I. THE USE OF NUMERICAL METHODS IN THE TREATMENT OF DATA FROM ENZYME KINETICS STUDIES

II. THE EFFECT OF ENZYME CONCENTRATION ON THE RATE OF A HYDROLYSIS CATALYZED BY ALPHA-CHYMOTRYPSIN

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ABSTRACT

Three methods have been developed for handling data from enzyme kinetics studies. The first method, which involves the use of Simpson's rule, is applicable when Michaelis-Menten (21) kinetics obtain and has the advantage of a short calculation time. The second method, which involves approximation of the data by a set of orthogonal polynomials, is applicable when the reaction mechanism is either unknown or so complicated that the mathematical formulation is intractable. The third method applies when the Michaelis-Menten mechanism holds but the steady state approximation is invalid. Use is made of Simpson's rule and of numerical differentiation.

The effect of enzyme concentration in the alpha-chymotrypsin catalyzed hydrolysis of L-tryptophanhydroxamide has been studied. All the work was done at 25°C. at an ionic strength of either 0.15 M. or 0.30 M. The experiments covered the pH range of 6 to 8. The buffer from pH 6 to pH 7 was cacodylic acid. The buffer used at pH 8 was tris-(hydroxymethyl)-aminomethane.

It is shown that the effect of changing enzyme concentration is understandable in terms of a reversible dimerization of the enzyme which makes one active site of the two enzyme molecules involved unavailable for bonding to substrate. This effect decreases with increasing pH, becoming negligible at pH 8.

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Part I

THE USE OF NUMERICAL METHODS IN THE TREATMENT OF DATA FROM ENZYME KINETICS STUDIES

THE EMPIRICAL EVALUATION OF THE INITIAL VELOCITIES OF ENZYME CATALYZED REACTIONS*

For the past four years we have been concerned with the development of procedures for the objective evaluation of the initial velocities of enzyme catalyzed reactions in those cases where a rate equation can be assumed on the basis of prior knowledge, or is established in the course of the evaluation procedure (1-6). However, if the reaction being studied cannot be described in terms of a relatively simple rate equation and this fact is not revealed by the evaluation procedure, or alternatively if it is revealed but the information at hand does not permit the development of a satisfactory rate equation, it is clear that the forced application of any of the above procedures (1-6) may lead to biased values of the initial velocities. Since we have encountered a number of situations where knowledge of the initial velocities was desirable but where the available data were not sufficiently extensive to permit the development of satisfactory rate

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equations we decided to direct our attention to the development of an empirical procedure, for the evaluation of the initial velocities of enzyme catalyzed reactions, which by definition does not require any information relative to the rate equation of the reaction under investigation, rather than to accept the risk of obtaining biased values of the initial velocities in these particular situations by the forced application of procedures based upon specific rate equations (1-6).

The procedure which has been developed is based upon fitting a set of orthogonal polynomials to the data of a particular experiment by the method of least squares (7), employing the "t" test (8) to determine the significance of the coefficients of each of the polynomials relative to the evaluated experimental error and to thus select a suitable polynomial for the representation of the experimental data, transforming the selected polynomial into a series of the form $C'_0 + C'_1(s) + C'_2(s)(s-1) + \cdots$, differentiating the above series to arrive at a value of the initial velocity and finally determining the variability of the latter value. The only limitation of this general procedure is that the observations provide for a point corresponding to time equal zero and that the experimental observations with respect to the extent of reaction be made at equal time intervals, or that such information be obtained from the actual observations by an unequivocal interpolation procedure.

In order to facilitate use of the above procedure it has been set up in a block form which will permit the necessary computations to be made with a desk calculator in about twenty minutes. With the aid of the tables of orthogonal polynomials for n + 1 equally spaced points which are given in Milne (7) the procedure may be employed for experiments involving from six to twenty-one points. It will be noted that the highest order polynomial considered in reference (10) is $P_{5,n}$. However, this is not a limitation since it has been found that even $P_{4,n}$ is rarely required.

In this communication we shall consider the development of the procedure for the evaluation of an experiment involving eight points, including the one corresponding to zero time, and shall give the necessary constants for the evaluation of a nine point experiment.* The notation of Milne (7) and of Bennett and Franklin (8) will be used throughout.

a. Evaluation Of The Coefficients $C_{m,n}$ Of The Tabulated Orthogonal Polynomials For n = 7

For the case where n = 7, i.e., for an experiment with eight points including the one corresponding to zero time, the first step is to evaluate the coefficients $C_{m,n}$ of the

*Eight and nine point runs have been selected because of their pertinence to experiments conducted in 10 ml. volumetric flasks and involving the withdrawal of 1 ml. aliquots at a selected time interval.

tabulated orthogonal polynomials selected for the representation of the experiment. $C_{m,n}$ are the coefficients of the tabulated orthogonal polynomials listed by Milne (7) and are the constants determined in line C of the example given by the same authors (10). The polynomials with which we will be concerned are those given below:

$$P_{0,7}(s) = 1$$

$$P_{1,7}(s) = 7 - 2(s)$$

$$P_{2,7}(s) = 7 - 16(s) + 1(s)(s-1)$$

$$P_{3,7}(s) = 7 - 12(s) + 6(s)(s-1) - 0.6666(s)(s-1)(s-2).$$

$$P_{4,7}(s) = 7 - 20(s) + 15(s)(s-1) - 4.6666(s)(s-1)(s-2) + 0.5833(s)(s-1)(s-2)(s-3).$$

$$P_{5,7}(s) = 7 - 30(s) + 35(s)(s-1) - 18.6666(s)(s-1)(s-2) + 5.25(s)(s-1)(s-2)(s-3) - 0.7(s)(s-1)(s-2)(s-3)(s-4).$$

These polynomials, which are the polynomials whose values are given by Milne (9) for n = 7, were obtained from the general polynomials given by Milne (11) except that each polynomial above $P_{0,7}(s)$ has been multiplied by the number at the top of its column in the table (9) for n = 7, i.e., in each instance by 7 except for the one noted above.

b. Test For Significance Of Each Coefficient $C_{m,n}$

The question of what order polynomial should be used is determined on the basis of the "t" test for significance (8) by considering the significance of each coefficient $C_{m,n}$ as it is evaluated in step a. above. Since, in the

case at hand $t_{(n-m)}$ is the ratio of $C_{m,7}$ to its standard deviation we shall regard a coefficient $C_{m,7}$ as significant if $t_{(n-m)}$ is larger than a prescribed value. As in the example considered in Bennett and Franklin (12), $t_{(n-m)}$ is computed on the basis of equations 1 and 2 where S_m is defined as in Milne (9),

$$t_{(n-m)} = (S_m)^{1/2} C_{m,n} / \sigma_{y,m} \qquad (1)$$

$$\sigma_{y,m} = \left\{ \sum_{i=0}^{n} (y_i)^2 - (n+1)(\bar{y})^2 - \sum_{k=1}^{m} S_k (C_{k,n})^2 / (n-m) \right\}^{1/2} \qquad (2)$$

$\sigma_{y,m}$ is the standard deviation of the experimental data calculated on the basis of an orthogonal polynomial representation of degree m, y_i is the value of the dependent variable and $\bar{y}$ is the arithmetic average of the values of y_i. It will be noted that the quantity (n-m) is equal to the number of degrees of freedom and also is equal to the number of points in the run, i.e., (n + 1), minus the number of coefficients to be determined for the polynomial, i.e., (m + 1). In practice values of $t_{(n-m)}$ computed on the basis of equations 1 and 2 are compared with values of $t_{(n-m),\alpha}$ which are given in a table of the percentage points of the Student "t" distribution (13). In our studies α has been taken as either 0.05 or 0.10. If $t_{(n-m)}$ is greater than $t_{(n-m),0.05}$ the risk in accepting $C_{m,n}$ as actually being different from zero is less than 5%.

A special case arises where sufficient information is available, e.g., through repeated observation of the characteristics of a given system, to recognize that the true value of $C_{m,n}$ is either positive or negative. In this instance instead of testing whether the coefficient is significantly different from zero one immediately rejects constants which have the wrong sign and then tests the remaining coefficients to determine whether they are significantly greater than zero (14). In this case $t_{(n-m)}$ is compared with $t_{(n-m),2\alpha}$ if the risk in accepting $C_{m,n}$ as being different from zero is to be less than α.

c. Transformation Of The Orthogonal Polynomial Representation Into A Series Representation

In order to facilitate subsequent differentiation it is convenient to transform the orthogonal polynomial representation into a series representation of the form given by equation 3. It is evident from equation 3 that C'_m is

$$\tilde{y} = C'_0 + C'_1(s) + C'_2(s)(s-1) + \ldots \qquad (3)$$

$$C'_0 = C_{0,7} + 7\,C_{1,7} + 7\,C_{2,7} + \ldots$$

$$C'_1 = -2\,C_{1,7} + 6\,C_{2,7} + 12\,C_{3,7} + 20\,C_{4,7} + 30\,C_{5,7}$$

$$C'_2 = C_{2,7} + 5\,C_{3,7} + 15\,C_{4,7} + 35\,C_{5,7}$$

$$C'_3 = -0.6666\,C_{3,7} + 4.6666\,C_{4,7} + 18.6666\,C_{5,7}$$

$$C'_4 = 0.5833\,C_{4,7} + 5.25\,C_{5,7}$$

$$C'_5 = -0.7\,C_{5,7}$$

obtained by summing the contribution of the m^{th} power terms in each polynomial to the final answer. Thus, the coefficient of (s)(s-1) is determined by first noting that it occurs in $P_{2,7}$, $P_{3,7}$, $P_{4,7}$ and $P_{5,7}$. The contribution from $P_{2,7}$ is 1 $C_{2,7}$, the contribution from $P_{3,7}$ is 6 $C_{3,7}$, etc.

d. Differentiation Of Series Representation And Evaluation Of The Initial Velocity

Differentiation of equation 3 leads to equation 4. It is seen from

$$d\tilde{y}/ds = C_1' + C_2'(2s-1) + C_3'(3s^2-6s+2) + C_4'(4s^3 - 18s^2 + 22s - 6) + C_5'(5s^4-40s^3-105s^2-100s+24) \quad (4)$$

equation 4 that the quantity $(d\tilde{y}/ds)_{s=C}$ is given by equation 5

$$(d\tilde{y}/ds)_{s=0} = 1\ C_1' - 1\ C_2' + 2\ C_3' - 6\ C_4' + 24\ C_5' \quad (5)$$

and that substituting for values of C_m' will lead to equation 6.

$$-(d\tilde{y}/ds)_{s=0} = (2C_{1,7} + 6C_{2,7} + 12C_{3,7} + 20C_{4,7} + 30C_{5,7}) + (C_{2,7} + 5C_{3,7} + 15C_{4,7} + 35C_{5,7}) + 2(0.6666\ C_{3,7} + 4.6666\ C_{4,7} + 18.6666\ C_{5,7}) - 6(0.5833\ C_{4,7} + 5.25\ C_{5,7}) + 24\ (0.7\ C_{5,7}) \quad (6)$$

We can now evaluate $(d\tilde{y}/ds)_{s=0}$ for any order polynomial up to an including the fifth order by collecting terms in equation 6 to give equation 7. If for example a third order

$$-(d\tilde{y}/ds)_{s=0} = 2\,C_{1,7} + 7\,C_{2,7} + 18.333\,C_{3,7} + 47.833\,C_{4,7} + 150.633\,C_{5,7} \qquad (7)$$

polynomial is selected on the basis of considerations outlined in step b. above only the first three terms of equation 7 are calculated. The initial velocity, i.e., v_o, is then evaluated from the relationship given in equation 8

$$v_o = -(dy/ds)_{s=0}/b\,\Delta\tau \qquad (8)$$

where $\Delta\tau$ is the time interval at which the observations were made and b is a factor which is used to convert values of y to values of concentration.

e. Determination Of The Variability Of The Value Of v_o

It has been shown (10) that the quantity C_m is given by equation 9 from which we may derive equation 10. Since v_o

$$C_m = \left(\sum_{s=0}^{n} P_{m,n}(s)\, f(s)\right)/S_m \qquad (9)$$

$$\sigma_{C_m} = \sigma_y/(S_m)^{1/2} \qquad (10)$$

is calculated from a linear combination of values of C_m we may evaluate σ_{v_o}, for n = 7, on the basis of equation 11

$$\sigma_{v_o} = (1/b\Delta\tau)(2^2\sigma^2 C_{1,7} + 7^2\sigma^2 C_{2,7} + \cdots + 150.633^2\sigma^2 C_{5,7})^{1/2} \qquad (11)$$

which may be transformed into equations 12 and 13. The

$$\sigma_{v_o} = (\sigma_y/b\Delta\tau)(2^2/168 + 7^2/168 + \cdots + 150.633^2/2184)^{1/2} \quad (12)$$

$$\sigma_{v_o} = \sigma_y \; L_{m,n}/b\,\Delta\tau \quad (13)$$

parameter $L_{m,n}$ in equation 13, which is the quantity given by the square root term of equation 12, is computed but once and values of $L_{m,7}$ and $L_{m,8}$ for values of m up to and including m = 5 are given in Table I. It will be seen from the above discussion that if σ for v_o given by a third order set of orthogonal polynomials is to be computed, the square root of the first three terms under the square root sign of equation 12, i.e., the value of $L_{3,7}$ given in Table I, is multiplied by the quantity $\sigma_y/b\Delta\tau$.

f. Application Of The Evaluation Procedure

In practice it has been found desirable to present the entire evaluation procedure in block form in order to facilitate computation. The form used in these laboratories is illustrated by the following example.

The α-chymotrypsin catalyzed hydrolysis of L-tryptophan-hydroxamide was studied in aqueous solutions at 25° and pH 6.93 and 0.3 M in the THAM component of a THAM-HCl buffer under conditions where [E] = 0.914 mg. protein-nitrogen per ml. of

an Armour preparation No. 00592 and $[S]_o = 21.79 \times 10^{-3}$ M. One ml. aliquots were withdrawn from the reaction mixture at four minute intervals and the L-tryptophanhydroxamide present in these samples was determined spectrophotometrically essentially by the same procedure that was used by Foster, Jennings and Niemann (15) for the determination of L-tyrosinhydroxamide. The primary experimental data so obtained, i.e., optical density, i.e., OD_i, as a function of time, i.e., τ in minutes, are given in Table II.

Table II is so organized that the part of the table above the first dashed line is devoted to the evaluation of the coefficients $C_{m,n}$ of the orthogonal polynomials tabulated for n = 7 and m = 0 to 5, cf., section a. above. The values of $P_0(s)$, $P_1(s)$, etc., are those given by Milne (9). The values of S_m in line (A) are the accumulative squares of the coefficients $P_0(s)$, $P_1(s)$, etc. The values of C_m in line (B) are the results of the accumulative multiplication of the values of OD_i by the corresponding coefficients, i.e., the values of $P_0(s)$, $P_1(s)$, etc. The values of C_m in line (C) are the quotients c_m/S_m, i.e., the quotients of each value in line (B) divided by the corresponding value in line (A), and are the desired coefficients $C_{m,7}$ of the orthogonal polynomials.

The second part of Table II, i.e., the part between the first and second dashed lines, is devoted to computations required for the "t" test, cf., section b. above. The values

of $(C_m)^2$ in line (D) are computed from the values of C_m in line (C) above. In line (E) the quantity $\sum_{i=0}^{7} (OD_i)^2 - \sum_{k=0}^{m} S_k (C_k)^2$ is evaluated by first computing the accumulative squares of OD_i, entering this value in the OD_i column of line (E) and then successively subtracting from this quantity the accumulative products represented by $\sum_{k=0}^{m} S_k (C_k)^2$ and computed from the values of S_m given in line (A) and the values of $(C_m)^2$ in line (D). The values of (n - m), in this instance (7 - m), are entered in line (F) and in line (G) the quantity $(\sigma_{OD})^2$ is evaluated as the quotient $(\sum_{i=0}^{7} (OD_i)^2 - \sum_{k=0}^{m} S_k (C_k)^2)/(n - m)$ of the values of the numerator given in line (E) and the corresponding values of the denominator given in line (F). From the values of $(\sigma_{OD})^2$ in line (G) the corresponding values of σ_{OD} are obtained and entered in line (H). The value of σ_{OD} in any particular column is the estimate of the σ of the observed values of OD based upon the residuals between the approximating polynomial and the primary data. Values of $(S_m)^{1/2}$, in this instance of $(S_{m,7})^{1/2}$, which may be obtained from Table III are entered in line (I) and in line (J) the quantity $t_{(n - m)}$ is evaluated from the relation $(S_m)^{1/2} C_m / \sigma_{OD}$ by dividing the product of the values given in lines (C) and (I) of any particular column by the corresponding value given in line (H). The individual values of $t_{(n - m)}$ in line (J) are now compared with the corresponding values of $t_{(n-m),\alpha}$ which are given by Bennett and Franklin (13) for a value of $\alpha = 0.05$, i.e., for an assumed risk of 5%,

and which are entered in line (K). It will be seen from the values of $t_{(n-m)}$ and $t_{(n-m),\alpha}$ given in Table II that the values of $t_{(n-m)}$ in columns $P_2(s)$ and $P_3(s)$ are greater than the corresponding values of $t_{(n-m),\alpha}$ and that the value of $t_{(n-m)}$ in column $P_4(s)$ is less than the corresponding value of $t_{(n-m),\alpha}$. Therefore, for the example at hand we shall represent the experimental data by the polynomials $P_0(s)$, $P_1(s)$, $P_2(s)$ and reject those of higher order.

The third part of Table II, i.e., between the second and third dashed lines, relates to the transformation of the orthogonal polynomial representation into a series representation and the subsequent evaluation of the initial velocity, cf., sections c. and d. above. In line (L) of Table II are the values of m and in line (M) the coefficients for v_o given by equation 7 and tabulated for values of n = 7 and n = 8 in Table IV. The quantity $(-d\tilde{y}/ds)_{s=0}$ is then computed from the relation $(-d\tilde{y}/ds)_{s=0} = \sum_{k=0}^{m} C_k \text{ (coeff. } v_o)_{m,n}$, in this case by the accumulative multiplication of the values given in lines (C) and (M) and the value of $(-d\tilde{y}/ds)_{s=0}$ so obtained is entered in line (N). It should be noted that in the case at hand the value given in line (N) is in units of OD and $(\text{min.}/4)^{-1}$. To convert this latter value into one based upon more general units, i.e., M/min., the value given in line (N) is divided by the factor $b\Delta\tau$, where b = 98.4 OD units/M to give the value of v_o in line (O) which is in units of M/min. x 10^{-4}. It will be seen that for the experiment being

considered $v_o = 14.20 \times 10^{-4}$ M/min.

The fourth part of Table II, i.e., below the third dashed line, is devoted to an estimate of the variability of the value of v_o obtained immediately above. The values of $L_{m,n}$ in line (P) are those given in Table I. The quantity $(-d\tilde{y}/ds)_{s=0}$ is evaluated as the product of the values given in line (H) and (P) of column $P_3(s)$ and is entered in line (Q). The quantity σ_{v_o} is then evaluated as the quotient of the value given in line (Q) divided by the factor $b\Delta\tau$. The value of σ_{v_o} entered in line (R) is seen to be $\pm$ 1.04 x 10^{-4} M/min. which leads to a final value of $v_o = 14.2 \pm 1.0 \times 10^{-4}$ M/min.

The primary experimental data employed in the example given immediately above are presented in Fig. 1 as is the curve which is a graphical representation of the polynomial selected to represent the experimental data in the above evaluation operation. A line of slope $v_o = 0.5558$ OD/(min./4) is also presented in Fig. 1 in order to demonstrate the difficulty of producing this latter line by visual inspection.

For an experiment involving nine observations, i.e., for the case where n = 8, a form similar to that described by Table II may be prepared. However, it should be noted that for the case of n = 8 all of the constant coefficients will have values different than those given in Table II. For the nine point experiment the necessary values of $P_0(s)$, $P_1(s)$, etc., are given in reference (9) and the values of $L_{m,n}$,

$(S_m)^{1/2}$ and (coeff. $v_o)_{m,n}$ in Tables I, III and IV. The values of m and of (n - m) will be obvious and the values of $t_{(n-m),\alpha}$ may be obtained from reference (13). The choice of the significance level, i.e., the value of α, is determined largely by experience.

During the past year the polynomial approximation procedure described in this communication has been used to evaluate the initial velocities of several hundred enzyme catalyzed reactions. It has been found to be particularly useful in those cases where sufficient experimental data were not available to evaluate the enzyme-reaction product dissociation constants, where systems were being studied under conditions which appeared to preclude the usual steady state approximations and where the nature of the study, e.g., the preliminary evaluation of the inhibition constants of competitive inhibitors or initial studies of the effect of extrinsic parameters upon certain enzyme catalyzed reactions, did not justify the development of a complete rate equation for the basic reaction system. With respect to future applications of the polynomial approximation procedure it should be noted that this procedure affords a basis for accepting or rejecting a particular reaction mechanism provided that the primary data are free of systematic errors. If a particular reaction mechanism is assumed and values of the initial velocities are calculated on the basis of the corresponding rate equation then if the postulated rate equation is a valid

one, one should observe lower values for σ_y and σ_{v_o} than are observed when the same experimental data are evaluated by the polynomial approximation procedure. If this is not found to be the case then the postulated reaction mechanism can be rejected.

Finally with regard to the labor involved in performing the computations required by the polynomial approximation procedure it has been our experience that the evaluation of a limited amount of experimental data with the aid of the block form described above and a desk calculator is no more time consuming than many graphical procedures (1-6). Furthermore, with larger quantities of experimental data the calculations required by the polynomial approximation procedure can be performed with the aid of a digital computor, such as the IBM 604, with a concomitant decrease in computational time and an increase in computational accuracy.

The Evaluation of the Kinetic Constants of Enzyme-catalyzed Reactions by Procedures Based upon Integrated Rate Equations. II[1]

By Keith A. Booman and Carl Niemann[2]

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Enzyme-catalyzed reactions that can be represented by equations 1, 2 and 3 are of sufficient

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

$$E_f + P_{1f} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EP_1 \quad (2)$$

$$E_f + P_{2f} \underset{k_7}{\overset{k_6}{\rightleftharpoons}} EP_2 \quad (3)$$

general interest as to encourage the continued development of more reliable and convenient methods for the evaluation of the kinetic constants of such reactions.

For zone A conditions[3-5] a reaction represented by equations 1, 2 and 3 can be formulated in terms of equation 4 where $k_3' = k_3K_P/(K_P - K_S)$,

$$-d[S]/dt = k_3'[E][S]/(K_S' + [S]) \quad (4)$$

$K_S' = K_S(K_P + [S]_0)/(K_P - K_S$, $K_S = (k_2 + k_3)/k_1$, $K_P = 1\Big/\sum_{j=1}^{n} 1/K_{Pj}$, $K_{P1} = k_5/k_4$ and $K_{P2} = k_7/k_6$.

Definite integration of equation 4 to time t followed by rearrangement gives equation 5. It is seen from equation 5 that a

$$\left(\int_0^t [S]dt\right)\Big/([S]_0 - [S]_t) = ((2K_S' + [S]_0)/2k_3'[E]) + ([S]_t/2k_3'[E]) \quad (5)$$

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

(3) O. H. Straus and A. Goldstein, *J. Gen. Physiol.*, **26**, 559 (1943).
(4) A. Goldstein, *ibid.*, **27**, 529 (1944).
(5) R. J. Foster and C. Niemann, This Journal, **77**, 1886 (1955).

plot of $\left(\int_0^t [S]dt\right)/([S]_0 - [S]_t)$ *vs.* $[S]_t$ will lead to a series of lines of slope $1/2k_3'[E]$ and ordinate intercept $(2K_S' + [S]_0)/2k_3'[E]$ for various values of $[S]_0$, *cf.*, Fig. 1.

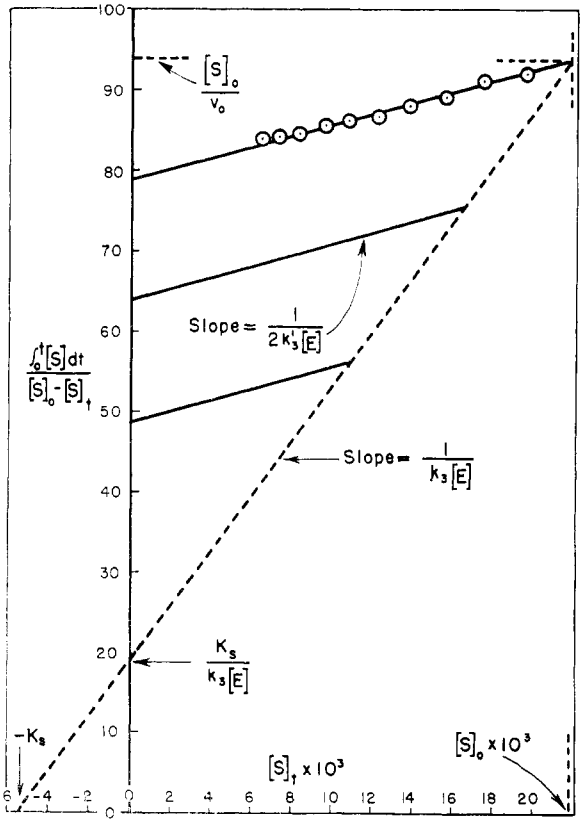

Fig. 1.—α-Chymotrypsin-catalyzed hydrolysis of L-tryptophanhydroxamide in aqueous solutions at 25° and *p*H 6.92 and 0.3 *M* in the THAM component of the THAM–HCl buffer; [E] = 0.0932 mg. protein nitrogen/ml.; $[S]_0$ = 21.8 × 10^{-3} *M*; Δt = 5 min.; $\int_0^t [S]\,dt/([S]_0 - [S]_t)$ in minutes; $[S]_t$ in *M* × 10^3, *i.e.*, moles per liter × 10^3.

For each of the lines of slope $1/2k_3'[E]$ and ordinate intercept $(2K_S' + [S]_0)/2k_3'[E]$ there is a point corresponding to $t = 0$, *i.e.*, when $[S]_t = [S]_0$. These points may be located as before[6-8] by the determination of the limits of the two parameters $\left(\int_0^t [S]dt\right)/([S]_0 - [S]_t)$ and $[S]_t$ as $t \rightarrow 0$. Since the limit of $\left(\int_0^t [S]dt\right)/([S]_0 - [S]_t)$ as $t \rightarrow 0$ is $[S]_0/(-d[S]/dt) = [S]_0/v_0$ and that of $[S]_t$ as $t \rightarrow 0$ is $[S]_0$ it is evident that the points at which the lines of slope $\frac{1}{2}k_3'[E]$ possess abscissa values of $[S]_t = [S]_0$ will be the points where $t = 0$. Furthermore, the initial velocities, *i.e.*, the values of v_0, will be given in terms of $[S]_0/v_0$ by their ordinate parameter $\left(\int_0^t [S]dt\right)/([S]_0 - [S]_t)$ for the condition that $t = 0$.

As the coördinates of the points corresponding to $t = 0$ are, respectively, $[S]_0/v_0$ and $[S]_0$ it follows[8] that a line drawn through these points will describe the behavior of the reaction system when $t = 0$. This line will have a slope of $1/k_3[E]$, an ordinate intercept of $K_S/k_3[E]$ and an abscissa intercept of $-K_S$. With K_S and k_3 so determined K_P may be evaluated from the slope and ordinate intercepts of the parallel lines of slope $1/2k_3'[E]$ and ordinate intercept $(2K_S' + [S]_0)/2k_3'[E]$ and the relation $K_P = k_3'K_S/(k_3' - k_3) = K_S(K_S' + [S]_0)/(K_S' - K_S)$.

The definite integral in equation 5 can be evaluated in several ways. However, it has been found that approximate integration through the use of Simpson's Rule is generally satisfactory when it is used in the form given in equation 6 where *m* is one-

$$\int_0^{2m} [S]dt = (h/3)([S]_0 + 4[S]_1 + 2[S]_2 + 4[S]_3 + 2[S]_4 + \cdots + 4[S]_{2m-1} + [S]_{2m}) - m[S]^{(4)}h^5/90 \quad (6)$$

half the number of intervals over which the integral is being evaluated, *h* is the time interval between successive observations and $[S]^{(4)}$ is a value of the fourth derivative of [S] with respect to *t* at some point between $[S]_0$ and $[S]_t$.[9] In practice the definite integral $\int_0^t [S]dt$ is first evaluated without regard to the contribution of the remainder term of equation 6. From this approximate value of $\int_0^t [S]dt$ for a particular value of $[S]_0$ values of k_3' and K_S' are obtained from a $\left(\int_0^t [S]/dt\right)/([S]_0 - [S]_t)$ *vs.* $[S]_t$ plot. With these values of k_3' and K_S' and the corresponding values of $[S]_0$ and [E] the quantity $m[S]_0^{(4)}h^5/90$ is evaluated to give a maximum estimate of the difference between the actual area under the experimental curve and the area given by equation 6 without regard to the remainder term. If this difference is within the limits of experimental error, as is frequently the case, the above values of k_3' and K_S' may be taken as the final values. However, if the above difference is observed to be greater than that ascribable to experimental error the definite integral is again evaluated with the inclusion of the remainder term obtained as above.

Under the conditions which usually obtain in studies of the α-chymotrypsin-catalyzed hydrolysis of L-tryptophanhydroxamide and acetyl-L-tyrosinhydrazide it has been observed[10] that when Δt is of the order of two to five minutes the remainder term of equation 6 is generally less than 0.5% of the total and therefore is well within the limits of experimental error. Finally, it should be noted that if the use of Simpson's Rule is contemplated for the approximate integration of the integral $\int_0^t [S]dt$ the experimental observations should be made at a constant and closely spaced time interval.

The data which are presented in Fig. 1 relate to the α-chymotrypsin-catalyzed hydrolysis of L-tryptophanhydroxamide in aqueous solutions at 25° and *p*H 6.92 and 0.3 *M* in the THAM[11] component of a THAM–HCl buffer for the condition

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(8) R. R. Jennings and C. Niemann, *ibid.*, **77**, 5432 (1955).

(9) W. E. Milne, "Numerical Calculus," Princeton University Press, Princeton, N. J., 1949, p. 121.

(10) Unpublished observations of K. A. Booman and W. Lands.

(11) Tris-(hydroxymethyl)-aminomethane.

that [E] = 0.0932 mg. protein-nitrogen/ml., $[S]_0$ = 21.8×10^{-3} M and Δt = 5.0 minutes with the reaction as represented by equations 1 to 3 inclusive being allowed to proceed to an extent of approximately 70%. These data when evaluated *via* a $\left(\int_0^t [S]dt\right)/([S]_0 - [S]_t)$ *vs.* $[S]_t$ plot, in which $\int_0^t [S]dt$ was approximated through the use of equation 6 without regard for the remainder term of this equation, gave a value of $k_3' = 7.86 \times 10^{-3}$ M/min./mg. protein nitrogen/ml., a value of $K_S' = 4.69 \times 10^{-3}$ M and a value of $v_0 = 2.32 \times 10^{-4}$ M/min. From the above values of k_3' and K_S' and the other known parameters of the system the quantity $m[S]_0^{(4)}h^5/90$ was evaluated and found to be but 6.86×10^{-6}% of the total area thus providing complete justification for ignoring the remainder term of equation 6 in the evaluation of $\int_0^t [S]dt$ in this particular instance.

It will be recognized that the plot of $\left(\int_0^t [S]dt\right)/([S]_0 - [S]_t)$ *vs.* $[S]_t$ described in this communication has many points in common with the plot of $t/(\ln([S]_0/[S]_t))$ *vs.* $([S]_0 - [S]_t)/(\ln([S]_0/[S]_t))$ described in an earlier communication from these laboratories[8] and that both of these plots are related to the $[S]_0/v_0$ *vs.* $[S]_0$ plot of Lineweaver and Burk.[12] However it should be noted that the latter plot requires the separate evaluation of the initial velocities and even if this operation is performed in an objective manner[13] this plot can be used only for the evaluation of data obtained in the initial stages of a reaction represented by equations 1 to 3 inclusive provided that K_P is substantially greater than K_S. As this latter information is not disclosed by a $[S]_0/v_0$ *vs.* $[S]_0$ plot, or by either of its two variants,[8] it is clear that the use of these three plots is accompanied by some uncertainty in the absence of knowledge of the relative magnitudes of K_P and K_S particularly since it has been observed[14] that with certain but not all specific substrates of α-chymotrypsin K_P may be substantially less than K_S when K_P is evaluated from experiments conducted in the absence of added hydrolysis products.

In principle a $\left(\int_0^t [S]dt\right)/([S]_0 - [S]_t)$ *vs.* $[S]_t$ plot should be equivalent to a $t/(\ln([S]_0/[S]_t))$ *vs.* $([S]_0 - [S]_t)/(\ln([S]_0/[S]_t))$ plot,[8] a $t/([S]_0 - [S]_t)$ *vs.* $(\ln([S]_0/[S]_t))/([S]_0 - [S]_t)$ plot,[8] or a $([S]_0 - [S]_t)/t$ *vs.* $(\ln([S]_0/[S]_t))/t$ plot.[6,7] Therefore, it is of interest to compare the values of k_3', K_S' and v_0 obtained from a $\left(\int_0^t [S]dt\right)/([S]_0 - [S]_t)$ *vs.* $[S]_t$ plot with the comparable values obtained from a $([S]_0 - [S]_t)/t$ *vs.* $(\ln([S]_0/[S]_t))/t$ plot[6,7] using in each instance the same experimental data. It was noted above that the experimental data represented in Fig. 1 gave, on the basis of a $\left(\int_0^t [S]dt\right)/([S]_0 - [S]_t)$ *vs.* $[S]_t$ plot, a value of $k_3' = 7.86 \times 10^{-3}$ M/min./mg. protein nitrogen/ml., a value of $K_S' = 46.9 \times 10^{-3}$ M and a value of $v_0 = 2.32 \times 10^{-4}$ M/min. When the same experimental data were evaluated through the use of a $([S]_0 - [S]_t)/t$ *vs.* $(\ln([S]_0/[S]_t))/t$ plot[6,7] it was found that $k_3' = 8.09 \times 10^{-3}$ M/min./mg. protein nitrogen/ml., $K_S' = 48.8 \times 10^{-3}$ M and $v_0 = 2.33 \times 10^{-4}$ M/min.

In practice it has been observed that for reactions which may be represented by equations 1 to 3 inclusive the plot based upon equation 5 is better suited for treating data that have been obtained during the initial stages of a given reaction, *i.e.*, for a lesser extent of reaction, than are the three alternative plots which are derived by indefinite integration and rearrangement of the common differential rate equation. In addition there is less numerical work involved in evaluating experimental data with the first plot than with the other three. However, as has been noted previously[8] this latter factor of convenience may be outweighed by other considerations.

As all of the plots considered above are useful only when the reaction in question has been allowed to proceed to an extent compatible with the evaluation of K_P, which will be determined not only by the magnitude of K_P but also by the relative magnitudes of K_P and K_S we are now engaged in exploring the possible use of methods involving numerical differentiation since such methods would be useful and desirable in those cases where reactions proceed only at very low velocities and where extended times of observation are not desirable.

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The *t*-Butylbenzenes. II. A High Melting Hydrocarbon from Friedel–Crafts Alkylation of 1,3,5-tri-*t*-butylbenzene with *t*-Butyl Chloride[1]

BY L. ROSS C. BARCLAY AND EILEEN E. BETTS[2]

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In part I[3] we reported a compound, $C_{22}H_{34}$, m.p. 218.5–219°, obtained by Friedel–Crafts alkylation of 1,4-di-*t*-butylbenzene with *t*-butyl chloride below 0°. The empirical formula $C_{22}H_{34}$, the ultraviolet absorption spectrum, and the high melting point indicated the presence of at least one alicyclic ring in this aromatic hydrocarbon. In this communication experimental evidence is presented which elucidates the structure of this compound.

Bartlett and co-workers[4] alkylated 1,4-di-*t*-butylbenzene with *t*-butyl chloride to form 1,3-di-*t*-butylbenzene, 1,3,5-tri-*t*-butylbenzene and a compound, m.p. 209–210°, which is probably the same as our high melting hydrocarbon.

1,1,4,4,5,5,8,8-Octamethyl-1,2,3,4,5,6,7,8-octahydroanthracene.—The high melting hydrocarbon was dehydrogenated with palladium at about 400° in a sealed tube and the ultraviolet spectrum of the product clearly showed the presence of anthracene or an anthracene derivative. Among the possible

(1) Taken in part from the M.Sc. thesis of Eileen E. Betts.
(2) Recipient of a National Research Council of Canada Bursary.
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THE EVALUATION OF THE KINETIC CONSTANTS OF ENZYME-CATALYZED REACTIONS WHEN THE STEADY STATE APPROXIMATION IS INVALID

If the mechanism of an enzyme catalyzed reaction, or of any other reaction, can be considered to be given by equation 1 where E_f represents free enzyme, S_f is free substrate, ES is the enzyme-substrate complex, and P is the product or

$$E_f + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} P + E_f \qquad (1)$$

products of the reaction, then at least two cases arise when an attempt is made to evaluate the kinetic constants k_1, k_2, and k_3. One case is that in which the rate concentration of the intermediate ES can be considered to be constant throughout the reaction. This situation is described by equation 2.

$$d[ES]/dt = 0 \qquad (2)$$

The derivation of equation 3, which indicates how the rate of change of product concentration is related to the total enzyme concentration and the total substrate concentration, from equations 1 and 2 will be given below. Equation 4 de-

$$d[P]/dt = k_3[E][S]/(K_s + [S]) \qquad (3)$$

$$K_s = (k_2 + k_3)/k_1 \qquad (4)$$

fines K_s. In this case k_2 and k_1 cannot be evaluated. A second case arises if the concentration of the intermediate ES varies appreciably during the course of the reaction. In this case k_1, k_2, and k_3 can be evaluated. The derivation of equation 3 will be preceded by an outline of the approach used by Laidler (16) to determine the conditions for which equation 3 is valid. Finally, the second case will be considered. Simple numerical methods will be applied to the problem of evaluating k_1, k_2, and k_3 when concentration-time data is available and the steady state approximation, i.e. equation 2, is invalid.

Equation 5 represents the rate of change of the concen-

$$(d[ES]/dt) = k_1[E]_f[S]_f - (k_2 + k_3)[ES] \qquad (5)$$

tration of the enzyme-substrate complex with time if the above mechanism is a true indication of the course of the reaction. Equations 6 and 7 relate the total concentration of enzyme and substrate, i.e. [E] and $[S]_o$ to $[S]_f$, [ES], and $[E]_f$,

$$[S]_o = [S]_f + [ES] + [P] \qquad (6)$$

$$[E] = [E]_f + [ES] \qquad (7)$$

and [P]. The next step is to eliminate $[S]_f$ and $[E]_f$ from equation 5 by using equations 6 and 7. The result is equation 8. Laidler observes that for short reaction times

$$(d[ES]/dt) = k_1([E] - [ES]) ([S]_o - [P] - [E]) - (k_2 + k_3)[ES] \quad (8)$$

$[S]_o - [P]$ can be replaced by an "average" substrate value $[\bar{S}]$, which is considered to be a constant. If this approximation is made, equation 5 integrates to equation 9. The symbols are defined in equations 10, 11, 12, and 13.

$$t = Q^{-\frac{1}{2}} \ln((b[ES] + 2c + [ES]Q^{\frac{1}{2}})/(b[ES] + 2c - yQ^{\frac{1}{2}})) \quad (9)$$

$$Q = b^2 - 4ac \quad (10)$$

$$a = k_1 \quad (11)$$

$$b = -(k_1[E] + k_2 + k_3 + k_1[\bar{S}] \quad (12)$$

$$c = k_1[E][S] \quad (13)$$

At the time when [ES] reaches a maximum value, the right hand member of equation 9 can be set equal to zero. The resulting equation can be solved for $[ES]_{max}$ to give equation 14. Equations 9 and 14 can be combined to show that

$$[ES]_{max} = (-b - Q^{\frac{1}{2}})/2a \quad (14)$$

the time, τ, that is required for [ES] to reach $[ES]_{max}$, is given by the inequality 15.

$$\tau < 5.3Q^{-\frac{1}{2}} \quad (15)$$

Equation 1 indicates that an upper limit to the amount of product formed during the induction period would be given by equation 16. Finally, inequalities 15 and 16 can be

$$[P] < k_3[ES]_{max}t \tag{16}$$

combined to give, after rearrangement and substitutions, inequality 17. This inequality applies to the time when

$$([P]/[S]_o) < (5.3k_3/2k_1[S]_o)((1-4ac/b^2)^{-\frac{1}{2}}-1) \tag{17}$$

$[ES] = 0.99[ES]_{max}$.

The ratio $[P]/[S]_o$ will be small if inequality 18 is

$$k_1[S]_o \gg k_3 \tag{18}$$

obeyed or if inequality 19 holds. Inequality 19 is equiva-

$$4ac \ll b^2 \tag{19}$$

lent to inequality 20. Inequality 20 will be true if any

$$2k_1^2[E][\overline{S}] \ll (k_2 + k_3)^2([E]^2 + [\overline{S}]^2) + 2(k_2 + k_3)k_1([E] + [\overline{S}]) \tag{20}$$

one or more of the inequalities 21, 22, 23, 24 is true.

$$[S]_o \gg [E] \tag{21}$$

$$[E] \gg [S]_o \tag{22}$$

$$(k_2 + k_3) \gg k_1[E] \tag{23}$$

$$(k_2 + k_3) \gg k_1[S]_o \tag{24}$$

The inequalities 18, 21, 22, 23, 24 give the conditions for a short transient phase. The steady state approximation will be a good one if any of these five inequalities is true.

When the steady state approximation applies, the right hand member of equation 4 is set equal to zero to give upon rearrangement equation 25. If K_s is defined by equation 4

$$((k_2 + k_3)/k_1)=([E]-[ES])([S]_o-[P] - [ES])/[ES] \tag{25}$$

$$K_s = (k_2 + k_3)/k_1 \tag{4}$$

and [ES] is negligible compared to $[S]_o$ - [P], equations 25 and 26 give rise to equation 27. This rate equation will

$$d[P]/dt = k_3[ES] \tag{26}$$

$$d[P]/dt = k_3[E]([S]_o-[P])/(K_s([S]_o-[P])) \tag{27}$$

describe the rate of formation of product as a function of enzyme concentration and substrate concentration if the reaction mechanism is that given by equation 1, if [ES] is negligible compared to [S], and if the steady state assumption is valid. The requirement that [ES] be small compared to [S] will be satisfied if any one of the inequalities 21, 23, 24 hold. An estimate of [ES] can be obtained by solving equation 25 for [ES] by the method of successive

approximations.

Many methods have been developed for evaluating k_3 and K_s when data pertaining to the steady state reaction are at hand (1-6). An approach will now be presented to the evaluation of k_1, k_2, and k_3 which can be applied to concentration-time data from a reaction which has a noticeable induction period.

The first step is to obtain a differential equation which contains only measurable quantities, i.e. $[E]$, $[S]_o$, $[P]$, or the time derivatives of these quantities, and the constants k_1, k_2, and k_3. Equation 26 is used to eliminate $[ES]$ from equation 5. The result is equation 28.

$$(1/k_s)\, d^2[P]/dt^2 = k_1[E][S]_o - k_1[E]\,[P] - (k_1/k_3)[E]\,(d[P]/dt) - (k_1/k_s)[S]_o(d[P]/dt) + (k_1/k_3)[P](d[P]/dt) + (k_1/k_3^2)\,(d[P]/dt)^2 - ((k_2 + k_3)/k_3)\,(d[P]/dt) \qquad (28)$$

In the next step equation 28 is divided through by $k_1[E]$ and the operation of integrating each term with respect to t from t equals zero to t is performed or indicated. Equation 29 is the result.

$$(1/k_1k_3[E])[d[P]/dt]_0^t - [S]_ot + \int_0^t [P]dt + (1/k_3)[P] + ([S]_o/k_3[E])[P] - (k_1/k_3^2)\int_0^t (d[P]/dt)^2dt - (1/k_3[E])[P]^2 + ((k_2 + k_3)/k_1k_3[E])[P]=0 \qquad (29)$$

Collecting terms gives equation 30. Equation 30 can be put

$$(1/k_1k_3[E])[d[P]/dt]_o^t - [S]_o t + \int_o^t [P]dt + ((k_1[E] + k_1[S]_o + k_2 + k_3)/k_1k_3[E])[P] - (1/k_3[E])[P]^2 - (1/k_3^2[E])\int_o^t (d[P]/dt)^2 dt = 0 \quad (30)$$

into a more concise form if we use the definitions shown in equations 31, 32, 33, and 34.

$$d[P]/dt = v \quad (31)$$

$$a = 1/k_1k_3[E] \quad (32)$$

$$b = (k_1[E] + k_1[S]_o + k_2 + k_3)/k_1k_3[E] \quad (33)$$

$$c = 1/k_3[E] \quad (34)$$

$$d = 1/k_3^2[E] \quad (35)$$

Equation 30 now becomes equation 36

$$a(v - v_o) - [S]_o t + \int_o^t [P]dt + b[P] - c[P]^2 - d\int_o^t v^2 dt = 0 \quad (36)$$

It is seen that the kinetic constants are then given by equations 37, 38 and 39.

$$k_1 = c/a \quad (37)$$

$$k_3 = c/d \tag{38}$$

$$k_2 = (bc^2[E]_o/ad) - (c/a)([E]_o - [S]_o) \tag{39}$$

Assuming that values of [P] have been observed at equally and closely spaced time intervals throughout the course of the reaction, there are several ways in which the velocities and integrals in equation 36 can be determined so that the only unknowns are a, b, c and d. The first method would be to use any one of a number of integrating and differentiating instruments. Another method would be to use Simpson's Rule and differentiation formulae. A third method would be to calculate the orthogonal polynomial representation of the data and then to integrate or differentiate the resulting polynomial. We shall discuss the second and third methods.*

The second method would start with the evaluation of $\int_o^t [P]\,dt$ by Simpsons rule (5). The value of $v = d[P]/dt$ may be calculated through the use of differentiation formulae. A number of these formulae are given by Milne (18) for cases in which the number of points used tc evaluate the derivative at a particular point varies from three to seven. It is suggested that the seven point formulae be used. The formulae which should be used whenever possible is the formula for

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*The possible role of numerical calculus in solving complicated kinetics problems has been suggested by Stabo (17).

the derivative at the fourth point since the error term in this case is at the minimum. The derivatives of the first three points would be calculated with the aid of the formulae for y_o', y_2' and y_2'. The derivatives at the last three points would be calculated with the aid of y_4', y_5', y_6'. The formula for y_3' should be used at the rest of the points. Simpson's Rule can be used again to evaluate $\int_o^t v^2 dt$.

Method 3 would involve fitting the orthogonal polynomials of Milne (11) to the [P], t data using only those polynomials whose coefficients were found to be significant (8) by the t test. Using the nomenclature of Booman and Niemann (19)

$$v = \left(\sum_{k=1}^{m} C_k P_k'(s) \right)/b\,\Delta t \text{ and } \int_o^t [P]\, dt = (1/b\,\Delta t) \sum_{k=0}^{m} C_k \int_o^t P_k(s) ds.$$

The quantities $\int_o^t v^2 dt$ would be obtained by finding the orthogonal polynomial representation of v^2 as a function of t and then integrating as in the case of $\int_o^t [P]dt$.

The next step, after v, $\int_o^t v^2 dt$, $\int_o^t [P]dt$ have been obtained for each point in the data, is to determine a, b, c, and d of equation 36 using the principle of least squares. k_s, k_1, and k_2 are then calculated from the relationships given in equations 32, 33, 34, and 35.

For the approach of this paper to succeed it will be necessary to average the values of [P] at t from n identical runs, where n might be in the neighborhood of five depending on the standard deviation of [P]. The reason for this is twofold. First, the method of least squares will not give the maximum likelihood estimates of a, b, c, and d when applied to equation 36 if there is scatter in the data. Obtaining maximum likelihood estimates of the constants when scatter in the data is present is the usual purpose for using least squares. The method falls down in this instance because equation 36 is of the form $[P] = f(t,[P])$ whereas it should be of the form $[P] = f(t)$, assuming that t is the errorless variable. Thus we must have values of [P] at t which are errorless in a practical sense. Then all that can be said when the method of least squares is applied is that a, b, c, and d have been so chosen that the sum of the squares of the residuals of equations 36 are at a minimum. No statement, for instance can be made concerning the standard deviations of a, b, c, and d.

The second reason for using the average value of [P] at t from n runs applies to method 2 where Simpson's Rule was used in conjunction with differentiation formulae. Since Simpson's Rule is of the form $\Sigma C_i[P]_i$ it will be seen that the standard deviation of the area becomes larger as more values of $[P]_i$ are used to evaluate the area from 0 to t even though the standard deviation of [P] is constant. Thus the

standard deviation of the calculated area will gradually increase from the beginning to the end of the run. Another type of consideration applies to the use of differentiation formulae. It is well known that numerical differentiation is not suitable when applied to data that contain scatter (20). The reason for this is the fact that although the form of the differentiation formulae is the same as that of Simpson's Rule, the sign of the constant which is a pre-multiplier for $[P]_i$ alternates from plus to minus to plus to minus, etc. Since the derivatives depend on differences between successive values of [P], the standard deviation of the derivative as calculated by this method can become hopelessly large when compared to the actual value of the derivative. Thus we see that the use of Simpson's Rule, differentiation formulae, and the use of the principle of least squares to calculate the constants a, b, c, and d all make it desirable to have data at hand which have as low a standard deviation as possible. This is accomplished by using the average [P] values at t from n different runs.

It is granted that either method 2 or method 3 will involve a good deal of work. Method 2 will involve less work but will also discard half of the points since Simpson's Rule gives values for the area at every other point. If it is easy to observe many values of [P] during a run, method 2 will be preferred.

Part II

THE EFFECT OF ENZYME CONCENTRATION ON THE RATE OF A REACTION CATALYZED BY ALPHA-CHYMOTRYPSIN

INTRODUCTION

It has been known (21-23) for approximately forty years that the rates of many enzyme catalyzed reactions can be explained in terms of the reaction mechanism given in equation 1

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

and the rate equation 2. The symbols used in the development

$$v_o = k_3[E][S]_o/(K_s + [S]_o) \qquad (2)$$

of the rate equations 2 and 10 are:

Ef = free enzyme

[E] = total enzyme concentration

S_f = free substrate

$[S]_o$ = initial substrate concentration

P_{if} = free product. P_{1f} may be amino acid and P_{2f} may be ammonia, hydroxylamine, hydrazine, etc.

$K_S = (k_2 + k_3)/k_1$

The work of Niemann (1-6) and his many coworkers has verified that equations 1 and 2 apply to alpha-chymotrypsin catalyzed hydrolyses of L-amino acid amides, hydroxamides, hydrazides, and esters. This work has been concerned for the most part with the dependency of v_o on $[S]_o$. The enzyme

concentration has always been less than 10^{-5}M. It was the purpose of this study to examine the behavior of alpha-chymotrypsin catalyzed hydrolyses at higher enzyme concentrations.

Information concerning the rate of catalyzed hydrolysis at high enzyme concentrations was desired since a number (24-27) of investigators had interpreted molecular weight studies in terms of the presence of monomer, dimer, and higher polymers. No experimental data were available which would allow any decision to be made as to the nature or magnitude of the effect of association of the enzyme on reactions which it catalyzed.

PRELIMINARY EXPERIMENTS

A set of experiments was devised in which the rate of hydrolysis of a 12 x 10^{-3} M solution of L-tryptophanhydroxamide was studied at enzyme concentrations of 8.88 x 10^{-6} M and at 8.88 x 10^{-4} M, at pH 6.0, pH 6.9, and at pH 7.9. Equation 2 indicates that the ratio $r = v_o/[E]$ should be constant over the entire range of enzyme concentration. If we designate the value of this ratio at the high enzyme concentration by r_H, and at the low enzyme concentration by r_L, then we see that for the ideal situation described in equation 2 that r_H/r_L is equal to unity. The experimental data are presented in Table VII. In figure 2, r_H/r_L is plotted against pH. While the system is ideal at pH 8, r_H/r_L drops as the pH is decreased, becoming one-half at pH 6.6.

This behavior can be understood in part if the equilibria represented in equations 3 and 4 are added to equation 1.

$$E_f + E_f \underset{k_5}{\overset{k_4}{\rightleftharpoons}} E_{2f} \qquad (3)$$

$$E_f + ES \underset{k_5}{\overset{k_4}{\rightleftharpoons}} E_2S \qquad (4)$$

These two equations express the fact that the enzyme can

dimerize. Equation 4 expresses the assumption that two enzyme molecules can be bound together even though one enzyme molecule is already bound to a molecule of substrate. Furthermore, the dissociation constant $K_D = k_5/k_4$ is assumed to be the same for the two equilibria. In other words, the dimerization process is assumed to be independent of the complexing of substrate. This means that the equilibria expressed in equations 5 and 6 also have the same equilibrium

$$E_f + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \tag{5}$$

$$E_{2f} + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_2S \tag{6}$$

constant. It is not expected that a small substrate molecule bonded at the active site of the enzyme will affect the dimerization process.

The association of two enzyme molecules to form the dimer is assumed to involve the binding area of the active site of the first enzyme molecule. The point of attachment of the second enzyme molecule may be some place along the peptide chain that is occupied by an amino acid such as tryptophan, phenylalanine, or tyrosine. The binding area of the active site of alpha-chymotrypsin is known to bond to synthetic substrates (28) containing these amino acids.

The last assumption is that the rate of hydrolysis of

E_2S is the same as the rate of hydrolysis of ES.

The mechanism that results when the equilibria of equations 3, 4, 5, and 6 are combined with equation 1 is given in equation 7.

$$\begin{array}{ccccccccc} E_f & + & S_f & \underset{k_2}{\overset{k_1}{\rightleftharpoons}} & ES & \xrightarrow{k_3} & E_f & + & P_{1f} & + & P_{2f} \\ + & & & & + & & & & & & \\ E_f & & & & E_f & & & & & & \\ k_4 \downarrow \uparrow k_5 & & & & k_4 \downarrow \uparrow k_5 & & & & & & \\ E_{2f} & + & S_f & \underset{k_2}{\overset{k_1}{\rightleftharpoons}} & E_2S & \xrightarrow{k_3} & E_{2f} & + & P_{1f} & + & P_{2f} \end{array} \qquad (7)$$

It may be noticed that if the complex ES and EP exist, then E_2P probably exists too. The added complication of treating the equilibria in equations 8 and 9 is not great.

$$P_{if} + E_f \underset{k_7}{\overset{k_6}{\rightleftharpoons}} EP_i \qquad (8)$$

$$P_{if} + E_{2f} \underset{k_7}{\overset{k_6}{\rightleftharpoons}} E_2P_i \qquad (9)$$

However, since the work described here involves initial velocities only, equations 8 and 9 may be ignored.

THE FORMULATION OF THE KINETIC EQUATION

The mechanism expressed in equation 7 gives rise to the rate equation 10. The derivation of equation 10

$$(v_o/[E][S]_o) = (k_3/2([S]_o + K_S)) - (k_3K_D/8[E]K_S)(1-(1+8[E]K_S/K_D(K_S + [S]_o))^{\frac{1}{2}}) \quad (10)$$

starts with equation 11. The rate of hydrolysis is propor-

$$v = k_3([ES] + [E_2S]) \quad (11)$$

tional to the concentration of ES plus the concentration o E_2S. Both complexes are attacked by water at the same rate. Equation 12 accounts for all the species in which enzyme

$$[E] = [E]_f + 2[E_2]_f + [ES] + 2[E_2S] \quad (12)$$

is present according to equation 7.

Equation 13, which is the equivalent of equations 3 and 4, and equation 14, which is the equivalent of equations 8 and 9, make it possible to eliminate $[E]_f$, $[E_2]_f$, [ES], $[E_2S]$ from equations 11 and 12.

$$K_D = ([E]_f^2/[E_2]_f) = ([E]_f[ES]/[E_2S] \quad (13)$$

$$K_S = ([E]_f[S]_f/[ES]) = ([E_2]_f[S]_f/[E_2S]) \qquad (14)^*$$

One path to equation 10 would be to transform equations 11 and 12 into equations in $[E]_f$ and $[S]_f$ and then to eliminate $[E]_f$. Equation 11 becomes equation 15 and equation 12

$$(vK_S/k_3[S]_f) = [E]_f + (1/K_D)[E]_f^2 \qquad (15)$$

becomes equation 16. Eliminating $[E]_f$ from equations 15 and

$$([E]K_S/([S]_f + K_S)) = [E]_f + (2/K_D)[E]_f^2 \qquad (16)$$

16 gives equation 17. Remembering that this equation is

$$(v/[E][S]_f) = (\tfrac{1}{2})k_3[E]/(K_S + [S]_f)$$

$$-(k_3K_D/8K_S)(1\pm(1+8[E]K_S(K_S+[S]_f))^{\frac{1}{2}}) \qquad (17)$$

valid only at time equals zero, v should be written v_o. Next, the material balance for substrate is given in equation 18. For situations in which $[ES] + [E_2S] \ll [S]_f$, $[S]_o$

$$[S]_o = [S]_f + [ES] + [E_2S] \qquad (18)$$

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*Equation 14 is valid if a steady state 9 exists, i.e., if d[ES]/dt and d[E_2S]/dt are practically zero. The experience of investigators working with alpha-chymotrypsin catalyzed reactions with a half-time of approximately one-half hour is that the steady state assumption is a good one.

can be substituted for $[S]_f$ in equation 17. Incorporating these two changes, equation 17 becomes equation 19. The question of whether the plus of the minus sign is correct

$$(v_o/[E][S]_o) = \tfrac{1}{2}k_3[E]/(K_S + [S]_o) \qquad (19)$$
$$-(k_3K_D/8K_S)(1\pm(1+8[E]K_S/K_D(K_S + [S]_o))^{\frac{1}{2}})$$

is resolved by noting that the right hand member must approach $k_3[E]/(K_S+[S]_o)$ at low enzyme concentrations or for high values of K_D. In other words, the second term of the left hand member of equation 19 must be positive. Therefore the minus sign is the correct choice and equation 10 results.

$$(v_o/[E][S]_o) = \tfrac{1}{2}k_3[E]/(K_S + [S]_o)$$
$$- (k_3K_D/8K_S)(1-(1+8[E]K_S/K_D(K_S+[S]_o))^{\frac{1}{2}}) \qquad (10)$$

Equation 10 applies to the situation in which:

1. E_2S hydrolyzes at the same rate as ES
2. E_2 has the same K_S as E
3. E_2S has the same K_D as E_2
4. the concentration of trimer and higher polymers is negligible.
5. $[S]_f \gg [ES] + [E_2S]$
6. the steady state assumption is valid.

All of these conditions must be kept in mind when equation 10 is used to correlate rate data.

EXPERIMENTAL RESULTS AND CALCULATIONS

It is customary to evaluate enzyme kinetic constants at the pH optimum for the reaction under study if the pH dependence of the rate of the reaction is of no immediate importance. Thus the first step in the study of a particular enzyme system is to locate the pH optimum, i.e., the pH at which the reaction proceeds at the highest rate. The results of experiments 3 through 19 are shown in figure 3. These experiments were carried out at a substrate concentration of 9.16×10^{-3} M and an enzyme concentration of 0.200 mg/ml. The only variable that was changed was pH. The pH optimum is 6.93.

The initial velocities were obtained by fitting the data to a first order rate equation by the method of averages (29). This method fits the equation to the data so that the sum of the residuals is equal to zero. The simplifying assumption that the reaction is first order is justified by the fact that the object of this set of experiments is to locate an optimum. The results are being compared within the set of experiments. The initial velocities were not evaluated by the orthogonal polynomial method since equal time intervals were not used throughout every experiment in this series. In this set of experiments the reactions went to approximately 16% completion. The results shown in figure 3 are taken from Table V.

Experiments 32 through 148 were designed to give information concerning the effect of enzyme concentration on the rate of the alpha-chymotrypsin catalyzed hydrolysis of L-tryptophanhydroxamide. Referring to equation 10, which states the proposed mathematical model for this system, it is seen that the experimental quantities involved are $[E]$, $[S]_o$, and v_o. The initial velocities were obtained from the concentration-time data (Table VI) by the method of orthogonal polynomials. The initial velocities have been listed in Table V.

The constants in equation 10 that are to be determined are k_3, K_S, and K_D. Since it would be very difficult to evaluate all three constants by fitting equation 10 to the experimental data directly, the following approach was devised. At low enzyme concentrations, equation 10 reduces to equation 2. When equation 2 is rearranged as suggested by Lineweaver and Burk (29), equation 20 is the result.

$$([S]_o[E]/v_o) = (K_S/k_3) + (1/k_3)[S]_o \qquad (20)$$

The constants k_3 and K_S can be evaluated by the method of least squares.* All experiments for which the enzyme concentration was 0.200 mg/ml or less were correlated in terms

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*It is recognized that the method of least squares will give biased values for k_3 and K_S (31,32). The magnitude of the bias has not been evaluated.

of equation 20. The results, as calculated by the method of least squares, are summarized in Table XII.

With k_3 and K_S known, equation 10 can be solved for K_D to give equation 21. Only experiments at enzyme concentrations higher than 1 mg/ml were considered.

$$K_D = 4K_S\, a^2/b. \tag{21}$$

The new parameters a and b are defined in equations 22 and 23.

$$a = (v_o/k_3[S]_o) - \tfrac{1}{2}([E]/K_S + [S]_o)) \tag{22}$$

$$b = ([E]/(K_S + [S]_o)) - v_o/k_3[S]_o \tag{23}$$

A value of K_D was calculated from the data from each experiment that was carried out at an enzyme concentration of 1 mg/ml or higher. The values which were obtained from experiments where the only variables that were allowed to change were [E] and $[S]_o$ were averaged. The average K_D values are listed in Table XII as a function of pH and of ionic strength.

The results of the experiments at an ionic strength of 0.300 M are presented in figures 4, 5, 6, 7, and 11. The supporting tables are numbers VIII, IX, and XI. In each one of these figures is drawn the line given by equation 20 with the appropriate constants for the particular pH. Figures 4 and 5 represent experiments at pH 6.0. On the average, the points from high enzyme concentration experiments

fall considerably above the line in figure 4. In figure 5 the high enzyme concentration points have been corrected by the factor predicted by equation 10. The experiments at pH 7.0 have been treated the same way in figures 6 and 7. Again, the points from experiments at the higher enzyme concentrations are above the line in Figure 6. The correction for the effect of dimerization as predicted by equation 10 is shown in figure 7. Only one graph, figure 11, is required at pH 8 since at this high pH all the points, regardless of enzyme concentration, are reasonably distributed about the line. This behavior was expected. The experiments correlated in figure 2 indicated that there was no dimerization at pH 8.

The situation at pH 6.9 and an ionic strength of 0.300 M has been depicted in figures 8, 9, and 10. The least squares fit of equation 11 to the low enzyme concentration data is shown in figure 8. The points taken at an enzyme concentration greater than 0.200 mg/ml lie above this line with a few exceptions as is seen in figure 9. In figure 10 the high enzyme concentration points have been corrected for the effect of dimerization as given by equation 10.

Turning to the actual values obtained for K_D in Table XII, two trends are noticed. As the pH increases, K_D increases. As the ionic strength increases, K_D decreases. The experimental error is too large to allow a definite statement about these trends to be made on the basis of these data.

However, the same two trends were noticed by Steiner (27) in his studies on the concentration dependence of the molecular weight of alpha-chymotrypsin by the light scattering method. Extrapolating his values for the association constant of alpha-chymotrypsin to pH 6.0 and an ionic strength of 0.300 M, a K_D of 1 mg prot N_2/ml is obtained. The K_D spare reported in Table XII of this thesis is 0.7 $\pm$ 0.4 mg prot N_2/ml.

To summarize, the hypothesis that the effect of the concentration of alpha-chymotrypsin on the rate of hydrolysis of L-tryptophanhydroxamide is due to a reversible dimerization of the enzyme in which one of the binding areas of the two enzyme molecules involved is made unavailable for complexing substrate is substantiated by the following items:

1. The rate of hydrolysis is one-half the expected fate at high enzyme concentrations. See figure 2.
2. Equation 10 correlates rate data from high enzyme concentration experiments in a satisfactory manner. See figures 5, 7, and 10.
3. The dissociation constant has the same pH and ionic strength as the constant determined by an independent method. See figure 12 and Table spare XII.
4. The dissociation constant obtained at pH 6.0 and an ionic strength of 0.300 M agrees with the value obtained by an independent method (27).

EXPERIMENTAL PROCEDURE

L-Tryptophan Methyl Ester Hydrochloride. This intermediate was prepared by the method of Brenner (33). The dropwise addition of 20 ml. of thionyl chloride to 100 ml. of reagent grade methanol was carried out during a half hour period. The temperature was kept at 0° during this time. The solution was stirred continuously from the start of the thionyl chloride addition until the point indicated below. After a fifteen minute interval, 50 g. of L-tryptophan was added in small portions. A period of twenty minutes was allowed for the addition of the amino acid. The reaction mixture temperature was then slowly raised to 50° and 100 ml of reagent grade methanol was added to the reaction flask.

The temperature of the reaction mixture was maintained at 50° for two hours. The reaction mixture was then stripped to dryness *in vacuo*. The solid light orange product was dissolved in 100 ml. of methanol plus 150 ml. of ethyl acetate at the boiling point of this solvent combination. The slow addition of 50 ml of n-hexane to the hot solution started the precipitation of product. The product was filtered, washed, and dried *in vacuo*. This material weighed 38.5 g. and melted at 212°.

n-Hexane was added to the hot filtrate until crystallization started again. By this procedure an additional 18 g.

was recovered. This material was impure and required another recrystallization.

L-Tryptophanhydroxamide. A suspension of 20 g. of L-tryptophan methyl ester hydrochloride and 11 g. hydroylamine hydrochloride in 50 ml. of methanol was neutralized by a methanolic sodium methoxide solution which had been prepared by dropping 5.5 g. of sodium in small portions into 100 ml. of methanol. The neutralization of the ester hydrochloride and hydroxylamine hydrochloride suspension was carried out at 0°.

The reaction mixture was kept at 5° for one week. The sodium chloride was then filtered off and n-hexane added to the boiling solution until a second phase separated. At this point isopropyl ether was added until the second phase disappeared. Crystallization occurred when this solution was kept at 5° overnight. The fine crystals were separated from the mother liquor by filtration and washed with a mixture of isopropyl ether and n-hexane. The produce was recrystallized from methanol, n-hexane, and isopropyl ether as above, and a final drying operation was carried out in an Abderhalden drier. The dessicant was P_2O_5. The drier was evacuated, heated to 60° for one-half hour, and opened the following morning.

The product, which weighed 12 g., melted at 157.0-157.5°. $[\alpha]_{25}^{D}$ is 93.1°. The concentration of the aqueous

solution for the measurement of the optical rotation was 0.4%.

Analysis. Calculated: C 60.3, H 6.0, N 19.2
Found: C 60.4, H 6.0, N 19.3

Buffer Solutions

The aqueous stock solutions of buffer were prepared from tris-(hydroxymethyl)-aminomethane hydrochloride for the pH range 7-9 and from cacodylic acid for the pH range 5-7. THAM* was purified by recrystallization from water and then from aqueous ethanol and then converted to the hydrochloride with concentrated HCl. Water was added to give a solution of the desired concentration, 3.75 F. To make a stock solution of a specified pH, 20 ml. of the 3.75 F THAM.HCl solution was pipetted into a 25 ml. volumetric flask and concentrated NaOH added until the solution had the desired pH. Water was then added until the total volume was 25 ml. This stock solution is 3.00 F. in THAM and has an ionic strength of 3.00 M.

Buffer solutions which were 3.00 F. in cacodylic acid with an ionic strength of 3.00 M. were made from 3.75 F sodium cacodylate and concentrated HCl. The cacodylic acid

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*Tris-(hydroxymethyl)-aminomethane.

which was used for this purpose was C.P. reagent. Titration with standardized base indicated that the cacodylic acid was 100.1% pure.

Enzyme Solutions

The enzyme solutions were prepared from alpha-chymotrypsin (Armour Co., Lot No. 00592). These solutions were kept at 25.0° for the time interval during which the aliquot portions were transferred to the reaction system. No aliquots were taken from stock solutions which had been at 25° for more than one hour. The protein nitrogen content of this lot is 14.1%.

Reaction Procedure

The following procedure is a modification of the procedure used by Jennings (15). A 10 ml. volumetric flask was used to contain the reaction mixture. One milliliter of 3.00 F. buffer was pipetted into the reaction flask. A measured volume of a stock solution of substrate (L-tryptophanhydroxamide) was then added. This was followed by the number of milliliters of water that would make the total volume of the reaction mixture 10 ml. when the desired volume of enzyme stock solution was added. All the stock solutions were sufficiently dilute that their volumes were strictly additive on mixing, as judged by the level of the meniscus in a 10 ml. volumetric flask.

At equal time intervals one milliliter aliquots of the

reaction mixture were pipetted into a 10 ml. volumetric flask which already contained 8 ml. of methanol and one milliliter of a solution which was prepared as follows: 54 g. $FeCl_3 \cdot 6H_2O$ was dissolved in water and 200 ml. of concentrated HCl was added. This solution was diluted to 500 ml. with water, and then to one liter with methanol.

In a number of instances the enzyme concentration was higher than 6 mg./ml. The one ml. aliquots from these reaction mixtures were pipetted into a 10 ml. volumetric flask containing 8 ml. of H_2O and 1 ml. of the acidic solution of $FeCl_3$ in methanol mentioned in the last paragraph. The substitution of H_2O for methanol was necessary to prevent turbidity at the higher enzyme concentrations.

The optical densities of the solutions of L-tryptophan-hydroxamide-ferric ion complex were measured in a one centimeter cell at 505 millimicrons in the Beckman Model B Spectrophotometer. The proportionality constant between optical density and concentration of the hydroxamide, while independent of enzyme concentration, was strongly dependent upon ionic strength, the solvent, the buffer, and the pH. The proportionality constants, listed in Table V, were determined each day under the conditions that would be encountered during that day.

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(12) Ref. 8, p. 258.

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Figure 1

Graphical representation of certain of the results given in Table II. Solid circles are the experimental points, curve is the polynomial used for representation of the experimental data, line is the line whose slope is equal to the computed initial velocity.

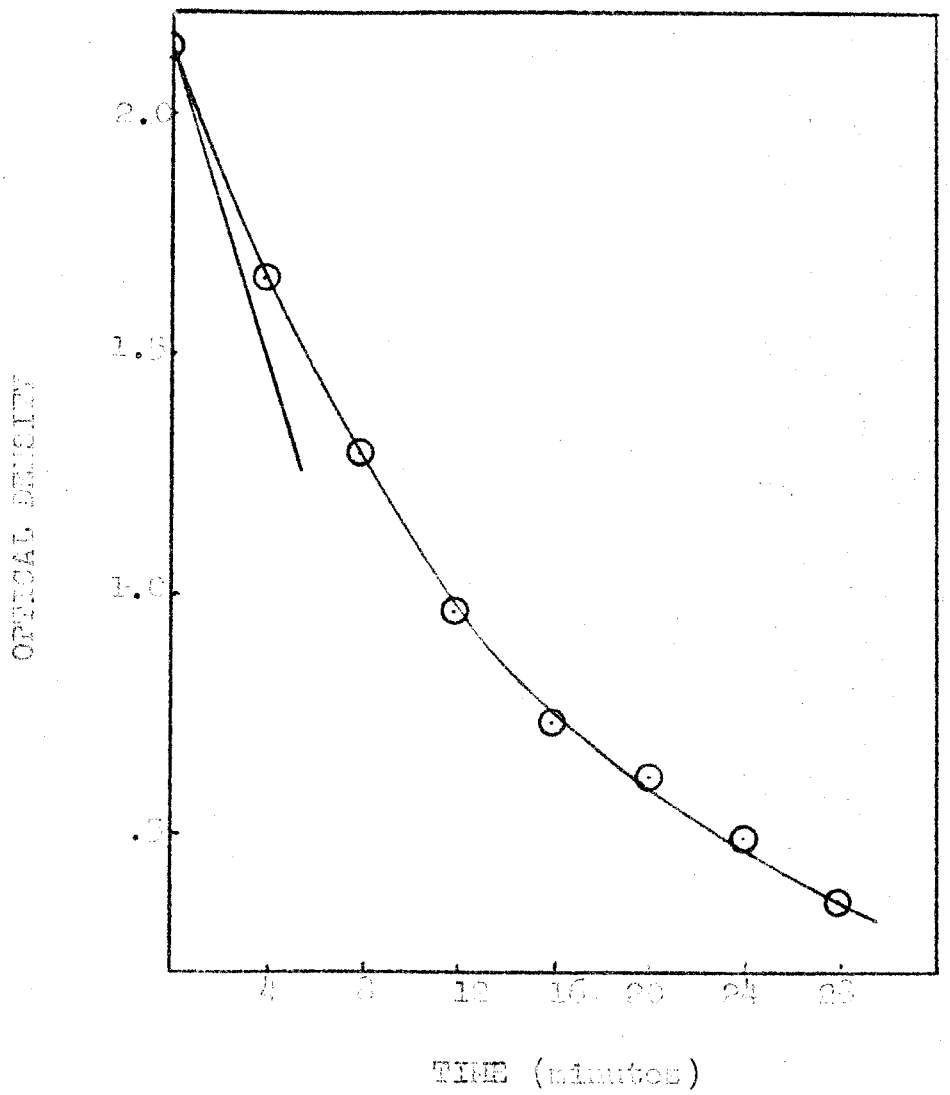


Figure 2

v_{HP}/v_L for the alpha-chymotrypsin catalyzed hydrolysis of L-tryptophanhydroxamide at 25.0° in aqueous solution at an ionic strength of 0.300 M.

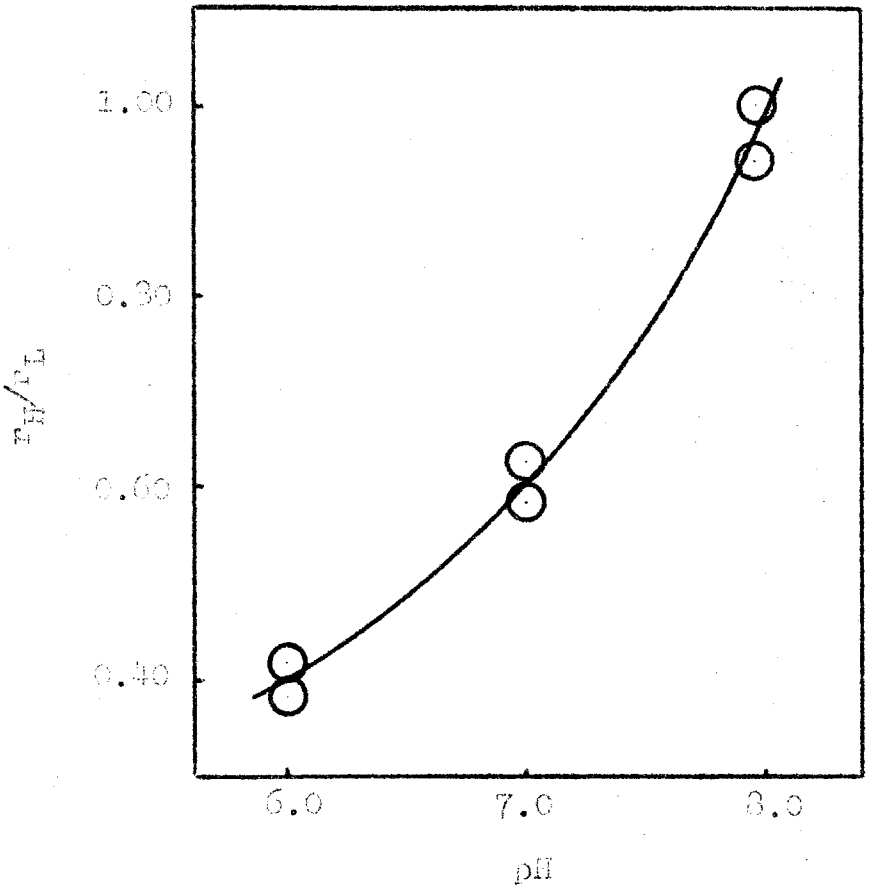


Figure 3

The pH optimum for the alpha-chymotrypsin catalyzed hydrolysis of L-tryptophanamydromanide at 25.0° and at an ionic strength of 0.300 M. The experiments represented by the three points at the right-hand end of the curve were done in an aqueous solution which was buffered by 0.300 F THAM. The rest of the experiments were done in 0.300 F cacodylic acid buffers.

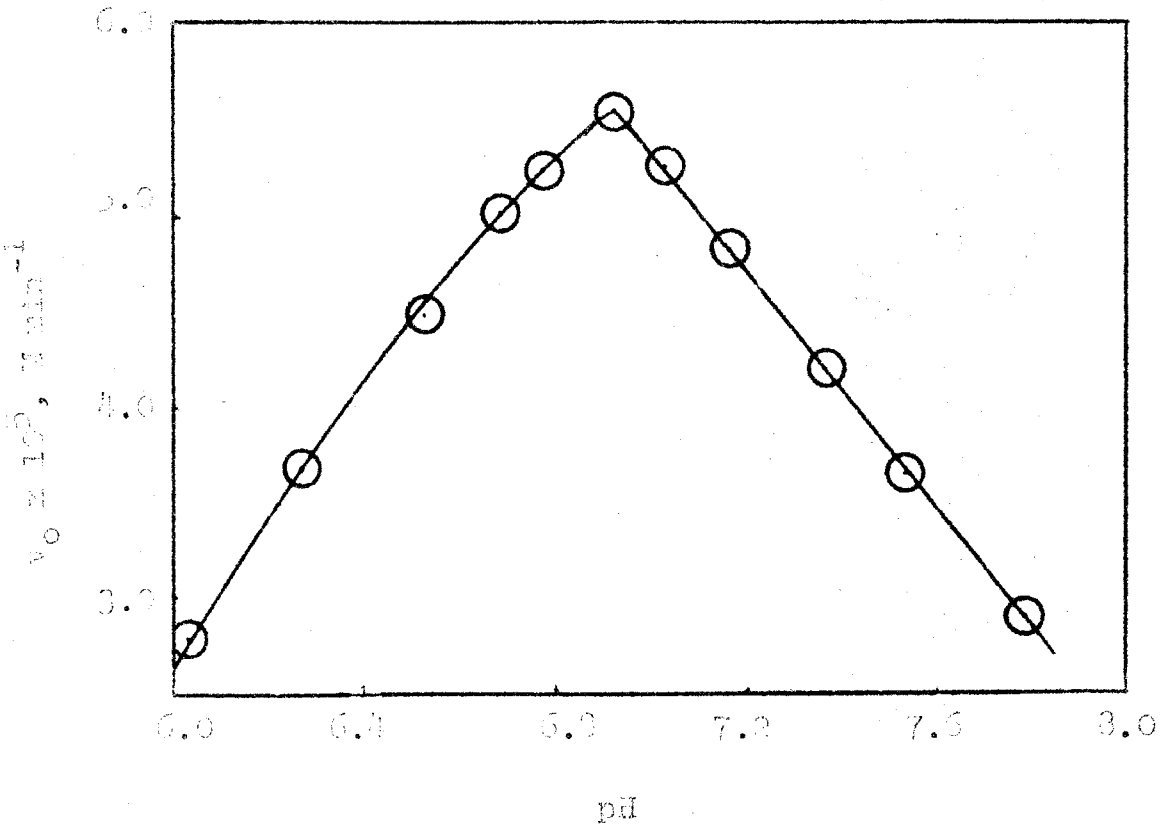


Figure 4

The alpha-chymotrypsin catalyzed hydrolysis of L-tryptophan-hydroxamide at pH 6.0. The enzyme concentration for the experiments represented by circles is 0.2 mg/ml. The triangles denote results obtained at higher enzyme concentrations. 25.0°, 0.300 F cacodylic acid buffer, ionic strength 0.300 M.

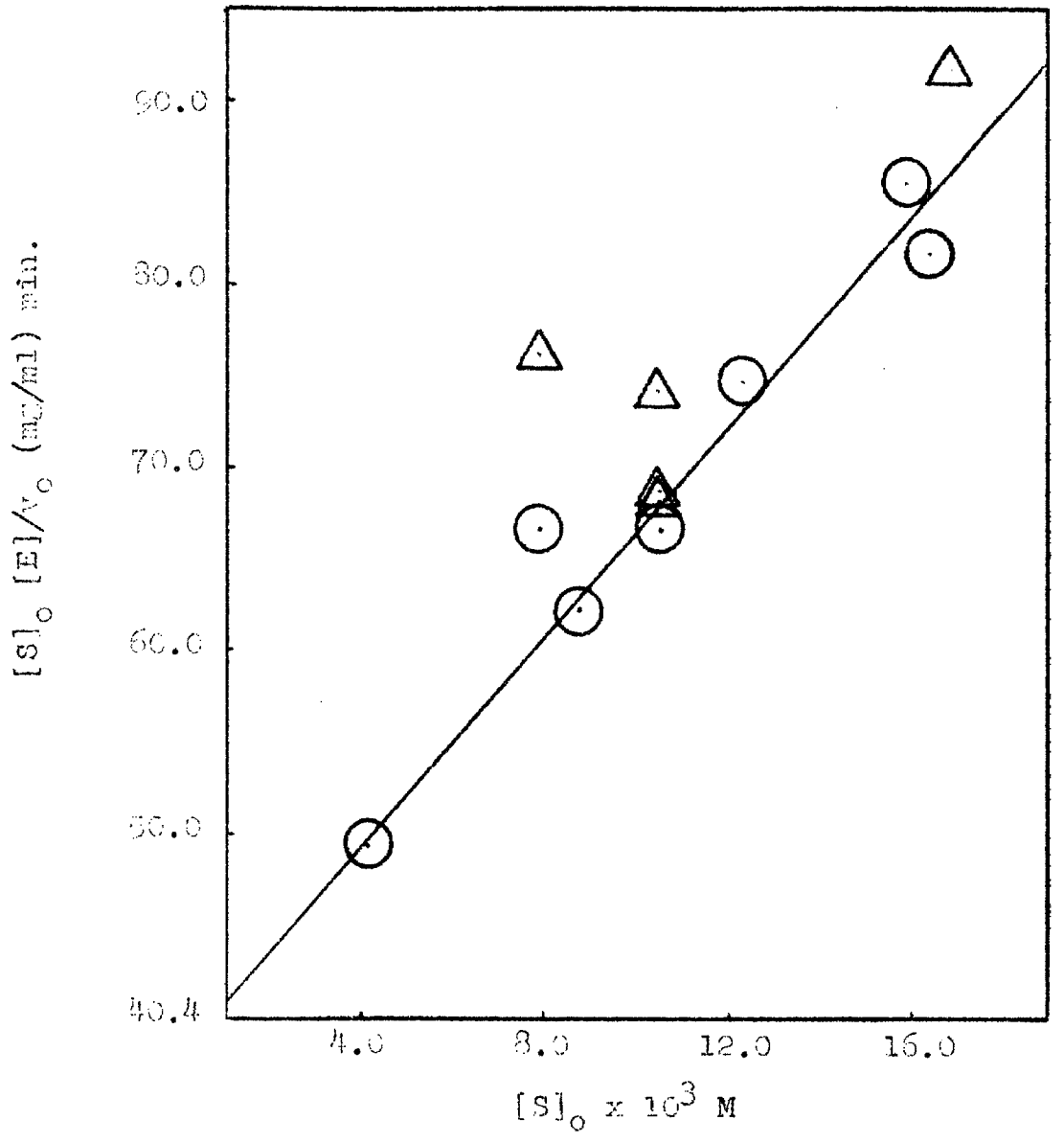


Figure 5

The alpha-chymotrypsin catalyzed hydrolysis of L-tryptophan-hydroxamide at pH 6.0. The enzyme concentration for the experiments represented by circles is 0.2 mg/ml. The triangles denote results obtained at higher enzyme concentrations which have been corrected for dimerization. The reaction conditions are: 25.0°, 0.300 F cacodylic acid buffer, ionic strength 0.300 M.

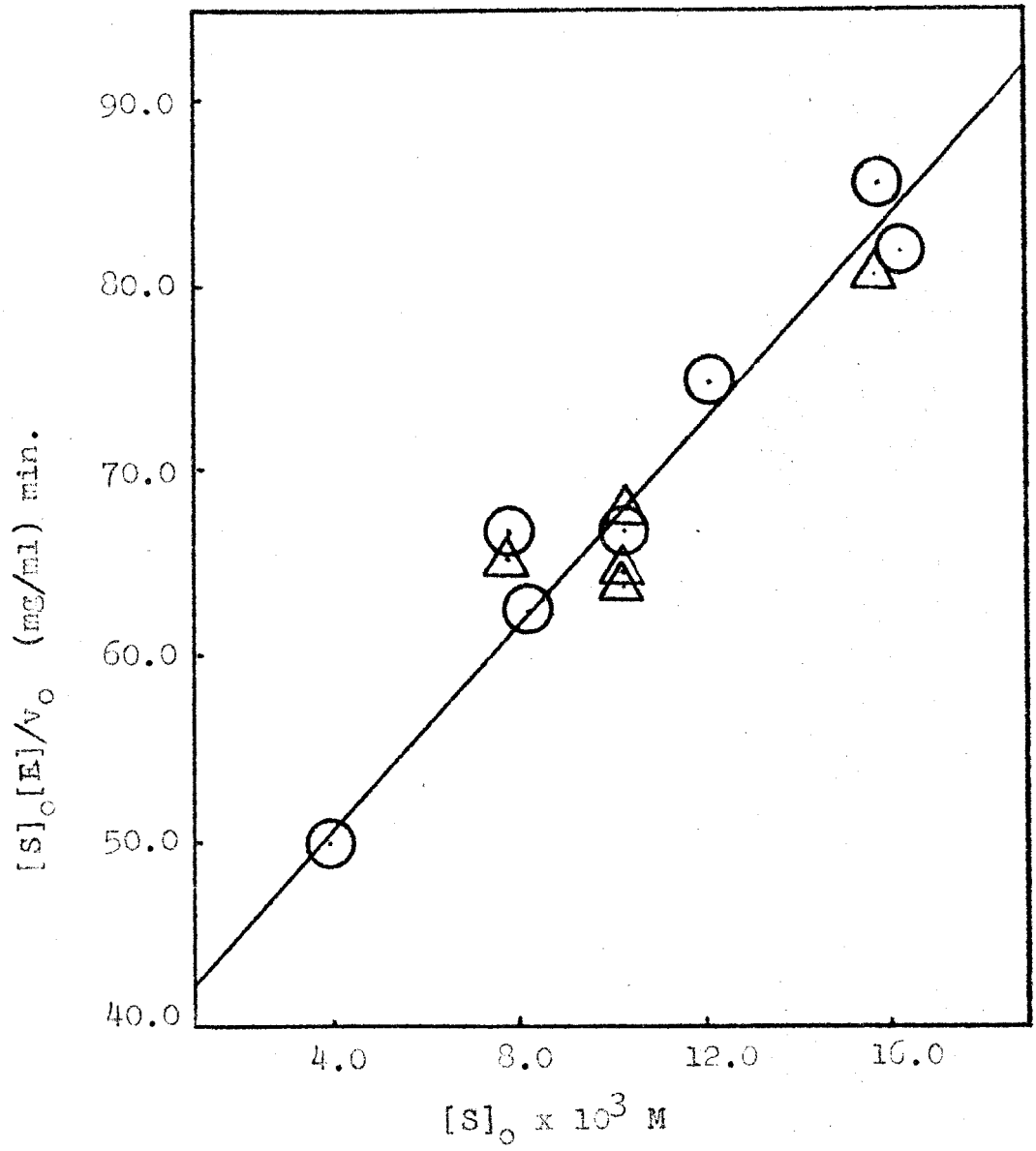


Figure 6

The alpha-chymotrypsin catalyzed hydrolysis of L-tryptophan-hydroxamide at pH 7.0. The enzyme concentration for the experiments represented by circles is 0.2 mg/ml. The triangles denote results obtained at higher enzyme concentrations. The reaction conditions are: 25.0°, 0.300 F cacodylic acid buffer, ionic strength 0.300 M.

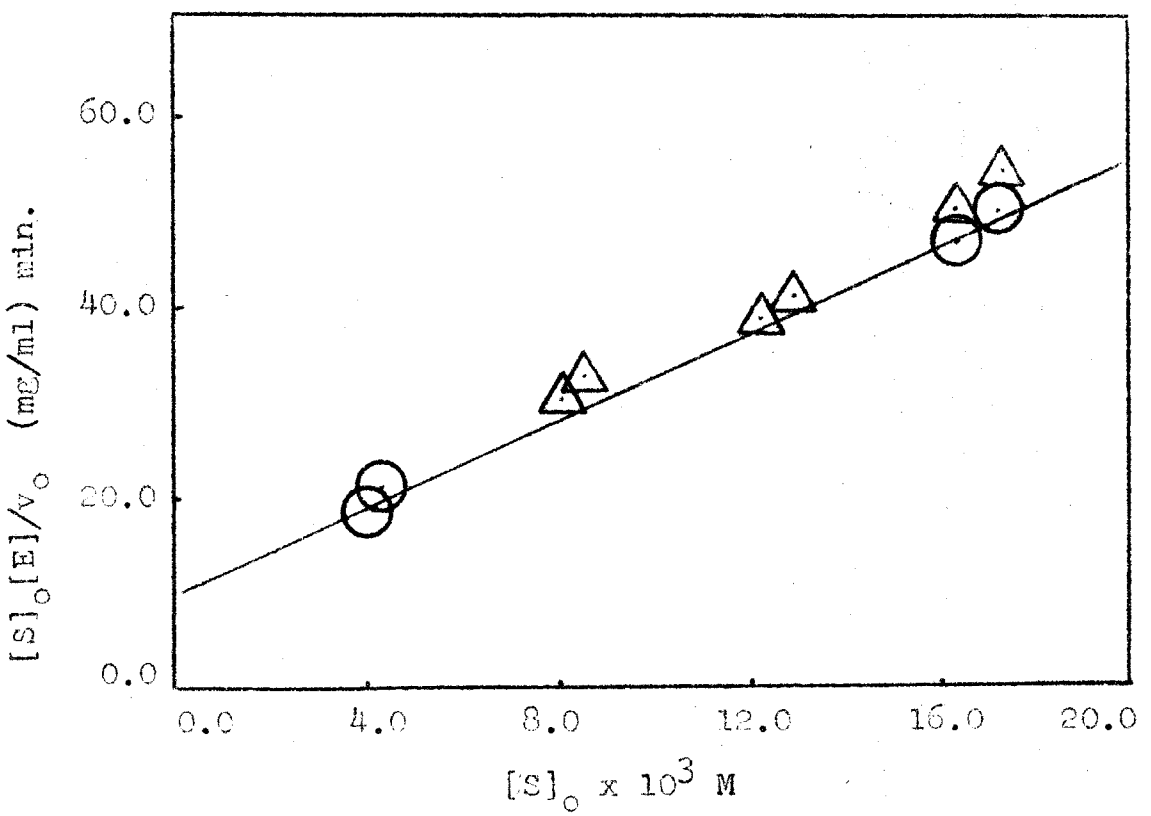


Figure 7

The α-chymotrypsin catalysed hydrolysis of L-tryptophanhydroxamide at pH 7.0. The enzyme concentration for the experiments represented by circles is 0.2 mg/ml. The triangles denote results obtained at 1.0 mg/ml which have been corrected for enzyme dimerization. The reaction conditions are: 25.0°, 0.300 F cacodylic acid buffer, ionic strength 0.300 M.

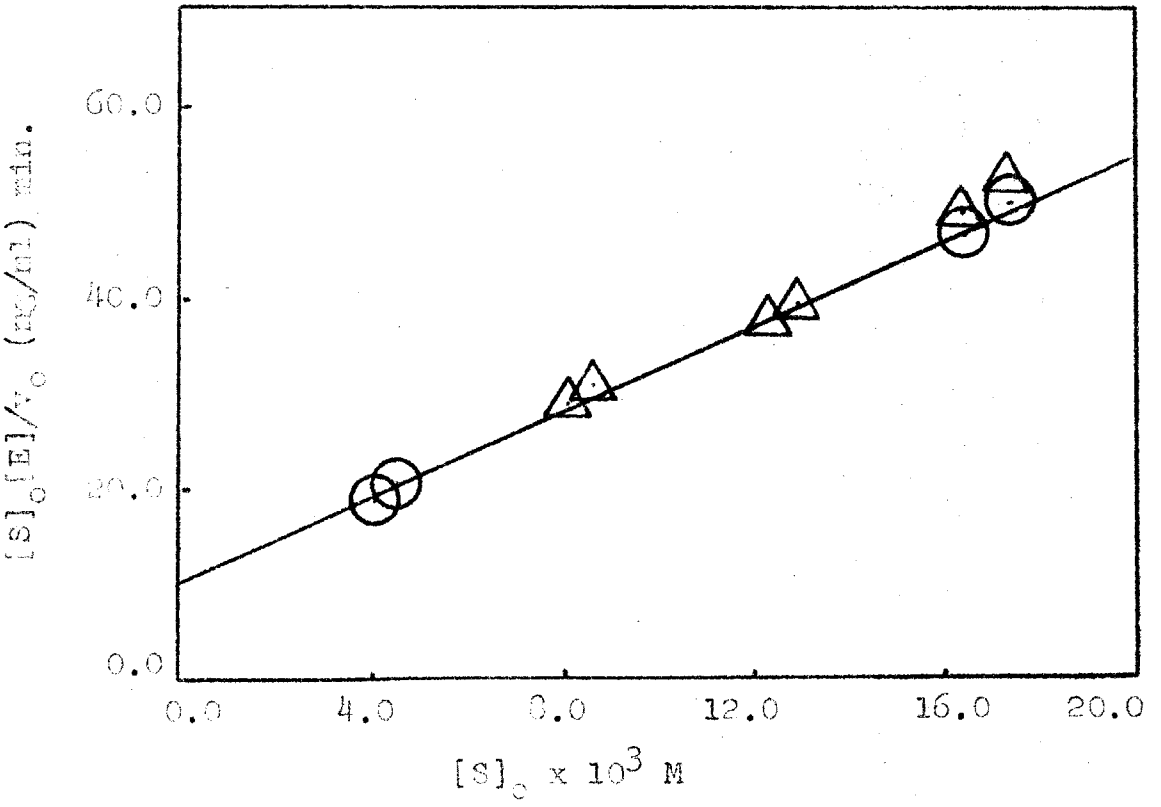


Figure 3

The alpha-chymotrypsin catalyzed hydrolysis of L-tryptophanhydroxamide at pH 6.[illegible]. The enzyme concentration is less than 0.200 mg/ml. The reaction conditions are: 25.0°, 0.150 F cacodylic acid buffer, ionic strength 0.150 M.

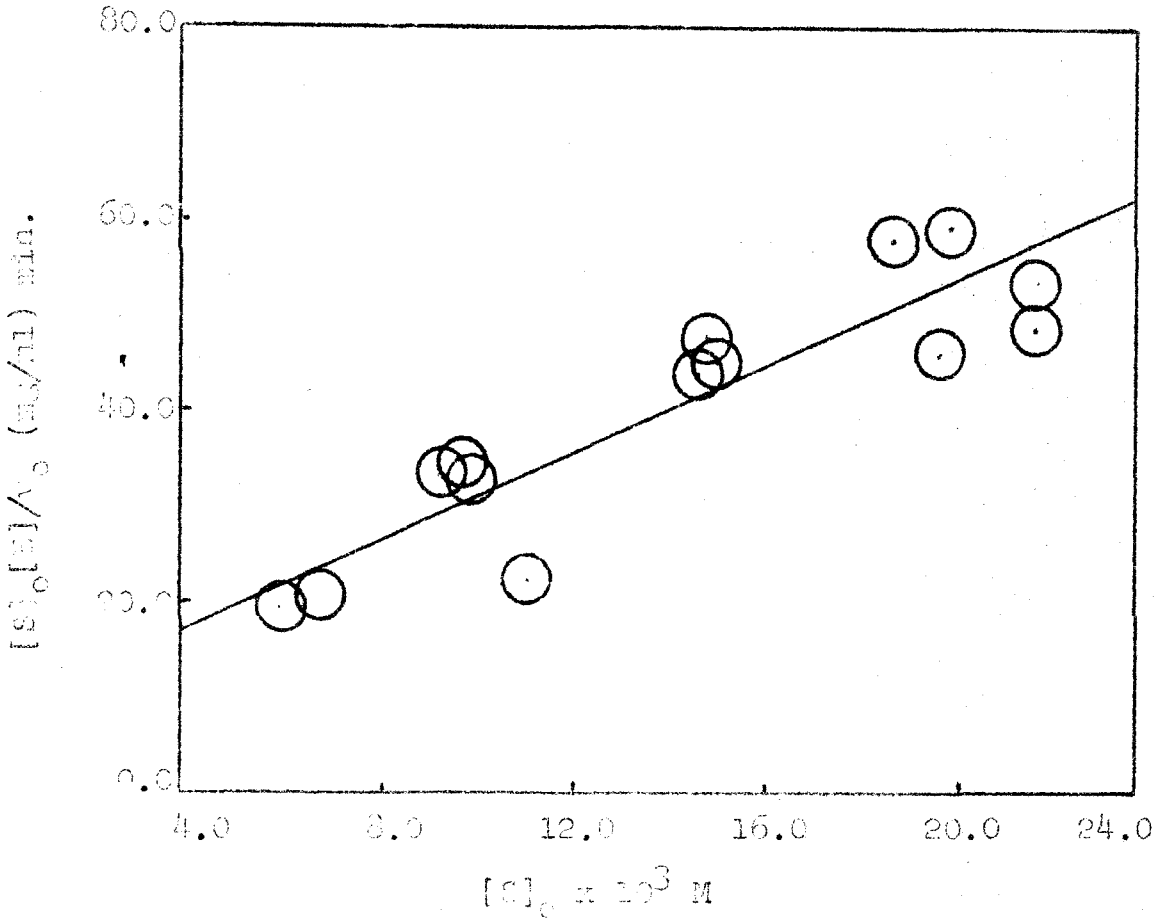


Figure 9

The alpha-chymotrypsin catalyzed hydrolysis of L-tryptophan-hydroxamide at pH 6.9. The circles represent results at enzyme concentrations higher than 1 mg/ml. The reaction conditions are: 25.0°, cacodylic acid buffer 0.150 F, ionic strength 0.150 M.

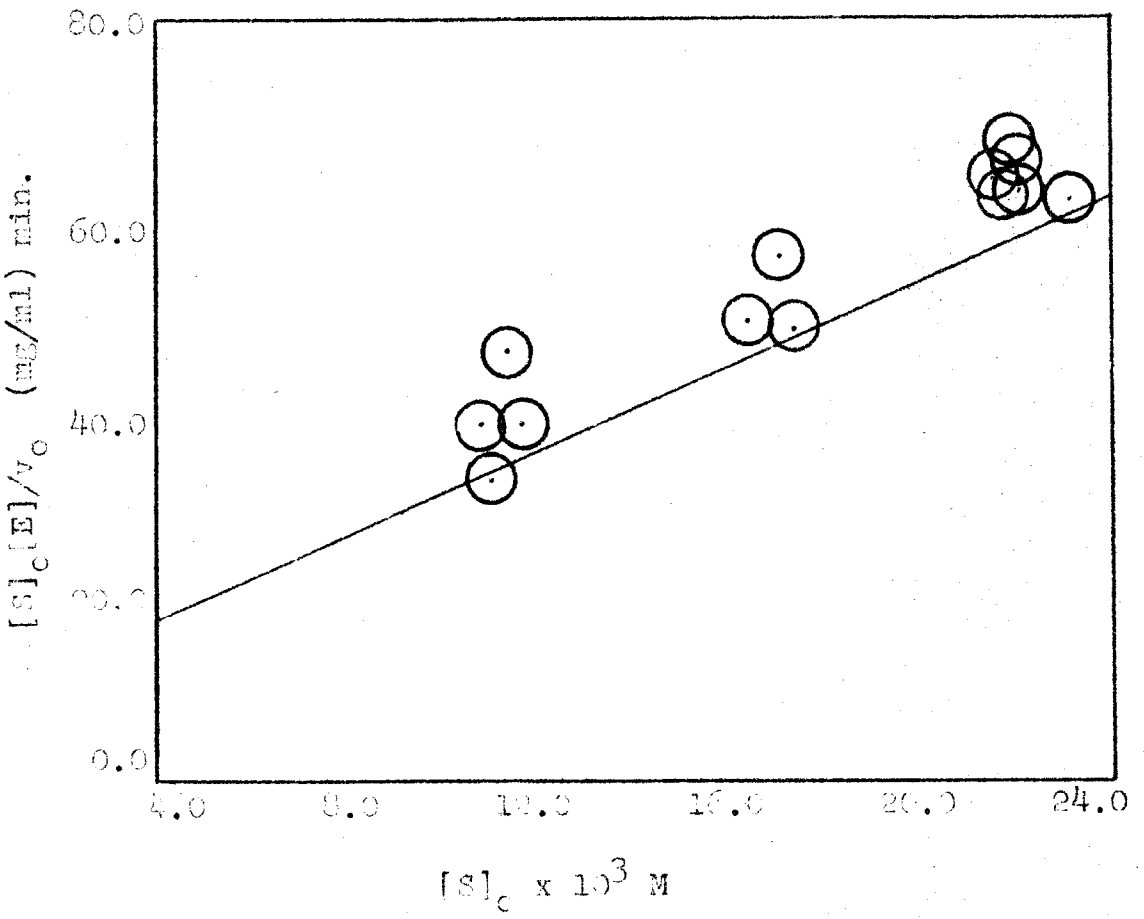


Figure 10

The alpha-chymotrypsin catalyzed hydrolysis of L-tryptophan-hydroxamide at pH 6.9. The circles represent results at enzyme concentrations higher than 1 mg/ml which have been corrected for the effect of dimerization. The reaction conditions are: 25.0°, cacodylic acid buffer 0.150 F, ionic strength 0.150 M.

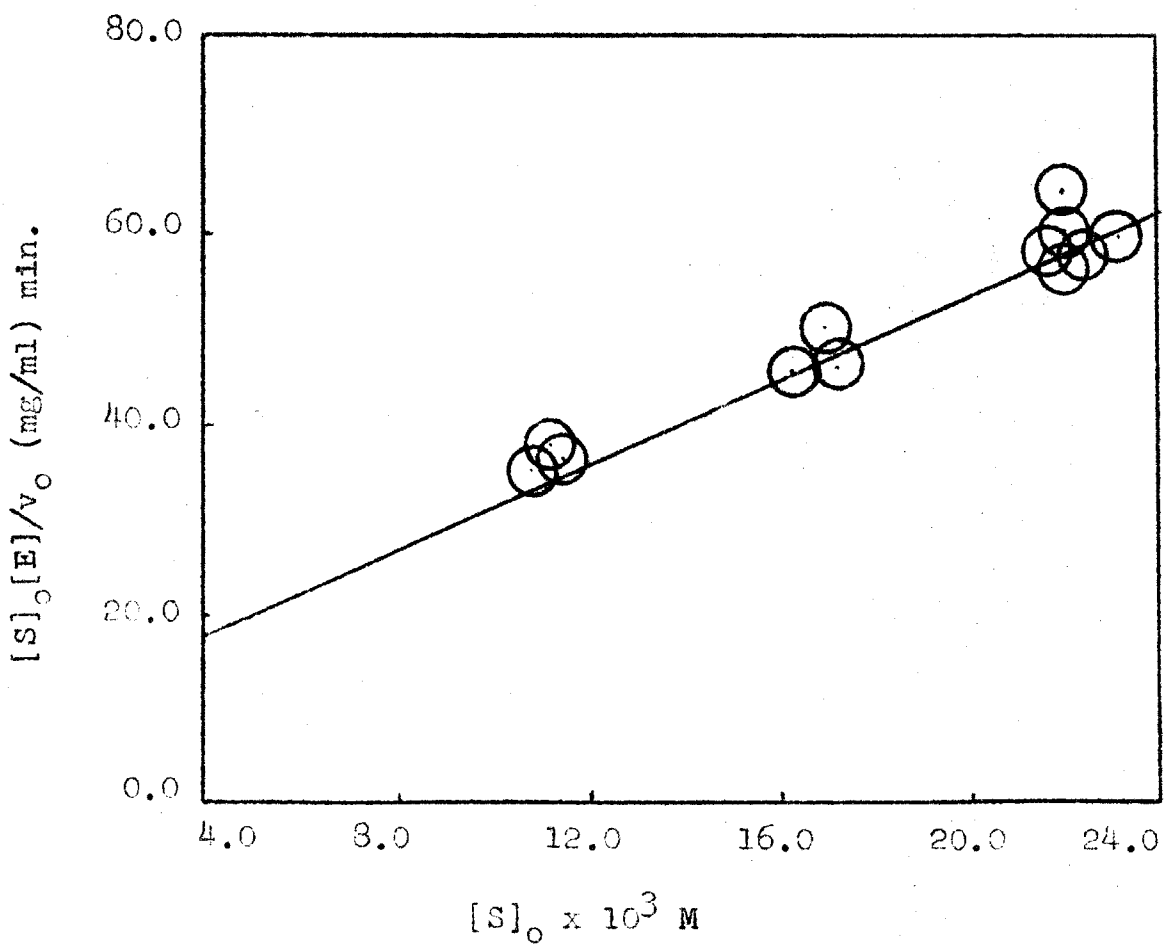


Figure 11

The alpha-chymotrypsin catalyzed hydrolysis of L-tryptophan-hydroxamide at pH 7.9. The reaction conditions are: 25.0°, THAM buffer 0.300 F, ionic strength 0.300 M.

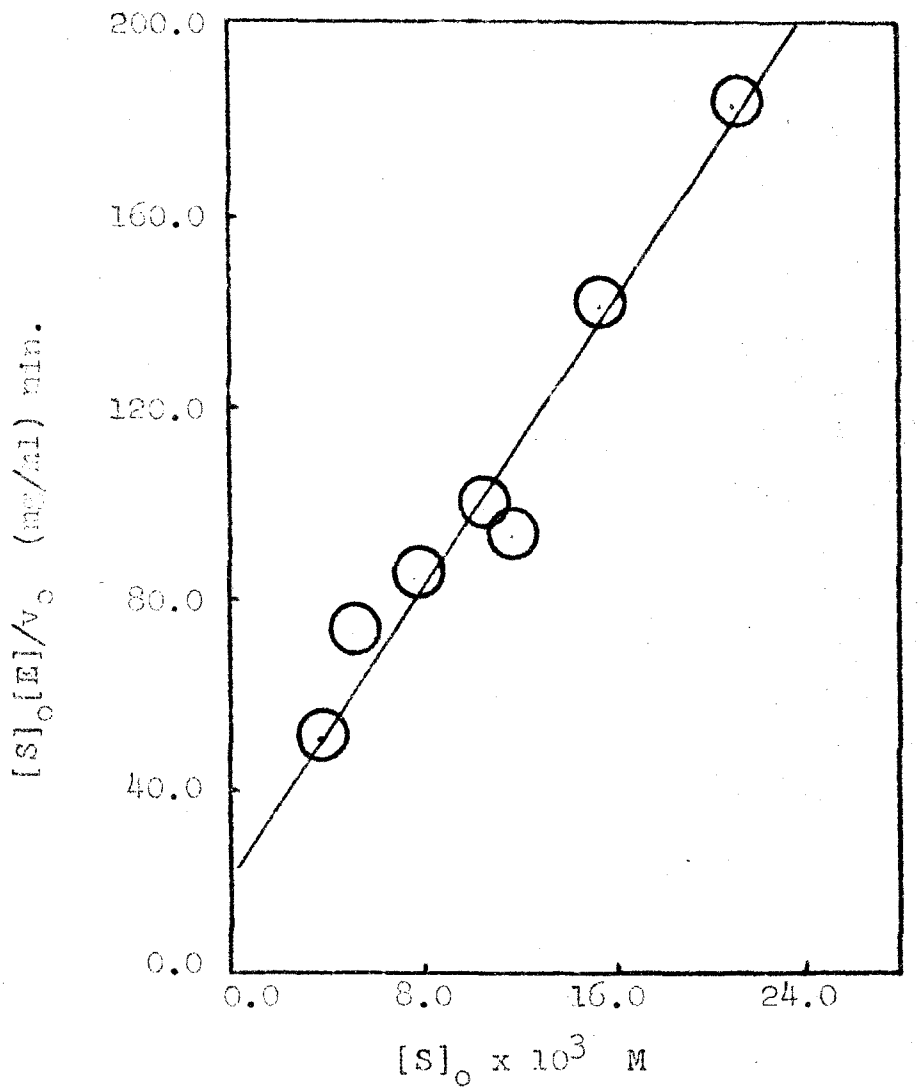
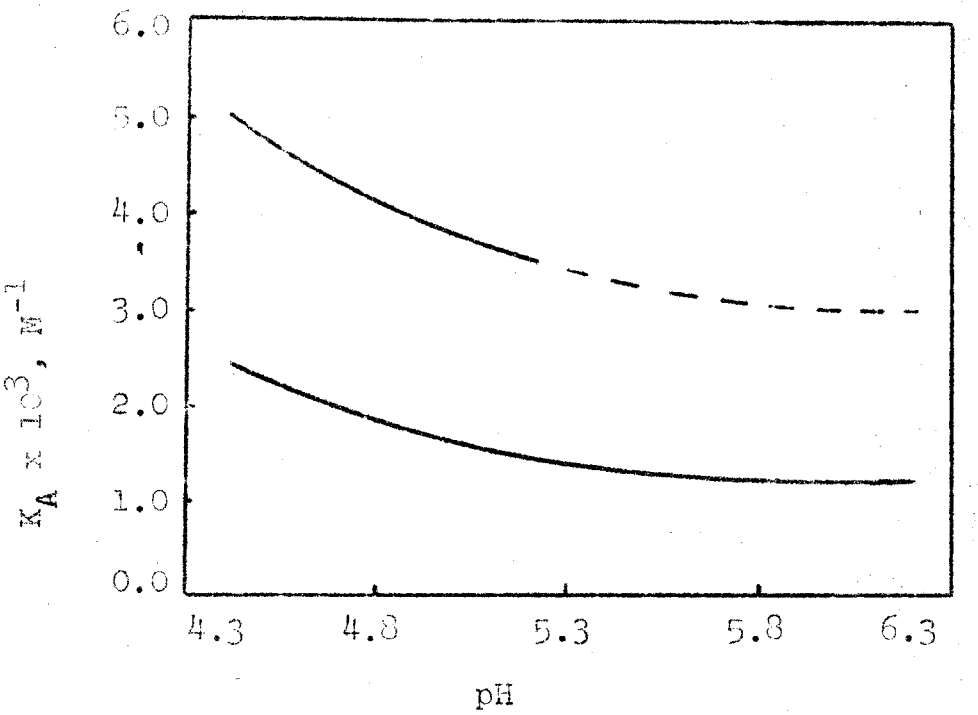


Figure 12

The association constant for alpha-chymotrypsin at 22° and in aqueous solution buffered by 0.1 F NaOAc. Data of Steiner (27).



The lower curve gives K_A as a function of pH at an ionic strength of 0.1 M. The upper curve, for an ionic strength of 0.3 M, has been extrapolated to pH 6.2.

Table I

Values of $L_{m,n}$ for n = 7 and n = 8

m	1	2	3	4	5
$L_{m,7}$	0.1542	1.139	1.605	2.510	4.100
$L_{m,8}$	0.1291	0.4543	1.061	1.940	2.876

Table III

Values of $(S_{m,n})^{1/2}$ for n = 7 and n = 8

m	1	2	3	4	5
$(S_{m,7})^{1/2}$	12.96	12.96	16.25	24.82	46.73
$(S_{m,8})^{1/2}$	7.75	52.65	31.46	44.74	21.63

Table IV

Values of $(\text{coeff. } v_o)_{m,n}$ for n = 7 and n = 8

m	1	2	3	4	5
$(\text{coeff. } v_o)_{m,7}$	2.000	7.000	18.333	47.833	150.633
$(\text{coeff. } v_o)_{m,8}$	1.000	24.000	30.166	72.666	45.933

Table II

Analysis of K. A. Booman Expt. No. 34

s	x_i (min.)	y_i (OD_i)	$P_0(s)$	$P_1(s)$	$P_2(s)$	$P_3(s)$	$P_4(s)$	$P_5(s)$
0	0	2.140	1	7	7	7	7	7
1	4	1.656	1	5	1	-5	-13	-23
2	8	1.291	1	3	-3	-7	- 3	17
3	12	0.960	1	1	-5	-3	9	15
4	16	0.724	1	-1	-5	3	9	-15
5	20	0.614	1	-3	-3	7	-3	-17
6	24	0.482	1	-5	1	5	-13	23
7	28	0.353	1	-7	7	-7	7	-7
(A) $S_m = \sum_{s=0}^{7} (P_m(s))^2$			8	168	168	264	616	2184
(B) $c_m = \sum_{s=0}^{7} P_m(s)f(s)$			8.220	20.646	5.454	1.192	-0.902	0.556
(C) $C_m = (B)/(A)$			1.027	1.228	3.246	4.515	-1.464	2.545
			5	928×10^{-1}	430×10^{-2}	152×10^{-3}	285×10^{-3}	790×10^{-4}
(D) $(C_m)^2$			1.055	1.510	1.053	2.038	2.144	6.481
			756	264×10^{-2}	931×10^{-3}	660×10^{-5}	130×10^{-6}	050×10^{-8}
(E) $\sum_{i=0}^{7} (y_i - y_i)^2 = \sum_{i=0}^{7} (OD_i)^2 -$								
$\sum_{k=0}^{m} S_k (C_k)^2$		11.168322	2.722	1.8503	7.9970	2.5873	1.2690	1.1273
			274	$\times 10^{-1}$	$\times 10^{-3}$	$\times 10^{-3}$	$\times 10^{-3}$	$\times 10^{-3}$

Table II (Continued)

	$P_0(s)$	$P_1(s)$	$P_2(s)$	$P_3(s)$	$P_4(s)$	$P_5(s)$
(F) $(n - m) = (7 - m)$	7	6	5	4	3	2
(G) $(\sigma_y)^2 = (E)/(F)$		3.0838×10^{-2}	1.5994×10^{-3}	6.4682×10^{-4}	4.2301×10^{-4}	5.6367×10^{-4}
(H) σ_y		175.60×10^{-3}	39.99×10^{-3}	25.43×10^{-3}	20.57×10^{-3}	
(I) $(S_m)^{1/2}$		12.96	12.96	16.25	24.82	46.73
(J) $t_{(n-m)} = (C)(I)/(H)$		9.070	10.520	2.888	1.762	
(K) $t_{(n-m),0.05}$			2.571	2.776	3.182	4.303
(L) m		1	2	3	4	5
(M) (coeff. v_o)		2.000	7.000	18.333	47.833	150.633
(N) $(-dy/ds)_{s=0} = \sum_{k=1}^{m} C_k$ (coeff. $v_o)_k$		0.2458	0.4730	0.5558		
(O) v_o(M/min. x 10^5) $= (N)/b\Delta t$				14.20×10^{-4}		
(P) L_m		0.1542	1.139	1.605	2.510	4.100
(Q) $\sigma (-dy/ds)_{s=0} = L_m \sigma_y$				0.04082		
(R) σv_o (M/min. x 10^5) $= (Q)/b\Delta t$				1.04×10^{-4}		

Table V

Results of Kinetic Studies on the alpha-Chymotrypsin Catalyzed Hydrolysis of L-Tryptophanhydroxamide

Expt. No.	$[S]_o \times 10^3$ M	[E] mg/ml	pH final	$\frac{[S]_o}{OD_o} \times 10^2$ M	Degree of OP*	$v_o \times 10^4$	$\sigma_{v_o} \times 10^4$
Ionic strength 0.300 M. v_o obtained by "method of averages" fit to a first order rate equation.							
Buffer is cacodylic acid except as noted.							
3 (a)	9.16	0.200	7.38			0.420	
6 (a)	9.16	0.200	7.78			0.292	
8	9.16	0.200	6.69			0.500	
10	9.16	0.200	6.05			0.279	
11	9.16	0.200	6.28			0.366	
14	9.16	0.200	7.19			0.483	
15	9.16	0.200	6.54			0.447	
16	9.16	0.200	7.05			0.525	
17	9.16	0.200	6.79			0.524	
18	9.16	0.200	6.94			0.554	
19 (a)	9.16	0.200	7.55			0.365	
Ionic strength 0.150 M. v_o obtained by the orthogonal polynominal method.							
32	21.81	0.635	6.89	1.10	2	2.64	0.13
34	21.79	6.312	6.84	1.02	2	11.9	1.0
35 (b)	21.86	0.0567	7.04	1.10	1	0.258	
36	21.96	2.94	6.89	1.06	3	9.96	0.22

Table V (Continued)

Expt. No.	$[S]_o \times 10^3$ M	[E] mg/ml	pH final	$\frac{[S]_o}{OD_o} \times 10^2$ M	Degree of CP*	$v_o \times 10^4$	$\sigma v_o \times 10^4$
37 (c)	21.85	0.197	6.97	2.87	2	0.810	0.05
38	21.96	4.30	6.81	1.05	3	15.2	0.24
39	21.89	1.01	6.83	1.05	2	3.32	0.02
40	10.95	0.508	6.83	1.02	3	1.89	0.02
41	10.95	0.248	6.85	1.03	2	0.888	0.04
43	11.27	0.0102	6.93	1.03	1	0.0533	0.005
45	15.10	0.999	6.85	1.04	2	3.70	0.06
47	15.20	0.100	6.99	1.04	1	0.339	0.06
48	6.08	0.100	6.88	1.04	2	0.318	0.02
49	6.85	0.0230	6.80	1.04	1	0.078	0.03
61	21.71	2.036	6.79	1.04	3	7.20	0.18
62	16.28	2.036	6.78	1.02	3	6.90	0.12
63	10.86	2.036	6.75	1.02	3	5.86	0.14
64	20.11	0.114	6.87	1.04	1	0.388	0.023
65	15.08	0.114	6.82	1.02	1	0.361	0.03
66	10.06	0.114	6.79	1.02	2	0.361	0.02
67	23.07	1.088	6.83	1.04	2	4.10	0.20
68	17.30	1.088	6.78	1.02	2	3.96	0.09
69	11.54	1.088	6.73	1.02	2	3.32	0.18
70	18.90	0.0584	6.90	1.03	1	0.191	0.021
71	14.18	0.0584	6.84	1.03	1	0.190	0.012
72	9.45	0.0584	6.80	1.02	1	0.167	0.011

Table V (Continued)

Expt. No.	$[S]_o \times 10^3$ M	[E] mg/ml	pH final	$\frac{[S]_o}{OD_o} \times 10^2$ M	Degree of OP*	$v_o \times 10^4$	$\sigma v_o \times 10^4$
73	22.40	3.142	6.79	1.04	3	11.10	0.16
74	16.99	3.146	6.76	1.03	3	9.84	0.46
75	11.29	3.142	6.73	1.02	2	7.92	1.3
76	19.28	0.284	6.85	1.03	2	1.02	0.08
77	14.46	0.284	6.80	1.01	2	1.01	0.07
78	9.64	0.284	6.75	1.01	1	0.59	±0.10
82	19.86	0.102	6.88	1.03	2	0.431	0.03
83	14.90	0.102	6.80	1.02	1	0.349	0.03
84	9.93	0.102	6.74	1.03	1	0.298	0.03
		Ionic strength 0.300 M.					
96 (d)	12.19	21.0	5.99	3.13	2	12	3
97	6.10	0.200	6.04	1.29	1	0.22	0.04
98	12.19	0.200	6.04	1.27	1	0.30	0.14
99 (d)	12.19	20.4	7.94	1.57	3	17.5	0.4
100	6.10	0.212	7.95	0.950	1	0.17	0.03
101	12.19	0.212	7.94	0.959	1	0.2	0.1
102	6.10	0.199	7.00	1.27	1	0.39	0.09
103	12.19	0.199	7.05	1.29	2	0.37	0.12
104 (d)	12.19	21.0	6.96	3.30	3	24.6	0.4
105 (d)	12.19	2.04	6.01	3.01	4	12.1	0.3
106	6.16	0.238	6.01	1.25	2	0.28	0.05
107	12.31	0.238	6.03	1.29	1	0.40	0.08

Table V (Continued)

Expt. No.	$[S]_o \times 10^3$ M	[E] mg/ml	pH final	$\frac{[S]_o}{OD_o} \times 10^2$ M	Degree of OP*	$v_o \times 10^4$	$\sigma v_o \times 10^4$
108 (d)	12.31	20.9	6.91	3.22	3	32	1
109	6.16	0.213	6.96	1.27	1	0.46	0.05
110	12.31	0.213	6.96	1.29	2	0.56	0.05
111 (d)	12.31	21.0	7.90	1.59	3	22.2	0.5
112	6.16	0.222	7.92	0.988	1	0.30	0.04
113	12.31	0.222	7.95	0.978	1	0.25	0.02
114	16.41	0.199	6.10	1.28	1	0.398	0.033
115	12.31	0.199	6.10	1.28	1	0.311	0.04
116	8.20	0.199	6.09	1.28	1	0.261	0.02
117	4.10	0.199	6.01	1.28	1	0.164	0.01
122 (a)	20.94	0.203	7.92	0.979	1	0.234	0.03
123 (a)	15.70	0.203	7.91	0.973	1	0.228	0.02
124 (a)	10.47	0.203	7.90	0.957	1	0.218	0.02
125 (a)	5.23	0.203	7.91	0.960	1	0.149	0.01
126	10.47	0.201	6.03	1.28	1	0.314	0.03
127	10.47	0.603	6.06	1.28	2	0.922	0.07
128	10.47	0.804	6.04	1.28	2	1.22	0.04
129	10.47	1.005	6.02	1.28	2	1.41	0.08
131 (a)	11.95	0.428	7.89	0.945	2	0.558	0.06
132 (a)	7.96	0.428	7.91	0.946	1	0.411	0.03
133 (a)	3.98	0.428	7.89	0.897	2	0.345	0.03
134	15.93	2.02	5.95	1.29	3	3.50	0.01
135	15.93	0.202	6.01	1.29	1	0.376	0.05

Table V (Continued)

Expt. No.	$[S]_o \times 10^3$ M	[E] mg/ml	pH final	$\frac{[S]_o}{OD_o} \times 10^2$ M	Degree of OP*	$v_o \times 10^4$	$\sigma_{v_o} \times 10^4$
136	7.96	2.02	6.01	1.29	3	2.10	0.06
137	7.96	0.202	5.99	1.29	1	0.241	0.03
138	11.95	2.02	6.01	1.29	2	2.50	0.1
139	16.42	0.200	7.06	1.27	2	0.706	0.05
140	16.42	1.00	6.98	1.27	3	3.32	0.07
141	12.31	1.00	6.98	1.26	3	3.20	0.05
142	8.21	1.00	6.98	1.26	3	2.74	0.02
143	4.10	0.200	6.98	1.25	2	0.448	0.05
144	17.27	0.200	6.99	1.29	2	0.700	0.05
145	17.27	1.00	6.99	1.29	2	3.21	0.1
146	12.95	1.00	6.98	1.28	3	3.17	0.02
147	8.64	1.00	6.95	1.27	3	2.69	0.06
148	4.32	0.200	6.95	1.26	2	0.413	0.02

*The highest degree orthogonal polynominal used in the representation of the experiment.

(a) THAM Buffer.

(b) OD measured at 475 millimicrons v_o from initial slope.

(c) 1 ml aliquot from reaction mixture diluted 1:25 instead of 1:10.

(d) Iron complex of the hydroxamide formed in water instead of methanol.

Table VI

Primary Data from Experiments on the alpha-Chymotrypsin Catalyzed Hydrolysis of L-Tryptophanhydroxamide at 25°.

Expt. No.	Δt, min.	Point No. 1	2	3	4	5	6	7
		Optical density at time equals Δt x (Point No.).						
3 (a)	4	0.746	0.644	0.590	0.519	0.458	0.415	0.363
6	5	0.802	0.774	0.761	0.753	0.741	0.732	
8	5	0.614	0.603	0.585	0.571	0.552	0.538	
11	5	0.618	0.597	0.590	0.576	0.566	0.558	
14 (b)	5	0.611	0.597	0.581	0.563	0.552	0.535	0.523
15	5	0.615	0.603	0.587	0.577	0.560	0.546	0.532
16	5	0.613	0.598	0.583	0.568	0.553	0.536	0.517
17	5	0.610	0.596	0.579	0.567	0.546	0.533	0.518
18	5	0.612	0.595	0.582	0.563	0.546	0.529	0.512
19	5	0.771	0.759	0.745	0.734	0.712	0.708	0.683
32	10	1.781	1.591	1.409	1.257	1.105	0.979	0.869
34	4	1.656	1.291	0.960	0.724	0.614	0.482	0.353
35	3	1.976	1.928	1.923	1.853	1.845	1.828	
36	4	1.734	1.442	1.224	1.016	0.772	0.700	0.580
37	10	0.734	0.704	0.686	0.658	0.632	0.612	0.592
38	4	1.606	1.203	0.899	0.681	0.511	0.378	0.284
39	10	1.772	1.514	1.391	1.163	0.963	0.800	0.663
40	10	0.897	0.756	0.632	0.533	0.446	0.373	0.308
41	10	0.982	0.899	0.838	0.768	0.685	0.652	0.612

Table VI (Continued)

Expt. No.	Δt, min.	Point No. 1	2	3	4	5	6	7
		Optical density at time equals Δt x (Point No.).						
43	10	1.076	1.066	1.065	1.062	1.054	1.052	1.046
45	4	1.320	1.184	1.079	0.962	0.857	0.768	0.691
47	4	1.429	1.438	1.420	1.395	1.390	1.375	1.367
48	4	0.575	0.566	0.556	0.547	0.536	0.526	0.514
49	4	0.651	0.653	0.655	0.651	0.652	0.641	0.643
61	6	1.728	1.405	1.121	0.902	0.711	0.574	0.458
62	6	1.234	0.948	0.718	0.550	0.409	0.311	0.232
63	6	0.765	0.545	0.374	0.267	0.182	0.126	0.090
64	15	1.910	1.851	1.788	1.731	1.682	1.631	1.573
65	15	1.441	1.378	1.319	1.275	1.210	1.169	1.119
66	15	0.946	0.896	0.851	0.801	0.759	0.722	0.686
67	6	2.019	1.802	1.595	1.438	1.258	1.115	0.978
68	6	1.468	1.271	1.094	0.941	0.806	0.694	0.586
69	6	0.930	0.790	0.640	0.522	0.430	0.351	0.290
70	15	1.802	1.780	1.762	1.726	1.701	1.665	1.650
71	15	1.360	1.332	1.310	1.274	1.251	1.227	1.196
72	15	1.905	1.887	0.859	0.837	0.810	0.790	0.760
73	6	1.613	1.175	0.855	0.614	0.442	0.316	0.227
74	6	1.176	0.763	0.513	0.339	0.233	0.149	0.098
75	6	0.681	0.413	0.252	0.155	0.092	0.107	0.035
76	15	1.756	1.611	1.477	1.347	1.231	1.127	1.025

Table VI (Continued)

Expt. No.	Δt, min.	Point No. 1	2	3	4	5	6	7
		Optical density at time equals Δt x (Point No.).						
77	15	1.288	1.156	1.026	0.912	0.818	0.737	0.639
78	15	0.840	0.725	0.630	0.539	0.478	0.392	0.350
82	15	1.861	1.808	1.758	1.715	1.656	1.602	1.554
83	15	1.389	1.339	1.289	1.248	1.193	1.140	1.101
84	15	0.929	0.874	0.840	0.800	0.745	0.716	0.671
96	2	0.318	0.243	0.196	0.160	0.126	0.100	0.083
97	4	0.468	0.460	0.450	0.448	0.440	0.429	0.430
98	4	0.940	0.920	0.912	0.910	0.901	0.905	0.879
99	2	0.583	0.421	0.298	0.214	0.153	0.112	0.086
100	4	0.632	0.628	0.616	0.610	0.604	0.600	0.589
101	4	1.256	1.258	1.244	1.239	1.228	1.223	1.212
102	4	0.475	0.461	0.458	0.429	0.421	0.409	0.400
103	4	0.919	0.903	0.912	0.895	0.872	0.866	0.859
104	2	0.245	0.156	0.096	0.063	0.044	0.034	0.020
105	2	0.322	0.260	0.209	0.170	0.136	0.113	0.095
106	4	0.482	0.475	0.461	0.456	0.447	0.437	0.430
107	4	0.956	0.942	0.928	0.921	0.917	0.892	0.880
108	2	0.218	0.110	0.059	0.035	0.024	0.020	0.017
109	4	0.473	0.456	0.441	0.420	0.415	0.399	0.385
110	4	0.938	0.919	0.903	0.881	0.867	0.848	0.838
111	2	0.540	0.351	0.223	0.142	0.096	0.071	0.060

Table VI (Continued)

Expt. No.	Δt, min.	Point No. 1	2	3	4	5	6	7
		Optical density at time equals Δt x (Point No.).						
112	4	0.638	0.623	0.609	0.599	0.588	0.581	0.566
113	4	1.249	1.238	1.229	1.219	1.210	1.195	1.189
114	8	1.265	1.231	1.212	1.186	1.166	1.139	1.116
115	8	0.949	0.928	0.910	0.890	0.865	0.850	0.840
116	8	0.635	0.615	0.597	0.581	0.568	0.551	0.538
117	8	0.319	0.309	0.298	0.288	0.278	0.268	0.259
122	8	2.122	2.108	2.088	2.072	2.047	2.022	2.009
123	8	1.601	1.582	1.567	1.543	1.526	1.500	1.489
124	8	1.072	1.051	1.028	1.019	1.000	0.985	0.961
125	8	0.540	0.525	0.511	0.499	0.485	0.475	0.461
126	8	0.799	0.781	0.758	0.740	0.717	0.700	0.682
127	8	0.769	0.707	0.664	0.615	0.576	0.535	0.503
128	8	0.739	0.674	0.616	0.560	0.511	0.473	0.434
129	8	0.738	0.656	0.582	0.522	0.474	0.417	0.378
131	6	1.238	1.200	1.171	1.134	1.109	1.080	1.057
132	6	0.824	0.798	0.768	0.739	0.719	0.689	0.664
133	6	0.422	0.403	0.378	0.361	0.342	0.326	0.309
134	10	1.000	0.794	0.640	0.532	0.438	0.366	0.307
135	10	1.219	1.181	1.157	1.115	1.096	1.051	1.045
136	10	0.477	0.357	0.269	0.207	0.160	0.126	0.100
137	10	0.604	0.581	0.565	0.545	0.518	0.505	0.493
138	10	0.728	0.587	0.446	0.356	0.288	0.235	0.202

Table VI (Continued)

Expt. No.	Δt, min.	Point No. 1	2	3	4	5	6	7
		Optical density at time equals Δt x (Point No.).						
139	10	1.222	1.184	1.131	1.090	1.041	1.000	0.961
140	10	1.047	0.848	0.683	0.538	0.430	0.338	0.269
141	10	0.743	0.569	0.428	0.320	0.239	0.175	0.128
142	10	0.462	0.317	0.215	0.145	0.095	0.064	0.039
143	10	0.292	0.264	0.228	0.215	0.190	0.171	0.156
144	10	1.294	1.242	1.191	1.150	1.111	1.062	1.026
145	10	1.119	0.906	0.729	0.579	0.468	0.370	0.292
146	10	0.795	0.614	0.466	0.353	0.262	0.193	0.142
147	10	0.496	0.348	0.241	0.166	0.113	0.074	0.060
148	10	0.312	0.280	0.255	0.228	0.208	0.187	0.166

(a) The points were actually observed at the following times:
3:00, 7:10, 11:00, 15:00, 19:00, 23:00, 27:00 minutes.

(b) The points were actually observed at the following times:
5:00, 10:30, 15:00, 20:50, 26:00, 30:00, 35:00, 40:00 minutes.

Table VII

The Effect of a Hundred-fold Change in Enzyme Concentration on the alpha-Chymotrypsin Catalyzed Hydrolysis of L-Tryptophanhydroxamide

Reaction conditions: 25.0°, $[S]_o$ 12 x 10^{-3} M, ionic strength 0.300 M.

Expt. No.	pH	[E] mg/ml	$v_o \times 10^4$ M min^{-1}	$v_o/[E]$ M min^{-1} $(mg/ml)^{-1}$	r_H/r_L
96	5.99	21.0	12	0.572	0.382
98	6.04	0.200	0.30	1.50	
99	7.94	20.4	17.5	0.858	1
101	7.94	0.212	0.2	0.9	
103	7.05	0.199	0.37	1.86	0.629
104	6.96	21.0	24.6	1.17	
105	6.01	20.4	12.1	0.694	0.413
107	6.03	0.238	0.40	1.68	
108	6.91	20.9	32	1.53	0.582
110	6.96	0.213	0.56	2.63	

Table VIII

Results of the alpha-Chymotrypsin Catalyzed Hydrolysis of L-Tryptophanhydroxamide at pH 6.0.

The buffer system is 0.300 F cacadylic acid. The ionic strength is 0.300 M.

Expt. No.	$[S]_o \times 10^3$ M	[E] mg/ml	$v_o \times 10^4$ M min^{-1}	$[S]_o[E]/v_o$ (mg/ml)min	$([S]_o[E]/v_o)^*$ (mg/ml)min
114	16.41	0.199	0.398	82.1	
115	12.31	0.199	0.311	75.0	
116	8.20	0.199	0.261	62.6	
117	4.10	0.199	0.164	49.9	
126	10.47	0.201	0.314	67.0	
127	10.47	0.603	0.922	68.4	64.7
128	10.47	0.804	1.22	68.9	63.8
129	10.47	1.005	1.41	74.5	68.0
134	15.93	2.02	3.50	92.0	80.7
135	15.93	0.202	0.376	85.7	
136	7.96	2.02	2.10	76.6	65.5
137	7.96	0.202	0.241	66.8	
138	11.95	2.02	2.50	96.8	

*Corrected for the effect of dimerization.

Table IX

Results of the alpha-Chymotrypsin Catalyzed Hydrolysis of L-Tryptophanhydroxamide at pH 7.0.

The buffer system is 0.300 F cacadylic acid. The ionic strength is 0.300 M.

Expt. No.	$[S]_o \times 10^3$ M	[E] mg/ml	$v_o \times 10^4$ M min^{-1}	$[S]_o[E]/v_o$ (mg/ml)min	$([S]_o[E]/v_o)^*$ (mg/ml)min
139	16.42	0.200	0.706	46.6	
140	16.42	1.00	3.32	49.6	48.2
141	12.31	1.00	3.20	38.5	37.2
142	8.21	1.00	2.74	30.0	28.7
143	4.10	0.200	0.448	18.4	
144	17.27	0.200	0.700	49.4	
145	17.27	1.00	3.21	53.8	52.2
146	12.95	1.00	3.17	40.9	39.5
147	8.64	1.00	2.69	32.1	30.7
148	4.32	0.200	0.413	20.9	

*Corrected for the effect of dimerization.

Table X

Results of the alpha- Chymotrypsin Catalyzed Hydrolysis of L-Tryptophanhydroxamide at pH 6.9.

Buffer system 0.150 F cacadylic acid. Ionic strength 0.150 M.

Expt. No.	$[S]_o \times 10^3$ M	[E] mg/ml	$v_o \times 10^4$ M min^{-1}	$[S]_o[E]/v_o$ (mg/ml)min	$([S]_o[E]/v_o)^*$ (mg/ml)min
32	21.81	0.635	2.64	52.5	51.3
35	21.86	0.0567	0.258	48.0	
36	21.96	2.94	9.96	64.8	59.4
37	21.85	0.197	0.810	53.2	
38	21.96	4.30	15.2	62.2	55.5
39	21.89	1.01	3.32	66.6	64.2
40	10.95	0.508	1.89	29.4	28.5
41	10.95	0.248	0.888	30.6	30.1
43	11.27	0.0102	0.0533	21.6	
45	15.10	0.999	3.70	40.8	38.9
47	15.20	0.100	0.339	44.8	
48	6.08	0.100	0.318	19.1	
49	6.85	0.0230	0.078	20.2	
61	21.71	2.04	7.20	61.4	57.5
62	16.28	2.04	6.90	48.0	44.3
63	10.86	2.04	5.86	37.7	34.1
64	20.11	0.114	0.388	59.1	
65	15.08	0.114	0.361	47.6	
66	10.06	0.114	0.361	31.8	

Table X (Continued)

Expt. No.	$[S]_o \times 10^3$ M	[E] mg/ml	$v_o \times 10^4$ M min^{-1}	$[S]_o[E]/v_o$ (mg/ml)min	$([S]_o[E]/v_o)^*$ (mg/ml)min
70	18.90	0.0584	0.191	57.8	
71	14.18	0.0584	0.190	43.7	
72	9.45	0.0584	0.167	33.1	
82	19.86	0.102	0.437	46.4	
83	14.90	0.102	0.349	43.5	
84	9.93	0.102	0.298	34.0	
67	23.07	1.088	4.10	61.2	59.0
68	17.30	1.088	3.96	47.5	45.4
69	11.54	1.088	3.32	37.8	35.6
73	22.40	3.14	11.1	63.4	57.0
74	16.99	3.14	9.84	55.3	49.7
75	11.29	3.14	7.92	44.8	37.0
76	19.28	0.284	1.02	53.7	53.1
77	14.46	0.284	1.01	40.7	40.1
78	9.64	0.284	0.59	46.4	45.5

*Corrected for the effect of dimerization.

Table XI

Results of the alpha-Chymotrypsin Catalyzed Hydrolysis of L-Tryptophanhydroxamide at pH 7.9.

Buffer system 0.300 F THAM. Ionic strength 0.300 M.

Expt. No.	$[S]_o \times 10^3$ M	[E] mg/ml	$v_o \times 10^4$ M min^{-1}	$[S]_o[E]/v_o$ (mg/ml)min
122	20.94	0.203	0.234	182
123	15.70	0.203	0.228	140
124	10.47	0.203	0.218	97.7
125	5.23	0.203	0.149	71.2
131	11.95	0.428	0.558	91.6
132	7.96	0.428	0.411	83.0
133	3.98	0.428	0.345	49.5

Table XII

Summary of the Kinetic Constants for the alpha-Chymotrypsin Catalyzed Hydrolysis of L-Tryptophanhydroxamide

Ionic strength 0.300 M

pH	6	7	8
$k_3 \times 10^3$, M/min/mg prot. N_2/ml	2.6 ± 0.1	3.1 ± 0.2	0.93 ± 0.07
$K_S \times 10^3$, M	15.4 ± 0.5	5 ± 2	3 ± 1
$K_D \times 10$, mg prot. N_2/ml	7 ± 4	10 ± 1	large

Ionic strength 0.150 M

$k_3 \times 10^3$, M/min/mg prot. N_2/ml	3.1 ± 0.4
$K_S \times 10^3$, M	4 ± 2
$K_D \times 10$, mg prot. N_2/ml	4 ± 4

PROPOSITIONS

1. It is proposed that calorimetry is at such a stage of refinement that this method may meet the need for a general and continuous means of following enzyme reactions (1, 2).

2. It is proposed that further information concerning the mode of action of proteolytic enzymes may be gained by studying the catalyzed reactions in solutions of heavy water.

3. The concept that the side-chain for a substrate of alpha-chymotrypsin must be aromatic was proven false when Jennings and Niemann (3) published data concerning the hydrolysis of acetyl-L-hexahydrophenylalaninamide.

It is proposed that cycloheptyl-, cyclopentyl-, cyclobutyl-, and cyclopropylalanine be investigated as substrates for alpha-chymotrypsin.

4. Dimerization should be suspected as a possible feature of mechanism for any enzyme-catalyzed system in which the reaction being catalyzed is that of a protein or a protein model.

5. A linear relationship between K_S and k_3 for the alpha-chymotrypsin-catalyzed hydrolysis of substrates derived from acetyl-L-tyrosine may exist.

It is proposed that more substrates derived from acetyl-L-tyrosine should be investigated.

6. It is proposed that compounds derived from 2-amino-4-phenylbutanoic acid and 3-amino-4-phenylbutanoic acid will give information concerning the requirements for substrates of alpha-chymotrypsin.

7. Acetylene has been copolymerized with a number of vinylidene compounds (4). Copolymerization of acetylene with ethylene may raise the softening point of the polyethylene product.

8. It is proposed that nitrophenyl esters of acylated amino acids may be prepared by heating the acylated amino acid with nitrophenyl acetate. A strong acid would be used as catalyst. The equilibrium would be displaced by the removal of acetic acid.

9. Dichlorocarbenes should be investigated as ring-expanding agents for the synthesis of azulenes (5) and tropilidenes (6).

10. It is proposed that the 1,6-cyclodecadione intermediate in the Pfau and Plattner synthesis (7) of azulene may be obtained by the reaction of lead tetraacetate with decalin-9,10-diol.

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