I. INVESTIGATIONS OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF AMINO ACID HYDRAZIDES

II. SYNTHESIS OF AMINO ACID DERIVATIVES

III. THE APPARENT IONIZATION CONSTANTS OF A SERIES OF PHENYLALANINE DERIVATIVES

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ABSTRACT

The effect of variation in the concentration of tris-(hydroxymethyl)-aminomethane buffer systems upon the kinetic constants of the alpha-chymotrypsin catalyzed hydrolysis of nicotinyl-L-tyrosinhydrazide has been determined at pH 7.9 and 25.0°C. This study indicates a linear relationship between the logarithm of values of k_3 and the square root of the ionic strength. It has been demonstrated, in the case of the hydrazide investigated, that the value of the Michaelis-Menten constant, K_S, is independent of the variations applied.

A confirmation of the activating influence of calcium ion has been made. An increase of approximately 75% in the initial velocity of the alpha-chymotrypsin catalyzed hydrolysis of nicotinyl-L-tyrosinhydrazide is noted upon the addition of 10^{-1} M calcium ion to the reaction system.

The colorimetric determination of hydrazine employed to determine the extent of hydrolysis in the above cases has been reinvestigated.

A reinvestigation of the kinetics of the alpha-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinhydrazide utilizing improved procedures of analysis indicated above has been made. The possibility of observing product inhibition depending upon values of initial substrate concentration and K_P is discussed.

The preparation of acetyl-, benzoyl-, trifluoracetyl-,

and methanesulfonyl-DL-phenylalanine salicylamide esters is described. Several attempts to synthesize the optically active esters are described. Reasons for racemization in the cases tried are briefly discussed.

During the course of attempted dehydration procedures with polyphosphoric acid several interesting dehydrocyclization products are isolated and described.

The preparation of trifluoracetyl-L-phenylalanine anthranilamide is described.

The synthesis of acetyl-DL-phenylalanine glycolamide and acetyl-L-phenylalanine glycolamide are described.

Preliminary investigations were conducted in the alpha--chymotrypsin catalyzed hydrolysis of the above compound.

The apparent ionization constants of the protonated amino group of a series of DL-phenylalanine derivatives have been determined. The expected differences in the pK of these derivatives due to changes in acid derivative were not realized except in the case of phenylalanine hydrazide. The use of an automatic pH titration and recording instrument is described.

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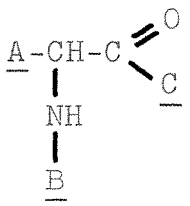
PART I

INVESTIGATIONS OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF AMINO ACID HYDRAZIDES

INTRODUCTION AND FORMULATION OF ENZYME KINETICS

The proteolytic enzyme, alpha-chymotrypsin, is a catalyst for the hydrolysis of proteins. One approach to the understanding of the nature of this reaction is the investigation of the kinetics of its hydrolysis of simple peptides or simple amino acid derivatives. If the alpha-amino acid derivatives are represented by the general formula:



the nature of the A, B, and C groups may be reviewed.

The bond upon which the alpha-chymotrypsin specifically acts is the bond between the carbonyl carbon and the group represented by C. Hydrolysis has been found to occur when this group is an ethyl ester (1), a methyl ester (1), an amide (1-7), a hydroxamide (1,8,12), a hydrazide (1,9,10,11), a salicylamide ester (22) or a glycolamide ester (22). The variations in B include carbobenzoxy (1), carbobenzoxyglycyl (1), chloracetyl (6), trifluoracetyl (6), acetyl (1,3,4,7,9,10), nicotinyl (1,3,4,5,8,10), benzoyl (1,10), acetylglycyl (11) and pyvaloyl (23). The group A has been the radical cor-

responding to the group found in L-tyrosine (1,2,6,8,9,10, 11,23), L-tryptophan (1,2,3,5,12), L-phenylalanine (6), L-hexahydrophenylalanine (8), L-methionine (1), L-arginine (1), L-norleucine (1), and L-norvaline (1).

Since a great deal of the investigations reported in this thesis involve kinetic studies of the alpha-chymotrypsin catalyzed hydrolyses, it is felt that a brief formulation of the basic kinetic equations should be presented.

The mathematical expressions of the kinetic equations for these hydrolysis reactions have been formulated previously and have been reviewed in a number of very extensive articles (1,13,14,15). The rate of reaction of simple hydrolysis and the slightly more complicated reaction involving inhibition by the reaction products or one of the reaction products will be covered briefly and referred to throughout the subsequent investigations.

Simple Hydrolysis

Consider an enzyme catalyzed reaction which may be represented by the following equation:

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

where

k_1, k_2, and k_3 are specific rate constants and

$[E_f]$ = formal concentration of free enzyme

$[S_f]$ = formal concentration of free substrate

$[ES]$ = formal concentration of enzyme-substrate complex

$[P_{1f}]$ and $[P_{2f}]$ = formal concentrations of products

$[S]$ = total concentration of substrate

$[E]$ = total concentration of enzyme

Further consider the case where all of the above possess unit activity.

The rate of formation of the complex:

$$\frac{d[ES]}{dt} = k_1 \left[([E]-[ES])\ [S] \right] - \left[(k_2 + k_3)\ [ES] \right] \qquad (2)$$

The rate of disappearance of the substrate:

$$-\frac{d[S]}{dt} = k_1 \left[([E] - [ES])\ [S] \right] - k_2[ES] \qquad (3)$$

The summation of (2) and (3) is the rate of the overall reaction:

$$\frac{d([ES] + [S])}{dt} = - k_3[ES] \qquad (4)$$

If the restricted case of steady state is considered where $\frac{-d[ES]}{dt} \ll - \frac{d[S]}{dt}$ the validity of the following equation is assumed:

$$\frac{d[ES]}{dt} \doteq 0 \doteq k_1 \Big[([E]-[ES])[S]\Big] - \Big[(k_2+k_3)[ES]\Big] \quad (5)$$

Utilizing this relationship, equations (2) and (3) reduce to equation 6:

$$-\frac{d[S]}{dt} = k_3\,[ES] \quad (6)$$

also from equation 2:

$$k_1([E]-[ES])[S] = (k_2+k_3)[ES]$$

Define K_S, the Michaelis-Menton constant, as,

$$K_S = \frac{([E]-[ES])[S]}{[ES]} = \frac{[E_f][S]}{[ES]} = \frac{k_2+k_3}{k_1} \quad (7)$$

Since $[ES] = \dfrac{[E_f][S]}{K_S}$, then $[E]-[ES] = [E_f]$

and

$$[E_f] = \frac{[E]}{1 + \dfrac{[S]}{K_S}} \quad (8)$$

Upon substitution of 8 into 6:

$$-\frac{d[S]}{dt} = \frac{k_3[E_f][S]}{K_S}$$

$$= \frac{k_3[E][S]}{K_S + [S]} \quad (9)$$

In equation 9, K_S approaches an equilibrium constant when $k_2 \gg k_3$. There are a number of forms of equation 9 from which the values of K_S and k_3 may be derived. Lineweaver and Burk obtained and utilized the following rearrangement:

$$\frac{1}{v_o} = \frac{K_S + [S]_o}{V_m[S]_o} = \frac{K_S}{k_3[E]}\left[\frac{1}{[S]_o}\right] + \frac{1}{k_3[E]} \qquad (10)$$

If $1/v_o$ is plotted versus $1/[S]_o$ a straight line results with a slope of $K_S/k_3[E]$ and an intercept of $1/k_3[E]$. Further rearrangements result in a number of other convenient plots (15-17). A plot of $[S]_o/v_o$ versus $[S]_o$ yields a straight line with slope of $1/k_3[E]$ and an intercept of $K_S/k_3[E]$ and another variant of v_o versus $v_o/[S]_o$ gives an ordinate intercept of $k_3[E]$, an abscissa intercept of $k_3[E]/K_S$ and a line of slope $-K_S$.

Hydrolysis Involving Inhibition by Product or Products

The hydrolysis of an alpha-amino acid derivative by alpha-chymotrypsin may be slowed down, inhibited, by some process which blocks the so-called "active-site" of the enzyme.

The theory that the hydrolysis of the substrate by the enzyme involves the interaction with three centers of the enzyme site has much evidence in its favor (3,5,6,18,

19,20,21). This means then that inhibition of the reaction involves blocking of one or more of these centers. There may be inhibition then at one, two, or three of these centers. In this regard one or theoretically both of the hydrolysis products may inhibit the reaction. It has been assumed, thus far, that the inhibition is due to the amino acid moiety and not the other hydrolysis product since inhibition by the latter has not been observed upon the addition of these substances to the system.

The problem of inhibition by products has been circumvented in a number of cases by consideration of the reaction at a low percent hydrolysis where the concentrations of the products are negligible and the assumption that very little product inhibition has taken place is a good one. However, Foster and Niemann (21) have considered, in detail, the case where product inhibition is evident.

For a reaction system where there is product inhibition one can derive the corresponding rate equation. If the system can be described by equations 1, 11, and 12:

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

$$E_f + P_{1f} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EP_1 \qquad (11)$$

$$E_f + P_{2f} \underset{k_7}{\overset{k_6}{\rightleftharpoons}} EP_2 \qquad (12)$$

and the steady state is assumed, as before, and the assumptions that $[S] \doteq [S_f]$ and $[P_1] \doteq [P_{1f}]$ are made it follows that

$$\frac{k_2 + k_3}{k_1} = \frac{([E]-[ES]-[EP_1])([S])}{[ES]} = K_S \qquad (13)$$

$$\frac{k_5}{k_4} = \frac{([E]-[ES]-[EP_1])\ ([S]_o-[S]_t)}{[EP_1]} = K_{P_1} \qquad (14)$$

$$-\frac{d[S]}{dt} = \frac{k_3[E][S]}{K_S\left[1 + \frac{[S]_o-[S]_t}{K_{P_1}}\right] + [S]} \qquad (15)$$

and

$$k_3[E]t = K_S\left[1 + \frac{[S]_o}{K_{P_1}}\right] \ln \frac{[S]_o}{[S]_t} + \left[1 - \frac{K_S}{K_{P_1}}\right]$$

$$([S]_o - [S]_t) \qquad (16)$$

A form of equation 16 that is often used is the following:

$$k_3[E]_t = K_S(1+[S]_o \sum_{j=1}^{n} 1/K_{P_j})\ln [S]_o/[S]_t +$$

$$(1-K_S \sum_{j=1}^{n} 1/K_{P_j} ([S]_o-[S]_t) \qquad (17)$$

The procedure used for the evaluation of K_P and K_S in most cases is the method of Foster and Niemann (2). For zone A conditions (24,25) and if $1/K_P = \sum_{j=1}^{n} 1/K_{P_j}$ equation 17 may be simplified and rearranged to give equation 18,

$$k_3[E]t = (1-K_S/K_P)([S]_o-[S]_t) + K_S(1+[S]_o/K_P)$$

$$\ln[S]_o/[S]_t \qquad (18)$$

A plot of $([S]_o-[S]_t)/t$ <u>versus</u> $\ln ([S]_o/[S]_t)/t$ in the case of a specific initial substrate concentration will be linear and have a slope of $-K_S(K_P + [S]_o)/(K_P-K_S)$. Straight lines drawn through the origin will have slopes equal to $[S]_o$ (25a) and therefore the intersection of a line drawn through the origin with a slope equal to a specific initial substrate concentration used in an experiment with a line defined by the experimental data will define in terms of the ordinate the corresponding initial velocity, i.e., v_o. A straight line drawn through a series of these inter-

sections, derived from a number of experiments at various substrate concentrations, will have a slope of $-K_S$, an ordinate intercept of $k_3[E]$ and an abscissa intercept of $k_3[E]/K_S$.

The different methods of evaluating the initial velocity, v_o, are discussed in the following section.

THE EFFECT OF VARIATION IN THE CONCENTRATION OF THAM* BUFFER SYSTEMS UPON THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF NICOTINYL-L-TYROSINHYDRAZIDE AT 25.0°C. AND pH 7.9

Introduction

The indication that the addition of salts, per se, or the consequent change in ionic strength accompanying this addition, would have a marked effect upon the proteolytic enzyme, alpha-chymotrypsin, arose with the report of Jandorf (26) in 1950. He observed as much as a five-fold increase in the esterase and amidase activity of the enzyme upon the addition of magnesium sulfate to the systems which he was studying. The extension of this primary salt effect to enzymatic cleavage rates of amide linkages was made in 1952. Shine and Niemann (6) noticed a 24 percent increase in the rate of hydrolysis of chloracetyl-L-tyrosinamide at 25°C. and pH 7.75 upon the addition of magnesium sulfate to the reaction solution. The system was approximately .1 M in magnesium sulfate. The early work of Neurath and co-workers (27) on the effect of calcium ion and other divalent cations upon the alpha-chymotrypsin catalyzed hydrolysis of either ester or amide

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*Tris-(hydroxymethyl)-aminomethane.

linkages has recently been substantiated by Laskowski (28). Neurath found the activity of alpha-chymotrypsin was increased to 150 percent of its initial level by the addition of 10^{-2} M calcium ion with phenylalanine ethyl ester as the substrate. Laskowski studied the effect of calcium ion on both alpha- and beta-chymotrypsins. His results were very similar to earlier ones and the substrate employed was also phenylalanine ethyl ester. It was stated in the work of Neurath that calcium was far more effective in producing the increase in rate than was magnesium or any of the other divalent cations investigated. These results would seem to indicate that the effect of calcium is a specific ion effect. Since these studies were all conducted with an ester as the substrate, the effect of calcium upon the hydrolysis rate of nicotinyl-L-tyrosinhydrazide was investigated and is reported in a later section of this part of the thesis.

Earlier investigations have shown that in the case of the hydroxamide substrates an increase in the initial velocity by calcium ion can be demonstrated. Jennings (29) determined the kinetic constants for the alpha-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide in the presence of 0.04 M $CaCl_2$ at pH 7.6. The k_3 value was shown to increase from 1.41×10^{-3} M/min. in the absence of added calcium to 1.61×10^{-3} M/min. in the presence of 4×10^{-2} M $CaCl_2$.

In 1955, Shine and Niemann (30) reported an investigation of the effect of added sodium and potassium chloride upon the alpha-chymotrypsin catalyzed hydrolysis of chloracetyl-L-tyrosinamide at 25.0°C. and pH 7.75. It was found that a straight line was obtained when the square root of the molarity of either the added sodium or potassium chloride was plotted versus the logarithm of the initial velocity. From a least squares fit of the data, in the case of added sodium chloride, to the equation $\log v_o/v_o^o = a + b\sqrt{M}$ it was possible to express the results by the simple relationship, $\log v_o/v_o^o = 0.304 \pm 0.005\sqrt{M}$ where v_o^o has replaced a and is the initial velocity in the absence of added sodium chloride. A second set of experiments with potassium chloride as the added salt gave the result, $\log v_o/v_o^o = 0.298 \pm 0.009\sqrt{M}$. It was concluded then that within the limits of experimental error the effect could be summarized by equation 19.

$$\log v_o/v_o^o = (0.30 \pm 0.01)\sqrt{M} \qquad (19)$$

where M, the molality of the added sodium or potassium chloride is within the limits of 0 and 1.5 M.

It was further shown in this investigation that the Michaelis-Menten constant, K_S, evaluated at .3 M and .4 M with respect to added sodium chloride were, respectively,

$23 \pm 3 \times 10^{-3}$ M and $26 \pm 2 \times 10^{-3}$ M. In the absence of added salt $K_S = 27 \pm 2 \times 10^{-3}$ M. It was concluded that K_S does not vary, within the limits of experimental error, with the addition of the named salts.

It is interesting to note in the case of a very simple substrate, methyl hydrocinnamate, hydrolysis by alpha-chymotrypsin is seemingly unaffected by inorganic ions and the effects of the buffers employed in this work seem to be ionic strength effects (31).

Bernhard (32) conducted a series of experiments with acetyl-L-tyrosinamide as the specific substrate. They were conducted at 25.0°C. and pH 7.9 in aqueous solutions and in the presence of varying amounts of phosphate buffers. The experimental data were evaluated by the method described by Foster and Niemann (21). The various K_S values determined were in good agreement, however there was apparent lack of agreement, well outside the limits of experimental error, between various k_3 values and between k_3 determined in presence of a less concentrated THAM-THAM·HCl buffer system. Apparently the k_3 values increase with increasing phosphate buffer and/or with increasing ionic strength. The adherence to a precise linear relationship was not found upon examining various relationships that might exist between the constant k_3 and ionic strength.

In previous investigations where the addition of buffer was required to maintain the pH of the reaction system

of various alpha-chymotrypsin catalyzed hydrolyses at the optimum, a frequently employed buffer system has been the univalent tris-(hydroxymethyl)-aminomethane-tris-(hydroxymethyl)-aminomethane-hydrochloride.* Since the concentration of buffer required to maintain this optimum pH varies depending upon the substrate employed, a range of different molalities have been used (e.g. most frequently employed is .02 M in the amine component, however, in the case of hydroxamides as high as 0.5 M has been employed) (7,33,34).

Since it was reasonable to suspect an effect of this variation, it was decided to look at the variation of THAM buffer systems over an extended range. It was the purpose of these experiments to ascertain if any simple relationship as postulated in the case of added sodium or potassium chloride might hold for the mentioned buffer system. Further investigation of the effect of variation in the concentration of the THAM-THAM.HCl buffer upon the enzymatic hydrolysis constants K_S and k_3 was needed. The value of such a relationship would lie in providing a means to correct k_3 values of the various substrates to a norm to facilitate the comparison of rate constants in the elucidation of the mode of action of the enzyme alpha-chymotrypsin.

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*Subsequently referred to in the text as THAM-THAM·HCl.

The extension of the activating effect of calcium ion upon the alpha-chymotrypsin catalyzed hydrolyses has been made to the hydrazides. Investigations at two ionic strengths have verified the earlier results of a specific ion effect (27,28,29). The possibility of the presence of calcium ion in the enzyme preparation employed in these investigations has been eliminated as a result of studies in which sufficient dihydrogen disodium versenate to sequester any existing calcium was added with no observable diminution in hydrolysis rate.

Discussion of Results Relative to the Variation of Concentration of THAM Buffer Systems

The substrate chosen for the investigations was nicotinyl-L-tyrosinhydrazide. This substrate was first shown to be hydrolyzed in 1949 (9). Despite earlier reports that benzoyl-L-tyrosinhydrazide was ineffective as an inhibitor, which leads to the conclusion that the hydrazides are not hydrolyzed by the enzyme, Niemann and MacAllister found 63 percent hydrolysis in 91 minutes at pH 7.9 and 25.0°C. The kinetic constants were previously determined by Lutwack (10) utilizing the THAM-THAM·HCl buffer system, with the formality of the amine component of the system held constant at 0.02 F. The values obtained were: $K_S = 9.1 \times 10^{-3}$ M, and $k_3 = 0.97 \times 10^{-3}$ M/min./mg.protein-nitrogen/ml. An estimation of probable

error was not given. The determination was at pH 7.8 and 25.0°C.

The parameters recognized and held constant in the experiments were the pH, the temperature, the solvent, the substrate, and the enzyme concentration.

Five sets of experiments were carried out at various concentrations of the THAM-THAM·HCl buffer system: .01 F, seven runs with $[S]_o$ values ranging from 1.1×10^{-4} M to 40.25×10^{-4} M; 0.1 F, seven runs with $[S]_o$ values ranging from 1.01×10^{-4} M to 60.10×10^{-4} M; 0.5 F, five runs with $[S]_o$ ranging from 2.01×10^{-4} M to 60.10×10^{-4} M; 0.75 F, five runs with $[S]_o$ ranging from 5.01×10^{-4} M to 60.10×10^{-4} M; and 0.9 F, six runs with $[S]_o$ ranging from 1.02×10^{-4} M and 40.72×10^{-4} M.

The extent of hydrolysis was determined by the quantitative estimation of the hydrazine liberated by the formation of bis-p-dimethylaminobenzalazine in a procedure analogous to that of Lutwack (10) and discussed more thoroughly in the next section.

From graphs of assumed first and zero order kinetics, i.e., $\ln [S]_o/[S]$ vs. time and $([S]_o-[S])$ vs. time, were obtained the values of v_o, the initial velocity, for each substrate concentration. Utilizing these preliminary values in equation 10, values of K_S and k_3 were tentatively evaluated. From the preliminary values of K_S and k_3 and the various substrate concentrations, the corrected time

scale was obtained by the method of Jennings and Niemann (8). Using the corrected time scales, values of the initial velocity for each substrate were re-evaluated. Finally, values of K_S and k_3 were obtained by a least squares fit of equation 10.

For each concentration of THAM used the pH was held constant within $\pm$ 0.1 of 7.9, the temperature was controlled at 25.0 $\pm$ 0.1°C., the solvent was water, the substrate was nicotinyl-L-tyrosinhydrazide, and the enzyme concentration was 0.1785 mg. protein-nitrogen per ml.

The kinetic data are shown in Tables II, III, IV, V, and VI. Figures* representing the results of these tables are at the end of each table. The results of a least squares fit of equation 10 for each of the five buffer concentrations are listed in Table I.

Recently, Booman and Niemann initiated the use of orthogonal polynomials for the determination of initial velocities (35). The procedure involves representing the data of an individual run by a set of orthogonal polynomials using the method of least squares. Each run in this investigation was evaluated a second time using the procedure outlined by Booman and Niemann. These initial velocities, signified by v_o, are given in Table I, along

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*Note that V_o has been used instead of v_o in the ordinate caption of the figures.

with the probable errors, σ_{v_o}', calculated by this method and the order of the polynomial, P_m, employed in the calculation. While direct comparison cannot be expected since an accurate zero time-blank correction is inherent in and essential to the evaluation of accurate and meaningful initial velocities, it is gratifying to see that for a majority of the substrate concentrations the initial velocities calculated by the two different methods are comparable within the limits of experimental error. A blank correction and better zero time calculation, as discussed in next section of this thesis, would correct this error and bring the initial velocities calculated by the two procedures into direct comparison. As can be seen from Table I, a least squares fit of the v_o' values gives K_S and k_3 values that are comparable with the ones determined by the former method but with a larger probable error.

The values of K_S and k_3 taken for further discussion are those determined by the method of Jennings and Niemann, since the experiments were not designed for the orthogonal polynomial calculation.

It is seen in Table I that the Michaelis constant, K_S, evaluated at the five concentrations of THAM-THAM·HCl buffer system seems to be essentially constant within the limits of experimental error. An arithmetic mean of the five values corresponds to a value of K_S equal to $8.0 \pm 1.6 \times 10^{-3}$ M. This value is in agreement with the earlier

determined value of 9.1×10^{-3} M. The fact that the constant was evaluated five separate times in this investigation would seem to indicate that the earlier value was slightly high even though still within the limits of experimental error. While K_S remains constant the k_3 value is seen to increase from 0.9×10^{-3} M/min./mg. protein-nitrogen/ml. at .02 F THAM to 2.2×10^{-3} M/min./mg. protein-nitrogen/ml. at 0.9 F THAM. This is an increase of approximately 130 percent.

Evidence has been accumulating that in reactions catalyzed by alpha-chymotrypsin where the hydrolytic reaction may be represented by equation 1,

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

that where $K_S = \frac{k_2 + k_3}{k_1}$, the K_S may be considered to be an apparent dissociation constant, $K_S \doteq k_2/k_1$. The work here would seem to support this view and provide additional verification for the fact that $k_2 \gg k_3$ and that K_S is truly a dissociation constant.

If one assumes that K_S remains constant at a value of $8.0 \pm 1.6 \times 10^{-3}$ M, values of v_o based on an arbitrarily chosen initial substrate concentration can be calculated for each of the THAM-THAM·HCl concentrations. These are shown in Table VII. Using these velocities,

various plots were made of functions which could exist between the values of ionic strength, or buffer concentration and the logarithm of the velocities or the velocities themselves. While it was very difficult to ascertain which function gave the most definite linear relationships, it appeared on the basis of standard deviation and inspection that a relationship existed between the logarithm of the velocity and the square root of the ionic strength or the molality of buffer to the first power. A least squares fit of the relationship $\log v_o = a + b\sqrt{\mu}$ gave a value of $a = .423 \pm .022$ and a value of $b = .467 \pm .032$. A least squares fit of the relationship $\log v_o = a + b\,(\underline{M})$ gave a value of $a = .473 \pm .030$ and a value of $b = .353 \pm .042$. The values of the least squares fit of all the reasonable relationships are listed in Table VII. Comparison of these values with those found in the THAM-THAM$\cdot H_2SO_4$ case will be discussed later. Figure 1 represents the relationship between $\log v_o$ and $\sqrt{\mu}$. The initial velocities calculated by means of this relationship are compared to the ones found experimentally in Table VIII.

In order to examine the effect of THAM buffers further, a set of experiments were carried out in which the buffer was not the uni-univalent buffer but the uni-divalent THAM-THAM$\cdot H_2SO_4$.* The parameters held constant were the

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*Tris-(hydroxymethyl)-aminomethane-tris-(hydroxymethyl)-aminomethane-sulfate.

pH at 7.9 ± 0.1, the temperature at 25.0°C., the solvent was water, the substrate concentration at 3.324×10^{-4} M, and the enzyme concentration at 0.1785 mg. protein-nitrogen per ml. As before the rate of hydrolysis was followed by the appearance of hydrazine as indicated by the quantitative formation of bis-p-dimethylaminobenzalazine.

The kinetic data are listed in Table IX. The initial velocities evaluated by the method of Jennings and Niemann and for comparison the initial velocities calculated by the orthogonal polynomial method are listed in Table X. Again the various relationships between the initial velocities and buffer concentration or ionic strength were plotted and least squares fits of the data were made. Again it appeared that the most linear relationship existed between the logarithm of the velocity and the square root of the ionic strength or the molality of the buffer to the first power. A least squares fit of the former gave a value of a = .373 ± .017 and a value of b = .461 ± .018. A least squares fit of the latter gave a value of a = .470 ± .016 and a value of b = .387 ± .030. The values of the least squares fits of all the reasonable relationships are listed in Table XI. Figure 2 shows the relationship between the logarithm of the initial velocity and the square root of ionic strength.

Since the initial substrate concentrations were different, the intercepts of the plots cannot be compared;

however, it is interesting to compare the various slopes of the relationships. While the values of the slope in the case of the logarithm v_o versus molality for the two types of THAM buffers are coincident, within the experimental error, i.e. b = .353 ± .042 in the case of THAM-THAM·HCl, and b = .387 ± .030 in the case of THAM-THAM·H_2SO_4, the agreement of the slopes when the logarithm v_o is plotted versus $\sqrt{\mu}$ in both cases would seem to indicate a relationship of this type. The two slopes are, in the case of THAM-THAM·HCl and THAM-THAM·H_2SO_4 respectively, equal to .467 ± .032 and .461 ± .018. It is recognized that ionic theory would not predict this linear relationship at the high ionic strengths employed, but in the absence of another definitive relationship, this would seem to indicate the adherence of the alpha-chymotrypsin catalyzed hydrolysis rates in the presence of THAM buffer species to the simple relationship:

$$\log v_o/v_o^{\circ} = (0.46 \pm 0.03)\sqrt{\mu} \qquad (20)$$

at least with the substrate used and over the concentration range studied.

There is apparent lack of agreement between the relationship postulated here and the one postulated earlier for the case of added sodium or potassium chloride. However, it is noteworthy that Shine and Niemann (36) have

noticed an acceleration of the hydrolysis rate by the addition of a non-electrolyte, sucrose. It is evident that a combination of effects are simultaneously acting upon the system. The apparent agreement with equation 20 may be fortuitous, or it may be that the effect of ionic strength masks the effect of molality.

Utilizing equation 20, where v_o° is the initial velocity in the absence of added buffer and is dependent upon the substrate concentration, initial velocities have been calculated for the buffers employed at their various concentrations. The results are presented in Table VIII. The agreement is within the limits of experimental error.

Since throughout the studies the assumption that the reaction in the presence of increased amounts of buffer could be described in terms of equation 21

$$k_3[E]t = K_S \ln [S]_o/[S]_t + ([S]_o-[S]_t) \qquad (21)$$

it could be expected that upon the addition of this amount of buffer to the system to the particular system investigated where K_S remains constant the specific rate would also increase with increasing ionic strength of buffer according to the same relationship,

$$\log k_3/k_3^\circ = 0.46 \pm 0.03 \sqrt{\mu} \qquad (22)$$

where k_3^o is the rate constant in the absence of any buffer. A least squares fit of the data according to this relationship between log k_3 and $\sqrt{\mu}$ gives a slope of 0.468 ± .064 and an intercept of .974 ± .088 in agreement with this theory. The intercept corresponds to the logarithm of the specific rate constant in the absence of buffer. The antilog of this corresponds to a k_3 of $0.94 \pm 0.16 \times 10^{-3}$ M/min./mg. protein-nitrogen/ml. This is in good agreement with the experimentally determined values of k_3 previously evaluated in the presence of THAM-THAM·HCl at a concentration of .02 F. The frequently employed concentration is seen then, within the limits of experimental error, to give a true specific rate constant.

The explanation of how the addition of the buffer and the subsequent increase in ionic strength could effect the change in k_3 while causing no significant change in the value of K_S requires more detailed knowledge of the active site of the enzyme and the environment in the neighborhood of the active site. The Michaelis constant has been looked upon as a measure of the extent of combination of the catalytically active site with the substrate in all possible modes (21). The fact that K_S does not change would seem to indicate that the concentration of the total enzyme-substrate complexes is not altered. However, if it is remembered that only a few species of the enzyme-substrate complex exist with the substrate in the proper

orientation for the formation of a transition state and subsequent breakdown into products, it may be that the increase in buffer and the consequent increase in ionic strength effects the equilibria involved in the formation of the active transition state. The specific rate constant, k_3, should be a direct reflection of changes in these equilibria. Or, it may be that the buffer changes the non-reactive enzyme substrate complexes by reaction with the complex, per se, or by alteration in the environment around these non-reactive complexes, so as to increase their susceptibility to further reaction.

In any case, it is seen, that the previous results where the buffer concentration used has been very much greater than the normal .02 *F* THAM-THAM·HCl, the specific rates constants have been over-estimated. It would seem that these results could be corrected by a relationship similar or perhaps equivalent to the one found in the case of the hydrazide used in the present investigation. Future investigators must be aware of the possibility of increased initial velocities and hydrolysis rates upon the addition of increased amounts of buffer as well as other electrolytes and non-electrolytes.

Enhancement of Rate of Hydrolysis by Addition of Calcium Ion to alpha-Chymotrypsin Catalyzed Reactions

The enhancing effect of calcium ion upon the activity of alpha-chymotrypsin has been reported previously (28,29,27). As stated above Neurath found the activity of alpha-chymotrypsin to increase 50 percent upon the addition of 10^{-2} M calcium ion to a system containing phenylalanine ethyl ester. During the course of the ionic strength investigations the results of Neurath were substantiated by the addition of calcium ion to the system alpha-chymotrypsin-nicotinyl-L-tyrosinhydrazide. Activation of trypsin has recently been studied by several workers (27,37,38,39).

The following experiments were designed primarily to substantiate the activating effect of calcium ion and secondly to demonstrate the absence of calcium ion in the enzyme preparations used. Three sets of experiments were carried out. The first set was conducted at a pH 7.9 ± 0.1, a THAM-THAM·HCl buffer concentration of .02 F, a concentration of nicotinyl-L-tyrosinhydrazide of 10.028 x 10^{-4} M, an enzyme concentration of .218 mg. protein-nitrogen/ml.,* water as the solvent, and a temperature of 25.0 ± 0.1°C. Calcium ion was added to the system as calcium chloride in concentrations varying from 10^{-6} M to

*Armour Lot No. 00592. Protein-nitrogen 14.6%. Analysis by Dr. Adalbert Elek.

10^{-1} M. The extent of hydrolysis was followed as before by determining the rate of liberation of hydrazine. The initial velocities were calculated both by the methods of Jennings and Niemann (8) and Booman and Niemann (35). The kinetic data are compiled in Table XIII. The initial velocities are summarized in Table X.

A second set of runs was carried out under the same conditions as above except the concentration of the buffer THAM-THAM·HCl was increased to 1.00 F in an effort to ascertain if the effect of calcium ion is one which is over and above the effect expected simply by the increase in ionic strength. The kinetic data are listed in Table XIV and the initial velocities are summarized in Table XII.

A third set of experiments designed to demonstrate the absence of calcium in the enzyme preparations used were made. Four runs were conducted with the addition of varying amounts of disodium dihydrogen versenate.* Kinetic data are compiled in Table XV and the initial velocities are summarized in Table XII.

A calculation of the initial velocities which would be expected by the increased ionic strength accompanying the added calcium salt can be made from equation 20.

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*The disodium salt of ethylenediamine tetraacetic acid. This sequestering agent complexes 179 mg. of calcium ion per gm. of versene.

If one assumes a v_o^o of 2.64×10^{-5} M/min., corresponding to the average velocity found in the absence of added calcium but in the presence of 0.02 F THAM-THAM·HCl, and also assumes equation 20 to hold in the case at hand these expected initial velocities are equal to the values listed in Table XII under v_o (calcd). The initial velocity is observed to increase approximately 75 percent at 10^{-1} M calcium ion, 65 percent at 10^{-2} M, 50 percent at 10^{-3} M, and 20 percent at 10^{-4} M. The increase at 10^{-2} M calcium ion agrees well with the increase of 50 percent at 10^{-2} M calcium ion in the case of the alpha-chymotrypsin catalyzed hydrolysis of benzoylalanine ethyl ester observed by Neurath.

The same type of calculation can be made for the case where the buffer concentration has been increased to 1.0 F THAM. The increase here is approximately 10 percent at 10^{-5} M, 20 percent at 10^{-4} M, 35 percent at 10^{-3} M, and 55 percent at 10^{-2} M. This set of increases is, allowing for error, of the same order as observed in the case of the .01 F THAM-THAM·HCl.

The values of the initial velocity calculated according to the ionic strength and the values of initial velocity observed are plotted versus the negative of the logarithm of the calcium ion concentration in Figures 8 and 9. It is interesting to note that the observed velocity begins to rise above the calculated velocity between 10^{-5} M and 10^{-4} M $[Ca^{++}]$ and reaches a maximum

at approximately 10^{-2} M [Ca^{++}]. At this point it seems to give about the initial velocities expected from ionic strength considerations. These results are similar to the results obtained by both Neurath and Laskowski. The effect of calcium is definitely a specific ion effect which is over and above the ionic strength effect.

An attempt was made to calculate a dissociation constant assuming that calcium reacts reversibly with alpha-chymotrypsin to form a calcium--alpha-chymotrypsin complex. A sufficient number of experiments were not conducted, however, to yield a definitive constant.

The formulation of a definitive mechanism for the effect of calcium upon the alpha-chymotrypsin catalyzed hydrolyses would require a great deal more information than is at the present, known.

Metal ions do not appear to be essential to the activity of alpha-chymotrypsin. Since Neurath (39) has shown that the addition of versene to the reaction completely reverses any activation by calcium ion, the results of the present investigation where the addition of disodium dihydrogen versenate caused no diminution in initial velocity (see Table XII) would seem to indicate the absence of any effective concentration of calcium in the present enzyme preparations.

While no completely satisfactory explanation of the activating effect of calcium can be given, in light of

the data at hand, it is of worth, to look at some of the postulated mechanisms.

Recently, Gorini (37,40-42) and Nord (38) in a series of investigations dealing with the inhibition of autolysis of trypsin have postulated that calcium exerts its activating influence by preventing denaturation of trypsin by itself and thus allows the effective concentration of trypsin to remain at a higher level. This explanation cannot explain the activation in the case of alpha-chymotrypsin since it has been shown in a number of cases that the activity of the enzyme does not decrease in the periods during which the experiments are conducted by Niemann and coworkers. Initial velocities of duplicate runs using the same enzyme preparation remain constant, within the limits of experimental error.

It has been postulated that calcium activation is exhibited due to the inhibition of dimerization and thus, effectively increasing the concentration of active monomer. Recent evidence has shown, however, no appreciable dimerization is observed at pH 7.9 (12).

In a number of the peptidases where the metal ion is known to be essential to enzymatic activity, it has been postulated that the electrons of the peptide bond are rearranged by the metal "through its combination with the protein centers which attract or bind R' and R" serve to orient the substrate and fix it into position so that

strong electronic attraction of the metal produces an electronic deformation. The simplest explanation of the resulting hydrolysis of the peptide bond is due to hydrogen and/or hydroxyl ion catalytic effects." (43). While the metal may not be essential to the activity, interaction with alpha-chymotrypsin in a similar manner would increase the hydrolysis rate.

If one assumes that there are a number of possible modes of combination of the substrate with the enzyme and that only a few lead to the transition state, the effect of calcium must be either to shift the equilibria between the unfavorable modes and the hydrolytically susceptible mode in favor of the latter by formation of a calcium-chymotrypsin complex, which is still active, or to alter the structure and shape of the active intermediate to allow the formation of complex or departure of products to proceed more readily.

In conclusion it might be stated that a complete investigation of the effect of calcium ion on the kinetic constants, K_S and k_3 would permit a more definitive interpretation of the problem.

Experimental and Figures*

Synthesis of substrate.--Esterification of 25 gms. of L-tyrosine (.014 mole) with ethanolic hydrogen chloride gave 24.1 gms. of L-tyrosine ethyl ester hydrochloride (.0112 mole - 80%). Liberation of the ester by slurrying the hydrochloride in chloroform followed by the addition of chloroform saturated with ammonia, removal of the ammonium chloride by filtration, and evaporation of the chloroform gave 19.4 gm. of L-tyrosine ethyl ester (.0108 mole - 67%), m.p. 106-108°C.

Acylation of the 19.4 gms. of L-tyrosine ethyl ester (.0108 mole) with nicotinyl azide gave 15.8 gms. of nicotinyl-L-tyrosine ethyl ester (.0056 mole - 52%), m.p. 145.4 - 147.5°C. The crude acylated ester was dissolved in ethanol and added slowly to an excess of hydrazine. The solution was refluxed for three hours and the white solid filtered and dried. Two crystallizations from one liter of water gave 14.5 gms. of nicotinyl-L-tyrosinhydrazide (.0053 mole - 95%), m.p. 245.5 - 246.1°C. $[\alpha]_D^{25.3^\circ}$ = + 24.8 ± 0.6 (C, 2.3% in methyl cellosolve).

Anal. Calcd. for $C_{15}H_{16}O_3N_4$: C, 60.00; H, 5.37; N, 18.66
Found C, 60.12; H, 5.42; N, 18.80
Found C, 60.20; H, 5.51; N, 18.62

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*All analyses were made by Dr. Adalbert Elek, Elek Microanalytical Laboratories, Los Angeles, California.

Buffer Solutions.--The tris-(hydroxymethyl)-amino-methane used was Matheson and Company practical grade decolorized with Norite and recrystallized three times from water, m.p. 169.3-169.6°C. In each case the stock solution was made up with 10 times the desired concentration by dissolving the required amount of the amine in water and bringing to pH 7.93 ± 0.05 with concentrated hydrochloric acid or concentrated sulfuric acid depending upon the system. The addition of one milliliter of these solutions to the ten milliliter reaction flasks gave the desired final concentration.

Enzyme Solutions.--The enzyme preparations used were obtained from the Armour Research Division, Chicago 9, Illinois. Lot No. 10705 was analyzed for protein-nitrogen by Dr. Adalbert Elek after precipitation with 5% trichloroacetic acid in the usual manner. Found: 11.9%. Lot No. 00592, used in the calcium investigations analysis, gave 14.6% protein-nitrogen. Solutions of seventy-five milligrams of enzyme in five ml. of water were prepared as follows: the enzyme was weighed on a small boat constructed of black weighing paper. The boat was inserted into the neck of a five ml. volumetric flask containing a small amount of water. The enzyme was tapped into the flask and placed in solution by gentle swirling to limit the formation of bubbles. One ml. of the above solution

in a ten ml. reaction flask gave the required final concentration of 1.5 mg./ml. This corresponds to 0.1785 mg. protein-nitrogen/ml. for Lot No. 10705, and 0.218 mg. protein-nitrogen/ml. for Lot No. 00592.

The enzyme was thermostatted at 25.0 ± 0.1°C. No aliquots were taken from stock solutions which had been kept for more than two hours. No drop in activity could be noted in this time.

Reaction and Analysis.--Solutions containing the desired amount of substrate in 8 ml. of water and 1 ml. of the buffer system used, and in the case of the addition of $CaCl_2$ the required calcium concentration, were equilibrated in a Sargent constant temperature bath for 15-20 minutes at 25.0 ± 0.1°C. Fifteen seconds before zero time, one ml. of the enzyme solution was withdrawn from the enzyme flask and pipetted into the reaction flask at zero time. The flasks were swirled gently five times and replaced in the bath. At equal time intervals of one, two, or three minutes, depending upon rate of hydrolysis, one milliliter aliquots were withdrawn from the reaction flask and pipetted into the suitable size color-developing flasks containing the acid and aldehyde solution described under next heading. The color was allowed to develop for twenty minutes and then the optical density was measured in 1 cm. quartz cells in the Beckmann

Model B spectrophotometer at 455 mμ against a water blank. The optical density in larger flasks than ten ml. was converted to its equivalent reading and recorded.

Acid and Aldehyde Solutions.--One hundred and forty-four ml. of Bakers Analyzed hydrochloric acid (spec. grav. 1.186, 36.8%) was diluted to one liter with distilled water. One milliliter of this solution in a 10 ml. color-developing flask results in a final acid concentration of 0.172 N.

A solution of one gm. of para-dimethylaminobenzaldehyde in 100 ml. of absolute ethanol was prepared. The aldehyde used was Matheson and Co. reagent grade, recrystallized from aqueous methanol, m.p. 75.5-75.9°C. One milliliter of this solution in a ten ml. color-developing flask corresponds to a final aldehyde concentration of 0.671×10^{-2} M.

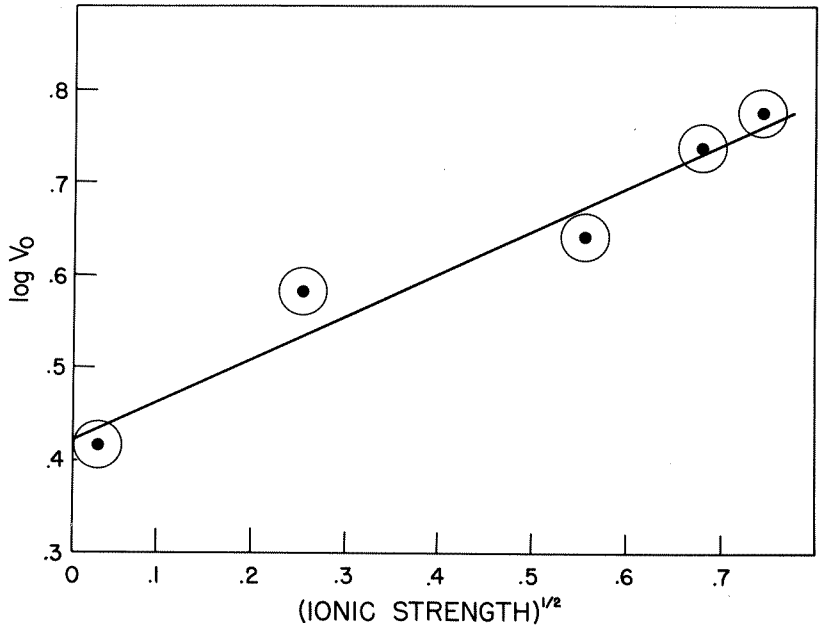

Fig. 1. Log v_o - $\sqrt{\mu}$ relationship of the system alpha-chymotrypsin-nicotinyl-L-tyrosinhydrazide at 25°C., pH 7.9, and at the various ionic strengths of THAM-THAM·HCl buffer systems used.

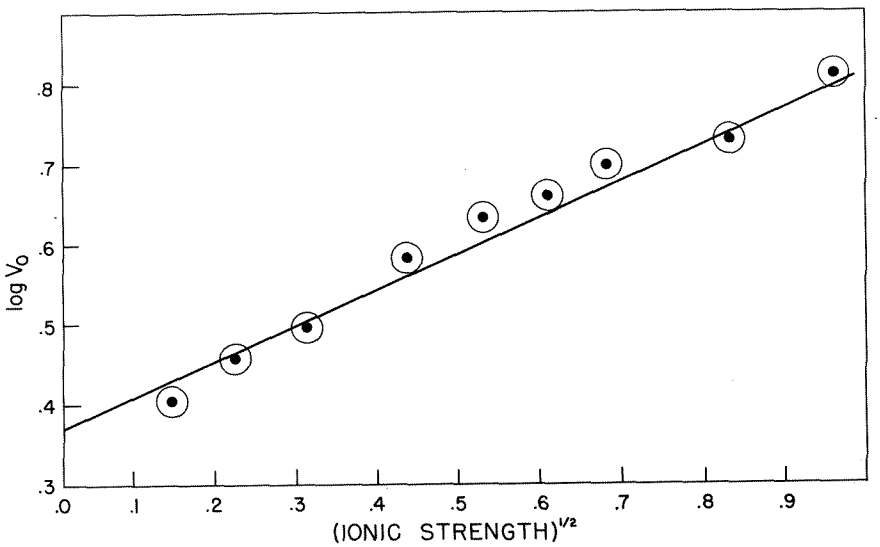

Fig. 2. Log v_o - $\sqrt{\mu}$ relationship of the system alpha-chymotrypsin-nicotinyl-L-tyrosinhydrazide at 25°C., pH 7.9, and at the various ionic strengths of THAM-THAM·H_2SO_4 buffer systems used.

Table I

Initial Velocities and Kinetic Constants of the alpha-Chymotrypsin Catalyzed Hydrolysis of Nicotinyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C. Using Various Concentrations of the Amine Component of a THAM-THAM·HCl Buffer System

[Buffer]	$[S]_o$ (a)	v_o (b)	v_o' (c)	$\sigma_{v_o'}$ (d)	P_m (e)
.01 F	1.01	0.24	0.22	±0.03	2
	2.52	0.44	0.43	±0.05	3
	5.03	1.04	1.05	±0.13	2
	10.06	1.95	1.85	±0.33	2
	20.12	3.54	3.24	±0.27	3
	20.12	3.56	3.34	±0.10	2
	30.10	4.62	4.20	±0.63	3
	40.24	5.95	5.67	±0.55	2
	$K_S=7.35\pm1.65\times10^{-3}$M		$K_S'=6.97\pm2.50\times10^{-3}$M		
	$k_3=0.91\pm0.16\times10^{-3}$		$k_3'=0.84\pm0.13\times10^{-3}$		
.10	1.01	0.30	0.34	±0.02	3
	2.52	0.70	0.67	±0.02	2
	5.03	1.39	1.35	±0.13	2
	10.06	2.71	2.72	±0.09	3
	20.12	4.91	4.98	±0.14	3
	30.08	6.36	6.00	±0.27	2
	40.24	8.08	8.59	±0.14	3
	$K_S=8.25\pm1.18\times10^{-3}$M		$K_S'=8.36\pm2.68$M		
	$k_3=1.37\pm0.30\times10^{-3}$		$k_3'=1.39\pm0.23$		
.05	20.12	5.20	5.27	±0.03	2
	30.00	7.98	7.08	±0.75	2
	40.10	9.15	10.02	±0.24	2
	50.10	10.70	10.41	±0.50	2
	60.10	11.57	11.41	±0.93	4
	$K_S=8.66\pm1.78\times10^{-3}$M		$K_S'=8.48\pm2.71\times10^{-3}$M		
	$k_3=1.57\pm0.29\times10^{-3}$		$k_3'=1.52\pm0.34\times10^{-3}$ (f)		

Table I.--Continued

[Buffer]	$[S]_o$(a)	v_o(b)	v_o'(c)	$\sigma_{v_o'}$(d)	P_m(e)
.75	5.01	1.40	1.41	±0.04	2
	20.00	5.74	6.17	±0.09	3
	30.00	8.16	8.42	±0.16	3
	50.10	10.75	11.05	±0.46	3
	60.10	12.16	12.05	±0.14	2
	$K_S=7.52\pm1.53\times10^{-3}$ M		$K_S'=7.66\pm1.72\times10^{-3}$ M		
	$k_3=1.97\pm0.30\times10^{-3}$		$k_3'=1.92\pm0.39\times10^{-3}$		
.90	1.02	0.43	0.42	±0.09	2
	2.56	1.16	1.15	±0.06	2
	5.03	2.33	2.12	±0.03	2
	10.22	4.28	4.36	±0.07	2
	20.36	7.29	7.55	±0.25	3
	40.72	12.60	12.60	±0.09	2
	$K_S=8.31\pm1.79\times10^{-3}$ M		$K_S'=8.57\pm2.62\times10^{-3}$ M		
	$k_3=2.15\pm0.32\times10^{-3}$		$k_3=2.30\pm0.40\times10^{-3}$		

(a) $[S]_o$ is in units of 10^{-4} M

(b) Initial velocities determined by the method of Jennings and Niemann. v_o is in units of 10^{-5} M $min.^{-1}$.

(c) Initial velocities determined by the method of Booman and Niemann. v_o' is in units of 10^{-5} M $min.^{-1}$.

(d) Probable error in (c). $\sigma_{v_o'}$ is in units of 10^{-5} M $min.^{-1}$.

(e) The order of the polynomial used in calculation of (c).

(f) k_3 and k_3' are in units of M/min./mg. protein-nitrogen/ml.

Table II

alpha-Chymotrypsin Catalyzed Hydrolysis of Nicotinyl-L-tyrosinhydrazide at pH 7.9 and 2.5°C.

.01 F with Respect to Amine Component of Tris-[hydroxymethyl]-aminomethane--Tris-[hydroxymethyl]-aminomethane-hydrochloride Buffer System

$[E]^{*}$ = .1785 mg. Protein Nitrogen ml.$^{-1}$

$[S]_o \times 10^{-4}$	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
1.01	2	.024	.06484	1.93	-
	4	.039	.10705	3.79	-
	6	.059	.16720	5.53	-
	8	.072	.20782	7.23	-
	10	.086	.25376	8.84	-
	12	.100	.30158	10.36	-
	14	.118	.36741	11.72	-
	16	.127	.40146	13.17	-
	18	.140	.45362	14.47	-

Dilution Factor 1:10 % Hydrolysis 36.5

$v_o = 0.24 \times 10^{-5}$ M min.$^{-1}$

2.52	1	.023	.02761	1.00	-
	3	.052	.05543	2.92	-
	6	.113	.12486	5.62	-
	9	.154	.17395	8.27	-
	12	.198	.23111	10.75	-
	15	.239	.28593	13.08	-
	18	.287	.35488	15.16	-
	21	.326	.41409	17.20	-

Dilution Factor 1:10 % Hydrolysis 33.9

$v_o = 0.44 \times 10^{-5}$ M min.$^{-1}$

Table II.--Continued

$[S]_o x10^{-4}$	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
5.03	2	.088	.04582	1.95	2.01
	4	.166	.08983	3.82	4.02
	6	.244	.13540	5.61	6.03
	8	.317	.17981	7.33	8.05
	10	.381	.22019	8.98	10.07
	12	.464	.27611	10.49	12.10
	14	.525	.31845	12.00	14.13
	16	.565	.34745	13.52	16.17
	18	.665	.42068	14.67	18.22
	Dilution Factor 1:10		% Hydrolysis 34.6		
	$v_o=1.04x10^{-5}\underline{M}$ min.$^{-1}$				
10.06	2	.150	.04018	1.96	2.01
	4	.291	.07880	3.86	4.02
	6	.429	.11866	5.68	6.03
	8	.576	.16211	7.51	8.05
	10	.720	.20782	9.11	10.09
	12	.833	.24451	10.74	12.13
	14	.958	.28593	12.34	14.18
	16	1.086	.33146	13.78	16.24
	18	1.212	.37844	15.21	18.32
	Dilution Factor 1:1		% Hydrolysis 31.5		
	$v_o=1.95 \times 10^{-5}\underline{M}$ min.$^{-1}$				
20.12	1	.137	.01488	1.00	0.99
	2	.274	.03536	2.01	1.96
	3	.415	.05543	3.02	2.93
	4	.542	.07324	4.03	3.88
	5	.690	.09440	5.05	4.81
	6	.807	.11064	6.06	5.73
	7	.954	.13278	7.08	6.63
	8	1.095	.15357	8.11	7.53
	Dilution Factor 1:10		% Hydrolysis 14.2		
	$v_o=3.54x10^{-5}\underline{M}$ min.$^{-1}$				

Table II.--Continued

$[S]_o x10^{-4}$	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
20.12	1	.155	.02078	0.99	1.00
	2	.290	.03825	1.96	2.01
	3	.440	.05921	2.92	3.02
	4	.564	.07603	3.87	4.03
	5	.730	.09984	4.80	5.04
	6	.846	.11689	5.72	6.06
	7	.984	.13627	6.62	7.08
	8	1.124	.15785	7.50	8.11
	9	1.234	.17479	8.39	9.14
Dilution Factor 1:10				% Hydrolysis 16.1	
$v_o=3.56x10^{-5}\underline{M}$ min.$^{-1}$					
30.10	1	.115	.00995	1.00	1.00
	2	.285	.02468	1.98	2.01
	3	.455	.04018	2.95	3.02
	4	.620	.05543	3.91	4.03
	5	.780	.07045	4.87	5.05
	6	.940	.08526	5.81	6.07
	7	1.090	.09984	6.74	7.08
	8	1.235	.11333	7.67	8.11
	9	1.375	.12751	8.59	9.14
Dilution Factor 1:50				% Hydrolysis 12.0	
$v_o=4.62x10^{-5}\underline{M}$ min.$^{-1}$					
40.24	1	.230	.01488	0.99	1.00
	2	.440	.02859	1.98	2.01
	3	.675	.04496	2.95	3.03
	4	.910	.06015	3.92	4.04
	5	1.145	.07696	4.87	5.06
	6	1.370	.09349	5.81	6.09
	7	1.620	.11154	6.74	7.13
	8	1.840	.12751	7.66	8.17
	9	2.045	.14236	8.59	9.21
Dilution Factor 1:50				% Hydrolysis 13.3	
$v_o=5.95x10^{-5}\underline{M}$ min.$^{-1}$					

*Armour Lot No. 10705. Protein Nitrogen 11.9%. Analysis by Dr. Adalbert Elek.

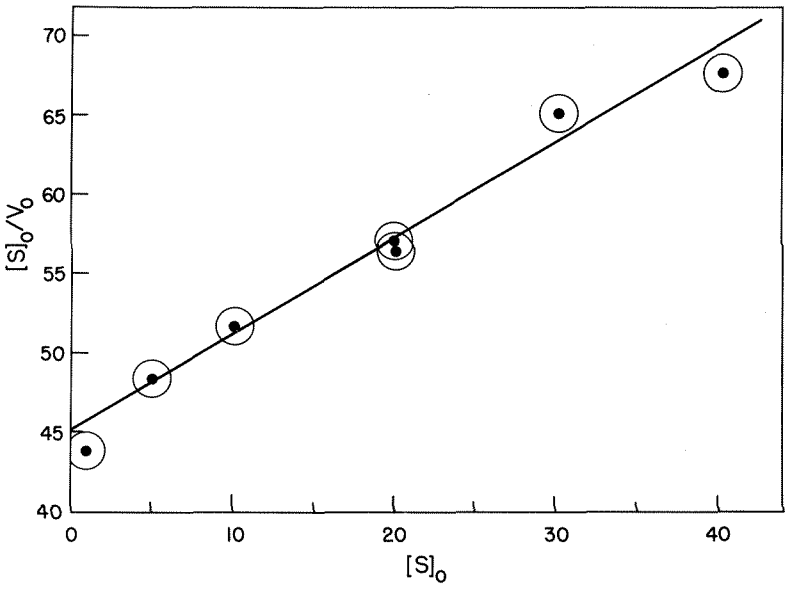

Fig. 3. $[S]_o/v_o$ vs. $[S]_o$ graph for the system of alpha-chymotrypsin-nicotinyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C.

.01 F with respect to the amine component of THAM-THAM·HCl buffer system.
$[S]_o$ is in units of 10^{-4} M. $[S]_o/v_o$ is in units of minutes.

Table III

alpha-Chymotrypsin Catalyzed Hydrolysis of Nicotinyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C.

0.1 F with Respect to the Amine Component of a Tris-(hydroxymethyl)-aminomethane--Tris-(hydroxymethyl)-aminomethane-hydrochloride Buffer System

$[E]^{*}$ = .1785 mg P.N. ml.$^{-1}$

$[S]_o \times 10^{-4}$	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
1.01	2	.033	.08983	1.91	2.00
	4	.053	.14929	3.72	4.00
	6	.075	.21729	5.41	6.00
	8	.094	.28065	6.98	8.01
	10	.111	.34145	8.48	10.02
	12	.130	.41343	9.81	12.03
	14	.157	.52591	10.88	14.06
	16	.172	.59493	12.05	16.08
	18	.184	.65233	13.23	18.11

Dilution Factor 1:10 % Hydrolysis 47.9

$v_o = 0.30 \times 10^{-5}$ M min.$^{-1}$

2.52	1	.033	.03536	0.98	1.00
	3	.093	.10165	2.85	3.00
	6	.169	.19309	5.47	6.01
	9	.240	.28743	7.83	9.04
	12	.303	.37844	10.00	12.07
	15	.355	.46121	11.93	15.12
	18	.403	.54392	13.90	18.18
	21	.450	.63180	15.58	21.23
	24	.492	.71734	17.18	24.29

Dilution Factor 1:10 % Hydrolysis 51.2

$v_o = 0.70 \times 10^{-5}$ M min.$^{-1}$

Table III.--Continued

$[S]_o x10^{-4}$	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
5.03	2	.121	.06484	1.93	2.00
	4	.227	.12574	3.76	4.01
	6	.329	.18813	5.48	6.02
	8	.420	.24686	7.10	8.04
	10	.502	.30305	8.63	10.06
	12	.586	.36394	10.06	12.10
	14	.657	.41871	11.44	14.13
	16	.748	.44348	12.66	16.16
	18	.810	.54754	13.86	18.22
Dilution Factor 1:10			% Hydrolysis 42.2		
$v_o=1.39x10^{-5}\underline{M}$ min.$^{-1}$					
10.06	2	.225	.06015	1.94	2.01
	4	.437	.12044	3.78	4.02
	6	.635	.18065	5.53	6.05
	8	.825	.24137	7.18	8.09
	10	.985	.29639	8.76	10.13
	12	1.160	.35976	10.22	12.19
	14	1.326	.42002	11.59	14.27
	16	1.470	.48243	12.93	16.34
	18	1.608	.54232	14.18	18.41
Dilution Factor 1:25			% Hydrolysis 41.9		
$v_o=2.71x10^{-5}\underline{M}$ min.$^{-1}$					
20.12	1	.209	.02761	0.99	1.00
	2	.409	.05448	1.95	2.01
	3	.589	.07973	2.90	3.02
	4	.780	.10705	3.83	4.04
	5	.996	.13889	4.73	5.06
	6	1.142	.16125	5.63	6.09
	7	1.326	.18979	6.49	7.12
	8	1.487	.21511	7.34	8.16
	9	1.655	.24216	8.18	9.21
Dilution Factor 1:50			% Hydrolysis 21.5		
$v_o=4.91x10^{-5}\underline{M}$ min.$^{-1}$					

Table III.--Continued

$[S]_o x10^{-4}$	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
30.08	2	.660	.05921	1.95	2.02
	3	.910	.08249	2.90	3.03
	4	1.140	.10436	3.84	4.06
	5	1.350	.12486	4.78	5.08
	6	1.560	.14583	5.69	6.11
	7	1.865	.17730	6.55	7.15
	8	2.070	.19885	7.44	8.20
	9	2.300	.22314	8.32	9.22
Dilution Factor 1:50			% Hydrolysis 20.0		
$v_o=6.36x10^{-5}$ M min.$^{-1}$					
40.24	1	.625	.04113	0.99	1.01
	2	.975	.06578	1.96	2.02
	3	1.300	.08801	2.91	3.04
	4	1.620	.11154	3.85	4.09
	5	1.880	.13015	4.79	5.11
	6	2.220	.15613	5.69	6.15
	7	2.470	.17479	6.60	7.20
	8	2.790	.20048	7.49	8.29
	9	3.050	.22081	8.36	9.26
Dilution Factor 1:50			% Hydrolysis 19.8		
$v_o=8.08x10^{-5}$ M min.$^{-1}$					

*Armour Lot No. 10705. Protein Nitrogen 11.9%. Analysis by Dr. Adalbert Elek.

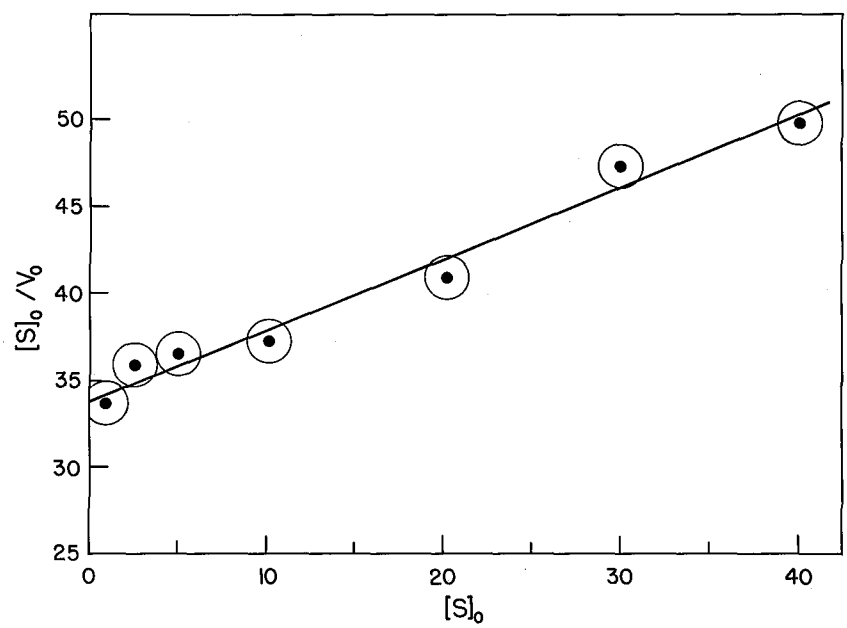

Fig. 4. $[S]_o/v_o$ vs. $[S]_o$ graph for the system of alpha-chymotrypsin-nicotinyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C.

0.1 F with respect to the amine component of THAM-THAM.HCl buffer system.
$[S]_o$ is in units of 10^{-4} M. $[S]_o/v_o$ is in units of minutes.

Table IV

alpha-Chymotrypsin Catalyzed Hydrolysis of Nicotinyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C.

0.5 F with Respect to the Amine Component of a Tris-(hydroxymethyl)-aminomethane--Tris-(hydroxymethyl)-aminomethane hydrachloride Buffer System

$[E]^{*}$ = .1785 mg. P. N./ml.

$[S]_o \times 10^{-4}$	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
20.12	2	.470	.06297	1.95	2.01
	3	.665	.08618	2.89	3.03
	4	.850	.11689	3.81	4.05
	5	1.025	.14323	4.72	5.07
	6	1.185	.16720	5.61	6.10
	7	1.350	.19309	6.48	7.13
	8	1.505	.21729	7.34	8.17
	9	1.645	.24059	8.18	9.21
	10	1.780	.26300	9.01	10.25

Dilution Factor 1:50 % Hydrolysis 23.2

$v_o = 5.20 \times 10^{-5}$ M min.$^{-1}$

30.00	2	.770	.06952	1.95	2.02
	3	1.090	.09984	2.89	3.04
	4	1.400	.13015	3.82	4.06
	5	1.710	.16125	4.72	5.10
	6	2.000	.19144	5.60	6.14
	7	2.280	.22081	6.47	7.19
	8	2.530	.24919	7.31	8.24
	9	2.770	.27687	8.15	9.30
	10	3.000	.30379	8.95	10.36

Dilution Factor 1:50 % Hydrolysis 26.2

$v_o = 7.98 \times 10^{-5}$ M min.$^{-1}$

Table IV.--Continued

$[S]_o x10^{-4}$	t(min	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
40.10	2	.850	.05733	1.96	2.02
	3	1.225	.08314	2.91	3.04
	4	1.600	.11064	3.85	4.07
	5	1.950	.13627	4.78	5.11
	6	2.290	.16211	5.69	6.16
	7	2.625	.18813	6.58	7.22
	8	2.940	.21349	7.46	8.28
	9	3.240	.23745	8.32	9.34
	10	3.540	.26300	9.16	10.42
Dilution Factor 1:50			% Hydrolysis 23.1		
$v_o=9.15x10^{-5}\underline{M}$ min^{-1}					
50.10	2	.985	.05259	1.97	2.02
	3	1.390	.07510	2.93	3.04
	4	1.785	.09803	3.88	4.08
	5	2.195	.12222	4.83	5.12
	6	2.700	.15186	5.74	6.17
	7	3.110	.17730	6.64	7.25
	8	3.460	.19885	7.55	8.32
	9	3.825	.22314	8.44	9.41
	10	4.175	.24608	9.31	10.48
Dilution Factor 1:50			% Hydrolysis 21.8		
$v_o=10.70x10^{-5}\underline{M}$ $min.^{-1}$					
60.10	2	1.090	.04879	1.97	2.02
	3	1.550	.06952	2.44	3.04
	4	2.000	.09075	3.89	4.07
	5	2.450	.11243	4.83	5.11
	6	2.900	.13540	5.77	6.16
	7	3.300	.15529	6.69	7.22
	8	3.790	.18065	7.58	8.28
	9	4.180	.20048	8.49	9.36
	10	4.650	.22632	9.37	10.45
Dilution Factor 1:50			% Hydrolysis 20.3		
$v_o=11.57x10^{-5}\underline{M}$ $min.^{-1}$					

*Armour Lot No. 10705. Protein Nitrogen 11.9%. Analysis by Dr. Adalbert Elek.

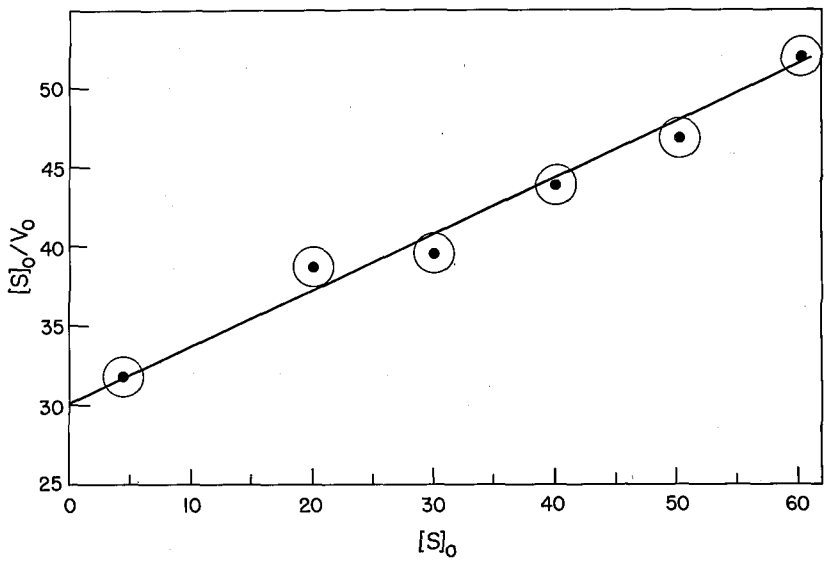

Fig. 5. $[S]_o/v_o$ vs. $[S]_o$ graph for the system of alpha-chymotrypsin-nicotinyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C.

0.5 F with respect to the amine component of THAM-THAM·HCl buffer system.
$[S]_o$ is in units of 10^{-4} M. $[S]_o/v_o$ is in units of minutes.

Table V

alpha-Chymotrypsin Catalyzed Hydrolysis of Nicotinyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C.

0.75 F with Respect to the Amine Component of a Tris-(hydroxymethyl)-aminomethane--Tris-(hydroxymethyl)-aminomethane-hydrochloride Buffer System

$[E]^{*}$ = .1785 mg. P.N./ml.

$[S]_o \times 10^{-4}$	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
5.01	2	.145	.07880	1.92	2.00
	4	.255	.14323	3.73	4.01
	6	.354	.20456	5.43	6.02
	8	.442	.26236	7.04	8.04
	10	.522	.31845	8.58	10.07
	12	.602	.37775	10.00	12.10
	14	.680	.43889	11.34	14.13
	16	.749	.49653	12.61	16.16
	18	.805	.54712	13.86	18.20

Dilution Factor 1:10 % Hydrolysis 42.1

$v_o = 1.40 \times 10^{-5}$ M min.$^{-1}$

20.00	2	.625	.08526	1.93	2.02
	4	1.090	.15357	3.76	4.06
	6	1.460	.21187	5.52	6.12
	8	1.865	.27914	7.17	8.21
	10	2.260	.35066	8.72	10.32
	12	2.585	.41277	10.21	12.44
	14	2.805	.45742	11.72	14.56
	16	3.015	.50138	13.17	16.70
	18	3.265	.55732	14.54	18.86

Dilution Factor 1:50 % Hydrolysis 42.7

$v_o = 5.74 \times 10^{-5}$ M min.$^{-1}$

Table V.--Continued

$[S]_o x10^{-4}$	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
30.00	2	.825	.07510	1.96	2.02
	3	1.150	.10615	2.92	3.04
	4	1.470	.13714	3.81	4.06
	5	1.790	.16973	4.70	5.10
	6.25	2.130	.20538	5.80	6.41
	7	2.350	.22952	6.44	7.20
	8	2.635	.26138	7.28	8.26
	9	2.885	.28968	8.10	9.32
	10	3.135	.31990	8.90	10.38
	Dilution Factor 1:50		% Hydrolysis 27.4		
	$v_o=8.16x10^{-5}\underline{M}$ min.$^{-1}$				
50.10	2	1.130	.06109	1.96	2.02
	3	1.605	.08801	2.92	3.05
	4	2.005	.11064	3.82	4.09
	5	2.420	.13540	4.80	5.13
	6	2.815	.15870	5.72	6.19
	7	3.185	.18232	6.63	7.25
	8	3.575	.20701	7.53	8.32
	9	3.975	.23269	8.42	9.41
	10	4.300	.25464	9.28	10.50
	Dilution Factor 1:50		% Hydrolysis 22.5		
	$v_o=10.75x10^{-5}\underline{M}$ min.$^{-1}$				
60.10	2	1.635	.07417	1.96	2.04
	3	2.130	.09712	2.92	3.07
	4	2.585	.12012	3.87	4.11
	5	3.060	.14328	4.82	5.16
	6	3.505	.16551	5.75	6.22
	7	3.965	.18979	6.67	7.29
	8	4.415	.21349	7.58	8.40
	9	4.835	.23666	8.47	9.49
	10	5.270	.26071	9.35	10.58
	Dilution Factor 1:50		% Hydrolysis 23.0		
	$v_o=12.16x10^{-5}\underline{M}$ min.$^{-1}$				

*Armour Lot No. 10705. Protein Nitrogen 11.9%. Analysis by Dr. Adalbert Elek.

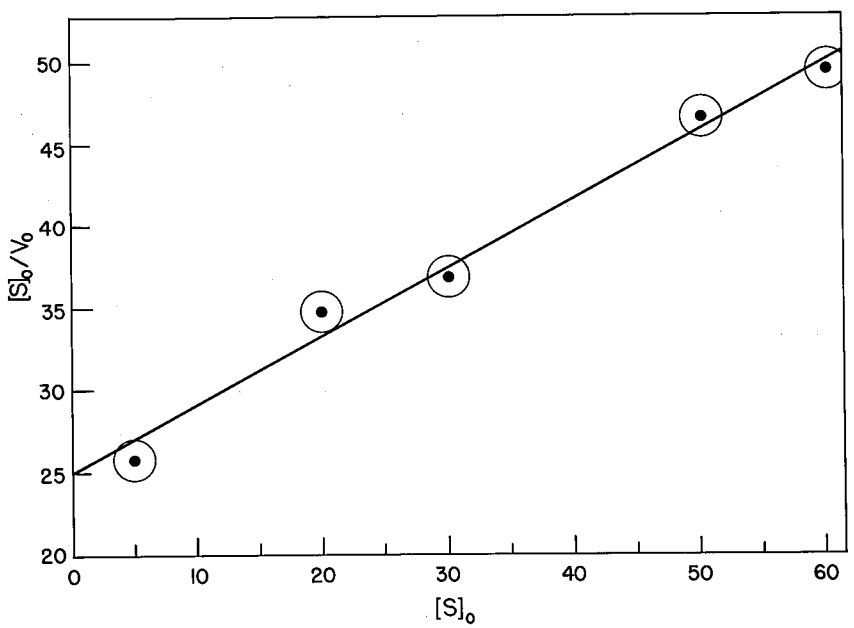

Fig. 6. $[S]_o/v_o$ vs. $[S]_o$ graph for the system of alpha-chymotrypsin-nicotinyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C.

0.75 F with respect to the amine component of THAM-THAM·HCl buffer system.
$[S]_o$ is in units of 10^{-4} M. $[S]_o/v_o$ is in units of minutes.

Table VI

alpha-Chymotrypsin Catalyzed Hydrolysis of Nicotinyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C.

0.90 F with Respect to the Amine Component of a Tris-(hydroxymethyl) aminomethane--Tris-(hydroxymethyl)-aminomethane hydrochloride Buffer System

$[E]^{*}$ = .1785 mg. P.N./ml.

$[S]_o \times 10^{-4}$	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
1.02	2	.095	.27990	1.75	-
	4	.124	.38389	3.32	-
	6	.167	.56076	4.58	-
	8	.176	.60213	6.02	-
	10	.194	.69064	7.23	
	12	.211	.78161	8.35	-
	14	.221	.83941	9.63	-
	16	.240	.95941	10.30	-
	18	.258	1.08822	10.94	-

Dilution Factor 1:10 % Hydrolysis 66.3

$v_o = 0.43 \times 10^{-5}$ M min.$^{-1}$

2.56	1	.067	.07138	0.96	1.00
	3	.157	.17562	2.75	3.01
	5	.243	.28668	4.36	5.02
	7	.303	.37156	5.85	7.04
	9	.380	.49348	7.09	9.05
	11	.430	.57885	8.34	11.08
	13	.477	.67039	9.49	13.10
	15	.524	.76965	10.47	15.14
	17	.567	.86961	11.39	17.17

Dilution Factor 1:10 % Hydrolysis 58.0

$v_o = 1.16 \times 10^{-5}$ M min.$^{-1}$

Table VI.--Continued

$[S]_o x10^{-4}$	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
5.03	2	.215	.11866	0.95	1.00
	4	.386	.22394	3.60	4.02
	6	.529	.32208	5.16	6.04
	8	.664	.42068	6.59	8.06
	10	.780	.52117	7.86	10.10
	12	.890	.62272	9.05	12.14
	14	.980	.71345	10.16	14.20
	16	1.081	.82724	11.04	16.26
	18	1.157	.92187	11.95	18.31
Dilution Factor 1:10			% Hydrolysis 60.2		
$v_o=2.33x10^{-5}\underline{M}$ min.$^{-1}$					
10.22	1	.191	.04974	0.98	1.00
	2	.372	.09984	1.91	2.01
	3	.520	.14323	2.81	3.02
	4	.659	.18647	3.68	4.04
	5	.825	.23745	4.50	5.05
	6	.955	.28065	5.29	6.07
	7	1.086	.32569	6.05	7.10
	8	1.198	.36672	6.79	8.14
	9	1.298	.40414	7.52	9.18
Dilution Factor 1:25			% Hydrolysis 33.2		
$v_o=4.28x10^{-5}\underline{M}$ min.$^{-1}$					
20.36	1	.305	.04018	0.98	1.01
	2	.580	.07788	1.93	2.02
	3	.875	.12012	2.86	3.04
	4	1.155	.16040	3.75	4.06
	5	1.410	.19967	4.62	5.10
	6	1.650	.23825	5.46	6.13
	7	1.895	.27914	6.27	7.18
	8	2.115	.31699	7.06	8.23
	9	2.365	.36255	7.81	9.29
Dilution Factor 1:50			% Hydrolysis 30.4		
$v_o=7.29x10^{-5}\underline{M}$ min.$^{-1}$					

Table VI.--Continued

$[S]_o x10^{-4}$	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
40.72	1	.645	.04209	0.99	1.01
	2	1.120	.07510	1.95	2.03
	3	1.640	.11154	2.89	3.05
	4	2.130	.14756	3.81	4.09
	5	2.585	.18149	4.71	5.14
	6	3.025	.21584	5.59	6.22
	7	3.425	.24842	6.45	7.28
	8	3.860	.28518	7.27	8.36
	9	4.330	.32641	8.06	9.46

Dilution Factor 1:50 % Hydrolysis 27.8

$v_o = 12.60 x 10^{-5} \underline{M}$ min.$^{-1}$

*Armour Lot No. 10705. Protein Nitrogen 11.9%. Analysis by Dr. Adalbert Elek.

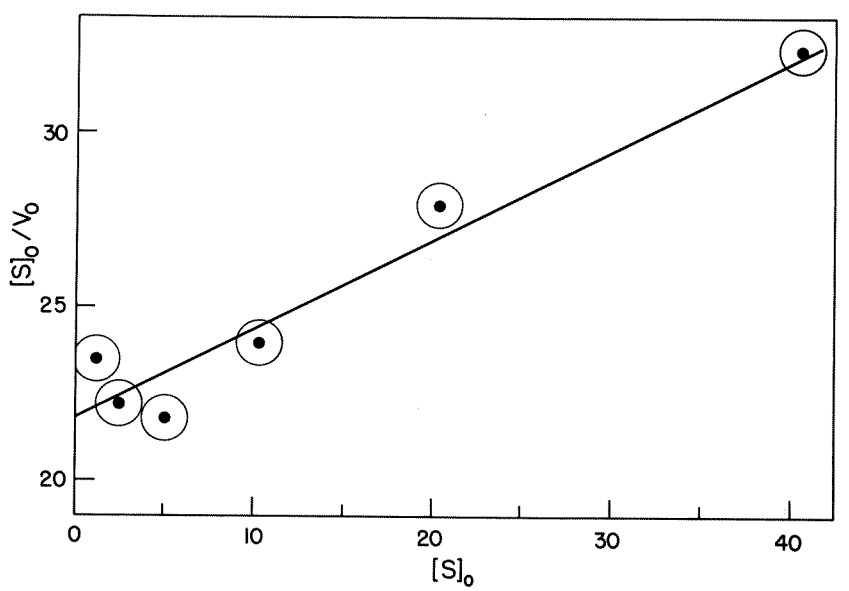

Fig. 7. $[S]_o/v_o$ vs. $[S]_o$ graph for the system of alpha-chymotrypsin-nicotinyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C.

0.90 F with respect to the amine component of THAM-THAM·HCl buffer system.
$[S]_o$ is in units of 10^{-4} M. $[S]_o/v_o$ is in units of minutes.

Table VII

Least Squares Constants for Various Relationships between Initial Velocities, Ionic Strength, and Molality of THAM-THAM·HCl Buffer Systems

v_o (a)	log v_o (b)	[THAM] (c)	$\sqrt{[THAM]}$	μ (d)	$\sqrt{\mu}$
2.56	.4082	.01	.100	.006	.025
3.85	.5855	.10	.316	.061	.247
4.42	.6454	.50	.707	.305	.552
5.51	.7412	.75	.866	.458	.677
6.05	.7812	.90	.949	.549	.741

A least squares fit of the relationship Y = a + bX where Y and X are the indicated functions, gave, within the limits indicated, the values of slope and intercept below:

Y	X	slope (b)	intercept (a) (e)
log v_o	$\sqrt{\mu}$	.467 ± .032	.423 ± .022
log v_o	μ	.578 ± .085	.473 ± .029
log v_o	$\sqrt{M}$	.390 ± .032	.404 ± .026
log v_o	M	.353 ± .052	.473 ± .030
v_o	μ	.557 ± .064	.294 ± .041 (f)

(a) Calculated by using the values of k_3 determined at each concentration of THAM and assuming a constant K_S = 8.0×10^{-3} M, and arbitrarily choosing a substrate concentration of 1×10^{-3} M. The value expressed as above is in units of 10^{-5} M/min.

(b) For purposes of calculation of the least squares fit the mantissa corresponding to 10^{-5} has been dropped.

(c) The concentration of the amine component of the buffer added. Includes both protonated and unprotonated buffer.

Table VII.--Continued

(d) The ionic strength at each buffer concentration was calculated by the equation below on the basis of the concentration of buffer species present and all other ions in the reaction medium. The following equation was employed:

$$\mu = 1/2 \sum_i m_i z_i^2$$

where μ is ionic strength, m_i, the molality of the ion and z_i, the valence or charge of ion.

(e) This is the characteristic of the logarithm of the velocity in the absence of added buffer at a $[S]_o$ of 1.0×10^{-3} M. The antilog must be multiplied by 10^{-5} M/min.

(f) In units of 10^{-4} M/min.

Table VIII

Comparison of Initial Velocities Experimentally Determined and Calculated by Equations Indicated

THAM-THAM·HCl			THAM-THAM·H_2SO_4		
[Buffer]	v_o(exp.)	v_o(calcd.)[a]	[Buffer]	v_o(exp.)	v_o(calcd.)[b]
.01	2.56	2.66	.02	2.43	2.66
.10	3.85	3.56	.05	2.89	2.96
.50	4.42	4.79	.10	3.16	3.26
.75	5.51	5.48	.20	3.86	3.72
.90	6.05	5.88	.30	4.36	4.12
			.40	4.63	4.49
			.50	5.05	4.85
			.75	5.43	5.69
			1.00	6.61	6.53

[a] Calculated from an assumed relationship between the logarithm of v_o and the square root of ionic strength:

$$\log v_o = .46\sqrt{\mu} + \log v_o^{\circ} \text{ where } \log v_o^{\circ} = .42$$

[b] Calculated from an assumed relationship between the logarithm of v_o and the square root of ionic strength:

$$\log v_o = .46\sqrt{\mu} + \log v_o^{\circ} \text{ where } \log v_o^{\circ} = .37.$$

Table IX

alpha-Chymotrypsin Catalyzed Hydrolysis of Nicotinyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C.

Variation in Concentration of Amine Component of Tris-(hydroxymethyl)-aminomethane--Tris-(hydroxymethyl)-aminomethane-sulfate Buffer System

$[S]_o^{(a)} = 3.324 \times 10^{-4}$ M

$[E]^{(b)} = .1785$ mg./protein-nitrogen/ml.

[Buffer]	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
.02	2	.208	.05733	1.95	2.00
	4	.413	.11511	3.80	4.02
	6	.586	.17226	5.55	6.05
	8	.715	.21430	7.26	8.08
	10	.829	.25298	8.93	10.12
	12	1.080	.34430	10.30	12.18
	14	1.205	.39271	11.76	14.24
	16	1.357	.45560	13.10	16.30
	18	1.585	.55732	13.65	18.40

Dilution Factor 1:25 % Hydrolysis 42.7

$v_o = 2.53 \times 10^{-5}$ M min.$^{-1}$

.05	2	.250	.06952	1.94	2.01
	3	.372	.10526	2.86	3.02
	4	.472	.13540	3.76	4.03
	5	.565	.16551	4.65	5.04
	6	.667	.19803	5.48	6.06
	7	.760	.22952	6.32	7.08
	8	.855	.26236	7.12	8.12
	9	.938	.29193	7.92	9.14
	10	1.022	.32208	8.65	10.16

Dilution Factor 1:10 % Hydrolysis 27.6

$v_o = 2.89 \times 10^{-5}$ M min.$^{-1}$

Table IX.--Continued

[Buffer]	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
.10	1	.129	.03536	0.98	1.00
	2	.252	.07045	1.94	2.01
	3	.365	.10346	2.86	3.02
	4				
	5	.584	.19309	4.63	5.04
	6	.709	.21187	5.46	6.06
	7	.806	.24842	6.26	7.08
	8	.904	.27914	7.06	8.12
	9	1.003	.31554	7.72	9.14
Dilution Factor 1:10			% Hydrolysis 27.3		
$v_o=3.16\times10^{-5}$ M min.$^{-1}$					
.20	1	.157	.04305	0.98	1.00
	2	.283	.07973	1.93	2.01
	3	.450	.12927	2.82	3.02
	4	.594	.17479	3.70	4.03
	5	.723	.21656	4.53	5.04
	6	.841	.25696	5.37	6.06
	7	.982	.30748	6.16	7.09
	8	1.107	.35417	6.88	8.12
	9	1.201	.39135	7.56	9.16
Dilution Factor 1:10			% Hydrolysis 32.4		
$v_o=3.86\times10^{-5}$ M min.$^{-1}$					
.30	1	.186	.05164	0.98	-
	2.1	.372	.10526	1.90	-
	3	.505	.14670	2.70	-
	4	.690	.20619	3.63	-
	5	.800	.24294	4.42	-
	6	.937	.29118	5.18	-
	7	1.067	.33932	5.88	-
	8	1.206	.39339	6.58	-
Dilution Factor 1:10			% Hydrolysis 32.5		
$v_o=4.36\times10^{-5}$ M min.$^{-1}$					

Table IX.--Continued

[Buffer]	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
.40	1	.161	.04401	0.98	-
	2	.345	.09803	1.90	-
	3	.512	.14843	2.79	-
	4	.675	.20048	3.63	-
	5	.835	.25541	4.42	-
	6	.976	.30527	5.18	-
	7	1.121	.35976	5.88	-
	8	1.235	.40481	6.58	
Dilution Factor 1:10				% Hydrolysis 33.3	
$v_o=4.63\times10^{-5}$ M min.$^{-1}$					
.50	1.0	.195	.05353	0.97	-
	2.0	.385	.10974	1.89	-
	3.0	.577	.16889	2.76	-
	4.0	.731	.21874	3.60	-
	5.0	.895	.27611	4.38	-
	6.0	1.074	.34217	5.08	-
	7.0	1.206	.39271	5.78	-
	8.5	1.434	.49164	6.72	-
	9.5	1.539	.53590	7.35	-
Dilution Factor 1:50				% Hydrolysis 41.5	
$v_o=5.05\times10^{-5}$ M min.$^{-1}$					
.75	1.00	.214	.05921	0.97	-
	1.75	.365	.10346	1.66	-
	2.50	.505	.14670	2.33	-
	3.25	.664	.19728	3.05	-
	4.00	.801	.24294	3.56	-
	4.75	.926	.28743	4.14	-
	5.50	1.079	.34359	4.66	-
	6.25	1.212	.39545	5.16	-
	8.25	1.501	.51875	6.44	-
Dilution Factor 1:50				% Hydrolysis 40.5	
$v_o=5.43\times10^{-5}$ M min.$^{-1}$					

Table IX.--Continued

[Buffer]	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
1.00	1.00	.240	.06672	0.97	-
	1.75	.424	.12132	1.65	-
	2.50	.585	.17142	2.30	-
	3.25	.766	.23111	2.91	-
	4.00	.949	.29564	3.51	-
	4.75	1.081	.38321	3.95	-
	6.00	1.326	.44211	5.03	-
	6.75	1.468	.50441	5.30	-
	7.50	1.644	.57642	5.68	-

Dilution Factor 1:50 % Hydrolysis 44.3

$v_o = 6.61 \times 10^{-5}$ M min.$^{-1}$

(a) Substrate concentration was maintained constant throughout investigations whose dataare listed in Table IX.

(b) Armour Lot No. 10705. Protein-nitrogen 11.9%. Analysis by Dr. Adalbert Elek.

Table X

Initial Velocities of the alpha-Chymotrypsin Catalyzed Hydrolysis of Nicotinyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C. Using Various Concentrations of the Amine Component of THAM-THAM·H_2SO_4 Buffer System

$[S]_o = 3.324 \times 10^{-4}$ M [E] = .218 mg. P.N./ml.

[Buffer]	$v_o \times 10^5$(a)	$v_o' \times 10^5$(b)	$\sigma_{v_o'}$(c)	P_m(d)
.02	2.43			
.05	2.89			
.10	3.16	3.16	± .05	2
.20	3.86	3.60	± .06	3
.30	4.36	4.35	± .14	2
.40	4.63	4.94	± .14	2
.50	5.05	5.00	± .12	3
.75	5.43	5.10	± .13	3
1.00	6.61	6.39	± .07	4

(a) Initial velocities determined by the method of Jennings and Niemann.

(b) Initial velocities determined by the method of Booman and Niemann.

(c) Probable error in (b).

(d) The order of the polynomial used in calculation of (b).

Table XI

Least Squares Constants for Various Relationships between Initial Velocities, Ionic Strength, and Molality of THAM-THAM·H_2SO_4 Buffer Systems

v_o (a)	log v_o (b)	[THAM] (c)	$\sqrt{[THAM]}$	μ (d)	$\sqrt{\mu}$
2.43	.4031	.02	.1414	.0183	.1353
2.89	.4609	.05	.2236	.0458	.2143
3.16	.4997	.10	.3162	.0915	.3025
3.86	.5866	.20	.4472	.1830	.4280
4.36	.6395	.30	.5477	.2745	.5240
4.63	.6656	.40	.6325	.3660	.6050
5.05	.7033	.50	.7071	.4575	.6764
5.43	.7348	.75	.8660	.6863	.8288
6.61	.8202	1.00	1.000	.9150	.9566

A least squares fit of the relationship Y = a + bX where Y and X are the indicated functions, gave, within the limits indicated, the values of slope and intercept below:

Y	X	slope (b)	intercept (a) (e)
log v_o	$\sqrt{\mu}$	.461 ± .018	.373 ± .017
log v_o	μ	.424 ± .062	.470 ± .029
log v_o	$\sqrt{M}$	.468 ± .016	.359 ± .010
log v_o	M	.387 ± .030	.470 ± .016
v_o	u	.427 ± .048	.283 ± .029 (f)

(a) In units of 10^{-5} M/min.

(b) For purposes of calculation of the least squares fit the mantissa corresponding to 10^{-5} has been dropped.

(c) The concentration of the amine component added.

(d) The ionic strength of each buffer concentration was calculated analogously to Table VII.

(e) This is the characteristic of the logarithm of the velocity in the absence of added buffer at a $[S]_o$ of 3.324×10^{-4} M. The antilog must be multiplied by 10^{-5} M/min. to obtain velocity.

(f) In units of 10^{-4} M/min.

Table XII

Summary of Initial Velocities from Tables XI-XIII

[Buffer]	$[Ca^{++}]$	v_o (a)	v_o (calcd.) (b)	v_o' (c)	σ_{v_o}' (d)	P_m (e)
0.02	0	2.62	2.64	2.95	± 0.22	3
	0	2.67	2.64	2.45	± 0.27	3
	10^{-6}	2.53	2.64	2.42	± 0.27	3
	10^{-5}	2.68	2.65	2.49	± 0.28	3
	10^{-4}	3.15	2.69	3.32	± 0.18	2
	10^{-3}	4.24	2.80	4.30	± 0.15	3
	10^{-2}	4.35	3.17	4.52	± 0.25	3
	10^{-1}	4.63	4.66	4.45	± 0.56	2
1.00	0	3.80	3.80	3.63	± 0.18	3
	10^{-5}	4.20	3.82	4.18	± 0.26	3
	10^{-4}	4.42	3.87	4.49	± 0.34	3
	10^{-3}	5.10	4.03	4.97	± 0.41	2
	10^{-2}	5.32	4.78	5.32	± 0.52	2
	Versene					
0.02	0	2.70	-	2.51	± 0.39	3
	5.3×10^{-6}	2.58	-	2.43	± 0.08	2
	8.9×10^{-6}	2.66	-	2.55	± 0.20	2
	15.1×10^{-6}	2.72	-	2.67	± 0.26	2

(a) Calculated by the method of Jennings and Niemann.

(b) Calculated from the relationship: $\log v_o/v_o^o = .46\sqrt{\mu}$ where v_o^o is the initial velocity in the absence of added calcium.

(c) Calculated by the method of Booman and Niemann.

(d) The probable error in (c).

(e) The order of the polynomial used in (c).

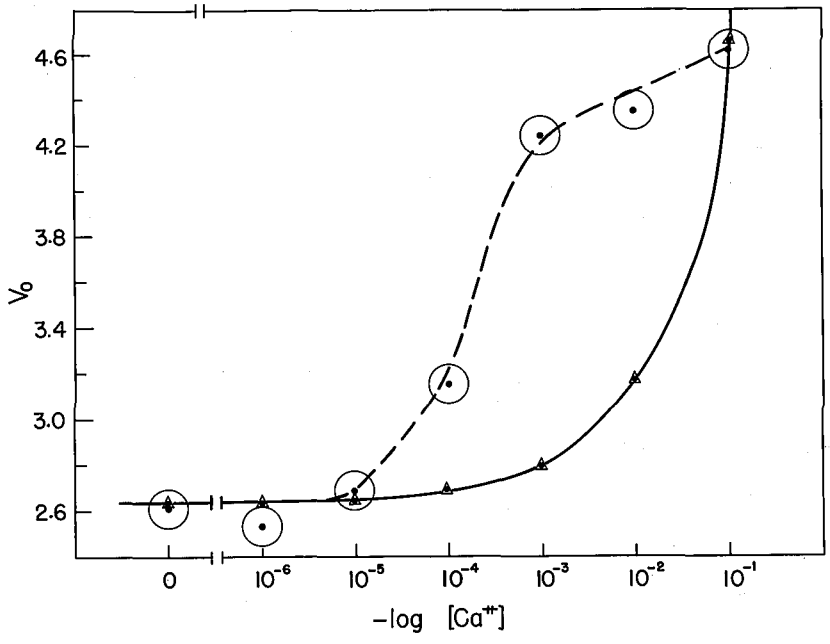

Fig. 8. v_o vs. -log [Ca^{++}] relationship for the system of alpha-chymotrypsin-nicotinyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C; ● - observed velocities; ▲ - calculated velocities.

.02 F With respect to the amine component of THAM-THAM·HCl buffer system.
v_o is in units of 10^{-5} M/min.

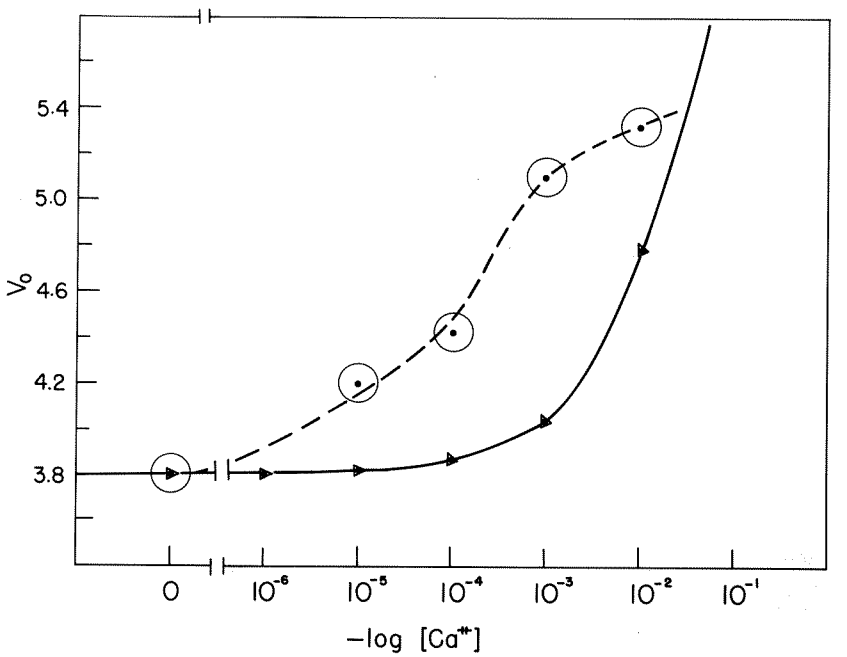

Fig. 9. v_o vs $-\log [Ca^{++}]$ relationship for the system alpha-chymotrypsin-nicotinyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C.; ● - observed velocities; ▲ - calculated velocities.

1.0 F with respect to the amine component of THAM-THAM·HCl buffer system.
v_o is in units of 10^{-5} M/min.

Table XIII

Effect of Addition of Calcium Ion on the alpha-Chymotrypsin Catalyzed Hydrolysis of Nicotinyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C. at .02 F Buffer Concentration

$[E]^*$ = .218 mg. P.N./ml.
$[S]_o$ = 10.028 M. D_{oo} = 4.125

$[Ca^{++}]$	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
0	2	.245	.06109	1.95	2.00
	3	.356	.08983	2.88	3.01
	4	.460	.11866	3.80	4.02
	5	.568	.14843	4.67	5.04
	6	.668	.17646	5.53	6.05
	7	.770	.20701	6.38	7.06
	8	.852	.23111	7.21	8.09
	9	.948	.26071	8.91	9.11

Dilution Factor 1:10 % Hydrolysis 22.9

$v_o = 2.62 \times 10^{-5}$ M min.$^{-1}$

0	2	.248	.06203	1.95	2.01
	3	.357	.09075	2.89	3.01
	4	.452	.11600	3.80	4.02
	5	.552	.14323	4.69	5.03
	6	.641	.16889	5.57	6.04
	7	.750	.20048	6.41	7.06
	8	.852	.23111	7.22	8.08
	9	.920	.25221	8.06	9.10
	10	1.013	.28141	8.82	10.14

Dilution Factor 1:10 % Hydrolysis 24.5

$v_o = 2.67 \times 10^{-5}$ M min.$^{-1}$

Table XIII.--Continued

$[Ca^{++}]$	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
10^{-6}	2	.218	.05448	1.95	2.00
	3	.314	.07880	2.89	3.01
	4	.413	.10526	3.80	4.02
	5	.508	.13013	4.71	5.03
	6	.588	.15357	5.60	6.04
	7	.668	.17646	6.68	7.06
	8	.752	.20130	7.32	8.08
	9	.829	.22473	8.15	9.11
	10	.930	.25541	8.95	10.16
Dilution Factor 1:10			% Hydrolysis 22.5		
$v_o=2.53\times10^{-5}$ M min.$^{-1}$					
10^{-5}	2	.230	.05733	1.94	2.00
	3	.328	.08249	2.89	3.01
	4	.426	.10884	3.80	4.02
	5	.520	.13452	4.71	5.03
	6	.599	.15700	5.67	6.04
	7	.692	.18398	6.46	7.06
	8	.773	.20782	7.30	8.09
	9	.860	.23348	8.12	9.13
	10	.950	.26138	8.92	10.17
Dilution Factor 1:10			% Hydrolysis 23.0		
$v_o=2.68\times10^{-5}$ M min.$^{-1}$					
10^{-4}	2	.267	.06672	1.94	2.01
	3	.392	.09984	2.87	3.01
	4	.507	.13103	3.77	4.02
	4.8	.597	.15613	4.47	5.04
	6	.742	.18979	5.51	6.05
	6.8	.827	.22314	6.17	7.08
	8	.941	.25927	7.14	8.11
	8.8	1.017	.28292	7.76	9.14
	10	1.100	.31041	8.74	10.18
Dilution Factor 1:10			% Hydrolysis 26.7		
$v_o=3.15\times10^{-5}$ M min.$^{-1}$					

Table XIII.--Continued

$[Ca^{++}]$	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
10^{-3}	2	.335	.08434	1.92	2.01
	3	.505	.13103	2.83	3.02
	4	.647	.17057	3.69	4.03
	5	.812	.21874	4.54	5.05
	6	.934	.25696	5.36	6.07
	7	1.077	.30231	6.17	7.10
	8	1.195	.34217	6.90	8.14
	9	1.283	.37225	7.65	9.18
	10	1.423	.42267	8.34	10.24
Dilution Factor 1:25			% Hydrolysis 34.5		
$v_o = 4.24 \times 10^{-5}$ M min.$^{-1}$					
10^{-2}	2	.382	.09712	1.90	2.01
	3	.534	.13889	2.82	3.02
	4	.707	.18813	3.68	4.03
	5	.834	.22553	4.53	5.06
	6	1.000	.27763	5.30	6.08
	7	1.105	.31188	6.10	7.11
	8	1.238	.35701	6.85	8.14
	9	1.376	.40613	7.54	9.19
	10	1.507	.45494	8.25	10.21
Dilution Factor 1:25			% Hydrolysis 36.5		
$v_o = 4.35 \times 10^{-5}$ M min.$^{-1}$					
10^{-1}	2	.349	.08801	1.92	2.01
	3	.525	.13627	2.82	3.02
	4	.682	.18065	3.68	4.03
	5	.842	.22791	4.51	5.05
	6	1.005	.27914	5.30	6.08
	7	1.127	.31917	5.98	7.11
	8	1.268	.36741	6.82	8.14
	9	1.407	.41673	7.49	9.17
	10	1.528	.45868	8.16	10.21
Dilution Factor 1:25			% Hydrolysis 37.2		
$v_o = 4.63 \times 10^{-5}$ M min.$^{-1}$					

*Armour Lot No. 00592. Protein-nitrogen 14.6%. Analysis by Dr. Adalbert Elek.

Table XIV

Effect of Addition of Calcium Ion on the alpha-Chymotrypsin Catalyzed Hydrolysis of Nicotinyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C. at 1 F Buffer Concentration

$[E]^*$ = .218 mg. P.N./ml.
$[S]_o$ = 10.028 M $\qquad D_{oo}$ = 4.125

Calcium	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
0	2	.366	.09258	1.92	2.01
	3	.512	.13278	2.83	3.02
	4	.657	.17311	3.70	4.04
	5	.795	.21430	4.56	5.05
	6	.909	.24919	5.38	6.07
	7	1.065	.29861	6.15	7.10
	8	1.165	.33218	6.92	8.13
	9	1.309	.38185	7.61	9.16
	10	1.368	.40279	8.38	10.20

Dilution Factor 1:25 % Hydrolysis 33.2

$v_o = 3.80 \times 10^{-5}$ M min.$^{-1}$

10^{-5}					
	2	.354	.08983	1.92	2.01
	3	.523	.13540	2.82	3.02
	4	.655	.16635	3.70	4.04
	5	.815	.21946	4.54	5.05
	6	.945	.26004	5.36	6.07
	7	1.060	.29713	6.15	7.10
	8	1.216	.34926	6.86	8.13
	9	1.322	.38662	7.45	9.16

Dilution Factor 1:25 % Hydrolysis 32.0

$v_o = 4.20 \times 10^{-5}$ M min.$^{-1}$

Table XIV.--Continued

Calcium	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
10^{-4}	2	.414	.10615	1.90	2.02
	3	.587	.15357	2.80	3.03
	4	.756	.20211	3.66	4.04
	5	.893	.24372	4.50	5.06
	6	1.058	.29639	5.27	6.08
	8	1.310	.38185	6.77	8.14
	9	1.420	.42201	7.50	9.18
	10	1.552	.47187	8.08	10.22
Dilution Factor 1:25			% Hydrolysis 37.6		
$v_o=4.42\times10^{-5}$ M min.$^{-1}$					
10^{-3}	2	.452	.11600	1.90	2.02
	3	.637	.16804	2.80	3.03
	4	.815	.21946	3.62	4.04
	5	.986	.27155	4.44	5.06
	6	1.161	.33074	5.19	6.08
	7	1.313	.38321	5.92	7.12
	8	1.462	.43760	6.62	8.16
	9	1.596	.48919	7.30	9.20
	10	1.754	.55389	7.90	10.24
Dilution Factor 1:25			% Hydrolysis 42.5		
$v_o=5.10\times10^{-5}$ M min.$^{-1}$					
10^{-2}	2	.491	.12663	1.89	2.02
	3	.685	.18149	2.77	3.03
	4	.884	.24137	3.60	4.05
	5	1.066	.29861	4.33	5.07
	6	1.245	.35906	5.11	6.10
	7	1.425	.42393	5.83	7.13
	8	1.582	.48369	6.51	8.18
	9	1.775	.56247	7.15	9.23
	10	1.840	.59080	7.78	10.27
Dilution Factor 1:25			% Hydrolysis 44.6		
$v_o=5.32\times10^{-5}$ M min.$^{-1}$					

*Armour Lot No. 00592. Protein Nitrogen 14.6%. Analysis by Dr. Adalbert Elek.

Table XV

Effect of Addition of Disodium Dihydrogen Versenate* to the alpha-Chymotrypsin Catalyzed Hydrolysis of Nicotinyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C.

$[E]^{**}$ = .218 mg. P.N./ml.
$[S]_o$ = 10.028 M $\quad D_{oo}$ = 4.125

Versene	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
0	2	.252	.06297	1.95	2.01
	3	.348	.08801	2.88	3.02
	4	.460	.11866	3.79	4.03
	5	.553	.14409	4.69	5.04
	6	.659	.17395	5.56	6.05
	7	.744	.19885	6.42	7.07
	8	.843	.22871	7.26	8.09
	9	.936	.25773	8.06	9.12
Dilution Factor 1:10			% Hydrolysis 22.7		
$v_o=2.70\times10^{-5}$ M min.$^{-1}$					
.0018 gm.	2	.250	.06298	1.95	2.01
	3	.352	.08892	2.88	3.02
	4	.451	.11600	3.80	4.02
	5	.549	.14323	4.70	5.04
	6	.623	.16381	5.57	6.05
	7	.714	.18979	6.43	7.06
	8	.817	.22019	7.26	8.09
	9	.906	.24764	8.07	9.12
Dilution Factor 1:10			% Hydrolysis 22.0		
$v_o=2.58\times10^{-5}$ M min.$^{-1}$					
.0030 gm.	2	.262	.06578	1.94	2.01
	3	.363	.09167	2.88	3.02
	4	.469	.12044	3.79	4.03
	5	.561	.14584	4.69	5.04
	6	.661	.17479	5.56	6.05
	7	.745	.19885	6.41	7.06
	8	.827	.21946	7.26	8.08
	9	.917	.25143	8.06	9.10
Dilution Factor 1:10			% Hydrolysis 22.2		
$v_o=2.66\times10^{-5}$ M min.$^{-1}$					

Table XV.--Continued

Versene	t(min)	D_t	$\ln \frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
.0051 gm.	2	.284	.07138	1.95	2.01
	3	.382	.09712	2.87	3.02
	4	.483	.12486	3.78	4.03
	5	.570	.14843	4.68	5.04
	6	.692	.18398	5.54	6.05
	7	.783	.21025	6.38	7.07
	8	.873	.23745	7.21	8.09
	9	.968	.26687	8.01	9.12

Dilution Factor 1:10 % Hydrolysis 23.4

$v_o = 2.72 \times 10^{-5}$ M min.$^{-1}$

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*The disodium dihydrogen salt of ethylene diamine tetracetic acid.

**Armour Lot No. 00592. Protein-nitrogen 14.6%. Analysis by Dr. Adalbert Elek.

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PART I

Section 2

INVESTIGATIONS IN THE COLORIMETRIC DETERMINATION OF HYDRAZINE

INVESTIGATIONS IN THE COLORIMETRIC DETERMINATION OF HYDRAZINE

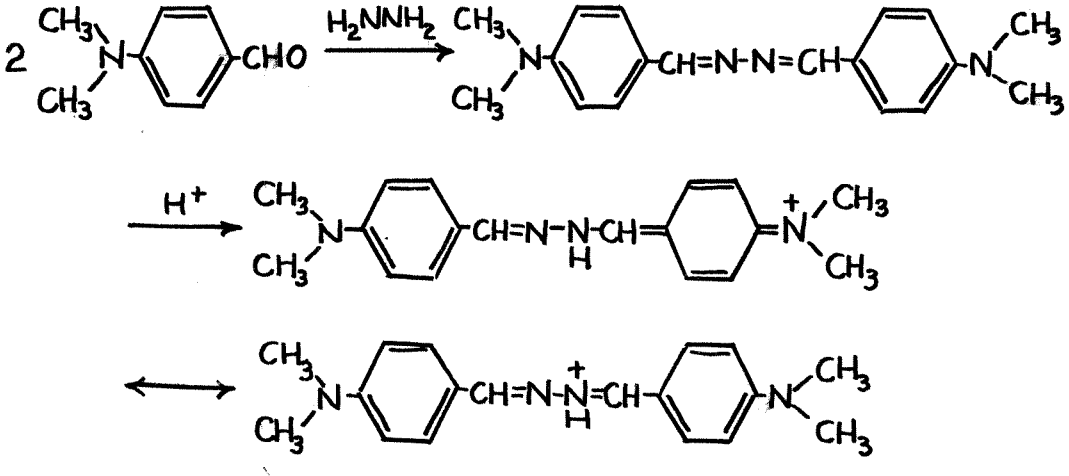
Introduction

The hydrazine liberated in the alpha-chymotrypsin catalyzed hydrolysis of certain acylated and non-acylated amino acid hydrazides (cf. the preceding section of this thesis) is quantitatively determined by a sensitive colorimetric method based upon the formation of an azine (1,2). p-Dimethylaminobenzaldehyde is a convenient reagent for this purpose, yielding a derivative whose U/V spectrum exhibits a rather broad maximum (in acid solution) at 455 mμ. Utilizing either the Beckmann Model B or Model DU Spectrophotometer, hydrazine concentrations as low as 10^{-7} M can be detected and determined.

The use of this method for the determination of enzymatically liberated hydrazine was initiated by Lutwack in the determination of the kinetic constants of a series of hydrazides (3). It was essentially the Lutwack procedure that was used in the determination of extent of hydrolysis while studying buffer effects in the preceding section. During the course of these latter investigations it became evident that a number of parameters other than those whose need for adequate control was already recog-

nized were introducing needless error into the determination. In view of the number of experimental operations involved in following the course of a typical enzymatic hydrolysis and the lack of a complete investigation, in this case, of the parameters involved in the analysis, an attempt has been made to define the conditions for maximum accuracy and reproducibility of the assay method. Specific attention has been directed to those factors involved when the method is applied to the determination of hydrazine liberated in the alpha-chymotrypsin catalyzed hydrolysis of hydrazides.

The procedure introduced by Lutwack consists of the addition of aliquots of reaction mixture, containing enzyme, buffer, unhydrolyzed substrate, and hydrolysis products to a series of acidic solutions of p-dimethylaminobenzaldehyde. The intensely-colored azine cation is formed according to equations given below:



The following experiments describe the reinvestigation of factors involved in the determination which needed closer examination and the study of other factors in order to standardize the procedure.

Discussion of the Results

The U/V spectrum, between 400 and 500 mμ, of the colored assay mixture is shown in figure 1. Curves I and Ia were produced by the simple solution containing aldehyde and known amounts of hydrazine sulfate at two different acid concentrations used in the Beer's Law calibration (see below). Curve II shows the spectrum of a typical sample containing all the components of the normal hydrolysis reaction solution. It is apparent that the position of the peak at 455 mμ is not affected by these changes in the constitution of the solution. It has also been established that a known amount of hydrazine gives rise to the same optical density whether or not enzyme and the other reactants are present in the solution. The calibration and use of the assay method is therefore justified.

The factors which have been examined in the system may be reviewed as follows:

(a) The choice of a blank.

(b) The effect of temperature upon the optical density of the azine solution.

(c) The time of development of the color.

(d) Dependence upon the composition of the color-developing solution.

(e) Adherence to Beer's Law.

The Choice of a Blank.--It has been found that the optical density at time t must be corrected for a zero-time optical density when ethanol, water, or an air-path is used as a blank. This zero-time optical density is due partly to intrinsic color of the acid and aldehyde solution and partly to the reaction of the acid and aldehyde solution with unhydrolyzed substrate or free hydrazine occluded by it. The most important contribution to this optical density, however, arises from turbidity produced when enzyme-containing aliquots are added to an acid solution of too high concentration. This turbidity may be alleviated to some extent by diluting the acid and aldehyde solution to about eighty percent of the final volume before the addition of the reaction aliquot.

However, if a blank containing the enzyme, the buffer, acid, aldehyde, and the substrate all in the same concentrations as in the typical solution is used, the zero-time correction is reduced to a random error of approximately $\pm$ 0.004 O.D. units.

The effect of temperature upon the optical density of the azine solution.--Since the enzymatic hydrolyses are carried out at 25°C., it has been convenient to equilibrate the color-developing solutions and the light absorption chamber of the Beckmann Model B spectrophotometer at this temperature. Optical densities in all the calibration curves were determined at 25°C. However, since some earlier investigations had not taken this into account, it was of interest to determine the relationship between optical density and temperature in some representative optical density ranges. This relationship is shown in three different regions, in figure 2. The results indicate that the optical density decreases at the rate of approximately 0.46 percent per degree centigrade rise in temperature in the range 15-40°C. In the highest optical density range investigated there is an indication of hydrolysis of the azine above 35°C. as shown by a drop in the observed optical density below the value expected from the temperature-optical density relationship.

The time of development of the color.--Pesez and Petit (1) reported that the color development in a similar system was complete in fifteen minutes. This was confirmed but a subsequent upward drift after twenty-five or thirty minutes was noted. This error was eliminated by standardization of the development time at twenty minutes.

Dependence upon the composition of the color-developing solution.--Using an iodate-standardized solution of hydrazine sulfate the optical relationships have been determined at three different aldehyde concentrations and various acid concentrations. The results are shown in figures 3, 4, and 5.

It is seen that the random error in optical density at a fixed acid concentration, is minimized when the aldehyde concentration is approximately 2.684×10^{-2} M. This observation conforms to the results of Wood (2), who obtained a hyperbolic type curve between optical density and aldehyde concentration at fixed hydrazine and acid concentrations.

The figures also show that the effects of variation in acid concentration are minimized in the solution of the higher aldehyde concentration; reduced acidity seems to lead to greater adherence to Beer's Law, although at acid concentrations slightly lower than the ones used, there is the risk of precipitation of the azine. In light of these results, it is suggested that the most satisfactory system is one in which the final aldehyde concentration is 2.684×10^{-2} M in absolute alcohol (4 gms. per 100 ml.), and which is 0.167 N with respect to hydrochloric acid. The obvious disadvantage of such a system (cf. figure 4 with figure 3) lies in the much higher optical density produced by a given amount of hydrazine.

While this allows the detection of a lower concentration of hydrazine, it produces far too intense a color at the higher concentrations. When prolonged or rapid reactions are being studied, this may necessitate dilution of the samples or use of thinner cells. The former procedure has been found to be valid provided dilution is carried out before the optical density of the sample exceeds 1.0-1.2. If the non-linear portion of the curve is too closely approached, dilution techniques will give rise to a stepped optical density-time relationship.

Adherence to a Beer's Law relationship.--Reference to figures 3 and 4 illustrates the adherence to a Beer's Law relationship between optical density and hydrazine concentration in the range of approximately 0-1.2 optical density units. The Beer's Law constants for these two different aldehyde concentrations are respectively 2.431 and 1.671×10^{-5} M per optical density unit.

Some of the factors to which the non-linearity of the curve might be due have been semi-quantitatively evaluated in an unsuccessful attempt to extend the linear portion of the curves for greater experimental convenience.

In particular, two ways by which a more or less constant proportion of the incident light could escape absorption have been examined. At increasing optical densities the deviation produced by these effects would be

almost exactly approximated if a constant 0.2 percent of the incident light escaped absorption as a result of light of other wavelengths of scattered light.

The band-width of the light beam supplied by the monochromator was approximately 2 mμ at the slit settings used; the rather broad shape of the peak at 455 mμ (see figure 1) suggests that only an extremely small contribution to the transmitted energy could be derived from light of wavelengths other than 455 mμ. The possibility of light scattering has also been investigated. This was accomplished by selecting a number of values above the linear portion of the Beer's Law curve and using open-ended cells and a transparent insert of calibrated optical path to bring the values down to where they would be expected to again adhere to Beer's Law. Comparison of the optical densities obtained indicates that scattered light makes a minimum contribution of 55-60% to the observed deviation in optical densities greater than 1.0-1.2. The use of the insert (or in effect of thinner cells) does not therefore eliminate these effects and it is recommended that optical densities be kept below the above value by suitable dilution techniques.

Discussion of Error

There have been a number of discussions of the accuracy attainable in spectrophotometric analysis (4-7). If the

optical density, O.D., is defined, O.D. = $\log_{10} I_o/I$, it has been shown (cf. 7) that

$$\frac{dc}{c} = -\frac{dI}{I} \times \frac{0.4343}{O.D.} \qquad (1)$$

If the percent absorbancy or optical density is plotted versus the logarithm of concentration the graph passes through a point of inflection where the slope is a maximum. The accuracy is the greatest when

$$\frac{dI}{dc/c} = \frac{dI}{d\ (\ln c)} = \frac{dI}{2.303\ d\ (\log c)} \qquad (2)$$

reaches this maximum. If the system obeys Beer's Law this maximum and hence, the minimum error, occurs when the optical density is 0.4343 corresponding to 63.2 percent absorbance where a 1 percent photometric error produces a relative analysis error of 2.72 percent. The variation of the photometric error with percent absorption or optical density for a system obeying Beer's Law is shown in figure 6. It is noteworthy that if the percent absorbancy is plotted against the logarithm of concentration for systems which do not adhere to Beer's Law, the graph is equally valuable since the determination of a point of inflection shows the optimum range for such a system also. It can be evaluated by rearranging equa-

tion 2,

$$\frac{dc/c}{dI} = \frac{2.303}{dI/d\ (\log c)} \qquad \text{or}$$

$$\frac{\%\ \text{relative analysis error}}{\%\ \text{absolute photometric error}} = \frac{230}{dI/d\ (\log c)} \qquad (3)$$

The relative analysis error for a 1 percent absolute photometric error can be obtained by dividing 230 by the slope of the curve.

If the data shown in figure 8 (the final complete Beer's Law calibration curve) are replotted as described above the characteristic curve is obtained (figure 7). The point of inflection corresponds to approximately 63.2 percent absorbency. This has been proven for a similar system by Watt and Chrisp (9).

A practical limit for the photometric reading error on the Beckmann Model B spectrophotometer can be given as $\pm$ 0.02 percent. The minimum relative analysis error due to this would be approximately $\pm$ 0.05 percent.

The color development system is most satisfactorily used in the hydrazine concentration range, in the final solution being read, of 2×10^{-6} M to 2×10^{-5} M where the analysis errors do not exceed 4 percent.

It may be added that the principles outlined above are very suitable for precise determinations where optical

densities show small variations about a mean value and by use of a number of methods including adjustment of concentration by dilution procedures, utilization of cells of varying thicknesses, adjustment of wave length, and measurement against solutions of known concentration the observed optical densities can be maintained around the desired value of 0.4343. It would seem to be more satisfactory to use the entire linear portion when rapid and several-fold changes in optical density occur. This is the case in the determination of the extent of hydrolysis in enzymatic studies. The optical density can be kept below 1.1-1.2 by suitable dilution techniques.

The concentrations as stated above are used in the next section to determine the enzymatic hydrolysis constants for N-acetyl-L-tyrosinhydrazide.

Experimental

Acid Reagent Solutions.--One hundred and forty ml. of Baker's Analyzed hydrochloric acid (spec. grav. 1.186, 35.8%) was diluted to one liter in distilled water. One ml. of this solution in a 10 ml. color-developing flask results in a final acid concentration of 0.167 N. The various other acid concentrations were prepared in a similar manner.

Aldehyde Reagent Solutions.--A solution of 4 gms. of p-dimethylaminobenzaldehyde in 100 ml. of absolute ethanol was prepared. The aldehyde used was reagent grade (Matheson and Co.) recrystallized from aqueous methanol, m.p. 75.5-75.9°C. One ml. of this solution in the developing flask corresponds to a final aldehyde concentration of 2.684×10^{-2} M. This solution is stable for periods up to a week if stored in a dark bottle. The color developing solutions of acid and aldehyde mixture should be made up as required. The other solutions of different aldehyde concentrations were prepared in the same manner.

Hydrazine Solutions.--The hydrazine solutions were made using reagent grade hydrazine sulfate $(N_2H_4)_2 \cdot H_2SO_4$ (Matheson and Co.). The approximate amount desired was weighed and placed in solution. The exact hydrazine concentration was determined using the standard potassium iodate titration to the amaranth endpoint according to procedure of Penneman and Audrieth (37). The various concentrations were prepared by dilution of the standard stock solution.

Determination of the Optical Density of the Solutions.--The hydrazine solutions were added to the color development flasks in varying amounts and were diluted to approximately 80 percent of the final volume. Shortly

before determination, equal quantities of acid and aldehyde of the desired concentration were mixed and 2 ml. aliquots were pipetted into the flasks. The solutions were equilibrated at 25.0 ± 0.1°C. in a Sargent constant temperature bath for twenty minutes. The optical densities of the solutions were then determined in a 1.0 cm. cell, at 455 mμ., in the Beckmann Model B spectrophotometer equipped with a reading chamber thermostatted at 25.0 ± 0.1°C. The blank used was a solution consisting of acid and aldehyde of the same concentration as in the developing flasks.

The investigations of development time and temperature effects were carried out in a similar manner maintaining the hydrazine concentration constant.

The U/V spectra of the azine with and without the presence of the other components were determined on the Cary Recording Spectrophotometer, Model 11M.

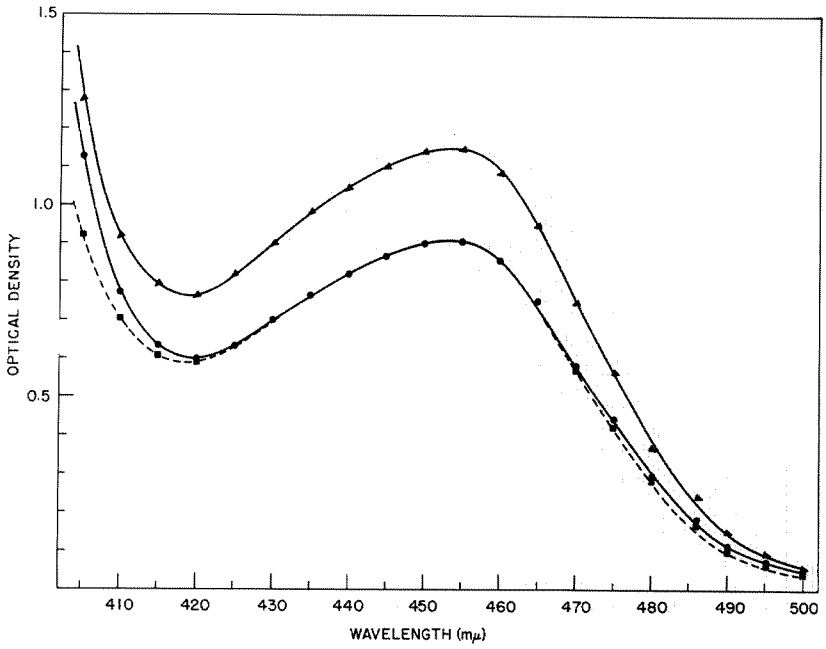


Fig. 1. U/V spectrum of color-developing solutions between 400 and 500 mμ. Acid concentrations: IA, ■ , 0.167 N; IB, ● , 0.287 N; II, ▲ , 0.167 N.

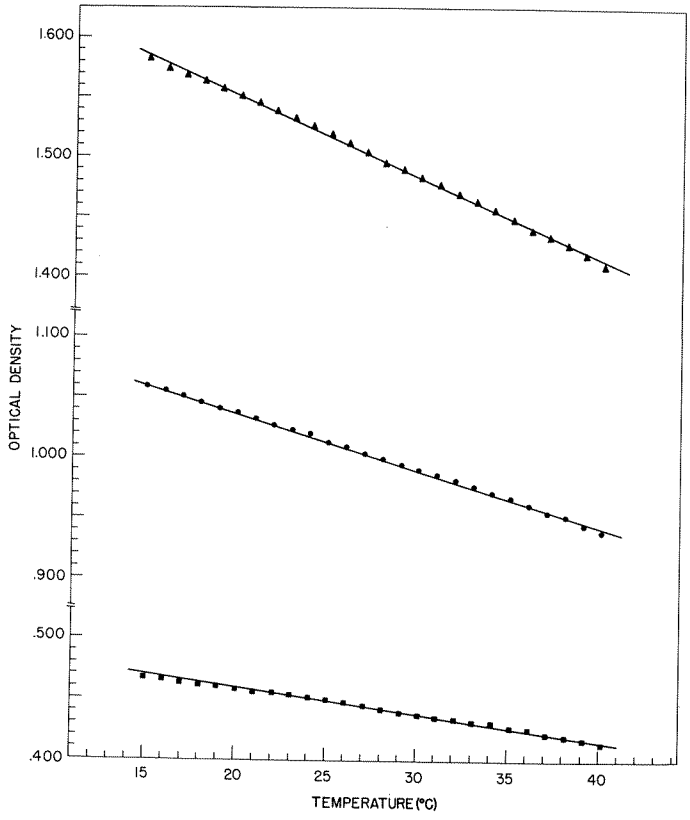


Fig. 2. The effect of temperature upon the optical density of the azine solution.

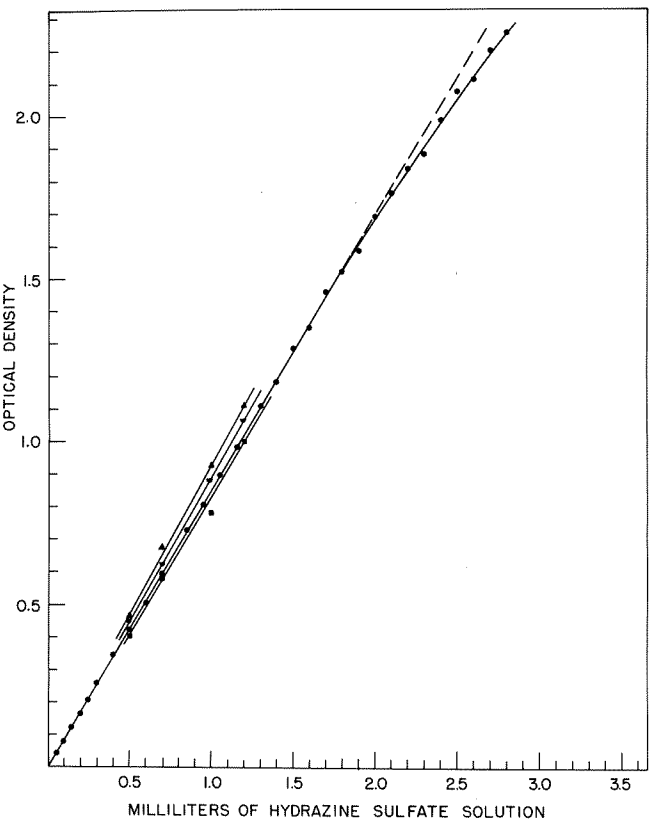


Fig. 3. Beer's Law calibration curve. Aldehyde concentration: 0.671 x 10^{-2} M; acid concentrations: ▲, 0.144 N; ▬, 0.167 N; ●, 0.192 N; ■, 0.240 N; hydrazine concentration: 2.083 x 10^{-4} M.

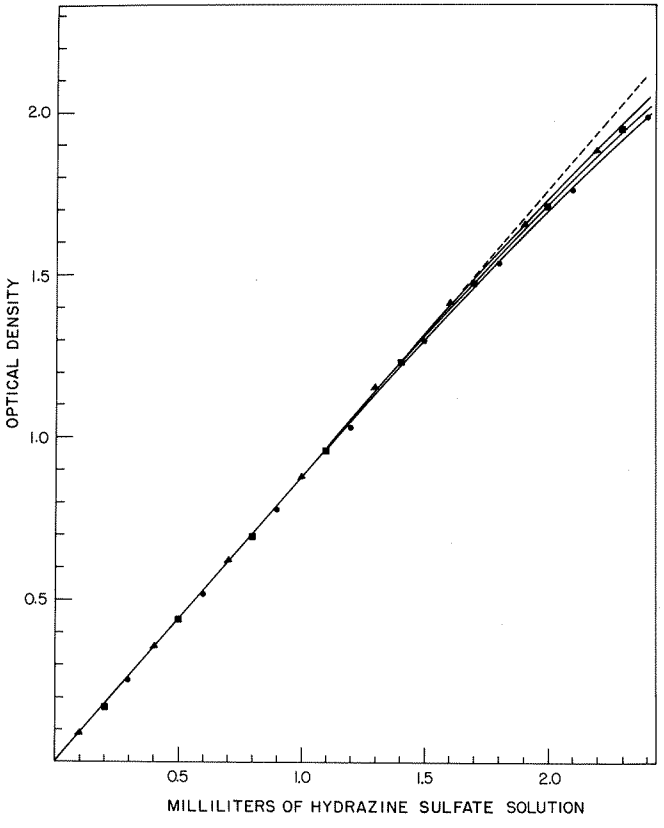


Fig. 4. Beer's Law calibration curve. Aldehyde concentration: 2.013×10^{-2} M; acid concentrations: ▲, 0.167 N; ■, 0.239 N; ●, 0.287 N; hydrazine concentration: 2.290×10^{-4} M.

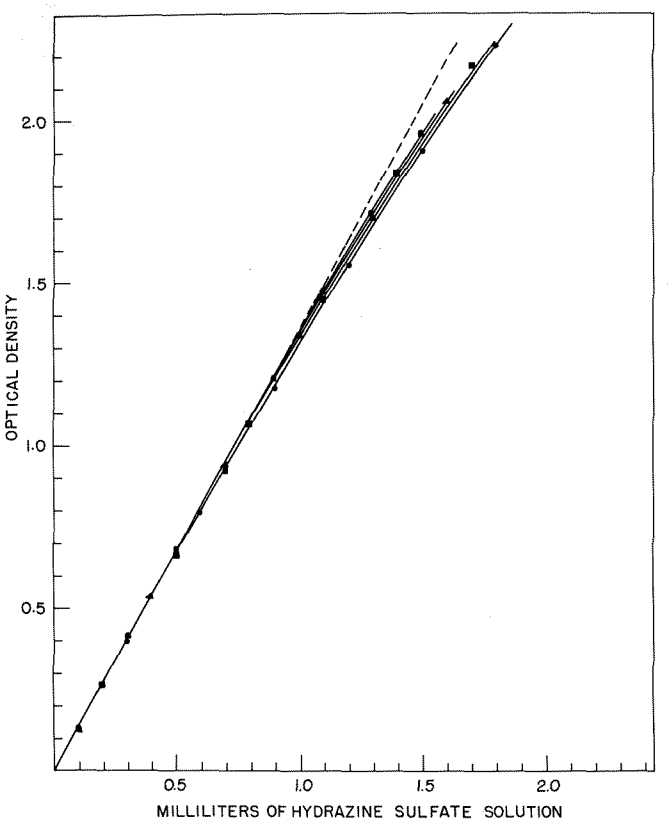


Fig. 5. Beer's Law calibration curve. Aldehyde concentration: 2.684×10^{-2} M; acid concentrations: ◗ 0.167 N, ▲ 0.192 N, ■ 0.240 N, ● 0.287 N; hydrazine concentration: 1.527×10^{-4} M.

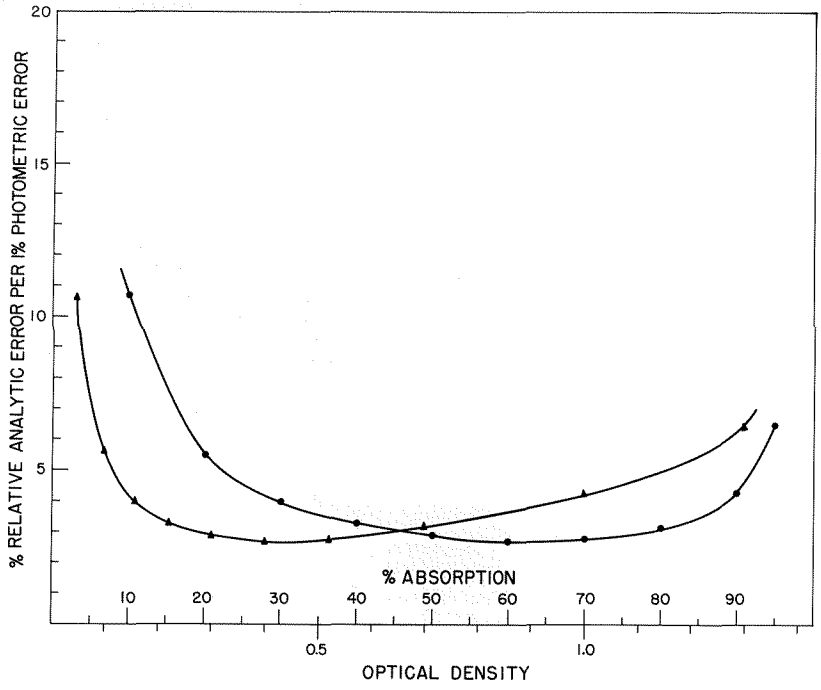


Fig. 6. Variation of analysis error with photometric error. ● Variation with % absorption. ▲ Variation with optical density.

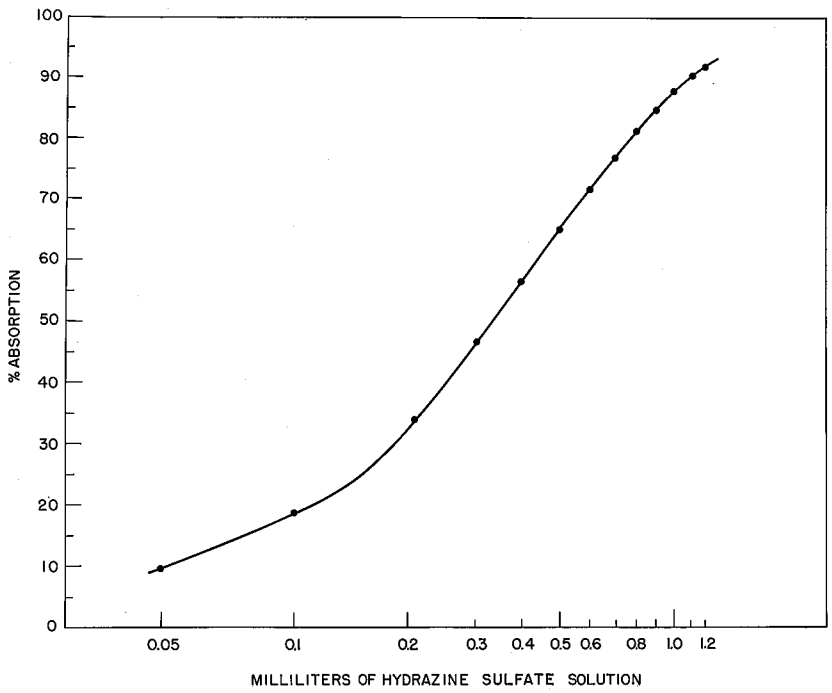


Fig. 7. Plot of percent absorption versus logarithm of concentration for azine system obeying Beer's Law. Hydrazine concentration: 1.527×10^{-4} M.

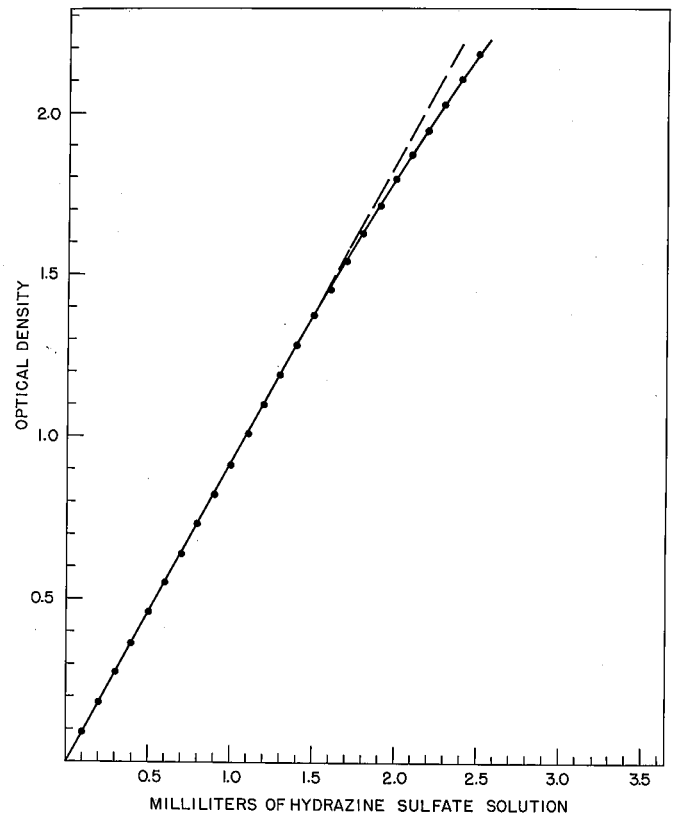


Fig. 8. Beer's Law calibration curve. Aldehyde concentration: 2.684×10^{-2} M; acid concentration: 0.167 N; hydrazine concentration: 1.527×10^{-4} M.

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PART I

Section 3

RE-EVALUATION OF THE KINETIC CONSTANTS FOR THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINHYDRAZIDE AT pH 7.9 AND 25.0°C.

RE-EVALUATION OF THE KINETIC CONSTANTS FOR THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINHYDRAZIDE AT pH 7.9 AND 25.0°C.

Introduction

The kinetic constants for the alpha-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinhydrazide were first determined by Lutwack (1). Shortly thereafter in the course of an investigation of the inhibition of the hydrolysis by a series of acylated amino acid methylamides in which acetyl-L-tyrosinhydrazide was used as the substrate, Lands (2) redetermined the kinetic constants. He found constants which were substantially different from those of the previous investigator. The development of the method of orthogonal polynomials for the evaluation of kinetic data and the improved analytical procedure gave impetus to a re-evaluation of the constants.

Discussion of Results

The first determination of the kinetic constants was made in 1954. The hydrolytic experiments were carried out at 25.0°C. and pH 7.9, in aqueous solutions and the solution was 0.02 F with respect to the amine component of the THAM-THAM·HCl buffer system. The enzyme concentration was 0.208 mg. protein-nitrogen per ml. and the sub-

strate concentration was varied over the range 12.7×10^{-4} M to 66.7×10^{-4} M. The initial velocities were determined by the method of Jennings and Niemann (3). From a typical $[S]_o/v_o$ versus $[S]_o$ plot the values of K_S and k_3 were determined. Values of the constants were; $K_S = 33.0 \times 10^{-3}$ M and $k_3 = 1.2 \times 10^{-3}$ M/min./mg. protein-nitrogen/ml. An attempt was made to evaluate the primary data by the method of orthogonal polynomials but due to the variation in zero time correction the calculations simply gave an estimate of the magnitude of the constants.

An examination of the reaction at an extended percent hydrolysis was not carried out by the first investigator. The examination which could result in an evaluation of the K_P, a measure of product inhibition, was not done for a number of reasons. The K_I of the amino acid moiety as the reaction product, acetyl-L-tyrosinate, has been determined as $115 \pm 15 \times 10^{-3}$ M and re-evaluated as $110 \pm 30 \times 10^{-3}$ M (4,5). Upon addition of the other product, hydrazine, to the reaction, there was no observation of inhibition. Furthermore, it would seem that concern about inhibition by the reaction products is obviated by the very low percent hydrolysis and consequently, the extremely low concentration of reaction products present.

In the evaluation of the inhibition constants of acetyl-, nicotinyl-, benzoyl-, and formyl-L-tyrosinmethylamide, Lands re-evaluated the constants for acetyl-

L-tyrosinhydrazide. The studies were carried out at pH 7.9 and 25.0°C., the solvent was water, the solution was 0.02 F with respect to the amine component of the THAM-THAM·HCl buffer system, the enzyme concentration was maintained constant at 0.145 mg. protein-nitrogen per ml. and the substrate concentration was varied from 0.45×10^{-4} M to 14.6×10^{-4} M. The hydrolyses were carried to between 30% and 75% hydrolysis and, consequently, the Foster-Niemann method of plotting the data was used (6). This method allows the simultaneous evaluation of K_S, k_3, and K_P. While the value of k_3 was in the range of 1×10^{-3} M/min./mg. protein-nitrogen/ml., the indication that K_S was of the order of $50\text{-}60 \times 10^{-3}$ M proved somewhat surprising in light of the earlier work. The K_P values were extremely variable, which is not surprising when one considers the low substrate concentrations employed in the majority of determination, i.e. values ranging from 0.5×10^{-3} M to 3×10^{-3} M were found. This indication that $K_P < K_S$ was very surprising in the light of the earlier determination that the two products would not seem to inhibit the reaction to any appreciable extent.

It was the purpose of this investigation to study the reaction at a low percent hydrolysis, minimizing the possibility of product inhibition, and to utilize the improved analytical procedure discussed in the previous section and the improved method of calculation of initial

velocities by the method of Booman and Niemann (7). The substrate was synthesized again and the system used was identical with that of the previous investigators except for the analytical procedure and the fact that the substrate concentration was varied over a much broader range, i.e., 4.5×10^{-4} M to 120.1×10^{-4} M. In no case was the percent hydrolysis over 9.2%. The initial velocities were determined by the orthogonal polynomial method. In no case was over a third order term in the polynomial needed and in every case the zero-time blank was less than the probable error in the optical density. The values of K_S and k_3 were obtained by a least squares fit of the data to the equation:

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

The kinetic data are listed in Table II. The initial velocities are summarized in Table I. A least squares fit of the data gave values of slope and intercept corresponding to $K_S = 29.5 \pm 6.0 \times 10^{-3}$ M and $k_3 = 1.1 \pm .2 \times 10^{-3}$ M/min./mg. protein-nitrogen/ml. These values agree within experimental error, with the values determined by Lutwack. A Lineweaver-Burke plot of the data is shown in figure 1.

The value obtained by Lands of $K_S = 60 \times 10^{-3}$ M would seem to be in error. An analysis of his rough data shows

that while a few of the runs in the investigation were carried out at high enough $[S]_o$ values to make the simultaneous determination of K_S and k_3 plausible, for the most part the concentration of substrate used was below the lower limit. This condition which is important to the simultaneous evaluation of K_S and k_3 by methods based on equations 2 and 3 relates to the relative magnitudes of K_S and $[S]_o$.

$$k_3[E]t = K_S \ln[S]_o/[S]_t + ([S]_o - [S]_t) \qquad (2)$$

$$-\frac{d[S]}{dt} = k_3[E][S]_o/(K_S + [S]_o) \qquad (3)$$

It is seen that if $[S]_o$ is very small relative to K_S, equation 3 may be approximated, within the limits of experimental error by equation 4,

$$-d[S]/dt = k_3[E][S]/K_S \ . \qquad (4)$$

Since solutions of equation 4 can only lead to values of K_S/k_3 or its reciprocal, it is obvious that care must be taken to choose values of $[S]_o$ so that they are not too small with respect to expected values of K_S. For an experimental error of $\pm$ 5.0% it is seen that $[S]_o/K_S$ should not be less than 0105 and a safe lower limit should be of the order of 0.1. If one assumes, in the case of acetyl-

L-tyrosinhydrazide, a $K_S = 30 \times 10^{-3}$ M then the lower limit of $[S]_o$ should be approximately 15×10^{-4} M. In the work of Lands this was the upper not the lower limit. Of course, values of $[S]_o/K_S$ of lower than 0.05 can be used if it is in combination with a number of values greater than this limit and the points so obtained are linear with the ones above the limit.

The very low values of K_P determined in the investigation above is very hard to account for in any reasonable way. It may be worthwhile, at this time, to look a little closer at the conditions wherein one could observe the inhibiting action of the products with regards to various K_P and $[S]_o$ values.

It has been shown in section one of this thesis (see pages 7-9) that a reaction that can be described by equations 5, 6, and 7 and under conditions when $-d[ES]/dt \doteq o$, $[S_f] \doteq [S]$, $[P_{1f}] \doteq [P_1]$, and $[P_{2f}] \doteq [P_2]$,

$$E_f + S_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_f + P_{1f} + P_{2f} \qquad (5)$$

$$E_f + P_{1f} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EP_1 \qquad (6)$$

$$E_f + P_{2f} \underset{k_7}{\overset{k_6}{\rightleftharpoons}} EP_2 \qquad (7)$$

can be described throughout the reaction by equation 8

$$-\frac{d[S]}{dt} = \left\{[E][S]\ (k_3K_P/(K_P-K_S))\right\} / \left\{(K_S(K_P+[S]))/(K_P-K_S) + [S]\right\} \qquad (8)$$

where $K_S = k_2+k_3/k_1$, $K_P = 1/\sum_{j=1}^{n} 1/K_{P_j}$, $K_{P_1} = k_5/k_4$, and $K_{P_2} = k_7/k_6$. It has been shown that upon integrating equation 8, one obtains equation 9 and from equation 9 the initial velocities will be described by the parameters $([S]_o-[S]_t)$ and $t'_{o(P)}$ where $t'_{o(P)}/t = f_{o(P)}$ is given by equation 10.

$$k_3[E]t = K_S(1+[S]_o/K_P)\ln([S]_o/[S]_t) + (1-K_S/K_P)([S]_o-[S]_t) \qquad (9)$$

$$f_{o(P)} = \left\{((K_S/[S]_o)+1)/(1-(K_S/K_P))\right\} / \left\{[S]_o\ln([S]_o/[S]_t))/[S]_o-[S]_t\right\} \left\{((K_S/[S]_o) + (K_S/K_P))/(1-(K_S/K_P)) + 1\right\} \qquad (10)$$

Lands and Niemann (8) have developed a procedure to evaluate $f_{o(P)}$ based upon the construction of a nomogram

where one can find the factor knowing the percent reaction, specific substrate concentration, and the constants K_S and K_P. Assuming a value of K_S of 30×10^{-3} M, values of $f_{o(P)}$ for various K_P values and various $[S]_o$ values have been determined. They are listed in Tables III, IV, and V.

When $K_P \gg K_S$ the inhibition by the hydrolysis product may be neglected. In this case the reaction may be represented by equation 11 and the initial velocities will be determined by the same parameters as those listed above except $f_o = t'_o/t$ is given now by equation 12,

$$k_3[E]_t = K_S \ln ([S]_o/[S]_t) + ([S]_o-[S]_t) \qquad (11)$$

$$f_o = ((K_S/[S]_o)+1)/\left\{(([S]_o \ln [S]_o/[S]_t))/\right.$$

$$\left.([S]_o-[S]_t)\ (K_S/[S]_o) + 1\right\} \qquad (12)$$

Jennings and Niemann (3) have developed a procedure to evaluate f_o based upon the construction of a family of curves based upon the parameters f_o, $([S]_o-[S]_t/[S]_o)$, and $K_S/[S]_o$. Assuming a value of K_S of 30×10^{-3} M, values of f_o for various $[S]_o$ values at the different percent reactions have been determined and are listed in Tables III, IV, and V.

Since $f_{o(P)} = t_{o(P)}/t$ and $f_o = \frac{t_o}{t}$ where $f_{o(P)}$ is the factor when product inhibition is not neglected and f_o is the factor when it can be neglected, it can be seen that

$$\frac{t_{o(P)}}{t_o} = \frac{f_o}{f_{o(P)}} \qquad (13)$$

where $t_{o(P)}$ is the time required to reach a certain percent hydrolysis with product inhibition and t_o the time without. By comparison of the ratio of these times, some estimation of the point where the difference will be greater than experimental error and thus, at what percent hydrolysis one could expect to observe this difference. The data in Tables III, IV, and V have been compared and the results are represented in figures 2, 3, and 4.

Figure 2 represents how the ratio is effected by various K_P values at $K_S = 30 \times 10^{-4}$ M and $[S]_o = 100 \times 10^{-4}$ M, which is a reasonable upper limit in the case of the hydrazide solubility. It is seen that unless the K_P of a reaction product is of the order of 1×10^{-3} M, the reaction must be allowed to proceed to above 70 to 80% hydrolysis before a great deal of difference is noted.

Figure 3 represents the change in the ratio caused by various $[S]_o$ values with $K_P = 100 \times 10^{-3}$ M and $K_S = 30 \times 10^{-3}$ M. Figure 4 is the same type except $K_P =$

10×10^{-3} M.

It might be well for future investigators of product inhibition to construct similar curves based upon expected parameters to yield an estimate of the $[S]_o$ value and the percent reaction required to evaluate the constant with regard to experimental error.

The figures point out the fact that if K_P is of the order of 100×10^{-3} M, as is the value determined for the acetyl-L-tyrosinate and the reaction is kept at a low percent hydrolysis, the effect upon the time, hence the reaction rate and the kinetic constants, can be neglected.

If, as reported by Lands, the K_P was as low as 1.0×10^{-3} M, then it certainly should have been noticed at high substrate concentrations. It is the opinion of the author, however, that at the low substrate concentrations used, i.e. of the order of 0.45×10^{-4} M to 14.6×10^{-4} M, and with the anomalies that might have arisen from analytical technique, the K_P determined may have been an artifact. Investigations of the K_I of certain inhibitors against the hydrolysis of acetyl-L-tyrosinhydrazide are currently being undertaken (9) and preliminary reports would seem to indicate agreement with the values determined using other specific substrates. This would seem to indicate that earlier reports of the K_P of the amino acid moiety were correct and that the low value

of K_p reported in the alpha-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinhydrazide was an anomaly.

Experimental and Figures

Synthesis of Substrate.--Fifteen and two-tenths ml. of redistilled thionyl chloride (26.2 gm. 0.22 mole) was added dropwise over a thirty-minute interval to 100 ml. of ethyl alcohol (1.6 mole) with continuous stirring and cooling. After allowing this to stir an additional fifteen minutes, 36.24 gms. of L-tyrosine (0.20 mole) was added portionwise. The first portion dissolved after which a slurry formed. The solution was allowed to equilibrate to room temperature and then warmed slowly to forty degrees. After stirring at this temperature for four hours the clear orange solution was stripped of excess ethanol in vacuo and the solid allowed to dry at 100°C. for one hour. Crude weight was 46.6 gms. of L-tyrosine ethyl ester hydrochloride (0.176 mole - 88.0% yield), m.p. 162-168°C.

The crude ester-hydrochloride was slurried in 100 ml. $CHCl_3$ and added to 500 ml. of NH_3-saturated $CHCl_3$. Removal of the NH_4Cl by filtration, removal of excess solvent under reduced pressure and drying the residue in vacuo over P_2O_5 gave 29.6 gms. of L-tyrosine ethyl ester (0.129 mole - 72.5% yield). This corresponds to a 59.4% overall yield. The method above is essentially

that of Brenner.

Acetylation of the crude ester above under Schotten-Bauman conditions gave 29.0 gms. of acetyl-L-tyrosine ethyl ester (0.107 mole - 83% yield).

The crude acylated ester above was dissolved in ethanol and added dropwise to an ethanol solution of 3.8 gms. of hydrazine (0.118 mole). The solution was heated under reflux for two hours and the white product crystallized out. Recrystallization twice from methanol and drying *in vacuo* over P_2O_5 gave 23.4 gms. of acetyl-L-tyrosinhydrazide (.089 mole - 83%), m.p. 227-228°C. (corr.). $[\alpha]_D^{25.3} = +40.80 \pm 0.6^\circ$ (C, 2.02% in methyl cellosolve).

Anal. Calcd. for $C_{11}H_{15}O_3N_3$: C, 55.68; H, 6.37; N, 17.71
Found: C, 55.60; H, 6.46; N, 17.76

Enzyme Solutions.--The enzyme used was Armour and Co., Lot No. 00592. Solutions were made up exactly the same as in section one with the exception that only fifty mgs. of enzyme were dissolved in five ml. of water. One ml. of this solution in the ten ml. reaction flasks led to a final concentration of 1 mg./ml. and based upon a nitrogen content of 14.5% and a molecular weight of 22,000 for monomeric alpha-chymotrypsin a final concentration of 0.145 mg. protein-nitrogen/ml. and 4.55×10^{-5} M.

Buffer Solution.--The solutions were made up analogously to those in section one with the solution 0.02 F with respect to the amine component of the THAM-THAM·HCl buffer system.

Reaction and Analysis.--The hydrolytic reaction was carried out in exactly the same manner as in section one. Changes in the analytical procedure were the following:

(a) The aldehyde was made up so as to give a final concentration of 2.684×10^{-2} M.

(b) The acid was made up so as to give a final concentration of 0.167 N.

(c) The acid and aldehyde mixture was prepared just before analysis and two ml. of the mixture was pipetted into a flask, the solution made up to nine ml. with distilled water to limit coagulation of the protein and consequent turbidity of the color-developing solution.

(d) The color-developing solutions were equilibrated at $25.0 \pm 0.1^{\circ}$C. for exactly twenty minutes.

(e) The optical density was read at 455 mμ in the Beckman Model B Spectrophotometer which was equipped with a reading chamber thermostatted at $25.0 \pm 0.1^{\circ}$C. The blank, however, now contained all the components of the reaction and analysis system in the analogous concentrations. It is noteworthy that by applying the equation calculated by the method of orthogonal polynomials to the

calculation of a zero time-optical density to every run, a random error of only ± 0.002 of an optical density unit is found. This is below the probable error in optical density.

The optical density was never allowed to get above 1.1 O.D. unit in the color developing flasks. When it approached this limit, larger flasks were substituted and the optical density corrected and recorded as its equivalent in the 10 ml. flasks.

Table I

Summary of Initial Velocities in the alpha-Chymotrypsin Catalyzed Hydrolysis of Acetyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C.

$[E]^{(a)}$ = 0.145 mg. protein-nitrogen per ml.

$[S]_o$ (b)	v_o (c)	σ_{v_o} (d)	P_m (e)	$1/v_o$ (f)	$1/[S]_o$ (g)
4.50	2.80	± 0.09	2	357.40	222.22
7.51	4.74	± 0.13	2	211.15	133.16
15.01	8.56	± 0.14	2	116.78	66.62
22.52	11.91	± 0.13	2	83.96	44.41
30.03	13.79	± 0.10	2	72.54	33.30
37.54	15.94	± 0.42	2	62.75	26.64
45.04	20.09	± 0.23	2	49.78	22.20
52.55	22.55	± 0.21	2	44.35	19.03
60.06	25.60	± 0.08	3	39.06	16.65
67.56	29.74	± 0.11	3	33.63	14.80
75.07	33.54	± 0.54	3	29.82	13.32
82.58	35.63	± 1.04	3	28.07	12.11
90.08	37.32	± 0.28	2	26.80	11.10
105.10	44.85	± 0.53	3	22.30	9.51
112.61	46.78	± 1.15	3	21.38	8.88
120.11	50.79	± 0.75	3	19.69	8.33

$K_S = 29.52 \pm 6.02 \times 10^{-3}$ M

$k_3 = 1.12 \pm 0.22 \times 10^{-3}$ M/min./mg. protein-nitrogen/ml.

(a) Armour Lot No. 00592.

(b) In units of 10^{-4} M.

(c) In units of 10^{-6} M/min.

(d) Probable error in v_o.

(e) Order of polynomial used in evaluating v_o.

(f) In units of 10^3 min./M.

(g) In units of 10^6 M^{-1}.

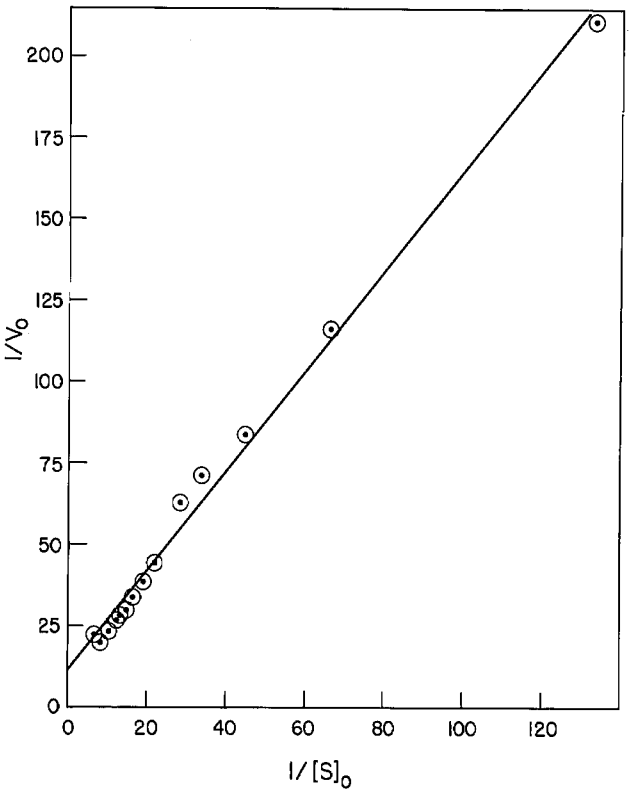

Fig. 1. $1/v_o$ vs. $1/[S]_o$ plot for the alpha-chymotrypsin catalyzed hydrolysis of nicotinyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C.

$1/v_o$ is in units of 10^3 min./M.

$1/[S]_o$ is in units of 10 M^{-1}.

Table II

alpha-Chymotrypsin Catalyzed Hydrolysis of Acetyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C.

[E] = 0.145 mg. protein-nitrogen per ml.

$[S]_o = 4.50 \times 10^{-4}$ M
Dilution Factor 1:10

Time (min.)	O.D.
2	.040
4	.073
6	.094
8	.120
10	.146
12	.163
14	.190
16	.207

$v_o = 2.80 \times 10^{-6}$ M/min.

$\sigma_{v_o} = .09 \times 10^{-6}$ M/min.

$P_m = 2$ % Hyd. = 7.8

$[S]_o = 7.51 \times 10^{-4}$ M
Dilution Factor 1:10

Time (min.)	O.D.
2	.065
4	.120
6	.175
8	.219
10	.263
12	.315
14	.362
16	.414

$v_o = 4.74 \times 10^{-6}$ M/min.

$\sigma_{v_o} = .13 \times 10^{-6}$ M/min.

$P_m = 2$ % Hyd. = 9.2

$[S]_o = 15.01 \times 10^{-4}$ M
Dilution Factor 1:10

Time (min.)	O.D.
2	.110
4	.204
6	.288
8	.377
10	.455
12	.532
14	.618
16	.672

$v_o = 8.56 \times 10^{-6}$ M/min.

$\sigma_{v_o} = 0.14 \times 10^{-6}$ M/min.

$P_m = 2$ % Hyd. = 7.5

$[S]_o = 22.52 \times 10^{-4}$ M
Dilution Factor 1:10

Time (min.)	O.D.
2	.145
4	.284
6	.404
8	.543
10	.655
12	.776
14	.883
16	1.001

$v_o = 11.91 \times 10^{-6}$ M/min.

$\sigma_{v_o} = 0.13 \times 10^{-6}$ M/min.

$P_m = 2$ % Hyd. = 7.4

Table II.--Continued

$[S]_o = 30.03 \times 10^{-4}$ M
Dilution Factor 1:10

Time(min.)	O.D.
2	.170
4	.322
6	.470
8	.609
10	.750
12	.873
14	1.007
16	1.117

$v_o = 13.79 \times 10^{-6}$ M/min.

$\sigma_{v_o} = 0.10 \times 10^{-6}$ M/min.

$P_m = 2$ % Hyd. = 6.2

$[S]_o = 37.54 \times 10^{-4}$ M
Dilution Factor 1:25

Time(min.)	O.D.
2	.205
4	.400
6	.583
8	.720
10	.908
12	1.093
14	1.230
16	1.420

$v_o = 15.94 \times 10^{-6}$ M/min.

$\sigma_{v_o} = 0.42 \times 10^{-6}$ M/min.

$P_m = 2$ % Hyd. = 6.3

$[S]_o = 45.04 \times 10^{-4}$ M
Dilution Factor 1:25

Time(min.)	O.D.
2	.238
4	.468
6	.690
8	.905
10	1.113
12	1.285
14	1.480
16	1.680

$v_o = 20.09 \times 10^{-6}$ M/min.

$\sigma_{v_o} = 0.23 \times 10^{-6}$ M/min.

$P_m = 2$ % Hyd. = 6.2

$[S]_o = 52.55 \times 10^{-4}$ M
Dilution Factor 1:25

Time(min.)	O.D.
2	.268
4	.528
6	.788
8	1.033
10	1.245
12	1.493
14	1.720
16	1.935

$v_o = 22.55 \times 10^{-6}$ M/min.

$\sigma_{v_o} = 0.21 \times 10^{-6}$ M/min.

$P_m = 2$ % Hyd. = 6.2

Table II.--Continued

$[S]_o = 60.06 \times 10^{-4}$ M
Dilution Factor 1:25

Time(min.)	O.D.
2	.315
4	.595
6	.858
8	1.120
10	1.383
12	1.650
14	1.915
16	2.160

$v_o = 25.60 \times 10^{-6}$ M/min.

$\sigma_{v_o} = 0.08 \times 10^{-6}$ M/min.

$P_m = 3$ % Hyd. = 6.0

$[S]_o = 67.56 \times 10^{-4}$ M
Dilution Factor 1:25

Time(min.)	O.D.
2	.355
4	.683
6	1.008
8	1.318
10	1.600
12	1.918
14	2.200
16	2.493

$v_o = 29.74 \times 10^{-6}$ M/min.

$\sigma_{v_o} = 0.11 \times 10^{-6}$ M/min.

$P_m = 3$ % Hyd. = 6.1

$[S]_o = 75.07 \times 10^{-4}$ M
Dilution Factor 1:25

Time(min.)	O.D.
2	.383
4	.748
6	1.108
8	1.443
10	1.745
12	2.090
14	2.418
16	2.745

$v_o = 33.54 \times 10^{-6}$ M/min.

$\sigma_{v_o} = 0.54 \times 10^{-6}$ M/min.

$P_m = 3$ % Hyd. = 6.1

$[S]_o = 82.58 \times 10^{-4}$ M
Dilution Factor 1:50

Time(min.)	O.D.
2	.410
4	.805
6	1.185
8	1.530
10	1.865
12	2.215
14	2.610
16	2.920

$v_o = 35.63 \times 10^{-6}$ M/min.

$\sigma_{v_o} = 1.04 \times 10^{-6}$ M/min.

$P_m = 3$ % Hyd. = 5.9

Table II.--Continued

$[S]_o = 90.08 \times 10^{-4}$ M
Dilution Factor 1:50

Time(min.)	O.D.
2	.440
4	.865
6	1.250
8	1.665
10	2.050
12	2.430
14	2.820
16	3.155

$v_o = 37.32 \times 10^{-6}$ M/min.

$\sigma_{v_o} = 0.28 \times 10^{-6}$ M/min.

$P_m = 2$ % Hyd. = 5.9

$[S]_o = 105.10 \times 10^{-4}$ M
Dilution Factor 1:50

Time(min.)	O.D.
2	.535
4	1.025
6	1.480
8	1.910
10	2.365
12	2.790
14	3.220
16	3.635

$v_o = 44.85 \times 10^{-6}$ M/min.

$\sigma_{v_o} = 0.53 \times 10^{-6}$ M/min.

$P_m = 3$ % Hyd. = 5.8

$[S]_o = 112.61 \times 10^{-4}$ M
Dilution Factor 1:50

Time(min.)	O.D.
2	.555
4	1.060
6	1.510
8	2.015
10	2.475
12	2.935
14	3.360
16	3.875

$v_o = 46.78 \times 10^{-6}$ M/min.

$\sigma_{v_o} = 1.15 \times 10^{-6}$ M/min.

$P_m = 3$ % Hyd. = 5.8

$[S]_o = 120.11 \times 10^{-4}$ M
Dilution Factor 1:50

Time(min.)	O.D.
2	.605
4	1.145
6	1.630
8	2.150
10	2.640
12	3.110
14	3.605
16	4.105

$v_o = 50.79 \times 10^{-6}$ M/min.

$\sigma_{v_o} = 0.75 \times 10^{-6}$ M/min.

$P_m = 3$ % Hyd. = 5.7

Table III

Comparison of Correction Factors at $[S]_o = 100 \times 10^{-4}$ M
$K_S = 30 \times 10^{-3}$ M and Variation in K_P

$K_P = 100 \times 10^{-3}$ M

% Hyd.	$f_{o(P)}$	f_o	$t'_{o(P)}/t'_o$
5	0.979	0.979	1.000
10	0.959	0.963	1.004
15	0.937	0.944	1.008
20	0.913	0.924	1.012
25	0.890	0.902	1.014
30	0.866	0.880	1.016
40	0.813	0.834	1.026
50	0.758	0.784	1.034
60	0.698	0.724	1.038
70	0.625	0.656	1.050
80	0.545	0.578	1.060

$a/b = -5.71$ $K_S/[S]_o = 3$

$K_P = 50 \times 10^{-3}$ M

% Hyd.	$f_{o(P)}$	f_o	$t'_{o(P)}/t'_o$
5	0.978	0.979	1.001
10	0.954	0.963	1.009
15	0.930	0.944	1.015
20	0.906	0.924	1.020
25	0.881	0.902	1.024
30	0.855	0.880	1.029
40	0.780	0.834	1.049
50	0.742	0.784	1.067
60	0.690	0.724	1.099
70	0.616	0.656	1.165
80	0.533	0.578	1.185

$a/b = -10.00$ $K_S/[S]_o = 3$

$K_P = 10 \times 10^{-3}$ M

% Hyd.	$f_{o(P)}$	f_o	$t'_{o(P)}/t'_o$
5	0.964	0.979	1.016
10	0.926	0.963	1.040
15	0.889	0.944	1.062
20	0.853	0.924	1.083
25	0.816	0.902	1.105
30	0.780	0.880	1.128
40	0.704	0.834	1.190
50	0.622	0.784	1.260
60	0.548	0.724	1.339
70	0.466	0.656	1.407
80	0.391	0.578	1.480

$a/b = 2.00$ $K_S/[S]_o = 3$

$K_P = 1 \times 10^{-3}$ M

% Hyd.	$f_{o(P)}$	f_o	$t'_{o(P)}/t'_o$
5	0.819	0.979	1.195
10	0.720	0.963	1.338
15	0.590	0.944	1.600
20	0.520	0.924	1.777
25	0.455	0.902	1.982
30	0.400	0.880	2.200
40	0.305	0.834	2.731
50	0.250	0.784	3.136
60	0.202	0.724	3.584
70	0.150	0.656	4.373
80	0.100	0.578	5.780

$a/b = 0.138$ $K_S/[S]_o = 3$

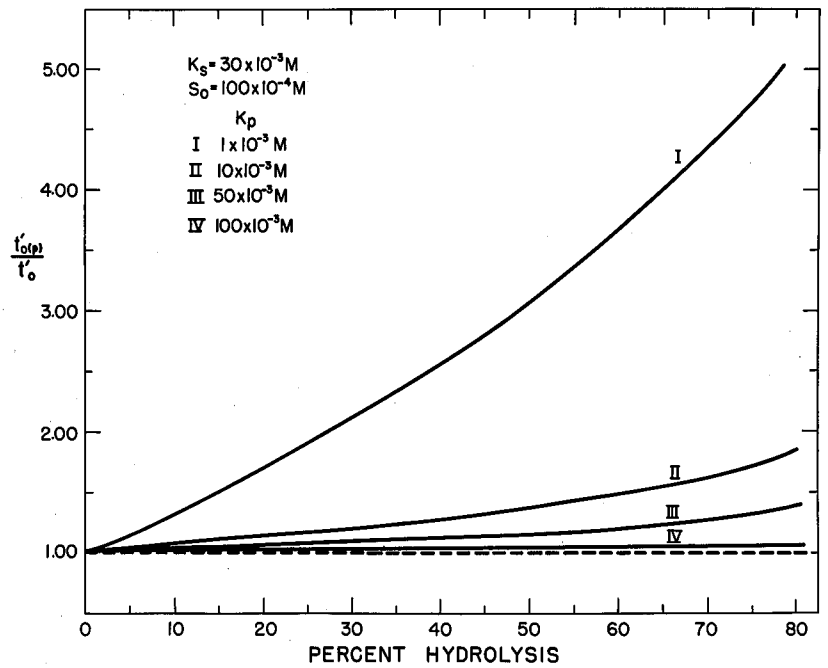

Fig. 2. $t'_{o(P)}/t'_o$ - percent hydrolysis relationship with $K_S = 30 \times 10^{-3}$ M, $[S]_o = 100 \times 10^{-4}$ M, and the various K_P values indicated.

Table IV

Comparison of Correction Factors at $K_P = 100 \times 10^{-3}$ M, $K_S = 30 \times 10^{-3}$ M and Variation in $[S]_o$ Values

$[S]_o = 5 \times 10^{-4}$ M

% Hyd.	$f_{o(P)}$	f_o	$t'_{o(P)}/t'_o$
5	0.975	0.975	1.000
10	0.950	0.950	1.000
15	0.924	0.924	1.000
20	0.897	0.900	1.002
25	0.870	0.873	1.003
30	0.842	0.846	1.005
40	0.782	0.787	1.006
50	0.722	0.727	1.007
60	0.657	0.672	1.007
70	0.582	0.586	1.008
80	0.498	0.502	1.009

$a/b = -87.14$ $K_S/[S]_o = 60$

$[S]_o = 25 \times 10^{-4}$ M

% Hyd.	$f_{o(P)}$	f_o	$t'_{o(P)}/t'_o$
5	0.976	0.976	1.000
10	0.952	0.952	1.000
15	0.927	0.928	1.001
20	0.902	0.904	1.002
25	0.876	0.878	1.003
30	0.849	0.852	1.004
40	0.792	0.797	1.006
50	0.733	0.739	1.008
60	0.666	0.674	1.012
70	0.594	0.598	1.014
80	0.510	0.518	1.016

$a/b = -18.59$ $K_S/[S]_o = 12$

$[S]_o = 50 \times 10^{-4}$ M

% Hyd.	$f_{o(P)}$	f_o	$t'_{o(P)}/t'_o$
5	0.978	0.978	1.000
10	0.954	0.954	1.000
15	0.930	0.932	1.002
20	0.906	0.909	1.003
25	0.881	0.884	1.004
30	0.855	0.859	1.006
40	0.800	0.808	1.010
50	0.742	0.752	1.014
60	0.679	0.690	1.016
70	0.608	0.616	1.018
80	0.522	0.533	1.021

$a/b = -10.00$ $K_S/[S]_o = 6$

$[S]_o = 100 \times 10^{-4}$ M

% Hyd.	$f_{o(P)}$	f_o	$t'_{o(P)}/t'_o$
5	0.979	0.979	1.000
10	0.959	0.963	1.004
15	0.937	0.944	1.008
20	0.913	0.924	1.012
25	0.890	0.902	1.014
30	0.866	0.880	1.016
40	0.813	0.834	1.026
50	0.758	0.784	1.034
60	0.698	0.724	1.038
70	0.625	0.656	1.050
80	0.545	0.518	1.060

$a/b = -5.71$ $K_S/[S]_o = 3$

Table IV.--Continued

$[S]_o = 500 \times 10^{-4}$ M				$[S]_o = 1000 \times 10^{-4}$ M			
% Hyd.	$f_{o(P)}$	f_o	$t'_{o(P)}/t'_o$	% Hyd.	$f_{o(P)}$	f_o	$t'_{o(P)}/t'_o$
5	0.986	0.989	1.003	5	0.989	0.994	1.005
10	0.972	0.980	1.008	10	0.976	0.987	1.011
15	0.956	0.969	1.014	15	0.964	0.980	1.017
20	0.939	0.958	1.020	20	0.949	0.974	1.026
25	0.921	0.946	1.027	25	0.936	0.966	1.032
30	0.904	0.934	1.033	30	0.920	0.958	1.041
40	0.864	0.906	1.049	40	0.885	0.944	1.067
50	0.820	0.873	1.065	50	0.848	0.919	1.084
60	0.757	0.836	1.104	60	0.805	0.885	1.099
70	0.705	0.792	1.123	70	0.745	0.866	1.163
80	0.630	0.730	1.159	80	0.680	0.816	1.216
a/b = -2.28		$K_S/[S]_o = 0.6$		a/b = -1.85		$K_S/[S]_o = 0.3$	

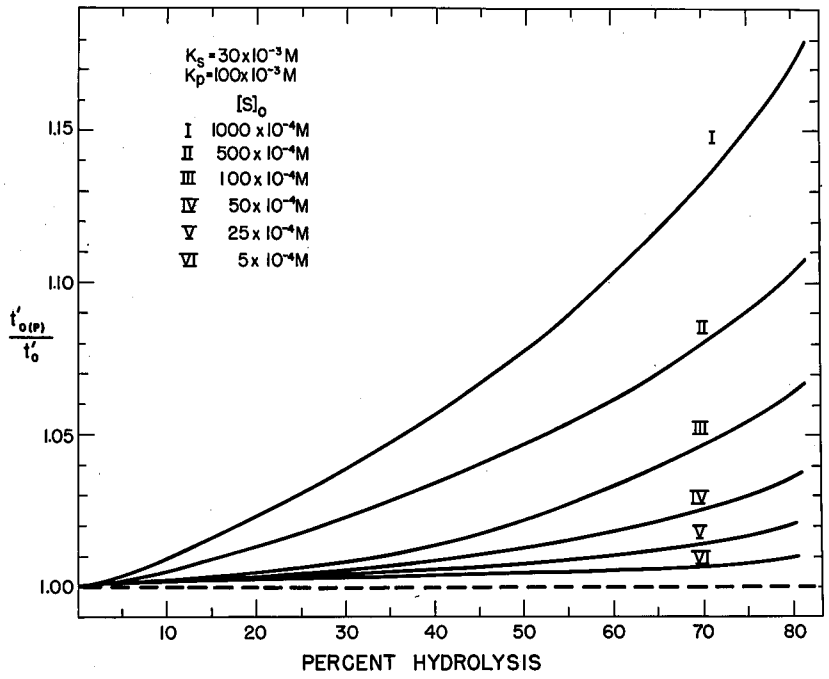

Fig. 3. $t'_{o(P)}/t'_o$ - percent hydrolysis relationship with $K_S = 30 \times 10^{-3}$ M, $K_P = 100 \times 10^{-3}$ M, and the various values of $[S]_o$ indicated.

Table V

Comparison of Correction Factors at $K_P = 10 \times 10^{-3}$ M, $K_S = 30 \times 10^{-3}$ M and Variation in $[S]_o$ Values

$[S]_o = 5 \times 10^{-4}$ M

% Hyd.	$f_{o(P)}$	f_o	$t'_{o(P)}/t'_o$
5	0.974	0.975	1.001
10	0.948	0.950	1.002
15	0.920	0.924	1.004
20	0.892	0.900	1.009
25	0.864	0.873	1.011
30	0.837	0.846	1.011
40	0.778	0.787	1.012
50	0.714	0.727	1.018
60	0.658	0.672	1.021
70	0.573	0.586	1.023
80	0.490	0.502	1.024

$a/b = 30.5$ $K_S/[S]_o = 60$

$[S]_o = 25 \times 10^{-4}$ M

% Hyd.	$f_{o(P)}$	f_o	$t'_{o(P)}/t'_o$
5	0.970	0.976	1.006
10	0.942	0.952	1.010
15	0.912	0.928	1.018
20	0.882	0.904	1.025
25	0.853	0.878	1.029
30	0.821	0.852	1.037
40	0.757	0.797	1.053
50	0.690	0.739	1.071
60	0.620	0.674	1.087
70	0.546	0.598	1.095
80	0.456	0.518	1.136

$a/b = 6.5$ $K_S/[S]_o = 12$

$[S]_o = 50 \times 10^{-4}$ M

% Hyd.	$f_{o(P)}$	f_o	$t'_{o(P)}/t'_o$
5	0.969	0.978	1.009
10	0.937	0.954	1.018
15	0.902	0.932	1.033
20	0.870	0.909	1.045
25	0.839	0.884	1.054
30	0.805	0.859	1.067
40	0.737	0.808	1.096
50	0.668	0.752	1.126
60	0.597	0.690	1.156
70	0.512	0.616	1.203
80	0.417	0.533	1.278

$a/b = 3.5$ $K_S/[S]_o = 6$

$[S]_o = 100 \times 10^{-4}$ M

% Hyd.	$f_{o(P)}$	f_o	$t'_{o(P)}/t'_o$
5	0.964	0.979	1.016
10	0.926	0.963	1.040
15	0.889	0.944	1.062
20	0.853	0.924	1.083
25	0.816	0.902	1.105
30	0.780	0.880	1.128
40	0.704	0.834	1.190
50	0.622	0.784	1.260
60	0.548	0.724	1.339
70	0.466	0.656	1.407
80	0.391	0.578	1.480

$a/b = 2.0$ $K_S/[S]_o = 3$

Table V.--Continued

$[S]_o$ = 500 x 10^{-4} M				$[S]_o$ = 1000 x 10^{-4} M			
% Hyd.	$f_{o(P)}$	f_o	$t'_{o(P)}/t'_o$	% Hyd.	$f_{o(P)}$	f_o	$t'_{o(P)}/t'_o$
5	0.944	0.989	1.048	5	0.940	0.994	1.057
10	0.889	0.980	1.102	10	0.880	0.987	1.122
15	0.840	0.969	1.154	15	0.830	0.980	1.181
20	0.791	0.958	1.211	20	0.770	0.974	1.265
25	0.742	0.946	1.275	25	0.724	0.966	1.334
30	0.694	0.934	1.346	30	0.680	0.958	1.409
40	0.610	0.906	1.485	40	0.582	0.944	1.622
50	0.512	0.873	1.705	50	0.502	0.919	1.831
60	0.460	0.836	1.817	60	0.422	0.885	2.097
70	0.390	0.792	2.031	70	0.363	0.866	2.386
80	0.312	0.730	2.340	80	0.290	0.816	2.814
a/b = 0.8		$K_S/[S]_o$ = 0.6		a/b = 0.65		$K_S/[S]_o$ = 0.3	

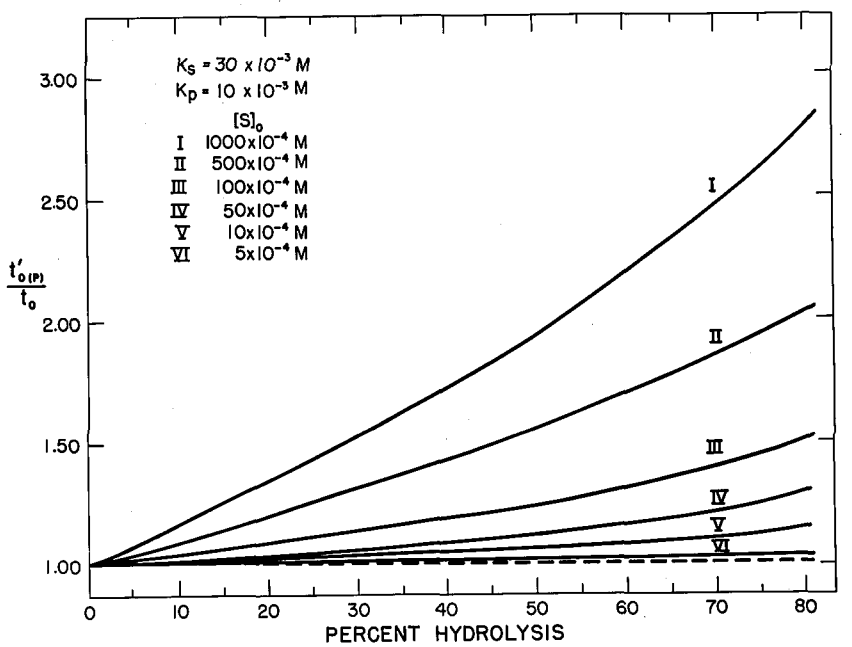

Fig. 4. $t'_{o(P)}/t'_o$ - percent hydrolysis relationship with $K_S = 30 \times 10^{-3}$ M, $K_P = 10 \times 10^{-3}$ M, and the various values of $[S]_o$ indicated.

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PART II

SYNTHESIS OF AMINO ACID DERIVATIVES

SYNTHESIS OF AMINO ACID DERIVATIVES

Introduction

The enzyme, alpha-chymotrypsin, specifically hydrolyzes a number of acylated and non-acylated alpha-amino acid derivatives. The extent of variation in the type of acid derivative thus far recognized and investigated was reviewed in part I of this thesis (see page 2). A number of esters, amides, hydrazides, and hydroxamides have been studied. Since the list is obviously not limited to the ones thus far recognized, the search for new substrates continues.

The properties of a compound, to be added to the list, should incorporate a number of characteristics. The first criterion is the possession of a bond which is susceptible to hydrolytic fission by the enzyme. Although work by Bock and Niemann (1) has indicated that the optically active D-isomer of an alpha-amino acid, in one case at least, is hydrolyzed at a slow rate, it is generally recognized that the L-enantiomorph is the substrate which is hydrolyzed. Secondly, the compound should possess sufficient solubility in water to allow for the simultaneous evaluation of both kinetic constants (2). This situation depends upon the relative magnitudes of K_S and $[S]_o$ and thus cannot be determined precisely in advance, however, with a K_S of

normal magnitude, in the neighborhood of 10^{-3} M, the solubility should be in the range of 10^{-3} - 10^{-4} M. A third requirement is the availability of a sufficiently quantitative method for the determination of extent of hydrolysis. Consideration of one or more of the above requirements has led to investigation of various types of acid derivatives.

In particular, the studies reported in this part of the thesis deal with the syntheses and attempted syntheses of alpha-amino acid derivatives where the amino acid moiety is esterified with either salicylamide or glycolamide or aminated with anthranilamide.

Salicylamide Esters of Amino Acids

Absorption spectroscopy in the ultraviolet region provides a convenient method for the determination of extent of hydrolysis if there exists a shift in the wave length when the substrate is converted to products. Several investigators have used the shift in the position of the maxima between O-substituted salicylic acid and salicylic acid. Edwards (3) used a method based upon the shift to follow the non-enzymatic hydrolysis of aspirin to acetic acid and salicylic acid. Brandenberger (4,5) and Hofstee (6,7,8) have described a method to follow the enzymatic hydrolysis of o-carboxyphenyl-phosphate by alkaline and acid phosphatases based upon the fact that salicylic acid strongly absorbs light at a wavelength between 298-300 mμ

while the esters have no absorption at that point. Detection of as little as 0.01 μmole of free salicylic acid has been reported and in a good case, accuracy nearing one part in one hundred can be attained.

In studies utilizing alpha-chymotrypsin as the catalyst, substrates which would exist as charged species at the pH optimum are not desired. The amide of salicylic acid, salicylamide, has one adsorption maximum at 202 mμ and another at 236 mμ at pH 6 (9,10). It seemed probable that esters of salicylamide would provide useful substrates for studies of enzymatic hydrolysis by alpha-chymotrypsin since upon cleavage of the ester linkage one of the products would be salicylamide.

The idea above was shown to be true in some preliminary investigations by Foster (11). He prepared the salicylamide ester of hydracinnamic acid by condensing the acid chloride with salicylamide in the presence of pyridine and showed that it had a spectrum different from that of salicylamide. While alpha-chymotrypsin and the ester exhibited a maxima at 210 mμ and 280 mμ, respectively, at pH 7.9, a peak began to appear at 300 mμ. upon hydrolysis of the substrate by alpha-chymotrypsin. The peak at 300 mμ. was the peak exhibited by free salicylamide at pH 7.9.

Foster, in an attempt to extend this method to amino acid derivatives tried, without success, to esterify acetyl-DL-phenylalanine with salicylamide. Among the methods tried

were the usual condensations of the phenol with acid chloride and acid azide. Transesterification between acetyl-DL-phenylalanine methyl ester and salicylamide was tried without success.

This author has tried numerous methods and the results of these studies are discussed in the sub-headings below.

Use of Trifluoracetic Acid Anhydride as a Condensing Agent.--The preparation of benzoyl-DL-phenylalanine α-napthyl ester using trifluoracetic acid anhydride as a condensing agent (12) suggested the use of this reagent in the preparation of the desired esters.

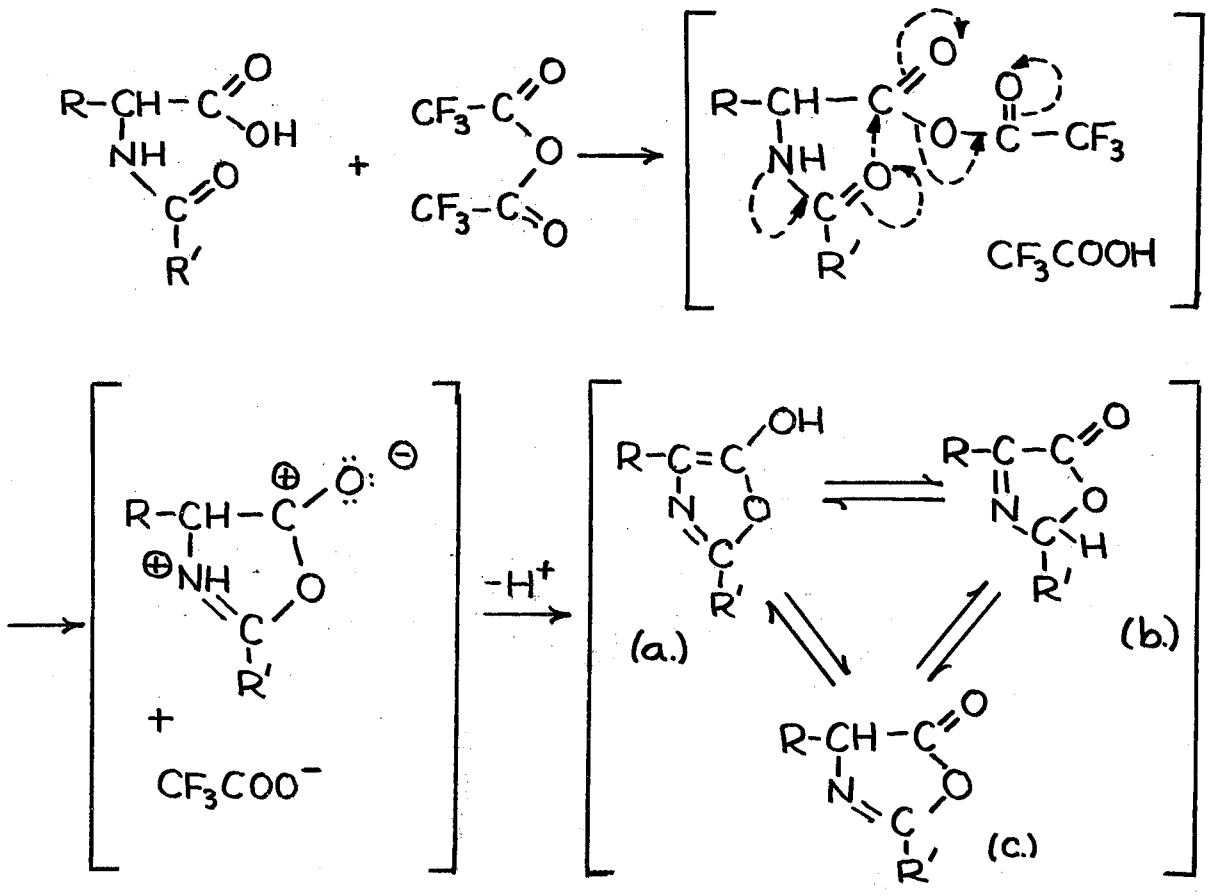
Benzoyl-DL-phenylalanine salicylamide ester (I) was prepared by the condensation of benzoyl-DL-phenylalanine with salicylamide in the presence of trifluoracetic acid anhydride. This procedure was also used successfully for the formation of acetyl-DL-phenylalanine salicylamide ester (II).

After the preparation of acetyl-L-phenylalanine the extension of the above method to the preparation of the optically active L-ester was attempted. The resultant compound was optically inactive and a mixed melting point with a sample of DL-compound prepared previously indicated that racemization had taken place.

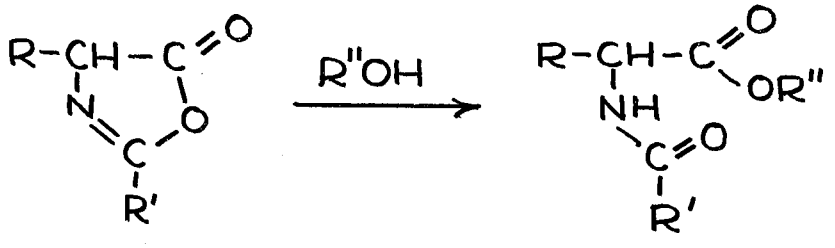
With the thought that operation at a lower temperature would reduce racemization two experiments were conducted at

sub-zero levels. The first trial at -40°C. to -50°C. did not lead to formation of the mixed anhydride and consequently the product was not formed. A second trial at -20°C. to -30°C. gave a small amount of racemic product.

The racemization observed above is not too surprising in light of earlier work (13,14) upon the effective racemization of alpha-acylamino acids in the presence of acetic anhydride. The racemization has been looked upon as due to formation of an intermediate mixed anhydride followed by formation of an oxazolone (azlactone) with consequent loss of optical activity (15). Bourne (16) has tentatively suggested that the active acylating agent in the esterification of carboxylic acids with alcohols and phenols in the presence of trifluoracetic anhydride is the mixed anhydride of trifluoracetic acid and the carboxylic acid. It is the feeling of the author that while the mixed anhydride is certainly an intermediate in the reaction, the observation of racemization in the cases studied as well as other examples (17-19) indicates the formation of an oxazolone as an intermediate and the reaction of the alcohol or phenol with this compound (see scheme below):



Loss of optical activity



Racemic product

In support of this mechanism was the recent isolation of 2-trifluormethyl-4-isopropyl-oxazolone-(5) from the reaction of trifluoracetic acid anhydride with alanine (20). It is interesting in this regard that N-nitroso-N-phenyl-glycine gives a 93% yield of N-phenylsydnone in presence of trifluoracetic acid anhydride at -5°C. (15).

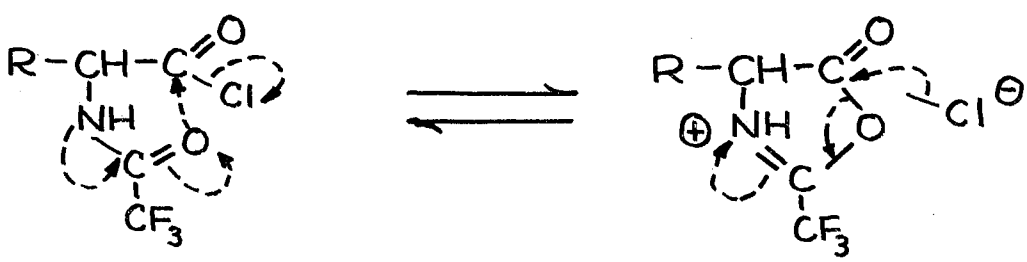
Use of Trifluoracetyl Amino Acid Chlorides as the Acylating Agent.--Following reports of Weygand (17-20) and Calvin (21) of the successful preparation, isolation, and utilization of trifluoracetyl amino acid chlorides as acylating agents for free amino groups without any apparent racemization, attention was directed towards these derivatives. Using a procedure modified from that of Weygand (18), trifluoracetyl-DL-phenylalanine was prepared. The acid chloride was prepared in the usual manner using phosphorous pentachloride. An attempt to condense this acid chloride with salicylamide in the presence of triethylamine failed so the condensation was carried out using the sodium salt of salicylamide. This led to the isolation of trifloracetyl-DL-phenylalanine salicylamide ester (V) in good yield. This procedure was repeated with the optically active acid. This led to the isolation of an ester which gave the analysis for the desired compound except it had a very small rotation. The rotation diminished upon recrystallization and the hydrolysis of

the compound gave back the optically inactive acid. This coupled with results of a mixed melting point with an authentic sample of the DL-ester indicates that extensive or complete racemization took place during the reaction. The procedure was repeated, this time isolating each intermediate and checking its optical integrity. It was found, surprisingly enough, that optical integrity was maintained until the condensation step. The isolation of the trifluoracetyl-L-phenylalanyl chloride (VII) has been reported by other workers (17-20, 21). Repetition of the final condensation step with triethylamine as the base also gave the inactive product.

The explanation of racemization may be approached in two ways in the case above. The first involves the formation of the desired ester and the subsequent abstraction of the active hydrogen on the alpha-carbon atom by base to yield a transient compound with a negative charge on this carbon. This compound can then add hydrogen to give the optically inactive mixture of D and L forms. This explanation seems highly unlikely since the isolation of trifluoracetyl-L-phenylalanylanilide has been accomplished (21) and also the anilide of trifluoracetyl-L-alanine has been isolated (18). Furthermore in a later section of this thesis the synthesis and isolation of trifluoracetyl-L-phenylalanine anthranilamide is described. In the case of this anilide-type derivative, triethylamine was used as

a base, without racemization.

The second, and most probable mechanism for the racemization involves the formation of an oxazolinium chloride. The question of whether alpha-acylamino acid halides exist as such or as oxazolinium chlorides or neither, i.e. an equilibrium between the two, is one of importance to investigators of the reactions of amino acids. It is felt that in the case at hand, an equilibrium exists between the two forms such as is illustrated in the scheme below:



It is felt that even though the optically active acid chloride can be isolated, in the condensation step, the chloride exists in a transition step in the form of the oxazolinium ion with consequent racemization. This may also exist in the case of reaction with amines (i.e.) aniline and anthranilamide; however, it can be seen that where reaction with an amino group is facile and may occur before any appreciable shift in equilibrium, the conditions

required to form an ester of salicylamide are more drastic and thus racemization occurs.

Utilization of Polyphosphoric Acid as a Dehydrating Agent.--Bader and Kontowicz (22) reported the successful preparation of phenyl esters by simply heating the acid with phenol in the presence of polyphosphoric acid. The extension of this method to the preparation of the desired ester was attempted.

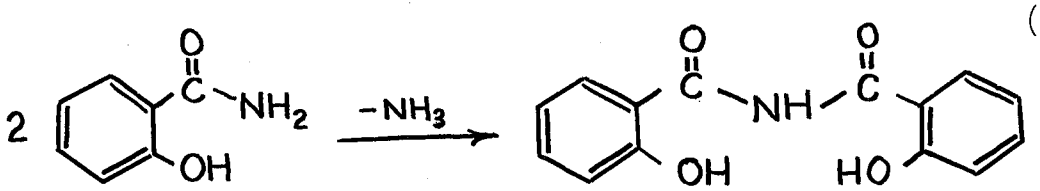
The first trial was the attempted preparation of hippuric acid salicylamide ester. Three compounds were isolated from the reaction mixture, none of which gave an analysis for or exhibited properties of the ester. The first product sublimed in the neck of the reaction flask and proved to be benzoic acid. The second compound proved identical with disalicylamide (IX) prepared later by treatment of salicylamide with polyphosphoric acid under the conditions of the reaction.

A third compound (X), of unknown structure, is described in the experimental section. A possible structure is proposed in proposition 7.

Since hydrocinnamic acid salicylamide ester had been prepared previously by Foster (11), the preparation of this as a model compound was attempted. Salicylamide and hydrocinnamic acid were treated under analogous conditions as above. Extraction of the reaction mixture with Na_2CO_3 and

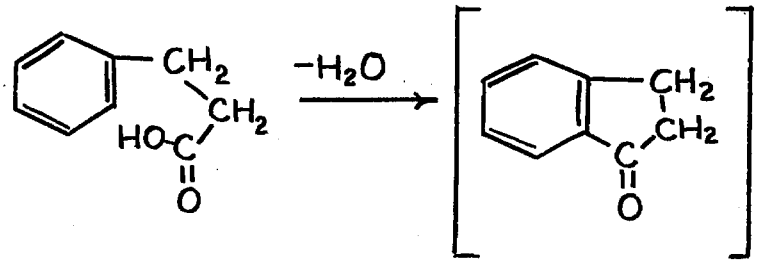
acidification of the extract gave a compound identical with disalicylamide (IX). The solid remaining was extracted with ethyl acetate. Removal of the solvent gave a compound, m.p. 142-144°C., which gave an elementary analysis corresponding to $C_{18}H_{14}O$ and was found to be a dimer of alpha-hydrindone, called by Kipping (23), "anhydrobishydrindone" (XI).

The brown solid remaining was recrystallized and gave pale yellow, silky needles, m.p. 376.7-378.5, determined with the aid of a copper-constantan thermocouple. The elementary analysis gave a formula of $C_{27}H_{18}$. The hydrocarbon was identified as alpha-truxene (tribenzylene benzene) (XII). The reactions taking place in the reaction mixture are illustrated by the equations below:



(1)

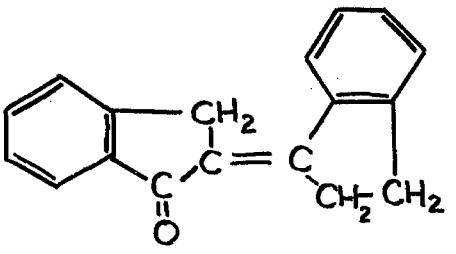
This was shown to be true by treatment of salicylamide alone with polyphosphoric acid. This treatment gave an 84.6 percent yield of $C_{14}H_{11}O_4\ N$, disalicylamide, m.p. 188-190°C. This compound was prepared previously (24) by the treatment of salicylamide with phosphorus pentoxide.



alpha-hydrindone
not isolated

(2a)

2 alpha-hydrindone $\xrightarrow{-H_2O}$



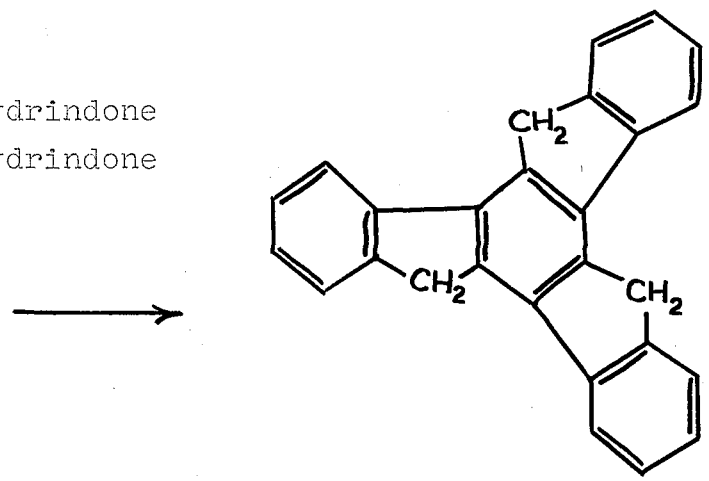
"anhydrobishydrindone" (XI)
isolated

(2b)

3 alpha-hydrindone

or

anhydrobishydrindone
and alpha-hydrindone



alpha-truxene
(tribenzylene benzene)(XII)

(2c)

Treatment of anhydrobishydrindone with polyphosphoric acid again yielded alpha-truxene. A search of the literature revealed that Snyder and Werber (25) had prepared alpha-hydrindone by treatment of hydrocinnamic acid with polyphosphoric acid. They also obtained a light yellow compound melting above 300°C. which they concluded was truxene. Liebermann and Kipping (26,23) prepared alpha-truxene (m.p. 365-368°C.) from alpha-hydrindone by treat-

ment with concentrated sulfuric acid. Kipping also isolated "anhydrobishydrindone" from the same reaction, m.p. 141-143°C.

Upon the discovery that acetyl-DL-phenylalanine had cyclicized and decarboxylated to yield 1-methyl isoquinoline (27), the method was abandoned as a method of preparing the desired esters.

Solid alpha-truxene exhibited fluorescence in "black light." Fluorescence measurements under various conditions were taken.* Under a strong U/V source, truxene appears quite fluorescent in the solid state with a color similar to uranium glass. In dimethyl formamide solution, however, the fluorescence is very weak. At a concentration of 0.075 mg./ml. the fluorescence is nil; however, at a concentration of 0.584 mg./ml. (almost saturated) it shows a fluorescence of about 4 percent of that of uranium glass. In the solid state, the fluorescence is about 49 percent of that of uranium glass. Qualitatively, at its maximum of 510-563 mμ. (0.1 mg./ml.) truxene is a great deal weaker in fluorescence than anthracene and about the same as fluorene.

The author acknowledges the spectra, taken by Osborne.*

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*Dr. Carter, Organic Chemicals Department, E. I. du Pont de Nemours and Company, arranged for the measurements. They were taken by Dr. Allan Osborne of that company with a Cary Model 14 U/V spectrophotometer appropriately modified for fluorescence and flame spectrophotometry. The intensity of the standard, uranium glass, could be set at any desired level. The comments above are essentially those of Dr. Osborne.

Other Synthetic Attempts in Preparation of Salicylamide Esters.--A number of short studies were conducted in an attempt to prepare the optically active salicylamide esters. These are discussed briefly below and the experimental details are given in the experimental section.

The reports of the usefulness of certain active esters (28-30) in peptide bond formation led to the preparation of two new phenylalanine derivatives, acetyl-DL-phenylalanine cyanomethylester (XV) and acetyl-L-phenylalanine cyanomethylester (XVII). The main feature of these esters is a strong negative substituent in the alcohol component enhancing the rate of aminolysis at the carbonyl carbon atom. The activated esters are prepared by the condensation of the amino acid and chloracetonitrile in the presence of triethylamine. While the worth of these compounds has been proven as acylating agents of amines and peptides, the fact that the compounds above could be recrystallized from ethanol gave an indication that the attempt to prepare salicylamide esters would fail. While they were not successful in acylating salicylamide they were used in the preparation of useful glycolamide derivatives discussed in a later section.

The fact that formation of the oxazolinium chloride and consequent racemization has been reported as not occurring when the acyl moiety of an acylamino acid chloride is the methanesulfonyl radical led to the preparation of

methanesulfonyl-DL-phenylalanine, conversion to the acid chloride and finally condensation with sodium salicylamide to yield XIII, methanesulfonyl-DL-phenylalanine salicylamide ester. Since the yield overall was of the order of 2 percent this method was abandoned in search of more productive methods.

An attempt to extend the reaction of diazonium borofluorate with acetic acid yielding phenylacetate to the preparation of the desired esters failed. While evidence of reaction was observed with propionic acid no product could be isolated. Reaction of the diazonium salt with the solvent in the case of the solid amino acids was a big factor in the failure. Acetonitrile and dimethyl formamide dissolved the diazonium borofluorate, but subsequent reaction led to formation of deep-red solutions and isolation procedures yielded dark tars.

The final attempt at synthesis of the salicylamide esters was a result of reports that esters and amides could be formed by bringing the acid and the alcohol or amine together in the presence of carbodiimides (31-37).

Sheehan (34) reported the successful formation of peptide linkages by simply mixing the acid and amine together in tetrahydrofuran in the presence of dicylohexylcarbodiimide. He later reports (35) the formation of peptide linkages involving hydroxy-amino acids without esterification of the primary or secondary alcohol groups.

A private communication from the author of these two papers expressed considerable doubt as to the possibility of formation of salicylamide esters with the reagent. Esterification of carboxylic acids has been reported, however, under somewhat more vigorous conditions (36). Both procedures were tried in an unsuccessful attempt to prepare the ester.

In conclusion, it might be stated that while the desired goal of isolation of an optically active salicylamide ester was not attained much interesting chemistry has been canvassed.

The difficulty of forming the phenol ester and the carboxyamide group in the ortho position have forced too drastic or too basic conditions which have led to racemization of the derivatives used.

The preparation of acetyl-, benzoyl-, trifluoracetyl-, and methanesulfonyl-DL-phenylalanine salicylamide esters has shown that they can be prepared, however, and a method where racemization is limited or absent is certainly not excluded.

Preparation of Trifluoracetyl-L-phenylalanine Anthranilamide

While the methods used in the preceding section failed to successfully yield an optically active salicylamide ester, it was felt that due to ease of formation of amide linkages, compared to phenol ester bonds, an optically active

anthranilamide amide could be obtained. It is noteworthy that the spectra of anthranilamide and N-substituted anthranilamides differ by an amount sufficient to suggest the usefulness of a compound of this type as a substrate for a spectral hydrolysis study (37).

While anthranilic acid and methyl anthranilate are commercially available, anthranilamide is not and thus the synthesis of anthranilamide was undertaken. The usual method of ammonolysis was found, surprisingly, to yield only 27 percent after five months standing. A search of the literature revealed the fact that Heilbron (38) obtained only a 5-10 percent yield on shaking methyl anthranilate with concentrated ammonium hydroxide for several days and Morris (39) obtained only a 36 percent yield by heating liquid ammonia and methyl anthranilate in a high pressure bomb at 1200 p.s.i. and 200°C. for ten hours.

It was decided to prepare o-nitrobenzamide from o-nitrobenzoic acid and then reduce the nitro group by means of Raney nickel and hydrazine (40).

Anthranilamide was synthesized over the path indicated with an overall yield of approximately 61 percent.

Anthranilamide was condensed with trifluoracetyl-L-phenylalanyl chloride in the presence of triethylamine to

give trifluoracetyl-L-phenylalanine anthranilamide (XIV). The compound was purified, analyzed and stored for possible use as a substrate for enzymatic hydrolysis studies.

Glycolamide Esters of Amino Acids

The hydrolysis of esters by alpha-chymotrypsin has long been known (page 2 of this thesis). The extent of reaction has usually been followed by titration of the liberated acid with standard base. This manual titration, at best, is accompanied by a great deal of error. The acquisition of an instrument which maintains the pH of a reaction solution at a pre-determined and pre-adjusted value by the addition of micro-amounts of standard base* and its ease of operation has led to the study of ester hydrolysis in these laboratories (41).

The solubility of glycolamide esters led to the examination of these esters as possible structures. A preliminary investigation of the solubility properties of benzoyl glycolamide indicated a solubility of the order of 10^{-1} M in water.

An extension of the preparation of cyanomethyl esters to the case at hand failed. While acetyl-DL-phenylalanine

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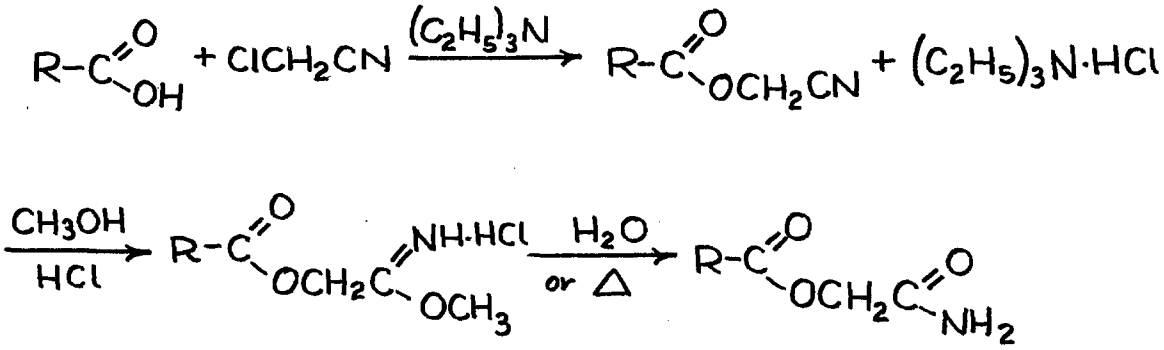
*The instrument is a pH stat designed and constructed by Dr. Mott Cannon of International Instruments, Canyon, California. The instrument is similar to the one described by Neilands and Cannon (46). The apparatus permits fully automatic determination of both ionization constants and volume of titrating fluid added as a function of time at constant pH.

glycolamide ester (XVI) could be prepared by heating the sodium salt of the acid with chloracetamide, a more convenient synthesis utilizing the cyanomethylester was used. The complete scheme is shown below:



R-, Acetyl-DL-phenylalanine XVI
Acetyl-L-phenylalanine XVIII

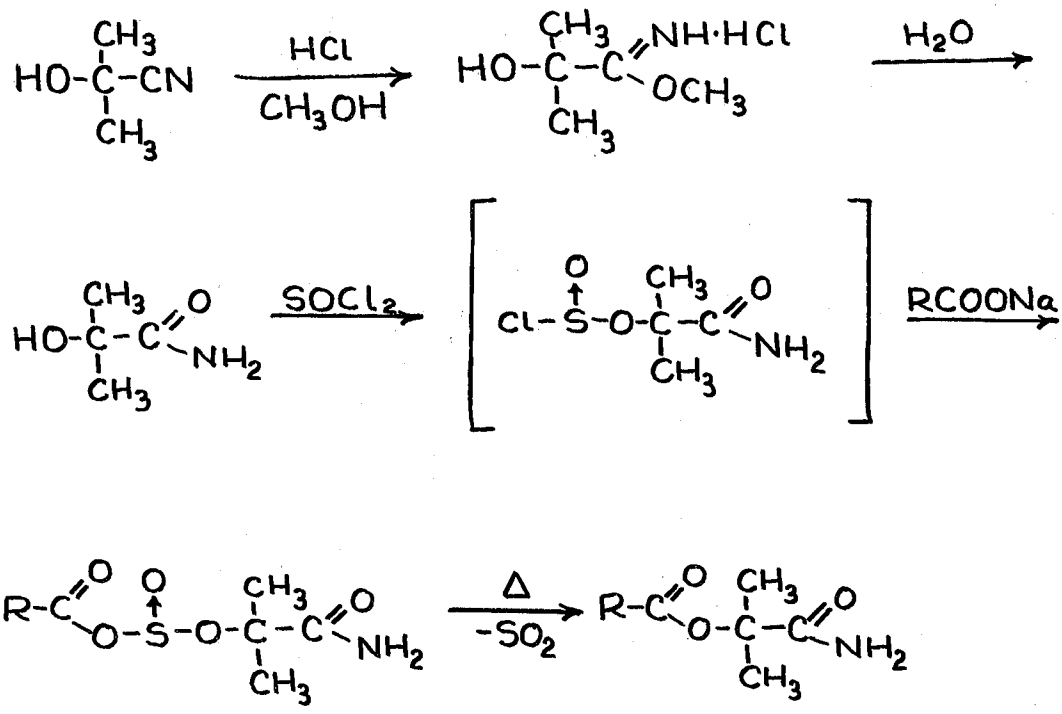
The scheme above gave good yields of both the DL-ester and the L-ester.

An attempt to prepare the glycolamide ester of hippuric acid by the same scheme gave hippuric acid and glycolamide. Since the hippurylcyanomethylester was isolated one must conclude that the glycolamide ester was formed but was hydrolyzed in isolation procedures.

The compound, acetyl-L-phenylalanine glycolamide ester was found to be very soluble in water. Preliminary investigations of the alpha-chymotrypsin catalyzed hydrolysis

of the compound gave the following results. In six minutes with [E] = 1 x 10^{-4} mg./ml., an extremely low value, and $[S]_o$ = 1 x 10^{-4} M, 8.0 percent hydrolysis was noted. In six minutes with [E] = 1 x 10^{-3} mg./ml. and $[S]_o$ = 1 x 10^{-3} M there was 37.0 percent hydrolysis. With the enzyme concentration any greater than 10^{-1} mg./ml. the titrimeter could not keep up with liberated acid.

An attempt was made to synthesize the ester between alpha-hydroxyisobutyric acid amide and acetyl-L-phenylalanine by the scheme below:



The condensation step between the chlorosulfite ester and the sodium salt of the acid, in this case acetyl-L-phenylalanine, resulted in a viscous tar.

Experimental*

Benzoyl-DL-phenylalanine Salicylamide Ester (I).-- Acylation of 66 gms. (0.40 mole) of DL-phenylalanine with 60 gms. (0.43 mole) of benzoyl chloride under Schotten-Bauman conditions gave 82.4 gms. (0.31 mole - 77.5% yield) of benzoyl-DL-phenylalanine, m.p. 179-182°C., lit. m.p. 181-182°C. (42).

A mixture of 10 gms. (.037 mole) of the above and 12 gms. (.057 mole) of trifluoracetic acid anhydride was warmed on a steam bath until a clear solution resulted. The excess trifluoracetic acid anhydride was removed under reduced pressure. To the now orange solution was added 5 gms. (.037 mole) of salicylamide and the mixture was warmed on the steam bath for one hour. The solution was cooled, 30 ml. of methanol added, and the mixture was allowed to stand overnight in the refrigerator. Since crystallization did not occur, the excess solvent was removed under reduced pressure, the residue washed with cold methanol and dried to give 10.4 gms. (.027 mole -

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*All analyses were made by Dr. Adalbert Elek, Elek Microanalytical Laboratories, Los Angeles, California. All melting points are corrected.

73.0%) of benzoyl-DL-phenylalanine salicylamide ester (I). Recrystallization from a mixture of dioxane and cyclohexane gave a product, m.p. 198-199°C.

Anal. Calcd. for $C_{23}H_{20}O_4N_2$: C, 71.12; H, 5.19; N, 7.21.
Found: C, 71.04; H, 5.26; N, 7.24.

The compound gave a positive ferric hydroxamate test (43), is soluble in dimethylformamide and dioxane, insoluble in water, methanol, and ethanol, and slightly soluble in diethyl ether.

Acetyl-DL-phenylalanine Salicylamide Ester (II).-- Acylation of 9 gms. (.054 mole) of DL-phenlylalanine with 19.2 ml. (20.8 gm. - 0.21 mole) of acetic anhydride under Schotten-Bauman conditions gave 9.4 gms. (.045 mole - 83.6%) of acetyl-DL-phenylalanine, m.p. 152-155°C.

The entire amount of crude acetyl-DL-phenylalanine (.045 mole) was mixed with 12 gms. (.060 mole) of trifluoracetic anhydride and heated on a steam bath. After removal of excess anhydride under reduced pressure, 5 gms. (.037 mole) of salicylamide was added and the solution heated for ten minutes on a steam cone. Upon cooling a white solid precipitated. The solid was washed with methanol and dried to give 9 gms. (.028 mole - 75.7%). of crude acetyl-DL-phenlylalanine salicylamide ester (II). Recrystallization twice from dimethylformamide gave a product, m.p. 208-209°C.

Anal.Calcd. for $C_{18}H_{18}O_4N_2$: C, 66.24; H, 5.56; N, 8.58.
Found: C, 66.25; H, 5.55; N, 8.67.

The compound gave a positive ferric hydroxamate test, is soluble in acetic acid, methyl cellosolve, and dimethylformamide, slightly soluble in acetonitrile, and insoluble in water, ethanol, and methanol.

Attempted Synthesis of Acetyl-L-phenylalanine Salicylamide Ester Using Trifluoracetic Acid Anhydride as a Condensing Agent.--Acylation of 7.5 gms. (.046 mole) of L-phenylalanine with 12 ml. (13.0 gms. - .128 mole) of acetic anhydride under Schotten-Bauman conditions gave, after one recrystallization from water, 9.0 gms. (.043 mole - 94%) of acetyl-L-phenylalanine, monoclinic crystals, m.p. 171-172°C.

Repeating the reaction used above for the DL-compounds, 5 gms. (.024 mole) of acetyl-L-phenylalanine gave 2.8 gms. (.009 mole - 37%) of a compound, m.p. 207-208°C. A small rotation disappeared upon repeated recrystallizations from dimethylformamide. Upon admixture with an authentic sample of the DL-compound the m.p. was not depressed. Racemization had occurred.

Attempts were made to run the above esterification at -40 to -50°C., maintaining the temperature with a dry ice and acetone system. The mixed anhydride would not form as indicated by the fact that the solution never cleared.

Attempts were made to run the above esterification at -20°C. In this case it appeared that the anhydride formed since a clear solution resulted. Upon the addition of salicylamide the solution remained thick for an extended period. A small amount of racemic product was isolated.

Trifluoracetyl-DL-phenylalanine Salicylamide Ester (V).-- Acylation of 20 gms. (.121 mole) of DL-phenylalanine with 18.8 ml. (.119 mole) of trifluoracetic acid anhydride as directed by Weygand (18), in acylating alanine, gave 30.4 gms. (.115 mole - 94.7%) of crude trifluoracetyl-DL-phenylalanine (III). Recrystallization from a mixture of benzene and hexane gave white crystals, m.p. 121-122°C., lit. m.p. 125.6 - 126.8°C. (21).

A solution of 5.2 gms. (.0191 mole) of III in 100 ml. of dry ether was cooled in an ice-salt bath. An excess of freshly-opened PCl_5 was added portionwise to the solution with vigorous shaking. After allowing to stand three hours, with intermittent shaking, the solvent was removed under reduced pressure, the residue was washed twice with petroleum ether (30-60°C.), collected, and dried to give 4.5 gms. (.0161 mole - 84.3%) of trifluoracetyl-DL-phenylalanylchloride (IV), m.p. 96-98°C., lit. m.p. 99-100°C. (18).

The sodium salt of salicylamide was prepared by dis-

solving salicylamide in an equimolar amount of base and evaporating the water in an air stream.

A solution of 4.5 gms. (.0161 mole) of the above in 50 ml. of dry benzene was added to a suspension of 2.4 gms. (.0161 mole) of the sodium salt of salicylamide in dry benzene and the resulting slurry was stirred vigorously at room temperature for 12 hours, and at 50°C. for one hour. The solvent was removed under reduced pressure, the solid washed repeatedly with water, and dried to give 3.6 gms. (.0095 mole - 59.0%) of V. Recrystallization from a mixture of ethanol and hexane gave a product, m.p. 223-224°C. (dec).

Anal. Calcd. for $C_{18}H_{15}O_4N_2F_3$: C,56.84; H, 3.98; N, 7.37.
Found: C,56.75; H, 3.94; N, 7.31

Compound V is soluble in hot ethanol and insoluble in water.

Attempted Synthesis of Trifluoracetyl-L-phenylalanine Salicylamide Ester by Way of the Acid Chloride.--The reaction conditions for preparation of trifluoracetyl-L-phenylalanine were changed from those used in synthesis of the DL-compound.

A solution of 10 gms. (.0605 mole) of L-phenylalanine in 100 ml. of dry benzene was cooled in a 3-necked flask fitted with a mechanical stirrer, a dropping funnel and a drying tube. Slightly less than a mole per mole ratio, 9.4 ml. (.0595 mole) of trifluoracetic acid anhydride was

added to the solution slowly with vigorous stirring. The solution was slowly warmed to 70°C. and then cooled to room temperature. The white solid was collected and dried to give 7.2 gms. (.0276 mole) of trifluoracetyl-L-phenylalanine (VI), m.p. 117-119°C. Removal of benzene and trifluoracetic acid under reduced pressure from the mother liquor gave 4.9 gms. of VI (.0188 mole), m.p. 116-118°C. Total yield; .0464 mole, 78%. Recrystallization twice from a mixture of benzene and hexane gave 10.4 gms. of pure VI, m.p. 119-120°C., lit. m.p. 119.4-120.6°C. (21). $[\alpha]_D^{25.0^\circ} = +35.2 \pm 0.6^\circ$ (C, 2.6% in glacial acetic acid); lit. $[\alpha]_D^{25.0^\circ} = +36.4^\circ$ (C, 0.4% in glacial acetic acid) (21).

A solution of 7.2 gms. (.0276 mole) of VI in 250 ml. of dry ether was treated in the usual manner with PCl_5. Recrystallization from a mixture of benzene and petroleum ether gave 6.5 gms. (0.232 mole - 84.2%) of trifluoracetyl-L-phenylalanylchloride (VII), m.p. 108-110°C., lit. m.p. 109.5-111.5°C.(21). $[\alpha]_D^{25.0^\circ} = +17.1 \pm 0.4^\circ$ (C, 3.3% in glacial acetic acid); lit. $[\alpha]_D^{28.3^\circ} = +15.5^\circ$ (C, 0.16% in glacial acetic acid) (21).

Treatment of a solution of 6.5 gms. (.0232 mole) of VII in 250 ml. of benzene in an analogous manner as above with 3.5 gms. (.0220 mole) of the sodium salt of salicylamide gave 7.6 gms. (.0198 moles - 90.0% of a compound m.p. 214.5-218°C. (dec.). Three recrystallizations from

ethanol gave a compound with no optical rotation, m.p. 218-219°C. The melting point was not depressed on admixture with an authentic sample of trifluoracetyl-DL-phenylalanine salicylamide ester, m.p. 223-224°C., prepared previously.

Anal. Calcd. for $C_{18}H_{15}O_4N_2F_3$: C, 56.84; H, 3.98; N, 7.37.
Found: C, 56.82; H, 3.95; N, 7.22.

An attempt to prepare the above compound by mixing the chloride and the salicylamide in the presence of triethylamine failed.

Reaction of Saliclyamide with Polyphosphoric Acid.--A mixture of 150 gms. of polyphosphoric acid and 41.1 gms. (0.3 mole) of salicylamide was heated on a steam cone for 24 hours. The deep red solution was diluted with 400 ml. of hot water, the solid remaining was collected and dried in vacuo. A white crystalline powder separated from the water on cooling, m.p. 142-144°C., which gave no depression upon admixture of a pure sample of salicylamide. Removal of most of the water gave 24 gms. of salicylamide. Thus, treatment of 17.1 gms. (0.125 mole) of salicylamide in the above manner gave 8.9 gms. (0.347 mole - 54%) of disalicylamide (IX). Three recrystallizations gave a product m.p. 188-190°C., lit. m.p. 197-199°C. (24).

Anal. Calcd. for $C_{14}H_{11}O_4N$: C, 65.36; H, 4.31; N, 5.45.
Found: C, 65.40; H, 4.44; N, 5.46

The compound is soluble in sodium carbonate, slightly soluble in ethyl acetate, dioxane, and acetic acid, and gives a bright red color with $FeCl_3$. It was previously prepared by heating salicylamide with P_2O_5 (24).

Reaction of Hippuric Acid with Polyphosphoric Acid.--Treatment of 36 gms. (0.2 mole) of hippuric acid in an analogous manner to that described above gave 26 gms. of the starting material. There also sublimed out of the reaction mixture 4 gms. of an acid, m.p. 121-123°. An anilide prepared from the acid had a melting point of 158-159.5°C., lit. m.p. of benzanilide 160°C. This means that, based upon the unrecovered starting material, 65% was converted to benzoic acid. Since this yield could have improved it seems logical to suspect that the unrecovered hippuric acid was hydrolyzed to benzoic acid and glycine. The exact nature of the hydrolysis is unclear.

Reaction of Salicylamide and Hippuric Acid in Presence of Polyphosphoric Acid.--A solution of 9 gms. (.05 mole) of hippuric acid in 150 gms. of polyphosphoric acid was heated on a steam bath and 10 gms. (.073 mole) of salicylamide were added. The mixture was heated on a steam bath for 24 hours during which 2.1 gms. of benzoic acid sublimed into the neck of the flask. Upon treatment of the mixture with water, and subsequent trituration with 5% Na_2CO_3 solution, the brown clay-like mass became friable. The dark brown

solid was collected and dried. Acidification of the 5% Na_2CO_3 solution gave a small amount of disalicylamide (IX), m.p. 186-189°C. The brown powder was extracted with ethyl acetate and the solution was filtered through 3 inches of activated alumina. Evaporation of the now yellow solution in air gave 3.8 gms. of a third compound (X). Compound X is very slightly soluble in 5% HCl, is oxidized readily by $KMnO_4$, readily decolorized Br_2 in CCl_4, gives a negative $FeCl_3$ test for the phenolic function and gives a negative ferric hydroxamate test. Recrystallization four times from ethanol did not remove the color, but gave light yellow needles, m.p. 131.5-132.5°C.

Anal. Calcd. for $C_{16}H_{10}O_2N_2$: C, 73.27; H, 3.84; N, 10.68.
Found (1): C, 73.14; H, 3.87; N, 10.15.
(2): C, 71.99; H, 3.88; N, 10.46

A colorless compound was obtained upon treatment of X with Br_2 in CCl_4. A U/V spectrum was taken with a Cary recording spectrophotometer, model 11 M, using spectral grade $CHCl_3$. The concentration used was 4 mg./100 ml. of solvent. The $E^{mol.}_{1\ cm.}$ values given below were calculated assuming $C_{16}H_{10}O_2N_2$ as the molecular formula. Three peaks were exhibited:

	λ max	$E^{1\%}_{1\ cm.}$	$E^{mol.}_{1\ cm} \times 10^{-3}$
(1)	247-8 mμ	740	19.4
(2)	264-5 mμ	380	9.9
(3)	347-8 mμ	90	2.4

The infrared spectrum was taken with a Perkin-Elmer self-recording infrared spectrophotometer, model 21, using spectral grade $CHCl_3$. A representation of this spectrum is given in the figure below.

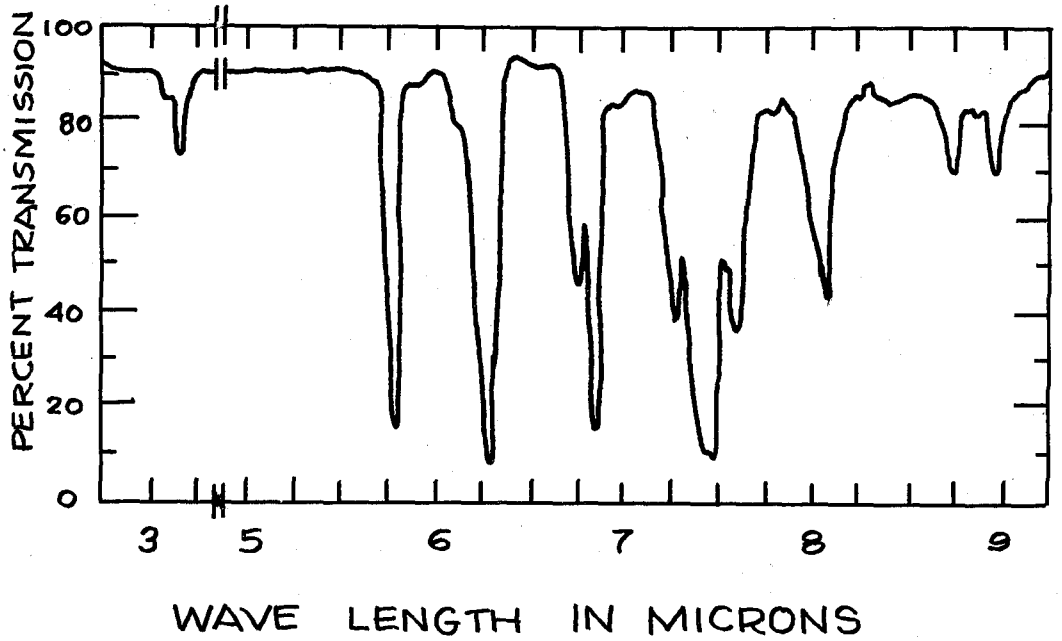

Fig. 1. Infrared spectrum of compound X.

Reaction of Salicylamide and Hydrocinnamic Acid in the Presence of Polyphoric Acid.--A mixture of 15 gms. (.100 mole) of hydrocinnamic acid and 20 gms. (.146 mole) of salicylamide in 150 gms. of polyphosphoric acid was treated in the same manner, as described above. Trituration of the green clay-like material resulting from the reaction, with 5% Na_2CO_3 gave a green solid and a clear

solution. The solution was acidified, a solid collected, and dried to give 10.2 gms. (.0398 mole - 55%) of disalicylamide, IX, m.p. 186-189°C.

The green solid was extracted with ethyl acetate to give a yellow solution and a greenish-yellow solid remained. Filtration of the solution through 3 inches of activated alumina and removal of the solvent under reduced pressure gave 2.1 gms. (.009 mole) of pale yellow compound XI, m.p. 140-143°C. Recrystallization twice from ethyl acetate gave a compound identified as a dimer of alpha-hydrindone, called by Kipping (23) "anhydrobishydrindone," m.p. 142-144°C., lit. m.p. 142-143°C. (23).

Anal. Calcd. for $C_{18}H_{14}O$: C, 87.77; H, 5.86.
Found: C, 87.55; H, 5.93.

The greenish-yellow solid remaining after extraction with ethyl acetate could be recrystallized from either tetrahydrofuran or dimethylformamide. Recrystallization four times from tetrahydrofuran gave 4.4 gms. of compound XII, silky yellow needles, m.p. 376.1-378.0°C. (obtained by means of a copper-constantan thermocouple). Compound XI could be converted to compound XII by heating with polyphosphoric acid. Compound XII was identified with alpha-truxene, m.p. 365-368° C. (23).

Anal. Calcd. for $C_{27}H_{18}$: C, 94.51; H, 5.32
Found: C, 94.22; H, 5.54

The fluorescence spectra of the compound was taken by Dr. Allan Osborne of the du Pont Company and is discussed in the text.

Methanesulfonyl-DL-phenylalanine Salicylamide Ester (XIII).--Acylation of 25 gms. (.152 mole) of DL-phenylalanine with 12 ml. (18 gms. - .52 mole) of methanesulfonyl chloride as directed by Grünert (44) gave an oil which solidified on standing one week to give 8.8 gms. (.036 mole) of product, m.p. 96-99°C. Recrystallization from a 1:5 mixture of benzene and acetic acid gave 7.2 gms. (.0296 mole - 19.4%) of methanesulfonyl-DL-phenylalanine, m.p. 101-102°C., lit. m.p. 104°C. (44).

Treatment of 7.2 gms. of the above with an excess of PCl_5 in the usual manner gave methanesulfonyl-DL-phenylalanylchloride, a yellow oil.

The oil above was placed in solution in 100 ml. of dry benzene and 3.9 gms. (.0226 mole) of the sodium salt of salicylamide were added. The suspension was vigorously stirred and heated at 40°C. overnight. The solid was collected, washed repeatedly with cold water, and recrystallized twice from a mixture of benzene and hexane to give 0.48 gms. (.0013 mole - 1% overall yield) of XIII, m.p. 153.8-155°C.

Anal. Calcd. for $C_{17}H_{18}O_5N_2S$: C, 56.34; H, 5.01; N, 7.73.
Found: C, 56.23; H, 5.42; N, 7.56.

Anthranilamide. A.--Repeated treatment of 100 gms. (0.66 mole) of methylanthranilate with methanol saturated with NH_3 over a 5 months period with intermittent removal of methanol under reduced pressure and addition of freshly saturated methanol gave an oil. The oil was taken up in ether and precipitation with hexane gave 22.2 gms. (.165 mole - 27.4%) anthranilamide, m.p. 108.5-111°C., lit. m.p. 109-111°C.

B.--A mixture of 33.4 gms. (0.2 mole) of o-nitrobenzoic acid and 43.6 gms. (0.22 mole) of PCl_5 was shaken by hand until reaction began. Then the semi-solid mass was stirred vigorously and heated slowly to 40°C. The deep brown solution, after cooling, was added dropwise from a separatory funnel into 200 ml. of conc. NH_4OH, precooled in an ice-salt bath. After the final addition the reaction solution was allowed to stir for an additional 30 minutes. The light brown solid was collected and dried to give 26.6 gms. (0.16 mole - 80%) of o-nitrobenzamide. Recrystallization from methanol gave a product, m.p. 173-176°C., lit. m.p. 174-176° C. (45).

A solution of 26.6 gms. (0.16 mole) of the above and 16 gms. (0.53 mole) of anhydrous hydrazine in 400 ml. ethanol was heated on a steam cone. When the solution was just warm a small amount of Raney-nickel was added and the solution was heated for 3 hours. More Raney-nickel was added and the solution was heated under refluxing conditions

for 30 minutes. The catalyst was removed by filtration, the solution heated with Norite and filtered, and the solvent was removed under reduced pressure. The resultant thick oil was cooled and the solid recrystallized from a mixture of ethanol and hexane to give 16.5 gms. (.123 mole - 76.7%) of anthranilamide, m.p. 109-110°C., lit. m.p. 109-111°C.

Trifluoracetyl-L-phenylalanine Anthranilamide (XIV).--

Trifluoracetyl-L-phenylalanine was prepared as before, $[\alpha]_D^{26.1}$ = + 17.3 ± 0.3 (C, 2% in ethanol), $[\alpha]_D^{25.0}$ = + 35.3 ± 0.4 (C, 2% in glacial acetic acid). Three and two tenths gms. (.0123 mole) of the above was converted to the acid chloride by PCl_5 in the usual manner. The acid chloride was placed in solution in ether, the ethereal solution was added to 1.36 gms. (.01 mole) of anthranilamide in dry ether, 2 gms. (.02 mole) of triethylamine was added, and the solution stirred at room temperature for 24 hours. The solution was filtered free of triethylamine-hydrochloride and the ether was removed under reduced pressure. Recrystallization of the residue from ethanol gave 1.86 gms. (.0049 mole - 49%) of XIV, m.p. 191.5-192.0°C. $[\alpha]_D^{25.0}$ = -48.8 ± 0.8 (C, 1.3% in dimethylformamide).

Anal. Calcd. for $C_{18}H_{16}O_3N_3F_3$: C, 56.99; H, 4.25; N, 11.08.
Found: C, 56.94; H, 4.23; N, 11.03.

Acetyl-DL-phenylalanine Cyanomethyl Ester (XV).--Treatment of 14.4 gms. (.0695 mole) of acetyl-DL-phenylalanine with 9.08 gms. (.121 mole) of redistilled chloroacetonitrile in the presence of triethylamine as directed by Schwyzer for similar compounds (29) gave 11.0 gms. (.0446 mole - 64%) of crude acetyl-DL-phenylalanine cyanomethyl ester (XV). Recrystallization twice from a mixture of ethanol and hexane gave white needles, m.p. 94-95°C.

Anal. Calcd. for $C_{13}H_{14}O_3N_2$: C, 63.40; H, 5.73; N, 11.38 .
Found: C, 63.52; H, 5.71; N, 11.42

The above was reacted with benzylamine to obtain acetyl-DL-phenylalanine benzamide, m.p. 161.5-162.9°C.

Anal. Calcd. for $C_{17}H_{18}O_2N_2$: N, 9.45
Found: N, 9.52

Acetyl-DL-phenylalanine Glycolamide Ester (XVI).--A solution of 2.46 gms. (.01 mole) of XV in 75 ml. of benzene to which 3.3 ml. of 3 M methanol (in benzene) had been added, was saturated with dry HCl gas. The solution was allowed to stand at room temperature for 15 minutes. The benzene was removed by distillation on a steam cone without vacuum and the white residue was taken up in 350 ml. of hot ethyl acetate and the solution cooled slowly. The solid was collected and dried to give 1.69 gms. (.064 mole - 64.0%) of XVI. Recrystallization from ethyl acetate gave a product, m.p. 158.5-159.5°C.

Anal. Calcd. for $C_{13}H_{16}O_4N_2$: C, 59.08; H, 6.10; N, 10.60
Found: C, 59.14; H, 6.07; N, 10.65

Acetyl-L-phenylalanine Cyanomethyl Ester (XVII).--Treatment of 7.2 gms. (.035 mole) of acetyl-L-phenylalanine under conditions analogous to the one described above for the DL-cyanomethyl ester gave 4.4 gms. (.018 mole - 51.5%) of crude XVII. Recrystallization twice from a mixture of ethanol and hexane gave white needles, m.p. 124.5-125.5°C. $[\alpha]_D^{25.3} = -11.2 \pm 0.4$ (C, 3.0% in acetone).

Anal. Calcd. for $C_{13}H_{14}O_3N_2$: C, 63.40; H, 5.73; N, 11.38
Found: C, 63.56; H, 5.80; N, 11.43.

Acetyl-L-phenylalanine Glycolamide Ester (XVIII).--Treatment of 2.46 gms. (.01 mole) of XVII under conditions analogous to the one described above for the DL-glycolamide ester gave 1.6 gms. (.061 mole - 61%) of crude XVIII. Recrystallization from a mixture of ethanol and hexane gave fine white needles, m.p. 120.5-121.5°C. $[\alpha]_D^{25.3} = +2.2 \pm 0.2^\circ$ (C, 2.3% in absolute ethanol).

Anal. Calcd. for $C_{13}H_{16}O_4N_2$: C, 59.08; H, 6.10; N, 10.60.
Found: C, 59.12; H, 6.05; N, 10.61

A mixed melting range with a sample of acetyl-DL-phenylalanine glycolamide ester, m.p. 160.5-161.5°C., was 132-162°C.

Qualitative observations on the enzymatic hydrolysis of the above compound were made and are reported in the text.

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PART III

THE APPARENT IONIZATION CONSTANTS OF A SERIES OF PHENYLALANINE DERIVATIVES

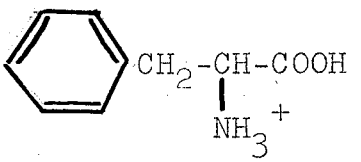
THE APPARENT IONIZATION CONSTANTS OF A SERIES OF PHENYLALANINE DERIVATIVES

Introduction

A number of problems being investigated in these laboratories including the specificity and action of alpha-chymotrypsin and the modification of the carboxyl function of amino acid derivatives (1) have required the synthesis and use of a number of non-acylated phenylalanine derivatives. It was of interest to have on hand more information as to the apparent ionization constants of a series of these derivatives. In this section the apparent ionization constants of the methyl ester, the amide, the thioamide, the hydrazide, and the amidoxime of DL-phenylalanine and that of the protonated amino group in DL-phenylalanine itself are reported.

It is well-known that the mono-basic, mono-carboxylic amino acids exist as dipolar ions in solution (2). In order that this may occur the molecule must have, in strongly acidic solution an uncharged group and a cationic group. This amphoteric compound, to exist at its isoelectric point as a dipolar ion must have an uncharged acid group stronger than the cationic group so that the compound upon losing a proton becomes a dipolar ion.

Phenylalanine exists in acid solution as the cation:



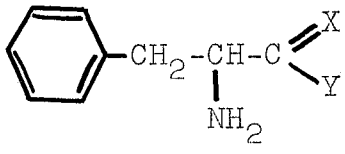
Adams (3) and Bjerrum (4) used an argument analogous to the following for phenylalanine to explain the dipolar ion structure of glycine.

The acid dissociation constants for DL-phenylalanine are $10^{-2.16}$ and $10^{-9.15}$ (5). The acid dissociation constant for beta-phenylpropionic acid is $10^{-4.66}$ (6). The presence of a positive charge on the alpha-amino group in phenylalanine might be expected to change the electrostatic environment of the carboxyl group, repelling a proton and thereby increasing acid dissociation. The constant for beta-phenylethylamine is $10^{-9.83}$ (7), over ten thousand times weaker than the first acid dissociation of phenylalanine. It is quickly seen that the value of $pK_1 = 2.16$ for phenylalanine is due to the carboxyl group and not the amino group. If one esterifies the carboxyl group the pK of the amino group is lowered almost two units (see Table I.). This is due to the removal of the carboxyl group and the difference between the pK of beta-phenylethylamine and phenylalanine methyl ester can be thought of as the effect of the carbonyl polarizability. A shift in electrons out of the amino group under the

influence of the carbonyl group was first termed the inductive effect by Lewis (8). If one changes the character of the carbonyl by modification, it might be expected that the effect of induction on the alpha-amino group would be different depending upon the modification. It was the purpose of this investigation to take a few of the available derivatives and determine their apparent dissociation constants with the above view in mind.

Discussion of Results

The series of DL-phenylalanine derivatives, I-VI, indicated below were either prepared or were available as indicated in the experimental section:



	X	Y
I	=O	-OH
II	=O	$-OCH_3$
III	=O	$-NH_2$
IV	=S	$-NH_2$
V	=O	$-NHNH_2$
VI	=NOH	$-NH_2$

The apparent ionization constants were determined as outlined in the experimental section and the results are tabulated in Table I. It was felt that since in the case of the derivatives capable of di-protonation; phenylalanine,

hydrazide, and amidoxime, the ratio of the apparent dissociation constants was in these cases greater than 10,000, and the ionic and molecular concentrations could be determined with certainty. This led to the consideration of a single equilibrium and direct evaluation of the apparent ionization constants of the protonated amino groups.

The pK value for the protonated amino group in phenylalanine was recorded as 9.17. This is in excellent agreement with the earlier report of 9.15 (5) and an earlier report of 9.13 (2).

An expected difference between the thioamide and the amide did not materialize as identical values, within the limits of experimental error, were found. The values for the methyl ester and the amidoxime were of the same order as the others.

The protonated amino group in DL-phenylalanylhydrazide was lower than the others, outside the limits of experimental error. Since it is felt that the inductive effect should be analogous in the amide and the hydrazide, the explanation must lie elsewhere. The inductive effect would predict a greater availability of the electrons in the hydrazide than in the methyl ester, yet the pK values show an opposite effect. The explanation may lie in a cyclic hydrogen-bonded structure (see proposition 3).

The results at three different salt concentrations indicate no particular dependence of the apparent ioniza-

tion constants upon the ionic strength over the range studied. Representative titration curves for each of the compounds are shown in figure 1.

The expected differences were either too small to be detected in the pK values or not noticeably important when separated by the one carbon atom. The determinations did serve to point out the ease of such titrations employing the automatic titration instrument described briefly in the experimental section.

The author is indebted to Dr. Paul Peterson for the ample samples of the thioamide and the amidoxime used in the above determinations.

Experimental*

DL-Phenylalanine (I).--Synthetic Dow Chemical Company DL-phenylalanine was recrystallized twice from water.

DL-Phenylalanine Methyl Ester (II).--This compound was prepared by the method employing thionyl chloride as described in the previous section for the preparation of L-tyrosine ethyl ester. It was isolated and used as the hydrochloride salt. Recrystallization from a 3:10 mixture of methanol and ether gave white needles, m.p. 158-159°C., lit. m.p. 158°C. (9).

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*All analyses are by Dr. Adalbert Elek.

DL-Phenylalanine Amide (III).--Ammonolysis of 3.6 gms. (.02 mole) of the free methyl ester of above gave 2.3 gms. (.014 mole - 70%) of III. Recrystallization from chloroform gave prisms, m.p. 138-140°C., lit. m.p. 138-139°C. (10).

DL-Phenylalanine Thioamide (IV).--This compound was prepared by Peterson (11), m.p. 135-136.3°C.

Anal. Calcd. for $C_9H_{12}N_2S$: C, 59.96; H, 6.71; N, 15.54
Found: C, 60.05; H, 6.72; N, 15.63

DL-Phenylalanyl Hydrazide (V).--Addition of 1.8 gms. (.01 mole) of DL-phenylalanine methyl ester in 25 ml. of absolute ethanol to 1 gm. of anhydrous hydrazine and heating the solution under refluxing conditions and cooling gave 1.1 gm. (.061 mole - 61%) of V, m.p. 87.5-89.0°C. Recrystallization from a mixture of ethanol and hexane gave colorless crystals, m.p. 88.5-90.0°C.

Anal. Calcd. for $C_9H_{13}O_3N$: C, 60.31; H, 7.31; N, 23.45
Found: C, 60.48; H, 7.42; N, 23.38

DL-Phenylalanine Amidoxime (α-amino-β-phenylpropioamidoxime) (VI).--This compound was prepared by Peterson (11). Recrystallization from water gave white plates, m.p. 117.5-118.5°C.

Anal. Calcd. for $C_9H_{13}O_3N_3$: C, 60.31; H, 7.31; N, 23.45
Found: C, 60.34; H, 7.39; N, 23.41

Potentiometric Determination of Apparent Ionization Constants.--All measurements were made with an automatic recording pH stat designed and build by M. D. Cannon, International Instruments Co., Canyon, California. The instrument is a modification of the instrument recently described by Neilands and Cannon (13). This model is a dual purpose instrument combining elements essential for either constant or variable-pH titrations. In the titrations described herein the instrument was calibrated and set for variable pH titration. When set up in this manner a unidirectional synchronous motor delivers the titrating fluid through an Agla* micrometer syringe. The motor operates at 6 r.p.m. and delivers the titrating fluid, in this case concentrated base, at the rate of 30×10^{-3} ml./min. The base employed in these titrations was of high enough concentration to render the change in volume of solution throughout the titration negligible. The delivery of .8534 N base corresponds to 25.63 milliequivalents/min. The base is delivered to the solution through a Dewitt and Herz Inc. stainless steel hypodermic needle with the tip bent up to limit diffusion of the base into the solution.

The cell used is of 25 ml. capacity and is enclosed in a water jacket designed to maintain the temperature at $25.0 \pm 0.1^{\circ}C$. The temperature inside the cell was maintained

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*Burroughs Wellcome and Co., London, England. Total Capacity--.5 ml.

by circulating water through the jacket from a Precision constant temperature bath and circulating system. The solution was vigorously stirred and a CO_2-free atmosphere was maintained by 'sweeping' the solution with nitrogen. The pH of the solution was measured using the Beckmann No. 4990-29 glass electrode and No. 4970-29 calomel reference electrode in conjunction with a Leeds and Northrup Model 7664-41 A.C.-operated pH indicator. The pH meter output is coupled directly with a Leeds and Northrup Speedomax Type G Recorder enabling one to obtain a titration curve directly.

In all cases the electrodes were standardized with buffer solution at pH 4, 7, and 10 before and after the titration and correction made for non-linearity of this calibration.

In all cases standard stock solutions were prepared in CO_2-free water as was the base and the acid used. The atmosphere was kept CO_2-free as stated above. Ten ml. aliquots of the stock solutions of amino acid derivatives containing approximately 75 milliequivalents were pipetted into the reaction cell, and 10 ml. of the various salt solutions (.4 N, .2 N, and .1 N) to make a final salt concentration of .2 N, .1 N, and .05 N, respectively, were added. One or two ml. of .0498 N acid was pipetted into the solution and the now acid solution was titrated as indicated above. An average of five titrations was made on each

derivative at the different salt concentrations. In no case could any apparent difference due to ionic strength be noted. The constants were evaluated from the curves considering a single equilibrium (14). The pK_a of the amino groups of the various derivatives are listed in Table I. Representative curves have been traced and are shown in Figure 1. The values given are believed to be accurate within .03 pK unit.

The author is indebted to Dr. Cannon for instruction in the operation of the titrimeter.

Table I

Apparent Ionization Constants of a Series of DL-Phenylalanine Derivatives at 25.0 ± 0.1°C.

Compound	[Salt]	pK' of $-NH_3^+$	[Salt]	pK' of $-NH_3^+$
I. DL-Phenylalanine	.05	9.21	.20	9.11
	.10	9.19	.20	9.13
	.20	9.17		
	pK'=9.17±.03			
II. DL-Phenylalanylmethyl Ester	.05	7.28	.20	7.33
	.10	7.25	.20	7.31
	.20	7.28	.20	7.35
	pK'=7.30±.03			
III. DL-Phenylalanine Amide	.05	7.41	.20	7.42
	.10	7.38	.20	7.46
	.20	7.36		
	pK'=7.40±.03			
IV. DL-Phenylalanine Thioamide	.05	7.37	.20	7.39
	.10	7.42	.20	7.36
	.20	7.40		
	pK'=7.39±.03			
V. DL-Phenylalanylhydrazide	.05	7.12	.20	7.16
	.10	7.13	.20	7.18
	.20	7.12		
	pK'=7.14±.02			
VI. DL-Phenylalanine Amidoxime	.05	7.24	.20	7.26
	.10	7.22	.20	7.24
	pK'=7.24±.02			

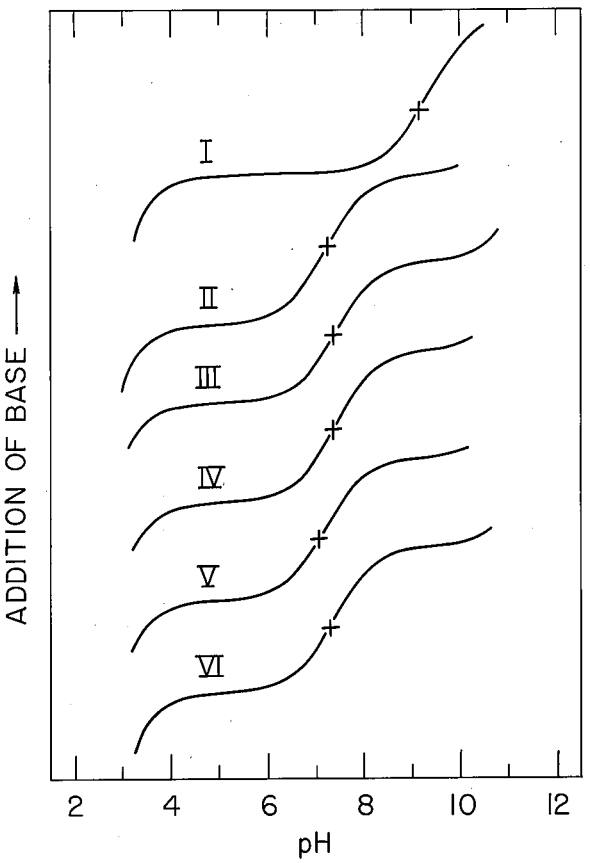

Fig. 1. Titration curves for a series of phenylalanine derivatives: I-acid, II-methyl ester, III-amide, IV-thioamide, V-hydrazide, VI-amidoxime.

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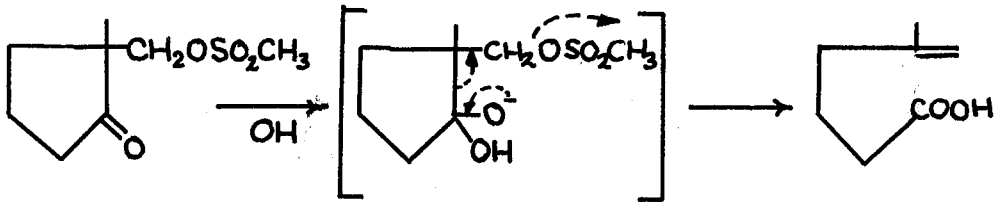
PART IV

PROPOSITIONS

PROPOSITIONS

1. In many cases in derivatization of α-amino acids it is very difficult to isolate the optically active L-enantiomorph which is desired for enzymatic hydrolysis studies. It is proposed that competitive hydrolysis between a substrate whose enzymatic hydrolysis constants are known and the optically inactive DL-compound will yield the desired constants for the L-isomer.

2. The following reaction was reported in 1952 (1):



Steroids oxygenated in the twelve position are available in the bile acid series. It is proposed that the same type of reaction as above be utilized in the conversion of the readily available steroids with the hydroxyl group in the C_{12} position into the important oxygenated C_{11} steroids.

3. The apparent ionization constant of the protonated amino group on phenylalanylhydrazide is 0.2 pK unit lower than that of phenylalanylamide (2). This difference also exists between glycylhydrazide and glycylamide (3,4).

(a) It is proposed that the difference is due to the possibility of a cyclic hydrogen-bonded molecule in the hydrazide whereas this is less likely in the amide.

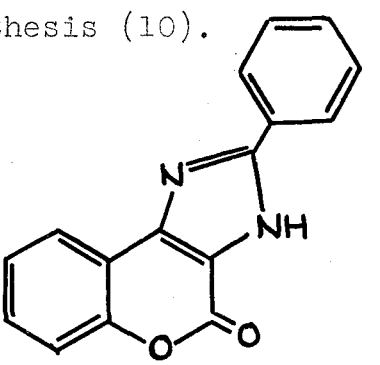
(b) It is proposed that preparation of phenylalanylmethylamide and the mono- and di-fluoromethylamides and subsequent determination of their apparent ionization constants would be interesting evidence in favor of the cyclic hydrogen bonded structure.

4. In the base-catalyzed decomposition of substituted α-(benzenesulfonamido)-carboxylic acid chlorides it has been suggested that the reaction mechanism involves a displacement at the α-carbon atom in which a hydroxyl ion displaces a $(:COCl)^-$ anion (5). A much more likely mechanism is one involving initial abstraction of the proton from the nitrogen followed by a shift of electrons leading to displacement of the anion. It is proposed that information to prove this mechanism would be gained from the base-catalyzed decomposition of the corresponding N-methyl compounds (6).

5. An interesting scheme has been advanced by Longuit-Higgins to explain the electronic structure of "sandwich molecules" such as $Fe(C_5H_5)_2$ and $Co(C_6H_6)_2$. In view of this scheme it is proposed that attempts be made to prepare molecules of this type with two different ring sizes in the same complex with the appropriate metal (7).

6. It is proposed that a study of the action of alpha-chymotrypsin upon 3-acetamido-4-(p-hydroxyphenyl)-2-butanone be undertaken (8). It contains all the requirements of the usual substrate except an easily hydrolysable group. The possibility exists that it would be bound to the enzyme strongly, inhibiting reaction of any other substrate. A second possibility is carbon-carbon cleavage since ethyl 5-(p-hydroxyphenyl)-3-ketovalerate is cleaved by alpha-chymotrypsin to yield p-hydroxyphenylpropionic acid (9).

7. From the experimental evidence obtained the following structure is proposed as a possible structure for compound X in this thesis (10).



8. The biochemical interconversions of α-aspartyl semi-aldehyde and aspartic acid as well as glyceraldehyde-3-phosphate and 3-phospho-glyceric acid proceed by way of phosphorylated intermediates (11-13). It is proposed that the biochemical interconversion of glutamic semi-aldehyde and glutamic acid involves a similar intermediate. Even though the γ-glutamyl phosphate has not been isolated one can test for the occurrence of such an intermediate by the detection of a glutamic acid dependent release of inorganic phosphate from P^{32} labeled adenosine triphosphate using known enzyme sources (14).

9. Thionalide (thioglycollic acid-β-aminonapthalide) has been used for the determination of the metals copper, mercury, silver, bismuth or lead (15,16). A volumetric method involving addition of an excess of the thionalide to the solution of the metal ion, removal of the precipitate by filtration and determination of excess thionalide with standard periodate is proposed.

10. An isomer of ethyl 4-phenylpiperidine-4-carboxylate (pethidine) where the substituents are moved around the piperidine ring one carbon, ethyl 3-phenyl-3-carboxylate (β-pethidine) (17) has been shown to possess a longer and more powerful effect than the

former. It also gives less euphoria, causes less respiratory depression, and shows less addiction properties (18). It is proposed that a similar isomer of morphine be synthesized and tested as possibly exhibiting some of these desirable properties over morphine itself.

11. (a) In attempts to prepare the imidic ethyl ester of benzoyl cyanide the only substances isolated were ethyl benzoate and decomposition products of HCN. It is proposed that the scope of acylation with acyl cyanides be investigated.
(b) It is further proposed that acyl cyanides where the acyl moiety is an acylated α-amino acid be investigated as substrates for enzymatic hydrolysis by alpha-chymotrypsin.

12. A solution of phenyliodosodichloride in pyridine allowed to stand at room temperature for one hour gives a good yield of 4-chloropyridine (19). A mechanism involving complex formation between the two reactants followed by a nucleophilic attack of chloride ion on the pyridine moiety is proposed.

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