Anal. Calcd. for $C_{33}H_{51}NO_{14}$: C, 57.79; H, 7.50; N, 2.04; $COCH_3$, 18.83. Calcd. for $C_{34}H_{53}NO_{14}$: C, 58.35; H, 7.63; N, 2.00; $COCH_3$, 18.45. Found: C, 57.77; H, 7.35; N, 2.00; $COCH_3$, 18.40.

Acknowledgments.—We are indebted to Mr. G. B. Hess for the titration and spectrophotometric data, to Dr. J. Means and Mr. T. Toolan for analyses and to Drs. T. Lees and R. Ottke for paper chromatographic studies. Mr. F. Leghorn developed the microbiological assay method and carried out the assays. We should also like to express our appreciation to Mr. M. Noseworthy, Miss J. Salvo and Mr. J. Catania for their technical assistance.

BROOKLYN 6, N. Y.

[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]

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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.

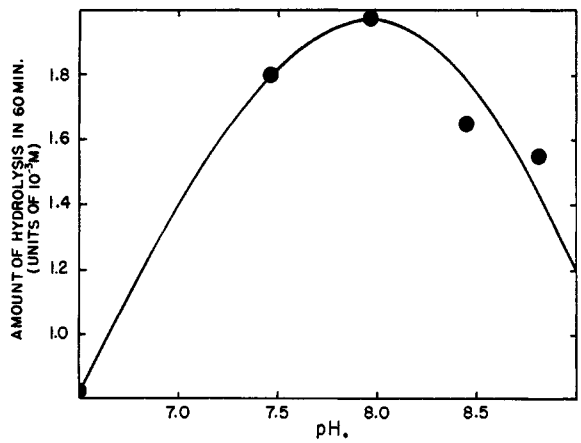

Fig. 1.—*p*H–activity relationship of the system α-chymotrypsin–acetyl-L-hexahydrophenylalaninamide in aqueous solutions at 25° and 0.02 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

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.

(2) United States Rubber Co. Postgraduate Fellow, 1952–1953.

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

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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.

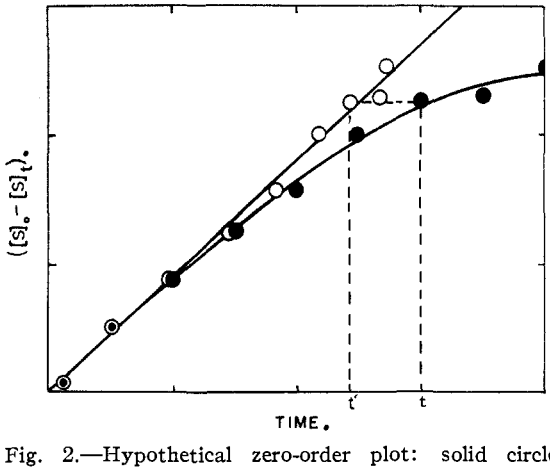

Fig. 2.—Hypothetical zero-order plot: solid circles, observed values; open circles, corresponding values if initial velocity were maintained. Curve through solid circles best fit to observed values; line through open circles the initial velocity.

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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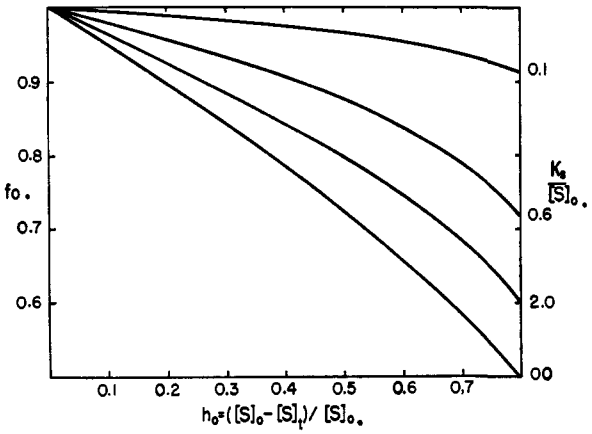

Fig. 3.—Graphical evaluation of the correction factor f_0 for zero-order plots.

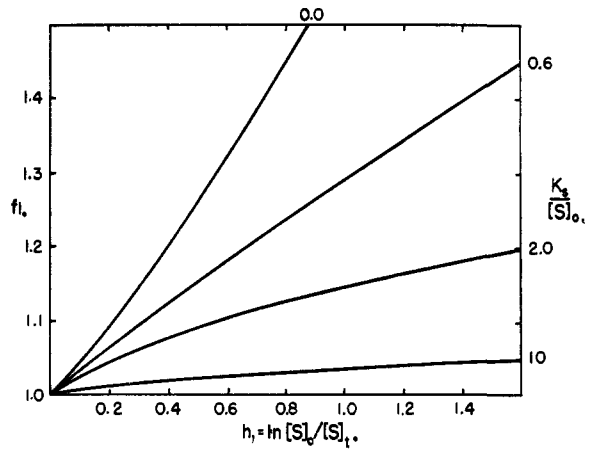

Fig. 4.—Graphical evaluation of the correction factor f_1 for first order plots.

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.

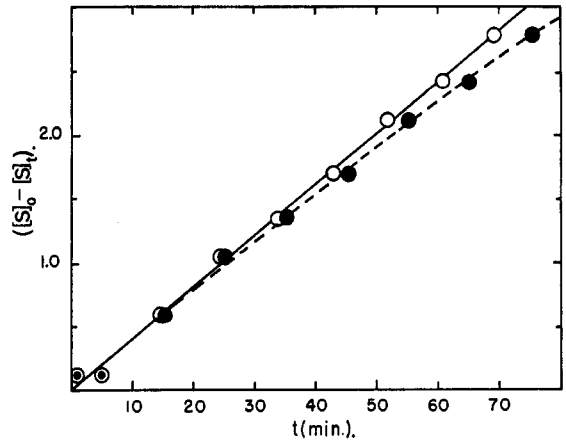

Fig. 5.—Zero-order plot of set of data obtained in experiment no. 33 and summarized in Table I: solid circles, observed values; dotted curve, best fit to observed values; open circles, corrected values; solid line, least squares fit to corrected values.

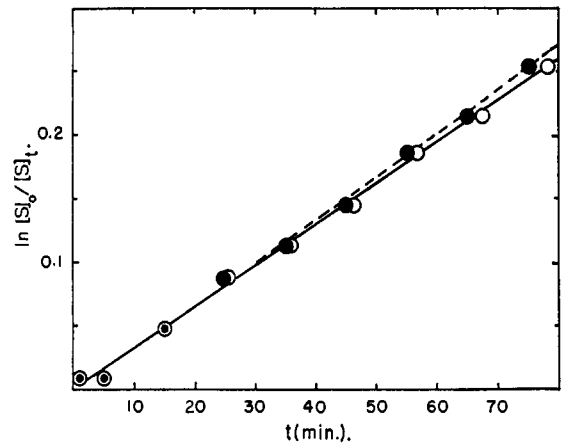

Fig. 6.—First-order plot of set of data obtained in experiment no. 33 and summarized in Table I: solid circles, observed values; dotted curve, best fit to observed values; open circles, corrected values; solid line, least squares fit to corrected values.

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

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

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

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

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

$$k_3[E] = K_S(1 + [I]/K_I + [S]_0/K_{P1})(\ln [S]_0/[S]_t)/t + (1 - K_S/K_{P1})([S]_0 - [S]_t)/t \quad (15)$$

Thus when [I] = 0 and K_{P1} 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_{P1} 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_1} 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_1}. 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_1} 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_1}. This approximate value of K_{P_1} 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_1} and the process repeated until satisfactory values of both K_S and K_{P_1} are obtained. When $K_{P_1} > K_S$ the correct values will be approached quite rapidly since in this case even an approximate value of K_{P_1} 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_1} 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_1} 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 pH 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 pH 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.

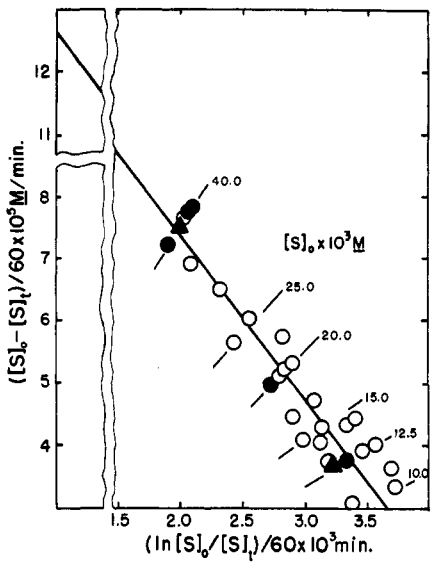

Fig. 7.—Walker–Schmidt plot of the results of the 38 experiments summarized in Table II: open circles, results of single experiments; solid circles, mean of duplicate experiments; solid triangles, mean of triplicate experiments. Line is a least squares fit.

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.

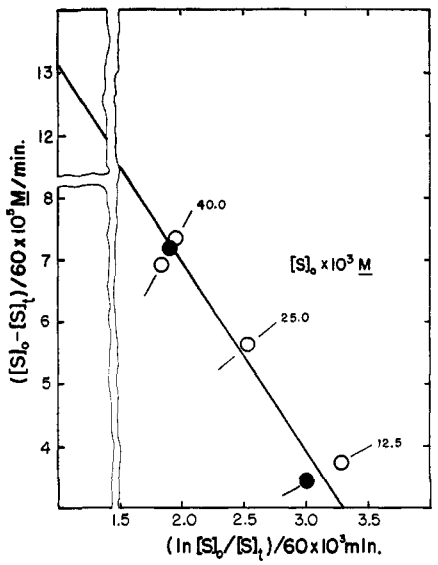

Fig. 8.—Walker–Schmidt plot of the results of the 8 experiments summarized in Table III: open circles, results of single experiments; solid circles, mean of duplicate experiments. Line is a least squares fit.

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).

PASADENA 4, CALIFORNIA

[CONTRIBUTION FROM THE INSTITUTE OF PAPER CHEMISTRY]

The Constitution of Sapote Gum. II. Components of the Methyl Ether Derivative

BY E. V. WHITE

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Methanolysis of sapote gum methyl ether furnishes a sirupy mixture of glycosidic products which can be separated into two fractions. The components of one of these fractions have been reported previously. The second fraction is composed of compounds containing both the glycosidic methyl group and the uronic acid ester function. Further treatment of this fraction has furnished, in part, the anomeric forms of methyl (methyl 3,4-di-O-methyl-D-glucopyranosid)-uronate. These, characterized by reduction of the methoxycarbonyl group followed by hydrolysis of the glycosidic function to provide 3,4-di-O-methyl-D-glucose, furnish crystalline 3,4-di-O-methyl-D-glucuronic acid upon removal of the ester and the aglycone methyl group by hydrolysis. The remainder of the second fraction has not been resolved completely. Prolonged aqueous hydrolysis thereof furnishes 3-O-methyl-D-xylose as the alkylated sugar moiety. The molar ratio of the components of sapote gum methyl ether has been estimated and some conclusions are drawn as to the molecular architecture of the macromolecule.

Methanolysis of sapote gum methyl ether produces a sirupy mixture of glycosidic products which can be separated into two fractions. The components of one of these fractions[1] have been identified as being derived from 3-O-methyl-D-xylose, 2,3,4-tri-O-methyl-D-xylose and 2,3,4-tri-O-methyl-L-arabinose. The components of the second fraction contain both the glycosidic methyl group and the uronic acid ester function. Upon further treatment of this fraction with methanolic hydrogen chloride and eventual vacuum distillation of the products of the reaction, methyl (methyl 3,4-di-O-methyl-D-glucopyranosid)-uronate in its anomeric forms was obtained as one of the components. The latter was characterized by reduction of the methoxycarbonyl group to the primary alcohol function followed by hydrolysis of the glycosidic methyl group to provide the known crystalline compound, 3,4-di-O-methyl-D-glucose. When both the ester group and the aglycone function are removed from the glycosiduronate by hydrolysis, the product of the reaction, namely, 3,4-di-O-methyl-D-glucuronic acid, may be obtained in crystalline form. It reacts in stoichiometric proportion with one mole of periodic acid establishing the presence

(1) E. V. White, THIS JOURNAL, **75**, 257 (1953).