of data extrapolated to pH 7.55. Heavy line is best estimate of the straight line through the points. Arrows indicate the average deviation in the value of $(1/60)\ln[S]_o/[S]_t$ where this could be determined. The dotted lines indicate reasonable limits of error in the data.

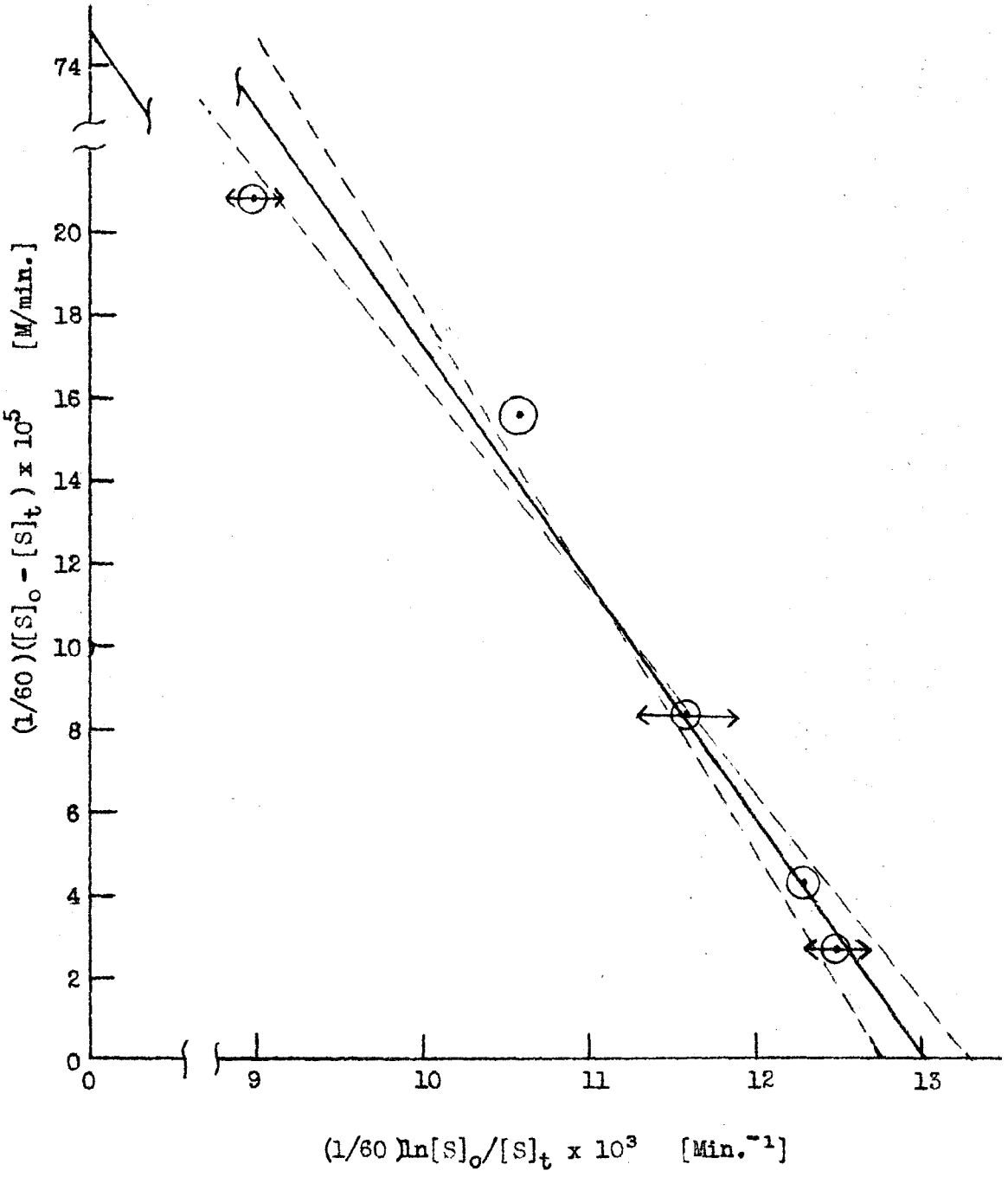


Figure 19. Walker-Schmidt plot of data extrapolated to pH 7.75. Heavy line is best estimate of the straight line through the points. Arrows indicate the average deviation in the value of $(1/60)\ln[S]_o/[S]_t$ where this could be determined. The dotted lines indicate reasonable limits of error in the data.

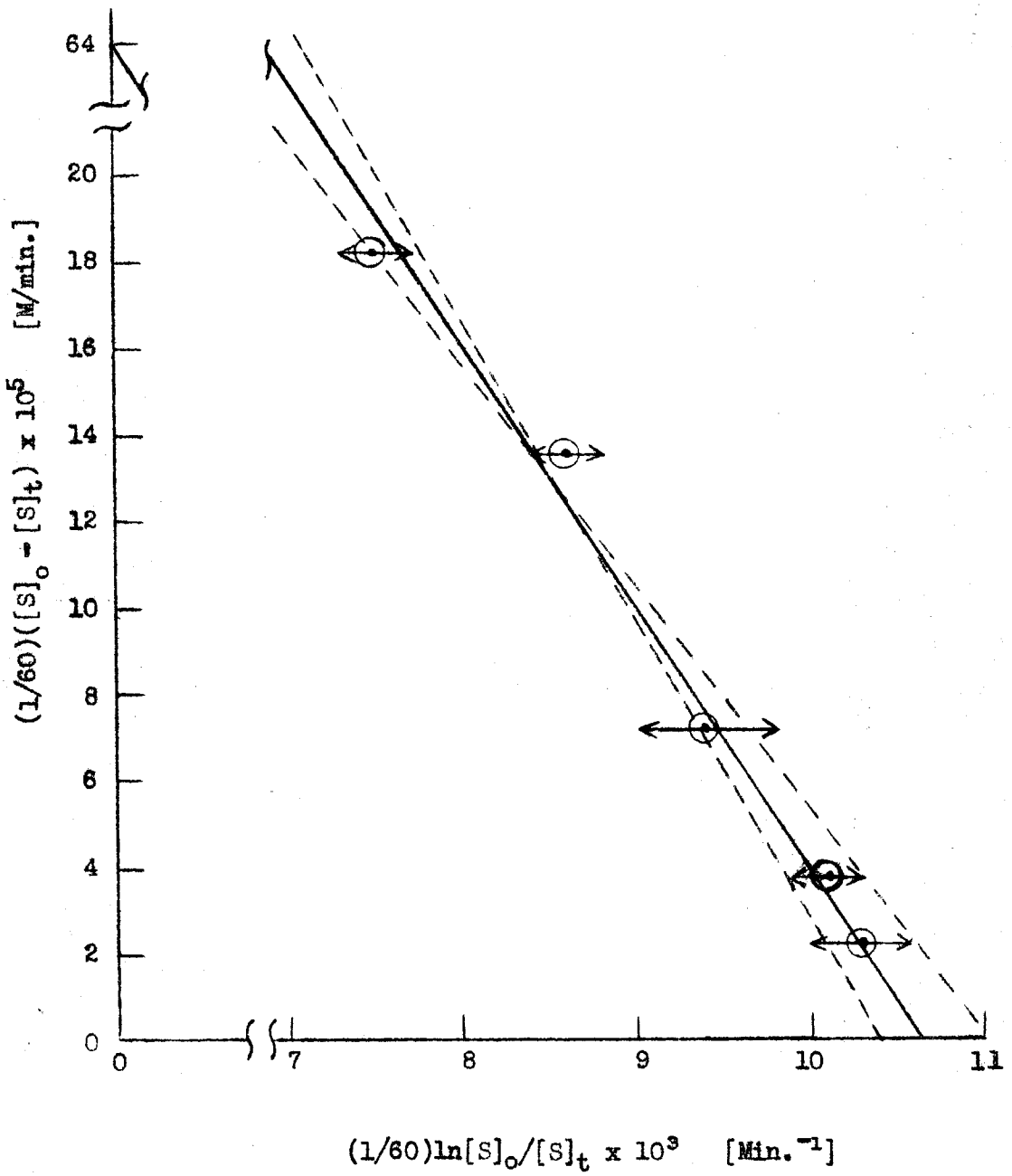


Figure 20. Walker-Schmidt plot of data extrapolated to pH 7.95. Heavy line is best estimate of the straight line through the points. Arrows indicate the average deviation in the value of $(1/60)\ln[S]_o/[S]_t$ where this could be determined. The dotted lines indicate reasonable limits of error in the data.

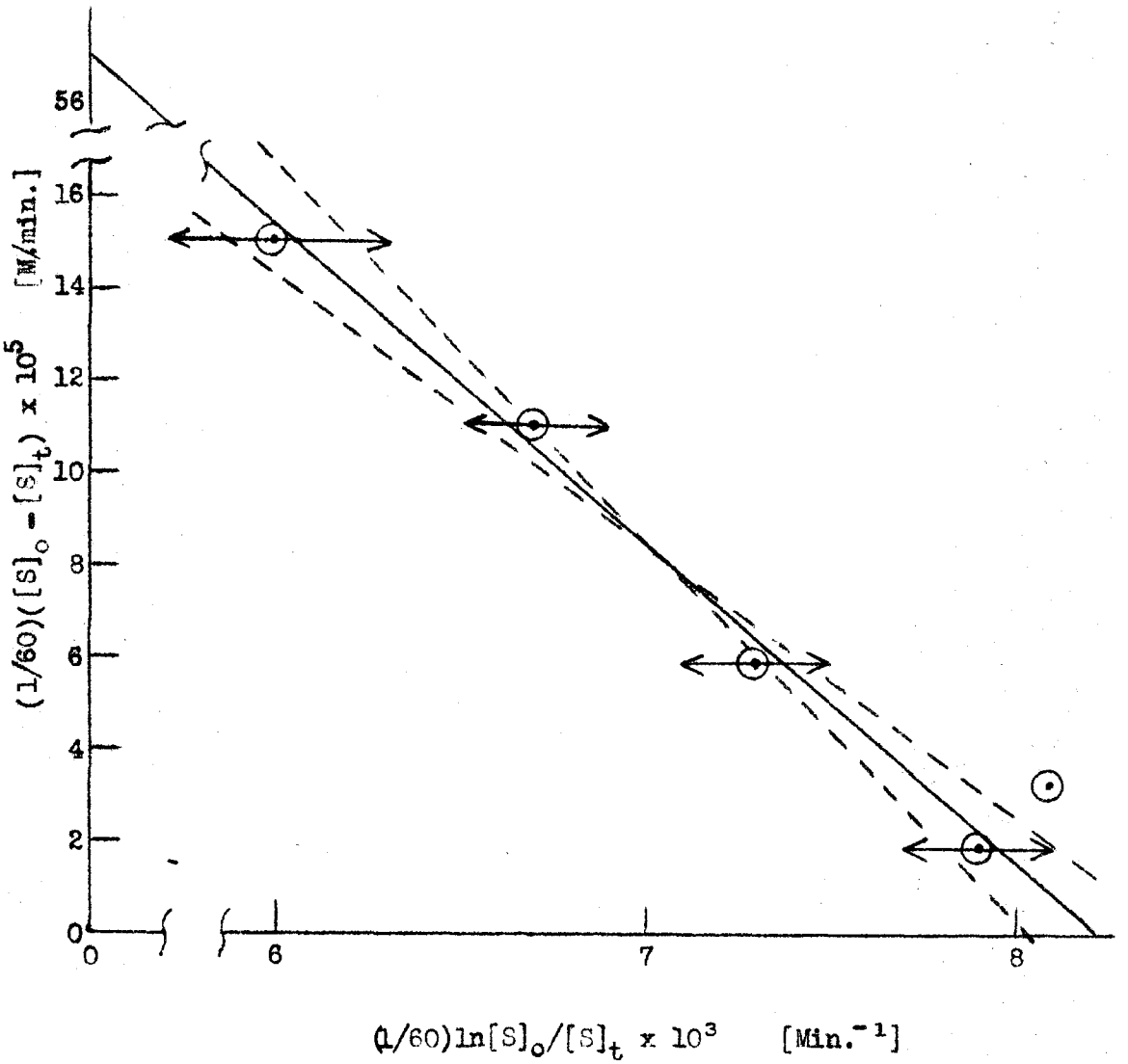


Figure 21. Plot of K_s values obtained for L-tyrosinhydroxamide vs. pH. The arrows indicate the estimated errors in the individual points.

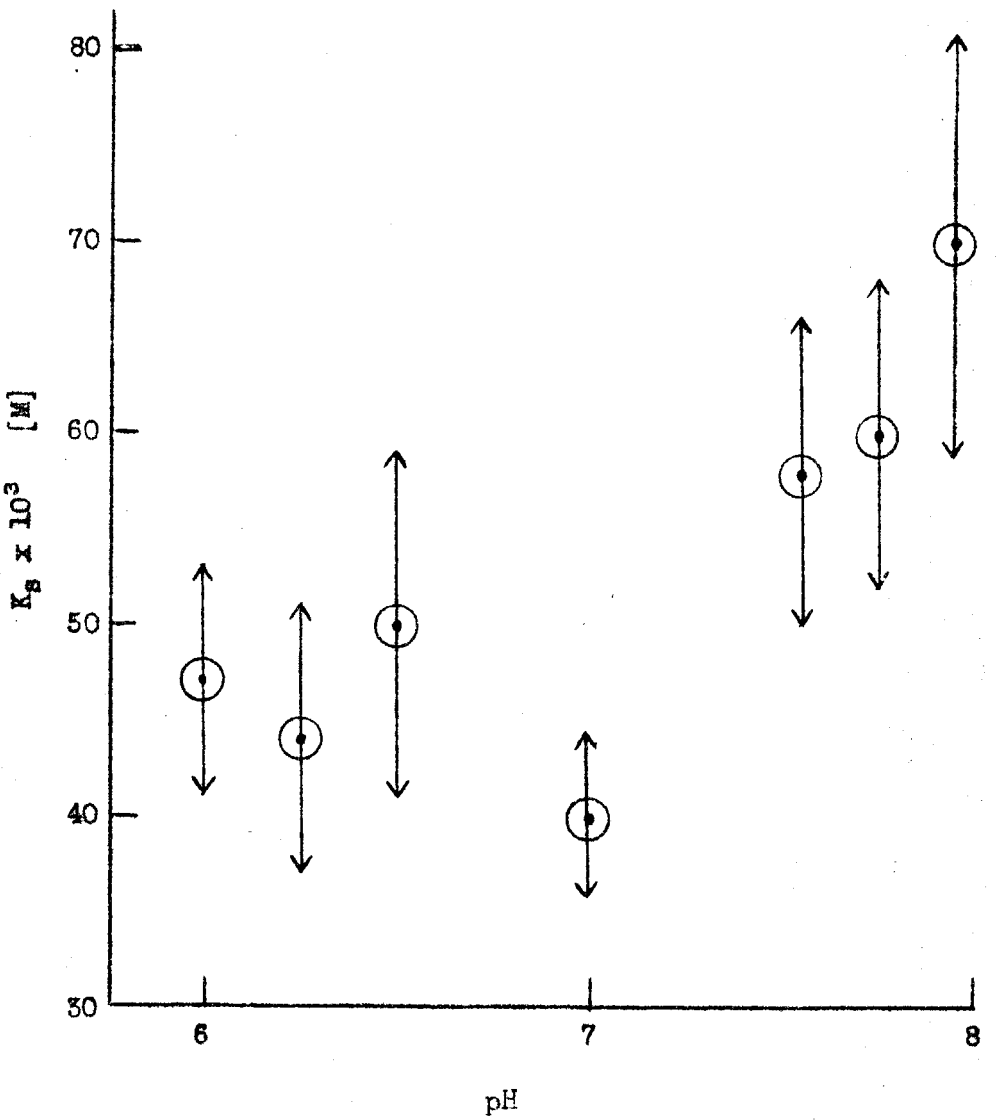


Figure 22. Plot of V_{max} for L-tyrosinhydroxamide vs. pH. Enzyme concentration = 0.208 mg. protein nitrogen per milliliter. The arrows indicate the estimated errors of the individual points.

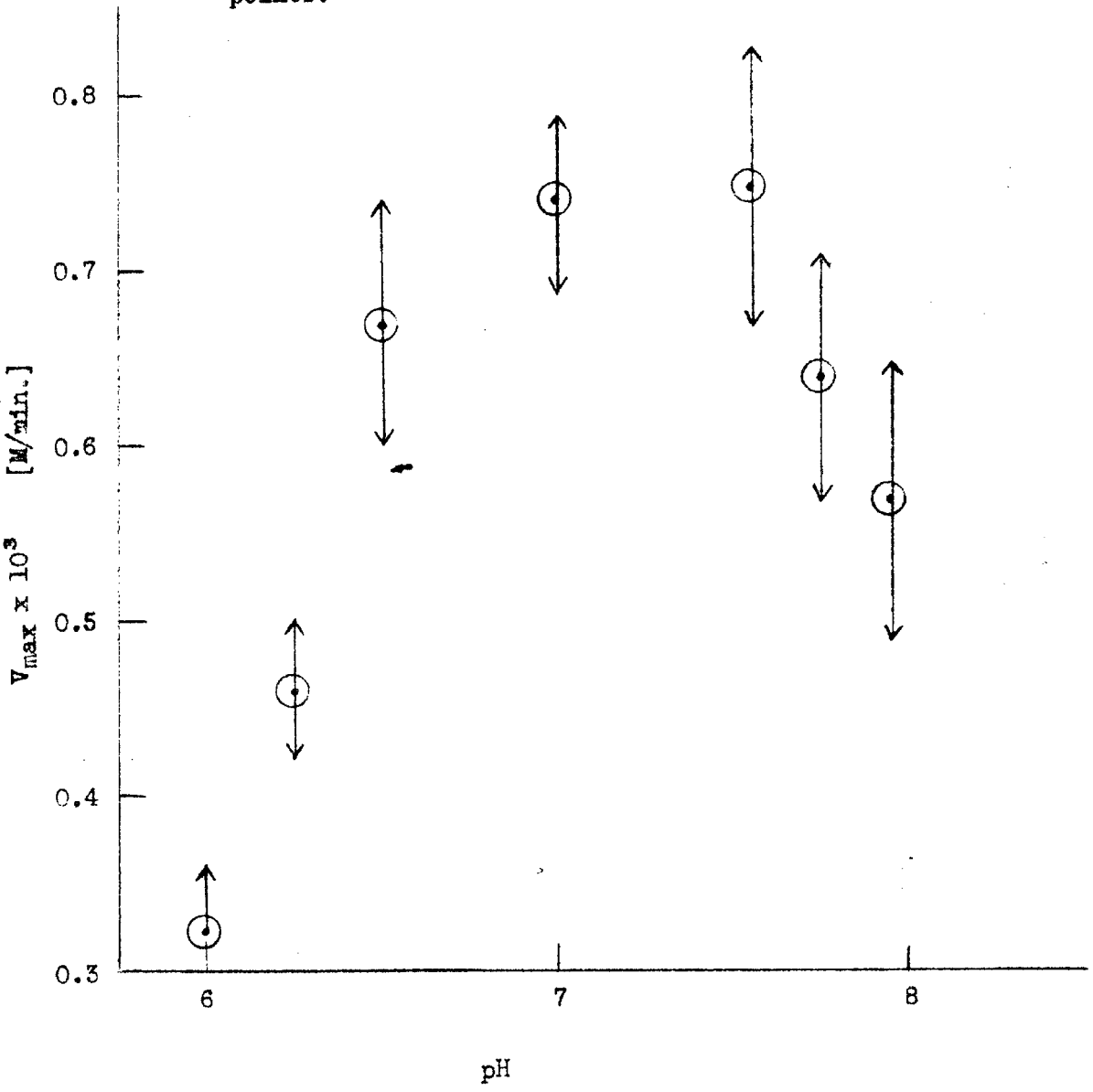


Figure 23. Plot of log K_s/V_{max} for L-tyrosinhydroxamide vs. pH.

• = obtained from runs with low initial substrate concentration.

x = obtained from the K_s and V_{max} values determined by Walker-Schmidt plots at various pH's.

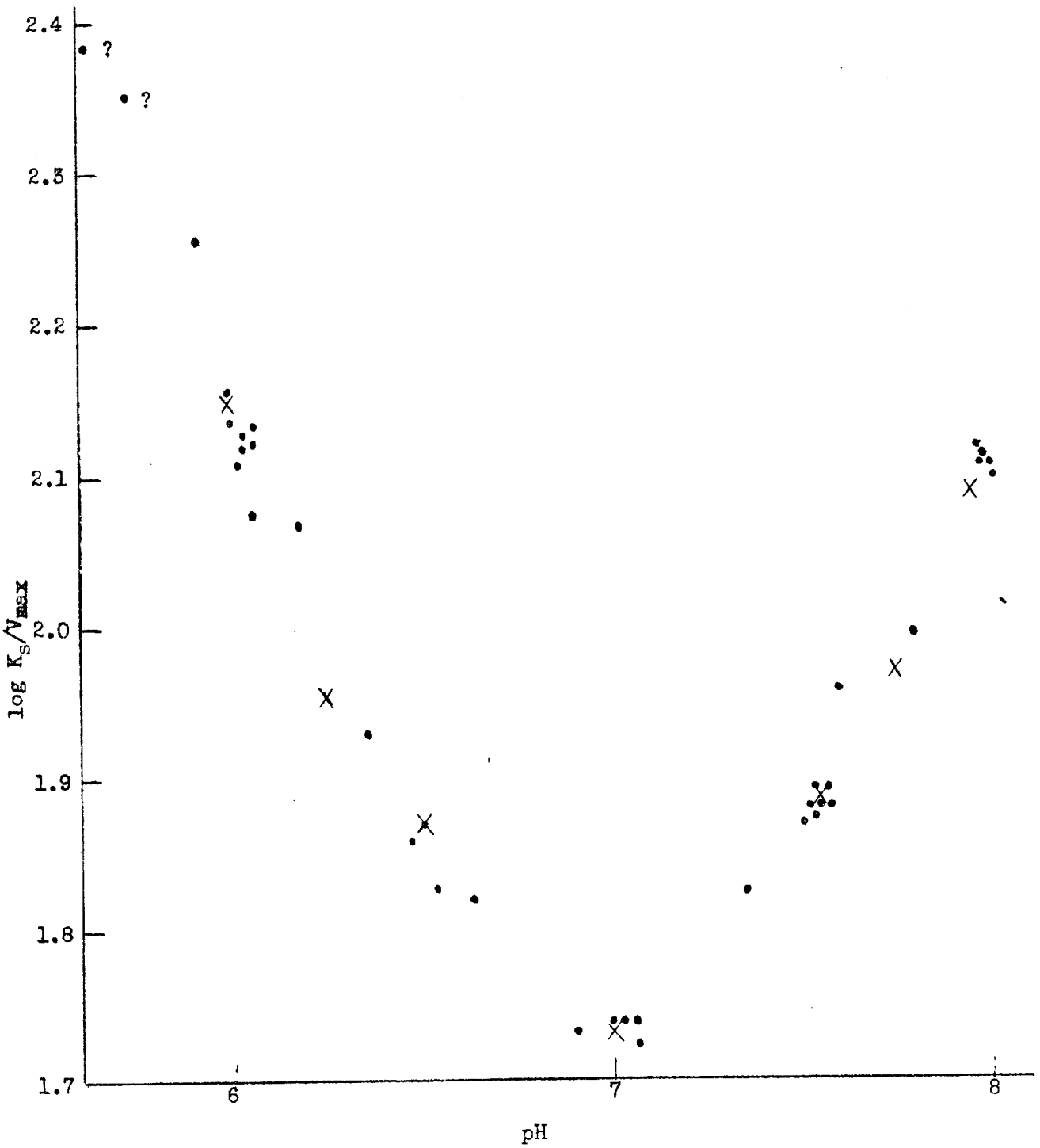


Figure 24. Results of runs made at pH 6.20. Cacodylic acid-NaOH buffer.

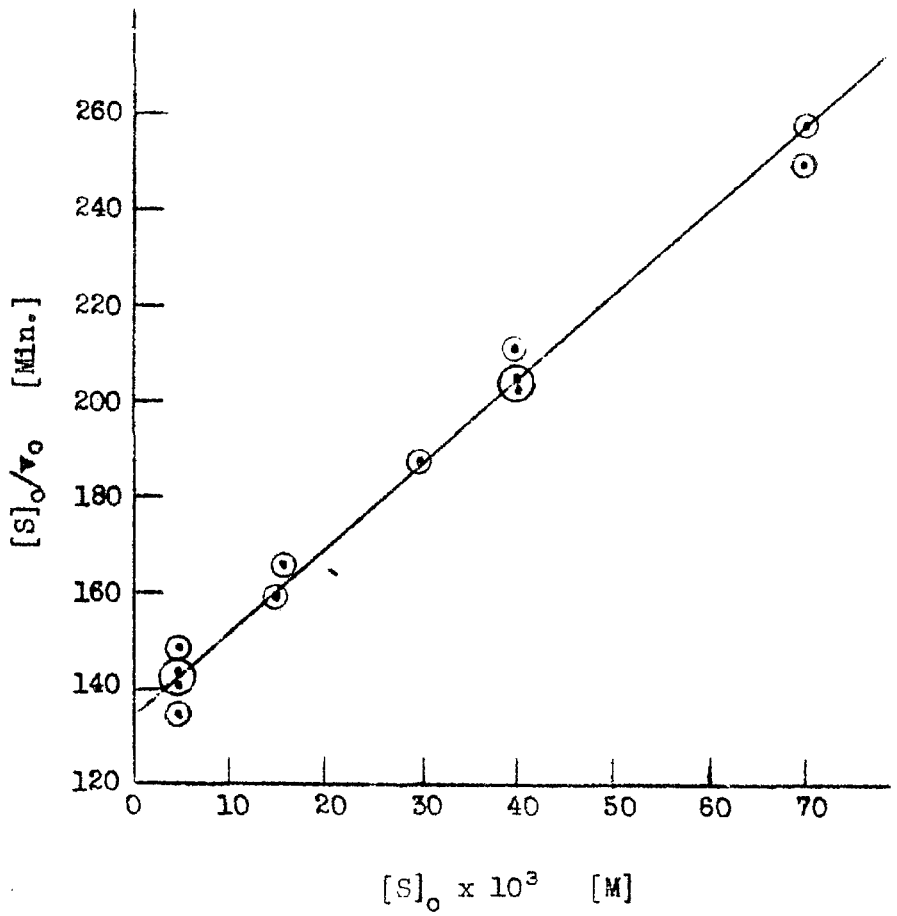


Figure 25. Lineweaver-Burk plot showing the action of various inhibitors on the alpha-chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide at pH 6.20. Cacodylic acid-NaOH buffer.

△ = Acetyl L tyrosinamide 30×10^{-3} M.

○ = Acetyl L tyrosine 45×10^{-3} M.

□ = Acetyl D tryptophanamide 2.46×10^{-3} M.

Lowest line = substrate alone.

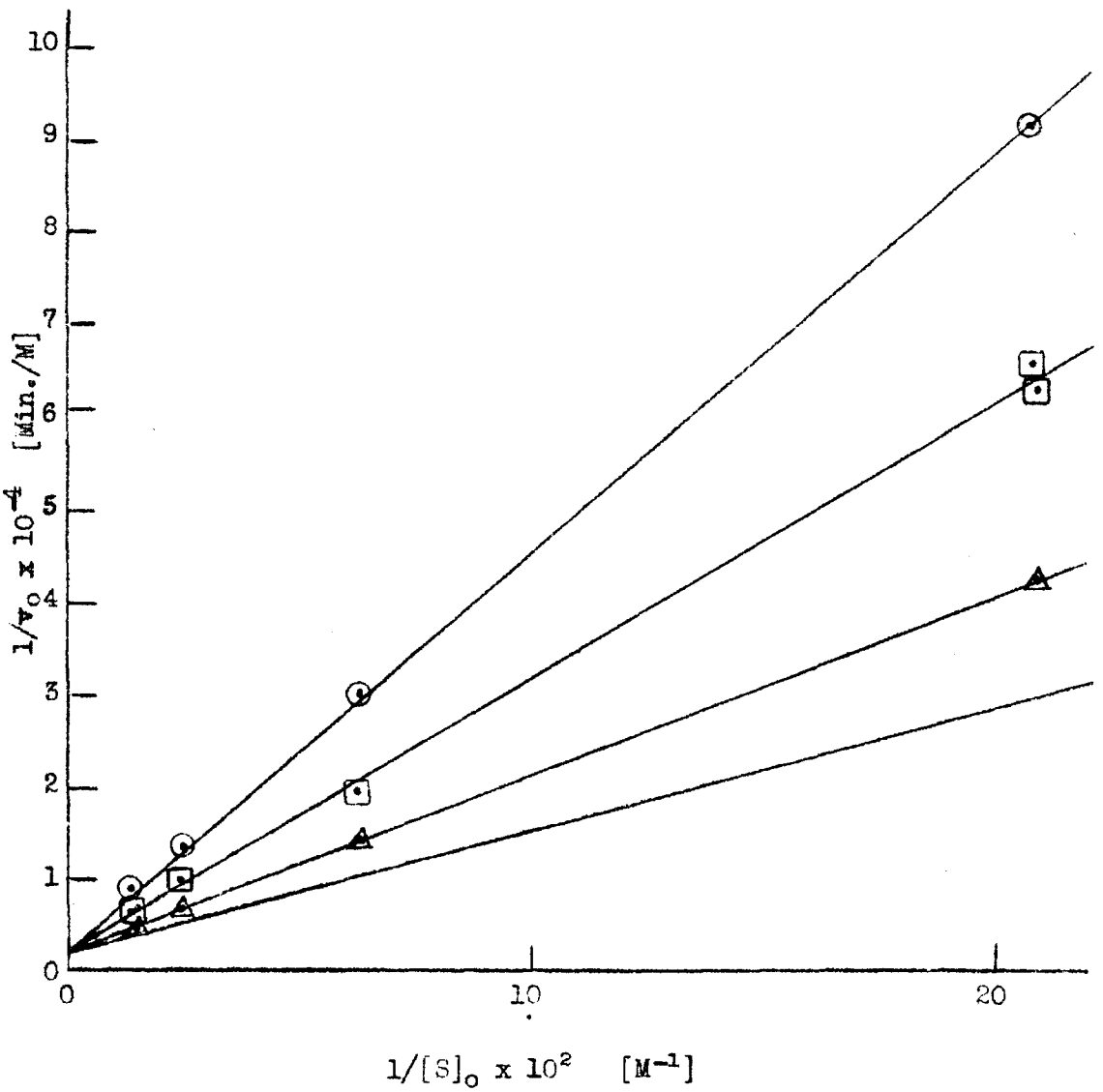


Figure 26. Results of runs made at pH 6.60. Cacodylic acid-NaOH buffer.

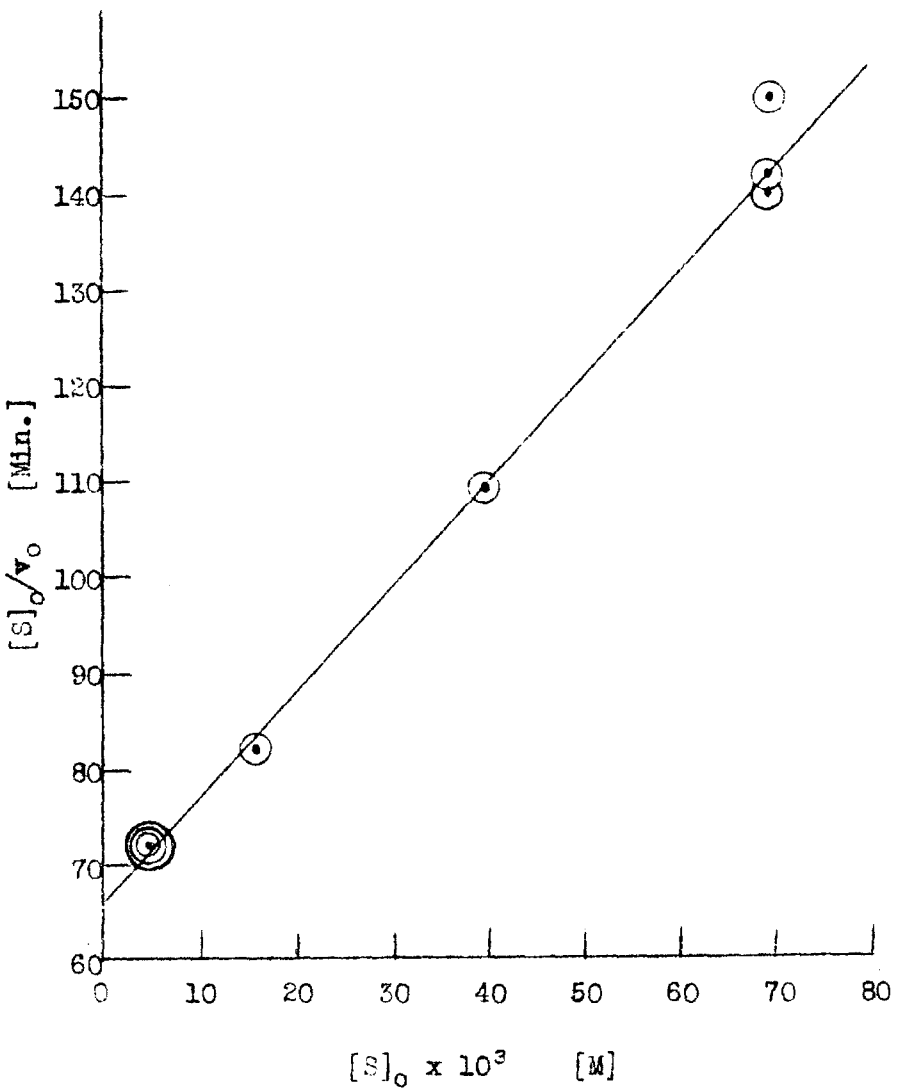


Figure 27. Results of runs made at pH 6.95. Cacodylic acid-NaOH buffer.

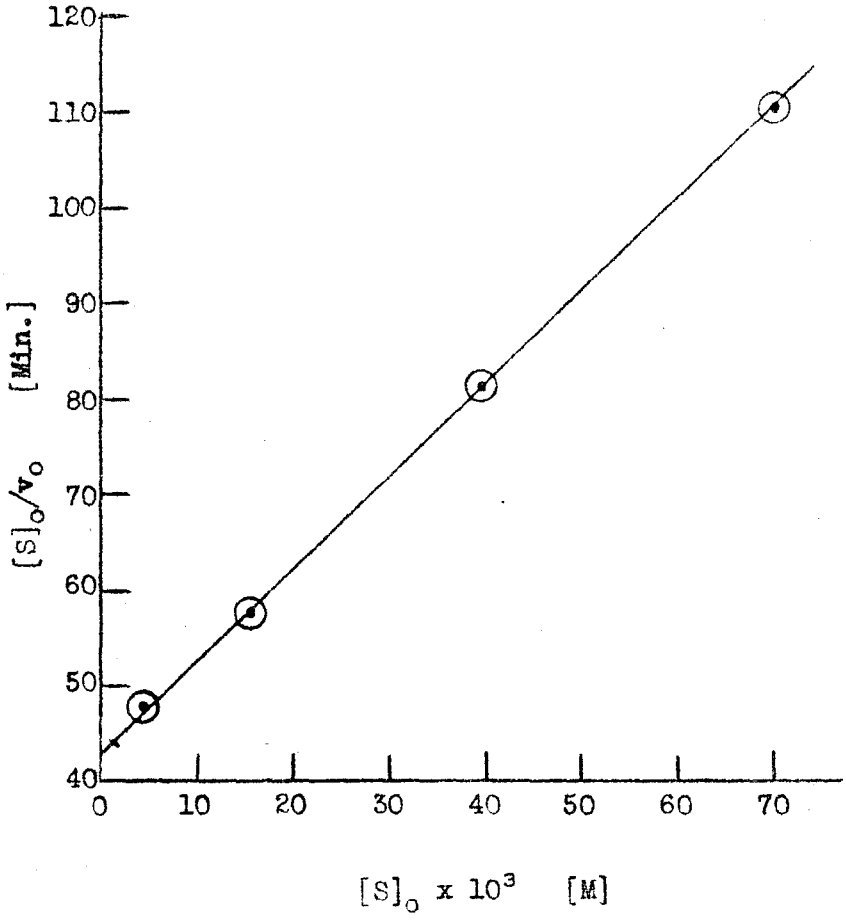


Figure 28. Results of runs made at pH 7.13. Phosphoric acid-KOH buffer.

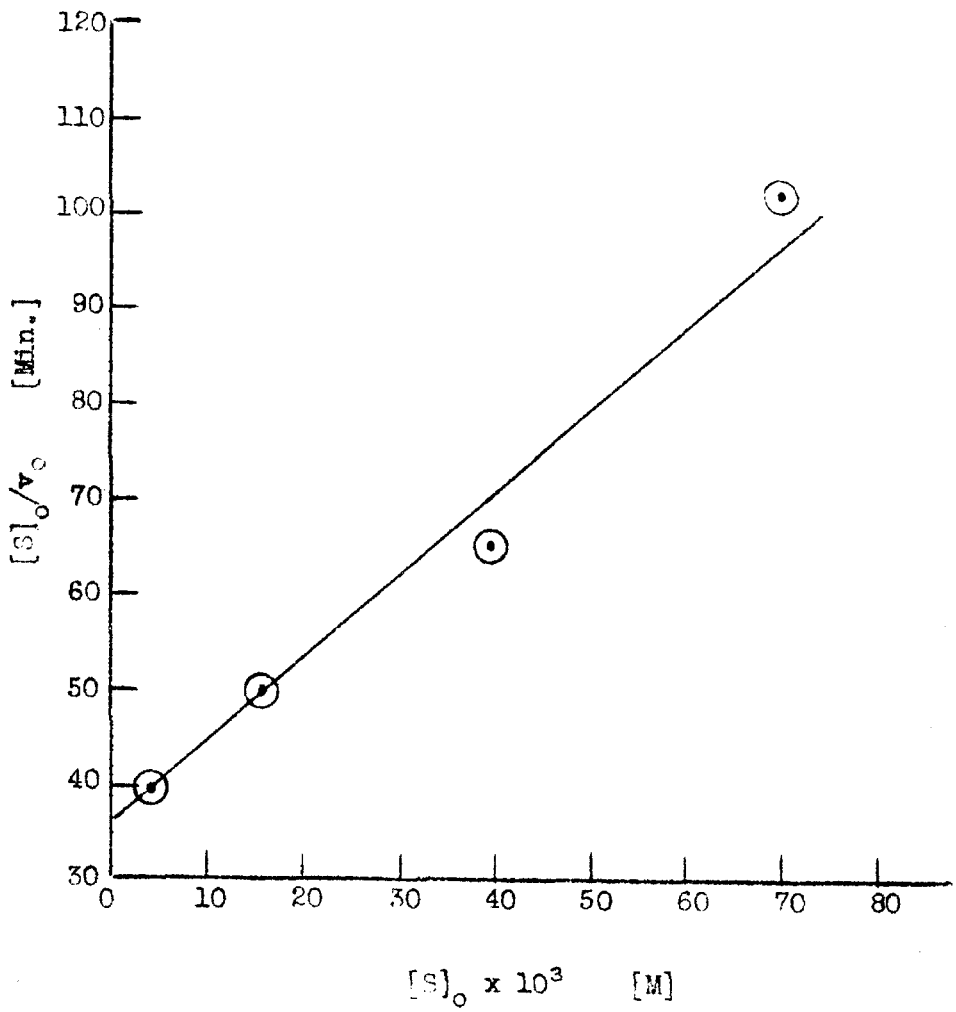


Figure 29. Results of runs made at pH 7.2. Tris-(hydroxymethyl)-aminomethane-HCl buffer.

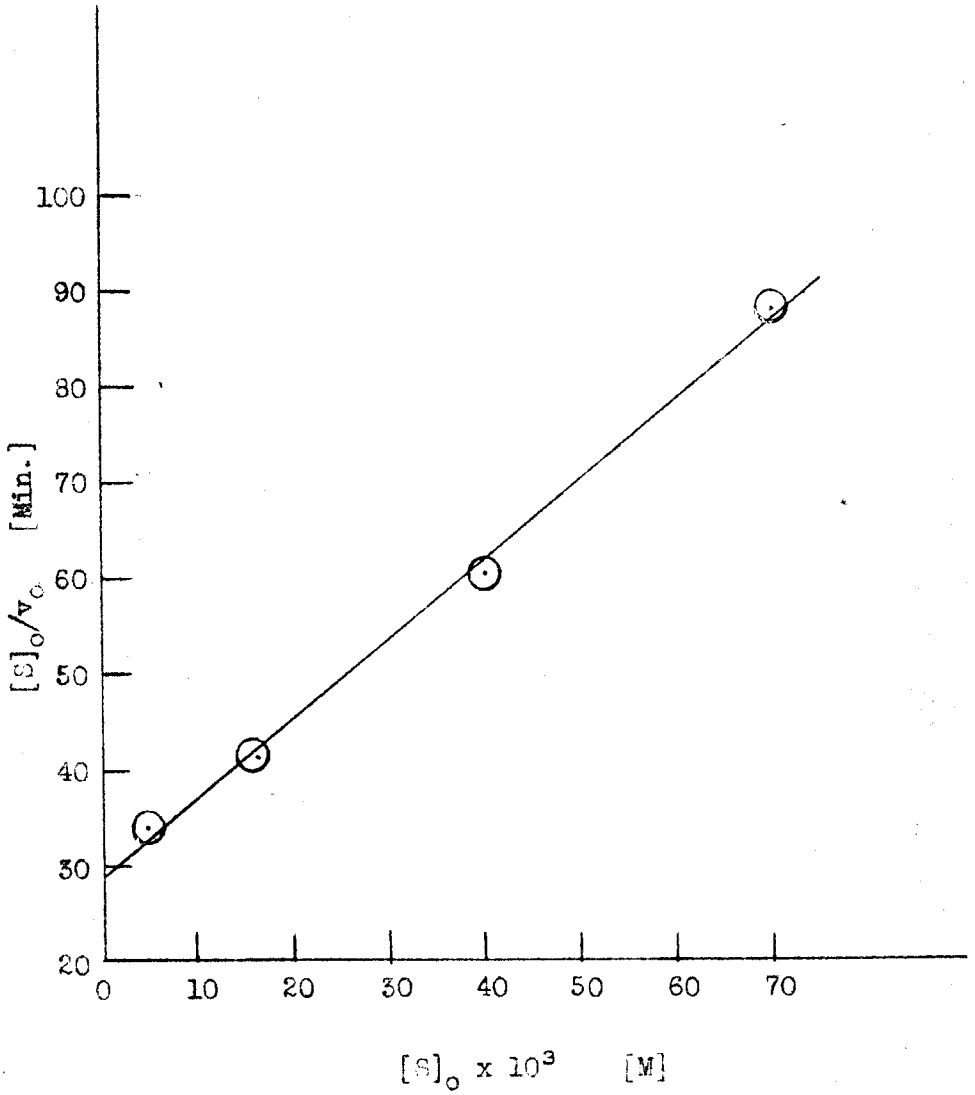


Figure 30. Results of runs made at pH 7.15. Hydroxylamine-HCl buffer.

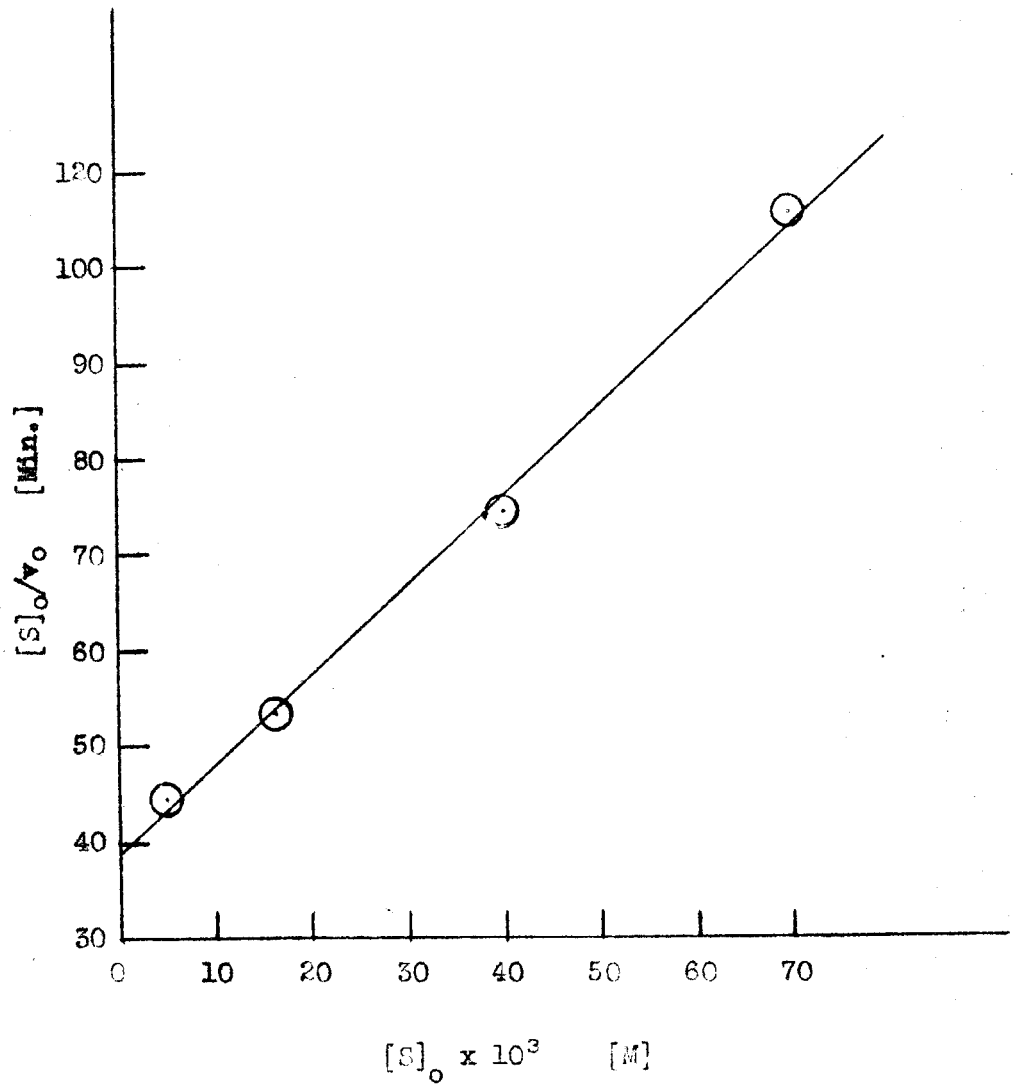


Figure 31. Results of runs at pH 7.30. Ethylenediamine-HCl buffer.

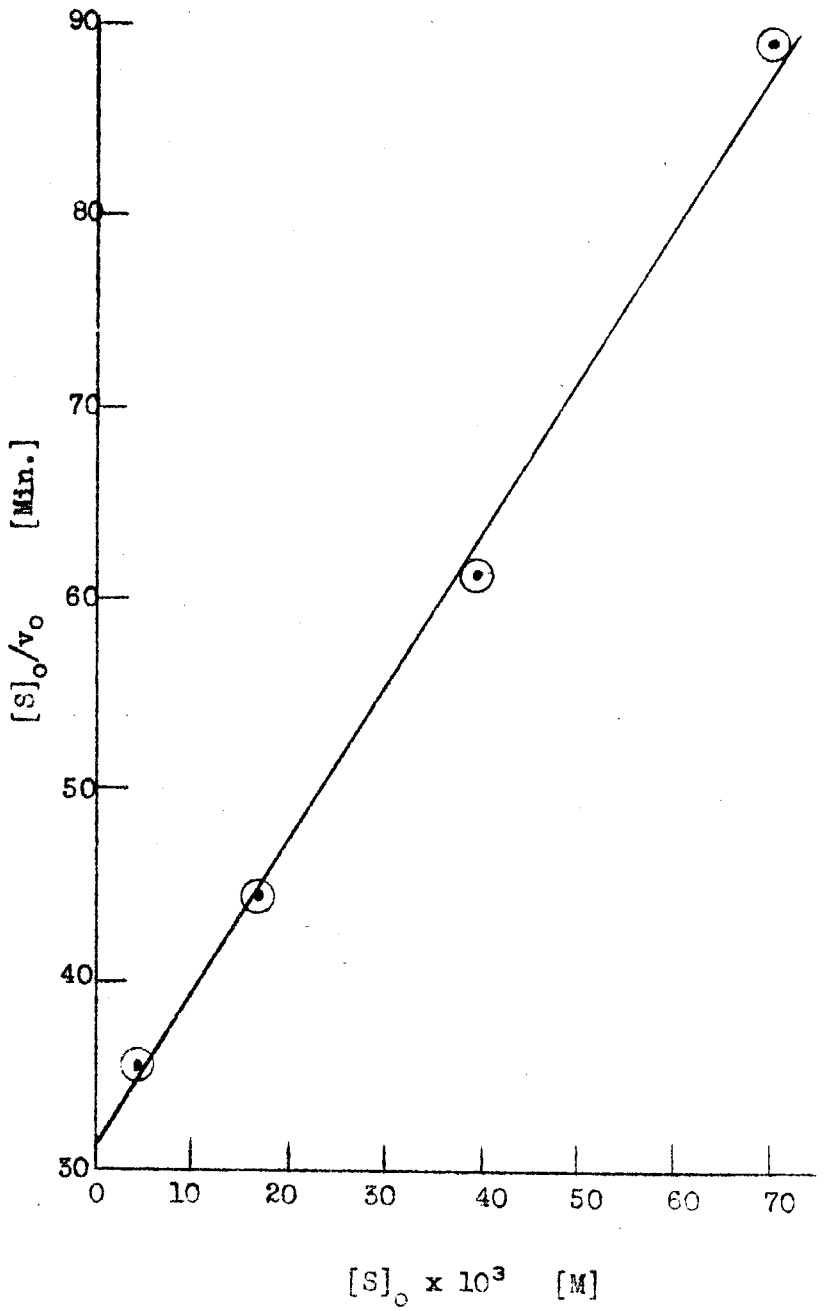


Figure 32. Results of runs at pH 7.60. Tris-(hydroxymethyl)-aminomethane-HCl buffer.

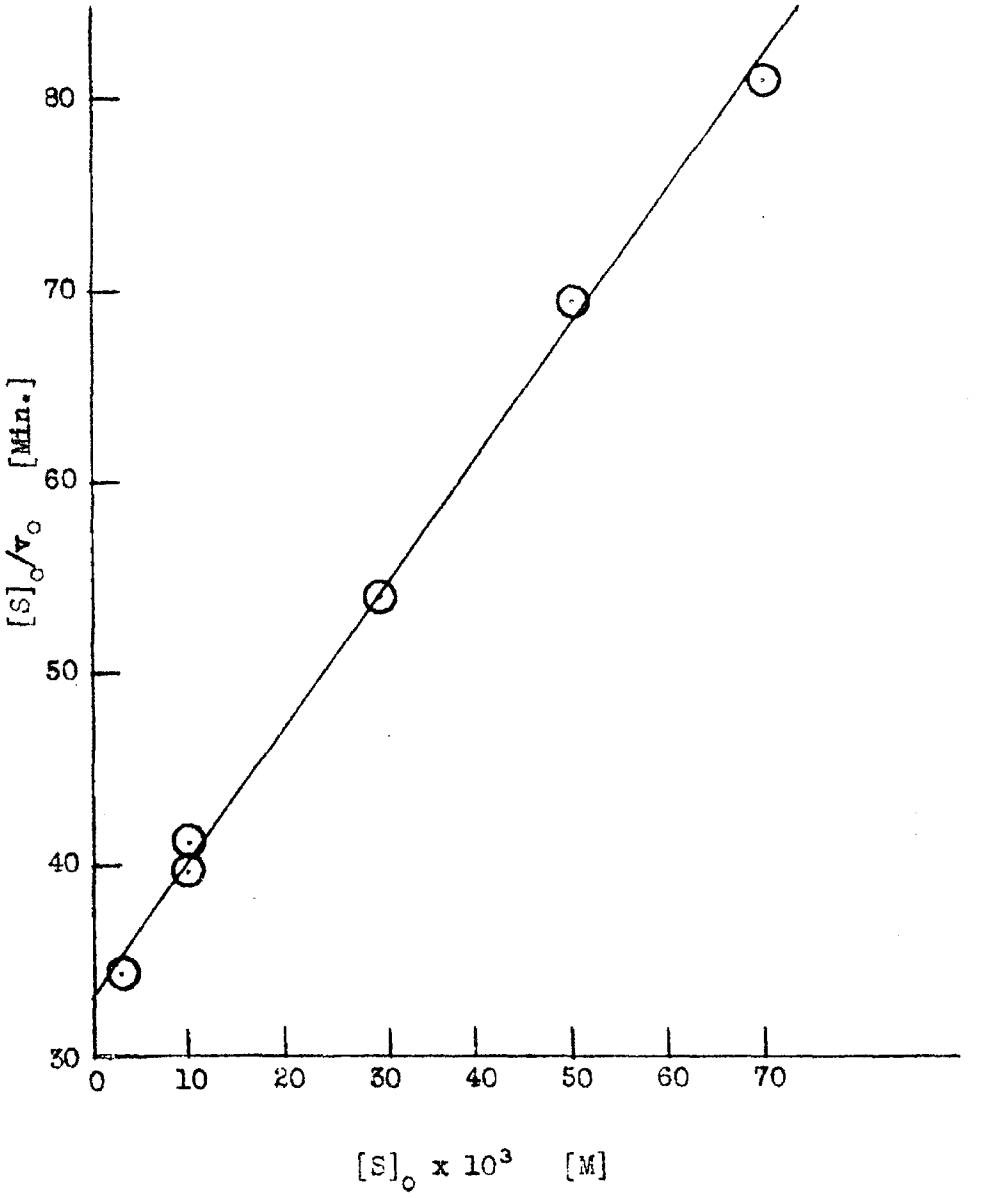


Figure 33. Results of runs made in the presence of 0.04 M $CaCl_2$ at pH 7.60. Tris-(hydroxymethyl)-aminomethane-HCl buffer.

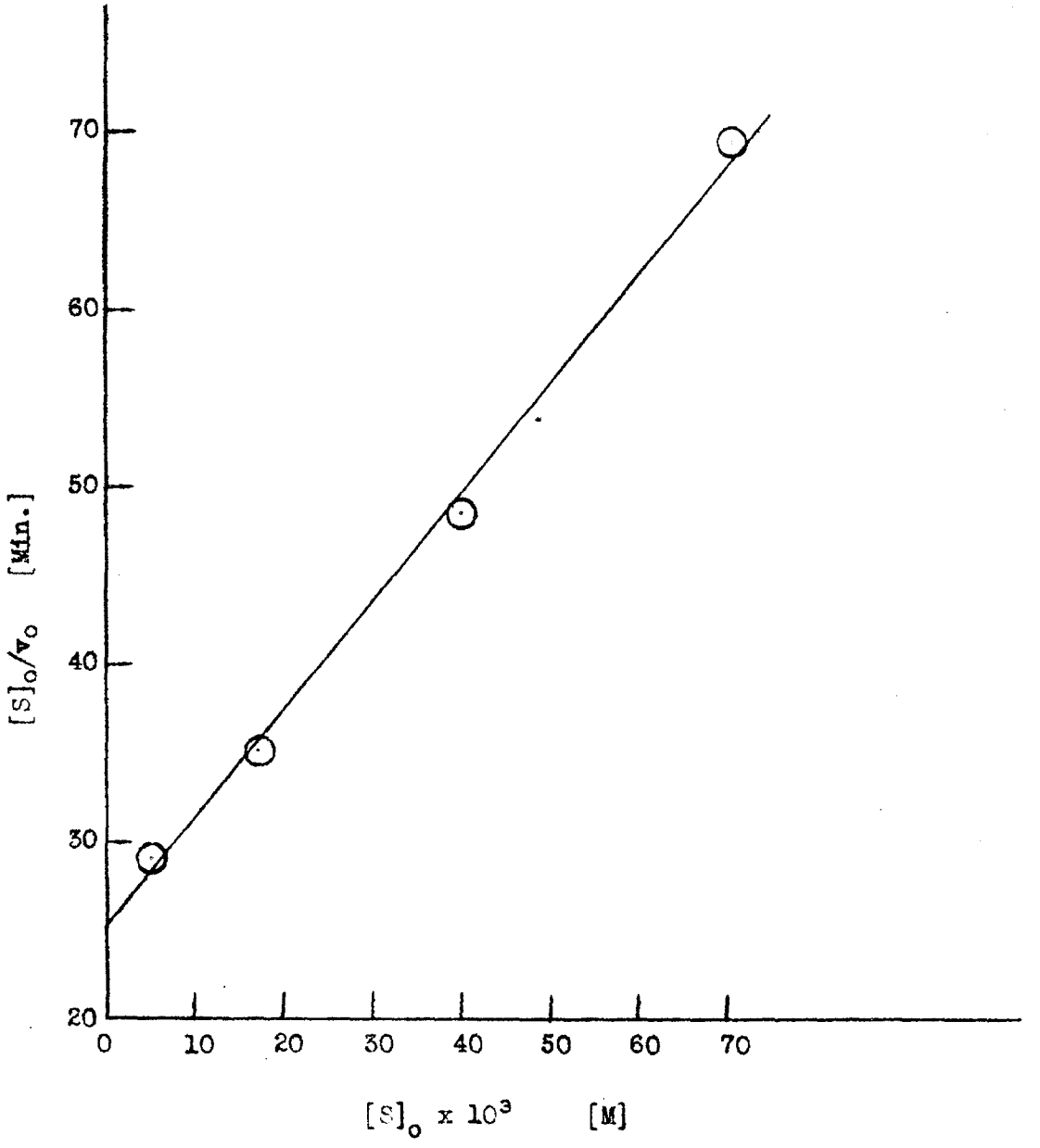


Figure 34. Results of runs made at pH 7.60 in the presence of 55.4×10^{-3} M L tyrosinamide. Tris-(hydroxymethyl)-aminomethane-HCl buffer.

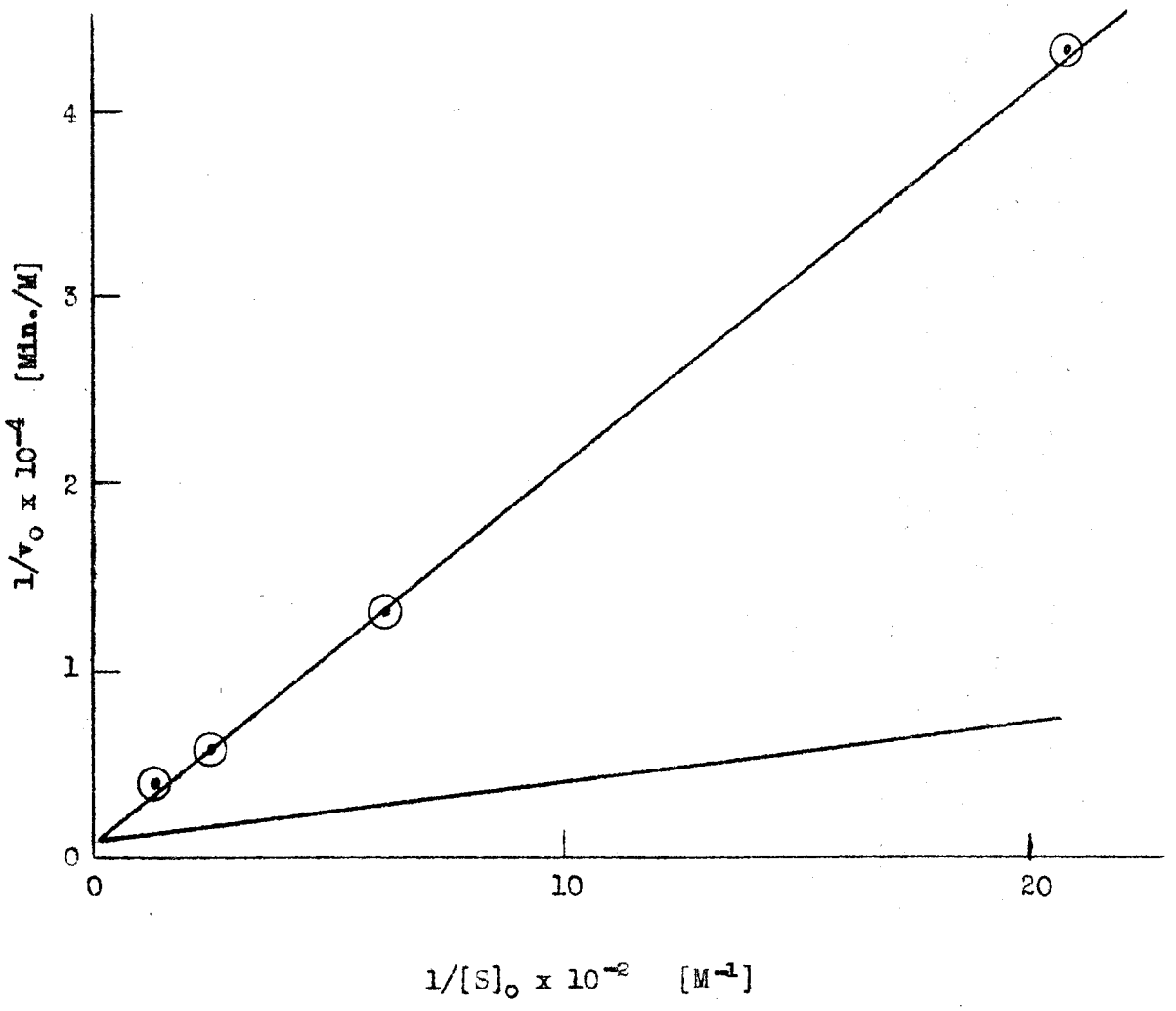


Figure 35. Results of runs made at pH 7.70. Tris-(hydroxymethyl)-aminomethane-HCl buffer.

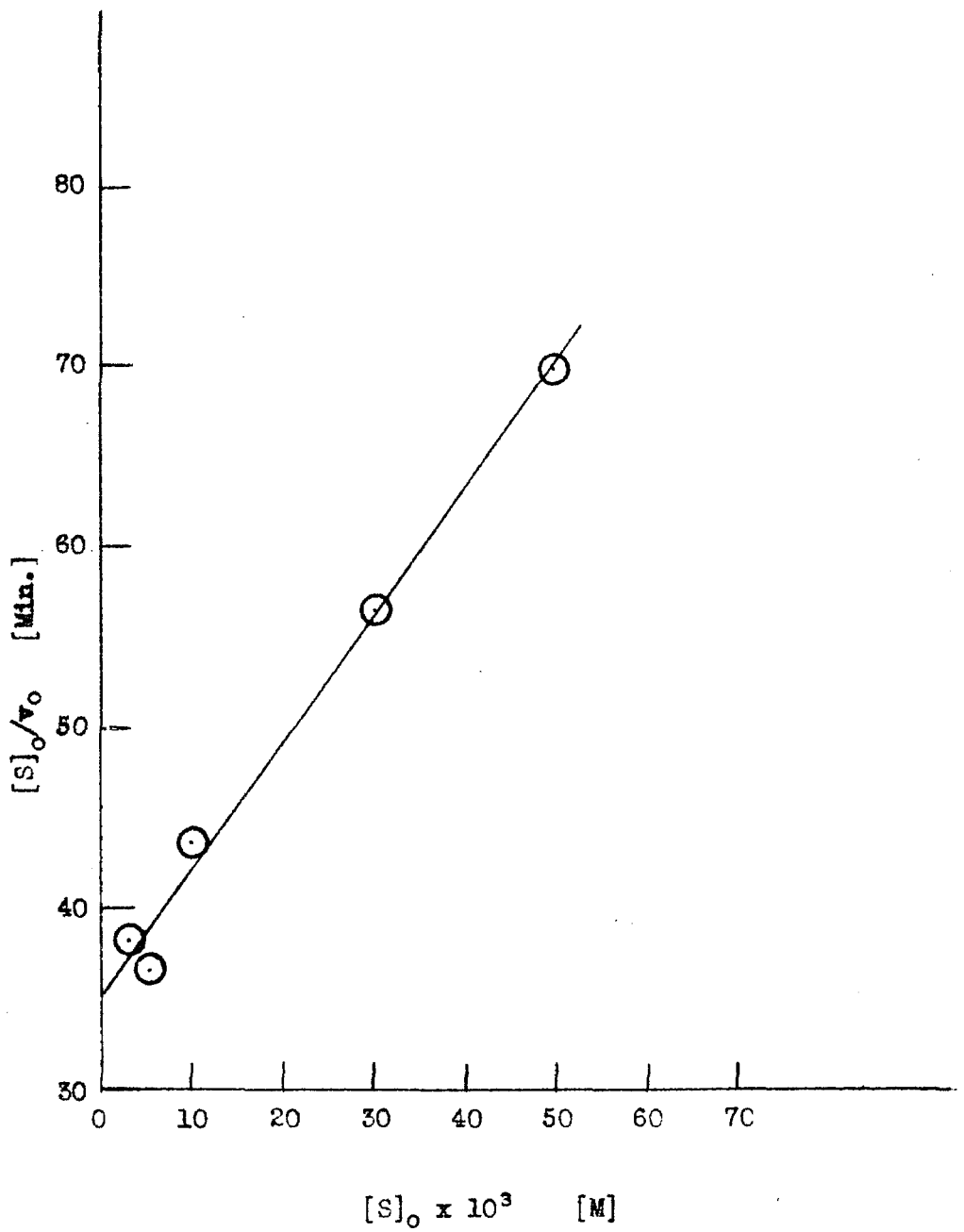


Figure 56. Runs made at pH 7.88. Tris-(hydroxymethyl)-aminomethane-HCl buffer.

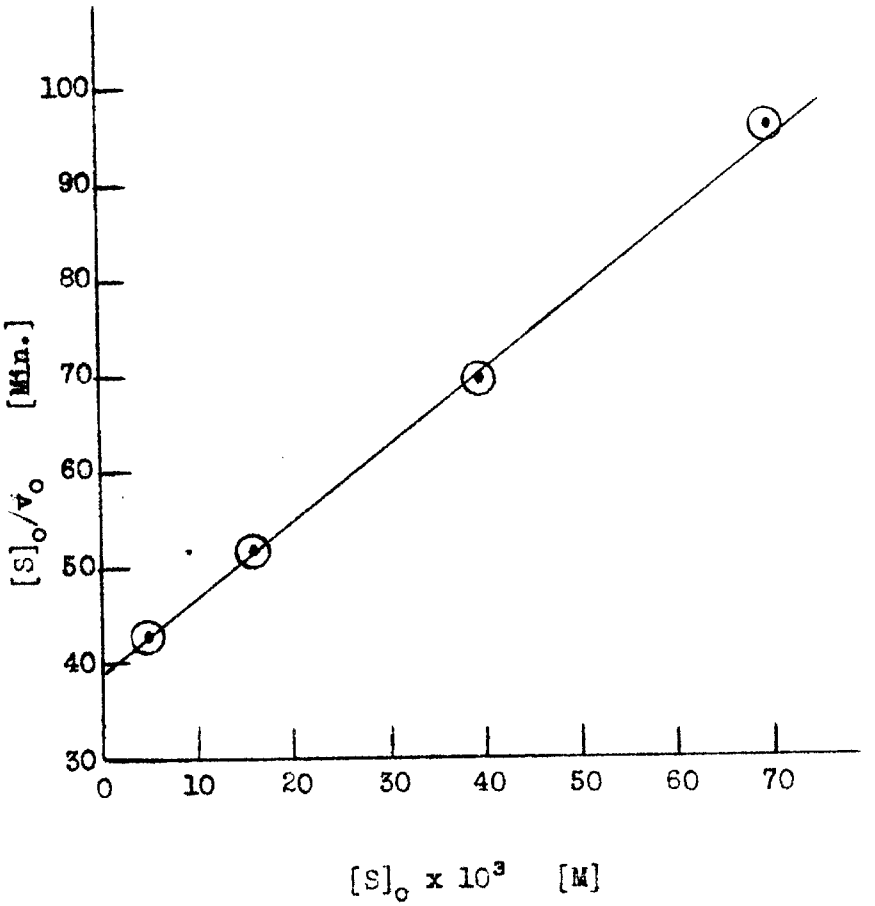


Figure 37. Inhibition of the alpha chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide by varying amounts of acetyl-L-tyrosinamide. $[S]_o = 16 \times 10^{-3}$ M. Tris-(hydroxymethyl)-aminomethane-HCl buffer.

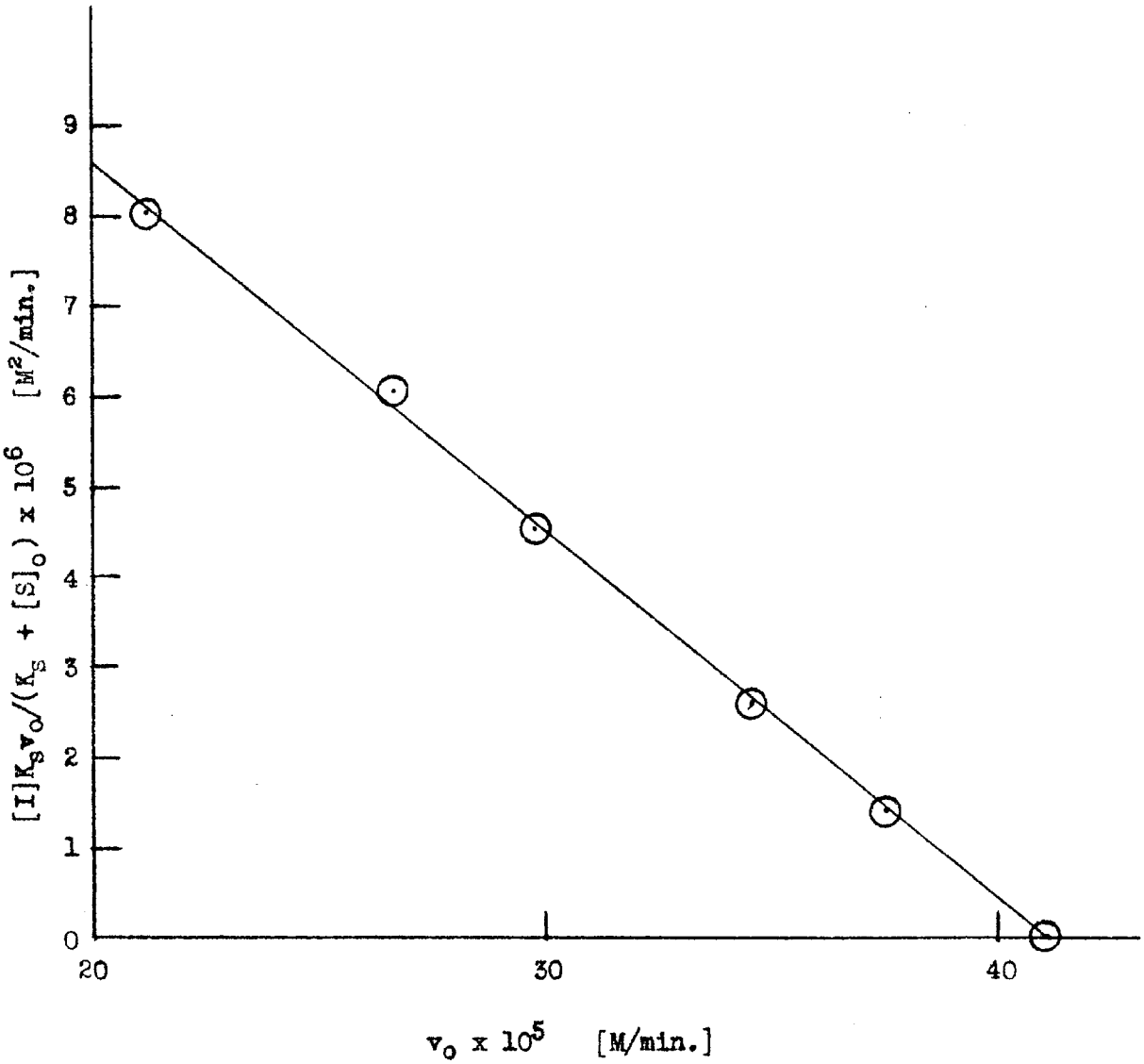


Figure 38. Results of runs made in the vicinity of pH 8.20, and extrapolated to pH 8.20. Tris-(hydroxymethyl)-amino methane-HCl buffer.

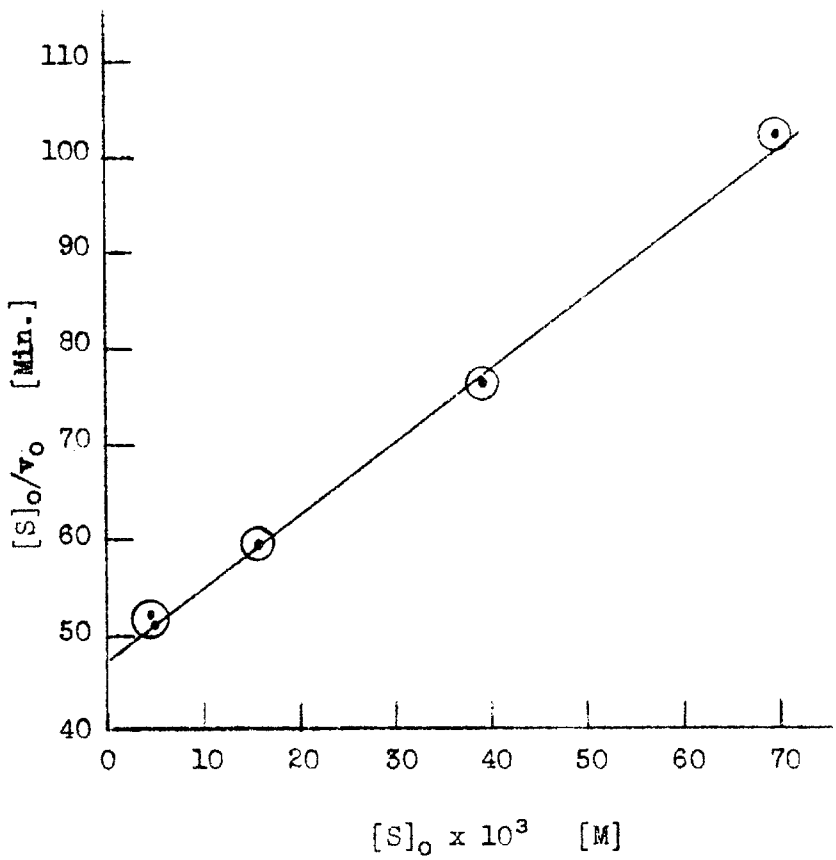


Figure 39a. Results of uninhibited runs made in the vicinity of pH 8.60. Runs 30 38 only. Tris-(hydroxymethyl)-aminomethane-HCl buffer.

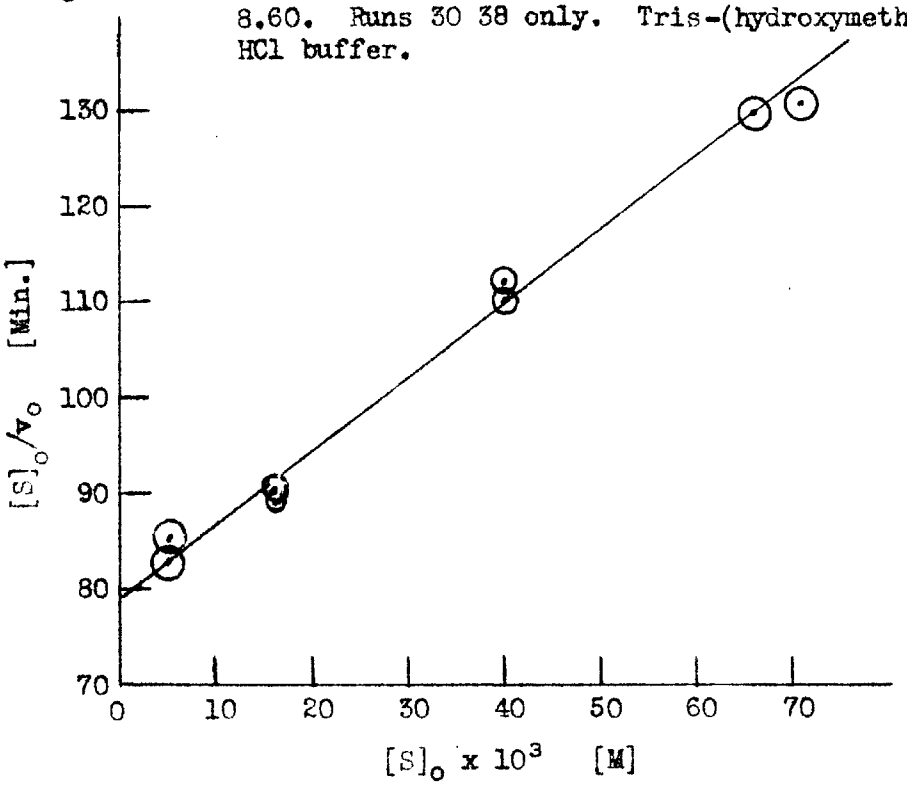


Figure 39b. Results of uninhibited runs made in the vicinity of pH 8.60. Runs 88, 91, 96b, 99a, 115, and 117. Tris-(hydroxymethyl)-aminomethane-HCl buffer.

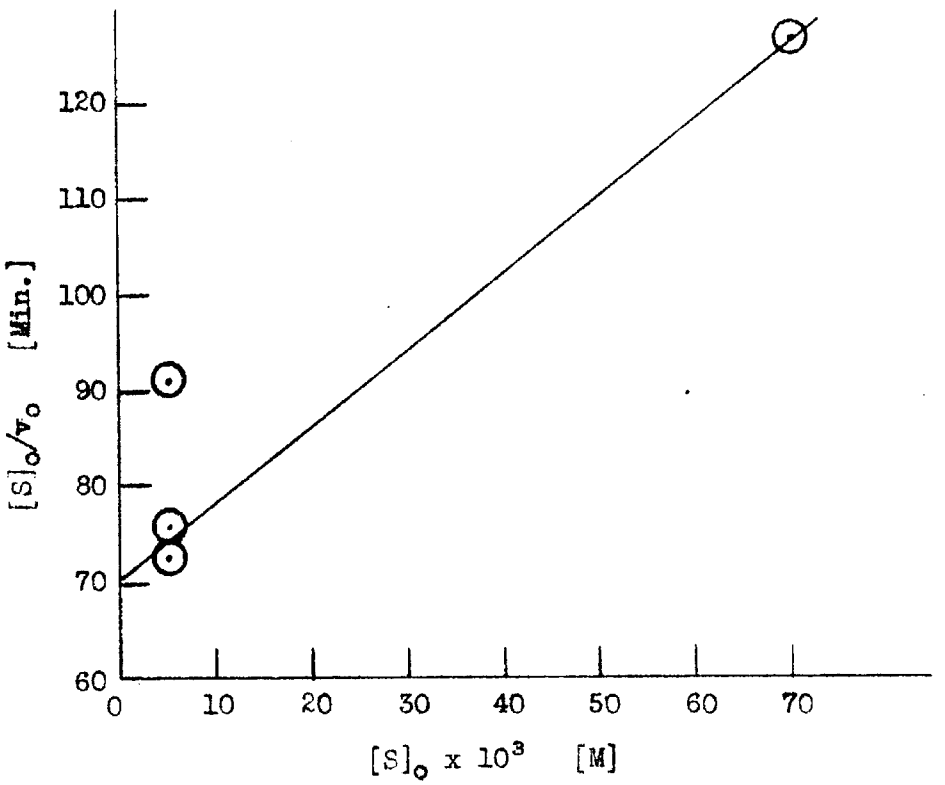


Figure 39c. Results of runs made in the vicinity of pH 8.60; all of the runs made in this vicinity are included. The figures indicate the number of individual runs represented by the adjacent points. The size of the diamond indicates the average deviation of the points where this could be determined. The solid line best represents the points; the dotted lines show possible extremes of error. Tris-(hydroxymethyl)-aminomethane-HCl buffer.

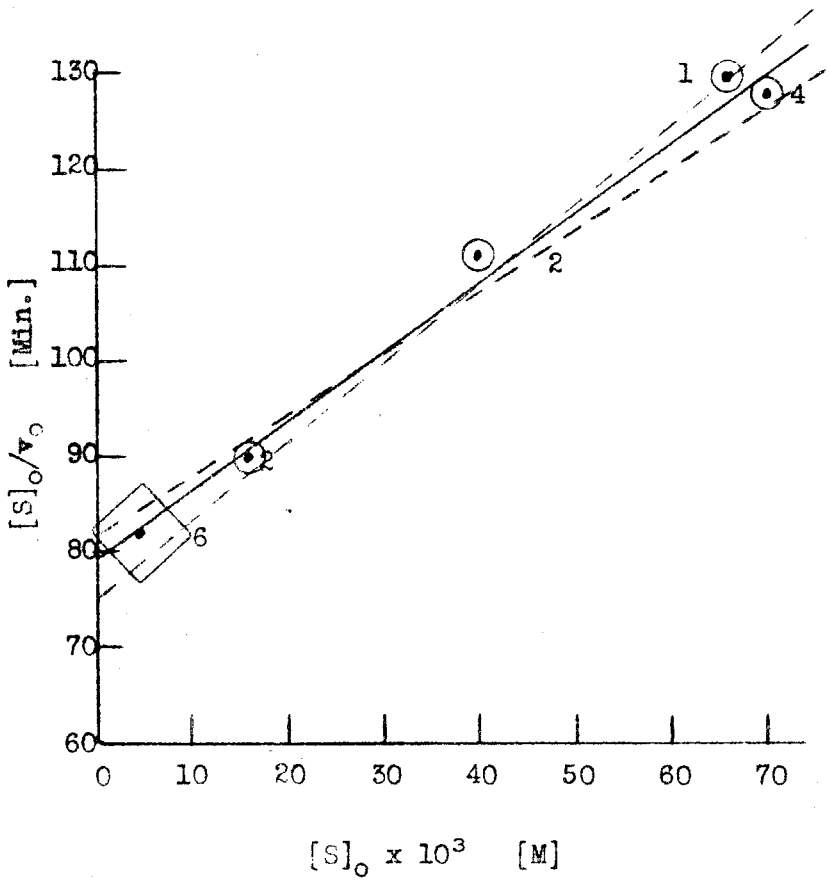
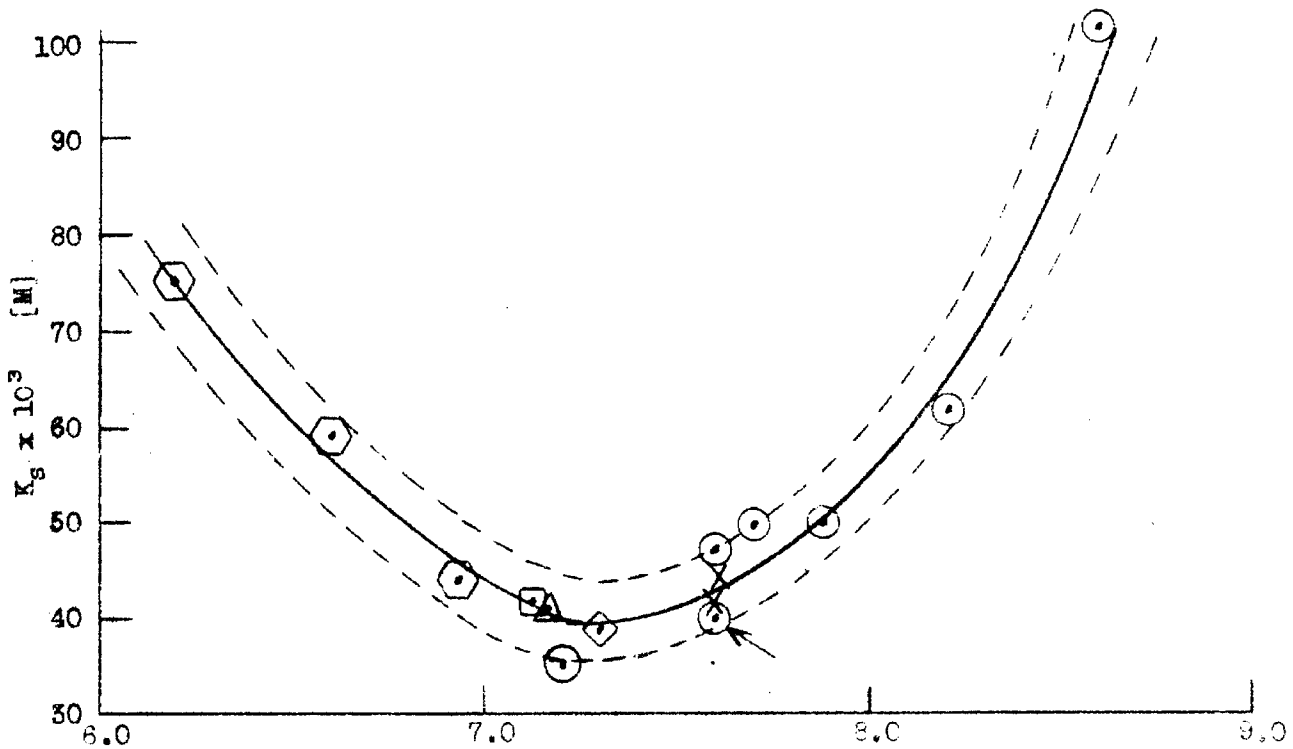


Figure 40. K_s vs. pH. Acetyl-L-tyrosinhydroxamide. Dotted envelope indicates approximately the maximum error in the solid curve.



Buffer systems.

- ⬡ Cacodylic acid–NaOH
- △ Hydroxylamine–HCl
- ○ Tris–[hydroxymethyl]–aminomethane–HCl
- ◇ Ethylenediamine–HCl
- □ Phosphoric acid–KOH
- X Results obtained by other investigators.

Figure 41. V_{max} vs. pH. Acetyl-L-tyrosinhydroxamide. Dotted envelope indicates approximately the maximum error in the solid curve. $[E]_o$ = 0.040 mg. protein nitrogen per ml.

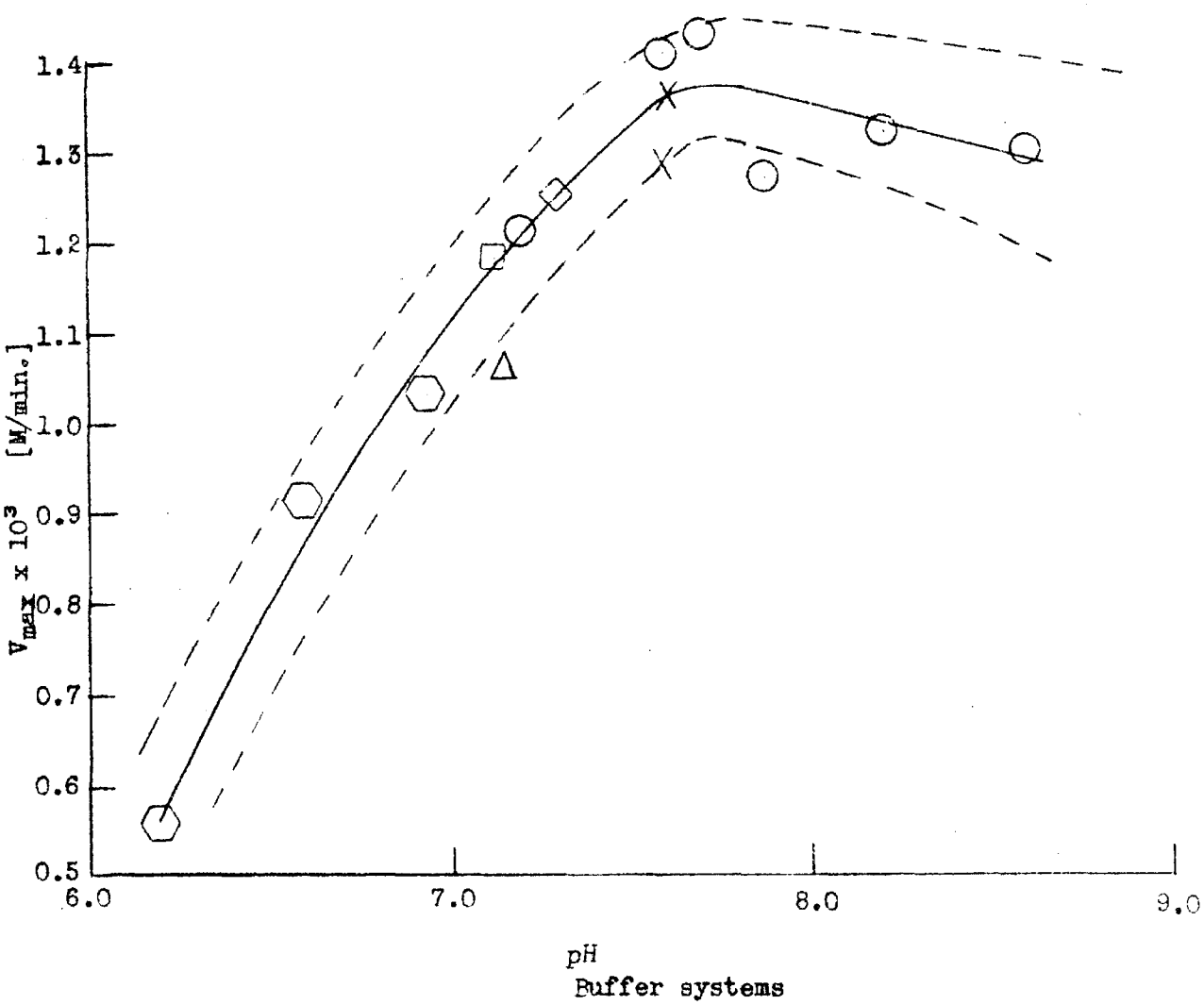


Figure 42. Plot of K_s/V_{max} vs. pH for acetyl-L-tyrosinhydroxamide.

• = estimated from runs made with low initial substrate concentrations.

X = estimated from values of the individual constants obtained at the indicated pH.

△ = values of other investigators.

⊙ = duplicate observations at low initial substrate concentrations.

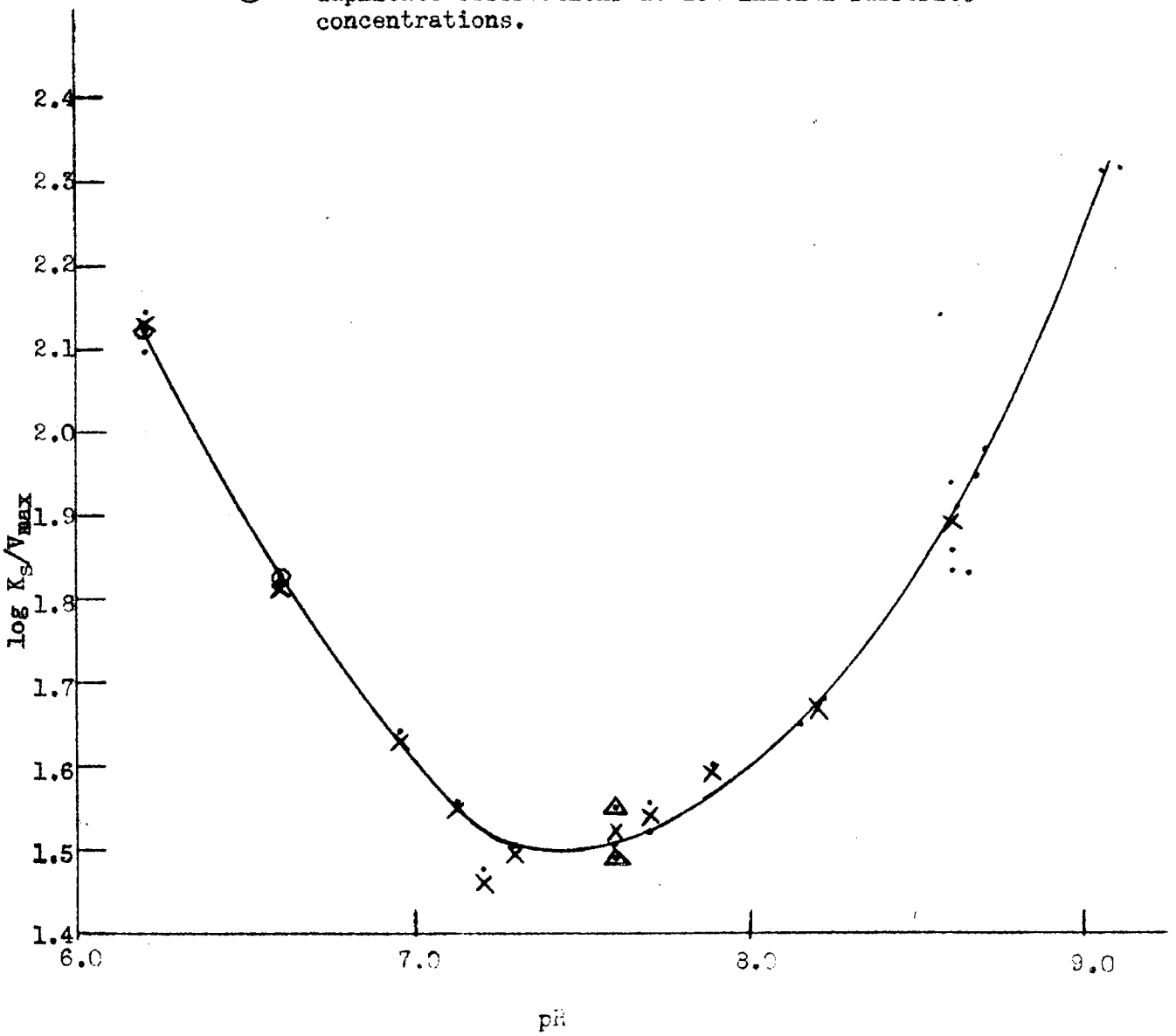


Figure 43. The effect of pH on the inhibition constant of acetyl-L-tyrosine.

◯ = observations with acetyl-L-tyrosinhydroxamide.

X = observations of other investigators.

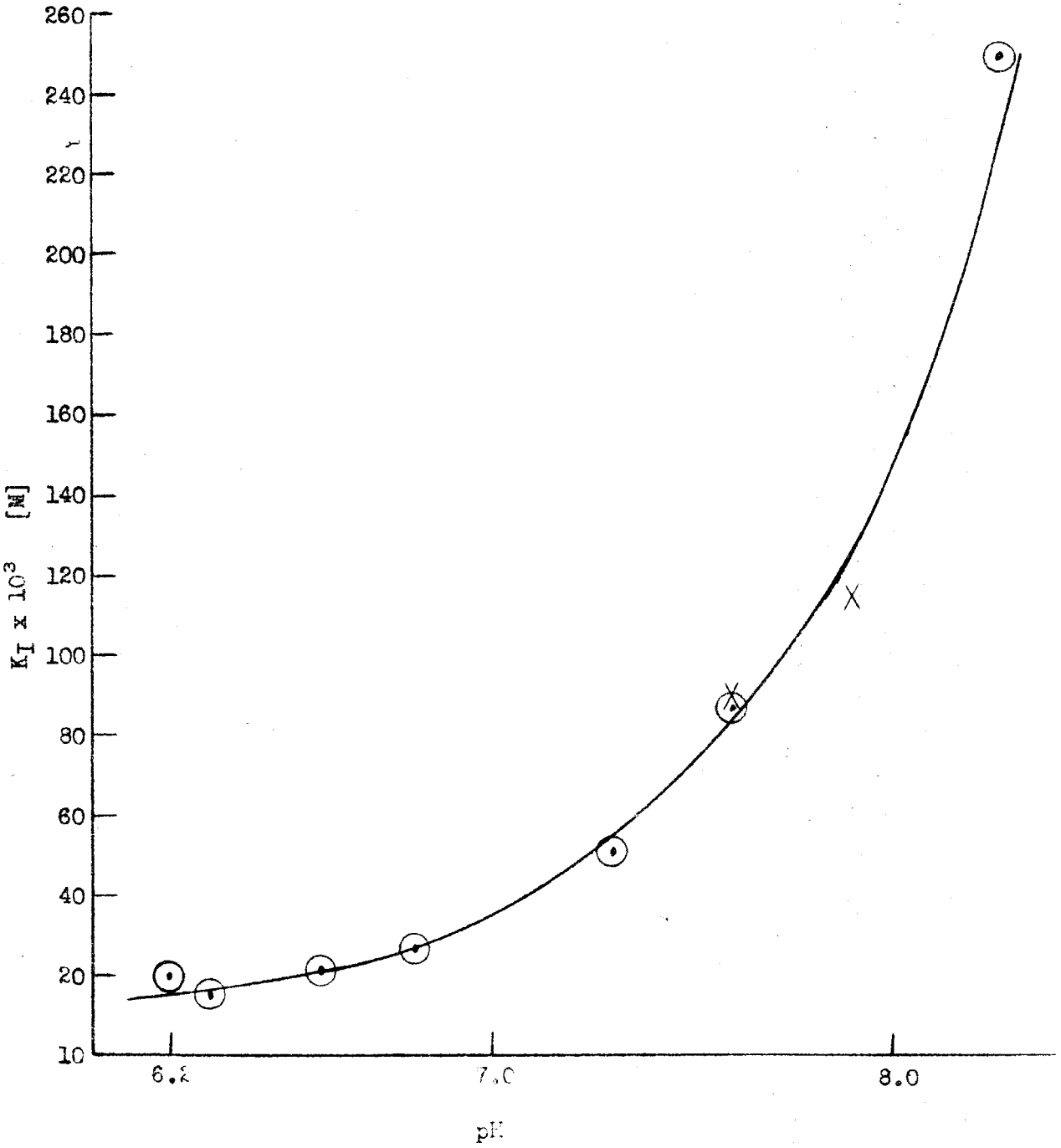


Figure 44. The variation in the inhibition constant of L-tyrosinamide with pH.

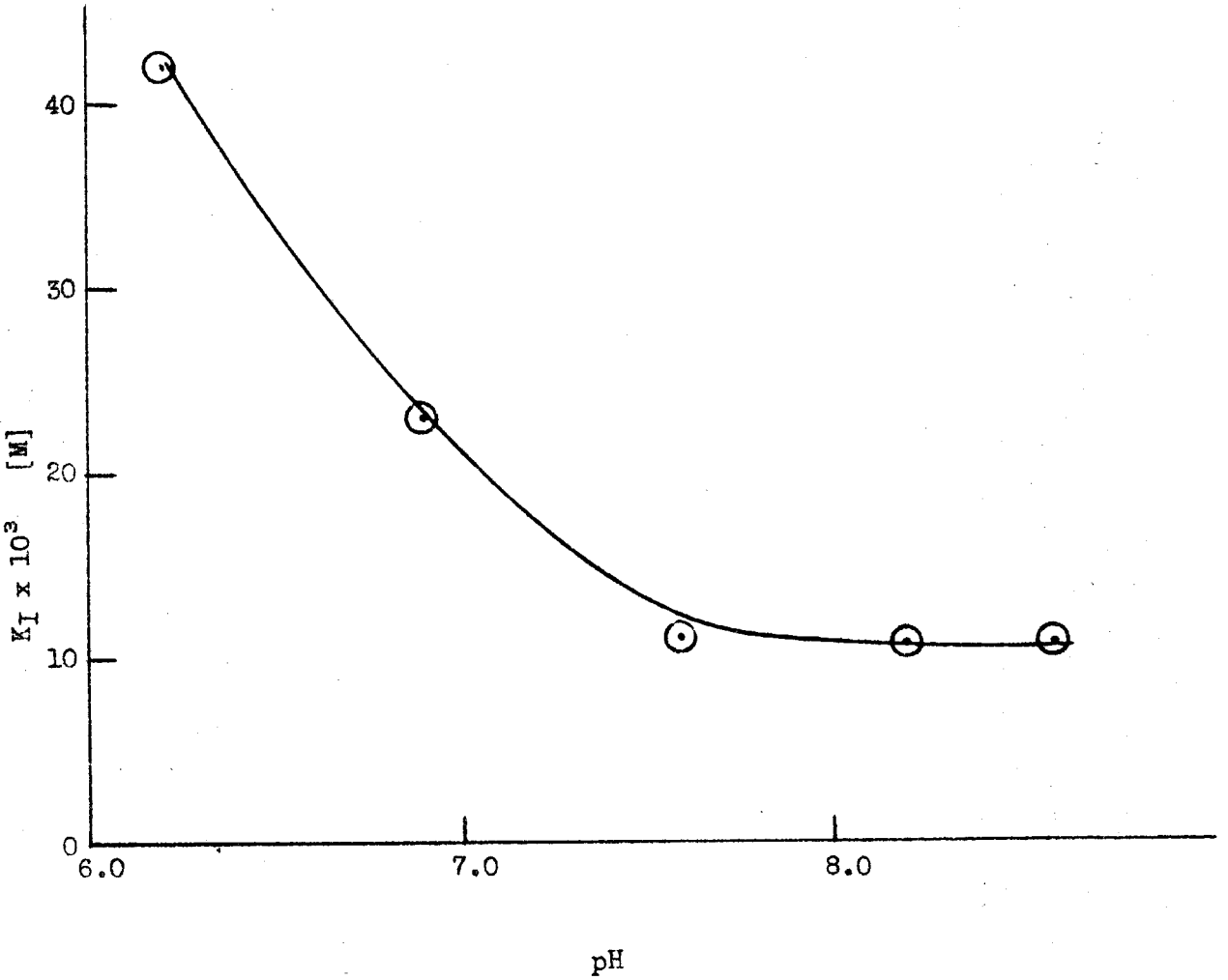


Figure 45. The determination of the value of K_c.

⊙ = present experiments.

X = determined by previous investigators.

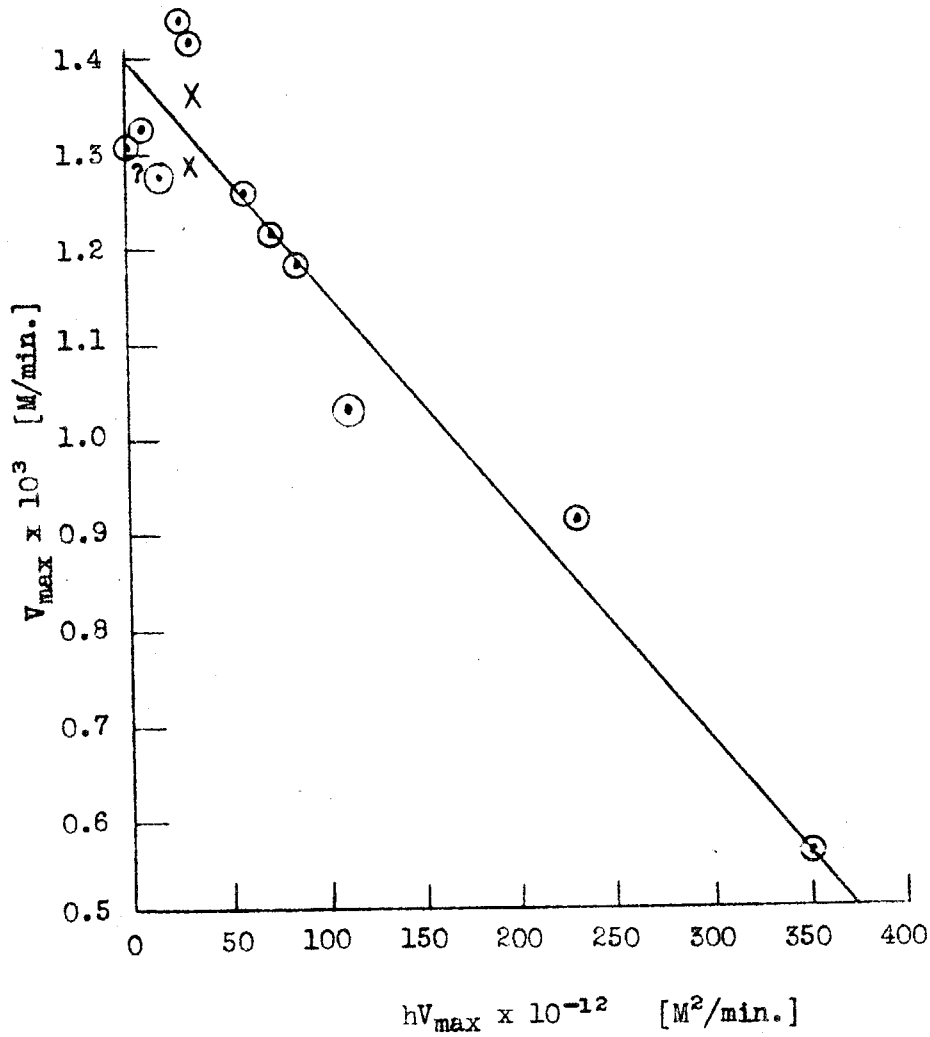
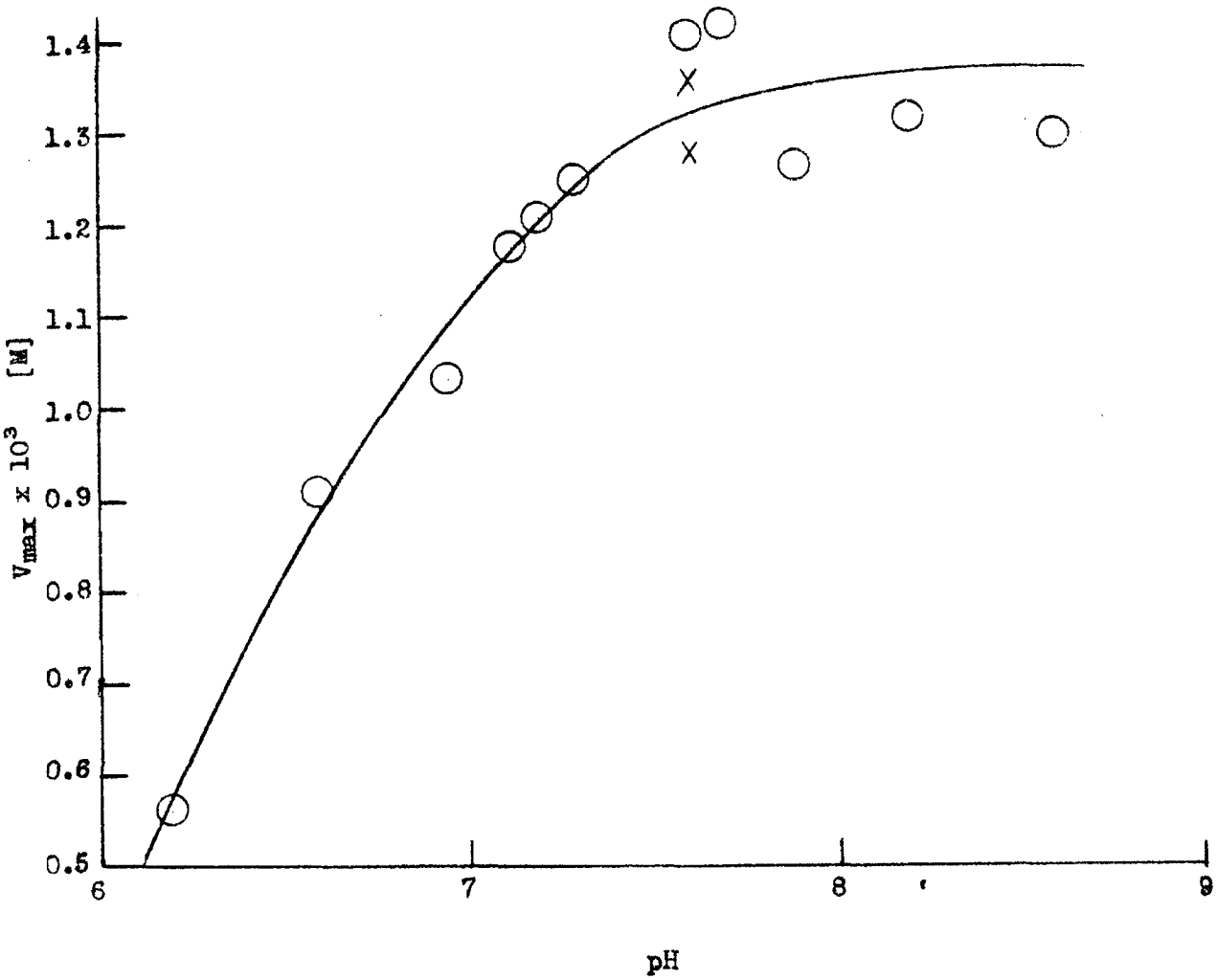


Figure 46. Representation of the behavior of V_{max} by the relationship derived in the text, and the constant evaluated in Fig. 45.

X = values obtained by previous investigators.



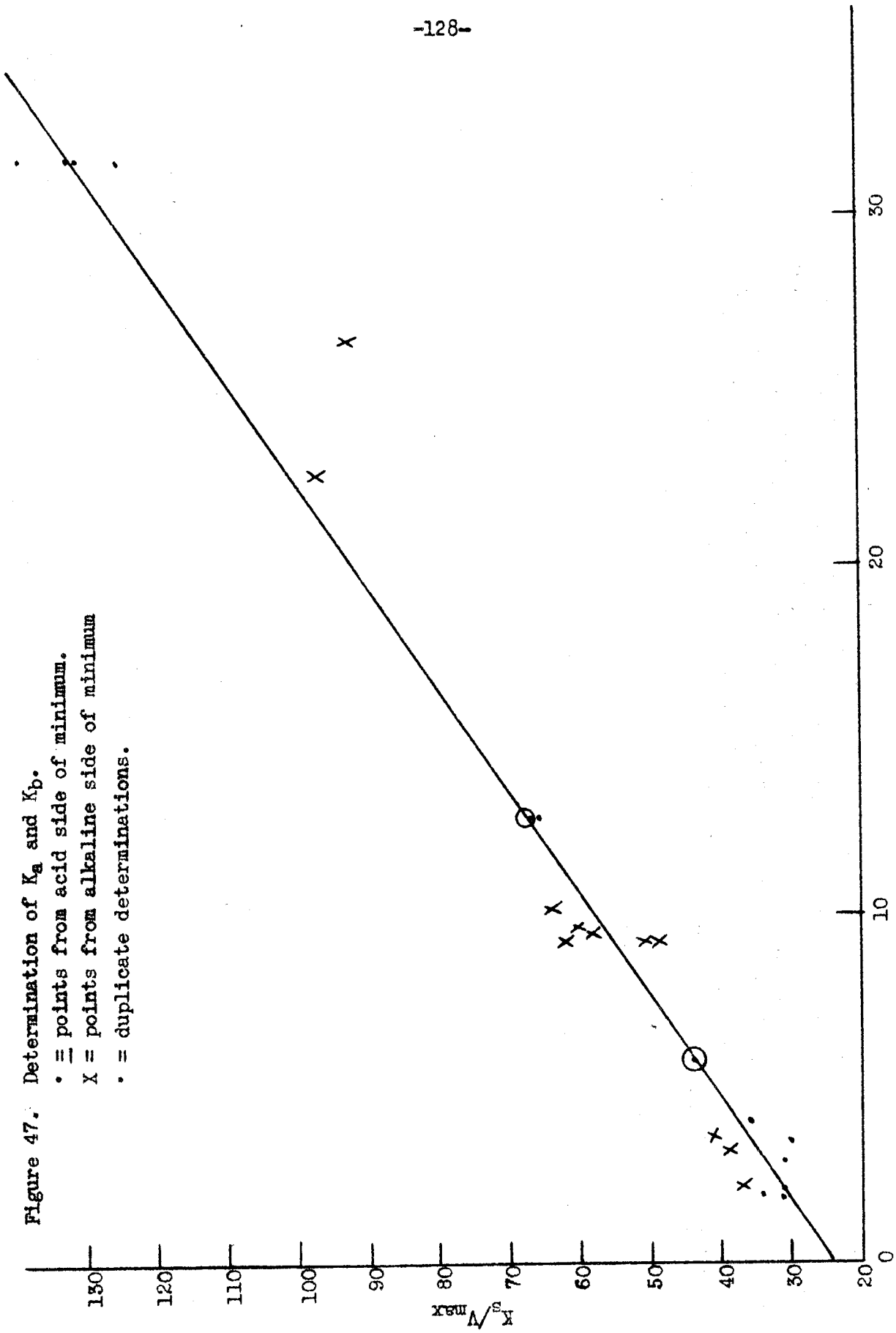


Figure 47. Determination of K_a and K_b.
• = points from acid side of minimum.
X = points from alkaline side of minimum
· = duplicate determinations.

Figure 48. Fitting of curve to the experimental data for K_s/V_{max}.

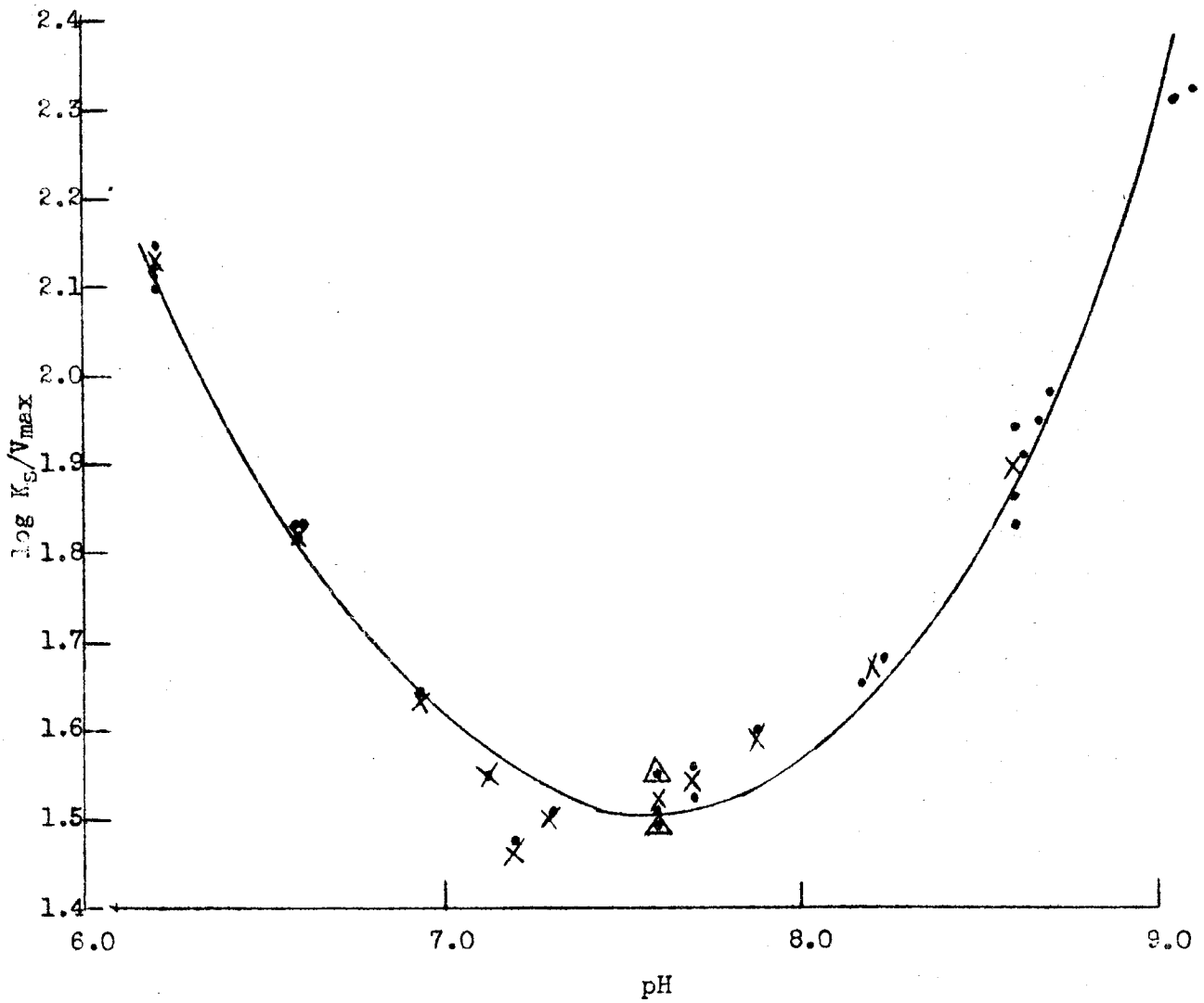


Figure 49. Fit of curve to experimental data for K_s.

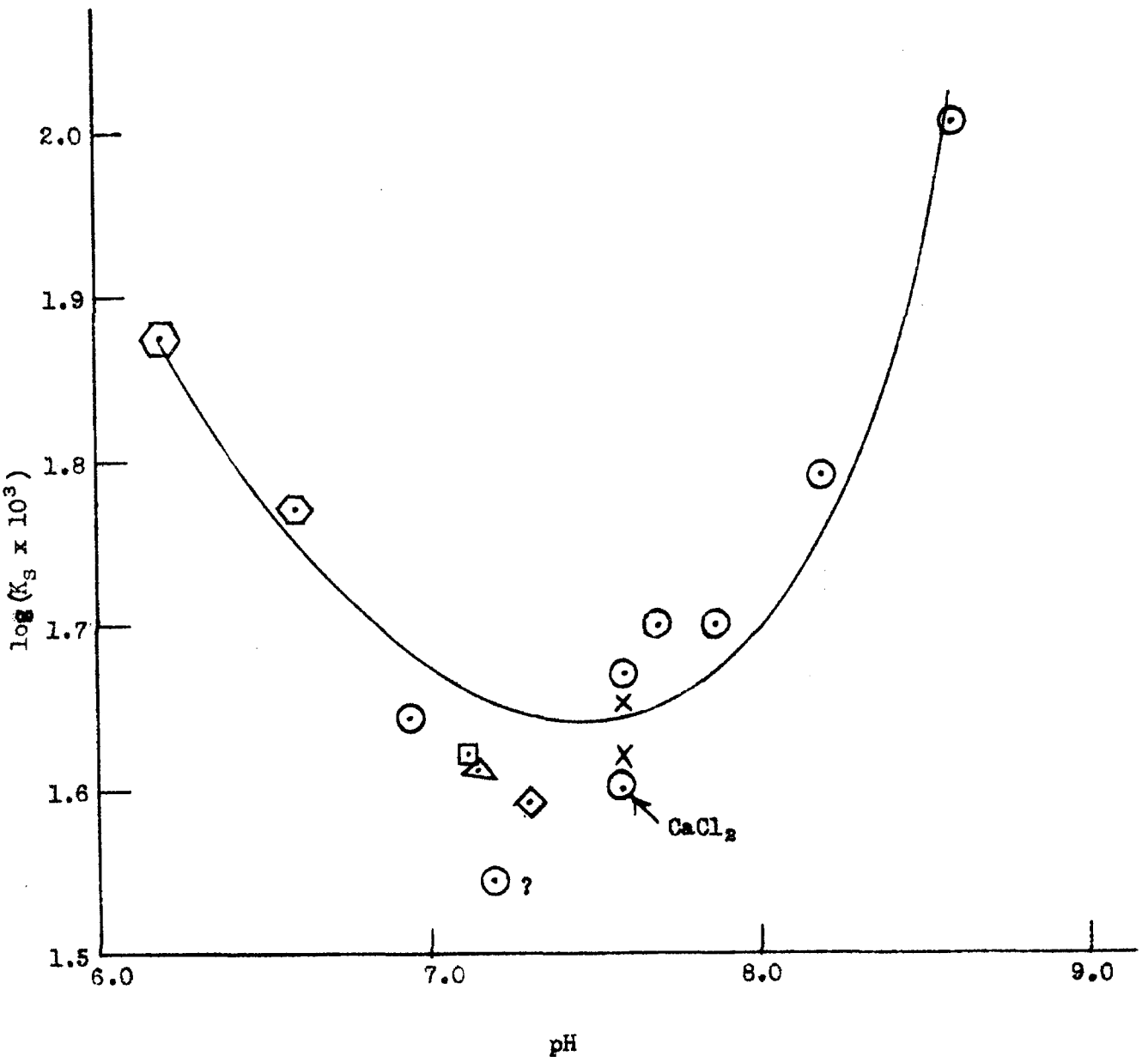


Figure 50. Behavior of K_s/V_{max} for L-tyrosinhydroxamide with changing pH.

• = from low substrate concentrations.

X = from ratio of constants determined from Walker-Schmidt plots.

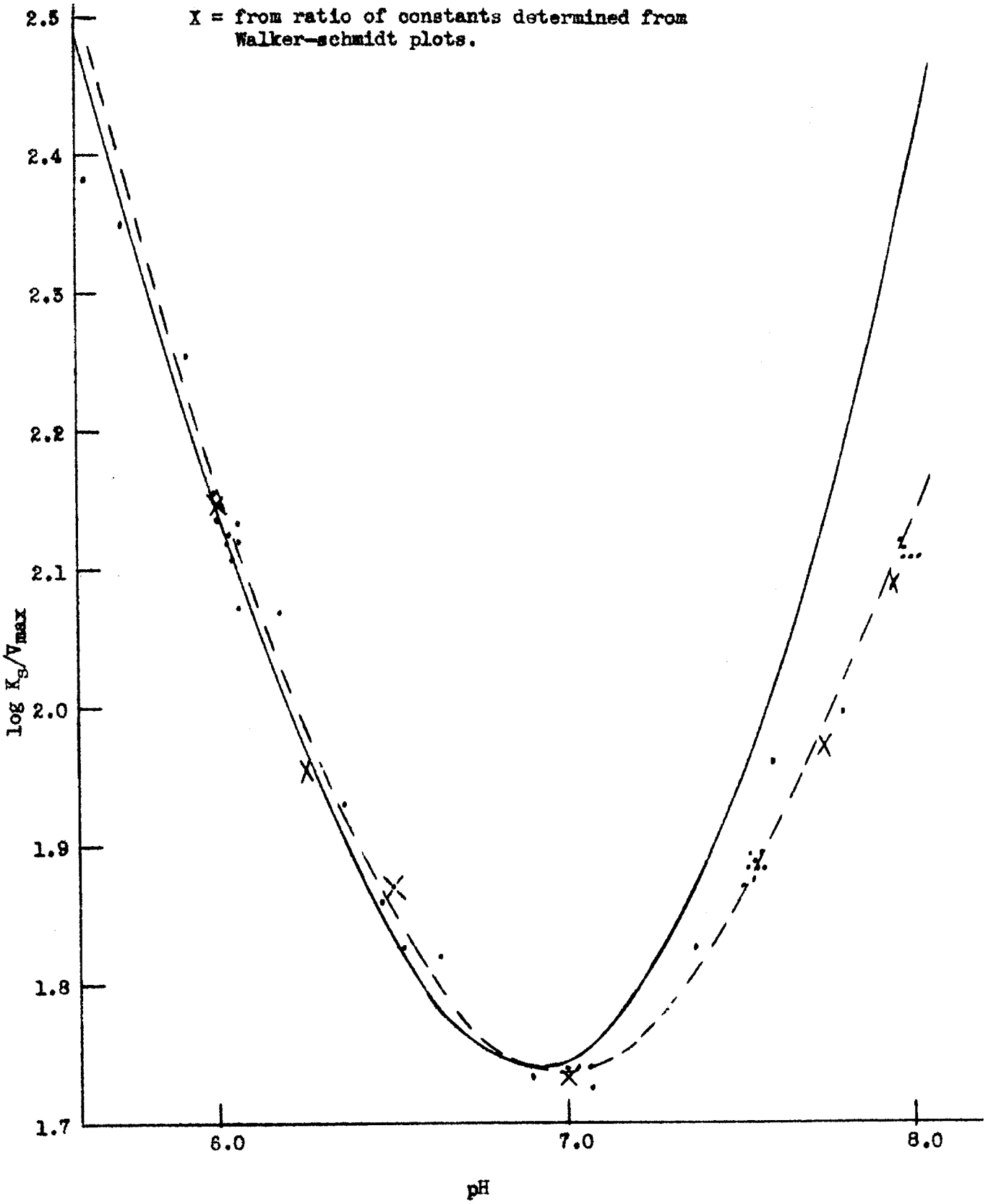


Figure 51. Determination of some constants appearing in equations which relate V_{max} for L-tyrosinhydroxamide to hydrogen ion concentration.

⊙ = points from acid side of optimum.

X = points from alkaline side of optimum.

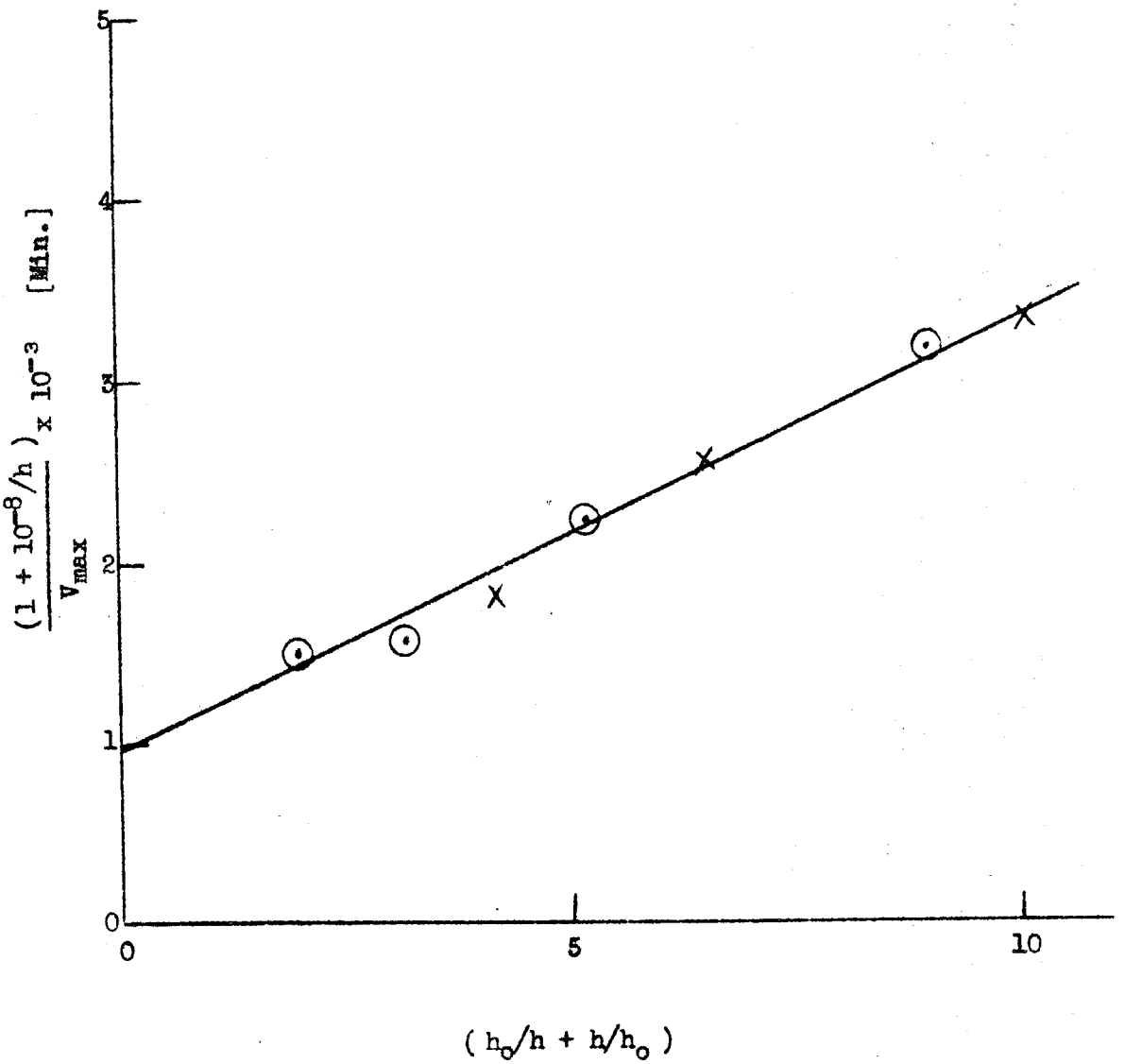


Figure 52. Fit of derived curve to experimental values of V_{max} for L-tyrosinhydroxamide.

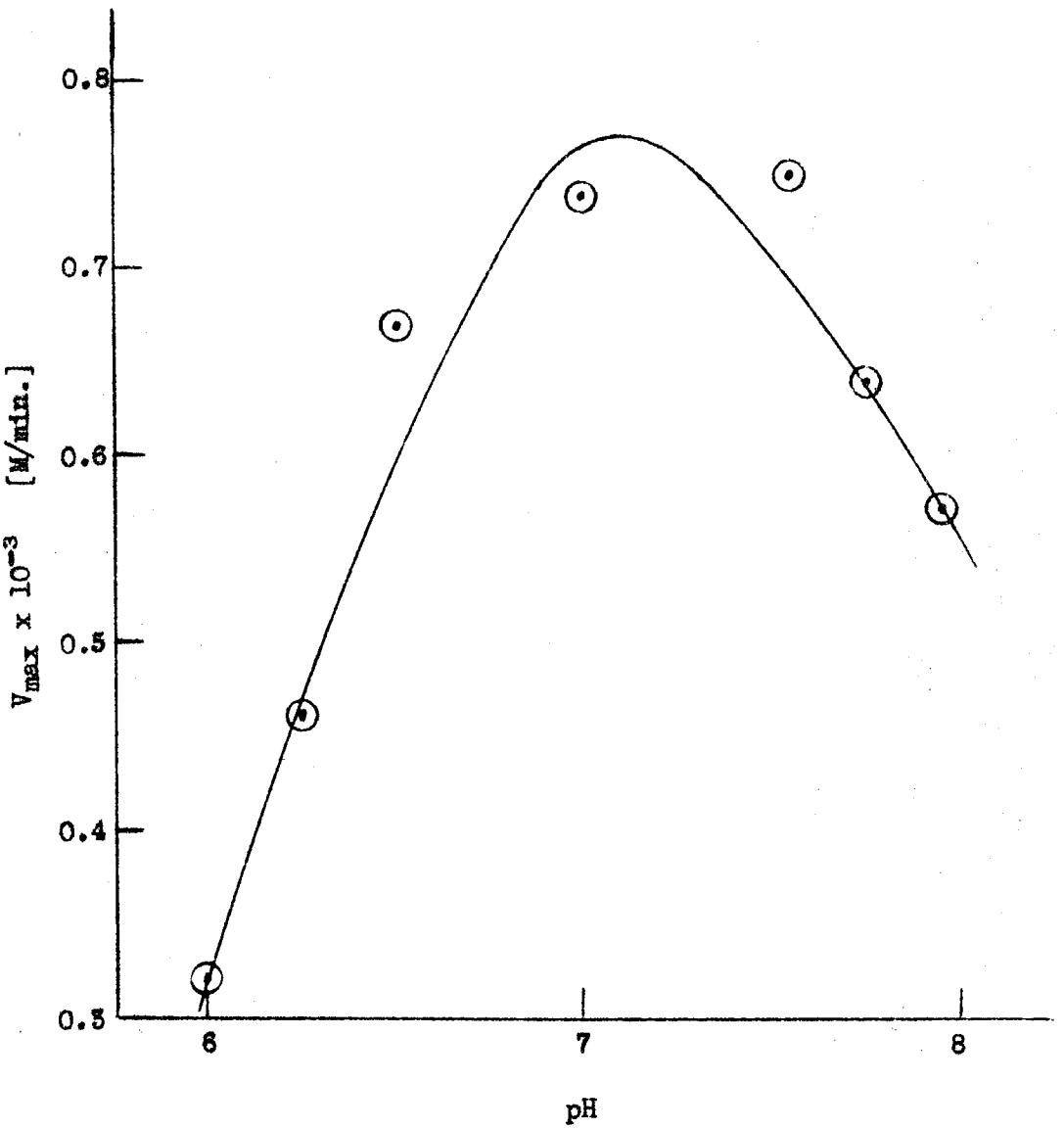


Figure 55. Fitting of curve to experimental values for K_s.

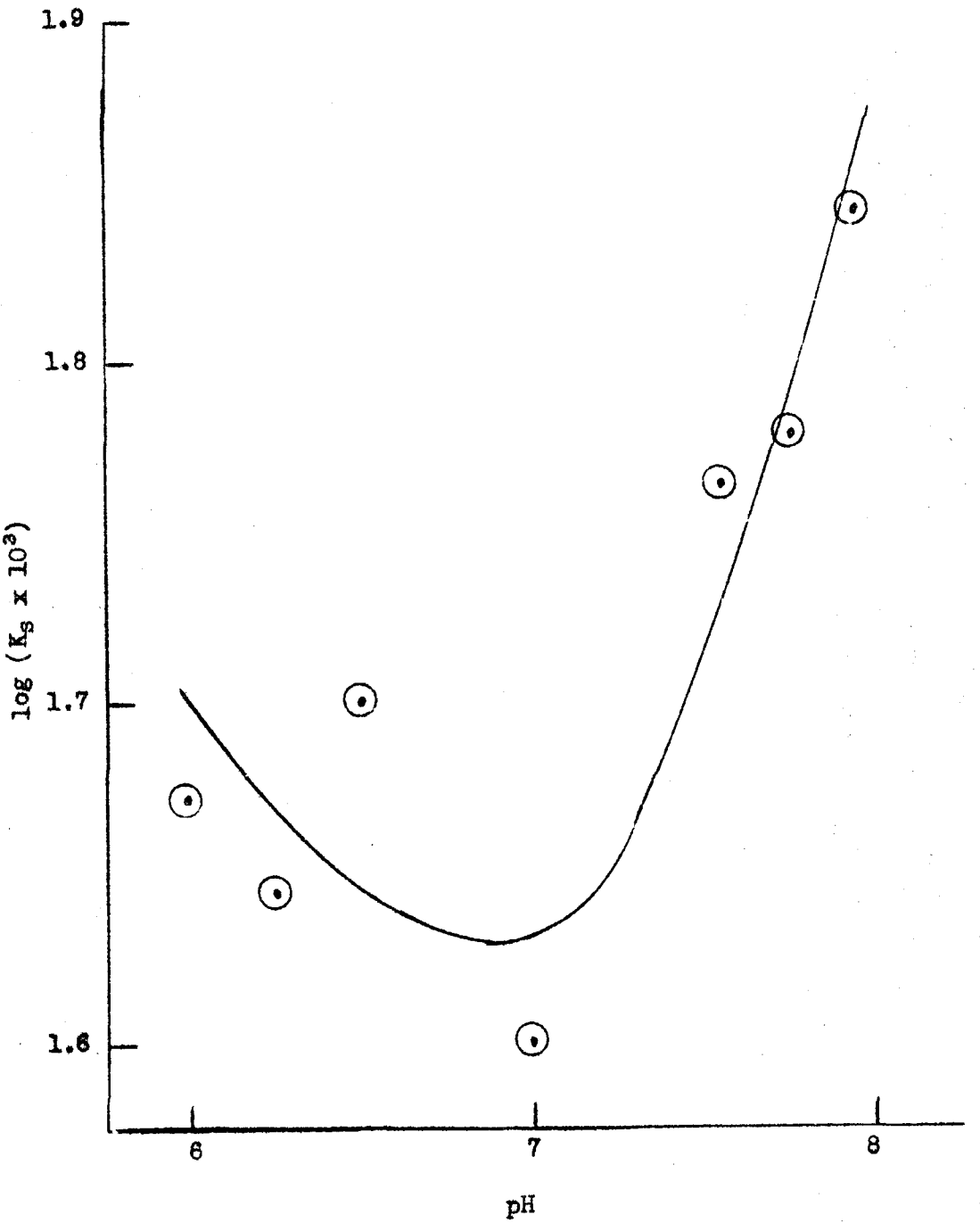


Figure 54. Determination of K_a from the behavior of the inhibition constant of acetyl-L-tyrosine with changing pH.

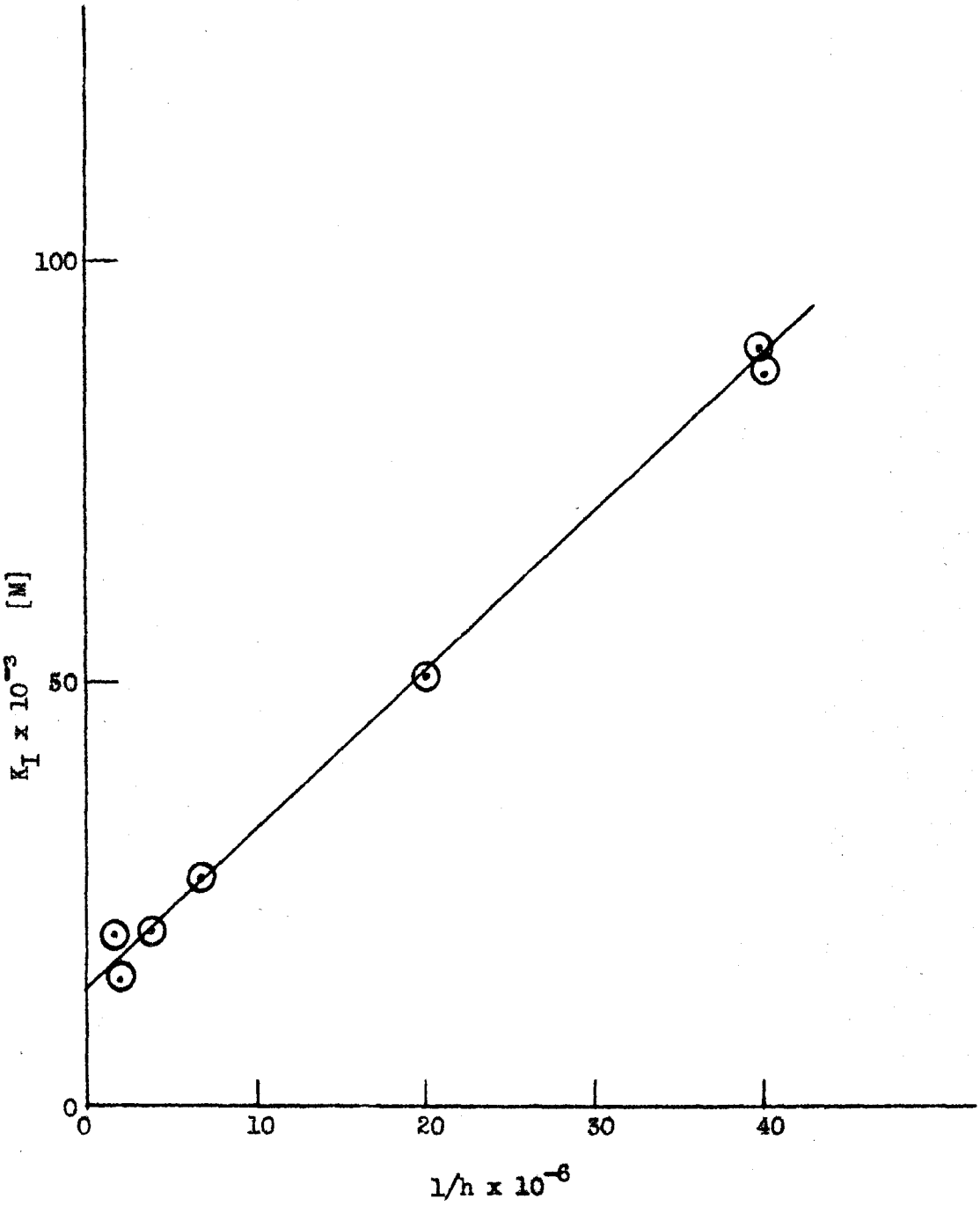


Figure 55. Titration of acethydroxamide with base.

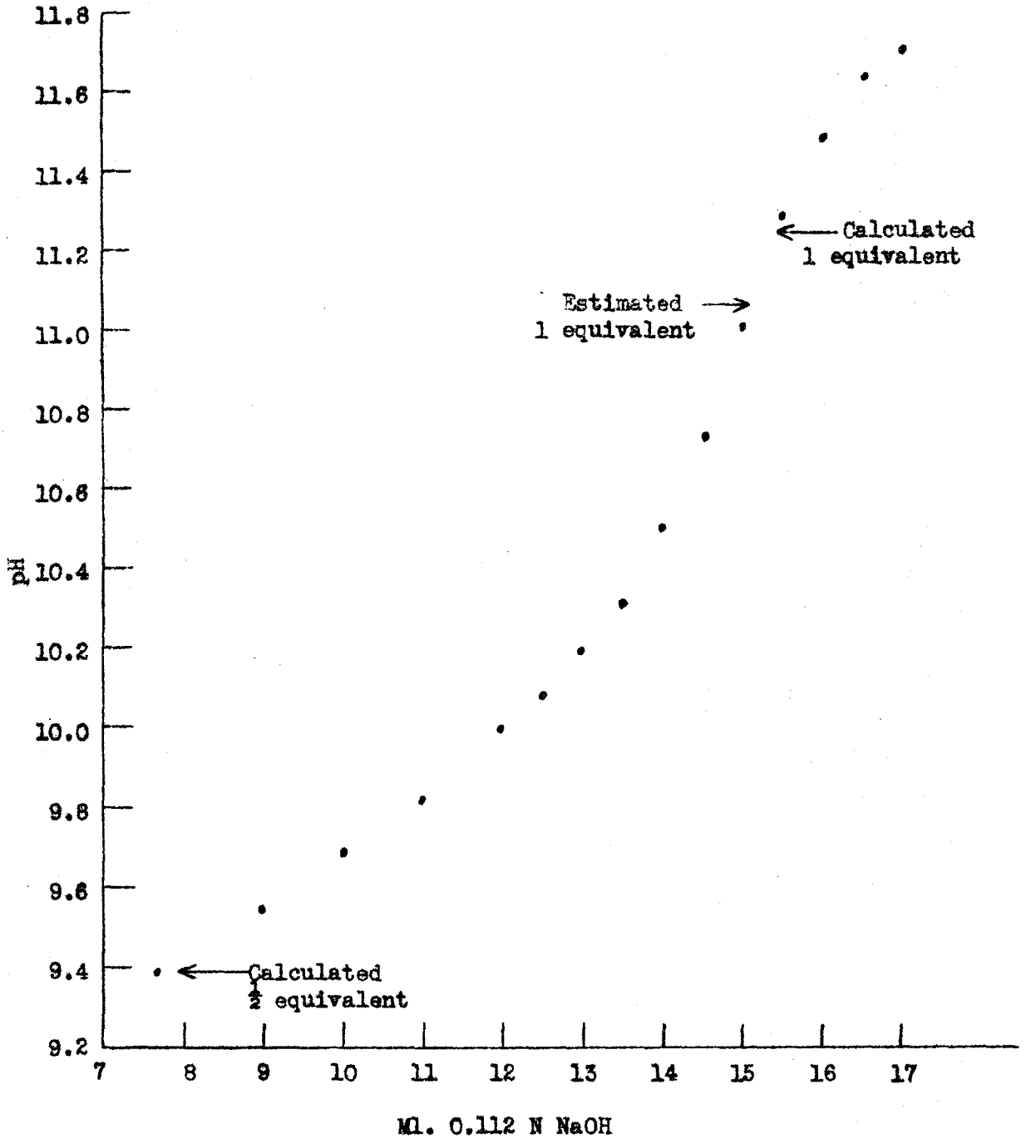


Figure 56. Back titration with 0.100N HCl of solution of acethydroxamide previously titrated with NaOH.

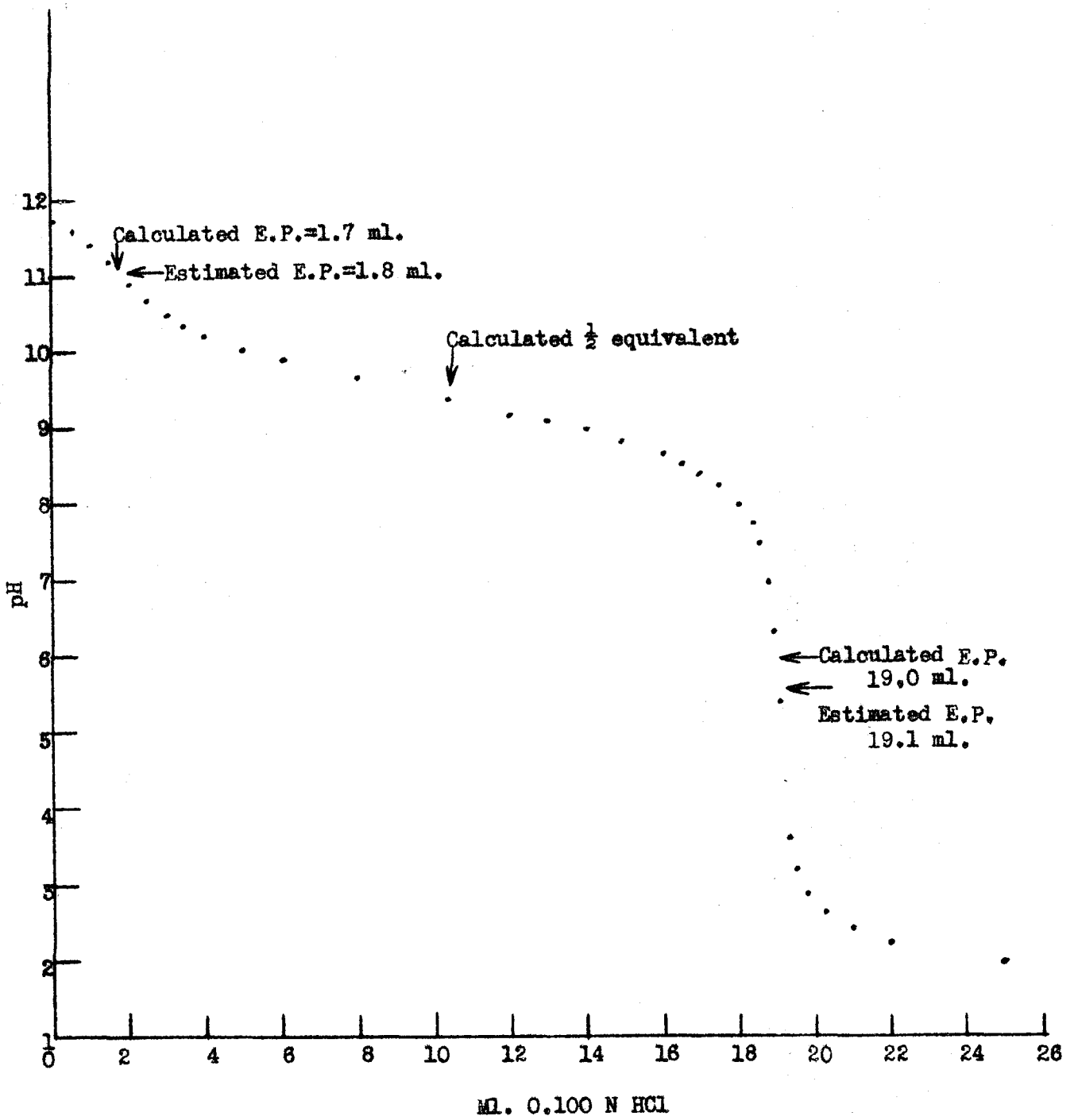


Figure 57. Titration of glycinhydroxamide with 0.100N HCl.

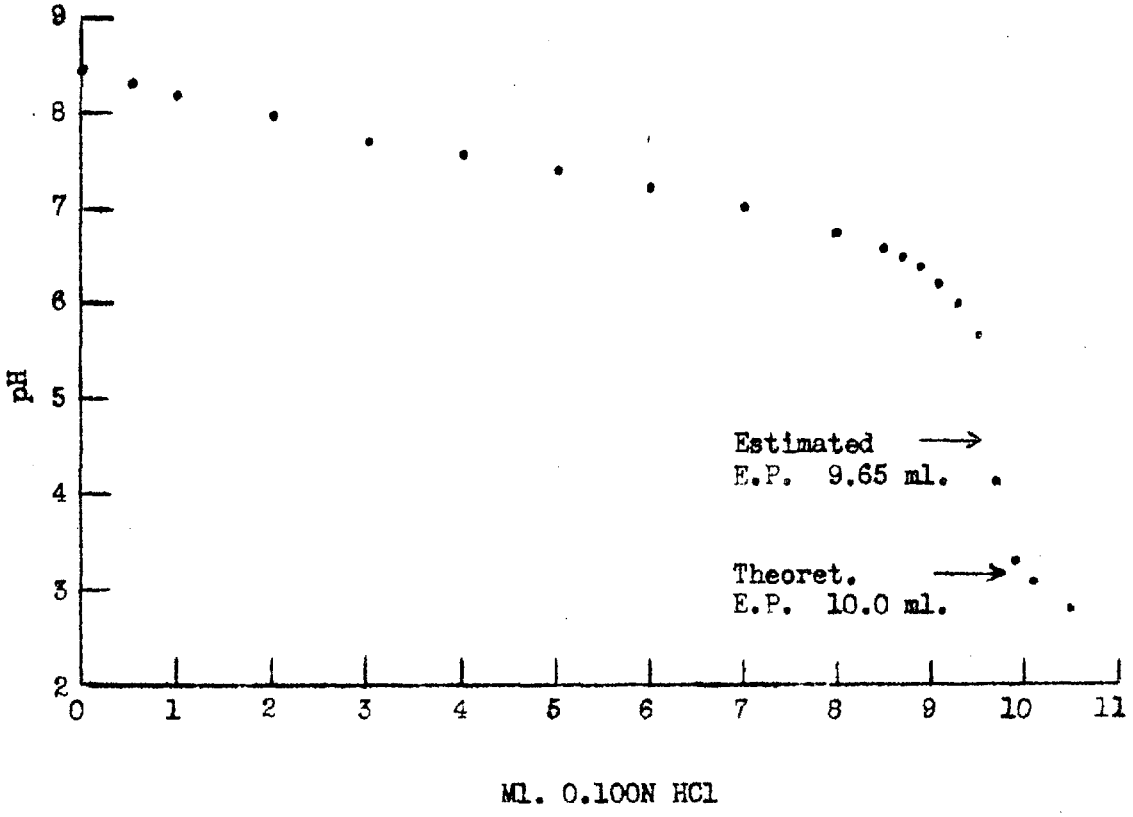


Figure 58. Back titration with 0.112N base of HCl titration of glycinhydroxamide.

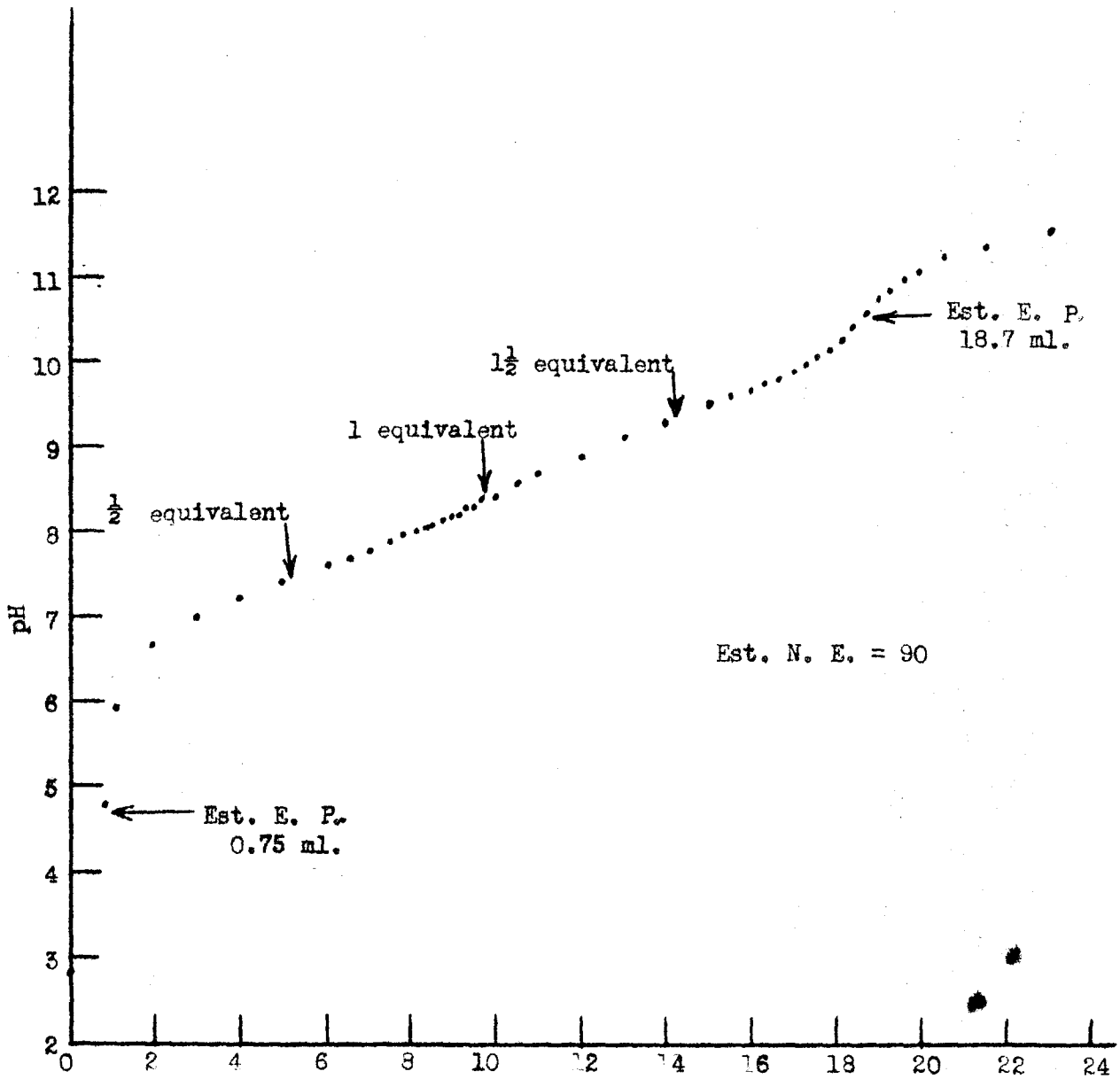


Figure 59. Titration of glycinhydroxamide with 0.112N base.

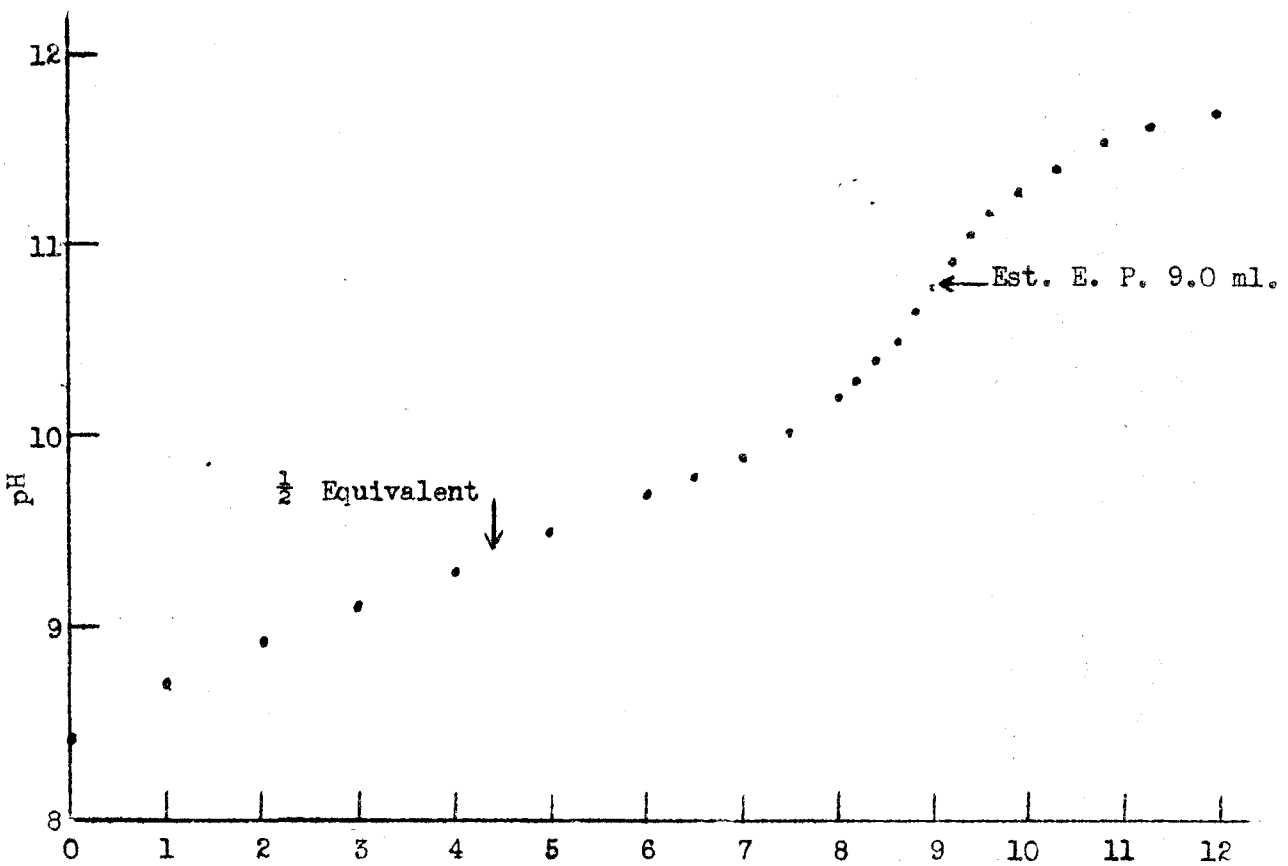
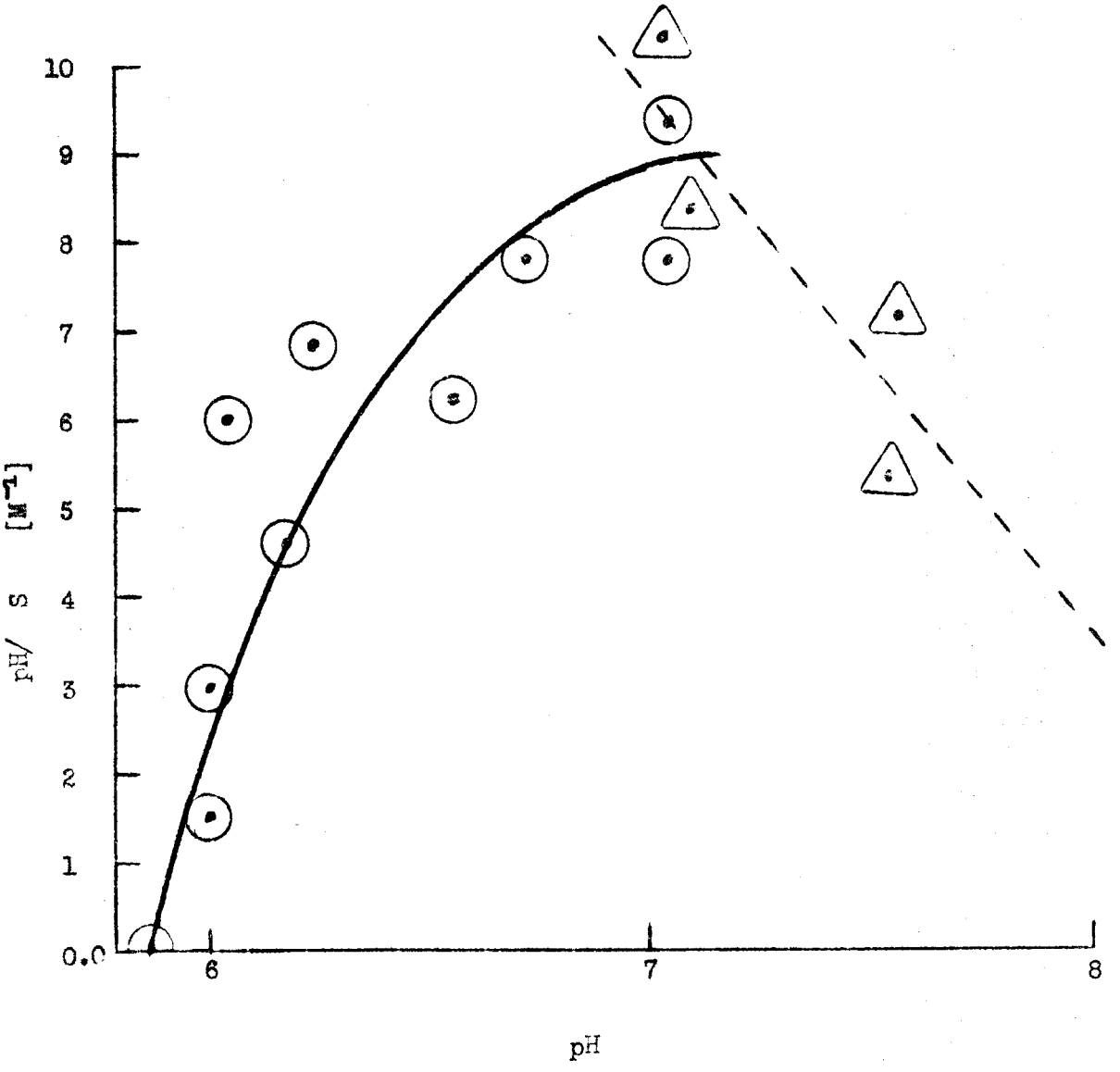


Figure 60. Change in pH with hydrolysis of L-tyrosinhydroxamide.



○ and solid line = cacodylic-acid NaOH buffer.

△ and broken line = tris-(hydroxymethyl)-aminomethane-HCl buffer.

TABLE VIII

Results of Kinetic Studies on the Alpha-chymotrypsin-catalyzed Hydrolysis of L-tyrosinhydroxamide

$[S]_o$ = initial substrate concentration.

$[S]_t$ = substrate concentration after 60 min. elapsed time.

Expt. No.	$[S]_o \times 10^3$ [M]	$(1/60) \ln[S]_o/[S]_t \times 10^3$ [Min.$^{-1}$]	Median pH
2	2.00	7.34	6.06
8	3.00	7.34	6.02
9	3.00	7.00	6.06
37 (a)	3.00	3.93 (?)	5.62
38 (a)	3.00	4.25 (?)	5.73
39	3.00	5.30	5.91
40	3.00	8.23	6.18
41	3.00	13.4	6.47
42	3.00	14.8	6.63
45	3.00	18.2	7.07
46	3.00	14.5	7.37
47	3.00	11.8	7.60
48	3.00	9.73	7.80
49	3.00	11.2	6.36
50	3.00	13.0	6.50
59	3.00	17.9	6.90
60	3.00	17.7	7.00
63	3.00	17.5	7.06
67	3.00	17.5	7.03
73	3.00	6.63	5.99
78	3.00	14.3	6.53
89	3.00	7.38	7.97
100	3.00	7.46	7.98
101	3.00	7.72	7.98
105	3.00	7.58	8.00
110	3.00	12.4	7.56
111	3.00	12.4	7.56
112	3.00	12.6	7.57
114	3.00	12.6	7.56
6	4.89	7.79	6.06
3	5.00	6.93	6.03
4	5.00	6.86	6.03
5	5.00	6.62	6.00

Expt. No.	$[S]_o \times 10^3$ [M]	$(1/60) \ln[S]_o/[S]_t \times 10^3$ [Min.$^{-1}$]	Median pH
107	5.00	7.47	8.01
120	5.00	12.5	7.54
121	5.00	12.4	7.53
123	5.00	12.4	7.54
124	5.00	12.6	7.51
1	10.0	6.58	6.03
7	10.0	7.30	6.10
22	10.0	3.21	5.76
23	10.0	3.90	5.87
24	10.0	5.29	6.00
25	10.0	8.08	6.28
26	10.0	11.3	6.55
27	10.0	13.4	6.72
28	10.0	15.1	6.94
29	10.0	14.4	6.84
30	10.0	12.6	7.44
31	10.0	11.9	7.51
32	10.0	14.2	7.24
33	10.0	14.5	7.19
34	10.0	5.90	8.08
57	10.0	15.4	6.96
58	10.0	15.4	7.10
62	10.0	15.8	7.02
65	10.0	15.6	7.04
71	10.0	15.5	7.04
74	10.0	5.57	5.98
79	10.0	11.5	6.51
86	10.0	15.6	7.12
91	10.0	7.27	7.96
95	10.0	5.39	5.99
96	10.0	7.30	7.91
102	10.0	7.06	7.98
106	10.0	7.22	7.99
113	10.0	11.7	7.58
118	10.0	11.6	7.57
122	10.0	11.9	7.56
127	10.0	11.7	7.53
11	20.0	7.50	6.25
68	20.0	13.9	7.07
72	20.0	5.15	5.99
77	20.0	10.5	6.51
84	20.0	14.1	7.05
90	20.0	6.65	7.95
99	20.0	6.74	7.90
104	20.0	6.64	7.98
109	20.0	6.53	7.99
115	20.0	10.3	7.58

Expt. No.	$[S]_o \times 10^3$ [M]	$(1/60) \ln[S]_o/[S]_t \times 10^3$ [Min.$^{-1}$]	Median pH
116	20.0	10.2	7.56
117	20.0	10.1	7.60
119	20.0	10.4	7.59
20	30.0	8.98	7.57
21	30.0	11.9	7.05
66	30.0	11.9	7.05
76	30.0	4.55	6.01
80	30.0	0.23	6.54
83	30.0	12.0	7.03
88	30.0	5.98	7.93
98	30.0	5.97	7.89
103	30.0	6.16	7.99
108	30.0	5.75	7.98
125	30.0	8.98	7.54
126	30.0	9.01	7.56
128	30.0	8.75	7.54
129	30.0	9.13	7.55
10	40.0	7.32	6.39
12	40.0	5.39	6.19
13	40.0	3.08	5.88
14	40.0	4.47	6.07
15	40.0	8.59	6.57
16	40.0	9.76	6.73
17	40.0	10.7	7.10
61	40.0	10.3	7.05
64	40.0	10.9	6.97
75	40.0	3.85	6.01
81	40.0	8.37	6.55
82	40.0	10.5	7.10

(a) very inaccurate values.

TABLE IX

Values Obtained at pH 6.0 by Extrapolation of Runs Made Below pH 6.25

Expt. No.	$[S]_o \times 10^3$ [M]	Extrapolated (1/60) $\ln[S]_o/[S]_t \times 10^3$ [Min.$^{-1}$]		Deviation	(1/60) $([S]_o - [S]_t) \times 10^5$ [M/min.]
2	2.00	6.54			1.08
8	3.00	7.07		0.65	
9	3.00	6.18		0.24	
39	3.00	6.51		0.09	
40	3.00	5.81		0.61	
73	3.00	6.50		0.08	
	Average	6.42	±	0.34	1.60
3	5.00	6.53		0.12	
4	5.00	6.46		0.19	
5	5.00	6.62		0.03	
6	4.89	6.99		0.34	
	Average	6.65	±	0.17	2.73
1	10.0	6.25		0.51	
7	10.0	6.21		0.47	
22	10.0	5.83		0.09	
23	10.0	5.87		0.42	
24	10.0	5.29		0.45	
74	10.0	5.79		0.05	
95	10.0	5.50		0.24	
	Average	5.74	±	0.32	4.85
11	20.0	4.87		0.16	
72	20.0	5.26		0.23	
	Average	5.03	±	0.20	8.70
76	30.0	4.46			11.68
12	40.0	3.78		0.10	
13	40.0	4.10		0.12	
14	40.0	3.87		0.01	
75	40.0	3.76		0.12	
	Average	3.88	±	0.09	14.00

TABLE X

Values Obtained at pH 6.25 by Extrapolation of Runs Made Between pH 6.0 and 6.5

Expt. No.	$[S]_o \times 10^3$ [M]	Extrapolated (1/60) $\ln[S]_o/[S]_t \times 10^3$ [Min.$^{-1}$]		Deviation	(1/60) $([S]_o - [S]_t) \times 10^5$ [M/min.]
2	2.00	9.88			1.49
8	3.00	10.42		0.61	
9	3.00	9.43		0.38	
40	3.00	9.17		0.64	
41	3.00	10.45		0.64	
49	3.00	9.73		0.08	
50	3.00	9.65		0.16	
	Average	9.81	±	0.42	2.22
3	5.00	9.88		0.12	
4	5.00	9.81		0.19	
5	5.00	9.97		0.03	
6	5.00	10.33		0.33	
	Average	10.00	±	0.17	3.77
1	10.0	8.98		0.50	
7	10.0	8.94		0.46	
24	10.0	8.02		0.46	
25	10.0	7.75		0.73	
79	10.0	8.73		0.25	
	Average	8.48	±	0.48	6.65
11	20.0	7.50			12.09
76	30.0	6.81			16.69
10	40.0	6.22		0.22	
12	40.0	5.90		0.10	
14	40.0	6.00		0.00	
75	40.0	5.89		0.11	
	Average	6.00	±	0.11	20.20

TABLE XI

Values Obtained at pH 6.50 by Extrapolation of Runs Made Between pH 6.0 and 6.5

Expt. No.	$[S]_o \times 10^3$ [M]	Extrapolated $(1/60)\ln[S]_o/[S]_t \times 10^3$ [Min.$^{-1}$]		Deviation	$(1/60)([S]_o - [S]_t) \times 10^5$ [M/min.]
41	3.00	13.8		0.4	
42	3.00	13.4		0.0	
49	3.00	13.1		0.3	
50	3.00	13.0		0.4	
78	3.00	13.9		0.5	
	Average	13.4	±	0.3	2.76
25	10.0	10.5		0.4	
26	10.0	10.7		0.2	
27	10.0	11.0		0.1	
79	10.0	11.5		0.6	
	Average	10.9	±	0.4	8.00
11	20.0	10.1		0.2	
77	20.0	10.4		0.1	
	Average	10.3	±	0.2	15.3
80	30.0	9.0			20.8
10	40.0	8.3		0.1	
15	40.0	8.0		0.2	
16	40.0	8.0		0.2	
81	40.0	8.1		0.1	
	Average	8.2	±	0.1	25.6

TABLE XII

Values Obtained at pH 7.00 by Extrapolation of Runs Made Between pH 6.75 and 7.25

Expt. No.	$[S]_o \times 10^3$ [M]	Extrapolated $(1/60)\ln[S]_o/[S]_t \times 10^3$ [Min.$^{-1}$]		Deviation	$(1/60)([S]_o - [S]_t) \times 10^5$ [M/min.]
45	3.00	18.2		0.4	
59	3.00	17.9		0.1	
60	3.00	17.7		0.1	
63	3.00	17.5		0.3	
67	3.00	17.5		0.3	
	Average	17.8	±	0.3	3.28
28	10.0	15.4		0.1	
29	10.0	15.3		0.2	
32	10.0	15.3		0.2	
33	10.0	15.2		0.3	
57	10.0	15.4		0.1	
58	10.0	15.4		0.1	
62	10.0	15.8		0.3	
65	10.0	15.6		0.2	
66	10.0	15.5		0.0	
86	10.0	15.6		0.1	
	Average	15.5	±	0.2	10.1
68	20.0	13.9		0.1	
84	20.0	14.1		0.1	
	Average	14.0	±	0.1	19.0
21	30.0	11.9			
66	30.0	11.9			
83	30.0	12.0			
	Average	11.9	±	0.1	25.5
17	40.0	10.7		0.1	
61	40.0	10.3		0.3	
64	40.0	10.9		0.3	
82	40.0	10.5		0.1	
	Average	10.6	±	0.2	31.3

TABLE XIII

Values Obtained at pH 7.55 by Extrapolation of Runs Made Between pH 7.25 and 7.50

Expt. No.	$[S]_o \times 10^3$ [M]	Extrapolated (1/60) $\ln[S]_o/[S]_t \times 10^3$ [Min.$^{-1}$]		Deviation	(1/60) $([S]_o - [S]_t) \times 10^5$ [M/min.]
46	3.00	12.3		0.2	
47	3.00	12.4		0.1	
48	3.00	12.6		0.1	
110	3.00	12.4		0.2	
111	3.00	12.2		0.3	
112	3.00	12.7		0.3	
114	3.00	12.7		0.2	
	Average	12.5	±	0.2	2.65
120	5.00	12.4		0.1	
121	5.00	12.3		0.0	
123	5.00	12.3		0.0	
124	5.00	12.2		0.1	
	Average	12.3	±	0.1	4.35
30	10.0	11.5		0.1	
31	10.0	11.5		0.1	
32	10.0	11.2		0.4	
34	10.0	11.4		0.2	
35	10.0	11.5		0.1	
113	10.0	12.0		0.4	
118	10.0	11.9		0.3	
122	10.0	12.0		0.4	
127	10.0	11.5		0.1	
	Average	11.6	±	0.3	8.33
115	20.0	10.6		0.0	
116	20.0	10.3		0.3	
117	20.0	10.6		0.0	
119	20.0	10.8		0.2	
	Average	10.6	±	0.1	15.6
20	30.0	9.1		0.1	
125	30.0	8.9		0.1	
126	30.0	9.1		0.1	
128	30.0	8.7		0.3	
129	30.0	9.1		0.1	
	Average	9.0	±	0.2	20.8

TABLE XIV

Values Obtained at pH 7.75 by Extrapolation of Runs Made Between pH 7.50 and 8.00

Expt. No.	$[S]_o \times 10^3$ [M]	Extrapolated (1/60) $\ln[S]_o/[S]_t \times 10^3$ [Min.$^{-1}$]		Deviation	(1/60) $([S]_o - [S]_t) \times 10^5$ [M/min.]
47	3.00	10.1		0.2	
48	3.00	10.1		0.2	
89	3.00	10.5		0.2	
100	3.00	10.1		0.2	
101	3.00	10.4		0.1	
105	3.00	10.5		0.2	
110	3.00	10.2		0.1	
111	3.00	9.9		0.4	
112	3.00	10.5		0.2	
114	3.00	10.4		0.1	
	Average	10.3	±	0.3	2.30
107	5.00	10.5		0.4	
120	5.00	10.1		0.0	
121	5.00	9.9		0.2	
123	5.00	10.0		0.1	
124	5.00	9.8		0.3	
	Average	10.1	±	0.2	3.80
31	10.0	8.5		0.9	
34	10.0	9.4		0.0	
35	10.0	8.5		0.9	
91	10.0	9.4		0.0	
96	10.0	8.9		0.5	
102	10.0	9.4		0.0	
106	10.0	9.6		0.2	
113	10.0	10.0		0.6	
118	10.0	9.8		0.4	
122	10.0	10.0		0.6	
127	10.0	9.5		0.1	
	Average	9.4	±	0.4	7.20
90	20.0	8.5		0.1	
99	20.0	8.1		0.5	
104	20.0	8.8		0.2	
109	20.0	8.8		0.2	
115	20.0	8.6		0.0	
116	20.0	8.3		0.3	
117	20.0	8.6		0.0	
119	20.0	8.8		0.2	
	Average	8.6	±	0.2	13.50

Expt. No.	$[S]_o \times 10^3$ [M]	Extrapolated (1/60) $\ln[S]_o/[S]_t \times 10^3$ [Min.$^{-1}$]		Deviation	(1/60) $([S]_o - [S]_t) \times 10^5$ [M/min.]
20	30.0	7.6		0.1	
88	30.0	7.4		0.1	
98	30.0	7.1		0.4	
103	30.0	8.1		0.6	
108	30.0	7.6		0.1	
125	30.0	7.3		0.2	
126	30.0	7.5		0.0	
128	30.0	7.1		0.4	
129	30.0	7.5		0.0	
	Average	7.5	$\pm$	0.2	18.2

TABLE XV

Values Obtained at pH 7.95 by Extrapolation of Runs

Greater Than pH 7.80

Expt. No.	$[S]_o$ x 10^3 [M]	Extrapolated (1/60) $\ln[S]_o/[S]_t$ x 10^3 [Min.$^{-1}$]		Deviation	(1/60) $([S]_o - [S]_t)$ x 10^5 [M/min.]
48	3.00	8.0		0.1	
89	3.00	7.6		0.3	
100	3.00	7.8		0.1	
101	3.00	8.1		0.2	
105	3.00	8.2		0.3	
	Average	7.9	±	0.2	1.90
107	5.00	8.1			3.20
35	10.0	7.5		0.2	
36	10.0	7.2		0.1	
91	10.0	7.4		0.1	
96	10.0	6.9		0.4	
102	10.0	7.4		0.1	
106	10.0	7.6		0.3	
	Average	7.3	±	0.2	5.91
90	20.0	6.7		0.0	
99	20.0	6.3		0.4	
104	20.0	6.9		0.2	
109	20.0	6.9		0.2	
	Average	6.7	±	0.2	11.0
88	30.0	5.8		0.2	
98	30.0	5.5		0.5	
103	30.0	6.5		0.5	
108	30.0	6.0		0.0	
	Average	6.0	±	0.3	15.0

TABLE XVI

K_s, V_{max}, and k_3 as Functions of pH

pH	$K_s \times 10^3$ [M]	$V_{max} \times 10^3$ [M/min.]	$k_3 \times 10^3$ [M/mg. protein N/ml./min.]
6.00	47 ± 6	0.32 ± 0.04	1.54 ± 0.16
6.25	44 ± 7	0.46 ± 0.04	2.21 ± 0.20
6.50	50 ± 9	0.67 ± 0.07	3.22 ± 0.35
7.00	40 ± 4	0.74 ± 0.05	3.56 ± 0.25
7.55	58 ± 8	0.75 ± 0.08	3.60 ± 0.40
7.75	60 ± 8	0.64 ± 0.07	3.08 ± 0.35
7.95	70 ± 11	0.57 ± 0.08	2.74 ± 0.40

TABLE XVII

Calculation of K_s/V_{max} from Runs Made With Low Initial Substrate Concentrations

t = 60 minutes.

Expt. No.	$[S]_o \times 10^3$ [M]	pH	$\frac{t}{\ln[S]_o/[S]_t}$ [Min]	Approx. $V_{max} \times 10^3$ [M/min.]	$\frac{1 - [S]_o-[S]_t}{t\ V_{max}}$	$\frac{K_s}{V_{max}}$ [Min]	$\log \frac{K_s}{V_{max}}$
2	2.00	6.06	136	0.36	0.97	132	2.120
3	5.00	6.03	141	0.33	0.91	131	2.118
4	5.00	6.03	146	0.33	0.91	133	2.124
5	5.00	6.00	151	0.32	0.91	137	2.136
6	4.89	6.06	128	0.36	0.92	118	2.072
8	3.00	6.02	136	0.33	0.94	128	2.107
9	3.00	6.06	143	0.36	0.95	136	2.134
37 (a)	3.00	5.62	254	0.20 (b)	0.95	224	2.350
38	3.00	5.73	236	0.23 (b)	0.95	224	2.350
39	3.00	5.91	189	0.29	0.95	180	2.255
40	3.00	6.18	122	0.46	0.96	117	2.068
41	3.00	6.47	75	0.63	0.96	72	1.858
42	3.00	6.63	69	0.67	0.96	66	1.820
45	3.00	7.07	55	0.75	0.96	53	1.724
46	3.00	7.37	70	0.75	0.96	67	1.826
47	3.00	7.60	93	0.71	0.97	90	1.960
48	3.00	7.80	103	0.63	0.96	99	1.996
49	3.00	6.36	89	0.56	0.96	85	1.930
50	3.00	6.50	77	0.63	0.96	74	1.870
59	3.00	6.90	56	0.74	0.96	54	1.732
60	3.00	7.00	57	0.74	0.96	55	1.740
63	3.00	7.06	57	0.75	0.96	55	1.740
67	3.00	7.03	57	0.74	0.96	55	1.740
73	3.00	5.99	151	0.32	0.95	143	2.155
78	3.00	6.53	70	0.63	0.96	67	1.826
89	3.00	7.97	136	0.55	0.97	132	2.120
100	3.00	7.98	134	0.55	0.97	130	2.114
101	3.00	7.98	130	0.55	0.97	126	2.100
105	3.00	8.00	132	0.54	0.97	128	2.108
107	5.00	8.01	134	0.53	0.94	126	2.100
110	3.00	7.56	81	0.72	0.96	78	1.892
111	3.00	7.53	81	0.72	0.96	78	1.892
112	3.00	7.57	79	0.71	0.96	76	1.882
114	3.00	7.56	79	0.72	0.96	76	1.882
120	5.00	7.54	80	0.72	0.94	75	1.875
121	5.00	7.53	81	0.72	0.94	76	1.882
123	5.00	7.54	81	0.72	0.94	76	1.882
124	5.00	7.51	79	0.73	0.94	74	1.870

(a) very inaccurate.

(b) extrapolated.

TABLE XVIII

The Effect of Ionic Strength and Calcium Ion on the Alpha-chymotrypsin-catalyzed Hydrolysis of L-tyrosinhydroxamide

The ionic strength attributed to the buffer is calculated on the basis of an acid constant of 10^{-6} for cacodylic acid.

Expt No.	$[S]_o \times 10^3$ [M]	pH	Added Substance	Ionic Strength from Added Substance	Ionic Strength from Buffer	Total Ionic Strength*	$1/60 \times \ln[S]_o/[S]_t \times 10^3$ $[Min.]^{-1}$
69	10.0	7.00	0.02f $MgCl_2$	0.06	0.09	0.15	16.4
70	10.0	7.00	0.02f $CaCl_2$	0.06	0.09	0.15	18.3
71	10.0	7.00			0.09	0.09	15.5
92	10.0	5.91	0.06f NaCl	0.06	0.05	0.10	5.50
93	10.0	5.89	0.02f $CaCl_2$	0.06	0.05	0.10	6.33
94	10.0	5.94	0.02f $MgCl_2$	0.06	0.05	0.10	5.21
95	10.0	5.99			0.05	0.05	5.39

* $MgSO_4$ present in the enzyme solution may cause significant increases in these values.

TABLE XIX

Results of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide at pH 6.2. Cacodylic Acid Buffer System.

Expt. No.	$[S]_o \times 10^3$ [M]	$v_o \times 10^5$ [M/min.]	$[S]_o/v_o$ [Min.]
9[b]	15.0	9.54	159
10	5.00	3.36	148
11	70.0	27.2	258
12	30.0	16.1	187
76	4.78	3.38	141
80	70.0	28.0	250
84	39.7	19.6	204
94[a]	40.0	19.7	203
93[b]	4.78	3.58	134
104	16.0	9.64	166
109	40.0	19.0	211
112	4.80	3.42	140

TABLE XX

Results of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide in the Presence of 45×10^{-3} M Acetyl-L-tyrosine at pH 6.20.

Cacodylic Acid Buffer System.

Expt. No.	$[S]_o \times 10^3$ [M]	$v_o \times 10^5$ [M/min.]	$1/[S]_o \times 10^2$ $[M]^{-1}$	$1/v_o \times 10^{-4}$ [Min./M]
107	70.8	11.3	1.41	0.885
108	40.0	7.29	2.50	1.372
110	16.0	3.38	6.25	2.98
111	4.80	1.09	20.80	9.17

TABLE XXI

Results of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide in the Presence of 2.46×10^{-3} M Acetyl-D-tryptophanamide at pH 6.20.

Cacodylic Acid Buffer System.

Expt. No.	$[S]_o \times 10^3$ [M]	$v_o \times 10^5$ [M/min.]	$1/[S]_o \times 10^2$ $[M]^{-1}$	$1/v_o \times 10^{-4}$ [Min./M]
78	4.78	1.59	20.9	6.30
79	15.94	5.02	6.26	1.99
81	66.4	14.4	11.51	0.70
82	39.8	10.0	2.52	1.00
83	70.0	15.3	1.43	0.65
85	4.81	1.52	20.8	6.58

TABLE XXII

Results of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide in the Presence of 30×10^{-3} M Acetyl-L-tyrosinamide at pH 6.20.

Cacodylic Acid Buffer System.

Expt. No.	$[S]_o \times 10^3$ [M]	$v_o \times 10^5$ [M/min.]	$1/[S]_o \times 10^2$ $[M]^{-1}$	$1/v_o \times 10^{-4}$ [Min./M]
92	70.0	20.2	1.43	0.50
93[a]	40.0	14.4	2.50	0.69
95[a]	4.78	2.37	20.9	4.22
96[a]	15.91	7.04	6.3	1.42

TABLE XXIII

Results of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide at pH 6.60. Cacodylic Acid Buffer System.

Expt. No.	$[S]_o \times 10^3$ [M]	$v_o \times 10^5$ [M/min.]	$[S]_o/v_o$ [Min.]
46[b]	70.0	46.4	150
47[b]	40.0	36.7	109
48[b]	4.80	6.74	71.4
49	16.0	19.5	82
50	4.80	6.70	71.7
51	4.80	6.64	72.3
52	70.1	50.0	140
53	70.1	49.2	142

TABLE XXIV

Results of Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide at pH 6.95. Cacodylic Acid Buffer System.

Expt. No.	$[S]_o \times 10^3$ [M]	$v_o \times 10^5$ [M/min.]	$[S]_o/v_o$ [Min.]
54	70.4	63.3	111
55	39.8	48.8	81.6
56	4.78	10.0	47.8
57	15.95	27.6	57.6

TABLE XXV

Results of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide at pH 7.12.

Phosphate Buffer System.

Expt. No.	$[S]_o \times 10^3$ [M]	$v_o \times 10^5$ [M/min.]	$[S]_o/v_o$ [Min.]
66	15.95	31.8	50
67	4.78	11.9	40
68	69.8	68.6	102
69	39.9	61.1	65

TABLE XXVI

Results of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide at pH 7.20.

Tris-(hydroxymethyl)-aminomethane-hydrochloric Acid Buffer System.

Expt. No.	$[S]_o \times 10^3$ [M]	$v_o \times 10^5$ [M/min.]	$[S]_o/v_o$ [Min.]
58	39.8	66.2	60.1
59	70.0	79.2	88.4
60	4.78	14.0	34.1
61	15.92	38.2	41.6

TABLE XXVII

Results of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide at pH 7.15.

Hydroxylamine-hydrochloric Acid Buffer System.

Expt. No.	$[S]_o \times 10^3$ [M]	$v_o \times 10^5$ [M/min.]	$[S]_o/v_o$ [Min.]
62	16.0	29.8	53.6
63	4.80	10.8	44.2
64	40.0	53.7	74.5
65	70.2	66.4	106

TABLE XXVIII

Results of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide at pH 7.30.

Ethylenediamine-hydrochloric Acid Buffer System.

Expt. No.	$[S]_o \times 10^3$ [M]	$v_o \times 10^5$ [M/min.]	$[S]_o/v_o$ [Min.]
70	15.92	22.6	44.3
71	4.77	13.4	35.5
72	39.8	65.0	61.1
73	70.0	78.4	89.3

TABLE XXIX

Results of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide at pH 7.60 Tris-(hydroxymethyl)-aminomethane-hydrochloric Acid Buffer System.

Expt. No.	$[S]_o \times 10^3$ [M]	$v_o \times 10^5$ [M/min.]	$[S]_o/v_o$ [Min.]
5	70.0	86.3	81.0
6	3.00	8.77	34.2
7	10.0	25.2	39.6
8	30.0	55.5	54.0
9[a]	50.0	72.0	69.5
39	10.0	24.3	41.1

TABLE XXX

Results of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide in the Presence of 0.04 M Calcium Ion at pH 7.60. Tris-(hydroxymethyl)-aminomethane-hydrochloric Acid Buffer System.

Expt. No.	$[S]_o \times 10^3$ [M]	$v_o \times 10^5$ [M/min.]	$[S]_o/v_o$ [Min.]
13	5.21	18.0	29.0
14	16.79	47.5	35.2
15	70.44	101.0	69.6
16	39.84	82.0	48.5

TABLE XXXI

Results of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide in the Presence of 55.4×10^{-3} M L-tyrosinamide at pH 7.60. Tris-(hydroxymethyl)-aminomethane-hydrochloric Acid Buffer System.

Expt. No.	$[S]_o \times 10^3$ [M]	$v_o \times 10^5$ [M/min.]	$1/[S]_o \times 10^2$ $[M]^{-1}$	$1/v_o \times 10^{-4}$ [Min./M]
132	4.80	2.31	20.8	4.33
133	16.00	7.60	6.25	1.315
134	70.0	25.0	1.43	0.40
135	40.0	16.9	2.50	0.59

TABLE XXXII

Results of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide at pH 7.70. Tris-(hydroxymethyl)-aminomethane-hydrochloric Acid Buffer System.

Expt. No.	$[S]_o \times 10^3$ [M]	$v_o \times 10^5$ [M/min.]	$[S]_o/v_o$ [Min.]
1	10.0	22.8	43.8
2	3.00	7.81	38.3
3	30.0	52.9	56.6
4	50.0	71.5	70.0
29	5.00	13.7	36.5

TABLE XXXIII

Results of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide at pH 7.88.

Tris-(hydroxymethyl)-aminomethane-hydrochloric Acid Buffer System.

Expt. No.	$[S]_o \times 10^3$ [M]	$v_o \times 10^5$ [M/min.]	$[S]_o/v_o$ [Min.]
45	15.98	31.0	51.6
46[a]	4.79	11.2	42.7
47[a]	70.0	73.1	95.6
48[a]	40.0	57.4	69.5

TABLE XXXIV

The Inhibition of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide by Varying Concentrations of Acetyl-L-tyrosinamide.

$[S]_o = 16 \times 10^{-3}$ throughout.

$K_s = 50$ in the calculation of the quantities in the last column (see Table XL).

pH = 7.83.

Tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer system.

Expt. No.	$[I] \times 10^3$ [M]	$v_o \times 10^5$ [M/min.]	$[I]v_oK_s/(K_s + [S]_o) \times 10^6$ [M^2/min.]
91[b]	50.0	21.2	8.03
100[a]	30.1	26.6	6.04
101	20.0	29.8	4.51
102	5.0	37.5	1.42
103	10.0	34.5	2.61
100[b]	0.0	41.1	0.00

TABLE XXXV

Results of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide at about pH 8.20.

Tris-(hydroxymethyl)-aminomethane-hydrochloric Acid Buffer System.

Expt. No.	$[S]_o \times 10^3$ [M]	pH	$v_o \times 10^5$ [M/min.]	$[S]_o/v_o$ [Min.]	$[S]_o/v_o$ at pH 8.20
27	5.00	8.16	10.25	48.8	51
41	39.6	8.20	52.2	76.0	76
42	70.2	8.18	69.5	101	102
43	4.75	8.22	9.06	52.3	52
44	15.84	8.21	26.5	60.0	60

TABLE XXXVI

Results of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide at about pH 8.60.

Tris-(hydroxymethyl)-aminomethane-hydrochloric Acid Buffer System.

Expt. No.	$[S]_o \times 10^3$ [M]	pH	$v_o \times 10^5$ [M/min.]	$[S]_o/v_o$ [Min.]	$[S]_o/v_o$ at pH 8.60
30	5.00	8.70	5.02	99.5	85
31	15.87	8.64	16.7	95.0	90
32	4.76	8.67	5.16	92.1	83
33	39.7	8.63	34.6	115.0	110
34	65.8	8.61	49.6	132	130
35	15.8	8.58	18.3	86.6	89
36	4.75	8.61	5.57	85.3	83
37	70.7	8.60	53.9	131	131
38	39.6	8.61	34.7	114.1	112
88	4.80	8.60	6.35	75.6	75.6
91	70.3	8.59	55.1	127	128
96[b]	4.81	8.60	5.31	90.5	90.5
99[a]	70.0	8.59	55.4	126	127
115	70.0	8.59	55.4	126	127
117	4.82	8.60	6.68	72.3	72.3

TABLE XXXVII

Inhibition of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide by Acetyl-D-tryptophanamide at pH 8.60.

The concentration of acetyl-D-tryptophanamide in the inhibited runs is 2.4×10^{-3} M.
The value for K_s used in calculating K_p is 102×10^{-3} M.
Tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer system.

Inhibited Runs			Control Runs			
Expt. No.	$[S]_o \times 10^3$ [M]	$v_o \times 10^5$ [M/min.]	Expt. No.	$[S]_o \times 10^3$ [M]	$v_o \times 10^5$ [M/min.]	$K_I \times 10^3$ [M]
86	4.79	3.12	88	4.80	6.35	2.3
87	15.98	10.3	88	4.80	6.35	2.5
89	39.9	22.2	91	70.3	55.0	2.5
90	70.0	34.4	91	70.3	55.0	2.4

Average 2.4×10^{-3} M.

TABLE XXXVIII

Inhibition of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide by Acetyl-L-tyrosinamide at pH 8.60.

The concentration of acetyl-L-tyrosinamide in the inhibited runs is 30×10^{-3} M.
The value for K_s used in calculating K_p is 102×10^{-3} M.
Tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer system.

Inhibited Runs			Control Runs			
Expt. No.	$[S]_o \times 10^3$ [M]	$v_o \times 10^5$ [M/min.]	Expt. No.	$[S]_o \times 10^3$ [M]	$v_o \times 10^5$ [M/min.]	$K_I \times 10^3$ [M]
94b	4.81	3.70	96b	4.81	5.31	65
95b	16.0	11.8	96b	4.81	5.31	72
97	70.3	43.7	99a	70.0	55.4	65
98	40.0	29.6	99a	70.0	55.4	72

Average 68×10^{-3} M.

TABLE XXXIX

Results of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide at about pH 9.0

Tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer system.

Expt. No.	$[S]_o \times 10^3$ [M]	pH [Initial]	$v_o \times 10^5$ [M/min.]
19	39.8	8.90	22.8
20	69.0	8.81	40.0
23	40.0	8.90	42.5
24	69.4	8.81	42.5
25	16.0	9.03	8.53
26	4.80	9.10	2.24
28	5.00	9.05	2.37

TABLE XL

Summary of K_S and V_{max} Values Obtained From the Studies of the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide.

Buffers are indicated as follows:

C = Cacodylic acid -- NaOH
T = Tris-(hydroxymethyl)-aminomethane -- HCl
P = Phosphoric acid -- KOH
E = Ethylenediamine -- HCl
H = Hydroxylamine -- HCl (+ NaCl)

pH	Buffer	$V_{max} \times 10^3$ [M/min.]	$K_S \times 10^3$ [M]	K_S/V_{max} [Min.]	$k_3 \times 10^3$ M/mg. protein nitrogen/ ml./min.
6.2	C	0.56 ± 0.06	75 ± 7	134	14.0
6.6	C	0.91 ± 0.07	59 ± 5	66	22.7
6.95	C	1.03 ± 0.07	44 ± 4	43	25.7
7.12	P	1.18 ± 0.09	42 ± 4	35.5	29.5
7.15	H	1.06 ± 0.08	41 ± 5	38.7	26.5
7.20	T	1.21 ± 0.10	35 ± 6	28.9	30.2
7.30	E	1.25 ± 0.06	39 ± 4	31.2	31.2
7.60	T	1.41 ± 0.05	47 ± 3	33.3	35.2
7.60	T	1.36 ± 0.08(a)	42 ± 2(b)	30.8(a)	34 ± 2(b)
7.60	T	1.28 ± 0.12(a)	45 ± 5(c)	35.0(a)	32 ± 3(c)
7.60	T + $CaCl_2$	1.61 ± 0.11	40 ± 5		40.2
7.70	T	1.43 ± 0.11	50 ± 6	35.0	35.8
7.88	T	1.27 ± 0.08(d)	50 ± 5	39.4	31.8
8.20	T	1.32 ± 0.08	62 ± 5	47.0	33.0
8.60	T	1.30 ± 0.10	102 ± 15	78.5	32.5

(a) Calculated from $V_{max} = k_3[E]_o$ for $[E]_o$ = 0.04 mg. protein nitrogen/ml.

(b) Reference (19).

(c) From experimental data obtained by Hogness (14), recalculated by Foster (20).

(d) Probably low activity of enzyme preparation.

TABLE XLI

Calculation of Values of K_s/V_{max} for Runs Made at Low Substrate Concentrations. V_{max} values from Figure 42.

Expt. No.	$[S]_o \times 10^3$ [M]	pH	$v_o \times 10^5$ [M/min.]	$[S]_o/v_o$ [Min.]	$V_{max} \times 10^3$ [M/min.]	$[S]_o/V_{max}$ [Min.]	K_s/V_{max} [Min.]
10	5.00	6.20	3.36	148	0.56	9	139
76	4.78	6.20	3.38	141	0.56	9	132
93b	4.78	6.20	3.58	134	0.56	9	125
112	4.80	6.20	3.42	140	0.56	9	131
48b	4.80	6.60	6.74	71	0.90	5	66
50	4.80	6.60	6.70	72	0.90	5	67
51	4.80	6.60	6.64	72	0.90	5	67
56	4.78	6.95	10.0	48	1.08	4	44
67	4.78	7.12	11.9	40	1.18	4	36
60	4.78	7.20	14.0	34	1.21	4	30
71	4.77	7.30	13.4	36	1.25	4	32
6	3.00	7.60	8.77	34	1.27	2	32
2	3.00	7.70	7.81	38	1.37	2	36
29	5.00	7.70	13.7	37	1.37	4	33
46a	4.79	7.88	11.2	43	1.37	3	40
27	5.00	8.16	10.3	49	1.33	4	45
43	4.75	8.22	9.1	52	1.33	4	48
30	5.00	8.70	5.02	100	1.28	4	96
32	4.76	8.67	5.16	92	1.28	4	88
36	4.75	8.61	5.57	85	1.29	4	81
88	4.80	8.60	6.35	76	1.29	4	72
96b	4.81	8.60	5.31	91	1.29	4	87
117	4.82	8.60	6.68	72	1.29	4	68
26	4.80	9.10	2.24	214	1.23	4	210
28	5.00	9.05	2.37	211	1.24	4	207

TABLE XLII

The Variation in the Inhibition Constant of Acetyl-L-tyrosine With pH.

Expt. No.	$[S]_o \times 10^3$ [M]	$[I] \times 10^3$ [M]	pH	$v_o \times 10^5$ [M/min.]	$K_s \times 10^3$ [M]	$V_{max} \times 10^3$ [M/min.]	$K_I \times 10^3$ [M]
			6.20				20(a)
105	16.0	45	6.30	3.54	70	0.66	15
123	4.80	90	6.57	1.35	58	0.87	21
124	4.80	90	6.81	2.22	49	1.01	27
125	4.80	90	7.30	5.17	40	1.25	51
126	4.80	90	7.60	7.05	43	1.36	87
127	4.80	90	8.26	6.54	68	1.32	250
			7.60				90(b)
			7.90				115(c)

(a) From data presented in Table XX and Figure 25.

(b) Reported by Foster (19); substrate acetyl-L-tyrosinhydroxamide.

(c) Reported by Thomas et al (21); substrate acetyl-L-tyrosinamide.

TABLE XLIII

The Variation in the Inhibition Constant of L-tyrosinamide With pH.

Expt. No.	$[S]_o \times 10^3$ [M]	$[I] \times 10^3$ [M]	pH	$v_o \times 10^5$ [M/min.]	$K_s \times 10^3$ [M]	$V_{max} \times 10^3$ [M/min.]	$K_I \times 10^3$ [M]
128	4.80	55.4	6.20	1.50	75	0.56	42
129	4.80	55.4	6.90	3.11	46	1.06	23
			7.60				11(a)
130	4.80	55.4	8.20	1.59	65	1.33	11
131	4.80	55.4	8.60	1.04	98	1.29	11

(a) From data presented in Table XXXI and Figure 34.

TABLE XLIV

Variation in the Inhibition Constant of Acetyl-D-trypto-phanamide With pH.

pH	$K_I \times 10^3$ [M]	Reference	Substrate
6.2	2.0(a)	---	acetyl-L-tyrosin-hydroxamide
6.9	2.0	(13)	L-tyrosinhydroxamide
7.9	2.7	(11)	acetyl-L-trypto-phanamide
8.6	2.4(b)	---	acetyl-L-tyrosin-hydroxamide

(a) From data presented in Figure 25 and Table XXI.

(b) From data presented in Table XXXVII.

TABLE XLV

Variation in the Apparent Inhibition Constant of Acetyl-L-tyrosinamide With pH.

pH	$K_I \times 10^3$ [M]	Reference
6.2	67(a)	---
7.83	41(b)	---
7.90	32(c)	(21, 20)
8.6	68(d)	---

(a) From data presented in Table XXII and Figure 25.

(b) From data presented in Table XXXIV and Figure 38.

(c) K_s obtained from kinetic studies of the chymotrypsin-catalyzed hydrodysis.

(d) From data presented in Table XXXVIII.

TABLE XLVI

Ionization Constants of Hydroxamic Acids.

Acid	Group	pK_A
Acethydroxamide	NHOH	9.4
Glycinhydroxamide	NHOH	9.4
Glycinhydroxamide	NH_3^+	7.4
L-tyrosinhydroxamide	NHOH	9.2
L-tyrosinhydroxamide	NH_3^+	7.0
L-tyrosinhydroxamide	OH	10.0
acetyl-L-tyrosinhydroxamide	NHOH	9.0
acetyl-L-tyrosinhydroxamide	OH	10.2

TABLE XLVII

Determination of K_c for Acetyl-L-tyrosinhydroxamide.

pH	h	$V_{max} \times 10^3$ [M/min.]	$V_{max}h \times 10^{12}$
6.20	6.3×10^{-7}	0.56	353
6.60	2.51×10^{-7}	0.91	228
6.95	1.12×10^{-7}	1.03	115
7.12	7.6×10^{-8}	1.18	90
7.20	6.3×10^{-8}	1.21	76
7.30	5.0×10^{-8}	1.25	63
7.60	2.51×10^{-8}	1.41	35
7.60	2.51×10^{-8}	1.36	34(a)
7.60	2.51×10^{-8}	1.28	33(b)
7.70	2.00×10^{-8}	1.43	29
8.20	6.3×10^{-9}	1.32	8
8.60	2.51×10^{-9}	1.30	3

(a) Reference (19).

(b) Reference (14, 20).

TABLE XLVIII

Calculation of K_a and K_b. pH Minimum = 7.7.

pH	h	K_s/V_{max} [Table XLI]	$\frac{1}{1+K'/h}$ $K'=10^{-9}$	$\frac{K_s/V_{max}}{1+K'/h}$	$\frac{f[pH]}{h_o = 2 \times 10^{-8}}$
6.20	6.31×10^{-7}	139	1.0	139	31.5
6.20	6.31×10^{-7}	132	1.0	132	31.5
6.20	6.31×10^{-7}	125	1.0	125	31.5
6.20	6.31×10^{-7}	131	1.0	131	31.5
6.60	2.51×10^{-7}	66	1.0	66	12.7
6.60	2.51×10^{-7}	67	1.0	67	12.7
6.60	2.51×10^{-7}	67	1.0	67	12.7
6.95	1.12×10^{-7}	44	1.0	44	5.78
7.12	7.59×10^{-8}	36	0.986	36	4.06
7.20	6.31×10^{-8}	30	0.985	30	3.48
7.30	5.01×10^{-8}	32	0.980	31	2.90
7.60	2.51×10^{-8}	32	0.960	31	2.06
7.70	2.00×10^{-8}	36	0.950	34	2.00
7.70	2.00×10^{-8}	33	0.950	31	2.00
7.88	1.32×10^{-8}	40?	0.930	37	2.17
8.16	6.92×10^{-9}	45	0.874	39	3.24
8.22	6.04×10^{-9}	48	0.859	41	3.61
8.70	2.00×10^{-9}	96	0.667	64	10.1
8.67	2.14×10^{-9}	88	0.682	60	9.56
8.61	2.46×10^{-9}	81	0.711	58	9.37
8.60	2.51×10^{-9}	72	0.714	51	9.21
8.60	2.51×10^{-9}	87	0.714	62	9.21
8.60	2.51×10^{-9}	68	0.714	49	9.21
9.10	7.95×10^{-10}	210	0.442	93	26.4
9.05	8.90×10^{-10}	207	0.470	97	22.5

TABLE XLIX

Experimental Observations for the Alpha-chymotrypsin-catalyzed Hydrolysis of L-tyrosinhydroxamide.

Enzyme concentration = 0.208 mg. protein nitrogen/ml.

Temperature = 25° C. Buffer 0.1f in organic component. The approximate amount of hydrolysis is indicated where this is important to the estimation of the median pH. The initial pH is indicated only where it is actually observed.

e = extinction observed at 505 millimicrons.

$[S]_o$ = initial substrate concentration.

Part A. Runs with cacodylic acid - sodium cacodylate buffer.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
1	10.00	2	0.965	2.267		6.03	6.03
		5	0.947	2.248			
		11	0.908	2.206			
		21	0.856	2.147			
		30	0.802	2.082			
		40	0.754	2.020			
		50	0.700	1.946			
		60	0.658	1.884			
			Interpolated Values				
		0	0.978	2.280			
		60	0.659	1.885			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
2	2.00	2	0.206	0.723		6.06	6.06
		10	0.190	0.642			
		20	0.178	0.577			
		30	0.167	0.513			
		40	0.156	0.445			
		50	0.144	0.365			
		60	0.134	0.293			
			Interpolated Values				
		0	0.208	0.732			
		60	0.134	0.292			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
3	5.00	2	0.475	1.558		6.03	6.03
		5	0.462	1.530			
		10	0.450	1.504			
		20	0.428	1.454			
		30	0.398	1.381			
		40	0.366	1.298			
		50	0.338	1.218			
		65	0.308	1.125			
		80	0.275	1.012			
			Interpolated Values				
		0	0.493	1.596			
		60	0.319	1.160			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
4	5.00	2	0.453	1.511		6.03	6.03
		5	0.448	1.500			
		10	0.430	1.459			
		20	0.403	1.394			
		30	0.372	1.314			
		42	0.348	1.247			
			Interpolated Values				
		0	0.462	1.529			
		60	0.305	1.117			

1 ml. aliquots placed in 10 ml. flasks for color reaction.
This run of doubtful value.

				ln 10e			
5	5.00	2	0.470	1.548		6.00	6.00
		5	0.460	1.526			
		10	0.448	1.500			
		20	0.414	1.421			
		30	0.390	1.361			
		40	0.362	1.287			
		50	0.342	1.230			
		60	0.323	1.173			
			Interpolated Values				
		0	0.476	1.560			
		60	0.320	1.163			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
6	4.89	2	0.460	1.526		6.06	6.06
		5	0.447	1.497			
		10	0.428	1.454			
		20	0.400	1.386			
		30	0.368	1.303			
		40	0.338	1.218			
		50	0.316	1.151			
		60	0.293	1.075			
			Interpolated Values				
		0	0.465	1.538			
		60	0.292	1.071			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
7	10	2	0.924	2.224		6.10	6.10
		5	0.906	2.204			
		10	0.877	2.171			
		20	0.810	2.092			
		30	0.752	2.018			
		40	0.702	1.949			
		52	0.640	1.856			
		60	0.606	1.802			
			Interpolated Values				
		0	0.938	2.238			
		60	0.605	1.800			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
8	3.00	5	0.262	0.963		6.02	6.02
		11	0.250	0.916			
		20	0.250	0.916			
		30	0.216	0.770			
		40	0.203	0.708			
		51	0.186	0.621			
		60	0.176	0.565			
			Interpolated Values				
		0	0.272	1.000			
		60	0.175	0.560			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
9	3.00	2	0.260	0.956		6.06	6.06
		5	0.258	0.948			
		10	0.243	0.888			
		20	0.229	0.829			
		30	0.213	0.756			
		40	0.200	0.693			
		50	0.188	0.631			
		60	0.172	0.542			
			Interpolated Values				
		0	0.266	0.972			
		60	0.174	0.551			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	$\underline{e}$	ln $10\underline{e}$	Initial pH	Final pH	Median pH [Estimated]
10	40	2	0.733	1.992		6.35	6.39
		6	0.754	2.020			
		10	0.699	1.944			
		14	0.680	1.917			
		18	0.672	1.905			
		22	0.667	1.898			
		26	0.632	1.844			
		30	0.627	1.836			
		35	0.603	1.797			
		39	0.575	1.749			
		43	0.576	1.751			
		47	0.547	1.699			
		51	0.525	1.658			
		55	0.516	1.641			
		59	0.503	1.615			
		60	0.495	1.599			
		61	0.490	1.589			
		62	0.474	1.556			
		80	0.440	1.482			
		81	0.433	1.466			
		Interpolated Values					
		0	0.742	2.040			
		60	0.496	1.600			

0.2 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 13 x 10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
11	20.0	1	0.380	1.335	6.28	6.23	6.25
		2	0.372	1.314			
		3	0.378	1.330			
		4	0.377	1.327			
		5	0.371	1.311			
		6	0.368	1.303			
		10	0.352	1.258			
		11	0.351	1.256			
		15	0.342	1.230			
		19	0.333	1.203			
		23	0.327	1.185			
		27	0.313	1.141			
		34	0.305	1.115			
		39	0.298	1.092			
		43	0.276	1.015			
		50	0.268	0.986			
		58	0.250	0.916			
		59	0.255	0.936			
		60	0.245	0.896			
		61	0.245	0.896			
		62	0.240	0.875			
			Interpolated Values				
		0	0.386	1.350			
		60	0.246	0.900			

0.2 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 7.3×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
12	40.0	1	0.770	2.041	6.22	6.17	6.19
		2	0.744	2.007			
		3	0.730	1.988			
		4	0.748	2.012			
		5	0.732	1.991			
		6	0.742	2.004			
		10	0.722	1.977			
		14	0.710	1.960			
		18	0.684	1.923			
		23	0.666	1.896			
		30	0.649	1.870			
		34	0.648	1.869			
		38	0.634	1.847			
		45	0.612	1.812			
		49	0.598	1.788			
		53	0.577	1.752			
		57	0.566	1.733			
		58	0.563	1.728			
		59	0.550	1.705			
		60	0.548	1.701			
		61	0.540	1.686			
		62	0.546	1.697			
		63	0.532	1.671			
			Interpolated Values				
		0	0.758	2.026			
		60	0.549	1.703			

0.2 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 11×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
13	40.0	1	0.770	2.041	5.88	5.88	5.88
		2	0.744	2.007			
		3	0.730	1.988			
		4	0.748	2.012			
		5	0.732	1.991			
		6	0.742	2.004			
		10	0.722	1.977			
		14	0.710	1.960			
		18	0.684	1.923			
		23	0.666	1.896			
		30	0.649	1.870			
		34	0.641	1.869			
		38	0.634	1.847			
		45	0.612	1.812			
		49	0.598	1.788			
		53	0.577	1.752			
		57	0.566	1.733			
		58	0.563	1.728			
		59	0.550	1.705			
		60	0.548	1.701			
		61	0.540	1.686			
		62	0.546	1.697			
		63	0.532	1.671			
			Interpolated Values				
		0	0.743	2.005			
		60	0.617	1.820			

0.2 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
14	40.0	1	0.738	1.999	6.10	6.04	6.07
		2	0.734	1.993			
		3	0.760	2.028			
		4	0.743	2.005			
		5	0.746	2.009			
		9	0.726	1.984			
		14	0.702	1.949			
		18	0.704	1.952			
		22	0.684	1.923			
		26	0.674	1.908			
		34	0.652	1.875			
		38	0.640	1.856			
		42	0.626	1.834			
		49	0.610	1.808			
		53	0.607	1.803			
		59	0.585	1.766			
		60	0.578	1.754			
		61	0.567	1.735			
		62	0.567	1.735			
		63	0.577	1.753			
		Interpolated Values					
		0	0.758	2.025			
		60	0.579	1.756			

0.2 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 10×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
15	40.0	1	0.762	2.031	6.62	6.52	6.57
		2	0.772	2.044			
		3	0.760	2.028			
		4	0.762	2.031			
		5	0.694	1.937			
		6	0.718	1.971			
		10	0.693	1.936			
		14	0.727	1.982			
		18	0.658	1.884			
		22	0.638	1.853			
		29	0.608	1.805			
		33	0.592	1.778			
		37	0.560	1.723			
		41	0.547	1.699			
		48	0.518	1.645			
		53	0.498	1.605			
		57	0.478	1.564			
		58	0.468	1.543			
		59	0.462	1.530			
		60	0.475	1.558			
		61	0.458	1.522			
		62	0.458	1.522			
			Interpolated Values				
		0	0.743	2.050			
		60	0.464	1.535			

0.2 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 16×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
16	40.0	1	0.740	2.001	6.80	6.66	6.73
		2	0.740	2.001			
		3	0.756	2.023			
		4	0.726	1.982			
		5	0.750	2.015			
		8	0.716	1.968			
		12	0.677	1.912			
		16	0.665	1.895			
		20	0.624	1.831			
		27	0.594	1.782			
		31	0.575	1.749			
		35	0.565	1.732			
		39	0.544	1.694			
		43	0.522	1.652			
		49	0.485	1.579			
		53	0.476	1.560			
		57	0.443	1.488			
		58	0.432	1.463			
		59	0.432	1.463			
		60	0.420	1.435			
		61	0.412	1.416			
		Interpolated Values					
		0	0.757	2.024			
		60	0.421	1.438			

0.2 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 18×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
22	10.0	2	0.952	2.253		5.76	5.76
		5	0.943	2.244			
		10	0.913	2.212			
		20	0.877	2.171			
		30	0.858	2.149			
		40	0.832	2.119			
		50	0.805	2.086			
		60	0.790	2.067			
			Interpolated Values				
		0	0.946	2.247			
		60	0.749	2.013			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
23	10.0	2	0.952	2.253		5.87	5.87
		5	0.922	2.221			
		10	0.906	2.204			
		20	0.872	2.166			
		30	0.847	2.137			
		40	0.807	2.088			
		50	0.776	2.049			
		60	0.748	2.012			
			Interpolated Values				
		0	0.946	2.247			
		60	0.749	2.013			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
24	10.0	2	0.960	2.262		6.00	6.00
		5	0.930	2.230			
		10	0.912	2.211			
		20	0.872	2.166			
		30	0.822	2.107			
		40	0.780	2.054			
		50	0.740	2.002			
		60	0.702	1.949			
			Interpolated Values				
		0	0.964	2.266			
		60	0.702	1.949			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
25	10.0	2	0.948	2.249		6.27	6.28
		5	0.926	2.226			
		10	0.882	2.177			
		20	0.813	2.096			
		30	0.752	2.018			
		40	0.694	1.937			
		50	0.642	1.859			
		60	0.591	1.777			
			Interpolated Values				
		0	0.961	2.263			
		60	0.592	1.778			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
26	10.0	2	0.950	2.251		6.54	6.55
		5	0.918	2.217			
		12	0.848	2.138			
		20	0.771	2.043			
		30	0.688	1.929			
		40	0.618	1.821			
		50	0.551	1.707			
		60	0.493	1.595			
			Interpolated Values				
		0	0.970	2.272			
		60	0.493	1.595			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 4.9×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
				ln 10e			
27	10	3	0.926	2.226		6.70	6.72
		5	0.908	2.206			
		12	0.828	2.114			
		22	0.718	1.971			
		32	0.632	1.844			
		42	0.544	1.694			
		52	0.482	1.573			
		62	0.420	1.435			
			Interpolated Values				
		0	0.965	2.267			
		60	0.432	1.462			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 5.5×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
28	10.0	2	0.958	2.260		6.92	6.94
		5	0.925	2.225			
		10	0.858	2.149			
		20	0.742	2.004			
		30	0.638	1.853			
		40	0.545	1.696			
		50	0.470	1.548			
		60	0.407	1.404			
			Interpolated Values				
		0	0.998	2.300			
		60	0.403	1.393			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 6×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
29	10.0	2	0.948	2.249		6.82	6.84
		5	0.900	2.197			
		10	0.838	2.126			
		20	0.733	1.992			
		30	0.630	1.841			
		40	0.542	1.690			
		50	0.468	1.543			
		60	0.410	1.411			
			Interpolated Values				
		0	0.970	2.272			
		60	0.410	1.410			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 5.8×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
32	10.0	2	0.960	2.262		7.22	7.24
		5	0.932	2.232			
		10	0.868	2.161			
		20	0.752	2.018			
		30	0.658	1.884			
		40	0.567	1.735			
		50	0.490	1.589			
		60	0.426	1.449			
			Interpolated Values				
		0	0.998	2.300			
		60	0.427	1.451			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 5.7×10^{-3} M.

				ln 10e			
37	3.00	2	0.286	1.051		5.62	5.62
		5	0.284	1.044			
		10	0.276	1.015			
		20	0.264	0.971			
		31	0.253	0.928			
		40	0.249	0.912			
		50	0.242	0.884			
		60	0.233	0.846			
			Interpolated* Values				
		0	0.289	1.060			
		60	0.228	0.824			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

* These values obtained by considering this run and run 38 together. Very poor accuracy is the result of the small amount of hydrolysis at this low pH.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
38	3.00	2	0.293	1.075		5.73	5.73
		5	0.279	1.026			
		10	0.278	1.023			
		20	0.266	0.978			
		30	0.255	0.936			
		40	0.243	0.888			
		50	0.238	0.867			
		60	0.236	0.859			
			Interpolated* Values				
		0	0.289	1.060			
		60	0.235	0.805			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

* Same considerations apply to this run as to run 37.

				ln 10e			
39	3.00	2	0.280	1.030		5.91	5.91
		5	0.277	1.019			
		10	0.267	0.982			
		20	0.257	0.944			
		30	0.240	0.876			
		40	0.230	0.833			
		50	0.218	0.779			
		60	0.207	0.728			
			Interpolated Values				
		0	0.284	1.045			
		60	0.207	0.727			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
40	3.00	2	0.280	1.030		6.18	6.18
		5	0.274	1.008			
		10	0.263	0.967			
		20	0.243	0.888			
		30	0.222	0.798			
		40	0.204	0.713			
		50	0.189	0.637			
		60	0.174	0.554			
			Interpolated Values				
		0	0.284	1.045			
		60	0.174	0.552			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
41	3.00	2	0.273	1.004		6.47	6.47
		5	0.260	0.956			
		10	0.245	0.896			
		20	0.217	0.775			
		30	0.186	0.621			
		40	0.163	0.489			
		50	0.140	0.337			
		60	0.128	0.247			
			Interpolated Values				
		0	0.280	1.030			
		60	0.125	0.223			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
42	3.00	2	0.272	1.001		6.63	6.63
		5	0.254	0.932			
		10	0.237	0.863			
		20	0.207	0.728			
		30	0.175	0.560			
		40	0.150	0.406			
		50	0.132	0.278			
		60	0.118	0.166			
			Interpolated Values				
		0	0.277	1.018			
		60	0.114	0.132			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
43	3.00	2	0.262	0.963		6.88	6.88
		5	0.248	0.908			
		10	0.227	0.820			
		20	0.192	0.652			
		30	0.163	0.489			
		40	0.138	0.322			
		50	0.120	0.182			
		60	0.102	0.020			
			Interpolated Values				
		0	0.268	0.986			
		60	0.101	0.010			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
44	3.00	2	0.260	0.956		7.14	7.14
		5	0.247	0.904			
		10	0.228	0.824			
		20	0.195	0.668			
		30	0.162	0.482			
		40	0.140	0.337			
		50	0.118	0.166			
		60	0.102	0.020			
			Interpolated Values				
		0	0.268	0.987			
		60	0.101	0.013			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
49	3.00	2	0.270	0.993		6.36	6.36
		5	0.262	0.963			
		10	0.250	0.916			
		20	0.220	0.788			
		30	0.200	0.693			
		40	0.180	0.588			
		50	0.158	0.457			
		60	0.138	0.322			
			Interpolated Values				
		0	0.277	1.018			
		60	0.141	0.347			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
50	3.00	2	0.272	1.006		6.50	6.50
		5	0.256	0.940			
		10	0.243	0.888			
		20	0.217	0.775			
		30	0.190	0.642			
		40	0.164	0.495			
		50	0.145	0.372			
			Interpolated Values				
		0	0.278	1.023			
		60	0.128	0.243			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
57	10.00	2	0.930	2.230		6.94	6.96
		5	0.890	2.186			
		10	0.822	2.107			
		20	0.704	1.952			
		30	0.606	1.802			
		40	0.523	1.654			
		50	0.443	1.488			
		60	0.380	1.335			
			Interpolated Values				
		0	0.955	2.257			
		60	0.381	1.338			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 6×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
58	10.0	2	0.907	2.205		7.08	7.10
		5	0.870	2.163			
		10	0.805	2.086			
		20	0.690	1.931			
		30	0.595	1.783			
		40	0.510	1.629			
		50	0.432	1.463			
		60	0.372	1.314			
			Interpolated Values				
		0	0.936	2.237			
		60	0.374	1.318			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 6×10^{-3} M.

				ln 10e			
59	3.00	2	0.276	1.015		6.90	6.90
		5	0.262	0.963			
		10	0.238	0.867			
		20	0.207	0.728			
		30	0.170	0.531			
		40	0.140	0.336			
		50	0.118	0.166			
		60	0.098	-0.020			
			Interpolated Values				
		0	0.289	1.060			
		60	0.098	-0.020			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
60	3.00	2	0.278	1.022		7.00	7.00
		5	0.263	0.967			
		10	0.243	0.888			
		20	0.202	0.703			
		30	0.171	0.536			
		40	0.144	0.365			
		50	0.119	0.174			
		60	0.098	-0.020			
			Interpolated Values				
		0	0.289	1.060			
		60	0.100	0.002			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
61	40.0	1	0.700	1.946	7.12	6.98	7.05
		2	0.693	1.936			
		3	0.695	1.939			
		4	0.685	1.924			
		5	0.670	1.902			
		8	0.657	1.883			
		11	0.651	1.873			
		15	0.618	1.821			
		18	0.600	1.792			
		22	0.570	1.740			
		26	0.552	1.708			
		29	0.550	1.705			
		32	0.523	1.654			
		37	0.502	1.613			
		40	0.480	1.569			
		45	0.447	1.497			
		49	0.430	1.459			
		53	0.420	1.435			
		57	0.408	1.406			
		58	0.393	1.369			
		59	0.389	1.358			
		60	0.378	1.330			
		61	0.378	1.330			
			Interpolated Values				
		0	0.710	1.960			
		60	0.382	1.341			

0.2 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 18×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
62	10.0	2	0.918	2.217		7.00	7.02
		5	0.910	2.208			
		10	0.813	2.096			
		20	0.690	1.932			
		30	0.598	1.788			
		40	0.505	1.619			
		50	0.433	1.466			
		60	0.370	1.308			
			Interpolated Values				
		0	0.949	2.250			
		60	0.370	1.308			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 6×10^{-3} M.

				ln 10e			
63	3.00	2	0.275	1.012		7.06	7.06
		5	0.253	0.928			
		10	0.238	0.867			
		20	0.200	0.693			
		30	0.167	0.513			
		40	0.138	0.322			
		50	0.120	0.182			
		60	0.098	-0.020			
			Interpolated Values				
		0	0.282	1.036			
		60	0.113	0.012			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
64	40.0	1	0.692	1.934	7.03	6.92	6.97
		2	0.678	1.914			
		3	0.690	1.932			
		4	0.685	1.924			
		5	0.678	1.914			
		60	0.363	1.289			
		61	0.368	1.303			
		62	0.342	1.230			
		63	0.350	1.253			
			Interpolated Values				
		0	0.705	1.953			
		60	0.366	1.297			

0.2 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 19 x 10^{-3} M.

				ln 10e			
65	10.00	1	0.918	2.217		7.02	7.04
		2	0.913	2.212			
		3	0.900	2.197			
		4	0.885	2.180			
		60	0.368	1.303			
		61	0.366	1.297			
		62	0.357	1.273			
		63	0.352	1.258			
			Interpolated Values				
		0	0.939	2.240			
		60	0.369	1.306			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 6 x 10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
66	30.0	1	0.503	1.615	7.12	6.98	7.05
		2	0.502	1.613			
		3	0.500	1.609			
		4	0.490	1.589			
		5	0.488	1.585			
		60	0.242	0.884			
		61	0.243	0.888			
		62	0.243	0.888			
		63	0.240	0.875			
		64	0.237	0.863			
			Interpolated Values				
		0	0.511	1.631			
		60	0.251	0.919			

0.2 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 15×10^{-3} M.

				ln 10e			
67	3.00	1	0.266	0.978		7.03	7.03
		2	0.266	0.978			
		3	0.258	0.948			
		4	0.253	0.928			
		60	0.094	-0.062			
		61	0.094	-0.062			
		62	0.092	-0.083			
		63	0.090	-0.105			
			Interpolated Values				
		0	0.273	1.003			
		60	0.095	-0.050			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
68	20.0	1	1.820	2.902		7.03	7.07
		2	1.788	2.884			
		3	1.765	2.871			
		4	1.758	2.867			
		60	0.790	2.067			
		61	0.790	2.067			
		62	0.778	2.052			
		63	0.773	2.045			
			Interpolated Values				
		0	1.843	2.915			
		60	0.801	2.081			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 11.3×10^{-3} M.

				ln 10e			
71	10.0	1	0.978	2.280		7.02	7.04
		2	0.963	2.265			
		3	0.950	2.251			
		4	0.930	2.230			
		60	0.392	1.366			
		61	0.386	1.351			
		62	0.379	1.332			
		63	0.373	1.316			
			Interpolated Values				
		0	0.989	2.292			
		60	0.391	1.363			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 6×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	$\underline{e}$	ln $\underline{e}$	Initial pH	Final pH	Median pH [Estimated]
72	20.0	1	2.000	0.693		5.99	5.99
		2	1.990	0.688			
		3	1.960	0.673			
		4	1.957	0.672			
		60	1.478	0.391			
		61	1.468	0.384			
		62	1.453	0.374			
		63	1.438	0.364			
			Interpolated Values				
		0	2.006	0.696			
		60	1.473	0.387			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10$\underline{e}$			
73	3.00	1	0.297	1.089		5.99	5.99
		2	0.292	1.072			
		3	0.290	1.065			
		4	0.287	1.054			
		60	0.200	0.693			
		61	0.196	0.673			
		62	0.196	0.673			
		63	0.195	0.668			
			Interpolated Values				
		0	0.296	1.085			
		60	0.199	0.687			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
74	10.0	1	0.985	2.287		5.98	5.98
		2	0.975	2.277			
		3	0.970	2.272			
		4	0.968	2.270			
		60	0.705	1.953			
		61	0.708	1.957			
		62	0.701	1.947			
		63	0.700	1.946			
			Interpolated Values				
		0	0.989	2.292			
		60	0.709	1.058			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
75	40.0	1	0.737	1.997	6.02	6.01	6.01
		2	0.761	2.029			
		3	0.740	2.001			
		4	0.742	2.004			
		5	0.748	2.012			
		60	0.605	1.800			
		61	0.595	1.783			
		62	0.599	1.790			
		63	0.589	1.773			
		64	0.582	1.761			
			Interpolated Values				
		0	0.754	2.020			
		60	0.599	1.790			

0.2 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
76	30.0	1	0.551	1.706	6.02	6.00	6.01
		2	0.552	1.708			
		3	0.560	1.723			
		4	0.552	1.708			
		5	0.548	1.701			
		60	0.423	1.442			
		61	0.427	1.452			
		62	0.438	1.477			
		63	0.422	1.440			
		64	0.417	1.428			
			Interpolated Values				
		0	0.560	1.723			
		60	0.426	1.450			

0.2 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln e			
77	20.0	1	1.975	0.680		6.48	6.51
		2	1.960	0.672			
		3	1.935	0.660			
		4	1.930	0.656			
		60	1.074	0.071			
		61	1.057	0.055			
		62	1.035	0.034			
		63	1.030	0.030			
			Interpolated Values				
		0	1.997	0.691			
		60	1.065	0.063			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 9.4×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
78	3.00	1	0.297	1.088		6.53	6.53
		2	0.288	1.058			
		3	0.283	1.040			
		4	0.276	1.015			
		60	0.126	0.231			
		61	0.124	0.215			
		62	0.122	0.199			
		63	0.120	0.182			
			Interpolated Values				
		0	0.296	1.086			
		60	0.125	0.226			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
79	10.0	1	0.985	2.287		6.49	6.51
		2	0.978	2.280			
		3	0.968	2.270			
		4	0.948	2.249			
		60	0.498	1.605			
		61	0.495	1.599			
		62	0.488	1.585			
		63	0.486	1.581			
			Interpolated Values				
		0	0.993	2.296			
		60	0.500	1.609			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 5×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln e	Initial pH	Final pH	Median pH [Estimated]
80	30.0	1	1.144	0.134		6.49	6.54
		2	1.142	0.133			
		3	1.126	0.119			
		4	1.122	0.115			
		60	0.675	-0.393			
		61	0.663	-0.411			
		62	0.650	-0.431			
		63	0.645	-0.438			
			Interpolated Values				
		0	1.162	0.150			
		60	0.668	-0.404			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 3×10^{-3} M.

				ln e			
81	40.0	1	1.518	0.417		6.50	6.55
		2	1.513	0.414			
		3	1.498	0.404			
		4	1.478	0.390			
		60	0.928	-0.075			
		61	0.922	-0.081			
		62	0.912	-0.092			
		63	0.900	-0.105			
			Interpolated Values				
		0	1.534	0.427			
		60	0.928	-0.075			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 16×10^{-3} M.

Part B. Runs with tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
17	40.0	2	0.736	1.996	7.18	7.02	7.10
		3	0.743	2.005			
		4	0.727	1.984			
		5	0.713	1.964			
		6	0.724	1.980			
		9	0.707	1.956			
		12	0.698	1.943			
		16	0.668	1.899			
		20	0.620	1.825			
		24	0.600	1.792			
		30	0.566	1.733			
		34	0.557	1.717			
		38	0.508	1.625			
		45	0.478	1.564			
		49	0.452	1.509			
		52	0.441	1.484			
		59	0.400	1.386			
		60	0.400	1.386			
		61	0.384	1.345			
		62	0.388	1.356			
		63	0.373	1.316			
			Interpolated Values				
		0	0.755	2.022			
		60	0.397	1.380			

0.2 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 19 x 10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
18	40.0	2	0.716	1.969	7.60	7.51	7.55
		3	0.710	1.960			
		4	0.713	1.964			
		5	0.704	1.952			
		6	0.718	1.971			
		10	0.702	1.949			
		14	0.680	1.917			
		18	0.652	1.875			
		22	0.630	1.841			
		26	0.610	1.808			
		32	0.575	1.749			
		36	0.562	1.726			
		40	0.539	1.685			
		47	0.498	1.605			
		51	0.472	1.552			
		54	0.476	1.560			
		58	0.448	1.500			
		59	0.438	1.477			
		60	0.440	1.482			
		61	0.428	1.454			
		62	0.426	1.449			
		Interpolated Values					
		0	0.761	2.030			
		60	0.435	1.470			

0.2 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 17×10^{-3} M.

Note: precipitate begins to form in the reaction flask after about 40 minutes.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
20	30.0	2	0.576	1.751	7.62	7.53	7.57
		3	0.570	1.740			
		4	0.567	1.735			
		5	0.567	1.735			
		6	0.554	1.712			
		9	0.568	1.737			
		13	0.532	1.671			
		17	0.520	1.649			
		21	0.500	1.610			
		24	0.490	1.589			
		28	0.467	1.541			
		34	0.448	1.500			
		38	0.427	1.452			
		42	0.413	1.418			
		48	0.390	1.361			
		52	0.382	1.340			
		58	0.348	1.247			
		59	0.332	1.200			
		60	0.338	1.218			
		61	0.337	1.215			
		62	0.336	1.212			
		Interpolated Values					
		0	0.585	1.766			
		60	0.341	1.227			

0.2 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 12.5×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	$\underline{e}$	ln $10\underline{e}$	Initial pH	Final pH	Median pH [Estimated]
21	30.0	2	0.542	1.690	7.13	6.97	7.05
		3	0.545	1.696			
		4	0.532	1.671			
		5	0.525	1.658			
		6	0.523	1.654			
		10	0.519	1.629			
		14	0.483	1.575			
		18	0.468	1.543			
		22	0.447	1.497			
		26	0.412	1.416			
		31	0.392	1.366			
		35	0.375	1.322			
		38	0.359	1.278			
		44	0.322	1.169			
		48	0.317	1.154			
		52	0.286	1.051			
		58	0.284	1.044			
		59	0.268	0.986			
		60	0.263	0.967			
		61	0.262	0.963			
		62	0.268	0.986			
		Interpolated Values					
		0	0.554	1.710			
		60	0.271	0.995			

0.2 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 15.5×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
30	10.0	2	1.014	2.317		7.42	7.44
		6	0.958	2.260			
		10	0.922	2.221			
		20	0.788	2.064			
		30	0.716	1.969			
		40	0.626	1.834			
		50	0.552	1.708			
		60	0.484	1.577			
			Interpolated Values				
		0	1.036	2.338			
		60	0.485	1.580			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 6×10^{-3} M.

				ln 10e			
31	10.0	2	1.018	2.320		7.49	7.51
		5	0.988	2.291			
		10	0.943	2.244			
		20	0.836	2.124			
		30	0.740	2.001			
		40	0.653	1.876			
		50	0.581	1.760			
		60	0.518	1.645			
			Interpolated Values				
		0	1.055	2.357			
		60	0.519	1.645			

1 ml. aliquots placed in 10 ml. flasksfor color reaction.

Approximate amount of hydrolysis in 60 min. = 5×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
33	10.0	2	1.014	2.317		7.17	7.19
		6	0.958	2.260			
		10	0.915	2.214			
		20	0.793	2.071			
		30	0.686	1.926			
		40	0.584	1.765			
		50	0.514	1.637			
		60	0.436	1.473			
			Interpolated Values				
		0	1.043	2.345			
		60	0.437	1.475			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 6×10^{-3} M.

				ln 10e			
34	10.0	2	1.006	2.309		7.60	7.61
		5	1.974	2.276			
		10	0.932	2.232			
		20	0.843	2.132			
		30	0.750	2.015			
		40	0.672	1.905			
		50	0.602	1.795			
		60	0.542	1.690			
			Interpolated Values				
		0	1.036	2.338			
		60	0.543	1.690			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 5×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
35	10.0	2	1.043	2.346		7.79	7.80
		5	1.003	2.306			
		10	0.972	2.274			
		20	0.890	2.186			
		30	0.810	2.092			
		40	0.742	2.004			
		50	0.674	1.908			
		60	0.617	1.820			
			Interpolated Values				
		0	1.057	2.360			
		60	0.619	1.820			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 4×10^{-3} M.

				ln 10e			
36	10.0	2	1.052	2.353		8.08	8.08
		5	1.016	2.319			
		10	1.000	2.303			
		20	0.942	2.243			
		30	0.898	2.195			
		40	0.842	2.131			
		50	0.788	2.064			
		60	0.727	1.984			
			Interpolated Values				
		0	1.059	2.360			
		60	0.743	2.006			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
45	3.00	2	0.280	1.030		7.07	7.07
		5	0.268	0.986			
		10	0.243	0.888			
		20	0.208	0.732			
		30	0.172	0.542			
		40	0.145	0.372			
		50	0.118	0.166			
		60	0.095	-0.051			
			Interpolated Values				
		0	0.294	1.080			
		60	0.099	-0.012			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
46	3.00	2	0.280	1.030		7.37	7.37
		5	0.268	0.986			
		10	0.248	0.908			
		20	0.218	0.779			
		30	0.184	0.610			
		40	0.156	0.445			
		50	0.148	0.392			
		60	0.122	0.199			
			Interpolated Values				
		0	0.290	1.065			
		60	0.120	0.185			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
47	3.00	2	0.288	1.058		7.60	7.60
		5	0.279	1.026			
		10	0.258	0.948			
		20	0.228	0.824			
		30	0.202	0.703			
		32	0.200	0.693			
		45	0.174	0.554			
		60	0.145	0.372			
			Interpolated Values				
		0	0.292	1.073			
		60	0.144	0.365			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
48	3.00	2	0.288	1.058		7.80	7.80
		5	0.278	1.023			
		10	0.267	0.982			
		28	0.227	0.820			
		36	0.204	0.713			
		41	0.199	0.688			
		50	0.182	0.599			
		60	0.165	0.501			
			Interpolated Values				
		0	0.294	1.080			
		60	0.164	0.496			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln e	Initial pH	Final pH	Median pH [Estimated]
82	40.0	1	1.626	0.486		7.02	7.10
		2	1.610	0.476			
		3	1.585	0.460			
		4	1.574	0.453			
		61	0.843	-0.171			
		62	0.840	-0.174			
		63	0.827	-0.190			
		64	0.822	-0.196			
			Interpolated Values				
		0	1.636	0.492			
		60	0.871	-0.138			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 19 x 10^{-3} M.

				ln e			
83	30.0	1	1.206	0.187		6.96	7.03
		2	1.203	0.185			
		3	1.188	0.172			
		4	1.180	0.166			
		60	0.583	-0.540			
		61	0.578	-0.548			
		62	0.580	-0.545			
		63	0.568	-0.566			
			Interpolated Values				
		0	1.228	0.205			
		60	0.600	-0.513			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 15 x 10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln e	Initial pH	Final pH	Median pH [Estimated]
84	20.0	1	2.090	0.736		7.00	7.05
		2	2.080	0.731			
		3	2.042	0.715			
		4	2.005	0.695			
		60	0.913	-0.091			
		61	0.900	-0.105			
		62	0.890	-0.117			
		63	0.868	-0.142			
			Interpolated Values				
		0	2.130	0.755			
		60	0.911	-0.093			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 11 x 10^{-3} M.

				ln 10e			
85	3.00	1	0.288	1.058		7.06	7.06
		2	0.278	1.022			
		3	0.271	0.997			
		4	0.268	0.986			
		60	0.086	-0.151			
		61	0.082	-0.198			
		62	0.082	-0.198			
		63	0.076	-0.274			
			Interpolated Values				
		0	0.291	1.067			
		60	0.085	-0.167			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
86	10.0	1	1.016	2.319		7.09	7.12
		2	1.003	2.306			
		3	1.002	2.305			
		4	0.968	2.270			
		60	0.408	1.406			
		61	0.398	1.381			
		62	0.392	1.366			
		63	0.390	1.361			
			Interpolated Values				
		0	1.038	2.340			
		60	0.406	1.402			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 6×10^{-3} M.

				ln 10e			
88	30.0	1	1.188	2.475		7.91	7.93
		2	1.202	2.487			
		3	1.180	2.468			
		4	1.190	2.477			
		60	0.850	2.140			
		61	0.840	2.128			
		62	0.827	2.113			
		63	0.830	2.116			
			Interpolated Values				
		0	1.208	2.492			
		60	0.844	2.133			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 9×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
89	3.00	1	0.317	1.154		7.97	7.97
		2	0.310	1.131			
		3	0.312	1.138			
		4	0.308	1.125			
		60	0.202	0.703			
		61	0.202	0.703			
		62	0.203	0.708			
		63	0.200	0.693			
			Interpolated Values				
		0	0.317	1.155			
		60	0.204	0.712			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln e			
90	20.0	1	2.125	0.754		7.94	7.95
		2	2.120	0.751			
		3	2.115	0.749			
		4	2.100	0.742			
		60	1.430	0.358			
		61	1.438	0.364			
		62	1.428	0.356			
		63	1.418	0.349			
			Interpolated Values				
		0	2.151	0.766			
		60	1.443	0.367			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 6.6×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
91	10.0	1	1.056	2.357		7.96	7.96
		2	1.056	2.357			
		3	1.046	2.348			
		4	1.042	2.344			
		60	0.696	1.940			
		61	0.687	1.927			
		62	0.678	1.914			
		63	0.675	1.910			
			Interpolated Values				
		0	1.069	2.370			
		60	0.692	1.934			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
96	10.0	1	1.064	2.365		7.91	7.91
		5	1.027	2.330			
		10	1.002	2.305			
		20	0.911	2.209			
		30	0.858	2.149			
		40	0.797	2.076			
		50	0.743	2.006			
		60	0.687	1.927			
			Interpolated Values				
		0	1.067	2.368			
		60	0.684	1.930			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	$\underline{e}$	ln $10\underline{e}$	Initial pH	Final pH	Median pH [Estimated]
97	3.00	1	0.321	1.166		7.91	7.91
		5	0.312	1.138			
		10	0.303	1.109			
		20	0.280	1.030			
		30	0.262	0.963			
		40	0.248	0.908			
		50	0.226	0.815			
		60	0.210	0.742			
			Interpolated Values				
		0	0.324	1.174			
		60	0.212	0.751			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln $10\underline{e}$			
98	30.0	1	1.202	2.487		7.87	7.89
		5	1.184	2.472			
		10	1.152	2.444			
		20	1.090	2.390			
		30	1.029	2.332			
		40	0.962	2.264			
		50	0.908	2.206			
		60	0.842	2.131			
			Interpolated Values				
		0	1.220	2.502			
		60	0.853	2.144			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 9×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
99	20.0	1	0.811	2.093		7.89	7.90
		5	0.798	2.077			
		10	0.776	2.049			
		20	0.718	1.971			
		30	0.673	1.907			
		40	0.630	1.841			
		50	0.583	1.763			
		60	0.552	1.708			
			Interpolated Values				
		0	0.823	2.108			
		60	0.549	1.702			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

				ln 10e			
100	3.00	1	0.322	1.169		7.98	7.98
		2	0.318	1.157			
		3	0.317	1.154			
		4	0.313	1.141			
		60	0.210	0.742			
		61	0.206	0.723			
		62	0.202	0.703			
		63	0.200	0.693			
			Interpolated Values				
		0	0.324	1.174			
		60	0.207	0.726			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
101	3.00	1	0.320	1.163		7.98	7.98
		2	0.318	1.157			
		3	0.316	1.151			
		4	0.312	1.138			
		60	0.204	0.713			
		61	0.201	0.698			
		62	0.200	0.693			
		63	0.198	0.683			
			Interpolated Values				
		0	0.322	1.171			
		60	0.203	0.708			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
102	10.0	1	1.059	2.360		7.98	7.98
		2	1.049	2.351			
		3	1.036	2.338			
		4	1.034	2.336			
		60	0.703	1.950			
		61	0.691	1.933			
		62	0.684	1.923			
		63	0.678	1.914			
			Interpolated Values				
		0	1.063	2.364			
		60	0.696	1.940			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
103	30.0	1	1.193	2.479		7.97	7.99
		2	1.178	2.467			
		3	1.182	2.470			
		4	1.172	2.462			
		60	0.822	2.107			
		61	0.828	2.114			
		62	0.818	2.102			
		63	0.818	2.102			
			Interpolated Values				
		0	1.200	2.485			
		60	0.829	2.115			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 9×10^{-3} M.

				ln 10e			
104	20.0	1	0.798	2.077		7.97	7.98
		2	0.802	2.082			
		3	0.795	2.073			
		4	0.783	2.058			
		60	0.548	1.701			
		61	0.542	1.690			
		62	0.532	1.671			
		63	0.527	1.662			
			Interpolated Values				
		0	0.808	2.089			
		60	0.543	1.691			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 7×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
105	3.00	1	0.330	1.194		8.00	8.00
		2	0.326	1.182			
		3	0.343	1.233			
		4	0.316	1.151			
		60	0.212	0.751			
		61	0.208	0.732			
		62	0.206	0.723			
		63	0.204	0.713			
			Interpolated Values				
		0	0.330	1.195			
		60	0.210	0.740			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
106	10.0	1	1.082	2.382		7.99	7.99
		2	1.080	2.380			
		3	1.068	2.369			
		4	1.054	2.356			
		60	0.708	1.957			
		61	0.704	1.952			
		62	0.696	1.940			
		63	0.693	1.936			
			Interpolated Values				
		0	1.091	2.390			
		60	0.708	1.957			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
107	5.00	1	0.528	1.664		8.01	8.01
		2	0.525	1.658			
		3	0.523	1.654			
		4	0.523	1.654			
		60	0.341	1.227			
		61	0.339	1.221			
		62	0.338	1.218			
		63	0.332	1.200			
			Interpolated Values				
		0	0.535	1.676			
		60	0.341	1.228			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
108	30.0	1	1.194	2.480		7.96	7.98
		2	1.195	2.481			
		3	1.192	2.478			
		4	1.188	2.475			
		60	0.862	2.154			
		61	0.850	2.140			
		62	0.848	2.138			
		63	0.837	2.125			
			Interpolated Values				
		0	1.210	2.493			
		60	0.857	2.148			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 9×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
109	20.0	1	0.808	2.089		7.98	7.99
		2	0.806	2.087			
		3	0.800	2.079			
		4	0.787	2.063			
		60	0.548	1.701			
		61	0.544	1.694			
		62	0.543	1.692			
		63	0.540	1.686			
			Interpolated Values				
		0	0.813	2.095			
		60	0.549	1.703			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

				ln 10e			
110	3.00	1	0.322	1.169		7.56	7.56
		2	0.318	1.157			
		3	0.312	1.138			
		4	0.308	1.125			
		60	0.155	0.438			
		61	0.153	0.425			
		62	0.149	0.399			
		63	0.148	0.392			
			Interpolated Values				
		0	0.324	1.177			
		60	0.154	0.434			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
111	3.00	1	0.318	1.157		7.53	7.53
		2	0.312	1.138			
		3	0.308	1.125			
		4	0.303	1.109			
		60	0.150	0.405			
		61	0.150	0.405			
		62	0.148	0.392			
		63	0.148	0.392			
			Interpolated Values				
		0	0.320	1.162			
		60	0.152	0.417			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
112	3.00	1	0.322	1.169		7.57	7.57
		2	0.318	1.157			
		3	0.312	1.138			
		4	0.308	1.125			
		60	0.154	0.432			
		61	0.153	0.425			
		62	0.146	0.378			
		63	0.145	0.372			
			Interpolated Values				
		0	0.325	1.178			
		60	0.151	0.412			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
113	10.0	1	1.042	2.344		7.57	7.58
		2	1.037	2.339			
		3	1.022	2.325			
		4	1.012	2.315			
		60	0.525	1.658			
		61	0.518	1.645			
		62	0.513	1.635			
		63	0.503	1.615			
			Interpolated Values				
		0	1.059	2.360			
		60	0.525	1.658			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
114	3.00	1	0.313	1.141		7.56	7.56
		2	0.318	1.157			
		3	0.312	1.138			
		4	0.303	1.109			
		60	0.154	0.432			
		61	0.149	0.399			
		62	0.145	0.372			
		63	0.145	0.372			
			Interpolated Values				
		0	0.322	1.168			
		60	0.154	0.433			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
115	20.0	1	0.808	2.089		7.55	7.58
		2	0.790	2.067			
		3	0.786	2.062			
		4	0.778	2.052			
		60	0.440	1.482			
		61	0.435	1.470			
		62	0.425	1.447			
		63	0.422	1.440			
			Interpolated Values				
		0	0.813	2.095			
		60	0.437	1.475			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 9×10^{-3} M.

				ln 10e			
116	20.0	1	0.789	2.066		7.53	7.56
		2	0.782	2.057			
		3	0.778	2.052			
		4	0.774	2.046			
		60	0.438	1.477			
		61	0.433	1.466			
		62	0.423	1.442			
		63	0.421	1.437			
			Interpolated Values				
		0	0.801	2.080			
		60	0.435	1.470			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 9×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
117	20.0	1	0.803	2.083		7.57	7.60
		2	0.782	2.057			
		3	0.787	2.063			
		4	0.780	2.054			
		60	0.443	1.488			
		61	0.436	1.472			
		62	0.430	1.459			
		63	0.427	1.452			
			Interpolated Values				
		0	807	2.088			
		60	0.446	1.484			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 9×10^{-3} M.

				ln 10e			
118	10.0	1	0.402	1.391		7.56	7.57
		2	0.401	1.389			
		3	0.395	1.374			
		4	0.388	1.356			
		60	0.208	0.732			
		61	0.203	0.708			
		62	0.198	0.683			
		63	0.195	0.668			
			Interpolated Values				
		0	0.408	1.407			
		60	0.204	0.714			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
119	20.0	1	0.790	2.067		7.56	7.59
		2	0.779	2.053			
		4	0.771	2.043			
		60	0.430	1.459			
		61	0.422	1.440			
		62	0.417	1.428			
		63	0.413	1.418			
			Interpolated Values				
		0	0.799	2.078			
		60	0.427	1.452			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 9×10^{-3} M.

				ln 10e			
120	5.00	1	0.526	1.660		7.54	7.54
		2	0.518	1.645			
		3	0.520	1.649			
		4	0.503	1.615			
		60	0.253	0.928			
		61	0.251	0.920			
		62	0.248	0.908			
		63	0.242	0.884			
			Interpolated Values				
		0	0.534	1.675			
		60	0.253	0.928			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
121	5.00	1	0.529	1.666		7.53	7.53
		2	0.519	1.647			
		3	0.515	1.639			
		4	0.506	1.621			
		60	0.253	0.928			
		61	0.252	0.924			
		62	0.248	0.908			
		63	0.243	0.888			
			Interpolated Values				
		0	0.533	1.674			
		60	0.254	0.931			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
122	10.0	1	1.035	2.337		7.55	7.56
		2	1.034	2.336			
		3	1.015	2.318			
		4	1.019	2.322			
		60	0.516	1.641			
		61	0.510	1.629			
		62	0.506	1.621			
		63	0.500	1.609			
			Interpolated Values				
		0	1.056	2.358			
		60	0.516	1.642			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	$\underline{e}$	ln $10\underline{e}$	Initial pH	Final pH	Median pH [Estimated]
123	5.00	1	0.531	1.670		7.54	7.54
		2	0.525	1.658			
		3	0.523	1.654			
		4	0.514	1.637			
		60	0.262	0.963			
		61	0.256	0.940			
		62	0.248	0.908			
		63	0.242	0.884			
			Interpolated Values				
		0	0.540	1.686			
		60	0.257	0.942			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln $10\underline{e}$			
124	5.00	1	0.525	1.658		7.51	7.51
		2	0.518	1.645			
		3	0.514	1.637			
		4	0.508	1.625			
		60	0.251	0.920			
		61	0.247	0.904			
		62	0.242	0.884			
		63	0.239	0.871			
			Interpolated Values				
		0	0.533	1.673			
		60	0.250	0.915			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
125	30.0	1	1.187	2.474		7.50	7.54
		2	1.168	2.458			
		3	1.160	2.451			
		4	1.145	2.438			
		60	0.700	1.946			
		61	0.689	1.930			
		62	0.683	1.921			
		63	0.665	1.895			
			Interpolated Values				
		0	1.189	2.476			
		60	0.694	1.937			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 12.5×10^{-3} M.

				ln 10e			
126	30.0	1	1.180	2.469		7.52	7.56
		2	1.166	2.457			
		3	1.167	2.457			
		4	1.160	2.451			
		60	0.694	1.937			
		61	0.690	1.932			
		62	0.680	1.917			
		63	0.674	1.908			
		70	0.626	1.834			
			Interpolated Values				
		0	1.195	2.481			
		60	0.694	1.937			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 12.5×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
127	10.0	1	0.410	1.411		7.52	7.53
		2	0.392	1.366			
		3	0.390	1.361			
		4	0.388	1.356			
		61	0.199	0.688			
		62	0.196	0.673			
		63	0.192	0.652			
			Interpolated Values				
		0	0.404	1.396			
		60	0.200	0.694			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

				ln 10e			
128	30.0	1	1.190	2.477		7.50	7.54
		2	1.178	2.467			
		3	1.165	2.456			
		4	1.170	2.460			
		60	0.713	1.964			
		61	0.710	1.960			
		62	0.697	1.942			
		63	0.689	1.930			
			Interpolated Values				
		0	1.204	2.488			
		60	0.712	1.963			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

Approximate amount of hydrolysis in 60 min. = 12.5×10^{-3} M.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
129	30.0*	1	1.121	2.417		7.51	7.55
		2	1.121	2.417			
		3	1.109	2.406			
		4	1.108	2.405			
		60	0.668	1.899			
		61	0.659	1.886			
		62	0.648	1.869			
		63	0.638	1.853			
			Interpolated Values				
		0	1.139	2.433			
		60	0.659	1.885			

1 ml. aliquots placed in 25 ml. flasks for color reaction.

* The extinction value for t = 0 indicates, by comparison with preceding runs, that the substrate concentration is about 6% lower than the value given. However, very little error is introduced if it is assumed that the run was made with a substrate concentration equal to 30×10^{-3} M.

Part C. Kinetic studies made in the presence of added salts.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
69	10.0	1	0.957	2.259		6.98	7.00
		2	0.940	2.241			
		3	0.923	2.222			
		4	0.910	2.208			
		60	0.366	1.297			
		61	0.353	1.261			
		62	0.350	1.253			
		63	0.342	1.230			
			Interpolated Values				
		0	0.970	2.272			
		60	0.362	1.286			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Run contains 0.02f $MgCl_2$. Cacodylic acid buffer system.

Approximate amount of hydrolysis in 60 min. = 6×10^{-3} M.

				ln 10e			
70	10.0	1	1.005	2.308		6.98	7.00
		2	0.985	2.287			
		3	0.975	2.277			
		4	0.953	2.254			
		60	0.342	1.230			
		61	0.333	1.203			
		62	0.328	1.188			
		63	0.323	1.172			
			Interpolated Values				
		0	1.020	2.323			
		60	0.341	1.227			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Run contains 0.02f $CaCl_2$. Cacodylic acid buffer system.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
92	10.0	1	0.947	2.248		5.91	5.91
		2	0.942	2.243			
		3	0.938	2.239			
		4	0.932	2.232			
		60	0.686	1.926			
		61	0.680	1.917			
		62	0.674	1.908			
		63	0.674	1.908			
			Interpolated Values				
		0	0.952	2.254			
		60	0.684	1.923			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Run contains 0.06f NaCl. Cacodylic acid buffer system.

				ln 10e			
93	10.0	1	0.943	2.244		5.89	5.89
		2	0.940	2.241			
		3	0.948	2.249			
		4	0.940	2.241			
		61	0.645	1.864			
		62	0.645	1.864			
		63	0.646	1.866			
		64	0.640	1.856			
			Interpolated Values				
		0	0.956	2.258			
		60	0.654	1.878			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Run contains 0.02f $CaCl_2$. Cacodylic acid buffer system.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min]	e	ln 10e	Initial pH	Final pH	Median pH [Estimated]
94	10.0	1	0.958	2.260		5.94	5.94
		2	0.954	2.255			
		3	0.945	2.246			
		4	0.933	2.233			
		60	0.702	1.949			
		61	0.702	1.949			
		62	0.690	1.932			
		63	0.690	1.932			
			Interpolated Values				
		0	0.960	2.262			
		60	0.702	1.949			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Run contains 0.02f $MgCl_2$. Cacodylic acid buffer system.

TABLE L

Check of Calculations of Initial pH

$[S]_o \times 10^3$ [M]	Ratio Amine:Salt	Observed pH	Calculated pH
80	10	8.78, 8.76	8.79
64	10	8.80, 8.81	8.82
32	10	8.92	8.94
21.3	10	8.98	9.00
12.8	10	9.04, 9.03	9.05
6.4	10	9.10	9.11
5	1.0	8.16	8.16
5	9.0	9.04	9.06
5	0.35	7.70	7.70
5	3.54	8.70	8.68
70	9	8.79	8.79
70	2	8.63	8.66
70	1.0	8.12	8.11
70	0.5	7.82	7.83

TABLE LI

Experimental Observations for the Alpha-chymotrypsin-catalyzed Hydrolysis of Acetyl-L-tyrosinhydroxamide.

Enzyme concentration = 0.040 mg. protein nitrogen/ml.

Temperature = 25°. Buffer 0.3f in organic component except where otherwise noted.

$\underline{e}$ = extinction observed at 515 millimicrons.

$[S]_o$ = initial substrate concentration.

f_1 = correction factor for first order plots as described in the section on experimental results and calculations. The "K_s", "K_p", and "K_I" values listed under f_1 refer to the approximate constants used in the computation of f_1. They are expressed in units of 10^{-3} M.

The initial pH is that obtained either from knowledge of the pH of the blank for low pH's, where the substrate does not ionize, or by calculation as described above for the pH regions where substrate ionization is important. The final pH is that obtained by direct observation.

The table is divided into several parts.

Part A. Runs made with cacodylic acid-sodium cacodylate buffer. No inhibitors added.

Part B. Runs made with cacodylic acid-sodium cacodylate buffer. Inhibitors added.

Part C. Runs made with potassium phosphate buffer. No inhibitors added.

Part D. Runs made with ethylenediamine-hydrochloric acid buffer. No inhibitors added.

Part E. Runs made with hydroxylamine-hydroxylamine hydrochloride buffer. No inhibitors added.

Part F. Runs made with tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer. No inhibitors added.

Part G. Runs made with tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer. Calcium chloride added.

Part H. Runs made with tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer. Inhibitors added.

Part A. Runs made with cacodylic acid-sodium cacodylate buffer. No inhibitors added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	$\underline{e}$	ln $\underline{e}$	f_1	Initial pH	Final pH
9b	15.0	2	1.800	0.588	$K_s = 70$	6.20	6.20
		5	1.756	0.564	$K_p = 20$		
		10	1.713	0.538			
		12	1.685	0.522			
		20	1.601	0.471	0.976		
		30	1.512	0.414	0.961		
		45	1.400	0.336	0.950		
		60	1.281	0.248	0.936		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10$\underline{e}$			
10	5.00	2	0.604	1.798	$K_s = 70$	6.20	6.19
		5	0.589	1.773	$K_p = 20$		
		10	0.565	1.732	0.995		
		20	0.531	1.690	0.990		
		30	0.499	1.607	0.985		
		40	0.465	1.537	0.985		
		50	0.438	1.477	0.980		
		60	0.410	1.411	0.970		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	$\underline{e}$	ln $\underline{e}$	f_1	Initial pH	Final pH
11	70.0	2	1.880	0.631	$K_s = 70$	6.20	6.18
		5	1.860	0.620	$K_p = 20$		
		10	1.817	0.597			
		20	1.762	0.566	0.965		
		30	1.694	0.526	0.936		
		40	1.637	0.493	0.920		
		50	1.588	0.462	0.905		
		60	1.549	0.437	0.900		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

				ln $\underline{e}$			
12	30.0	2	1.577	0.456	$K_s = 70$	6.20	6.19
		5	1.554	0.441	$K_p = 20$		
		10	1.516	0.416	1.00		
		20	1.430	0.358	0.964		
		30	1.363	0.310	0.944		
		40	1.321	0.278	0.936		
		50	1.243	0.218	0.930		
		60	1.196	0.179	0.869		

1 ml. aliquots placed in 25 ml. flasks for color reaction.

				ln $\underline{e}$			
46b	70.0	2	1.833	0.606	$K_s = 60$	6.62	6.51
		5	1.783	0.578	$K_p = 20$		
		10	1.724	0.545			
		20	1.668	0.511	0.960		
		30	1.524	0.421	0.910		
		40	1.459	0.378	0.903		
		50	1.388	0.328	0.891		
		60	1.320	0.278	0.875		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
47b	40.0	2	1.045	2.347	$K_s = 60$	6.62	6.55
		5	1.014	2.317	$K_p = 20$		
		10	0.960	2.262	0.997		
		20	0.893	2.189	0.943		
		30	0.821	2.105	0.920		
		40	0.770	2.041	0.896		
		50	0.708	1.957	0.880		
		60	0.656	1.881	0.861		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

				ln 10e			
48b	4.80	2	0.566	1.733	$K_s = 60$	6.62	6.59
		5	0.543	1.692	$K_p = 20$		
		10	0.503	1.615			
		20	0.441	1.484	0.984		
		30	0.389	1.358	0.975		
		40	0.338	1.218	0.966		
		50	0.293	1.075	0.961		
		60	0.258	0.948	0.956		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln e			
49	16.0	2	1.890	0.636	$K_s = 60$	6.62	6.55
		5	1.828	0.603	$K_p = 20$		
		10	1.719	0.541	0.980		
		20	1.537	0.430	0.960		
		30	1.371	0.316	0.941		
		40	1.227	0.204	0.926		
		50	1.110	0.104	0.911		
		60	1.008	0.008	0.901		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
50	4.80	2	0.560	1.723	$K_s = 60$	6.63	6.60
		4½	0.547	1.699	$K_p = 20$		
		6	0.535	1.677			
		10	0.506	1.621			
		20	0.439	1.479	0.890		
		30	0.383	1.343	0.973		
		40	0.337	1.215	0.969		
		50	0.296	1.085	0.960		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
51	4.80	2	0.561	1.725	$K_s = 60$	6.63	see run 50
		5	0.545	1.696	$K_p = 20$		
		10	0.505	1.619			
		20	0.440	1.482	0.983		
		30	0.381	1.345	0.975		
		40	0.336	1.212	0.967		
		50	0.297	1.089	0.961		
		60	0.259	0.952	0.955		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Same buffer solution as that used for Run 50.

				ln e			
52	70.1	2	1.850	0.615	$K_s = 60$	6.63	See run 53
		5	1.786	0.580	$K_p = 20$		
		10	1.752	0.561	0.966		
		20	1.626	0.486	0.935		
		30	1.540	0.432	0.908		
		40	1.450	0.372	0.896		
		50	1.390	0.330	0.879		
		60	1.322	0.279	0.871		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Same buffer proportion as that used in Run 53.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
53	70.1	2	1.841	0.611	$K_s = 60$	6.63	6.52
		5	1.795	0.585	$K_p = 20$		
		10	1.736	0.552	0.950		
		20	1.618	0.481	0.933		
		30	1.534	0.428	0.911		
		40	1.461	0.379	0.902		
		50	1.390	0.330	0.881		
		60	1.329	0.284	0.865		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

				ln e			
54	70.4	2	1.821	0.600	$K_s = 55$	6.95	6.80
		5	1.768	0.570	$K_p = 35$		
		10	1.684	0.521	0.970		
		15	1.613	0.478	0.975		
		20	1.544	0.434	0.973		
		25	1.493	0.401	0.970		
		35	1.368	0.314	0.955		
		45	1.280	0.246	0.950		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

				ln 10e			
55	39.8	2	1.028	2.330	$K_s = 55$	6.95	6.83
		5	0.982	2.284	$K_p = 35$		
		10	0.923	2.222	0.989		
		15	0.870	2.163	0.981		
		20	0.812	2.094	0.974		
		25	0.767	2.037	0.966		
		35	0.696	1.940	0.960		
		45	0.625	1.833	0.951		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
56	4.78	2	0.554	1.712	$K_s = 55$	6.95	6.92
		5	0.520	1.649	$K_p = 35$		
		10	0.475	1.558			
		15	0.420	1.435			
		20	0.379	1.332			
		25	0.343	1.233	0.990		
		35	0.281	1.033	0.988		
		45	0.228	0.824	0.981		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln e			
57	15.95	2	1.871	0.626	$K_s = 55$	6.95	6.91
		5	1.770	0.571	$K_p = 35$		
		10	1.616	0.480			
		15	1.490	0.399			
		20	1.368	0.314	0.980		
		25	1.268	0.238	0.975		
		35	1.072	0.070	0.965		
		45	0.912	-0.092	0.960		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
76	4.78	2	0.596	1.785	$K_s = 70$	6.20	6.20
		10	0.566	1.733	$K_p = 20$		
		20	0.522	1.653			
		30	0.489	1.587	0.986		
		45	0.439	1.479	0.980		
		60	0.399	1.384	0.971		
		75	0.360	1.291	0.966		
		90	0.328	1.188	0.960		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
80	70.0	2	1.880	0.631	$K_s = 70$	6.20	6.14
		5	1.841	0.611	$K_p = 20$		
		10	1.804	0.591			
		20	1.741	0.554	0.968		
		30	1.678	0.517	0.936		
		40	1.616	0.480	0.918		
		50	1.577	0.456	0.901		
		60	1.527	0.423	0.895		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
84	39.7	2	1.090	2.389	$K_s = 70$	6.20	6.20
		5	1.075	2.375	$K_p = 20$		
		10	1.046	2.348			
		20	0.998	2.301	0.965		
		30	0.951	2.252	0.936		
		40	0.920	2.219	0.929		
		50	0.886	2.182	0.920		
		60	0.840	2.128	0.906		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
94a	40.0	2	1.058	2.359	$K_s = 70$	6.20	6.19
		5	1.046	2.348	$K_p = 20$		
		10	1.020	2.323			
		20	0.969	2.271	0.959		
		30	0.928	2.228	0.941		
		40	0.895	2.192	0.926		
		50	0.860	2.152	0.919		
		60	0.819	2.103	0.902		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
93b	4.78	2	0.596	1.785	$K_s = 70$	6.20	6.21
		5	0.581	1.760	$K_p = 20$		
		10	0.560	1.723			
		20	0.520	1.649	0.986		
		30	0.484	1.577	0.984		
		40	0.448	1.500	0.980		
		50	0.418	1.430	0.975		
		60	0.391	1.364	0.970		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln e			
104	16.0	5	1.903	0.644	$K_s = 70$	6.20	6.21
		20	1.747	0.558	$K_p = 20$		
		40	1.561	0.444	0.955		
		60	1.397	0.334	0.939		
		80	1.262	0.232	0.921		
		100	1.131	0.123	0.905		
		120	1.034	0.033	0.890		
		153	0.882	-0.126	0.876		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
109	40.0	2	1.068	2.369	$K_s = 70$	6.20	6.18
		5	1.056	2.357	$K_p = 20$		
		10	1.032	2.335	1.00		
		20	0.987	2.280	0.965		
		30	0.941	2.242	0.936		
		40	0.903	2.201	0.931		
		50	0.871	2.164	0.919		
		60	0.835	2.122	0.910		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
112	4.80	2	0.593	1.780	$K_s = 70$	6.20	6.19
		5	0.586	1.768	$K_p = 20$		
		10	0.565	1.732			
		20	0.526	1.660	0.985		
		30	0.491	1.591	0.984		
		40	0.457	1.520	0.979		
		50	0.427	1.452	0.976		
		60	0.400	1.386	0.971		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Part B. Runs made with cacodylic acid-sodium cacodylate buffer.

Inhibitors added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
74	39.8	2	1.072	0.0695	$K_s = 75$	6.20	6.17
		10	1.063	0.0610	$K_p = 20$		
		20	1.045	0.044	$K_I = 2$		
		30	1.038	0.037			
		45	1.005	0.005			
		60	0.982	-0.018	0.991		
		75	0.966	-0.035	0.980		
		90	0.943	-0.059	0.975		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

6.0×10^{-3} M acetyl-D-tryptophanamide added.

				ln e			
75	70.4	2	1.900	0.641	$K_s = 75$	6.20	6.20
		10	1.866	0.624	$K_p = 20$		
		20	1.852	0.616	$K_I = 2$		
		30	1.823	0.601			
		45	1.792	0.583	0.994		
		60	1.767	0.569	0.988		
		75	1.731	0.549	0.976		
		90	1.700	0.531	0.976		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

6.0×10^{-3} M acetyl-D-tryptophanamide added.

				ln 10e			
77	4.78	2	0.604	1.798	$K_s = 75$	6.20	6.20
		10	0.598	1.788	$K_p = 20$		
		20	0.584	1.765	$K_I = 2$		
		30	0.579	1.756			
		45	0.562	1.726			
		60	0.551	1.707	0.998		
		75	0.548	1.701	0.998		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

6.0×10^{-3} M acetyl-D-tryptophanamide added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	$\underline{e}$	ln $10\underline{e}$	f_1	Initial pH	Final pH
78	4.78	4	0.586	1.768	$K_s = 75$	6.20	6.20
		10	0.576	1.751	$K_p = 20$		
		20	0.560	1.723	$K_I = 2$		
		40	0.522	1.653			
		60	0.489	1.587			
		80	0.460	1.526			
		100	0.427	1.452	0.990		
		120	0.400	1.386	0.988		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

2.46×10^{-3} M acetyl-D-tryptophanamide added.

			ln $\underline{e}$				
79	15.94	4	1.963	0.675	$K_s = 75$	6.20	6.22
		10	1.935	0.660	$K_p = 20$		
		20	1.870	0.626	$K_I = 2$		
		40	1.760	0.565			
		60	1.648	0.500	0.979		
		80	1.560	0.445	0.977		
		100	1.468	0.384	0.971		
		120	1.392	0.330	0.960		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

2.46×10^{-3} M acetyl-D-tryptophanamide added.

			ln $\underline{e}$				
81	66.4*	4	1.760	0.565	$K_s = 75$	6.20	6.16
		10	1.743	0.555	$K_p = 20$		
		20	1.695	0.527	$K_I = 2$		
		40	1.611	0.477	0.971		
		60	1.554	0.441	0.952		
		80	1.505	0.409	0.944		
		100	1.450	0.372	0.934		
		120	1.400	0.336	0.926		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

2.46×10^{-3} M acetyl-D-tryptophanamide added.

* Extrapolation to zero time indicated that a weighing error had been made, and that the actual concentration was less than that intended (70×10^{-3} M). The value given is that estimated from a comparison of the zero time extinction of this run with those of other runs made at the same time (80 and 82).

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
82	39.8	4	1.060	0.058	$K_s = 75$	6.20	6.20
		10	1.042	0.041	$K_p = 20$		
		20	1.012	0.012	$K_I = 2$		
		40	0.962	-0.039	0.981		
		60	0.918	-0.086	0.966		
		80	0.880	-0.128	0.960		
		100	0.843	-0.171	0.950		
		120	0.806	-0.216	0.939		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

2.46×10^{-3} M acetyl-D-tryptophanamide added.

				ln e			
83	70.0	4	1.941	0.664	$K_s = 75$	6.20	6.14
		10	1.915	0.650	$K_p = 20$		
		20	1.880	0.631	$K_I = 2$		
		40	1.800	0.588	0.977		
		60	1.720	0.542	0.952		
		80	1.658	0.506	0.940		
		100	1.602	0.471	0.930		
		120	1.545	0.435	0.921		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

2.46×10^{-3} M acetyl-D-tryptophanamide added.

				ln 10e			
85	4.81	4	0.591	1.777	$K_s = 75$	6.20	6.21
		10	0.585	1.766	$K_p = 20$		
		20	0.563	1.728	$K_I = 2$		
		40	0.528	1.664			
		60	0.497	1.603			
		81	0.466	1.539			
		100	0.440	1.482	0.988		
		120	0.411	1.413	0.987		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

2.46×10^{-3} M acetyl-D-tryptophanamide added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
92	70.0	4	1.849	0.614	$K_s = 75$	6.20	6.15
		10	1.812	0.595	$K_p = 20$		
					$K_I = 60$		
		20	1.762	0.566	0.979		
		40	1.663	0.509	0.948		
		60	1.588	0.462	0.920		
		80	1.507	0.410	0.911		
		100	1.453	0.374	0.895		
		120	1.393	0.322	0.876		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

30 x 10^{-3} M acetyl-L-tyrosinamide added.

				ln 10e			
93a	40.0	5	1.054	2.355	$K_s = 75$	6.20	6.19
		10	1.034	2.336	$K_p = 20$		
					$K_I = 60$		
		20	0.996	2.299	0.948		
		40	0.935	2.235	0.952		
		60	0.870	2.163	0.935		
		80	0.820	2.104	0.920		
		100	0.770	2.014	0.906		
		120	0.737	1.997	0.894		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

29.9 x 10^{-3} M acetyl-L-tyrosinamide added.

				ln 10e			
95a	4.78	4	0.592	1.778	$K_s = 75$	6.20	6.21
		10	0.575	1.749	$K_p = 20$		
		20	0.548	1.701	$K_I = 60$		
		40	0.474	1.556			
		60	0.450	1.504	0.988		
		80	0.409	1.409	0.980		
		100	0.372	1.314	0.976		
		120	0.340	1.224	0.975		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

30 x 10^{-3} M acetyl-L-tyrosinamide added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
96a	15.91	4	1.952	0.670	$K_s = 75$	6.20	6.20
		10	1.900	0.641	$K_p = 20$		
					$K_I = 2$		
		20	1.822	0.600	0.978		
		40	1.658	0.505	0.975		
		60	1.534	0.428	0.958		
		80	1.422	0.352	0.951		
		100	1.307	0.268	0.945		
		120	1.213	0.193	0.930		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

30 x 10^{-3} M acetyl-L-tyrosinamide added.

				ln e			
105	16.0	4	1.956	0.670	$K_s = 75$	6.30	6.30
		10	1.931	0.658	$K_p = 22$		
		20	1.890	0.636	$K_I = 22$		
		40	1.809	0.592			
		60	1.730	0.548	0.991		
		80	1.655	0.504	0.988		
		100	1.585	0.461	0.986		
		120	1.526	0.422	0.980		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

44.8 x 10^{-3} M acetyl-L-tyrosine added.

				ln e			
107	70.8	4	1.897	0.640	$K_s = 75$	6.20	6.18
		10	1.887	0.632	$K_p = 20$		
		20	1.848	0.614	$K_I = 20$		
		40	1.783	0.578	0.980		
		60	1.742	0.550	0.976		
		80	1.688	0.523	0.961		
		100	1.653	0.502	0.959		
		120	1.605	0.473	0.950		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

44.8 x 10^{-3} M acetyl-L-tyrosine added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
108	40.0	4	1.983	2.383	$K_s = 75$	6.20	6.20
		10	1.067	2.368	$K_p = 20$		
		20	1.052	2.354	$K_I = 20$		
		40	1.010	2.304			
		60	0.975	2.277	0.983		
		80	0.948	2.249	0.973		
		100	0.915	2.214	0.970		
		120	0.888	2.184	0.965		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

45.1 x 10^{-3} M acetyl-L-tyrosine added.

				ln e			
110	16.0	4	1.972	0.680	$K_s = 75$	6.20	6.20
		10	1.950	0.668	$K_p = 20$		
		20	1.910	0.647	$K_I = 20$		
		40	1.825	0.601			
		60	1.760	0.565	0.990		
		80	1.680	0.519	0.985		
		100	1.624	0.485	0.985		
		120	1.553	0.440	0.983		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

45.0 x 10^{-3} M acetyl-L-tyrosine added.

				ln 10e			
111	4.80	4	0.595	1.783	$K_s = 75$	6.20	6.21
		10	0.589	1.773	$K_p = 20$		
		20	0.575	1.749	$K_I = 20$		
		40	0.551	1.707			
		60	0.525	1.658			
		80	0.504	1.617	0.996		
		100	0.478	1.564	0.993		
		120	0.459	1.524	0.902		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

44.6 x 10^{-3} M acetyl-L-tyrosine added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
123	4.80	4	0.586	1.768	$K_s = 60$	6.60	6.57
		10	0.574	1.747	$K_p = 20$		
		20	0.557	1.717	$K_I = 20$		
		40	0.530	1.668			
		60	0.496	1.601			
		80	0.471	1.550			
		100	0.448	1.500	0.996		
		120	0.422	1.440	0.991		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

90.2 x 10^{-3} M acetyl-L-tyrosine added.

				ln 10e			
124	4.80	4	0.571	1.742	$K_s = 50$	6.81	6.81
		10	0.555	1.714	$K_p = 30$		
		20	0.532	1.671	$K_I = 30$		
		40	0.480	1.569			
		60	0.440	1.482			
		80	0.402	1.391			
		100	0.369	1.303			
		120	0.337	1.215	0.994		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

90 x 10^{-3} M acetyl-L-tyrosine added.

				ln 10e			
128	4.80	4	0.594	1.782	$K_s = 50$	6.20 ?	6.15
		10	0.582	1.761	$K_p = 20$		
		20	0.561	1.725	$K_I = 40$		
		40	0.528	1.664			
		60	0.498	1.605			
		80	0.466	1.539			
		100	0.440	1.482	0.990		
		120	0.412	1.416	0.991		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

55.4 x 10^{-3} M L-tyrosinamide added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
129	4.80	4	0.576	1.751	$K_s = 44$	6.89	6.89
		10	0.552	1.708	$K_p = 30$		
		20	0.518	1.645	$K_I = 22$		
		40	0.450	1.504			
		60	0.399	1.384			
		80	0.356	1.270			
		100	0.311	1.135	0.996		
		120	0.270	0.993	0.998		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

55.4×10^{-3} M L-tyrosinamide added.

Part C. Runs made with 0.3f potassium phosphate buffer. No inhibitors added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
66	15.95	2	1.181	2.469	$K_s = 50$	7.12	7.11
		5	1.119	2.415	$K_p = 50$		
		10	1.005	2.308	No cor-		
		15	0.915	2.214	rections		
		20	0.830	2.116			
		25	0.750	2.015			
		30	0.672	1.905			
		34½	0.611	1.810			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
67	4.78	2	0.362	1.287	No cor-	7.12	7.12
		5	0.332	1.200	rections		
		10	0.292	1.072			
		15	0.254	0.932			
		20	0.211	0.747			
		25	0.199	0.688			
		30	0.178	0.577			
		35	0.160	0.470			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
68	69.8	2	1.071	2.372	$K_s = 50$	7.12	7.05
		5	1.045	2.348	$K_p = 50$		
		10	0.998	2.301	No cor-		
		15	0.938	2.239	rections		
		20	0.900	2.197			
		25	0.858	2.147			
		30	0.822	2.107			
		35	0.778	2.052			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
69	39.9	2	0.620	1.825	K_s 50	7.12	7.05
		5	0.601	1.793	K_p 50		
		10	0.563	1.728	No cor-		
		15	0.514	1.637	rections		
		25	0.432	1.463			
		30	0.412	1.416			
		35	0.379	1.332			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Part D. Runs made with ethylenediamine-hydrochloric acid buffer.

No inhibitors added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
70	15.92	2	1.970	0.678	$K_s = 50$	7.31	7.27
		5	1.857	0.619	$K_p = 50$		
		10	1.652	0.501	No cor-		
		15	1.473	0.387	rections		
		20	1.318	0.276			
		25	1.178	0.164			
		30	1.046	0.045			
		35	0.923	-0.080			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
71	4.77	2	0.581	1.760	$K_s = 50$	7.31	7.29
		5	0.535	1.677	$K_p = 50$		
		10	0.465	1.537	No cor-		
		15	0.403	1.394	rections		
		20	0.352	1.258			
		25	0.304	1.112			
		30	0.266	0.978			
		35	0.230	0.833			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
72	39.8	2	1.013	2.316	$K_s = 50$	7.31	7.20
		5	0.958	2.260	$K_p = 50$		
		10	0.890	2.186	No cor-		
		15	0.824	2.109	rections		
		20	0.758	2.026			
		25	0.689	1.930			
		30	0.637	1.852			
		35	0.593	1.780			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
73	70.1	2	1.794	0.584	K_s 50	7.31	7.16
		5	1.738	0.552	K_p 50		
		10	1.640	0.494	No cor-		
		15	1.551	0.439	rections		
		20	1.465	0.382			
		25	1.382	0.324			
		30	1.309	0.269			
		35	1.238	0.214			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Part E. Runs made with hydroxylamine-hydrochloric acid buffer.

No inhibitors added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
62	16.0	2	2.112	0.748	$K_s = 40$	7.15	7.01
		5	1.890	0.636	$K_p = 40$		
		10	1.709	0.536	No cor-		
		15	1.550	0.438	rections		
		20	1.417	0.348			
		25	1.305	0.266			
		30	1.180	0.165			
		35	1.073	0.070			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
63	4.80	2	0.615	1.816	$K_s = 40$	7.15	7.15
		5	0.571	1.742	$K_p = 40$		
		10	0.509	1.627	No cor-		
		15	0.457	1.520	rections		
		20	0.407	1.404			
		25	0.360	1.281			
		30	0.325	1.179			
		35	0.290	1.065			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
64	40.0	$2\frac{1}{2}$	1.026	2.329	$K_s = 40$	7.15	6.92
		$5\frac{1}{2}$	0.981	2.283	$K_p = 40$		
		$10\frac{1}{2}$	0.918	2.217	No cor-		
		$15\frac{1}{2}$	0.848	2.138	rections		
		$20\frac{1}{2}$	0.798	2.077			
		$25\frac{1}{2}$	0.753	2.019			
		$30\frac{1}{2}$	0.704	1.952			
		$35\frac{1}{2}$	0.666	1.896			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
65	70.2	2	1.812	0.595	$K_s = 40$	7.15	6.88
		5	1.758	0.564	$K_p = 40$		
		10	1.678	0.517	No cor-		
		15	1.602	0.471	rections		
		20	1.527	0.423			
		25	1.485	0.396			
		30	1.422	0.352			
		35	1.358	0.306			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Part F. Runs made with tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer. No inhibitors added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
1	10.0	2	1.269	2.541	$K_s = 43$	7.70	7.67
		5	1.180	2.468	$K_p = 85$		
		10	1.045	2.347			
		20	0.839	2.127	1.017		
		25	0.745	2.008	1.020		
		30	0.658	1.884	1.025		
		35	0.581	1.760	1.030		
		40	0.518	1.645	1.032		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
2	3.00	2	0.398	1.381	$K_s = 43$	7.70	7.70
		5	0.358	1.275	$K_p = 85$		
		10	0.314	1.144	No cor-		
		15	0.279	1.026	rections		
		20	0.242	0.884			
		25	0.215	0.765			
		30	0.190	0.642			
		35	0.165	0.501			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln e			
3	30.0	2	1.565	0.448	$K_s = 43$	7.70	7.58
		5	1.490	0.399	$K_p = 85$		
		10	1.357	0.305	1.016		
		15	1.240	0.215	1.025		
		20	1.130	0.122	1.033		
		25	1.026	0.026	1.040		
		30	0.928	-0.075	1.050		
		35	0.837	-0.178	1.059		

1 ml. aliquots placed in 25 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
4	50.0	2	1.345	0.296	$K_s = 43$	7.70	7.50
		5	1.278	0.245	$K_p = 85$		
		10	1.190	0.174	1.018		
		15	1.099	0.091	1.030		
		20	1.022	0.022	1.040		
		25	0.950	-0.051	1.048		
		30	0.880	-0.128	1.059		
		35	0.808	-0.213	1.063		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

				ln e			
5	70.0	2	1.888	0.635	$K_s = 43$	7.62	7.30
		5	1.818	0.697	$K_p = 85$		
		10	1.717	0.541	1.022		
		15	1.594	0.466	1.030		
		20	1.491	0.400	1.040		
		25	1.400	0.336	1.048		
		30	1.321	0.278	1.057		
		35	1.227	0.204	1.065		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

				ln 10e			
6	3.00	2	0.387	1.353	$K_s = 43$	7.62	7.59
		5	0.353	1.261	$K_p = 85$		
		10	0.308	1.125			
		15	0.262	0.963	1.010		
		20	0.230	0.833	1.010		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
7	10.0	2	1.309	2.572	$K_s = 43$	7.62	7.56
		5	1.217	2.499	$K_p = 85$		
		10	1.060	2.361			
		15	0.940	2.241	1.020		
		20	0.817	2.100	1.020		
		25	0.721	1.975	1.027		
		30	0.634	1.847	1.027		
		35	0.560	1.723	1.034		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
8	30.0	2	1.576	0.455	$K_s = 43$	7.62	7.43
		5	1.490	0.399	$K_p = 85$		
		10	1.353	0.302	1.017		
		15	1.230	0.207	1.025		
		20	1.114	0.108	1.036		
		25	1.002	0.002	1.041		
		30	0.910	-0.094	1.051		
		35	0.830	-0.186	1.060		

1 ml. aliquots placed in 25 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
9a	50.0	2	1.323	2.589	$K_s = 43$	7.62	7.33
		5	1.273	2.545	$K_p = 85$		
		10	1.176	2.465	1.018		
		15	1.095	2.394	1.029		
		20	1.007	2.310	1.040		
		25	0.937	2.238	1.048		
		30	0.870	2.163	1.055		
		35	0.808	2.089	1.065		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
19	39.84	2	1.082	2.382	$K_s = 120$	8.90	8.80
		5	1.064	2.365			
		10	1.038	2.340	Values		
		20	0.969	2.271	from		
		30	0.923	2.222	Fig. 4		
		40	0.869	2.162			
		50	0.811	2.093			
		60	0.758	2.026			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
20	69.02	2	1.860	0.620	$K_s = 120$	8.81	8.65
		5	1.850	0.615			
		10	1.790	0.582	Values		
		20	1.683	0.521	from		
		30	1.582	0.459	Fig. 4		
		40	1.493	0.401			
		50	1.379	0.334			
		60	1.288	0.253			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

				ln 10e			
23	40.0	2	1.061	2.362	$K_s = 120$	8.90	8.82
		5	1.043	2.345			
		10	1.003	2.306	Values		
		20	0.951	2.252	from		
		30	0.890	2.186	Fig. 4		
		40	0.839	2.127			
		50	0.780	2.054			
		60	0.739	2.000			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

				ln e			
24	69.4	2	1.848	0.615	$K_s = 120$	8.81	8.70
		5	1.810	0.593			
		10	1.767	0.570	Values		
		20	1.644	0.497	from		
		30	1.550	0.438	Fig. 4		
		40	1.451	0.372			
		50	1.350	0.300			
		60	1.260	0.231			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
25	16.00	2	2.160	0.770	$K_s = 120$	9.03	8.98
		5	2.125	0.753			
		10	2.060	0.722	Values		
		20	1.940	0.663	from		
		30	1.858	0.620	Fig. 4		
		40	1.762	0.566			
		50	1.650	0.500			
		60	1.580	0.457			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
26	4.80	2	0.640	1.856	$K_s = 120$	9.10	9.09
		5	0.635	1.848			
		10	0.615	1.816	Values		
		20	0.589	1.773	from		
		30	0.564	1.730	Fig. 4		
		40	0.535	1.677			
		50	0.510	1.629			
		60	0.490	1.589			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
27	5.00	2	0.641	1.858	$K_s = 70$	8.16	8.12
		5	0.595	1.783			
		10	0.541	1.688	Values		
		20	0.440	1.482	from		
		30	0.353	1.261	Fig. 4		
		40	0.282	0.937			
		50	0.230	0.833			
		60	0.186	0.621			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
28	5.00	2	0.666	1.896	$K_s = 120$	9.05	9.01
		5	0.661	1.889			
		10	0.638	1.853	Values		
		25	0.595	1.783	from		
		40	0.558	1.719	Fig. 4		
		55	0.518	1.645			
		70	0.478	1.564			
		85	0.448	1.400			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

			ln 10e				
29	5.00	2	0.629	1.839	$K_s = 43$	7.70	7.68
		5	0.578	1.754	$K_p = 85$		
		10	0.502	1.613			
		15	0.437	1.472			
		20	0.380	1.335	1.011		
		25	0.333	1.203	1.013		
		30	0.287	1.054	1.017		
		35	0.249	0.912	1.020		

1 ml. aliquots placed in 10 ml. flask for color reaction.

			ln 10e				
30	5.00	2	0.660	1.887	$K_s = 105$	8.70	8.69
		5	0.633	1.845			
		10	0.605	1.800	Values		
		20	0.551	1.707	from		
		30	0.495	1.599	Fig. 4		
		40	0.448	1.499			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

			ln e				
31	15.87	2	2.060	0.723	$K_s = 105$	8.64	8.60
		5	2.030	0.708			
		10	1.903	0.644	Values		
		20	1.708	0.535	from		
		30	1.535	0.428	Fig. 4		
		40	1.377	0.320			
		50	1.220	0.199			
		60	1.098	0.094			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
32	4.76	2	0.606	1.802	K_s = 105	8.67	8.66
		5	0.602	1.795			
		10	0.560	1.723	Values		
		20	0.505	1.619	from		
		30	0.452	1.509	Fig. 4		
		40	0.405	1.399			
		50	0.365	1.295			
		60	0.322	1.169			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
33	39.67	2	1.016	2.319	K_s = 105	8.63	8.53
		5	0.987	2.290			
		10	0.947	2.248	Values		
		20	0.866	2.159	from		
		30	0.787	2.063	Fig. 4		
		40	0.722	1.977			
		50	0.650	1.872			
		60	0.592	1.778			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

				ln e			
34	65.8*	2	1.710	0.536	K_s = 105	8.61	8.50
		5	1.678	0.517			
		10	1.616	0.480	Values		
		20	1.502	0.406	from		
		30	1.378	0.320	Fig. 4		
		40	1.268	0.238			
		50	1.160	0.148			
		60	1.054	0.053			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

* Estimated from a comparison of the zero time extinction of this run with those of other runs made at the same time.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
35	15.84	2	2.078	0.731	K_s = 105	8.58	8.50
		5	1.995	0.690			
		10	1.892	0.639	Values		
		20	1.670	0.513	from		
		30	1.492	0.400	Fig. 4		
		40	1.323	0.280			
		60	1.034	0.033			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
36	4.75	2	0.618	1.821	K_s = 105	8.61	8.57
		5	0.593	1.780			
		10	0.567	1.735	Values		
		20	0.505	1.619	from		
		30	0.446	1.495	Fig. 4		
		40	0.397	1.379			
		50	0.350	1.253			
		60	0.310	1.131			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln e			
37	70.65	2	1.833	0.606	K_s = 105	8.60	8.42
		5	1.807	0.591			
		10	1.713	0.538	Values		
		20	1.586	0.461	from		
		30	1.472	0.386	Fig. 4		
		40	1.352	0.302			
		60	1.130	0.122			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

				ln 10e			
38	39.59	2	1.012	2.315	K_s = 105	8.61	8.49
		5	0.992	2.295			
		10	0.945	2.246	Values		
		20	0.862	2.153	from		
		30	0.790	2.067	Fig. 4		
		40	0.720	1.974			
		50	0.647	1.867			
		60	0.579	1.756			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
39	10.0	2	1.271	2.543	$K_s = 43$	7.60	7.49
		5	1.176	2.465	$K_p = 85$		
		10	1.042	2.344			
		15	0.920	2.219	1.020		
		20	0.809	2.091	1.022		
		25	0.708	1.957	1.026		
		30	0.634	1.847	1.030		
		35	0.556	1.716	1.030		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

				ln e			
40	140.6	2	1.845	0.613	$K_s = 43$	7.60	7.13
		5	1.820	0.599	$K_p = 85$		
		10	1.751	0.560	1.023		
		15	1.703	0.532	1.029		
		20	1.642	0.496	1.028		
		25	1.589	0.463	1.035		
		30	1.539	0.431	1.041		
		35	1.472	0.387	1.051		

1 ml. aliquots placed in 100 ml. flasks for color reaction.

				ln 10e			
41	39.59	2	1.034	2.336	$K_s = 70$	8.20	8.08
		5	0.986	2.288			
		10	0.926	2.226	Values		
		15	0.862	2.154	from		
		20	0.810	2.092	Fig. 4		
		25	0.750	2.015			
		30	0.696	1.940			
		35	0.644	1.863			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
42	70.2	2	1.838	0.609	$K_s = 70$	8.18	8.00
		5	1.783	0.578			
		10	1.700	0.531	Values		
		15	1.598	0.469	from		
		20	1.511	0.413	Fig. 4		
		25	1.448	0.370			
		30	1.352	0.302			
		35	1.288	0.253			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

				ln 10e			
43	4.75	2	0.618	1.821	$K_s = 70$	8.21	8.18
		5	0.584	1.765			
		10	0.526	1.660	Values		
		15	0.478	1.564	from		
		20	0.438	1.477	Fig. 4		
		25	0.399	1.384			
		30	0.357	1.276			
		35	0.328	1.188			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln e			
44	15.84	2	2.022	0.705	$K_s = 70$	8.21	8.14
		5	1.930	0.657			
		10	1.761	0.566	Values		
		15	1.630	0.488	from		
		20	1.476	0.389	Fig. 4		
		25	1.348	0.298			
		30	1.241	0.216			
		35	1.135	0.127			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
45	15.98	2	2.040	0.713	$K_s = 50$	7.88	7.78
		5	1.930	0.658	$K_p = 160$		
		10	1.750	0.560			
		15	1.578	0.456	1.020		
		20	1.433	0.360	1.030		
		25	1.280	0.247	1.040		
		30	1.155	0.144	1.045		
		35	1.041	0.040	1.050		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
46a	4.79	2	0.612	1.812	$K_s = 50$	7.88	7.86
		5	0.569	1.739	$K_p = 160$		
		10	0.505	1.619			
		15	0.451	1.506			
		20	0.400	1.386			
		25	0.356	1.270	1.017		
		30	0.314	1.144	1.019		
		35	0.280	1.030	1.020		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln e			
47a	70.0	2	1.839	0.609	$K_s = 50$	7.88	7.64
		5	1.791	0.583	$K_p = 160$		
		10	1.692	0.526	1.020		
		15	1.602	0.471	1.035		
		20	1.507	0.410	1.040		
		25	1.428	0.356	1.051		
		30	1.350	0.300	1.060		
		35	1.270	0.239	1.071		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
48a	39.95	2	1.036	2.338	$K_s = 50$	7.88	7.72
		5	0.994	2.297	$K_p = 160$		
		10	0.912	2.210	1.025		
		15	0.850	2.140	1.032		
		20	0.792	2.069	1.043		
		25	0.724	1.980	1.053		
		30	0.675	1.910	1.065		
		35	0.624	1.831	1.078		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

				ln 10e			
58	39.8	2	1.019	2.322	$K_s = 42$	7.20	6.78
		5	0.968	2.270	$K_p = 42$		
		10	0.897	2.194			
		15	0.822	2.107	No cor-		
		20	0.756	2.023	rections		
		25	0.707	1.956			
		30	0.660	1.887			
		35	0.623	1.829			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

				ln e			
59	70.0	2	1.808	0.592	$K_s = 42$	7.20	6.62
		5	1.748	0.558	$K_p = 42$		
		10	1.636	0.492			
		15	1.557	0.443	No cor-		
		20	1.480	0.392	rections		
		25	1.413	0.346			
		30	1.356	0.304			
		35	1.302	0.264			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
60	4.78	2	0.586	1.768	K_s = 42	7.20	7.12
		5	0.541	1.688	K_p = 42		
		10	0.459	1.524			
		15	0.397	1.379	No cor-		
		20	0.347	1.244	rections		
		25	0.298	1.092			
		30	0.258	0.948			
		35	0.223	0.802			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln e			
61	15.92	2	1.990	0.688	K_s = 42	7.20	6.98
		5	1.830	0.604	K_p = 42		
		10	1.636	0.492			
		15	1.443	0.366	No cor-		
		20	1.293	0.257	rections		
		25	1.137	0.128			
		30	1.022	0.022			
		35	0.916	-0.088			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln 10e			
88	4.80	2	0.629	1.839	K_s = 105	8.60	8.60
		5	0.605	1.800			
		10	0.565	1.732	Values		
		20	0.494	1.597	from		
		30	0.430	1.459	Fig. 4		
		40	0.379	1.332			
		50	0.330	1.194			
		60	0.287	1.054			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	$\frac{e}{}$	ln $\frac{e}{}$	f_1	Initial pH	Final pH
91	70.3	2	1.868	0.624	K_s = 105	8.59	8.47
		5	1.821	0.600			
		10	1.750	0.560	Values		
		20	1.624	0.485	from		
		30	1.489	0.398	Fig. 4		
		40	1.362	0.309			
		50	1.242	0.216			
		60	1.118	0.112			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

				ln $\frac{10e}{}$			
96b	4.81	2	0.637	1.852	K_s = 105	8.60	8.60
		5	0.615	1.816			
		10	0.582	1.761	Values		
		20	0.524	1.656	from		
		30	0.466	1.539	Fig. 4		
		40	0.414	1.421			
		50	0.373	1.316			
		60	0.331	1.197			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln $\frac{e}{}$			
99a	70.0	2	1.869	0.625	K_s = 105	8.59	8.42
		5	1.825	0.602			
		10	1.753	0.561	Values		
		20	1.610	0.476	from		
		30	1.481	0.392	Fig. 4		
		40	1.350	0.300			
		50	1.230	0.207			
		60	1.119	0.112			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
100b	16.05	2	2.043	0.715	$K_s = 50$	7.80	7.80
		5	1.889	0.636	$K_p = 160$		
		10	1.652	0.502			
		15	1.457	0.376	1.020		
		20	1.273	0.242	1.021		
		25	1.110	0.104	1.028		
		30	0.968	-0.033	1.031		
		35	0.840	-0.174	1.038		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln e			
115	70.0	2	1.871	0.626	$K_s = 105$	8.59	8.40
		5	1.822	0.600			
		10	1.752	0.561	Values		
		20	1.613	0.478	from		
		30	1.485	0.396	Fig. 4		
		40	1.350	0.300			
		50	1.224	0.202			
		60	1.108	0.102			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

				ln 10e			
117	4.82	4	0.610	1.808	$K_s = 105$	8.60	8.57
		10	0.558	1.719			
		20	0.485	1.579	Values		
		40	0.368	1.303	from		
		60	0.276	1.015	Fig. 4		
		80	0.206	0.723			
		100	0.155	0.438			
		120	0.118	0.166			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
120	70.4	2	1.857	0.619	$K_s = 50$	7.83	7.61
		5	1.778	0.575	$K_p = 160$		
		10	1.661	0.507	1.025		
		15	1.555	0.441	1.033		
		20	1.448	0.370	1.050		
		25	1.348	0.299	1.062		
		30	1.247	0.221	1.080		
		35	1.164	0.152	1.089		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Part G. Runs made with tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer. 0.04 M calcium chloride added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	$\underline{e}$	ln $10\underline{e}$	f_1	Initial pH	Final pH
13	5.21	2	0.650	1.872	$K_s = 45$	7.57	7.54
		5	0.584	1.765	$K_p = 90$		
		10	0.498	1.605			
		15	0.417	1.428	1.010		
		20	0.348	1.247	1.015		
		25	0.291	1.068	1.018		
		30	0.241	0.880	1.020		
		35	0.201	0.698	1.023		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln $\underline{e}$			
14	16.79	2	2.120	0.751	$K_s = 45$	7.57	7.47
		5	1.936	0.660	$K_p = 90$		
		10	1.680	0.519			
		15	1.440	0.364	1.023		
		20	1.240	0.215	1.032		
		25	1.055	0.054	1.046		
		30	0.918	-0.086	1.049		
		35	0.787	-0.240	1.057		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

				ln $\underline{e}$			
15	70.44	2	1.850	0.615	$K_s = 45$	7.57	7.20
		5	1.777	0.575	$K_p = 90$		
		10	1.636	0.492	1.018		
		15	1.516	0.416	1.033		
		20	1.406	0.341	1.043		
		25	1.300	0.262	1.050		
		30	1.206	0.187	1.062		
		35	1.114	0.108	1.073		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
16	39.84	2	2.080	0.732	$K_s = 45$	7.57	7.29
		5	1.933	0.659	$K_p = 90$		
		10	1.740	0.554	1.020		
		15	1.559	0.444	1.038		
		20	1.398	0.335	1.048		
		25	1.239	0.214	1.058		
		30	1.116	0.110	1.070		
		35	0.993	-0.007	1.076		

1 ml. aliquots placed in 25 ml. flasks for color reaction.

Part H. Runs made with tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer. Inhibitors added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
86	4.79	4	0.632	1.844	No corrections	8.60	8.60
		10	0.611	1.810			
		20	0.566	1.733			
		40	0.499	1.607			
		60	0.440	1.482			
		80	0.381	1.338			
		100	0.338	1.218			
		120	0.296	1.085			

1 ml. aliquots placed in 10 ml. flasks for color reaction.
2.47×10^{-3} M acetyl-D-tryptophanamide added.

				ln e			
87	15.99	4	2.105	0.745	$K_s = 200$	8.60	8.52
		10	2.016	0.701			
		20	1.898	0.640	Values		
		40	1.675	0.516	from		
		60	1.478	0.390	Fig. 4		
		80	1.272	0.240			
		100	1.116	0.110			
		120	0.980	-0.020			

1 ml. aliquots placed in 10 ml. flasks for color reaction.
2.47×10^{-3} M acetyl-D-tryptophanamide added.

				ln 10e			
89	39.9	4	1.048	2.350	$K_s = 200$	8.60	8.51
		10	1.009	2.312			
		20	0.961	2.263	Values		
		40	0.858	2.149	from		
		60	0.770	2.041	Fig. 4		
		80	0.676	1.911			
		100	0.598	1.788			
		120	0.530	1.668			

1 ml. aliquots placed in 50 ml. flasks for color reaction.
2.47×10^{-3} M acetyl-D-tryptophanamide added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
90	70.0	4	1.833	0.606	K_s = 200	8.60	8.40
		10	1.790	0.582			
		20	1.705	0.534	Values		
		40	1.540	0.433	from		
		60	1.379	0.322	Fig. 4		
		80	1.247	0.221			
		100	1.120	0.113			
		120	0.992	-0.008			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

2.47×10^{-3} M acetyl-D-tryptophanamide added.

				ln 10e			
94b	4.81	4	0.635	1.848	K_s = 150	8.60	8.56
		10	0.605	1.800			
		20	0.561	1.725	Values		
		40	0.481	1.571	from		
		60	0.418	1.430	Fig. 4		
		80	0.354	1.264			
		100	0.301	1.102			
		120	0.258	0.948			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

29.9×10^{-3} M acetyl-L-tyrosinamide added.

				ln e			
95b	16.03	4	2.120	0.751	K_s = 150	8.60	8.51
		10	2.025	0.705			
		20	1.890	0.636	Values		
		40	1.633	0.490	from		
		60	1.400	0.336	Fig. 4		
		80	1.198	0.181			
		100	1.019	0.019			
		120	0.870	-0.139			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

30.0×10^{-3} M acetyl-L-tyrosinamide added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
97	70.3	4	1.850	0.615	$K_s = 150$	8.60	8.39
		10	1.780	0.576			
		20	1.675	0.516	Values		
		40	1.471	0.386	from		
		60	1.291	0.256	Fig. 4		
		80	1.112	0.113			
		100	0.950	-0.051			
		120	0.810	-0.210			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

30.0 x 10^{-3} M acetyl-L-tyrosinamide added.

				ln 10e			
98	40.0	4	1.058	2.359	$K_s = 150$	8.60	8.49
		10	1.012	2.315			
		20	0.940	2.241	Values		
		40	0.810	2.092	from		
		60	0.688	1.929	Fig. 4		
		80	0.584	1.765			
		100	0.493	1.595			
		120	0.410	1.411			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

29.9 x 10^{-3} M acetyl-L-tyrosinamide added.

				ln e			
99b	16.05	2	2.096	0.740	$K_s = 50$	7.88	7.80
		5	2.035	0.710	$K_p = 90$		
		10	1.899	0.641	$K_I = 30$		
		20	1.654	0.503			
		30	1.449	0.371	1.010		
		40	1.268	0.238	1.010		
		50	1.109	0.103	1.010		
		60	0.957	-0.044	1.015		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

50.0 x 10^{-3} M acetyl-L-tyrosinamide added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
100a	16.01	2	2.100	0.743	$K_s = 50$	7.88	7.77
		5	1.998	0.691	$K_p = 90$		
		10	1.841	0.610	$K_I = 30$		
		20	1.566	0.449			
		30	1.318	0.276	1.011		
		40	1.110	0.104	1.012		
		50	0.931	-0.071	1.020		
		60	0.779	-0.250	1.021		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

30.1 x 10^{-3} M acetyl-L-tyrosinamide added.

				ln e			
101	16.05	2	2.083	0.735	$K_s = 50$	7.88	7.81
		5	1.970	0.678	$K_p = 90$		
		10	1.790	0.582	$K_I = 30$		
		15	1.634	0.491			
		20	1.486	0.396	1.010		
		25	1.350	0.300	1.018		
		30	1.228	0.206	1.020		
		35	1.108	0.103	1.022		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

20.0 x 10^{-3} M acetyl-L-tyrosinamide added.

				ln e			
102	16.05	2	2.060	0.723	$K_s = 50$	7.88	7.82
		5	1.910	0.646	$K_p = 90$		
		10	1.695	0.527	$K_I = 30$		
		15	1.504	0.408	1.017		
		20	1.340	0.292	1.018		
		25	1.182	0.167	1.023		
		30	1.048	0.047	1.026		
		35	0.920	-0.083	1.032		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

5.00 x 10^{-3} M acetyl-L-tyrosinamide added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
103	16.05	2	2.065	0.723	$K_s = 50$	7.88	7.83
		5	1.938	0.663	$K_p = 90$		
		10	1.734	0.550	$K_I = 30$		
		15	1.561	0.445			
		20	1.396	0.334	1.017		
		25	1.245	0.219	1.020		
		30	1.112	0.106	1.025		
		35	0.996	-0.004	1.026		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

10.0×10^{-3} M acetyl-L-tyrosinamide added.

				ln e			
106	16.0	2	2.100	0.742	$K_s = 105$	8.60	8.50
		5	2.035	0.710			
		10	1.936	0.660	Values		
		20	1.730	0.548	from		
		30	1.540	0.432	Fig. 4		
		40	1.365	0.312			
		50	1.218	0.197			
		60	1.075	0.072			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

45.0×10^{-3} M acetyl-L-tyrosine added.

				ln e			
113	69.7	4	1.839	0.609	$K_s = 105$	8.60	8.39
		10	1.755	0.562			
		20	1.638	0.494	Values		
		40	1.388	0.328	from		
		60	1.173	0.160	Fig. 4		
		80	0.977	-0.023			
		100	0.807	-0.214			
		120	0.656	-0.421			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

45.0×10^{-3} M acetyl-L-tyrosine added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
114	40.1	4	1.057	2.358	$K_s = 105$	8.60	8.44
		10	1.003	2.306			
		20	0.911	2.209	Values		
		40	0.753	2.019	from		
		60	0.606	1.802	Fig. 4		
		80	0.492	1.593			
		100	0.392	1.366			
		120	0.310	1.131			

1 ml. aliquots placed in 50 ml. flasks for color reaction.

45.0 x 10^{-3} M acetyl-L-tyrosine added.

				ln 10e			
116	4.81	4	0.624	1.831	$K_s = 105$	8.60	8.56
		10	0.580	1.758			
		20	0.515	1.639	Values		
		40	0.409	1.409	from		
		60	0.320	1.163	Fig. 4		
		80	0.251	0.920			
		100	0.198	0.683			
		120	0.156	0.445			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

44.8 x 10^{-3} M acetyl-L-tyrosine added.

				ln 10e			
118	40.0	2	1.049	2.351	$K_s = 50$	7.88	7.71
		5	1.006	2.309	$K_p = 160$		
		10	0.951	2.252			
		20	0.837	2.125	1.029		
		30	0.739	2.000	1.041		
		45	0.609	1.807	1.059		
		60	0.502	1.613	1.076		
		75	0.411	1.413	1.091		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

89.9 x 10^{-3} M acetyl-L-tyrosine added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
119	70.3	2	1.868	0.625	$K_s = 50$	7.88	7.60
		5	1.809	0.592	$K_p = 160$		
		10	1.723	0.544	1.018		
		20	1.572	0.451	1.025		
		30	1.422	0.352	1.045		
		45	1.232	0.208	1.065		
		60	1.066	0.064	1.083		
		75	0.918	-0.086	1.103		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

89.9×10^{-3} M acetyl-L-tyrosine added.

				ln e			
121	16.03	2	2.083	0.734	$K_s = 50$	7.88	7.82
		5	1.990	0.688	$K_p = 160$		
		10	1.848	0.614			
		20	1.576	0.455	1.019		
		30	1.343	0.295	1.023		
		45	1.058	0.056	1.032		
		60	0.830	-0.186	1.045		
		75	0.652	-0.427	1.050		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

90.0×10^{-3} M acetyl-L-tyrosine added.

				ln 10e			
122	4.80	2	0.627	1.836	$K_s = 50$	7.88	7.82
		5	0.591	1.777	$K_p = 160$		
		10	0.547	1.699			
		20	0.457	1.520			
		30	0.388	1.356	1.010		
		45	0.301	1.102	1.012		
		60	0.237	0.863	1.016		
		75	0.182	0.599	1.015		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

89.6×10^{-3} M acetyl-L-tyrosine added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	$\underline{e}$	ln $10\underline{e}$	f_1	Initial pH	Final pH
125	4.80	4	0.561	1.725	$K_s = 50$	7.30	7.30
		10	0.523	1.654	$K_p = 50$		
		20	0.467	1.541			
		40	0.378	1.330	No cor-		
		60	0.309	1.128	rections		
		80	0.243	0.888			
		100	0.198	0.683			
		120	0.160	0.470			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

90.0×10^{-3} M acetyl-L-tyrosine added.

				ln $10\underline{e}$			
126	4.80	2	0.571	1.742	$K_s = 43$	7.60	7.60
		5	0.546	1.697	$K_p = 90$		
		10	0.506	1.621			
		20	0.438	1.477			
		30	0.380	1.335			
		40	0.326	1.182			
		50	0.281	1.033			
		60	0.243	0.888	1.010		

1 ml. aliquots placed in 10 ml. flasks for color reaction.

90.0×10^{-3} M acetyl-L-tyrosine added.

				ln $10\underline{e}$			
127	4.80	2	0.573	1.746	$K_s = 80$	8.26	8.26
		5	0.552	1.708			
		10	0.514	1.637	No cor-		
		20	0.449	1.502	rections		
		30	0.391	1.364			
		40	0.340	1.224			
		50	0.297	1.089			
		60	0.258	0.945			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

89.5×10^{-3} M acetyl-L-tyrosine added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln 10e	f_1	Initial pH	Final pH
130	4.80	4	0.639	1.856	No corrections	8.21	8.21
		10	0.630	1.841			
		20	0.608	1.805			
		40	0.567	1.735			
		60	0.534	1.675			
		80	0.500	1.609			
		108	0.451	1.506			
		120	0.436	1.472			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

55.4 x 10^{-3} M L-tyrosinamide added.

				ln 10e			
131	4.80	4	0.646	1.866	No corrections	8.57	8.57
		10	0.639	1.855			
		20	0.624	1.831			
		40	0.597	1.787			
		60	0.574	1.747			
		80	0.552	1.708			
		86	0.540	1.686			
		120	0.502	1.613			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

55.4 x 10^{-3} M L-tyrosinamide added.

				ln 10e			
132	4.80	4	0.637	1.852	No corrections	7.60	7.59
		10	0.616	1.818			
		20	0.591	1.777			
		40	0.536	1.679			
		60	0.486	1.581			
		80	0.445	1.493			
		100	0.401	1.389			
		120	0.362	1.286			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

55.4 x 10^{-3} M L-tyrosinamide added.

Expt. No.	$[S]_o \times 10^3$ [M]	Time [Min.]	e	ln e	f_1	Initial pH	Final pH
133	16.0	4	2.110	0.746	No corrections	7.60	7.60
		10	2.066	0.725			
		20	1.968	0.676			
		40	1.788	0.581			
		60	1.621	0.483			
		80	1.478	0.390			
		100	1.341	0.294			
		120	1.222	0.200			

1 ml. aliquots placed in 10 ml. flasks for color reaction.

55.4 x 10^{-3} M L-tyrosinamide added.

				ln e			
134	70.0	4	1.868	0.624	$K_s = 45$	7.60	7.58
		10	1.814	0.596	$K_p = 90$		
		20	1.752	0.561	$K_I = 10$		
		40	1.630	0.489			
		60	1.516	0.416	1.010		
		80	1.408	0.342	1.010		
		100	1.317	0.275	1.018		
		120	1.223	0.202	1.020		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

55.4 x 10^{-3} M L-tyrosinamide added.

				ln 10e			
135	40.0	4	1.055	2.357	$K_s = 45$	7.60	7.58
		10	1.033	2.335	$K_p = 90$		
		20	0.994	2.297	$K_I = 10$		
		40	0.908	2.206			
		60	0.842	2.131			
		80	0.776	2.049			
		100	0.709	1.959			
		108	0.685	1.924	1.012		

1 ml. aliquots placed in 50 ml. flasks for color reaction.

55.4 x 10^{-3} M L-tyrosinamide added.

PART III

SOME GENETIC STUDIES IN *NEUROSPORA CRASSA*

A. Some tests designed to indicate optimal conditions for the expression of the phenotype dotted.

It has frequently been observed that the cultivation of Neurospora crassa on liquid media deficient in sulfur leads to the formation of black areas, usually about 1 mm. in diameter, in the mycelial mass. The ability to form such spots is apparently inherited since many stocks do not show spotting when grown on low sulfur medium. As a preliminary to possible genetic study, it was deemed advisable to determine the conditions required for good expression of the phenotype.

In the following series of experiments, the cultures were grown on liquid medium which was the same as the standard minimal medium (1) in every respect except for sulfate, which was added only in the amounts noted below. The spore cultures tested were 0-312 and 0-112, which previous evidence indicated would not give dotted, and 4-431 and 4-433, progeny of a cross of 0-312 and 0-112. These latter cultures had been observed to show dotted on low sulfate medium.

Each of these stocks was set up in duplicate cultures under the following conditions of volume of medium, amount of sulfate, temperature, and illumination (those cultures designated dark were covered with opaque paper; those designated light were subjected to continuous illumination by a 100 watt incandescent lamp.)

TABLE LII

Various Conditions Used in Obtaining Indications of Optimal Conditions for the Expression of the Phenotype Dotted

Condition	Test Number				
	1	2	3	4	5
Total volume of medium (ml.)	20	20	20	20	10
ml. of $MgSO_4$ solution (3.14 mg. $MgSO_4 \cdot 7H_2O$ per ml.)	0.03	0.03	0.03	1.25	0.015
Temperature	25	35	35	35	35
Illumination	Dark	Dark	Light	Dark	Dark

The results of these tests may be summarized in tabular form.

TABLE LIII

Summary of Tests to Determine Satisfactory Conditions of Growth for Expression of Dotted

Age (days)	Test Condition	Spore No.	Results and Comments
5	All	All	No appearance of dotted
7	1	0-112	No appearance of dotted
7	1	0-312	Dotted
7	1	4-431	Dotted
7	1	4-433	Dotted
7	Other than 1	All	No result
10	1	0-112	No result
10	1	0-312	Heavily dotted
10	1	4-431	Heavily dotted
10	1	4-433	Heavily dotted
10	2	0-112	No result
10	2	0-312	No result
10	2	4-431	A few spots
10	2	4-433	A few spots
10	Other than 1 and 2	All	No results
18	1	0-112	No result
18	1	0-312	Heavily dotted
18	1	4-431	Heavily dotted
18	1	4-433	Heavily dotted

Age (days)	Test Condition	Spore No.	Results and Comments
18	2	0-112	No result
18	2	0-312	No result
18	2	4-431	Dotted
18	2	4-433	Dotted
18	5	All	Dried up
18	3 and 4	All	No result

These results indicate the better conditions to be low sulfate, about 20 ml. volume, 25°, darkness, and about 7 days growing time.

In order to obtain a better idea of the effect of low sulfate on the expression of dotted, the following experiment, summarized in Table LIV , was carried out with 4-433.

TABLE LIV

Test Designed to Obtain Information Regarding the Influence of Sulfate Concentration on the Expression of Dotted

All flasks inoculated with 4-433.

Volume of medium = 20 ml.

Cultures grown in darkness.

Total mg. of $MgSO_4$ added to flask	Temperature of cultivation	Result after eight days
0.0	25	Inconclusive, some dark mycelium
0.0	35	No results
0.0195	25	Spots and darkened mycelium
0.0195	35	No results
0.0390	25	Spots and darkened mycelium
0.0390	35	No results
0.078	25	Spots and darkened mycelium
0.078	35	No results
0.156	25	A few spots
0.156	35	No results
0.314	25	No spots; some dark mycelium
0.314	35	No results
0.628	25	A little black mycelium
0.628	35	No results

Total mg. of $MgSO_4$ added to flask	Temperature of cultivation	Result after eight days
0.942	25	No results
0.942	35	No results
1.884	25	No results
1.884	35	No results

These data again indicate that a temperature of 25° is to be preferred to 35°. Optimum sulfate concentration seems to be in the region 0.02 to 0.08 mg. of $MgSO_4$ per 20 ml. of medium.

These tests are not conclusive insofar as true optimum conditions are concerned. They do, however, indicate some preferred conditions of culture. Further testing is necessary to establish whether or not these conditions will guarantee the expression of dotted. Some of the evidence seems to indicate quantitative differences between stocks which exhibit the dotted phenotype.

B. Genetic studies to determine the linkage and centromere distance of the gene controlling tyrosinase thermostability in Neurospora crassa.

The tyrosinase activity in Neurospora is greatly enhanced by cultivation on low sulfur media (2). This high activity permits the study of the tyrosinase enzyme in some detail (2, 3). It has been found that some stocks of Neurospora, when cultivated on low sulfur media, yield a tyrosinase preparation which is resistant to heating; while other stocks yield a thermolabile tyrosinase preparation under identical conditions. This behavior can be attributed to the action of a single pair of alleles (3). The present series of experiments is concerned with the determination of the linkage and centromere distance of the gene governing the thermostability of the Neurospora tyrosinase.

In the following discussion the alleles are designated as

T^L -- gene for thermolability of tyrosinase

T^S -- gene for thermostability of tyrosinase

Al -- albino 15300 mutant

Adp -- adenineless purple mutant 35203

A and a -- refer to mating types

Dissections of asci resulting from the two crosses T^L Al A x T^S adp a and T^L Al a x T^S Adp A were carried out and the resulting spores were germinated in the usual manner. Best results were obtained when some time was allowed for spontaneous germination before heat treatment. Spores numbered without prefixing number result from dissection by R. Jennings; those with prefixing numbers are the result of dissections by M. Fling.

The cultures were scored for Al and Adp on the basis of their appearance while growing on the complete medium. In order to obtain information regarding the centromere distance of T^L, only two spore pairs of the ascus needed to be tested; consequently, only the spores wild type with respect to Adp were analyzed for T^L and T^S (with a few exceptions). Transfers of these cultures were made to a low sulfate medium (3) and grown for 5 - 7 days. Two pads were grown for each spore pair in order that enough material might be obtained for testing. The testing method has been presented in detail elsewhere (3). In the present case, visual observation of the color development resulting from the action of tyrosinase on tyrosine was employed as a qualitative indication of tyrosinase activity.

In brief outline, then, the pads were harvested, washed several times, dried, weighed, and then ground with sand in the presence of a phosphate buffer. The sand and debris were centrifuged and a cloudy enzyme solution was obtained. The grinding and centrifuging were carried out at 5 - 10°C. The enzyme preparation resulting from a culture of a single spore was then divided into two portions. One portion was placed in a 60° water bath for 20 minutes, while the other was maintained at a low temperature. Both portions were brought to room temperature, and a solution of tyrosine was added to each. The development of color was observed after two hours, and the conclusions reached were checked after 12 hours. If color develops in both the heat treated and the refrigerated portions, the spore is scored T^S, if color develops in only the chilled portion, the spore is scored T^L. If color develops in neither of the two portions, the results are, of course, inconclusive.

The results did not indicate any differences in the behavior of the products of the two crosses described above, so the data have been treated together. Spores numbered from 0 to 348 and all of those designated 25- are from the cross T^L Al A x T^S Adp a; the other spores are from the cross T^L Al a x T^S Adp A. These data are summarized in Table LV. It might be noted that the visually observable phenotypes of the spores designated with prefixing number have been scored by two independent observers (Jennings and Fling) and agreement was obtained. A few of the observations on T^L and T^S were also duplicated. Only observed phenotypes are indicated in the table. In a few cases, Adp spores were tested for thermostability (naturally adenine was added to the cultivation medium).

Linkage is shown by the absence of the independent classes Al Al T^L T^L and Adp Adp T^S T^S.

The sequence of genes on the chromosome is shown by calculations of the centromere distances of the various genes made without taking into account the observed double crossover classes. These values may be compared with values which have been determined by previous investigators who were not able to detect double crossover products in the absence of markers. The distances thus computed are

Adp (classes 4, 5, and 6) = 3.6 (lit. 2.1 (4)).

Al 15300 (classes 2, 3, 4, 6, and 7) = 28.5 (lit. 24.6 (4)).

T^L (classes 3, 4, 5, 6, and 7) = 21.4 (lit. 30 (3)).

Minimum values for the various distances along the chromosome may be calculated from the observed crossovers. Crossovers between the centromere and Adp (classes 4, 5, and 6) give 3.6 for this distance.

TABLE LV

Results of Testing of Asci Resulting From the Cross

Al T^L x T^S Adp

Ascus	Spore Pair				Class
	1	2	3	4	
19	T^S	T^L Al	Al Adp	---	3
20	Al T^L	Al T^L	---	Adp	1
22	Al T^L	Al T^L	Adp	Adp	1
27	T^S	Al T^L	Al Adp	Adp	3
32	Adp	Al Adp	Al T^L	T^L	2
34	Adp	Adp	Al T^L	Al T^L	1
35	Adp	Adp	Al T^L	Al T^L	1
37	Adp	Al Adp	Al T^L	T^S	3
39	Adp	T^L	Al Adp	Al T^L	5
40	Adp	Adp	Al T^L	Al T^L	1
41	Adp	Al Adp	T^L	Al T^L	2
42	T^L	Al T^L	Adp	Al Adp	2
46	Al T^L	Al T^L	Adp	Adp	1
47	Al T^L	T^S	Adp	Al Adp	3
48	Adp	Adp	Al T^L	Al T^L	1
49	T^S	Al T^L	Al Adp	Adp	3
57	Al T^L	T^S	Adp	Al Adp	3
58	Al T^L	Al T^L	Adp	Adp	1
61	Adp	Adp	Al T^L	Al T^L	1
24-11	Al Adp	Adp	T^S	Al T^L	3
24-12	Al Adp	Adp	T^S	Al T^L	3
24-14	Adp	T^L	Al Adp	Al T^L	5

Ascus	Spore Pair				Class
	1	2	3	4	
24-31	T^S	Al T^L	Adp	Al Adp	3
24-32	Al T^L	Al T^L	Adp	Adp	1
24-44	Al T^L	Al T^L	Adp	Adp	1
24-51	Al T^L	Adp	---	T^S	6
24-61	Al Adp	Adp	Al T^L	T^S	3
24-62	T^L	Al T^L	Al Adp	Adp	2
24-63	Adp	Adp	Al T^L	Al T^L	1
24-71	Adp	---	Al T^L	Al T^L	1
24-81	T^L	Al T^L	Al Adp	---	2
24-102	Adp	Adp	Al T^L	Al T^L	1
24-103	Adp	Adp	Al T^L	Al T^L	1
24-104	Adp	Al Adp	Al T^L	T^L	2
24-105	Adp	Adp	Al T^L	Al T^L	1
24-107	Adp	Al Adp	T^S	Al T^L	3
24-108	Adp	Adp	Al T^L	Al T^L	1
24-109	Adp	Adp	Al T^L	Al T^L	1
24-1011	Al T^L	Adp	Al T^L	---	4
25-11	Adp	Al Adp T^L	T^S	Al T^L	3
25-12	Al Adp	Adp	Al T^L	T^S	3
25-13	Al Adp	---	T^L	Al T^L	2
25-14	Al T^L	T^S	Adp	Al Adp	3
25-15	Al Adp	Adp	Al T^L	T^S	3
25-23	Al Adp	Adp	Al T^L	T^S	3
25-41	Al T^L	Al T^L	Adp	Adp	1
25-42	Al T^L	T^S	Adp	Al Adp	3
25-43	Adp	Al Adp	T^L	Al T^L	2

Ascus	Spore Pair				Class
	1	2	3	4	
25-51	Al T^L	Al T^L	Adp	----	1
25-52	Adp	Adp	Al T^L	Al T^L	1
25-53	Adp	Al Adp T^L	Al T^L	T^S	3
25-54	Al Adp T^S	Adp	T^L	Al T^L	2
25-55	Adp	Adp	Al T^L	Al T^L	1
25-56	Al Adp	Adp	Al T^L	T^S	3
25-57	Al T^S	T^L	Al Adp	Adp	8
25-58	Al T^L	T^L	Al Adp T^S	Adp	2

TABLE LVI

Summary of Asci Resulting From the Cross Al T^L x T^S Adp

Class of Ascus	Spore Pair				No. of Asci
	1	2	3	4	
1	Al T^L	Al T^L	Adp	Adp	22
2	T^L	Al T^L	Adp	Al Adp	10
3	Al T^L	T^S	Al Adp	Adp	19
4	Al T^L	Adp	Al T^L	Adp	1
5	Adp	T^L	Al Adp	Al T^L	2
6	Al T^L	Adp	Al Adp	T^S	1
7	Al T^S	T^L	Al Adp	Adp	1
				Total	56

Crossovers between Adp and T^S (classes 3, 6, and 7) give 18.8 as the distance from Adp to T^S. Crossovers between T^L and Al (classes 2, 5, and 7) give 11.6 as the distance from T^L to Al. On this basis, the minimum centromere distance which can be obtained from these data are Adp = 3.6, T^S = 22.4, and Al = 34.0.

Two attempts were made to obtain data with respect to mating type. In both cases the spore cultures listed above were employed as the perithecial parents and inoculated with wild type (A in one instance, a in the other). These crosses proved to be rather infertile, particularly those involving the adenineless purple mutant, and meaningful data could not be obtained. Since, however, it has been previously established that both Adp 35203 and Al 15300 are in the sex chromosome, it appears evident that the gene controlling thermostability in tyrosinase is also in that chromosome.

REFERENCES

(1) Horowitz, N. H., J. Biol. Chem. 171, 255 (1947).

(2) Horowitz, N. H., and San-Chium Shen, J. Biol. Chem. 197, 513 (1952).

(3) Horowitz, N. H., and Fling, M., Genetics, 38, 360 (1953).

(4) Houlahan, M., Beadle, G., and Calhoun, H., Genetics, 34, 493 (1949).

PROPOSITIONS

1. The progress of the hydrolysis of half-esters of hydroquinone might be followed continuously at constant pH by measurements of the potential of a quinone-hydroquinone electrode immersed in the reaction solution. This technique might be applied to enzyme catalyzed hydrolyses.

   1) Glasstone, S. Physical Chemistry. New York: D. Van Nostrand Co., 1946.

2. The unintegrated expression for the velocity of an enzyme reaction leads to the use of plots of v_o vs. $v_o/[S]_o$, $1/v_o$ vs. $1/[S]_o$, and $[S]_o/v_o$ vs. $[S]_o$ for the evaluation of kinetic constants. I propose that there are two more plots related to the Walker-Schmidt Plot, and two more plots related to the Foster-Niemann plot. Furthermore, these plots may have some advantages over the ones currently in use.

   1) Walker and Schmidt, Arch. Biochem., 5, 445 (1944).

   2) Foster and Niemann, Proc. Nat. Acad. Sci., 39, 999 (1953).

3. a. I propose that results obtained in this thesis regarding the effect of pH on the kinetics of alpha-chymotrypsin-catalyzed hydrolyses of simple substrates provide a qualitative, and perhaps quantitative, explanation of the pH optimum curves which have been obtained for many substrates, including those observed for derivatives of tryptophan, which have heretofore appeared to be anomalous.

   b. I propose that, on the basis of experimental results noted in the thesis, the position of the pH optimum observed for a neutral substrate should vary with the substrate concentration.

   1) Huang and Niemann, J. Am. Chem. Soc., 73, 1541 (1951).

4. I propose that it might be worthwhile to attempt to inhibit the action of digestive enzymes on insulin by 1) adding a compound such as acetyl-D-tryptophan to all of the available free amino groups of insulin, 2) adding all of the free carboxyl groups of insulin to a compound such as D-tryptophan methyl ester. This preparation should be called "oralin".

   1) Huang and Niemann, J. Am. Chem. Soc., 73, 3228 (1951).

5. I propose that alpha-amino acid hydroxamides may exist as zwitter ions. Indications of this possibility may be found in the changes in acid constants resulting when 1) acetic acid is substituted with an alpha-ammonium group, 2) glycine is converted to its amide.

   1) Cohn and Edsall, "Proteins, Amino Acids, and Peptides," New York: Reinhold Publ. Corp., 1943.

6. I propose that suitable O,N-diacylated DL-serine derivatives might be resolved with alpha-chymotrypsin. In addition, the optically active serine thus obtained might be substituted at the oxygen atom to yield a series of substrates worthy of kinetic study.

   1) Huang and Niemann, J. Am. Chem. Soc., 73, 1541 (1951).

7. I propose that the expression of the phenotype dotted in Neurospora crassa should be tested to see whether or not it will result from dietary deficiencies other than sulfur.

8. I propose that a gene is composed of a complex system of interacting enzymes, and that as a consequence:

   (1) Gene mutation rates are affected by the number of enzymes present in the gene and the stability of the individual enzymes.

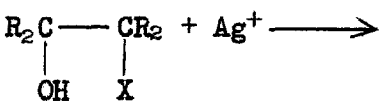
(2) These enzyme systems may produce other enzymes; substrates for the enzyme systems of other genes or external enzyme systems; inhibitors of the enzyme systems of other genes or external enzymes; or activators or coenzymes for these systems.

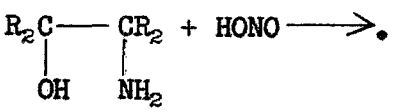
(3) These gene enzyme systems would be influenced by 1) activators necessary to start the gene action, 2) coenzymes, activators, or inhibitors of the individual members of the system, 3) concentration levels of substrates and other substances influencing enzyme action.

(4) The sensitivity of such a system to the products of other genes and external enzyme systems, concentration levels, etc. might provide the basis for a hypothesis concerning the operation of cell differentiation mechanisms.

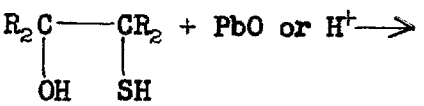
9. The possibility of using blocking groups which may be susceptible to selective oxidation in peptide synthesis should be investigated. Derivatives of oxalic acid might be of value in this way.

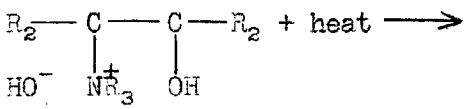
10. Rearrangements similar to the pinacolone rearrangements are given by the following treatments

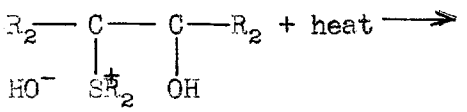




I propose that the following classes of compounds might undergo similar rearrangements:



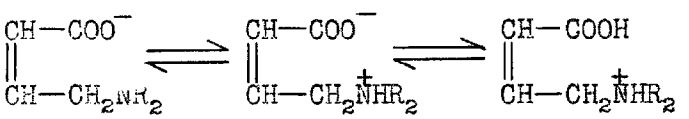


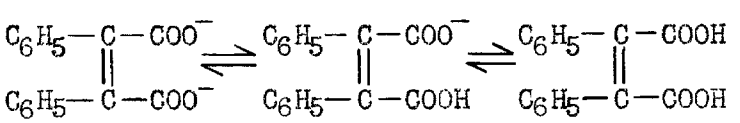


1) Wheland, G. W. Advanced Org. Chem. New York: Wiley, 1949 p. 478 ff.

2) Fuson. Advanced Organic Chemistry. New York: John Wiley and Sons, 1950. p. 578.

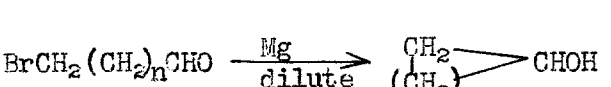
11. In the vast majority of cases the trans isomer of a compound containing an ethylenic double bond is more stable than the cis compound. It would appear that the equilibrium position for the cis–trans interconversion would be governed both by steric and by electrostatic interactions. I propose that some compounds, by virtue of strong electrostatic interactions, might be more stable in the cis configuration. Interesting examples for study might be





12. I propose that suitably halogenated aldehydes, ketones, esters, etc. might be reacted with active metals to effect ring closures of synthetic interest. Illustrative examples might be

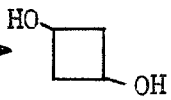
(1)



(2) $BrCH_2CHO + BrMgCH_2CH(OR)_2 \rightarrow BrCH_2CH(OH)CH_2CH(OR)_2$

$\rightarrow \rightarrow BrCH_2CH(OAc)CH_2CHO$

$\xrightarrow[\text{dilute}]{Zn}$ 

(3) $BrCH_2CHO \xrightarrow[Mg]{?} BrCH_2\overset{OMgBr}{C}H-CH_2CHO \xrightarrow{Mg}$ 

(4) cis— $BrCH_2CH=CH-CHO \xrightarrow[Mg]{?}$