STUDIES ON THE PURIFICATION AND PROPERTIES OF L-LEUCINE

STUDIES ON THE MODE OF ACTION OF TRYPSIN AND CHYMOTRYPSIN

Thesis by

Dudley W. Thomas

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1951

Acknowledgements

To Professor Carl Niemann, whose friendly guidance and encouragement were enjoyed throughout the course of my graduate studies, I am deeply grateful. It has been a pleasure to work with Professor Niemann and his group on many interesting problems.

I wish to acknowledge the help and advice that were always willingly offered by other members of the faculty and by other graduate students.

I am indebted to the California Institute of Technology for Teaching Assistantship from 1947-1949, and to the Allied Chemical and Dye Corp. for their 1949-1950 Predoctoral Fellowship.

To my wife, Ann, who has shared in the trials and tribulations of graduate study, this Thesis is appreciatively dedicated.

Abstract

A procedure for the preparation of L-leucine is described and certain physical properties, useful for establishing the identity or purity, have been redetermined. The behavior of L-leucine in sulfuric acid and glacial acetic acid solutions has been investigated.

Preliminary investigations of the tryptic hydrolysis of acetyl- and benzoyl-L-argininamide have been conducted. It was found that aqueous trypsin solutions are quite unstable, and thus not ideally suited for kinetic studies. L-Arginine-methyl ester was found to be rapidly split at pH 4.0, this pH being far removed from the expected optimum (i.e. pH 7-8).

The kinetics of the α-chymotrypsin catalyzed hydrolysis of acetyl- and nicotinyl-L-tyrosinamide has been investigated at 25° C. and pH 7.8-8.0. Suitable rate expressions have been developed and the rate constants determined. The effect of various competitive inhibitors of α-chymotrypsin has been measured, and conclusions have been drawn relative to structure and affinity for the enzyme. It has been shown that the α-chymotryptic hydrolyses, so far investigated quantitatively, can be described in terms of the classical Michaelis-Menten enzyme-substrate complex theory.

TABLE OF CONTENTS

PART I

THE PREPARATION OF L(-)LEUCINE AND ITS BEHAVIOR IN SOME NON-AQUEOUS SOLVENTS

By Dudley W. Thomas and Carl Niemann

(From the Gates and Crellin Laboratories of Chemistry*, California Institute of Technology, Pasadena.)

* Contribution No. 1175.

The Preparation of L(-)Leucine and Its Behavior in Some Non-aqueous Solvents

Some time ago Bergmann and Stein[1] developed a procedure for the isolation and purification of naturally occurring L(-)leucine based upon the precipitation and the recrystallization of the slightly soluble salt of this amino acid with napthalene-β-sulfonic acid. The product so obtained was stated to be 97% pure and to be free of methionine, a common contaminant of less pure preparations of L(-)leucine[2]. In these laboratories, using a more sensitive method[3] than was available to Bergmann and Stein, it was found that naturally occurring leucine purified as described above contained significant amounts of one or more of those amino acids which are oxidized by bromine in acid solution, e.g., cysteine, cystine, methionine, tyrosine, etc. As a preparation of maximum purity was desired, means were sought to improve the original procedure of Bergmann and Stein.

It was found that preliminary treatment of a 1 formal hydrochloric acid solution of crude leucine with bromine water resulted in the isolation of a napthalene-β-sulfonate from which impurities could be removed more rapidly and completely, as judged by polarimetric measurements, than from a salt prepared directly from the crude leucine. The use of the preliminary bromine oxidation and of more dilute solutions, than were used by Bergmann and Stein, for the recrystallization of the napthalene-β-sulfonate led to the

isolation of L(-)leucine preparations of exceptional purity. No sulfur could be detected in 500 mg. samples of these preparations though the method used[4] was capable of detecting as little as 2-3 μg. of sulfur. The absence of tyrosine, and other aromatic amino acids, was indicated by colorimetric tests and by the lack of specific absorption in the 260-270 mμ. region. Since values obtained for total nitrogen were in close agreement with those required for L(-)leucine (99.3% ± 0.8%), the only contaminant likely to escape detection by the above methods was isoleucine. Since this latter amino acid is more soluble in water than is leucine, solubility measurements were relied upon as a final test of purity. Such measurements gave no indication of the presence of isoleucine or other impurities in the L(-)leucine preparations obtained with the improved procedure. Characteristic physical constants of the L(-)leucine so prepared are given elsewhere in this paper.

In the determination of the optical rotation of α-amino acids in aqueous acid solutions it has been assumed at times that the concentration of the aqueous acid is not particularly critical provided that sufficient acid has been added to assure reasonably complete formation of the amino acid cation. Dunn[5] has pointed out that the specific rotation of solutions of L(+) and D(-) alanine in hydrochloric acid is dependent upon the hydrochloric acid concentration, and the data presented in Fig. I clearly shows a

similar dependency in the case of either hydrochloric or sulfuric acid solutions of L(-)leucine.

Optically active aliphatic monoaminomonocarboxylic acids in aqueous acid solutions show little or no tendency to racemize even at elevated temperatures[6]. Since racemization would be expected to involve at one stage the loss of a proton from the optically active α-carbon atom, an attempt was made to find conditions which might be more favorable for this reaction than those obtaining in aqueous acid solutions. The addition of a proton to an amino acid cation would lead to a structure $[RCHNH_3CO_2H_2]^{++}$, from whihh one might expect a proton to be lost from the α-carbon atom more readily than in the case of the singly charged cation $[RCHNH_3CO_2H]^{+}$. Since the formation of the double charged cation would not be expected to occur in aqueous solutions, attention was directed to the behavior of L(-)leucine in 100 per cent sulfuric acid solutions.

It was found that the optical activity of solutions of L(-)leucine in 100 per cent sulfuric acid remained unaltered when these solutions were allowed to stand at 25° for periods exceeding one month. Furthermore, L(-)leucine preparations recovered from these sulfuric acid solutions were indistinguishable from L(-)leucine preparations not so treated. Since either the lack of formation of the doubly charged cation or the stability of this ion, in respect to the loss of a proton from the α-carbon atom in sulfuric acid solutions,

may have been responsible for the optical stability noted, the cryoscopic properties of sulfuric acid solutions of L(-)leucine were investigated. It was found that the freezing point depression of these solutions was 2.2 times that of a non-electrolyte which would indicated that while the reaction

$$RCH\overset{+}{N}H_2CO_2^- + H_2SO_4 \longrightarrow RCH\overset{+}{N}H_3CO_2H + HSO_4^-$$

is essentially complete there is relatively little ionization of the type

$$RCH\overset{+}{N}H_3CO_2H + H_2SO_4 \longrightarrow RCH\overset{+}{N}H_3C\overset{+}{O}_2H_2 + HSO_4^-.$$

The effectiveness of the positively charged ammonium group in preventing appreciable protonation of the carboxyl group of the L(-)leucine cation in 100% sulfuric acid can be appreciated when it is remembered that both acetic acid and monochloroacetic acid are completely ionized in this solvent[7]. It is interesting to note that in aqueous solutions dichloroacetic acid is a stronger acid than the amino acid cation by a factor of 10[8], whereas in sulfuric acid solutions the difference in acid strength of these two compounds is small. Indeed, if one can accept Hantzsch's values[9], one might conclude that the amino acid cation is the stronger acid.

It was observed that where L(-)leucine was titrated

with perchloric acid in glacial acetic acid solutions[10] the specific rotation of the solution increased rapidly with added increments of perchloric acid until one equivalent of the acid had been added, the further addition of perchloric acid resulting in a gradual and asymptotic decrease in the specific rotation of the solution (Fig. II). Kolthoff and Willman[11] have argued that a dipolar ion, such as glycine, would be expected to behave as a strong base in glacial acetic acid solutions principally on the grounds of the greater acid strength of acetic acid as compared to water and because of an assumed near equivalence of the acid strengths of the amino acid cation and acetic acid in solutions of the latter substance. These authors conclude, on the basis of conductivity measurements, that glycine dissolved in glacial acetic acid is present as $[CH_2NH_3CO_2H]^+$ $[Ac]^-$ and that the degree of dissociation of this ion pair is of the same order as that of potassium or ammonium acetate in the same solvent. The observed change in the specific rotation of a glacial acetic acid solution of L(-)leucine upon the addition of perchloric acid in the same solvent and particularly the abrupt change in the trend of the specific rotation noted at the equivalence point is compatible with the idea of negligible or limited dissociation of the ion pair $[C_4H_9CHNH_3CO_2H]^+$ $[Ac]^-$ in glacial acetic acid where present in relatively high concentrations. If dissociation of the ion pair were

extensive, one would not expect the specific rotation of the solution to be markedly altered, at least not in the manner observed, by the replacement of acetate ion by perchlorate ion.

If one assumes that the amino acid is completely ionized in glacial acetic acid solutions, the observed changes in the specific rotation of these solutions upon the addition of perchloric acid may be interpreted as being due to the replacement of acetate ion by perchlorate ion in the undissociated ion pair. That such an effect is possible is clearly demonstrated by the polarimetric titration of a partially resolved sample of d-α-phenylethylamine in glacial acetic acid by perchloric acid in the same solvent (Fig. III). In this latter case there can be no doubt that the α-phenylethylamine is completely ionized in glacial acetic acid solutions. Although rigorous evidence as to the nature of the ion species present in a glacial acetic acid solution of L(-)leucine is lacking, present knowledge[7,8,12] would appear to offer little support to the view that the amino acid could exist in glacial acetic acid solutions as the dipolar ion.

In the course of these studies it was observed that the specific rotation of L(-)leucine in glacial acetic acid was strikingly dependent not only upon temperature but also upon the amino acid concentration (Fig. IV). Furthermore it was observed that the molal freezing point lowering of

solutions of L(-)leucine in glacial acetic acid varied with the amino acid concentration as shown in Fig. V. These observations suggest that, even at relatively low concentrations, there is extensive association of the ion pairs present in a glacial acetic acid solution of L(-)leucine.

Experimental

Bromometric Titration of Leucine Preparations - One hundred mg. samples of technical leucine (Lemke), a commercially available purified leucine (La Roche) and leucine isolated and purified as described by Bergmann and Stein[1] were titrated in the apparatus described by Sease et. al.[3]. The values found, expressed as moles of Br_2 consumed per gm. of sample, were for the Lemke preparation, 1.31×10^{-3}; for the LaRoche sample, 3.66×10^{-4}; and for leucine prepared by the method of Bergmann and Stein, 1.11×10^{-5}. In the latter case, repeated recrystallization of the napthalene-β-sulfonate, beyond that advocated by Bergmann and Stein, did not prove effective in removing bromine oxidizable impurities. Samples of L(-)leucine prepared as described below contained no detectable amounts of bromine oxidizable material.

Preparation of L(-)Leucine - Saturated bromine water was added, slowly and with stirring, to a 10% (wt./vol.) solution of technical leucine (Lemke) in 1 formal hydrochloric acid until the bromine color persisted for twenty minutes after the last addition. The excess bromine was destroyed

with sodium bisulfite prior to the addition of napthalene-β-sulfonic acid as directed by Bergmann and Stein[1]. The napthalene-β-sulfonate so obtained, m.p. 189-191.5°, $[\alpha]_D^{25}$ = 13.3° ± 0.1°, 4.3% (wt./vol.) in methyl cellosolve, was recrystallized four times from a 7.5% (wt./vol.) aqueous solution and the L(-)leucine, obtained by the decomposition[1] of the four times recrystallized napthalene-β-sulfonate, m.p. 191-192°, $[\alpha]_D^{25}$ = 13.2° ± 0.1°, 3/8% (wt./vol.) in methyl cellosolve, was recrystallized twice from 33% (vol./vol.) aqueous ethanol. The yield of the twice recrystallized L(-)leucine, based upon the weight of the starting material varied between 10 - 14%. Qualitative tests for the presence of sulfur[4] and of tyrosine[13] in the twice recrystallized L(-)leucine were negative. Examination of the absorption spectra of a 0.4% (wt./vol.) aqueous solution of the amino acid revealed no specific absorption in the 260-270 mμ. region. Analysis for total nitrogen gave 10.60% ± 0.09% or 99.3% ± 0.8% of theory.

Solubility of L(-)Leucine in Water - An excess of twice recrystallized L(-)leucine was added to 125 ml. glass stoppered bottles containing 60 ml. of redistilled water and the solutions equilibrated from both sides in a bath maintained at 25.05 ± 0.05°. The solvent was removed from aliquots, withdrawn over a period extending from five to thirty days, by evaporation at 105°. Seventeen determinations gave a mean value of 2.152 ± 0.006 gm./100 gm. of water for the

solubility of the twice recrystallized L(-)leucine at 25°. The undissolved leucine remaining from the above series of solubility measurements was utilized for a second series of measurements and in this latter instance twelve determinations, conducted as described above, gave a mean value of 2.15 ± 0.01 gm./100 gm. of water at 25°. The value of 2.15 ± 0.01 gm./100 gm. of water for the solubility of L(-)leucine in water at 25° obtained in these studies is to be compared with the value of 2.19 gm./ 100 gm. of water at 25° reported by Stoddard and Dunn[14] and the value of 2.20 gm./100 gm. of water at 25° reported by Hlynka[15]. Attention is called to the fact that the solubility of isoleucine in water is approximately twice that of leucine.

The Optical Rotation of L(-)Leucine In Aqueous Systems - The optical rotation of the twice recrystallized L(-)leucine was determined in 6.02 formal hydrochloric acid with the leucine concentration varying from 2.0 - 5.0% (wt./vol.) and the temperature from 18°-35°. The specific rotation of L(-)leucine in 6.02 formal hydrochloric acid was found to be independent of the leucine concentration between the limits studied. The variation of the specific rotation with temperature was found to be linear within the above limits and the temperature coefficient was found to have a value of 0.07°/degree $\overline{C}$ which is in reasonable agreement with the value of 0.063°/degree $\overline{C}$ reported by Stoddard and Dunn[14] for L(-)leucine in 6.08 formal hydrochloric

acid. The mean value of $[\alpha]_D^{25}$ for L(-)leucine in 6.02 formal hydrochloric acid was found to be $14.85^\circ \pm 0.04^\circ$.

The dependence of the specific rotation of L(-)leucine upon the concentration of the aqueous hydrochloric or sulfuric acid used as a solvent was studied and the data obtained are presented in Fig. I. In sulfuric acid the specific rotation rose from a minimum value in water to a maximum value in approximately 10-11 formal sulfuric acid and then declined to a second minimum in 100% sulfuric acid. With solutions containing sulfur trioxide the specific rotation rose rapidly with increasing sulfur trioxide concentration to values which were higher than the maximum value observed in aqueous sulfuric acid solutions.

In order to compare values for the specific rotation of L(-)leucine in aqueous hydrochloric acid solutions reported by others with those obtained in this study the literature values were interpolated to a temperature of 25° and a hydrochloric acid concentration of 6.0 formal. The values so obtained are given in Table I.

Behavior of L(-)Leucine in Sulfuric Acid - The optical stability of L(-)leucine in 100% sulfuric acid was determined by preparing a 0.1524 formal solution of this amino acid in 100% sulfuric acid and observing the specific rotation of the solution immediately after preparation and after it had stood at room temperature in an air tight container for eight weeks. No significant change in the specific

rotation was observed during this period. The sulfuric acid solution of the amino acid was then diluted with water, the amino acid recovered by precipitation with napthalene-β-sulfonic acid and compared with a L(-)leucine napthalene-β-sulfonate of known purity. The determination of the mixed melting point, nitrogen content and specific rotation failed to disclose any differences between these two preparations.

The cryoscopic properties of L(-)leucine in 100% sulfuric acid were studied using the method described by Hammett and Deyrup[17]. The freezing point depression of 0.03-0.12 weight formal solutions of L(-)leucine in 100% sulfuric acid was found to be 13.5° ± 0.2° per mole of amino acid, or approximately 2.2 ± 0.05 times greater than the depression caused by a non-electrolyte.

Titration of L(-)Leucine with Perchloric Acid In Glacial Acetic Acid - Anhydrous perchloric acid in glacial acetic acid was prepared by adding standardized 70% (wt./vol.) perchloric acid to the required amount of acetic anhydride to give a solution free of both water and acetic anhydride. Solutions of varying perchloric acid concentration were then prepared by diluting the concentrated solution with glacial acetic acid. The titration was carried out by dissolving 1.0 g. of the twice recrystallized L(-)leucine in 50 g. of each of the acetic acid - perchloric acid solutions and determining the rotation and density of these

solutions at 26°. The results of this titration are shown in Fig. II.

Titration of d-α-Phenylethylamine With Perchloric Acid In Glacial Acetic Acid - Solutions of d-α-phenylethylamine, $[\alpha]_D^{24} = 37.6$, were prepared by dissolving 0.50 g. of the amine in 25 ml. of acetic acid - perchloric acid solutions of varying perchloric acid concentrations and determining the specific rotation of these solutions at 24°. The results of this titration are given in Fig. III.

Behavior of L(-)Leucine in Glacial Acetic Acid - The specific rotation of twice recrystallized L(-)leucine in glacial acetic acid was determined at 25° using solutions 0.012 - 0.147 formal in L(-)leucine. The data so obtained are given in Fig. IV. Observations made at other temperatures indicated that the specific rotation of L(-)leucine in glacial acetic acid is demonstrably dependent upon temperature.

Varying amounts of twice recrystallized L(-)leucine were dissolved in 100 g. of glacial acetic acid, f.p. 16.6°, and the freezing point depression of each of these solutions determined with the aid of a Beckmann thermometer. The results of these cryoscopic measurements are given in Fig. V.

Summary

An improved procedure for the preparation of L(-)leucine is described and certain physical properties, useful for establishing identity or purity, have been redetermined. The

behavior of L(-)leucine in sulfuric acid and glacial acetic acid solutions has been investigated.

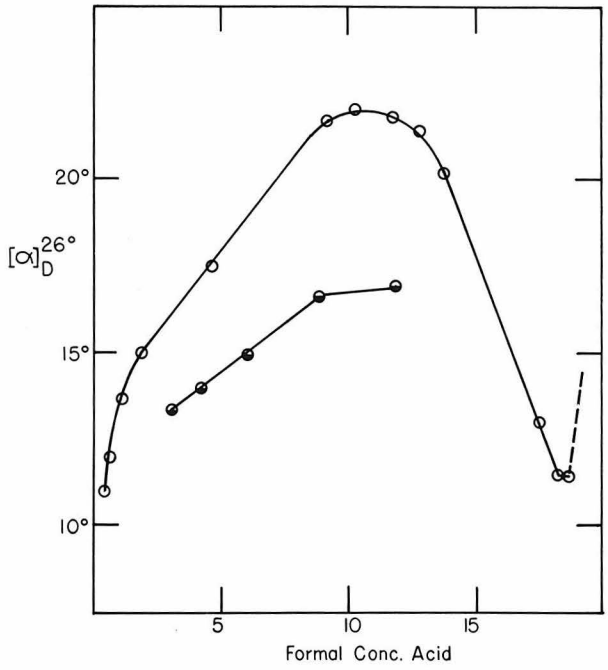


Fig. I

Specific Rotation of L(-)Leucine In Aqueous Hydrochloric Acid and Sulfuric Acid Solutions

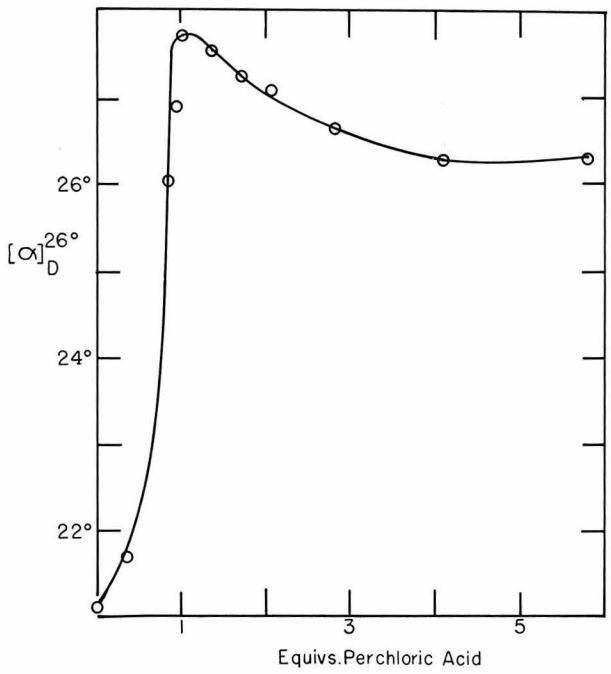


Fig. II

Polarimetric Titration of L(-)Leucine With Perchloric Acid In Glacial Acetic Acid

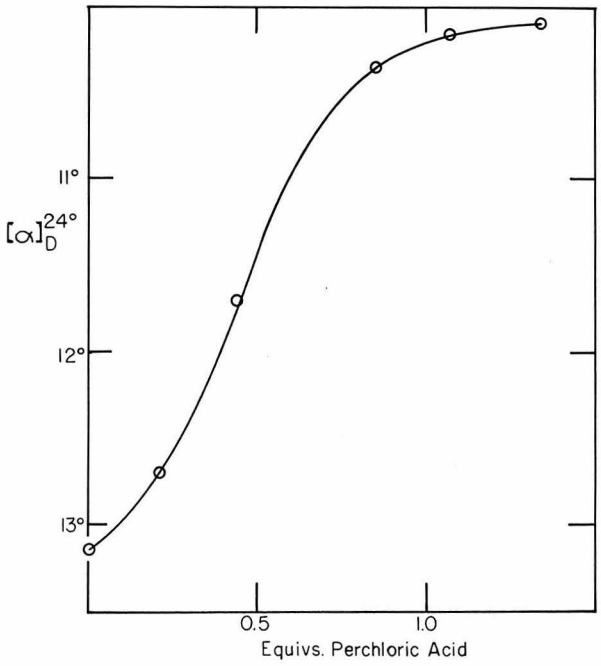


Fig. III

Polarimetric Titration of d-α-Phenylethylamine
With Perchloric Acid In Glacial Acetic Acid

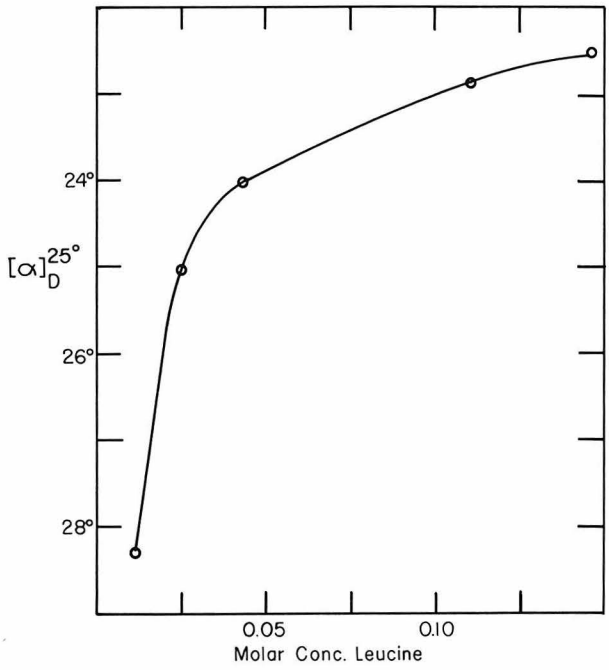


Fig. IV

Specific Rotation of L(-)Leucine In Glacial Acetic Acid at 25°

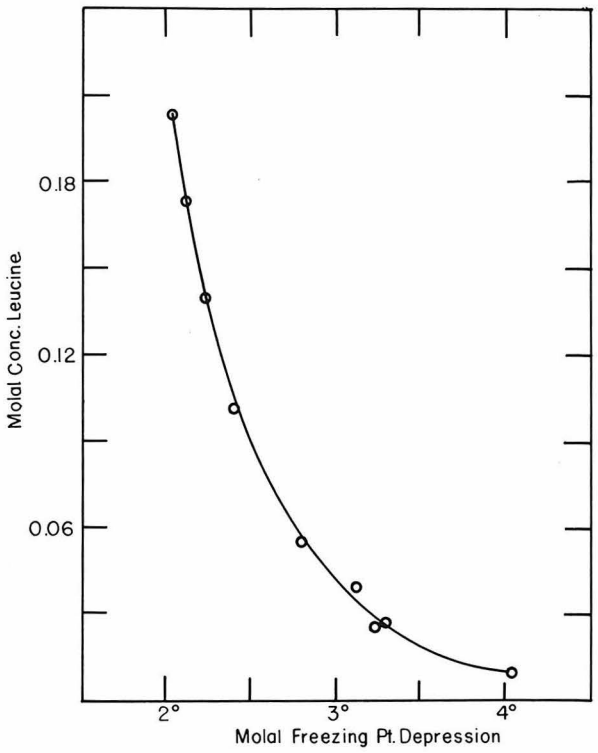


Fig. V

Freezing Point Depression of Solutions Of
L(-)Leucine In Glacial Acetic Acid

Table I

Specific Rotation Of L(-)Leucine

In 6.0 Formal Hydrochloric Acid and 25°

Author	Lit. Value	Interpolated Value*	Ref.
Bergmann and Stein	15.33°(21%HCl, 24°)	15.24	1
Stoddard and Dunn	15.21°(6.08N HCl,25°)	15.16	14
Dunn and Courtney	15.1°(6.0N HCl,25.9°)	15.03	16
Thomas and Niemann	14.85°(6.02E HCl,25°)	14.84	--

* Interpolated to 6.00 N(formal) HCl and 25°.

Bibliography

1. M. Bergmann and W. H. Stein, J. Biol. Chem., 129, 609 (1939).

2. M. S. Dunn and L. B. Rockland, Advances in Protein Chemistry, 3, 295, Academic Press, New York (1947).

3. a.) J. W. Sease, C. Niemann and E. H. Swift, Ind. Eng. Chem., Anal. Ed., 19, 197 (1947); b.) R. J. Myers,Jr., and E. H. Swift, J. Amer. Chem. Soc., in press.

4. E. L. Bennett, C. W. Gould, Jr., E. H. Swift and C. Niemann, Ind. Eng. Chem., Anal. Ed., 19, 1035 (1947).

5. M. S. Dunn, M. P. Stoddard, L. B. Rubin and R. C. Bovie, J. Biol. Chem., 151, 1241 (1943).

6. C. L. A. Schmidt, "The Chemistry of the Amino Acids and Proteins", Thomas, Springfield (1944).

7. L. P. Hammett, "Physical Organic Chemistry", McGraw-Hill, New York (1940).

8. E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides", Reinhold, New York (1943).

9. A. Hantzsch, Z. physik. Chem., 61, 257 (1908).

10. G. F. Nadeau and L. E. Branchen, J. Amer. Chem. Soc., 57, 1363 (1935).

11. I. M. Kolthoff and A. Willman, J. Amer. Chem. Soc., 56, 1014 (1934).

12. L. P. Hammett, Chem. Rev., 13, 61 (1933).

13. O. Folin and V. Ciocalteu, J. Biol. Chem., 73, 627 (1929).

14. M. P. Stoddard and M. S. Dunn, J. Biol. Chem., 142, 329 (1942).

15. I. Hlynka, Ph. D. Thesis, Calif. Inst. Tech., Pasadena (1939).

16. M. S. Dunn and G. Courtney, "Handbook of Chemistry and Physics", Chemical Rubber Publishing Co., Cleveland (1946).

17. L. P. Hammett and A. J. Deyrup, J. Amer. Chem. Soc., 55, 1900 (1933).

PART II

STUDIES ON THE MODE OF ACTION OF TRYPSIN AND CHYMOTRYPSIN

Studies on the Mode of Action of Trypsin and Chymotrypsin

Introduction

It has been known for many years that pancreatic juice possesses the property of digesting proteins. However, extracts of fresh pancreas or freshly secreted pancreatic juice have no proteolytic activity, the preparation becoming active when mixed with the contents of the small intestine, or when the pancreas is allowed to stand in slightly acid solution. Thus it was early concluded that the enzymes are present in an inactive form.

The enzymes chiefly responsible for the proteinase activity of pancreatic juice are trypsin and chymotrypsin, and these enzymes are secreted in the form of their inactive precursors, or zymogens - trypsinogen and chymotrypsinogen. They act best on denatured proteins, neither alone causing digestion to proceed very far, but the combination of the two carries the hydrolysis to the polypeptide stage.

The mode of activation and the nature of the enzymes was a subject of great controversy for many years, and it wasn't until 1931 that the first truly significant advance was made. It was during that year that Northrop and Kunitz[1] reported the crystallization of trypsin. In the decade that followed they were also able to obtain in crystalline form trypsinogen, chymotrypsinogen, and α-, β-, and γ-chymotrypsins, and to elucidate the mechanism of activation of these enzymes, as well as to show that they consisted entirely of protein material[2].

It was first believed that the activation of the pancreatic proteases consisted in the combination of zymogen with activator, but Kunitz[3] demonstrated unambiguously that the activation process is one of enzymatic hydrolysis. That is, enterokinase, a material early obtained from the small intestine, enzymatically converts trypsinogen to trypsin, and the trypsin thus formed can in turn convert chymotrypsinogen to chymotrypsin. Both of these reactions follow apparent first-order kinetics. Also traces of trypsin can catalyze the conversion of trypsinogen to trypsin, the reaction in this case being autocatalytic.

The nature of the products obtained from the activation of the zymogens depends greatly on the conditions employed, especially that of pH. There is only one known form of trypsin, but varying amounts of inert protein are obtained simultaneously to the activation process. In the case of chymotrypsin, there are three known forms, and several other forms whose existence has been postulated. α-Chymotrypsin is obtained catalytically from chymotrypsinogen at pH 7-9, and if this form of the enzyme is allowed to stand at pH 4 for a period of one month, it undergoes autolytic conversion into β- and γ-chymotrypsins plus inert protein[4]. α-Chymotrypsin is also autolytically converted to γ-chymotrypsin after standing at pH 8 and 35° C. for one hour[4]. A brief summary of the present knowledge of these activation processes is given in an

extensive review article on the pancreatic proteases by Neurath and Schwert[5].

The intestinal activator, enterokinase, has not as yet been crystallized. Kunitz[6] was able to concentrate the material from 5,000-50,000 fold, but could draw no conclusions as to its chemical nature. His purified material contained variable amounts of protein, carbohydrate, and glucosamine. The only definitely known action of enterokinase is the conversion of trypsinogen to trypsin.

Following the crystallization of trypsin and chymotrypsin, the next significant advance in the study of the specificity and mode of action of these enzymes was made by Bergmann and his co-workers[7-15]. They showed that synthetic peptides and peptide derivatives can be split as well as the immensely more complex protein substrates. Previously, the proteinases had been supposed to act exclusively on substrates of high molecular weight, but not to act on lower polypeptides and dipeptides. This erroneous concept required that enzymes be able to differentiate between substrates on the basis of molecular weight alone.

From this early work, it was established that certain amino acids must be incorporated into the substrates for the enzymes to be effective. Of a large number of substrates tested, only those containing L-arginine and L-lysine residues were split by trypsin, and only those containing L-tyrosine and L-phenylalanine residues were split by chymotrypsin. In all cases splitting occurred only at amide

or peptide linkages involving the carboxyl groups of the amino acids mentioned above. Thus, such simple substrates as benzoyl-L-argininamide, benzoylglycyl-L-argininamide, benzoyl-L-lysinamide, and benzoylglycyl-L-lysinamide were found to be rapidly split by trypsin into the free acids and ammonia. Glycyl- and carbobenzoxy-L-tyrosinamides, and glycyl- and carbobenzoxy-L-phenylalaninamides likewise were found to be split into the free acids and ammonia by chymotrypsin. Also internal peptide bonds were found to be split, a representative chymotrypsin substrate being carbobenzoxyglycyl-L-tyrosylglycinamide. In this case the hydrolysis products were found to be carbobenzoxyglycyl-L-tyrosine and glycinamide. In all cases, hydrolyses with trypsin and chymotrypsin were carried out at about pH 7·1-7·7, this having been qualitatively established as near the pH optimum for these enzymes.

More recently the list of essential amino acid residues for tryptic hydrolysis has been expanded to include one L-citrulline and one L-ornithine derivative[16]. Also for chymotryptic hydrolysis, L-methionine and L-tryptophane are now known to satisfy the structural requirements of the essential amino acid residue, or so-called "backbone"[17,18]. As well as increasing the number of effective substrates, recent work has also greatly increased the list of compounds that are resistant to hydrolysis, or that are split only at insignificant rates.

Along with the efforts to establish the nature of the specific amino acid residues for trypsin and chymotrypsin substrates, numerous studies have been undertaken to determine the effect of modifications in these substrates, such as variations in the substituents on the α-amino and carboxyl groups. The results that have been obtained will be discussed qualitatively in the paragraphs to follow, and a more quantitative treatment will be attempted after the presentation of kinetic data in the next section.

As has already been mentioned, N-α-benzoyl- and N-α-benzoylglycyl-L-lysin- and L-argininamides are rapidly hydrolyzed through the action of trypsin. N-α-toluenesulfonyl derivatives were first reported to be resistant to tryptic action[10], but these results were later found to be erroneous[11], and it has now been shown[19] that toluenesulfonyl-L-argininamide is split even more rapidly than benzoyl-L-argininamide at a single substrate concentration. That the substituent on the α-amino group can also be acetyl, or else omitted entirely, is evident from the work to be described in the next section. Also, it has been shown[20] that the amino group itself need not be present, α-hydroxy-δ-guanidinovalerate being split at a measurable, though slow, rate. There is reason to believe that many other modifications could be made on the α-amino group without destroying susceptibility to tryptic hydrolysis, but little work has been done as yet in this direction.

A contribution of major importance to the understanding of the mode of action of trypsin, was the discovery that this enzyme possesses an esterase as well as an amidase activity[19]. That is, an ester group can be substituted for the terminal amide group, with a resultant large increase in susceptibility to enzymatic hydrolysis. Schwert and Eisenberg[21] tested six esters of benzoyl-L-arginine, and found that they are all hydrolyzed at the same rate, this rate being one hundred fold greater than for benzoyl-L-argininamide. This equality of rates for ester hydrolysis would seem to indicate that, in this case, steric factors, due to the size of the alcohol moiety, are relatively unimportant. Evidence has been presented[19,22] that the esterase and amidase activities of trypsin reside in the same active centers of the molecule.

The only other modifications that have been tested on trypsin substrates are acylations of the terminal amino groups of L-lysine and L-ornithine. Thus it was found[11] that carbobenzoxylation of benzoyl- and benzoylglycyl-L-lysinamides causes almost complete loss of sensitivity to hydrolysis. This result, combined with the fact that norvaline and norleucine derivatives are also resistant to hydrolysis[5], led Neurath and Schwert to the belief that the effect is due to loss of positive charge at the terminal group, rather than to unfavorable steric factors[5]. However, α-benzoyl-L-citrullinamide and α-benzoyl-δ-acetyl-L-ornithinamide, neither of which is positively charged at

pH 7-8, are hydrolyzed by trypsin, while α-benzoyl-L-ornithinamide is resistant. Thus, it would appear from the work of Brand, that steric factors are of major importance, while charge effects are not significant. Until further data are available, this point cannot be settled.

Studies of the optical specificity of trypsin have not as yet been carried out, but it would be predicted, by analogy with other enzyme systems that have been studied, that the α-carbon atom must be of the L- configuration for substrate activity.

Investigations with chymotrypsin substrates have been far more extensive, and allow more adequate comparisons to be made. From the extensive investigations of Neurath and co-workers[5,18,23,24,25] and Niemann and collaborators[17,26,27] it now appears that the decreasing order of affinity of the specific amino acids for chymotryptic hydrolysis is tryptophane > tyrosine > phenylalanine > methionine. Numerous other amino acids have been tested for activity, but the rates of hydrolysis have never been significant. Even if such a small change is made as eliminating the β-carbon atom of phenylalanine, to give α-aminophenylacetic acid, activity is entirely lost. However, if the amino group of phenylalanine is replacedby -OH, -Cl, or omitted entirely, activity is greatly diminished but not eliminated. Also substituents

can be made in the phenyl group with varying effects on activity, but such modifications have not been studied carefully enough to allow any conclusions to be drawn with certainty.

Similarly, as with trypsin substrates, the α- amino group must also be acylated for maximum chymotryptic activity. A wide range of substitutions may be made without causing loss of activity. The known substituents that may be used are benzoyl[9], acetyl[23], glycyl[8], carbobenzoxyl[8], carbobenzoxyglycyl[8], carbobenzoxyglutamyl[14], nicotinyl[17], picolinyl[26], isonicotinyl[26], formyl[26], chloracetyl[28], and triflouracetyl[28]. Undoubtedly, other useful substituents will be found in the near future, as work is being pursued along these lines. On the basis of evidence, that is not too conclusive in all cases, the following tentative order of susceptibility for some of the above groups can be given[5]: benzoyl > nicotinyl > acetyl > carbobenzoxyglycyl > glycyl. Experiments are in progress in these laboratories to determine unambiguously the relative effects of most of the above substituents.

As was early shown, the hydrolyzable bond of low molecular weight chymotrypsin substrates can be amide[13], glycinamide[8], and glycylglycinamide[8], but secondary and tertiary amides have not been found to be split. More recently, it has become known that ester[24], hydrazide[29], and hydroxamide bonds[30] can also be hydrolyzed at

sufficiently high rates to be useful for specificity studies. All available evidence indicates that the same reactive sites on the enzyme molecule are involved in all of these hydrolyses[22]. On the basis of incomplete data, the order of susceptibility to hydrolysis may be tentatively listed as: ester > hydroxamide > glycinamide > amide > hydrazide > glycylglycinamide[5].

Unlike the trypsin case, careful studies have been made on the antipodal specificity of chymotrypsin, and it has been found that only amino acids of the L-configuration satisfy the structural requirements for specific amino acids. However, where the α-amino group is replaced by -Cl or -OH, this antipodal specificity is not retained. For example, both the D- and L- isomers of methyl-DL-α-chloro-β-phenyl-propionate appear to be split at the same rate, and methyl-D-β-phenyllactate is split at a measurable rate, but one that is less than for the L- isomer[29]. These substances, however, cannot be considered as true chymotrypsin substrates as they are hydrolyzed at rates considerably slower than for the corresponding amino acid derivatives, but their study, nevertheless, furnishes further information as to the mode of action of this enzyme.

In an early study of antipodal specificity, Bergmann and Fruton[9] came to the conclusion that while benzoyl-L-tyrosylglycinamide is split by chymotrypsin, neither the D- antipode nor the racemate is. They thus concluded that

the affinity of the L- isomer for the D- isomer was far greater than for the enzyme, and postulated that an extremely stable DL- compound existed in solution. A reinvestigation of this phenomenon[31] showed these results to be erroneous, the L- isomer of benzoyl-DL-tyrosylglycinamide being rapidly split by chymotrypsin, and no interaction between D- and L- isomers was apparent. It was also observed that the D- isomer apparently competes with the L- isomer for the reactive sites on the enzyme molecule, thus causing a decrease in rate over that observed for the L- isomer alone.

This important observation of Niemann and collaborators has led to an extensive investigation of the effect of D- antipodes of substrates on the rates of hydrolysis of the L- antipodes, and it has been shown in all cases that they inhibit in a competitive manner, and have an even greater affinity for the enzyme surfaces than the L- antipodes. This phenomenon will be discussed in greater detail in the following section.

Not only do the D- antipodes of substrates act as competitive inhibitors, but also the products of hydrolysis, as well as certain structural analogues. For example, in the chymotryptic hydrolysis of acetyl- and nicotinyl-L-tryptophanamide, the liberated acetyl- and nicotinyl-L-tryptophane have been shown to cause a decrease in rate, while the simultaneously formed ammonia has no effect[32].

Although inhibition is not great, it is of considerable significance in many cases, and its existence must be taken into consideration.

Kaufman and Neurath[33,34] have investigated a series of inhibitors, and have found that certain amino acids which cannot function as substrates are fairly active as inhibitors. For example, certain derivatives of methionine and possibly glycine are said to be effective. Also if the α-amino or carboxyl group is removed from one of the amino acids specific for chymotrypsin, the substances may function as potent inhibitors; e.g. β-phenylethylamine and β-phenylpropionic acid. This is in agreement with the results of Huang[27], who found tryptamine and three acylated derivatives of tryptamine to be very active inhibitors. In addition, it has been found that if the carboxyl group of a specific amino acid is replaced by a methyl ketone group, the affinity of the resultant compound for the enzyme is very great[34].

Investigations with the above types of specific inhibitors have not as yet been extensive enough to allow for broad generalizations, but it is evident that the structural requirements for competitive inhibitors are not as rigid as for specific substrates. This problem is being actively pursued as it is felt that the study of specific inhibitors offers one of the most promising routes to an understanding of the mode of action of proteolytic enzymes, it being possible to measure the true affinity of a competitive inhibitor for an enzyme.

Kinetics of Enzymatic Reaction - The overall kinetics of enzymatic reactions is usually determined by measurements of either the rate of disappearance of the substrate or the rate of formation of the reaction products, with time as the independent variable.

With the exception of systems that are complicated by inhibition due to the reaction products or the substrate itself, enzymatic reactions are usually characterized by two important features[5]: (1) Over a relatively wide range of enzyme concentrations, the rate, at constant initial substrate concentration, is directly proportional to the enzyme concentration. (2) If the enzyme concentration is maintained constant, the reaction rate increases with increasing initial substrate concentration according to the equation of a rectangular hyperbola, approaching a maximum value asymptotically. These relations were recognized early in the study of enzymatic reactions and led Michaelis and Menten[35] to formulate a theory in terms of an intermediate enzyme-substrate complex. This theory, with its subsequent extensions[36-42], has proven very satisfactory in the interpretation of the kinetics of enzymatic reactions. The following treatment of the theory is essentially that given by Huang and Niemann[32].

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

Where $[E]$ = molar concentration of total enzyme,

$[E_f]$ = " " " free enzyme,

$[S]$ = " " " total substrate,

$[S_f]$ = " " " free substrate,

$[ES]$ = " " " enzyme-substrate complex,

and $[P_{1f}], [P_{2f}]$ = " " " the two free hydrolysis products, it can be shown that for the case where all reactants possess unit activity

$$\frac{d[ES]}{dt} = k_1 [E_f] [S_f] - (k_2 + k_3) [ES], \quad (2)$$

$$- \frac{d[S_f]}{dt} = k_1 [E_f] [S_f] - k_2 [ES], \text{ and} \quad (3)$$

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

For the condition that

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

i.e., where $\frac{d[S]}{dt} \gg \frac{d[ES]}{dt}$ and $[S] \doteq [S_f] \gg [ES]$,

it follows that

$$\frac{k_2 + k_3}{k_1} = \frac{([E] - [ES])\ S}{[ES]} = K_s, \quad (6)$$

$$- \frac{d[S]}{dt} = \frac{k_3 [E] [S]}{K_s + [S]}, \quad (7)$$

$$k_3 [E]\ t = 2.3\ K_s \log \frac{[S]_o}{[S]} + ([S]_o - [S]), \quad (8)$$

where $[S] = [S]_o$ when $t = 0$.

The constant, K_s, appearing in equations (6), (7), and (8) is the so-called Michaelis constant, and can be evaluated from the relation

$$\frac{1}{v} = \frac{K_s}{V}\left(\frac{1}{[S]}\right) + \frac{1}{V} \quad , \tag{9}$$

where $V = k_3 [E]$ = the maximum velocity which is reached when all of the enzyme is bound in the enzyme-substrate complex, and $v = -\frac{d[S]}{dt}$, by the customary plot of $\frac{1}{v_o}$ versus $\frac{1}{[S]_o}$ (43).

The above relationships hold only if there is no inhibition due to the products of hydrolysis. However, competitive inhibition is often apparent, and the equations must be modified accordingly. Only one of the products of hydrolysis will be considered as it has been shown(32,44) that moderate concentrations of added ammonia produce no effect upon the rates of hydrolysis of the acylated amino acid amide substrates dealt with in the present studies with trypsin and chymotrypsin.

For the reaction system

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

$$E_f + P_{1f} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EP_1 \tag{10}$$

where $[P_{1f}]$ = molar concentration of free inhibitory hydrolysis product and $[EP_1]$ = molar concentration of enzyme-inhibitor complex, with the condition as before that

$\frac{d\,[ES]}{dt} \doteq 0$, and that $[S] \doteq [S_f]$ and $[P_1] \doteq [P_{1f}]$, it follows[37-45] that

$$\frac{k_2 + k_3}{k_1} = \frac{([E] - [ES] - [EP_1])\,[S]}{[ES]} = K_s, \qquad (11)$$

$$\frac{k_5}{k_4} = \frac{([E] - [ES] - [EP_1])\,([S]_o - [S])}{[EP_1]} = K_{P_1}, \qquad (12)$$

$$-\frac{d\,S}{dt} = \frac{k_3\,[E][S]}{K_s\left(1 + \frac{([S]_o - [S])}{K_{P_1}}\right) + [S]}, \text{ and} \qquad (13)$$

$$k_3[E]t = 2.3\,K_s\left(1 + \frac{[S]_o}{K_{P_1}}\right)\log\frac{[S]_o}{[S]} + \left(1 - \frac{K_s}{K_{P_1}}\right)([S]_o - [S]). \qquad (14)$$

The constant, K_{P_1}, appearing in equations (12), (13), and (14) can be evaluated, as before, by plots of $\frac{1}{v_o}$ versus $\frac{1}{[S]_o}$, taking advantage of equation (15).

$$\frac{1}{v} = \frac{K_s}{V}\left(1 + \frac{[P_1]}{K_{P_1}}\right)\frac{1}{[S]} + \frac{1}{V} \qquad (15)$$

While it has been shown[32,44] that, within the limits of experimental error, equation (14) is a satisfactory rate equation for certain enzyme catalyzed hydrolyses, it should be remembered that in the derivation, in addition to the assumption that $\frac{d\,[ES]}{dt} \doteq 0$, it was also assumed that

$$K_s = \frac{([E] - [ES])\,[S]}{[ES]} \text{ and } K_{P_1} = \frac{([E] - [ES] - [EP_1])\,[P_1]}{[EP_1]}$$

In view of the fact that the exact relationships are

$$K_s = \frac{([E] - [ES])\,[S_f]}{[ES]} \quad \text{and} \quad K_{P_1} = \frac{([E]-[ES]-[EP_1])\,[P_{1f}]}{[EP_1]},$$

it is apparent that the second assumption is valid only if $[S] \doteq [S_f] \gg [ES]$ and $[P_1] \doteq [P_{1f}] \gg [EP_1]$, or, in the terminology of Straus and Goldstein[40,41], if the systems under consideration are in zone A. Straus and Goldstein have shown that for a system containing enzyme and substrate, or enzyme, substrate, and inhibitor, if the maximum experimental error in the measurement of $\frac{v}{V}$ is taken as 0.01, the upper boundary of zone A corresponds to a specific enzyme concentration

$$E_s^1 = \frac{[E]}{K_s} \quad \text{or} \quad E_{P_1}^1 = \frac{[E]}{K_{P_1}}, \text{ of 0.1.}$$

The systems to be described in the following section will be shown to be in zone A, thus assuring the validity of the above assumptions.

When an inhibitor, e.g., the D-antipode of a specific substrate is knowingly added to an enzyme-substrate system, it is reasonable to postulate, on the basis of present knowledge, the following equilibria:

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

$$E_f + P_{1f} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EP_1 \qquad (10)$$

$$E_f + I_f \underset{k_7}{\overset{k_6}{\rightleftharpoons}} EI \qquad (16)$$

With $[I]$ = molar concentration of added inhibitor, $[EI]$ = molar concentration of the enzyme-added inhibitor complex, $\frac{d[ES]}{dt} \doteq 0$, $[S_f] \doteq [S]$, $[P_{1f}] \doteq [P]$, and $[I_f] = [I]$, it follows that

$$\frac{k_2 + k_3}{k_1} = \frac{([E] - [ES] - [EP_1] - [EI])\,[S]}{[ES]} = K_s , \qquad (17)$$

$$k_5/k_4 = ([E] - [ES] - [EP_1] - [EI])\,([S]_o - [S])/[EP_1] = K_{P_1} \qquad (18)$$

$$k_6/k_7 = ([E] - [ES] - [EP_1] - [EI])\,[I]/[EI] = K_I \qquad (19)$$

$$\frac{-d[S]}{dt} = \frac{k_3 [E]\,[S]}{K_s \left(1 + \frac{[I]}{K_I} + \frac{[S]_o}{K_{P_1}} + [S]\right)} , \text{ and} \qquad (20)$$

$$k_3[E]t = 2.3\, K_s \left(1 + [I]/K_I + [S]_o/K_{P_1}\right) \log [S]_o/[S] + \left(1 - K_s/K_{P_1}\right)([S]_o - [S]) \qquad (21)$$

In the absence of significant inhibition by one of the hydrolysis products, or for initial rates, equations (20) and (21) are reduced to

$$-d[S]/dt = k_3 [E]\,[S]/K_s (1 + [I]/K_I) + [S] , \text{ and} \qquad (22)$$

$$k_3[E]t = 2.3\, K_s (1 + [I]/K_I) \log [S]_o/[S] + ([S]_o - [S]). \qquad (23)$$

The constant, K_I, appearing in equations (22) and (23) can be evaluated as before by plots of $1/v_o$ versus $1/[S]_o$, making use of equation (24).

$$\frac{1}{v} = \frac{K_S}{V}\left(1 + \frac{[I]}{K_I}\right)\frac{1}{[S]} + \frac{1}{V} \qquad (24)$$

The above kinetic treatment will be shown to be valid, within experimental error, for the systems to be described in the pages to follow.

Discussion of Results

A. Trypsin Studies - As has been mentioned previously, it is known that acylated amides of L-arginine, where the acyl group is benzoyl, toluenesulfonyl, or benzoylglycyl, are rapidly split by trypsin at pH 7-8. The early kinetic studies with these substrates[12,19,46] led to the conclusion that the reactions are first-order with respect to both enzyme and substrate, and that for any single experiment, the rate of hydrolysis can be described by the equation

$$-\frac{dS}{dt} = CES, \qquad (25)$$

where E = the total enzyme concentration, S = the substrate concentration, t = the time, and C = the proteolytic coefficient[47] of the system.

A reinvestigation, by Harmon and Niemann[44], of the kinetics of the system, benzoyl-L-argininamide-trypsin, over a greater range of initial substrate concentrations, revealed that the initial reaction velocity is essentially independent of the initial substrate concentration, for concentrations from 0.01 - 0.05 M, and that, for these and smaller substrate concentrations, the initial reaction rates obey equation (7)*.

$$-\frac{d[S]}{dt} = \frac{k_3 [E][S]}{K_s + [S]} \qquad (7)$$

---

* See page 32ff of the previous section for the derivation of equations.

Thus, by use of the methods previously discussed, these authors were able to calculate an apparent K_s for benzoyl-L-argininamide of approximately 2.0×10^{-3} M. They also noted that the reaction rates decreased more rapidly with time than would be expected, and this observation led to the demonstration of inhibition by the hydrolysis product, benzoyl-L-arginine, but not by ammonia. It was shown that their data are consistent with equation (13), if a K_{P_1} of approximately 1.0×10^{-3} M is assumed for benzoyl-L-arginine.

In an independent investigation, Schwert and Eisenberg[21] obtained similar results. They demonstrated that K_s for benzoyl-L-argininamide is small enough, so that, at substrate concentrations of the order of 0.01 M, the reaction initially follows zero-order kinetics*, and that deviations from zero-order kinetics are the result of inhibition by benzoyl-L-arginine. This inhibition was shown to be of an intermediate type, being essentially non-competitive at low concentrations of added benzoyl-L-arginine, but becoming more competitive as the concentration of added benzoyl-L-arginine is increased.

As a continuation of the experiments of Harmon and Niemann, it was felt desirable to undertake a more extensive kinetic study of trypsin-catalyzed hydrolyses, and to investigate systematically the effect of substituents at the α-amino group on the rate of hydrolysis of L-argininamide.

---

* Inspection of equations (8), (14), (21), and (23) shows both a zero-order and a first-order term. If the initial substrate concentration is significantly larger than the value of K_s, the reaction should closely approximate zero-order kinetics, at least during the early stages of hydrolysis.

Also, due to the difficulty of accurately measuring the K_S value of benzoyl-L-argininamide, and the resultant disagreement as to the nature of the inhibition by benzoyl-L-arginine, it was hoped that a more suitable substrate might be found. The fact that benzoyl-L-arginine has a very low solubility at 25° C. and pH 8, limits the usefulness of benzoyl-L-argininamide as a substrate.

Consequently, a program was undertaken to synthesize the known trypsin substrates, as well as the amides of acetyl-, benzenesulfonyl- , carbamyl- , and formyl-L- and D-arginine, none of which has previously been recorded in the literature.

Upon completion of the synthesis of acetyl-L-argininamide, this substance was immediately tested as a substrate, and was found to be rapidly split by trypsin at 25° C. and pH 7.7. The rate followed apparent first-order kinetics (eq. 25), as can be seen from the results of a typical hydrolysis given in Table I and Fig. I. However, in attempting to duplicate the results of a given hydrolysis, it was found that there was a large variation in the calculated first-order rate constants, far greater than could normally be ascribed to experimental error. Therefore, it was deemed wise to turn to the system benzoyl-L-argininamide-trypsin, for which rate constants had previously been determined[12,19,46,48], and thus perform a check on experimental technique.

Due to the small amount of trypsin required for each hydrolysis, the practice in use at this time was to prepare a stock aqueous solution of enzyme (pH approx. 4.8), and

then to withdraw 1 ml. aliquots for each hydrolysis. Such a stock solution was kept in the ice box, except for the times at which it was brought to 25° C for withdrawal of individual aliquots. It was soon discovered, that for a given initial substrate concentration, the first-order rate constants decreased significantly with the age of the trypsin solution. However, the constants determined with fresh enzyme solutions (10-20 min. old) agreed well with some of the values given in the literature(12,19).

It had previously been shown(19,49) that trypsin is rapidly inactivated by a second-order autolytic reaction at pH 7-8, but the degree of instability at pH 5 had not been recognized. In Table II and Fig. II are given the results of an experiment with the system benzoyl-L-arginamide-trypsin, which illustrate this loss of proteolytic activity of trypsin solutions at approximately pH 4.8. The age given for the trypsin solution is the approximate length of time this solution remained at 25° C before being used.

A rough calculation of the rate of inactivation of trypsin at pH 4.8 and 25° C indicated the reaction to be intermediate between first-order and second-order. This is the result to be expected if the inactivation process is due to both autolysis and denaturation. The data, however, are not quantitative enough to be at all conclusive.

After recognizing this instability of trypsin solutions, it was possible to obtain fairly satisfactory results by

using only fresh solutions. It was thus possible to semi-quantitatively compare the relative rates of hydrolysis of benzoyl-L-argininamide and acetyl-L-argininamide at a given enzyme and initial substrate concentration. In Table III and Fig. III are given the data for one such experiment, and it can be seen that the specific first-order rate constant for acetyl-L-argininamide is approximately 1.9 times greater than for benzoyl-L-argininamide, at an initial substrate concentration of 0.01 M. A comparison of first-order rate constants is not conclusive, for the reasons already given, but such a comparison does offer an indication of relative rates at a given substrate and enzyme concentration.

It was also possible to demonstrate qualitatively inhibition due to the products of hydrolysis of acetyl-L-argininamide. In Table IV and Fig. IV are given the data for the hydrolysis of acetyl-L-argininamide with added acetyl-L-arginine plus ammonia. These data are to be compared with those of Table I. No attempt has been made to estimate the magnitude of this inhibition, as the data are not extensive enough.

Further kinetic studies of tryptic hydrolyses were not attempted as it was felt that the instability of trypsin introduces too great a source of difficulty and error, while chymotrypsin, which remains stable in solution for relatively long periods of time, is a more suitable enzyme for studies of proteolytic hydrolyses.

However, before leaving the discussion of tryptic hydrolysis, one further observation of interest should be mentioned. It was found that if a trypsin solution of pH 4.0 was added to a solution of arginine methyl ester, also of pH 4.0, extremely rapid hydrolysis occurred. This observation was surprising, as it had previously been shown[19] that the methyl esters of benzoyl-L-arginine and toluene-sulfonyl-L-arginine fail to be split at pH<5. A pH - activity curve has not been determined for L-arginine methyl ester-trypsin, but it has recently been reported[50] that the pH optimum for the chymotryptic hydrolysis of L-tyrosine ethyl ester is 6.2, which is to be compared with the pH optimum of 7.8 for the chymotryptic hydrolysis of benzoyl-L-tyrosine ethyl ester[24]. It has also been claimed[22] that these two esters of L-tyrosine are hydrolyzed at comparable rates at their respective pH maxima.

The data for a representative hydrolysis of L-arginine methyl ester at 25° C and pH 4.0 are given in Table V and Fig. V. The course of the hydrolysis was followed by using the continuous titration procedure of Neurath *et al* [19], and no correction was made for the changes in volume upon addition of standard alkali. However, the data do satisfactorily illustrate the rapidity of hydrolysis at this pH.

TABLE I

The Hydrolysis of Acetyl-L-Argininamide
by Trypsin at 25° C and pH 7.7

S_o = 10 μM/ml

E = 0.0187 mg. enzyme N/ml

t (min.)	% Hydrolysis	S	log S
0	0	10.00	1.000
2.5	4	9.60	.982
5.0	6	9.40	.972
10.5	14	8.60	.934
20.5	24.3	7.57	.878
30.0	32.0	6.80	.832
40.5	43.5	5.65	.751
60.0	58.5	4.15	.618

FIG. I

Hydrolysis of Acetyl-L-Argininamide
by Trypsin at 25° C. and pH 7.7

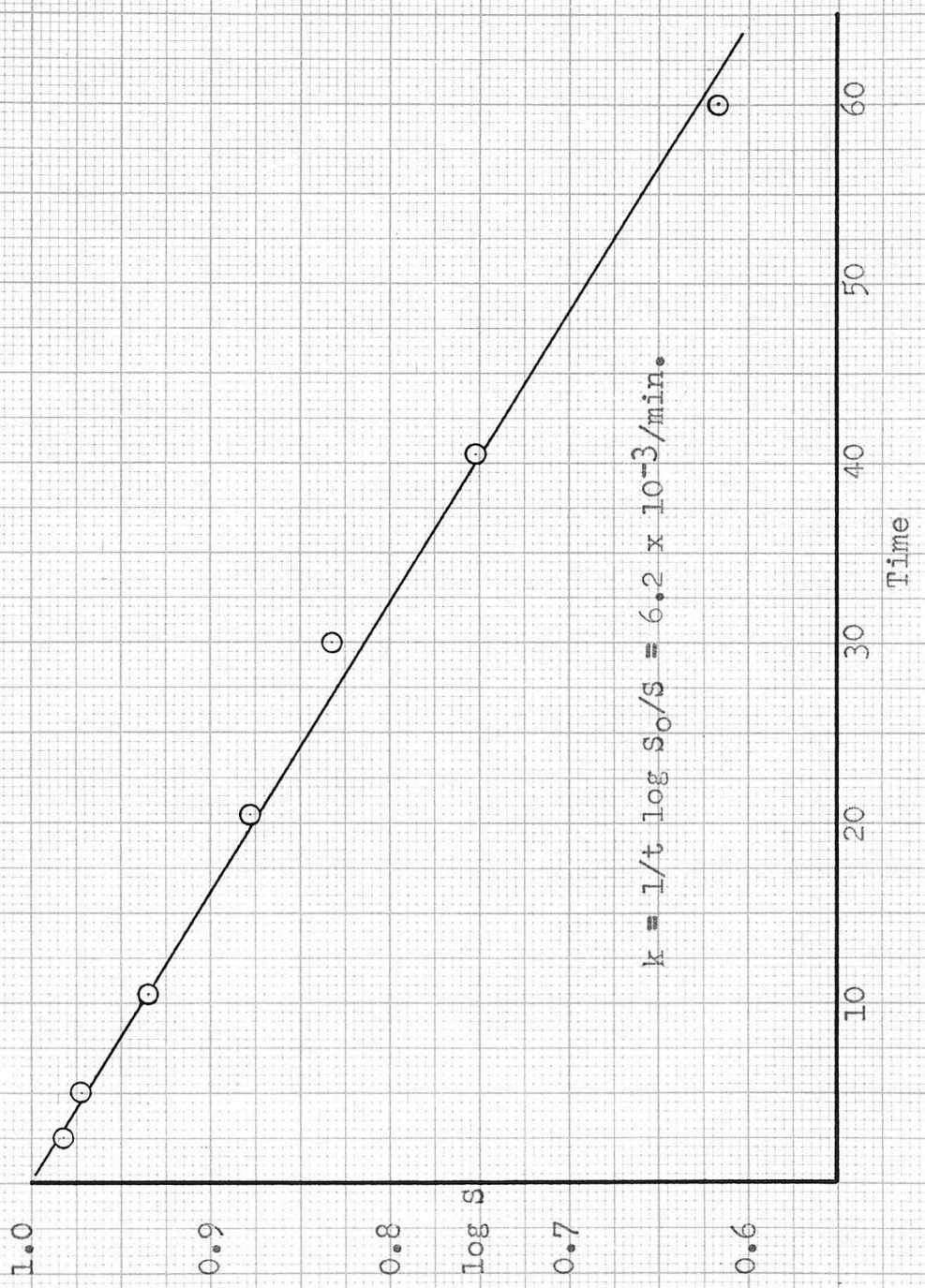


TABLE II

Hydrolysis of Benzoyl-L-Argininamide
by Trypsin at 25° C. and pH 7.8

S_o = 10μM/ml

E = 0.055 mg. enzyme N/ml

Age of Trypsin Solution (hrs)	t (min.)	S	log S
0.2	0	10.00	1.000
	3.0	9.75	.989
	10.5	7.75	.890
	24.0	5.80	.763
	35.0	4.55	.658
	45.5	3.60	.556
2.6	0	10.00	1.000
	3	9.50	.978
	11	7.90	.898
	22	6.30	.800
	33	4.90	.690
	45	4.00	.602
5.1	0	10.00	1.000
	3.0	9.45	.975
	11.0	8.20	.914
	22.0	6.55	.816
	33.5	5.60	.748
	43.0	4.70	.672
27.0	0	10.00	1.000
	2	9.80	.991
	11	9.15	.961
	19	8.20	.914
	28.5	7.50	.875
	38.5	6.70	.826

FIG. II

Hydrolysis of Benzoyl-L-Argininamide
by Trypsin at 25° C. and pH 7.8

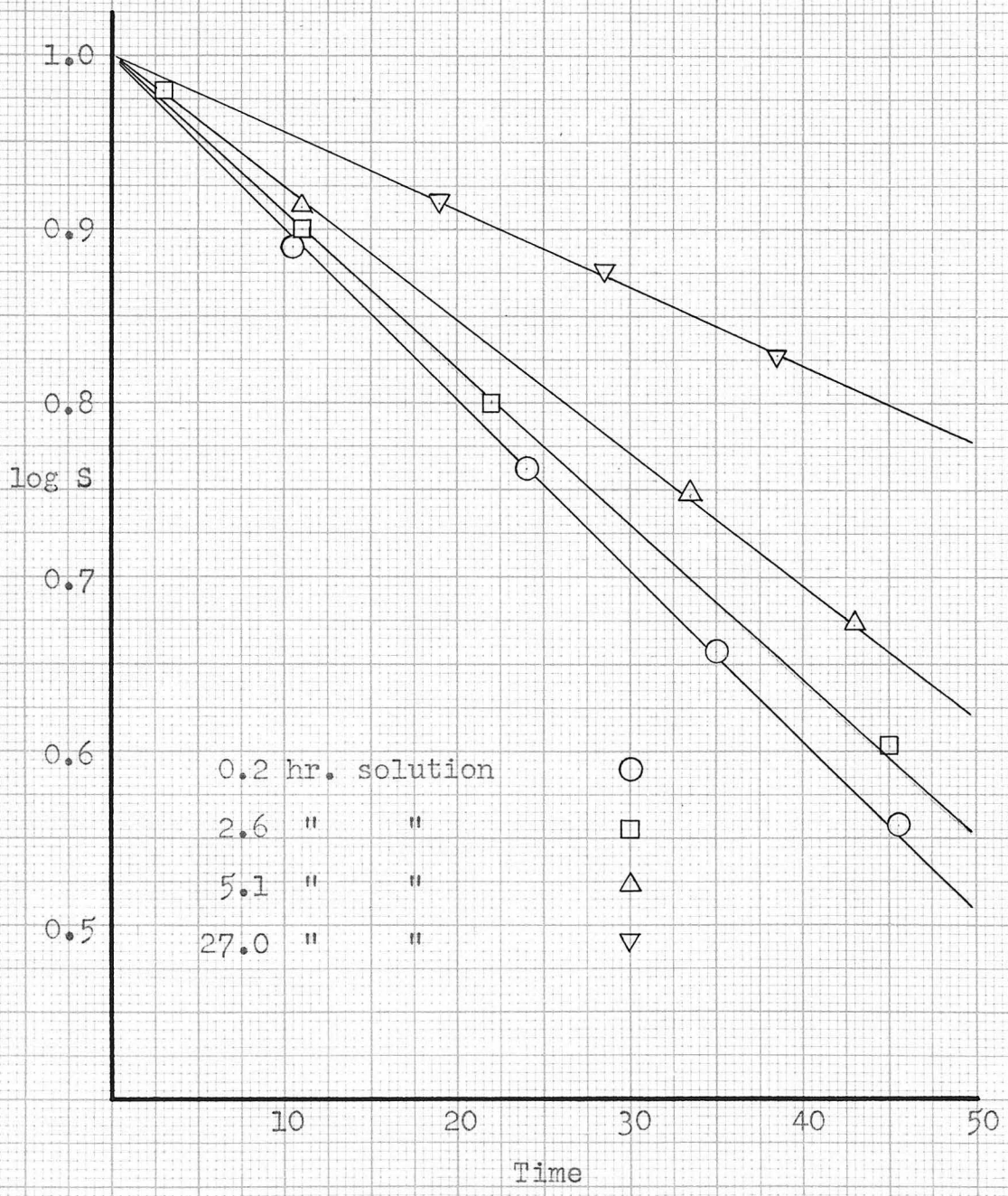


TABLE III

Relative Rates of Hydrolysis of
Benzoyl- and Acetyl-L-Argininamides
by Trypsin at 25° C and pH 7.7

S_o = 10 μM/ml.

E = 0.0187 mg. enzyme N/ml.

	t (min.)	% Hydrolysis	S	log S
	0	0	10.00	1.000
	2.5	4.0	9.60	.982
	5.0	6.0	9.40	.972
Acetyl	10.5	14.0	8.60	.934
	20.5	24.3	7.57	.878
	40.5	43.5	5.65	.751
	60.0	58.5	4.15	.618
	0	0	10.00	1.000
	1.5	1.0	9.90	.995
	5.0	7.5	9.25	.966
	10.0	8.0	9.20	.963
	20.0	16.0	8.40	.924
Benzoyl	30.0	20.1	7.99	.902
	40.0	25.5	7.45	.872
	60.0	35.0	6.50	.812
	90.0	49.0	5.10	.707

FIG. III

Hydrolysis of Benzoyl- and Acetyl-L-Argininamide by Trypsin at 25° C. and pH 7.7

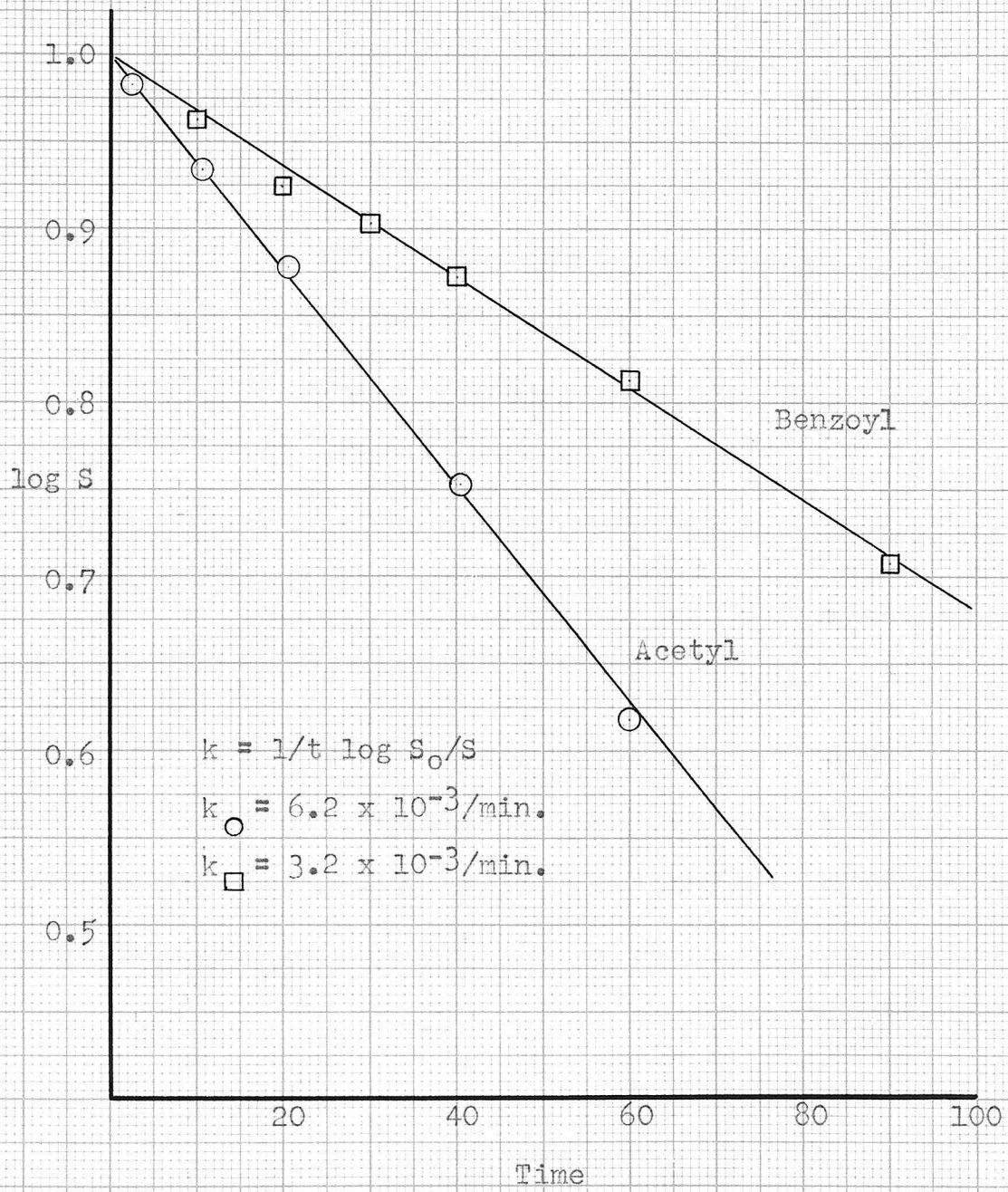


TABLE IV

Inhibition of Hydrolysis of Acetyl-L-Argininamide by the Products of Hydrolysis

S_o = 10 μM/ml.

P_1 = 10 μM/ml.

P_2 = 10 μM/ml.

E = 0.0187 mg enzyme N/ml.

t (min.)	% Hydrolysis	S	log S
0	0	10.00	1.000
2.0	3.0	9.70	.987
10.0	10.6	8.94	.951
30.0	29.9	7.01	.846
40.0	37.7	6.23	.794
60.0	44.9	5.51	.741
75.0	52.9	4.71	.672

FIG. IV

Inhibition of Hydrolysis of Acetyl-L-Argininamide
by the Products of Hydrolysis

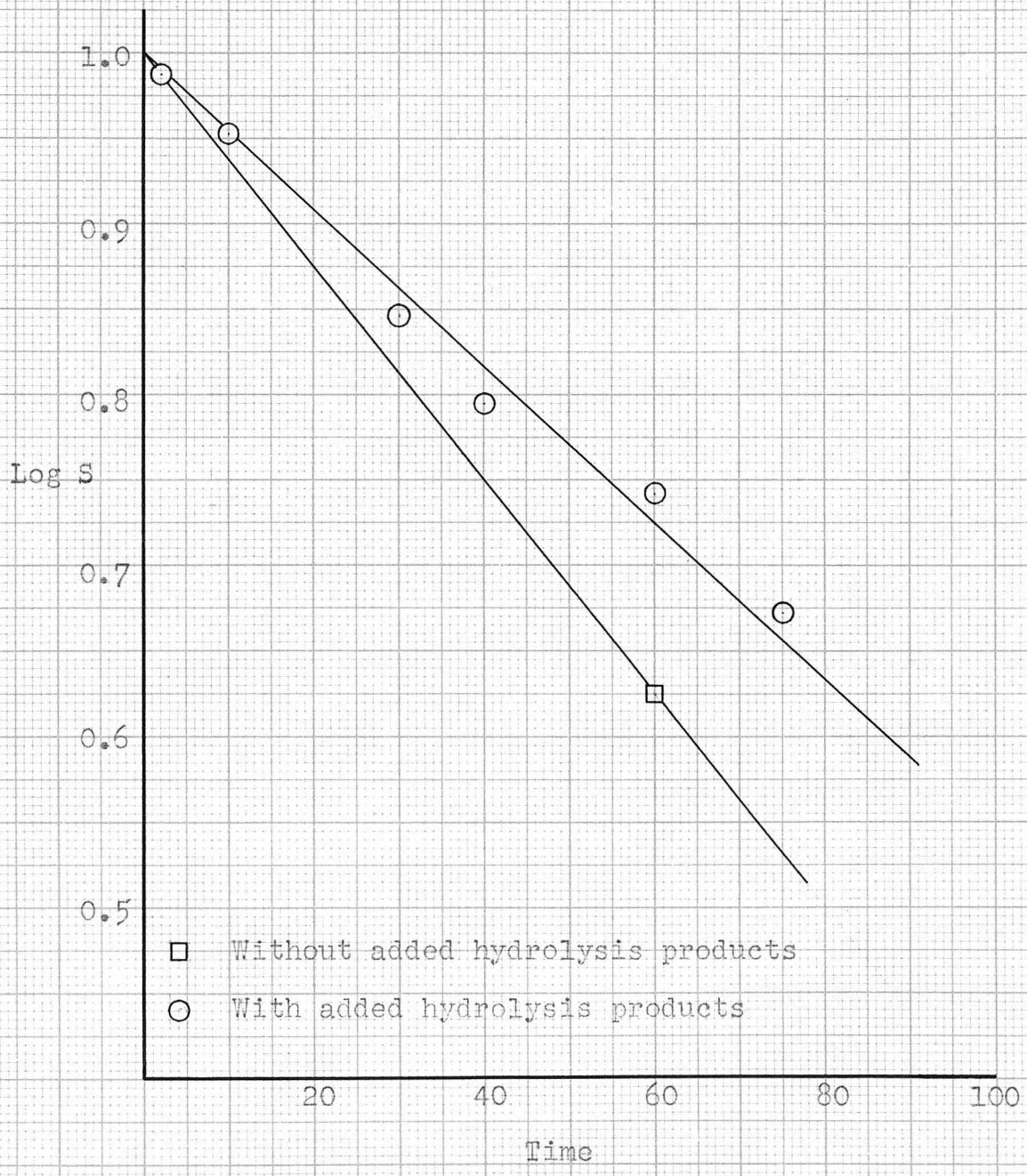


TABLE V

Hydrolysis of L-Arginine Methyl Ester
by Trypsin at 25° C and pH 4.0

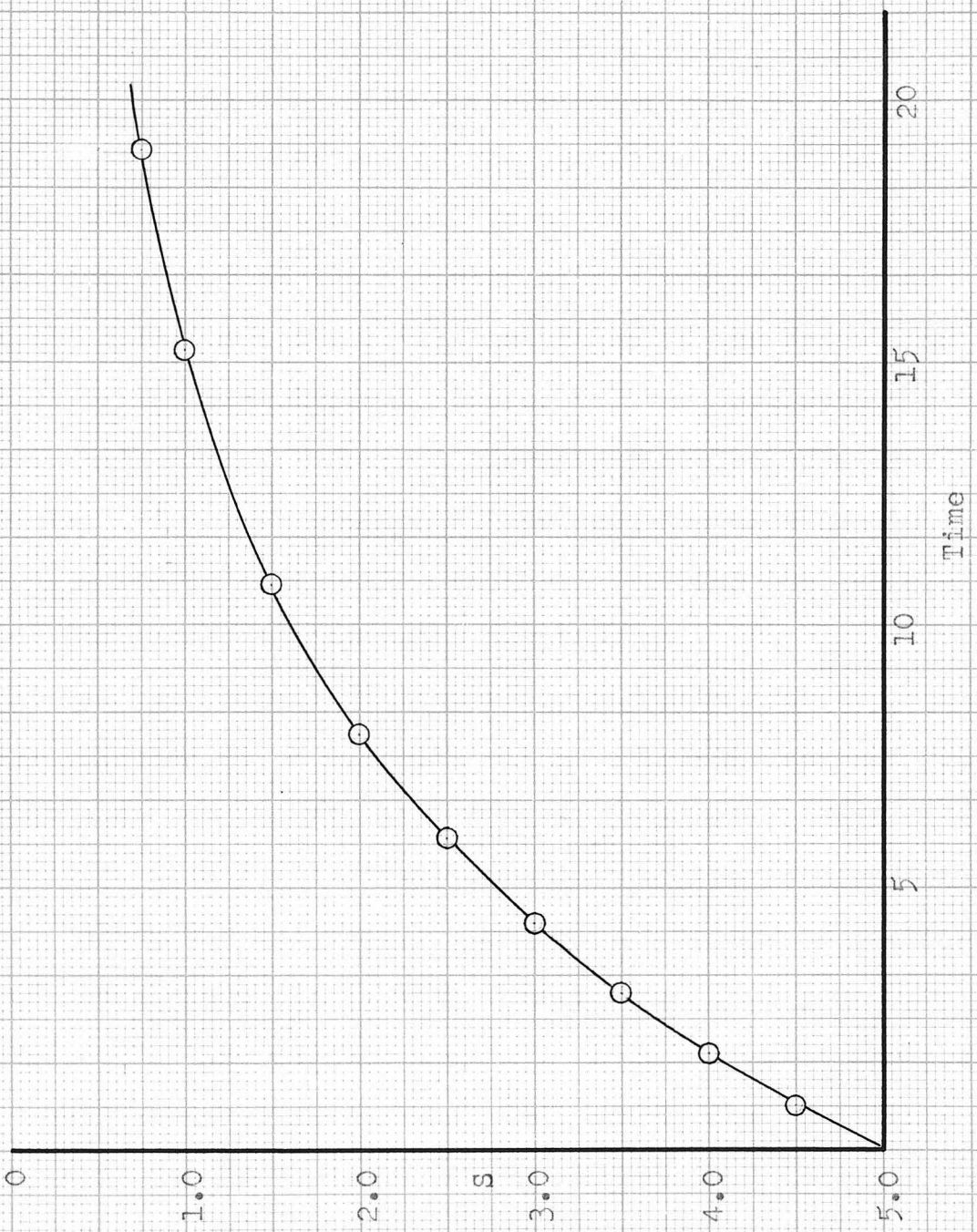
S_o = 5 μM/ml

E = 0.092 mg enzyme N/ml

t	S
0	5.0
50"	4.5
1'50"	4.0
3'	3.5
4'20"	3.0
5'55"	2.5
7'53"	2.0
10'47"	1.5
15'15"	1.0
19'5"	.75

FIG. V

Hydrolysis of L-Arginine Methyl Ester
by Trypsin at 25° C. and pH 4.0



B. Chymotrypsin Studies - As part of the program that is being actively pursued in these laboratories to determine the mode of action of proteolytic enzymes, the kinetics of the α-chymotrypsin catalyzed hydrolysis of acetyl- and nicotinyl-L-tyrosinamide, at 25° C. and pH 7.8-8.0, has been carefully examined. In these studies consideration was given to (a) the effect of the buffer components on the reaction system; (b) the stability of the enzyme preparations; (c) the dependence of the activity of the enzyme upon the pH of the reaction system; (d) inhibition of the hydrolytic reaction by the hydrolysis products; (e) the development of suitable rate expressions, and the determination of rate constants; (f) interpretation of the reaction kinetics in terms of a probable reaction mechanism; and (g) the inhibition of the substrates by their D-antipodes.

It has been shown[51] that α-chymotryptic activity, in systems buffered at pH 7.8 with either a tris(hydroxymethyl)-aminomethane-hydrochloric acid buffer, or an ethylenediamine-hydrochloric acid buffer, is independent of the nature of the buffer. Also, the activity is independent of the concentration of the above buffers, when varied from 0.005 M to 0.05 M with respect to the amine component. In the present studies only the two above buffers were used, and in the concentration of 0.02 M, and thus, it can be assumed that the only function of the buffers was to maintain a constant pH. It will be shown later that K_s, for acetyl-L-tyrosinamide, is

independent, within experimental error, of the buffer employed.

Since the practice employed was to prepare stock aqueous solutions of chymotrypsin, and then to store them at 4° C. between experiments, it was felt necessary to test the stability of such solutions. Consequently, the activity of a fresh solution was determined at pH 7.9 with acetyl-L-tyrosinamide as substrate, and then redetermined after 7 days storage in the ice-box. As can be seen from Table VI and Fig. VI, there was no change in activity during this period. Therefore, since stock enzyme solutions are rarely kept over 24 hours, it is safe to assume constancy of activity.

Extensive studies have not been made on the effect of pH on the α-chymotryptic hydrolysis of synthetic substrates, but there is evidence that the maximum activity is not exhibited at the same pH with all substrates[32,50]. Therefore, so-called pH-activity curves have been determined for both acetyl- and nicotinyl-L-tyrosinamide in aqueous solution at 25° C. As can be seen from the data in Tables VII and VIII and Figs. VII and VIII, maximum activity is obtained, with both substrates, in the region between pH 7.8-8.0, the region in which all the following experiments were performed. These pH-activity curves are similar to those obtained by Kaufman, Neurath and Schwert[24] for α-chymotrypsin-benzoyl-L-tyrosinamide and α-chymotrypsin-benzoyl-L-tyrosine ethyl ester in 30 volume % aqueous-ethanol at 25° C. The fact that the pH-activity curves observed with

substrates derived from L-tyrosine are different from those observed with substrates derived from L-tryptophane[32], suggests that the nature of the characteristic amino acid side chain, even though it bears no formal charge, is of considerable importance in determining the nature of the pH-activity curve.

As a first approximation, the kinetics of hydrolysis of acetyl- and nicotinyl-L-tyrosinamide were formulated in terms of equations (1-9), and K_S values determined for each substrate by use of equation (9). Initial velocities were either estimated from a plot of $([S]_o - [S])$ versus time, or from a plot of log $([S]_o - [S])$ versus time, depending on whether the early stages of the hydrolysis more closely approximated zero order or first order kinetics with respect to the substrate concentration. Five* independent determinations, with three different enzyme concentrations and two different buffer systems, gave a mean value of

$$K_S = 30.5 \pm 1.0 \times 10^{-3} \text{ M}$$

for acetyl-L-tyrosinamide, and three* independent determinations, with two different enzyme concentrations and two different buffer systems, gave a mean value of

$$K_S = 15.0 \pm 0.5 \times 10^{-3} \text{ M}$$

---

* One determination made by R. V. MacAllister[26,52].

for nicotinyl-L-tyrosinamide. Representative data from these determinations are given in Tables IX-XI and Fig. IX-XIV.

In the absence of competitive inhibition of the hydrolytic reaction by the hydrolysis products, the rate is given by eq. (8).

$$k_3 [E] t = 2.3 K_s \log \frac{[S]_o}{[S]} + ([S]_o - [S]) \qquad (8)$$

Provided $d[ES]/dt \doteq 0$ and $[S_f] \doteq [S] >> [ES]$, this equation can be taken as the exact rate expression for the reaction given in eq. (1)[42], and the approximate rate expression for the initial stages of the two simultaneous reactions described by eqs. (1) and (10). The data obtained from experiments, in which the reactions of acetyl-L-tyrosinamide and nicotinyl-L-tyrosinamide were allowed to proceed to near completion, were tested for congruity with eq. (8). It can be seen from Tables XII and XIII, and Figs. XV and XVI, where $F(S) = 2.3 K_s \log [S]_o/[S] + ([S]_o - [S])$, that, after approximately 50% hydrolysis, the reaction velocity for acetyl-L-tyrosinamide is less than that expected on the basis of eq. (8), while the reaction velocity for nicotinyl-L-tyrosinamide is within experimental error of that to be expected on the basis of this equation. Thus, it would appear that there is probably competitive inhibition due to the products of hydrolysis, at least in the case of acetyl-L-tyrosinamide.

The fact that the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide, at 25° and pH 7.8-8.0, is competitively inhibited by acetyl-L-tyrosine* is obvious from the data given in Tables XIV and XV, and Fig. XVII. K_{P_1} was evaluated by the use of eq. (15), from the usual plot of $1/v_o$ versus $1/S$[43], and a mean value of $K_{P_1} = 115 \pm 15 \times 10^{-3}$ M was obtained. Preliminary results of Dr. H. T. Huang[27] show that K_{P_1} for nicotinyl-L-tyrosine is approximately 60×10^{-3} M.

With the above values of K_S and K_{P_1} for acetyl-L-tyrosinamide and acetyl-L-tyrosine, and with the same experimental data as are given in Table XII, the values of

$$F(S) = 2.3\ K_S \left(1 + \frac{[S]_o}{K_{P_1}}\right) \log \frac{[S]_o}{[S]} + \left(1 - \frac{K_S}{K_{P_1}}\right)([S]_o - [S])$$

were calculated (Table XVI), and then plotted as a function of time (Fig. XVIII). It is clear that the experimental data are in excellent agreement with those predicted on the basis of equations (1), (10) and (14), and thus, equation (14) is a satisfactory rate equation for the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide at 25° C. and pH 7.8-8.0. The fact that eq. (8), rather than eq. (14), satisfactorily describes the hydrolysis of nicotinyl-L-tyrosinamide, is due to the difficulty of experimentally detecting inhibition by the hydrolysis products when the K_{P_1} values relatively are high.

---

* As has been mentioned previously, only the liberated acylated amino acids, and not the ammonia, inhibit chymotryptic activity[32].

The k_3 values for the above reactions were evaluated either from eq. (8), or for initial rates, from eq. (14)*, and a mean value of $k_3 = 2.4 \pm 0.1 \times 10^{-3}$ moles/liter/min./mg. enzyme N/ml. was obtained for acetyl-L-tyrosinamide, and a mean value of $k_3 = 6.2 \pm 0.2 \times 10^{-3}$ moles/liter/min./mg. enzyme N/ml. was obtained for nicotinyl-L-tyrosinamide. Representative data are given in Tables IX - XI and Figs. XIX and XX.

On the basis of previous observations it was expected that the α-chymotrypsin catalyzed hydrolysis of acetyl- and nicotinyl-L-tyrosinamide would be competitively inhibited by the D-antipodes. This was indeed found to be the case, and the dissociation constants, K_I, were calculated with the use of eq. (24), giving a value of $K_I = 12.0 \pm 1.0 \times 10^{-3}$ M for acetyl-D-tyrosinamide**, and a value $K_I = 6.4 \times 10^{-3}$ M for nicotinyl-D-tyrosinamide, at 25° C. and pH 7.8-8.0. Data for these determinations are given in Table XVII, and Figs. XXI and XXII.

From the data given in Tables XVIII-XX, and Fig. XXIII, it will be seen that acetyl-D-tyrosine ethyl ester is also a competitive inhibitor of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide, and in this case the dissociation constant, K_I, was found to be $3.5 \pm 0.5 \times 10^{-3}$ M

---

* This practice is permissible in this particular case because of the relatively large values of K_{P_1}.

** The primary data for inhibition by acetyl-D-tyrosinamide were obtained by R. V. MacAllister[26]. These data were recalculated using K_S, for acetyl-L-tyrosinamide, equal to 30.5×10^{-3} M.

at 25° C. and pH 7.8-8.0. Preliminary results of Dr. H. T. Huang[27] indicate that K_I for nicotinyl-D-tyrosine ethyl ester is approximately 1.0×10^{-3} M.

As has been mentioned previously (p. 35), in the derivations of the equations used to describe the kinetics of the above hydrolyses, in addition to the assumption that $d[ES]/dt \doteq 0$, it was also assumed that $[S] \doteq [S_f] \gg [ES]$, $[P_1] \doteq [P_{1f}] \gg [EP_1]$, and $[I] \doteq [I_f] \gg [EI]$, these latter assumptions being valid only if the systems under consideration are in zone A. Since an error of 0.01, for values of v/V, appears to be acceptable for these experiments, the upper boundary of zone A corresponds to a specific enzyme concentration, $E'_S = [E]/K_S$, or $E'_{P_1} = [E]/K_{P_1}$, or $E'_I = [E]/K_I$, of 0.1. As can be seen from Table XXI, the maximum specific enzyme concentrations never exceeded this value. Therefore, it can be concluded that the experimental conditions used in these investigations were such as to ensure the validity of the assumptions made above.

With the information now available, it is apparent that the α-chymotrypsin catalyzed hydrolysis of both acetyl- and nicotinyl-L-tyrosinamide (as well as of acetyl- and nicotinyl-L-tryptophanamide[32]), at 25° C. and pH 7.8-8.0, can be described in terms of the classical Michaelis-Menten theory. The observed dependence of the initial velocities upon the initial substrate concentrations, and the biphasic course

of the hydrolysis of the above substrates (cf. Tables IX-XI, and Figs. XXIV and XXV) provide further evidence in support of this formulation. It should be pointed out, as is evident from equations (8), (14) and (21), that one effect of competitive inhibition by the hydrolysis products, or by the D-antipode of a substrate, is to increase the importance of the first-order term. Thus, with the above types of competitive inhibition, apparent congruity to a first-order rate expression may be observed throughout the course of the reaction[18,24,25], though more extended observations would reveal the biphasic character of the reaction.

It is often not valid to compare the relative affinities of two substrates for an enzyme on the basis of K_s values, since $K_s \doteq k_2/k_1$ only when $k_2 \gg k_3$. However, if the respective K_s values for two substrates are in inverse order of their respective k_3 values, it then follows[5,20,53] that the substrate with the smaller K_m and larger k_3 will have the smaller value of k_2/k_1, or, in other words, the greater affinity for the enzyme. Therefore, on the basis of the summarized kinetic constants given in Table XXII, it can be concluded that nicotinyl-L-tyrosinamide is bonded more strongly to α-chymotrypsin, at 25° C. and pH 7.9, than is acetyl-L-tyrosinamide, and that nicotinyl-L-tryptophanamide is bonded more strongly to α-chymotrypsin than is acetyl-L-tryptophanamide. However, a tryptophane substrate cannot be compared to a tyrosine substrate on the basis of K_s and k_3 values.

In contrast to K_S, K_{P_1} and K_I, when determined with systems in zone A[40,41], are true equilibrium constants, and quantitative comparisons can thus be made. In Table XXIII are given the ratios of various K_{P_1}, K_I and K_s values, and from these it can be concluded that inhibitors derived from D- or L-tryptophane are bonded more firmly to the enzyme than are the corresponding inhibitors derived from D- or L-tyrosine. Also the replacement of an acetyl group by a nicotinyl group results in an increased affinity for the enzyme. Finally, the replacement of an $-NH_2$ group by an $-OC_2H_5$ group produces a more strongly bound inhibitor.

Inspection of Table XXIII shows that all the given ratios of acetyl-inhibitors to nicotinyl-inhibitors are approximately 2.0, and also the given ratios of acetyl-substrates to nicotinyl-substrates are approximately 2.0. This fact strongly suggests[32] that in the systems under discussion, the values of k_3 are very much smaller than those of k_2, and thus $K_s \doteq k_2/k_1$. Also, the fact that no lag has ever been observed in the establishment of the steady state in these systems, supports this view[45]. Further evidence in favor of the relative smallness of the k_3 values is the low turnover number; approximately 2 molecules/enzyme molecule/min. for acetyl-L-tryptophanamide[32], approximately 7 for nicotinyl-L-tryptophanamide[32], approximately 10 for acetyl-L-tyrosinamide, and approximately 27 for nicotinyl-L-tyrosinamide. These values are considerably smaller than those for fifteen representative enzyme

substrate systems listed by McIlwain[54], where the values varied from approximately 8 x 10^2 to 26 x 10^5 molecules/enzyme molecule/min.

A significant finding, based on the validity of the assumption that $K_s \doteq k_2/k_1$, is that the D-antipodes, of the substrates discussed above, appear to be more firmly bound to the enzyme than are the corresponding L- substrates (cf. Table XXIII). Also it has been shown that for two enantiomorphic competitive inhibitors, the one possessing the D-configuration is bonded more strongly to the enzyme than is the one possessing the L-configuration[27]. It is possible that an explanation of the apparent preference of α-chymotrypsin for a molecule of the unnatural or D-configuration may also provide at least a partial answer to the question as to the mode of action of this enzyme[52,55].

TABLE VI

Stability of Aqueous α-Chymotrypsin Solutions at 4° C.

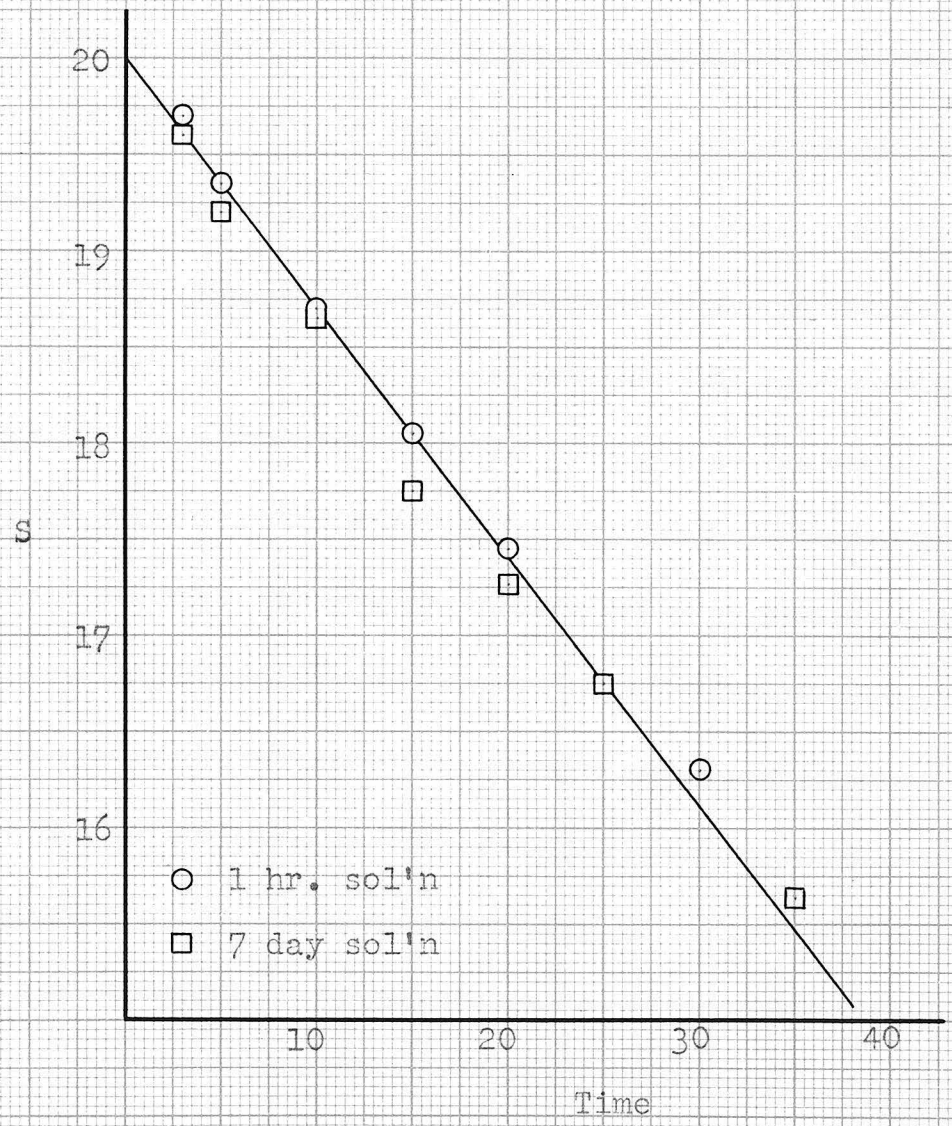
S_o = 20 μM/ml of Acetyl-L-tyrosinamide

E = 0.139 mg. enzyme N/ml

Age of Enzyme Solution	t (min)	S
	0	20.00
	3	19.70
	5	19.35
1 hour	10	18.70
	15	18.05
	20	17.45
	30	16.30
	0	20.00
	3	19.60
	5	19.20
7 days	10	18.65
	15	17.75
	20	17.27
	25	16.73
	35	15.63

FIG. VI

Stability of Aqueous α-Chymotrypsin Solutions at 4° C.



CHARLES BRUNING COMPANY, INC.
10 x 10 to the half inch.
NO. 700G
CONSTRUCTOR'S CROSS SECTION
PRINTED IN U. S. A.

TABLE VII

pH Optimum for the α-Chymotryptic Hydrolysis of Acetyl-L-Tyrosinamide at 25° C.

S_o = 20 μM/ml

E = 0.125 mg. enzyme N/ml

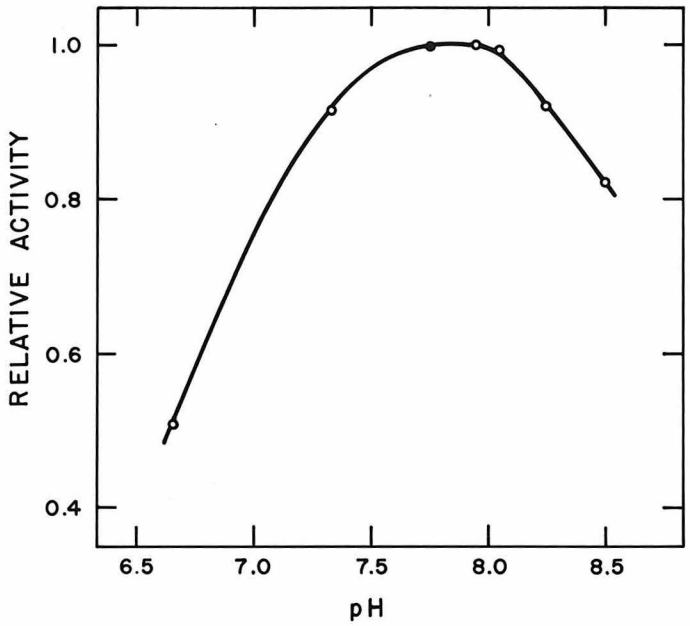
Buffer = tris (hydrolxymethyl) Aminomethane-hydrochloric acid, except for pH 7.75 where ethylenediamine-hydrochloric acid buffer was used.

t	S	log S	pH	$k^{ist.}$ x min.
0	20.00	1.301		
2.5	19.80	1.297		
5	19.60	1.292	6.63	14×10^{-4}
10	19.25	1.284		
15	19.00	1.279		
20	18.60	1.270		
25	18.45	1.266		
0	20.00	1.301		
3.3	19.65	1.293		
5	19.35	1.287		
10	18.85	1.275	7.32	25×10^{-4}
15	18.15	1.259		
20	17.70	1.248		
25	17.15	1.234		
0	20.00	1.301		
5	19.40	1.288		
10	18.70	1.272	7.75	27.4×10^{-4}
15	18.25	1.261		
20	17.60	1.246		
25	17.10	1.233		
0	20.00	1.301		
2.5	19.70	1.294		
5	19.20	1.284		
10	18.70	1.272	8.03	27.2×10^{-4}
15	18.20	1.260		
21	17.50	1.242		
25	17.10	1.233		
0	20.00	1.301		
2.5	19.70	1.293		
5	19.40	1.287		
10	18.75	1.275	8.22	25.2×10^{-4}
14.5	18.25	1.259		
20	17.82	1.248		
25	17.30	1.234		

TABLE VII (Cont)

t	S	log S	pH	$k^{ist.}$ x min.
0	20.00	1.301		
2.5	19.75	1.296		
5	19.40	1.288		
10	18.90	1.276	8.50	22.6×10^{-4}
15	18.40	1.265		
20	17.90	1.253		
25	17.55	1.244		

FIG. VII



Acetyl-L-tyrosinamide, $[S]_o$ = 20 x 10^{-3} M; [E] = 0.125 mg. enzyme N per ml.; ○ 0.02 M tris(hydroxymethyl)aminomethane-hydrochloric acid buffer; ● 0.02 M ethylenediamine-hydrochloric acid buffer.

TABLE VIII

pH Optimum for the α-Chymotryptic Hydrolysis of Nicotinyl-L-Tyrosinamide at 25° C

S_o = 12.5 μM/ml.

E = 0.047 mg enzyme N/ml.

buffer = 0.02 M tris(hydroxymethyl)aminomethane-hydrochloric acid.

t	S	pH	k^o/ M/ml./min
0	20.00		
2.3	19.90		
5	19.55		
10	19.15	6.75	8.55×10^{-2}
15	18.75		
20	18.25		
24.5	18.10		
30	17.45		
0	20.00		
3	19.75		
6	19.35		
10.3	19.05	7.02	10.0×10^{-2}
15	18.45		
20	18.05		
25	17.55		
30	16.95		
0	20.00		
2.5	19.90		
5	19.40		
10	18.80	7.40	11.75×10^{-2}
14.7	18.30		
20	17.60		
25.5	17.00		
30	16.45		
0	20.00		
2.3	19.75		
5	19.35		
10	18.75	7.60	12.3×10^{-2}
15	18.25		
19.5	17.55		
24.5	17.00		
30	16.65		

TABLE VIII (Cont)

t	S	pH	k^{o}/ M/ml./min
0	20.00		
2.3	19.85		
5	19.35		
10	18.75	7.96	12.5×10^{-2}
15	18.15		
20	17.50		
25	16.90		
0	20.00		
2.5	19.65		
4.5	19.50		
10	18.85	8.12	11.8×10^{-2}
15.5	18.15		
20	17.65		
25	17.10		
30	16.55		
0	20.00		
2.3	19.75		
5	19.45		
10	18.85	8.3	11.25×10^{-2}
14	18.35		
20	17.75		
24.5	17.25		
30	16.65		
0	20.00		
2.3	19.80		
5	19.50		
10	19.00	8.54	10.25×10^{-2}
14.5	18.65		
20	18.05		
25	17.60		
30	17.00		

FIG. VIII

pH Optimum for the α-Chymotryptic Hydrolysis of Nicotimyl-L-Tyrosinamide at 25° C

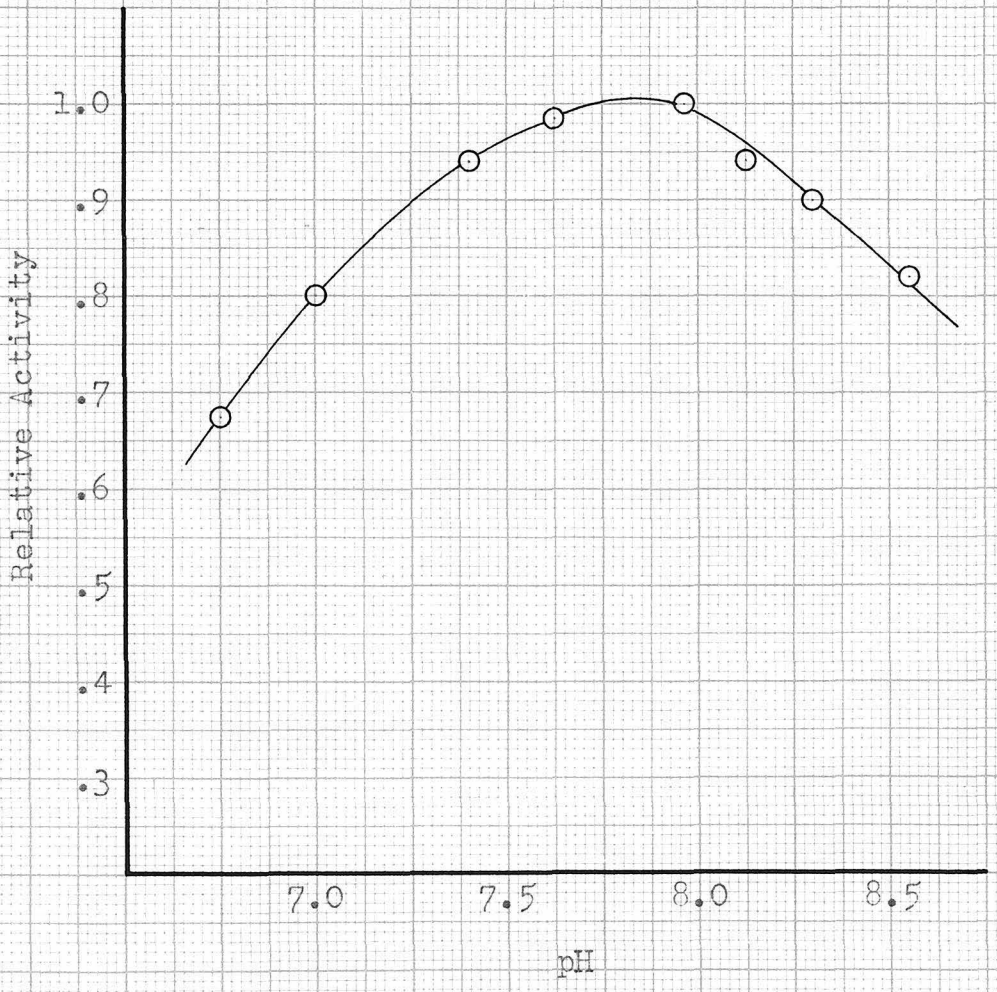


TABLE IX

Determination of K_s for

Acetyl-L-tyrosinamide at 25° C. and pH 7.9 ± 0.1

$K_s = 31.5 \times 10^{-3}$ M

E = 0.204 mg. enzyme N/ml

Buffer = 0.02 M ethylene diamino-hydrochloric acid

t (min)	S($\frac{\mu M}{ml}$)	log S	log $\frac{S_o}{S}$	2.3 K_s log $\frac{S_o}{S}$	(S_o-S)	$\underline{F}(\underline{S}) \times 10^3$
0	40.0	1.602	0	0	0	0
2	39.5	1.597	.005	0.37	0.50	0.87
5	38.7	1.588	.014	1.03	1.30	2.33
10	37.5	1.574	.028	2.06	2.50	4.56
20	34.6	1.539	.063	4.63	5.40	10.03
30	32.1	1.507	.095	6.98	7.90	14.88
40	29.8	1.474	.128	9.41	10.20	19.61
60	25.5	1.407	.195	14.32	14.50	28.82
90	20.4	1.310	.292	21.45	19.60	41.05
0	30.0	1.477	0	0	0	0
5.5	28.9	1.461	.016	1.18	1.10	2.28
10	28.0	1.447	.030	2.20	2.00	4.20
20	26.0	1.415	.062	4.55	4.00	8.55
30	24.0	1.380	.097	7.13	6.00	13.13
40	22.2	1.346	.131	9.63	7.80	17.43

TABLE IX (Cont)

t (min)	$S(\frac{\mu M}{ml})$	log S	log $\frac{S_o}{S}$	2.3 K_s log $\frac{S_o}{S}$	(S_o-S)	$\underline{F}(\underline{S})$ x 10^3
0	20.0	1.301	0	0	0	0
2	19.6	1.292	.009	0.66	0.40	1.06
5	19.3	1.286	.015	1.10	0.70	1.80
10	18.4	1.265	.036	2.65	1.60	4.25
20	16.8	1.224	.077	5.67	3.20	8.87
30	15.5	1.189	.112	8.23	4.50	12.73
40	13.9	1.143	.158	11.60	6.10	17.70
0	25.0	1.398	0	0	0	0
3	24.5	1.389	.009	0.66	0.50	1.16
5	24.0	1.380	.018	1.32	1.00	2.32
10	23.2	1.365	.033	2.43	1.80	4.23
20.5	21.1	1.324	.074	5.44	3.90	9.34
30	19.8	1.296	.102	7.50	5.20	12.70
40	18.2	1.259	.139	10.21	6.80	17.01
0	5.00	.699	0	0	0	0
2	4.95	.694	.005	0.37	0.05	0.42
5	4.50	.653	.046	3.40	0.50	3.90
10	4.25	.628	.071	5.20	0.75	5.95
20.5	3.75	.574	.125	9.20	1.25	10.45
30	3.17	.501	.198	14.55	1.83	16.40
40	2.80	.447	.252	18.50	2.20	20.70

FIG. IX

Determination of K_s for Acetyl-L-Tyrosinamide at 25° C. and pH 7.9 ± 0.1

K_s = 31.5 x 10^{-3} M

E = 0.204 mg. enzyme N/ml.

Buffer = 0.02 M ED-HCl

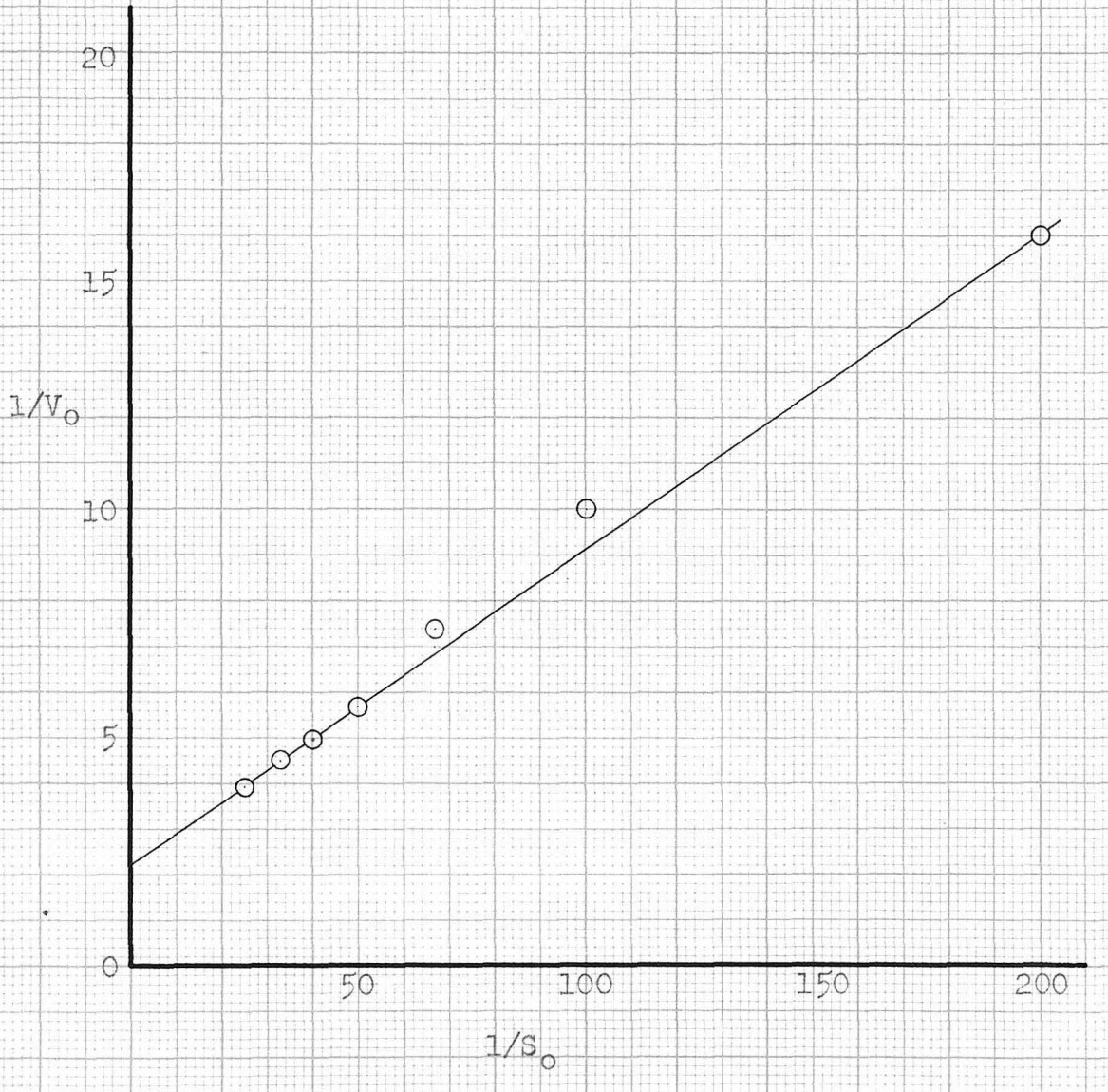


TABLE X

Determination of K_s for Acetyl-L-Tyrosinamide at 25° C. and pH 7.9 ± 0.1

$K_s = 31.5 \times 10^{-3}$ M

E = 0.139 mg. enzyme N/ml

Buffer = 0.02 M tris(hydroxymethyl)aminomethane-hydrochloric acid

t (min)	S ($\frac{\mu M}{ml}$)	log S	log $\frac{S_o}{S}$	2.3 K_s log $\frac{S_o}{S}$	(S_o-S)	F(S) x 10^3
0	10.0	1.000	0	0	0	0
2.3	9.8	.991	.009	.65	.20	.85
5	9.6	.982	.018	1.31	.40	1.71
10	9.4	.972	.028	2.03	.60	2.63
15.5	8.9	.948	.052	3.77	1.10	4.87
22	8.7	.940	.060	4.35	1.30	5.65
30	8.1	.908	.092	6.67	1.90	8.57
40.5	7.3	.865	.135	9.79	2.70	12.49
53	6.5	.813	.187	13.55	3.50	17.05
63	5.9	.771	.229	16.60	4.10	20.70
0	15.00	1.176	0	0	0	0
3	14.65	1.166	.010	.72	.35	1.07
7	14.40	1.158	.018	1.30	.60	1.90
13.5	13.90	1.143	.033	2.39	1.10	3.49
21	13.05	1.116	.060	4.35	1.95	6.30
30.5	12.25	1.088	.088	6.38	2.75	9.13
40	11.25	1.051	.125	9.07	3.75	12.82

TABLE X (Cont)

t (min)	$S(\frac{\mu M}{ml})$	log S	$\log \frac{S_o}{S}$	$2.3\ K_s \log \frac{S_o}{S}$	(S_o-S)	$F(S) \times 10^3$
51	10.25	1.011	.165	11.95	4.75	16.70
60	9.70	0.987	.189	13.70	5.30	19.00
0	20.0	1.301	0	0	0	0
4	19.5	1.290	.011	.80	.50	1.30
7.5	19.2	1.283	.018	1.31	.80	2.11
15	18.1	1.258	.043	3.12	1.90	5.02
24	17.2	1.236	.065	4.72	2.80	7.52
34	16.3	1.211	.090	6.53	3.75	10.28
42.5	15.2	1.180	1.210	8.78	4.85	13.63
51	14.4	1.158	1.430	10.37	5.60	15.97
60	13.5	1.129	1.720	12.48	6.55	19.03
0	30.0	1.477	0	0	0	0
2	29.6	1.471	.006	.44	.40	.84
5	29.3	1.467	.010	.73	.70	1.43
12	28.3	1.452	.025	1.81	1.70	3.51
20	27.1	1.432	.045	3.26	2.95	6.21
30	25.4	1.405	.072	5.22	4.60	9.82
41	23.9	1.378	.099	7.18	6.10	13.28
50	22.7	1.356	.121	8.77	7.30	16.07
58	21.8	1.338	.139	10.08	8.20	18.28

TABLE X (Cont)

t (min)	S($\frac{\mu M}{ml}$)	log S	log $\frac{S_o}{S}$	2.3 K_s log $\frac{S_o}{S}$	(S_o-S)	F(S) x 10^3
0	40.0	1.602	0	0	0	0
3	39.4	1.596	.006	.44	.60	1.04
7	38.8	1.589	.013	.94	1.20	2.14
13	37.5	1.574	.028	2.03	2.50	4.53
21	36.1	1.558	.044	3.19	3.90	7.09
30	34.2	1.534	.068	4.93	5.80	10.73
40.5	32.4	1.511	.091	6.60	7.60	14.20
61	29.1	1.464	.138	10.00	10.90	20.90

FIG. X

Determination of K_s for Acetyl-L-Tyrosinamide at 25° C. and pH 7.9 ± 0.1

$K_s = 31.5 \times 10^{-3}$ M

E = 0.139 mg. enzyme N/ml.

Buffer = 0.02 M T(HM)AM-HCl

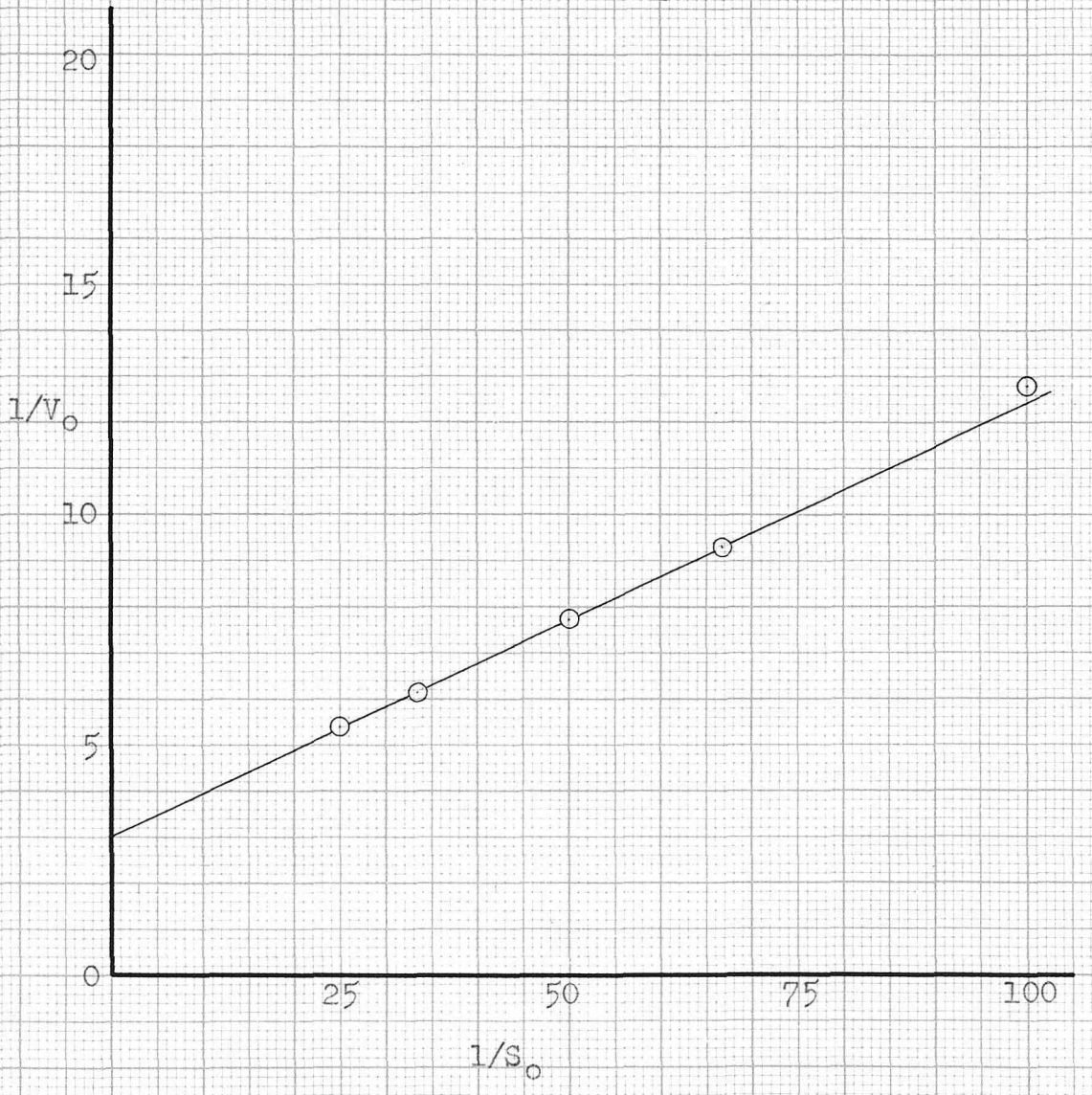


FIG. XI

Determination of K_s for Acetyl-L-Tyrosinamide at 25° C. and pH 7.9 ± 0.1

$K_s = 29.5 \times 10^{-3}$ M

E = 0.139 mg. enzyme N/ml.

Buffer = 0.02 M T(HM)AM-HCl

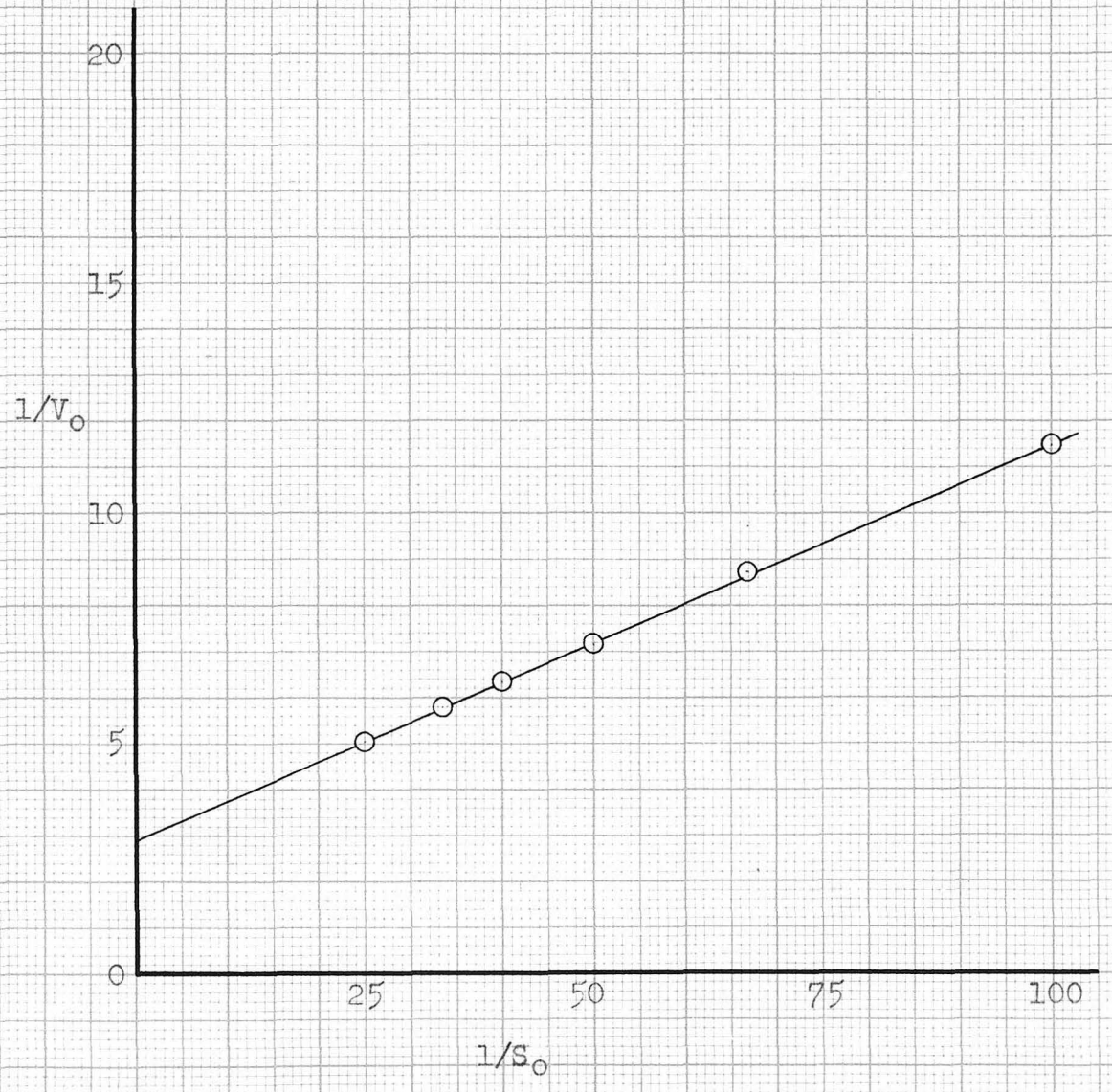


FIG. XII

Determination of K_s for Acetyl-L-Tyrosinamide at 25° C. and pH 7.9 ± 0.1

$K_s = 29.5 \times 10^{-3}$ M

E = 0.139 mg. enzyme N/ml

Buffer = 0.02 M ED-HCl

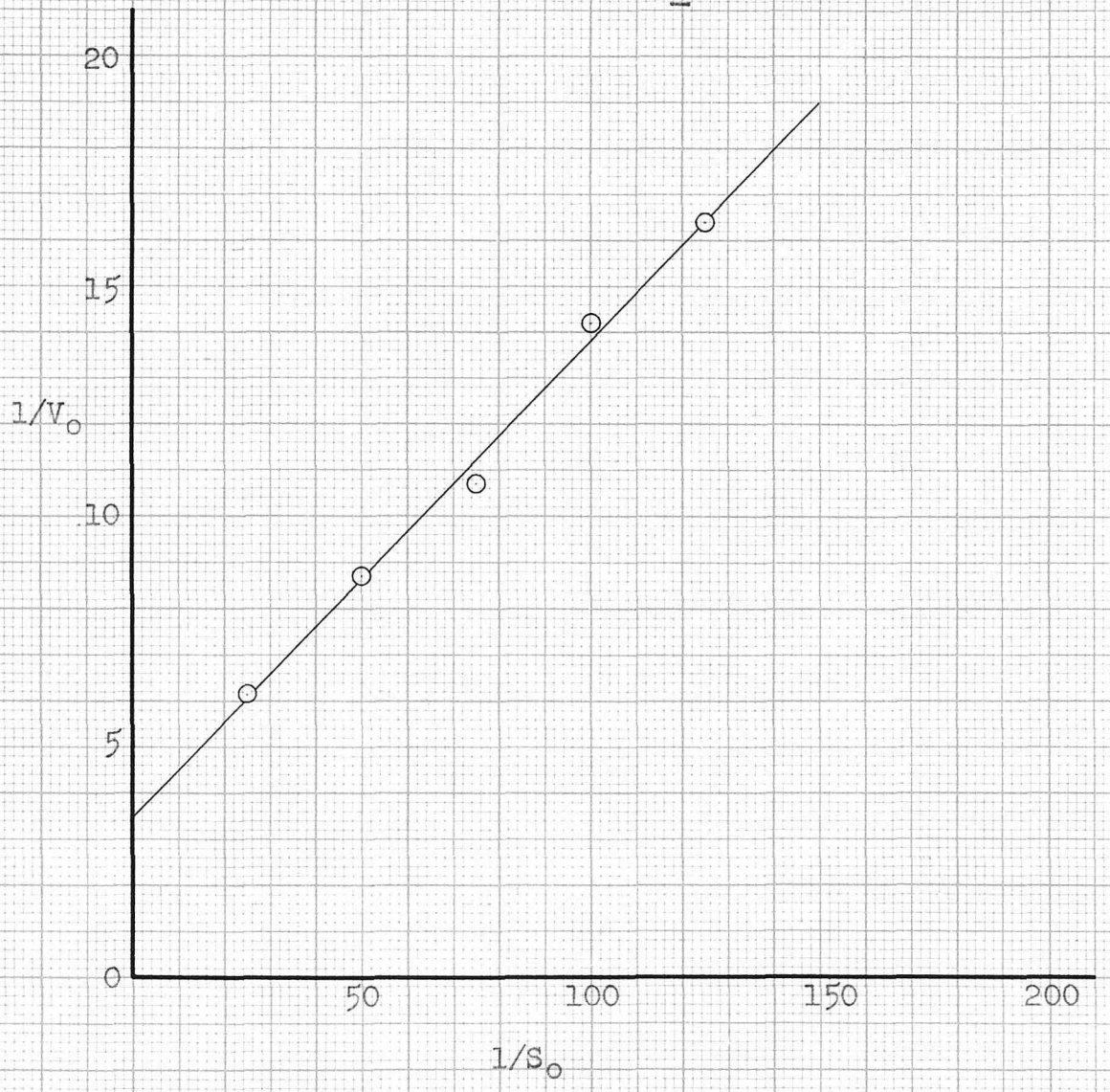


TABLE XI

Determination of K_s for Nicotinyl-L-Tyrosinamide at 25° C. and pH 7.9 ± 0.1

$K_s = 15.0 \times 10^{-3}$ M

E = 0.047 mg. enzyme N/ml

Buffer = 0.02 M tris(hydroxymethyl)aminomethane-hydrochloric acid

t (min)	$S(\frac{\mu M}{ml})$	log S	$\log \frac{S_o}{S}$	2.3 $K_s \log \frac{S_o}{S}$	(S_o-S)	F(S)
0	7.5	.875	0	0	0	0
2.5	7.3	.863	.012	.41	.20	.61
5	7.05	.848	.027	.93	.45	1.38
10	6.7	.826	.049	1.69	.80	2.49
16	6.15	.789	.086	2.96	1.35	4.31
20	5.75	.759	.116	4.00	1.75	5.75
0	10.00	1.000	0	0	0	0
2.3	9.85	.994	.006	.21	.15	.36
5	9.40	.974	.026	.90	.60	1.50
10	8.85	.947	.053	1.83	1.15	2.98
15	8.25	.916	.084	2.90	1.75	4.65
20	7.70	.887	.113	3.90	2.30	6.20
25	7.20	.857	.143	4.93	2.80	7.73
30	6.55	.816	.184	6.34	3.45	9.79
40	5.75	.760	.240	8.28	4.25	12.53

TABLE XI (Cont)

t (min)	$S(\frac{\mu M}{ml})$	log S	$\log \frac{S_o}{S}$	2.3 $K_s \log \frac{S_o}{S}$	(S_o-S)	$\underline{F}(S)$
0	12.50	1.097	0	0	0	0
2.5	12.20	1.086	.011	.38	.30	.68
5	11.90	1.076	.021	.73	.60	1.33
10	11.25	1.051	.046	1.59	1.25	2.84
15	10.55	1.023	.074	2.55	1.95	4.50
21	9.95	.998	.099	3.42	2.55	5.97
25	9.20	.964	.133	4.58	3.30	7.88
30	8.55	.932	.165	5.69	3.95	9.64
0	15.00	1.176	0	0	0	0
2.5	14.55	1.163	.013	.45	.45	.90
5	14.25	1.154	.022	.76	.75	1.51
10	13.65	1.135	.041	1.41	1.35	2.76
15	12.90	1.111	.065	2.24	2.10	4.34
20	12.15	1.085	.091	3.14	2.85	5.99
25	11.45	1.059	.117	4.03	3.55	7.58
30	10.95	1.039	.137	4.73	4.05	8.78
0	17.50	1.243	0	0	0	0
3	17.05	1.232	.011	.38	.45	.83
5	16.75	1.224	.019	.66	.75	1.41
10	16.15	1.208	.035	1.21	1.35	2.56
15	15.25	1.183	.060	2.07	2.25	4.32
20	14.40	1.158	.085	2.93	3.10	6.03
25	13.65	1.135	.108	3.73	3.85	7.58
30	12.95	1.112	.131	4.52	4.55	9.07

FIG. XIII

Determination of K_s for Nicotinyl-L-Tyrosinamide at 25° C. and pH 7.9 ± 0.1

$K_s = 15.0 \times 10^{-3}$ M

E = 0.047 mg. enzyme N/ml.

Buffer = 0.02 M T(HM)AM-HCl

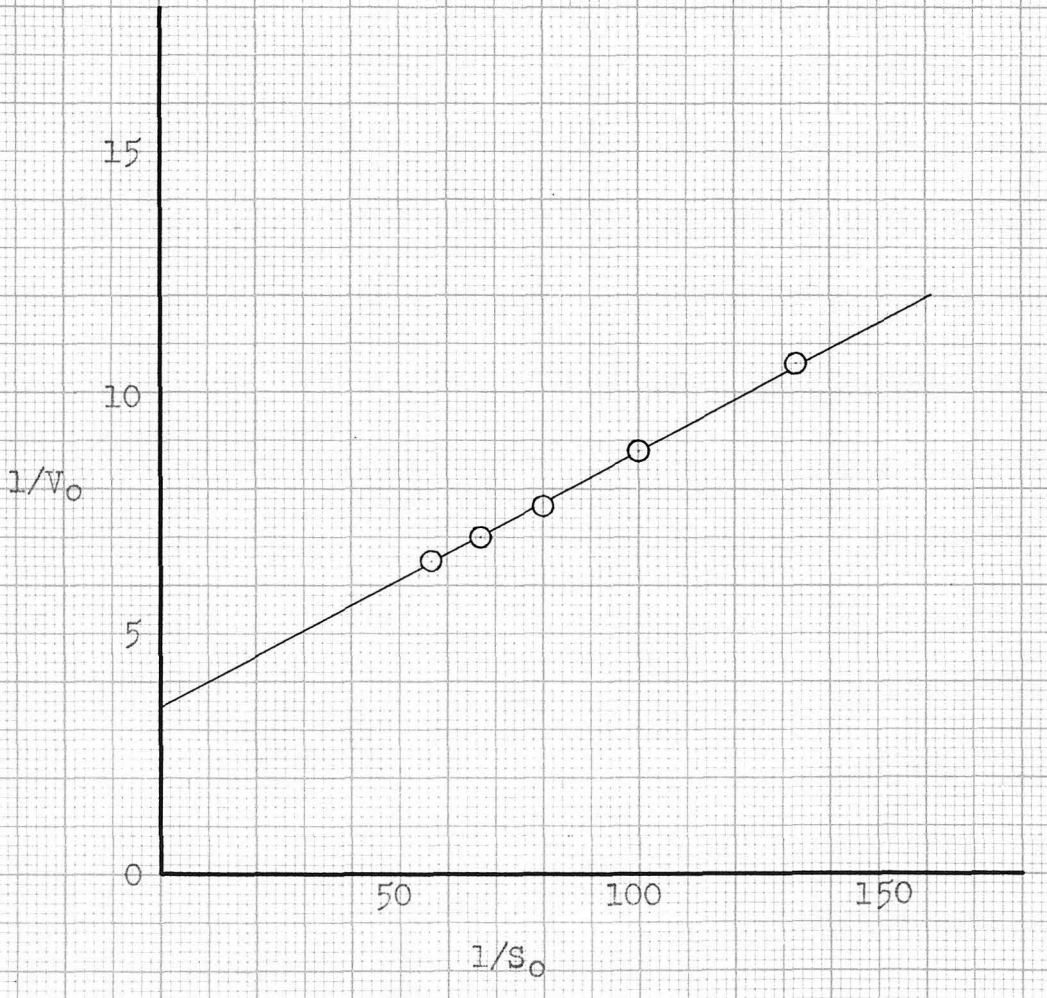


FIG. XIV

Determination of K_s for Nicotinyl-L-Tyrosinamide at 25° C. and pH 7.9 ± 0.1

$K_s = 14.5 \times 10^{-3}$ M

E = 0.047 mg. enzyme N/ml

Buffer = 0.02 M T(HM)AM-HCl

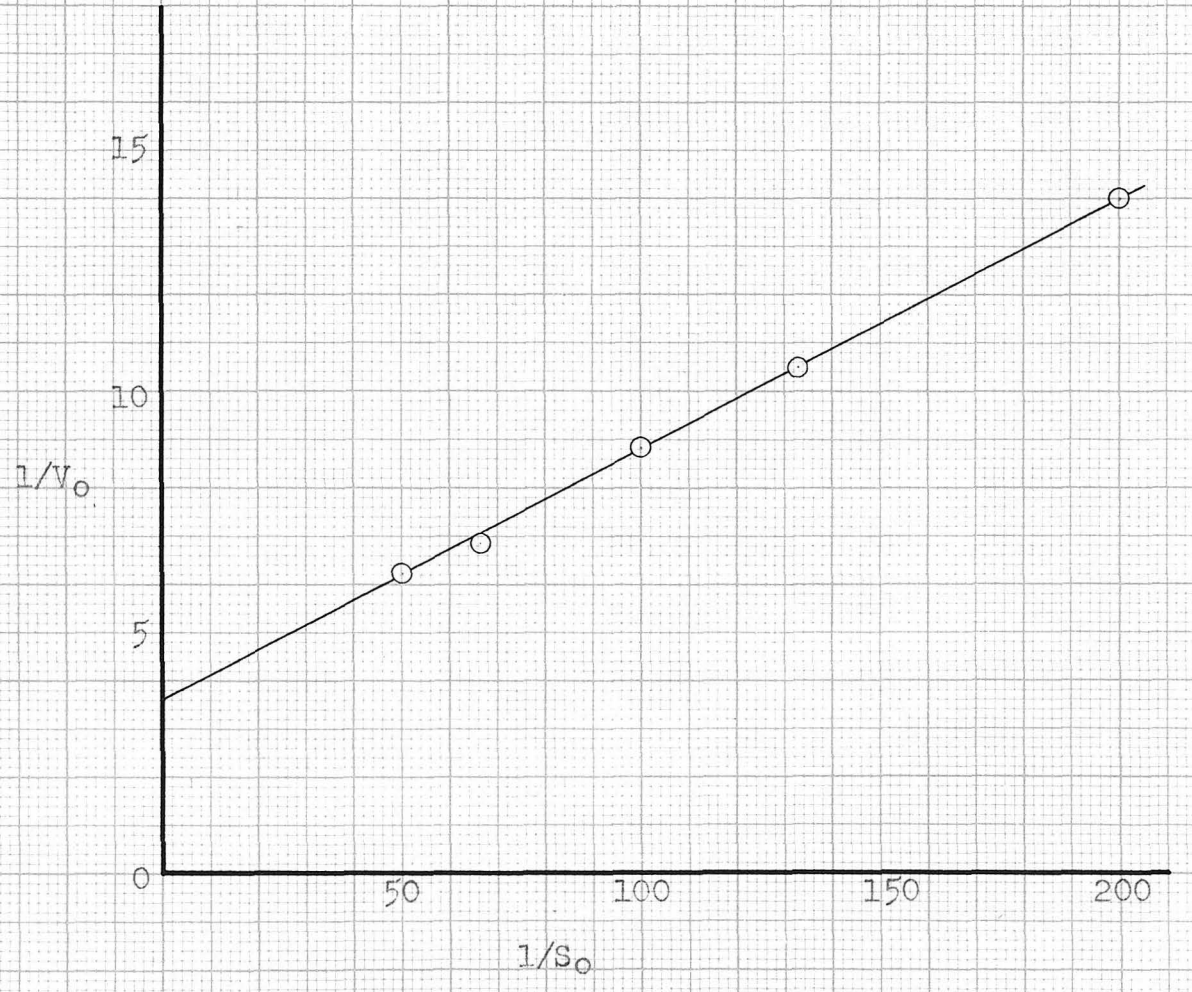


TABLE XII

Course of α-Chymotryptic Hydrolysis of Acetyl-L-Tyrosinamide

S_o = 20 μM/ml

E = 0.312 mg. enzyme N/ml

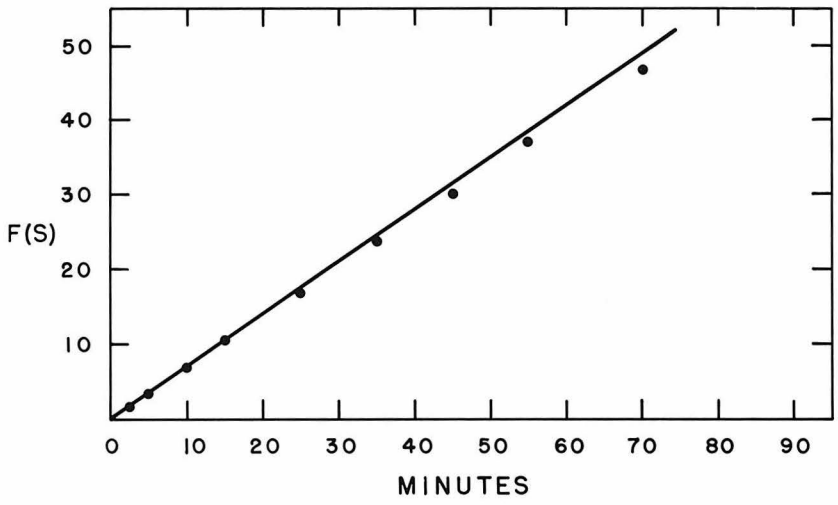
K_s = 30.5 x 10^{-3} M

Buffer = 0.02 M tris(hydroxymethyl)aminomethane-hydrochloric acid

pH = 7.9

t (min)	S ($\frac{\mu M}{ml}$)	log S	log $\frac{S_o}{S}$	2.3 K_m log $\frac{S_o}{S}$	(S_o-S)	F(S)
0	20.00	1.301	0	0	0	0
2.5	19.35	1.287	.014	.98	.65	1.63
5	18.65	1.271	.030	2.10	1.35	3.45
10	17.45	1.242	.059	4.13	2.55	6.68
15	16.10	1.207	.094	6.58	3.90	10.48
25	14.00	1.146	.155	10.85	6.00	16.85
35	12.00	1.079	.222	15.53	8.00	23.53
45	10.30	1.013	.288	20.16	9.70	29.86
55	8.55	0.932	.369	25.83	11.45	37.28
70	6.65	0.823	.478	33.46	13.35	46.81

FIG. XV



$\underline{F}(\underline{S})$ in units of 10^{-3} $\underline{M}$; acetyl-L-tyrosinamide, $[S]_o$ = 20×10^{-3} $\underline{M}$; $[E]$ = 0.312 mg. enzyme N/ml.; 0.02 $\underline{M}$ tris(hydroxymethyl)-aminomethane-hydrochloric acid buffer.

TABLE XIII

Course of α-Chymotryptic Hydrolysis of Nicotinyl-L-Tyrosinamide

S_o = 15 μM/ml

E = 0.104 mg. enzyme N/ml

K_s = 15.0 x 10^{-3} M

Buffer = 0.02 M tris(hydroxymethyl)aminomethane-hydrochloric acid

pH = 8.0

t (min)	S (μM/ml)	log S	log $\frac{S_o}{S}$	2.3 K_m log $\frac{S_o}{S}$	(S_o-S)	F(S)
0	15.00	1.176	0	0	0	0
2	14.40	1.158	.018	.62	.60	1.22
5	13.50	1.130	.046	1.59	1.50	3.09
10	12.00	1.079	.097	3.34	3.00	6.34
15	10.68	1.028	.148	5.11	4.33	9.44
25	8.48	..928	.248	8.55	6.53	15.08
35	6.35	.803	.373	12.87	8.65	21.52
45	4.55	.658	.518	17.90	10.45	28.35
55	3.20	.505	.671	23.15	11.80	34.95
70	1.98	.295	.881	30.40	13.03	43.43

FIG. XVI

Course of α-Chymotryptic Hydrolysis of Nicotinyl-L-Tyrosinamide

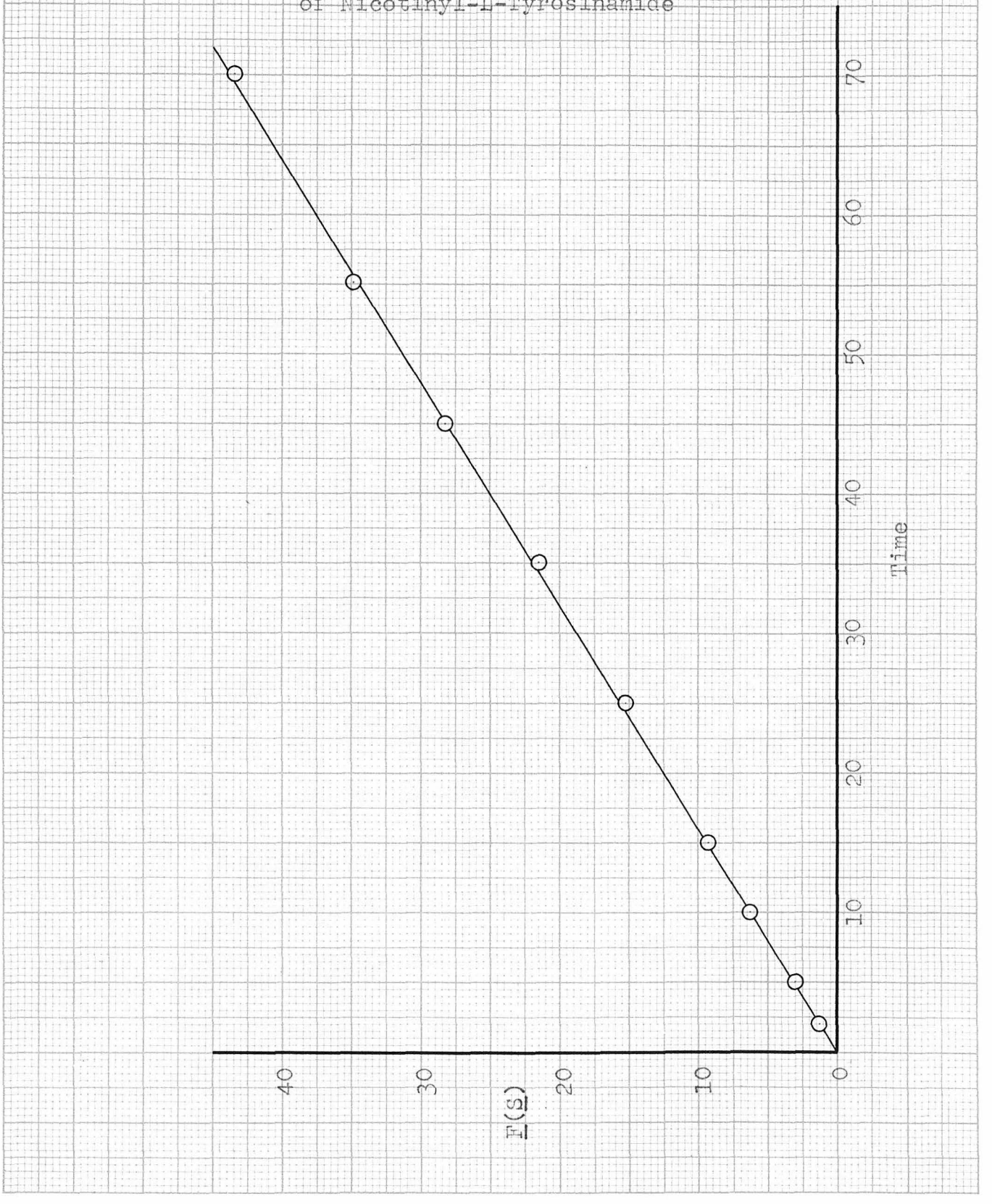


TABLE XIV

Determination of K_{P_1} for Acetyl-L-Tyrosine at 25° C. at pH 7.9 ± 0.1

$K_{P_1} = 115 \times 10^{-3}$ M

$[P_1]_o = 25 \mu M/ml$

$[E] = 0.139$ mg. enzyme N/ml

t(min)	S(μM/ml)	log S
0	15.00	1.176
2.5	14.85	1.172
5	14.50	1.161
10	14.15	1.151
15	13.70	1.137
20	13.15	1.119
30	12.25	1.088
40	11.65	1.066
0	20.00	1.301
2.5	19.85	1.297
5	19.40	1.288
10	18.85	1.275
15	18.30	1.262
20	17.70	1.248
25	17.10	1.233
30	16.60	1.220
40	15.60	1.193

TABLE XIV (Cont)

t(min)	S(μM/ml)	log S
0	30.00	1.477
2.5	29.65	1.472
5	29.15	1.465
10	28.45	1.454
15	27.55	1.440
20	26.75	1.427
25	26.00	1.415
31	25.15	1.400
41.5	23.75	1.376
0	40.00	1.602
2	39.70	1.599
5	39.00	1.591
10	38.30	1.583
14.5	37.30	1.571
20	36.40	1.561
25	35.50	1.550

TABLE XV

Determination of K_{P_1} for Acetyl-L-Tyrosine at 25° C. and pH 7.9 ± 0.1

$K_{P_1} = 115 \times 10^{-3}$ M

$[P_1]_o = 40 \mu M/ml$

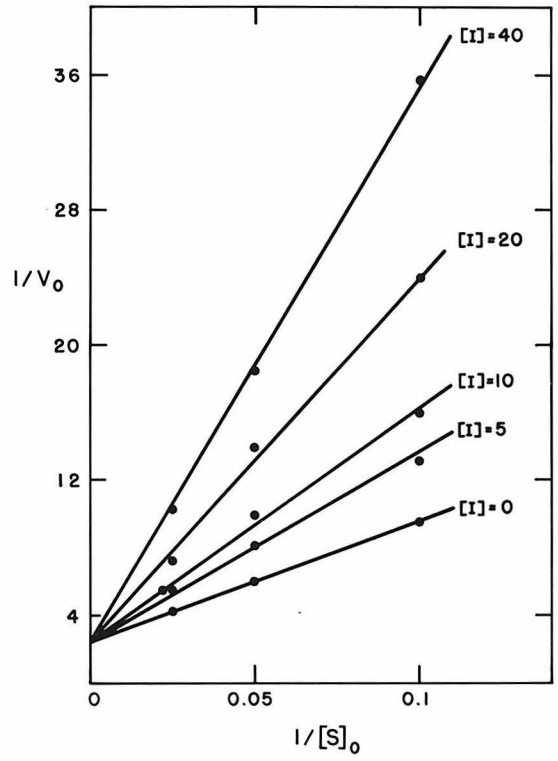
$[E] = 0.139$ mg. enzyme N/ml

t(min)	S(μM/ml)	log S
0	12.50	1.097
2.3	12.30	1.090
5	12.00	1.079
9	11.75	1.070
14	11.45	1.059
20	10.90	1.037
24.5	10.50	1.021
30.3	10.20	1.009
40	9.50	.978
0	15.00	1.176
2.5	14.80	1.170
5	14.50	1.162
10	14.10	1.149
15	13.65	1.135
20	13.20	1.121
25	12.75	1.105
32	12.20	1.086
45	11.20	1.049

TABLE XV (Cont)

t(min)	S(μM/ml)	log S
0	20.20	1.305
2.5	19.90	1.299
4.5	19.60	1.292
9	19.05	1.280
15	18.40	1.265
20	17.90	1.253
25	17.40	1.241
30	16.75	1.224
40	15.95	1.203
0	30.00	1.477
2.5	29.70	1.473
5.3	29.20	1.466
9.8	28.58	1.456
15	27.80	1.444
20	27.10	1.433
25	26.30	1.420
32	25.40	1.405
46	23.70	1.375
0	40.00	1.602
2.5	39.55	1.597
5	39.10	1.592
9.5	38.25	1.583
15	37.20	1.571
20.5	36.25	1.559
26	35.25	1.547
32	34.05	1.532
45	32.35	1.510

FIG. XVII



$[S]_o$ in moles x 10^{-3} per liter of acetyl-L-tyrosinamide; V_o (initial velocities) in moles x 10^{-3} per liter per min; inhibition by acetyl-L-tyrosine, $[P_1]_o$ in moles x 10^{-3} per liter; $[E]$ = 0.139 mg. enzyme-nitrogen per ml.; 0.02 M tris(hydroxymethyl)aminomethane-hydrochloric acid buffer.

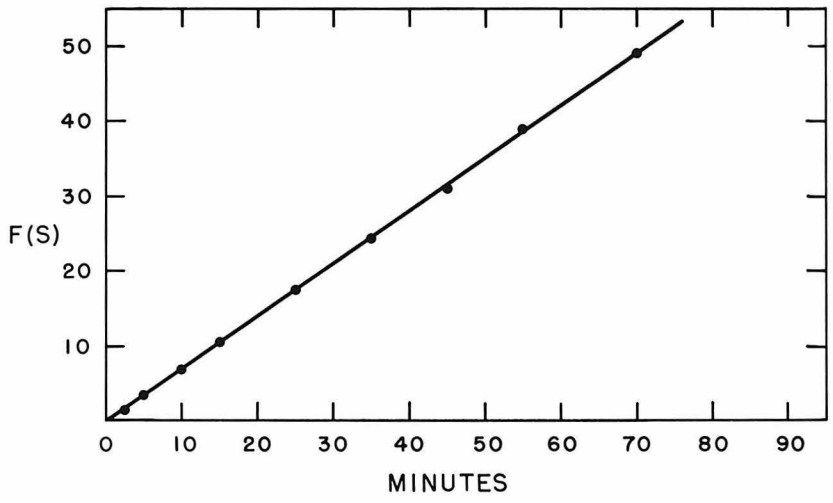
TABLE XVI

Course of the α-Chymotryptic Hydrolysis of Acetyl-L-Tyrosinamide

t (min)	$2.3\ K_s\ (1 + \frac{[S]_o}{K_{P_1}})\ \log \frac{[S]_o}{[S]}$	$(1 - \frac{K_s}{K_{P_1}})([S]_o - [S])$	$F(S)$
25	12.75	4.43	17.18
35	18.25	5.92	24.17
45	23.65	7.17	30.82
55	30.40	8.70	39.10
70	39.30	9.87	49.17

The first four F(S), from Table XII, were not recalculated as the difference is insignificant.

FIG. XVIII



F(S) in units of 10^{-3} M; acetyl-L-tyrosinamide, $[S]_o$ = 20×10^{-3} M; [E] = 0.312 mg. enzyme N/ml.; 0.02 M tris(hydroxymethyl)-aminomethane-hydrochloric acid buffer.

FIG. XIX

Determination of k_3 for Acetyl-L-Tyrosinamide Hydrolysis

$k_3 = 2.5 \times 10^{-3}$ moles/liter/min/mg. enzyme N/ml

$[S]_o = 40 \mu M/ml$

$[E] = 0.139$ mg. enzyme N/ml

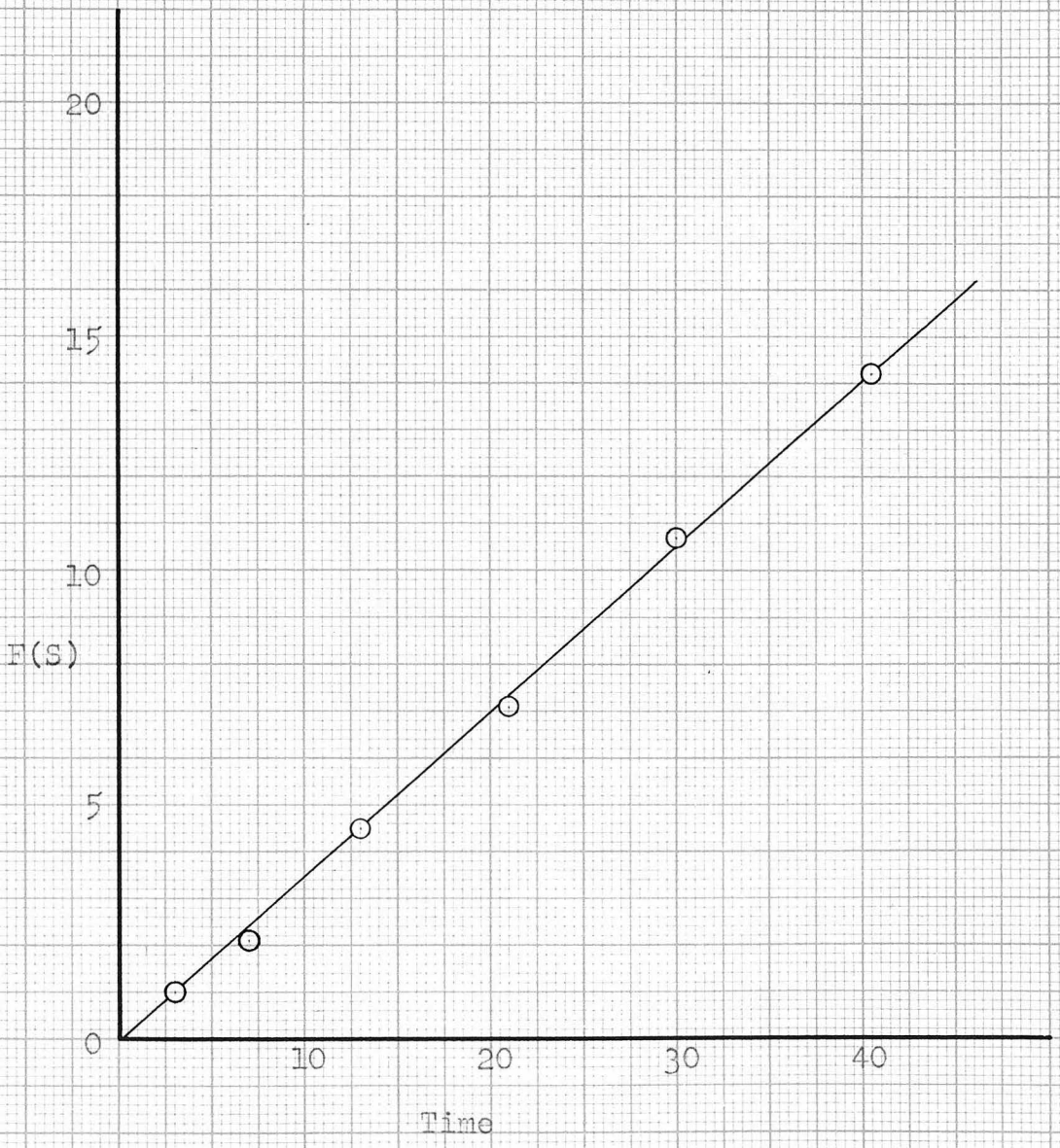


FIG. XX

Determination of k_3 for Nicotinyl-L-Tyrosinamide Hydrolysis

$k_3 = 6.3 \times 10^{-3}$ moles/liter/min/mg enzyme N/ml

$[S]_o = 15 \mu M/ml$

$[E] = 0.047$ mg. enzyme N/ml

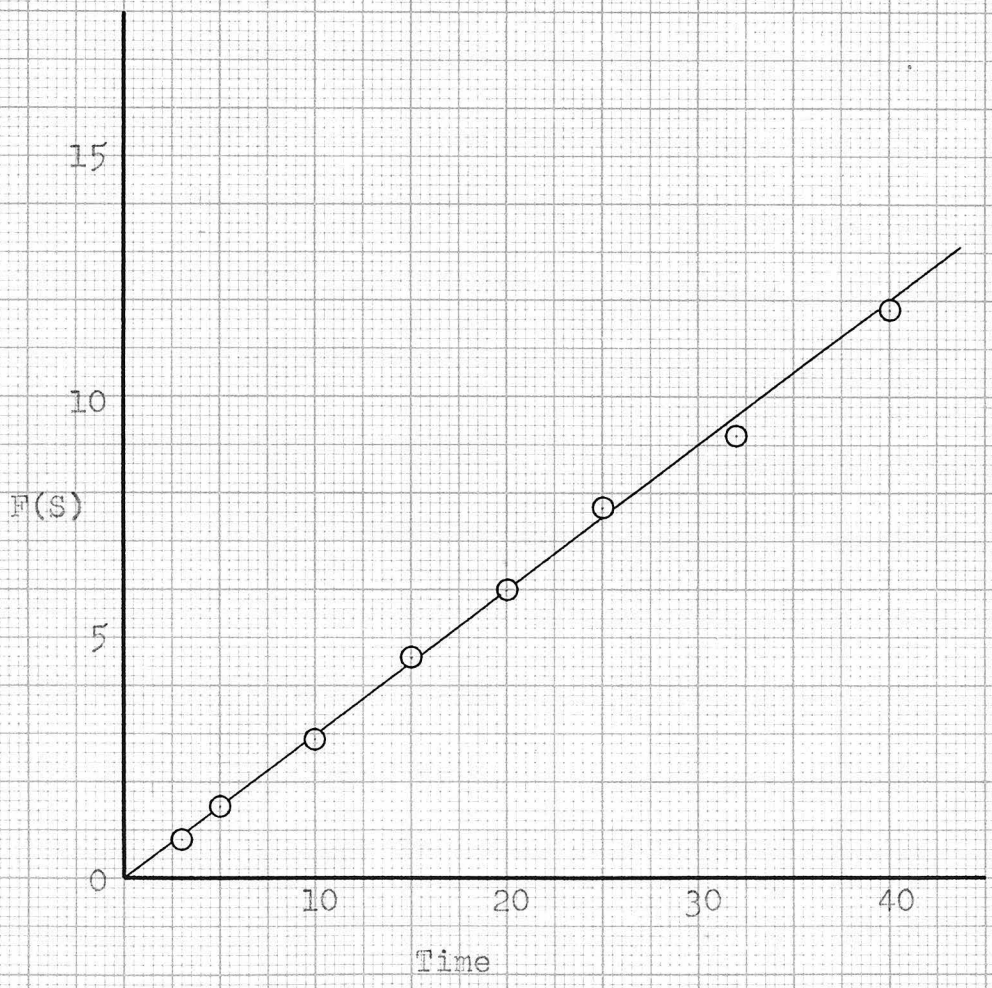
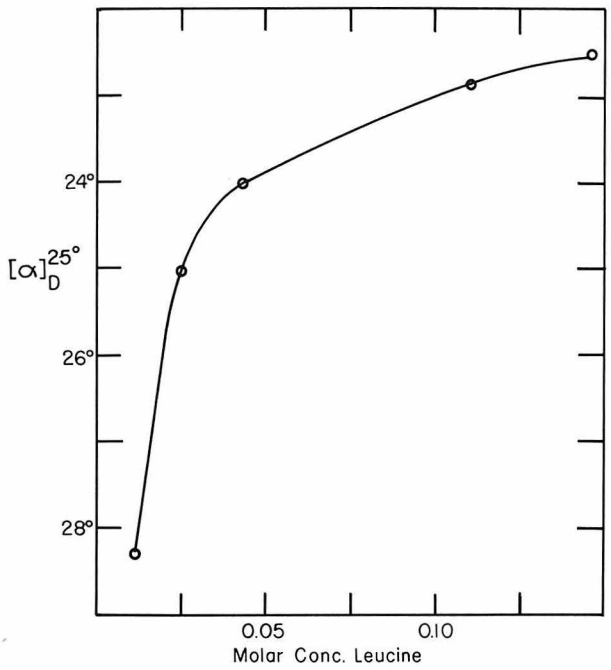


FIG. XXI



$[S]_o$ in moles x 10^{-3} per liter of acetyl-L-tyrosinamide; V_o (initial velocities) in moles x 10^{-3} per liter per min.; inhibition by acetyl-D-tyrosinamide, $[I]$, in moles x 10^{-3} per liter; $[E]$ = 0.150 mg. enzyme-nitrogen per ml.; 0.02 M ethylene diamine-hydrochloric acid buffer.

TABLE XVII

Determination of K_I for Nicotinyl-D-Tyrosinamide

$K_I = 6.4 \times 10^{-3}$ M

[I] = 2.5 μM/ml

[E] = 0.047 mg. enzyme N/ml

Buffer = 0.02 M tris(hydroxymethyl)aminomethane-hydrochloric acid

t(min)	S(μM/ml)
0	12.50
2.5	12.45
5.0	11.95
10	11.40
15	10.90
20	10.35
25	9.75
30	9.30
0	15.00
2.5	14.80
5	14.35
10	13.80
15	13.15
20	12.50
25	11.90
30	11.30
0	18.25
2.5	18.05
5	17.55
15	16.25
25	15.00
30	14.45
0	20.00
2.5	19.80
5	19.25
10	18.60
15	18.00
21	17.25
25	16.45
31	15.85

FIG. XXII

Determination of K_I for Nicotinyl-D-Tyrosinamide

$K_I = 6.4 \times 10^{-3}$ M

[I] = 2.5 μM/ml

[E] = 0.047 mg. enzyme N/ml

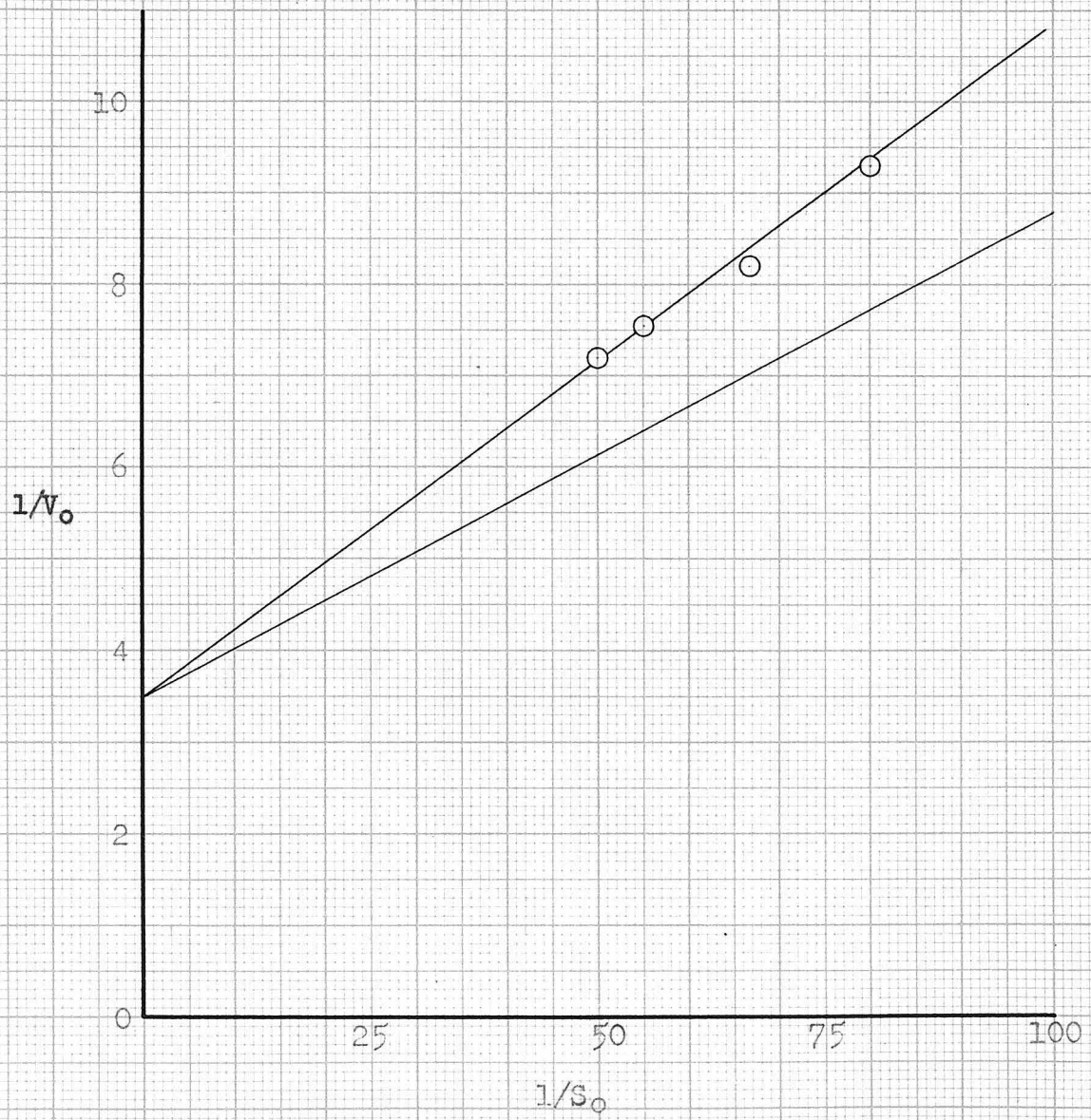


TABLE XVIII

Determination of K_I for Acetyl-D-Tyrosine Ethyl Ester

$K_I = 3.5 \times 10^{-3}$ M

[I] = 5 μM/ml

[E] = 0.139 mg. enzyme N/ml.

t(min)	S(μM/ml)	log S
0	20.00	1.301
2.3	20.00	1.301
4.5	19.65	1.293
10	19.30	1.285
17	18.75	1.273
25	18.25	1.261
34	17.65	1.247
45	17.15	1.234
60	16.25	1.211
0	15.00	1.176
2	14.90	1.173
4.8	14.68	1.167
9.5	14.33	1.156
16	13.95	1.145
21.5	13.55	1.132
27	13.30	1.124
35	12.90	1.110
45	12.55	1.099
0	35.15	1.546
2	34.95	1.543
6	34.45	1.537
10	33.95	1.531
16	33.35	1.523
27	32.15	1.507
35	31.35	1.496
50	30.20	1.480

TABLE XIX

Determination of K_I for Acetyl-D-Tyrosine Ethyl Ester

$K_I = 3.5 \times 10^{-3}$ M

[I] = 10 μM/ml.

[E] = 0.139 mg enzyme N/ml

t(min)	S(μM/ml)	log S
0	15.00	1.176
2.5	15.00	1.176
5	14.70	1.167
9.5	14.50	1.161
15	14.30	1.155
20	14.10	1.149
30	13.75	1.138
40	13.45	1.129
55	12.85	1.109
0	20.00	1.301
2.7	19.85	1.298
5	19.65	1.293
10	19.50	1.290
15	19.20	1.283
20	18.90	1.276
30	18.45	1.266
40	18.00	1.255
60	17.10	1.233
0	30.00	1.477
2.7	29.90	1.475
5	29.50	1.470
10	29.20	1.465
15	28.85	1.460
20	28.60	1.456
30	28.00	1.447
40	27.40	1.438
60	26.45	1.422
0	40.00	1.602
3	39.70	1.599
6	39.35	1.595
10	39.00	1.591
16	38.40	1.584
25	37.80	1.577
40	36.50	1.562
60	35.20	1.547
75	34.15	1.533

TABLE XX

Determination of K_I for Acetyl-D-Tyrosine Ethyl Ester

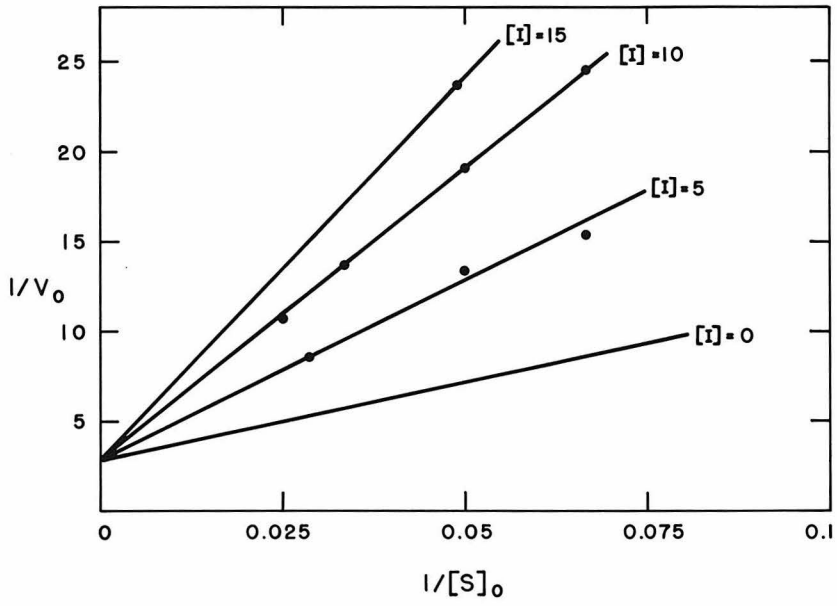
$K_I = 3.6 \times 10^{-3}$ M

[I] = 15 μM/ml

[E] = 0.139 mg enzyme N/ml

t(min)	S(μM/ml)	log S
0	20.40	1.310
3.3	20.30	1.307
7	20.10	1.303
12	19.90	1.299
20	19.65	1.293
30	19.45	1.289
40	18.80	1.274
50	18.45	1.266
60	18.00	1.255

FIG. XXIII



$[S]_o$ in moles x 10^{-3} per liter of acetyl-L-tyrosinamide; V_o (initial velocities) in moles x 10^{-3} per liter per min.; inhibition by acetyl-D-tyrosine ethyl ester, [I] in moles x 10^{-3} per liter; [E] = 0.139 mg. enzyme N per ml.; 0.02 M tris(hydroxymethyl)aminomethane-hydrochloric acid buffer.

TABLE XXI

Maximum Specific Enzyme Concentrations

Constant x 10^3 M	[E] x 10^5 M	E' x 10^2
K_S (Acetyl-L-tyrosinamide) = 30.5	4.8	0.16
K_{P_1} (Acetyl-L-tyrosine) = 115.0	3.2	0.03
K_I (Acetyl-D-tyrosinamide) = 12.0	3.5	0.30
K_I (Acetyl-D-tyrosine ethyl ester) = 3.5	3.2	0.90
K_S (Nicotinyl-L-tyrosinamide) = 15.0	1.1	0.07
K_I (Nicotinyl-D-tyrosinamide) = 6.4	1.1	0.17

* Calculated on the basis of the reasonable assumption that α-chymotrypsin has a molecular weight of 27,000, and contains but one reactive site per molecule[32].

FIG. XXIV

Biphasic Character of the Course of Hydrolysis of Acetyl-L-Tyrosinamide

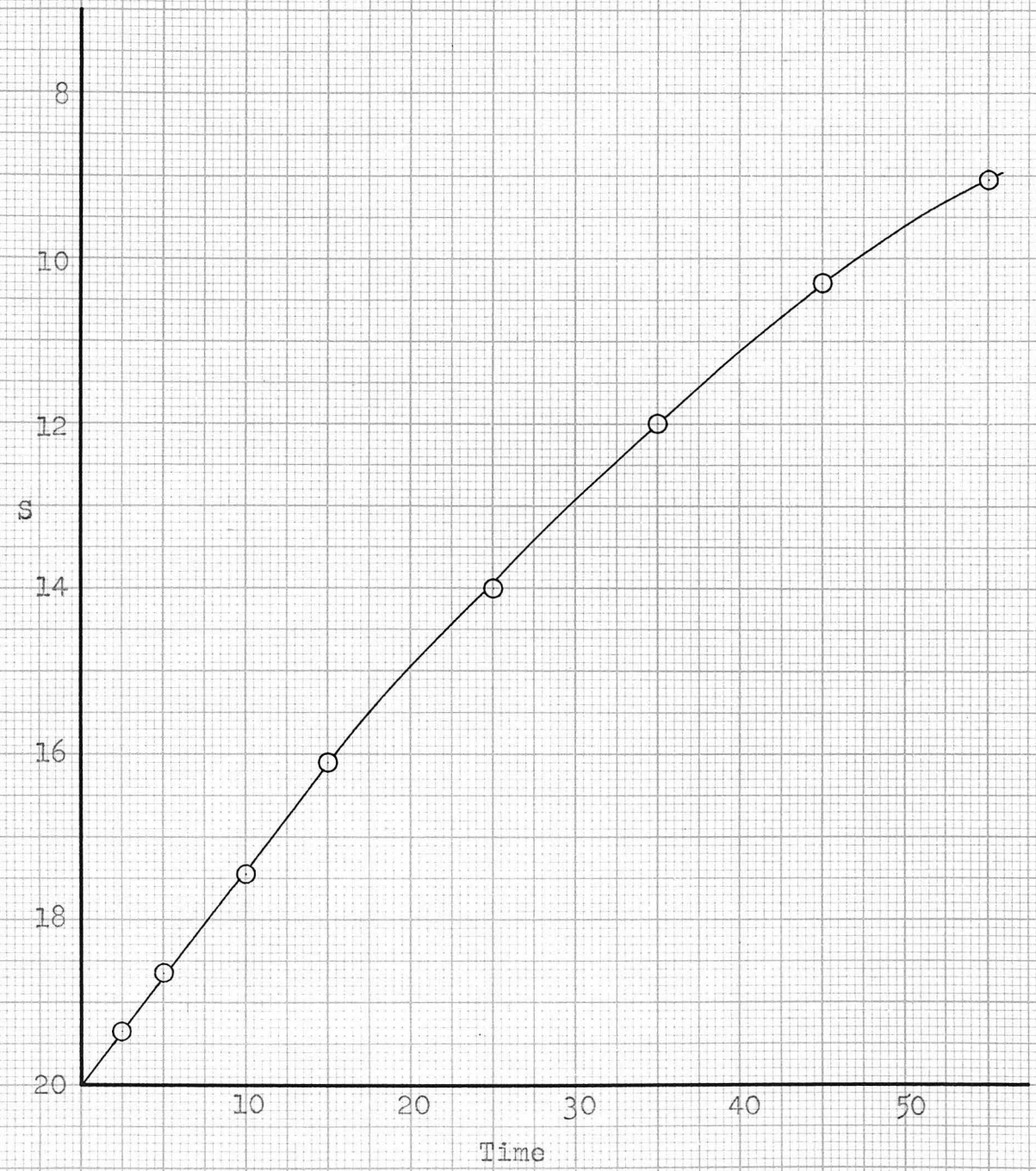


FIG XXV

Biphasic Character of the Course of
Hydrolysis of Nicotinyl-L-Tyrosinamide

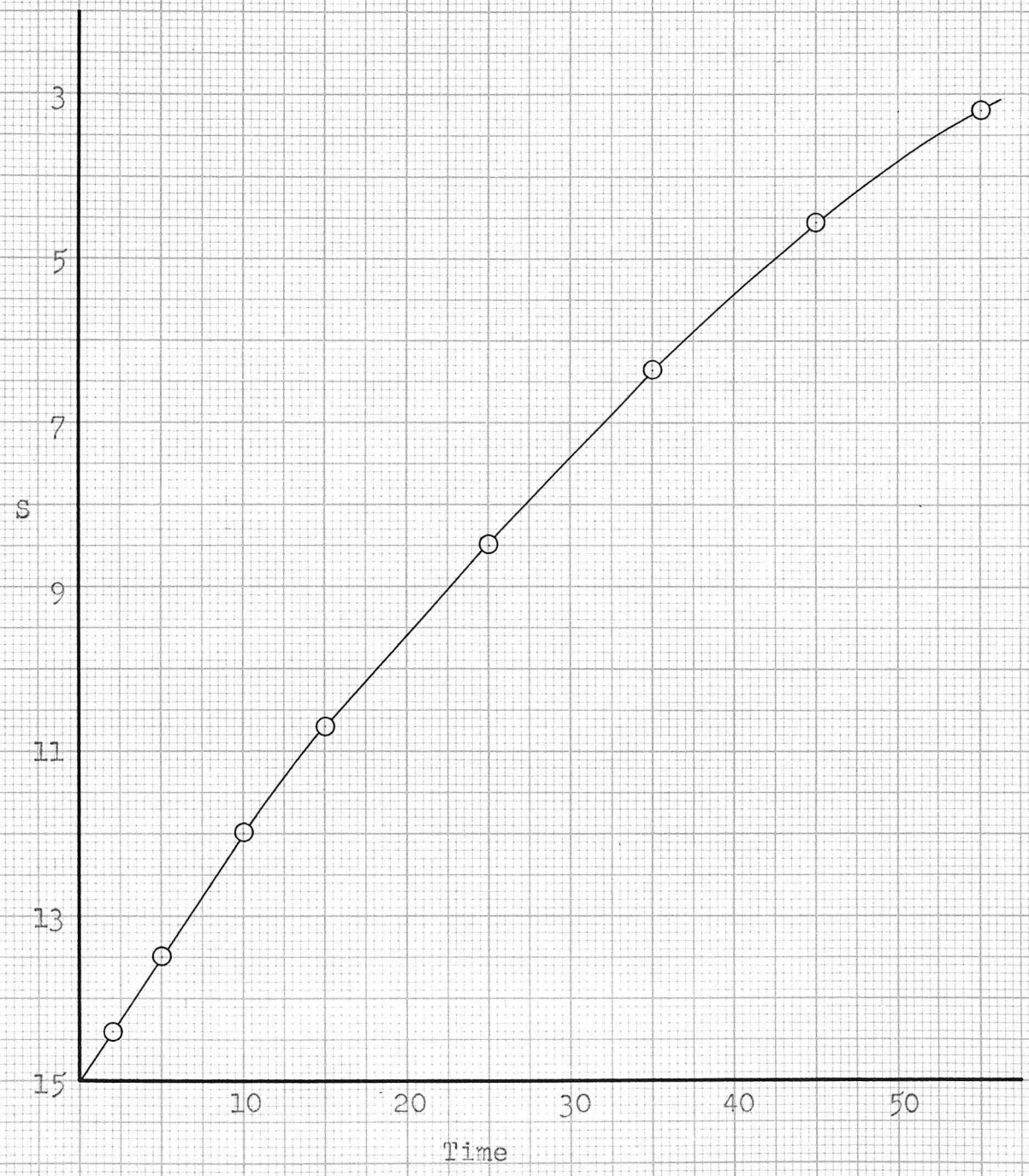


TABLE XXII

Kinetic Constants of Several Substrates and Competitive Inhibitors of α-Chymotrypsin

Compound	K x 10^3 M Value	Constant	$k_3 x 10^3$***
Acetyl-L-tyrosinamide	30.5 ± 1.0	K_S	2.4
Acetyl-L-tryptophanamide*	5.3 ± 0.2	K_S	0.5
Acetyl-L-tyrosine	115 ± 15	K_{P_1}	
Acetyl-L-tryptophane*	17.5 ± 1.5	K_{P_1}	
Acetyl-D-tyrosinamide	12.0 ± 1.0	K_I	
Acetyl-D-tryptophanamide*	2.7 ± 0.2	K_I	
Acetyl-D-tyrosine ethyl ester	3.5 ± 0.5	K_I	
Nicotinyl-L-tyrosinamide	15.0 ± 1.0	K_S	6.2
Nicotinyl-L-tryptophanamide*	2.7 ± 0.2	K_S	1.6
Nicotinyl-L-tyrosine**	60 ± 5	K_{P_1}	
Nicotinyl-L-tryptophane*	8.8 ± 1.0	K_{P_1}	
Nicotinyl-D-tyrosinamide	6.4 ± 0.5	K_I	
Nicotinyl-D-tryptophanamide*	1.4 ± 0.1	K_I	
Nicotinyl-D-tyrosine ethyl ester**	1.0 ± 0.4	K_I	

* cf. reference[32].

** cf. reference[27].

***In aqueous solution at pH 7.9 ± 0.1 and 25° C.

TABLE XXIII

Ratios of Various K_{P_1}, K_I, and K_S Values

$$\frac{\text{Acetyl-L-tyrosine}}{\text{Acetyl-L-tryptophane}} = \frac{115}{17.5} = 6.6$$

$$\frac{\text{Nicotinyl-L-tyrosine}}{\text{Nicotinyl-L-tryptophane}} = \frac{60}{8.8} = 6.8$$

$$\frac{\text{Acetyl-D-tyrosinamide}}{\text{Acetyl-D-tryptophanamide}} = \frac{12.0}{2.7} = 4.4$$

$$\frac{\text{Nicotinyl-D-tyrosinamide}}{\text{Nicotinyl-D-tryptophanamide}} = \frac{6.4}{1.4} = 4.6$$

$$\frac{\text{Acetyl-L-tyrosine}}{\text{Nicotinyl-L-tyrosine}} = \frac{115}{60} = 1.9$$

$$\frac{\text{Acetyl-L-tryptophane}}{\text{Nicotinyl-L-tryptophane}} = \frac{17.5}{8.8} = 2.0$$

$$\frac{\text{Acetyl-D-tyrosinamide}}{\text{Nicotinyl-D-tyrosinamide}} = \frac{12.0}{6.4} = 1.9$$

$$\frac{\text{Acetyl-D-tryptophanamide}}{\text{Nicotinyl-D-tryptophanamide}} = \frac{2.7}{1.4} = 1.9$$

$$\frac{\text{Acetyl-D-tyrosinamide}}{\text{Acetyl-D-tyrosine ethyl ester}} = \frac{12.0}{3.5} = 3.4$$

$$\frac{\text{Nicotinyl-D-tyrosinamide}}{\text{Nicotinyl-D-tyrosine ethyl ester}} = \frac{6.4}{1.0} = 6.4$$

$$\frac{\text{Acetyl-L-tyrosinamide}}{\text{Nicotinyl-L-tyrosinamide}} = \frac{30.5}{15} = 2.0$$

$$\frac{\text{Acetyl-L-tryptophanamide}}{\text{Nicotinyl-L-tryptophanamide}} = \frac{5.3}{2.7} = 2.0$$

$$\frac{\text{Acetyl-L-tyrosinamide}}{\text{Acetyl-D-tyrosinamide}} = \frac{30.5}{12.0} = 2.5$$

$$\frac{\text{Nicotinyl-L-tyrosinamide}}{\text{Nicotinyl-D-tyrosinamide}} = \frac{15}{6.4} = 2.4$$

$$\frac{\text{Acetyl-L-tyyptophanamide}}{\text{Acetyl-D-tryptophanamide}} = \frac{5.3}{2.7} = 2.0$$

$$\frac{\text{Nicotinyl-L-tryptophanamide}}{\text{Nicotinyl-D-tryptophanamide}} = \frac{2.7}{1.4} = 1.9$$

Experimental

Enzyme Solutions - Crystalline α-chymotrypsin containing magnesium sulfate (Armour, Lot No. 90402), and crystalline trypsin also containing magnesium sulfate (Armour, Lot No. 8011X) were used in these studies. The chymotrypsin preparation contained 10.4% N, and the trypsin preparation 7.9% N. Stock solutions of enzyme were prepared daily and were kept in the ice box, except when it was necessary to bring them to 25° C. to allow withdrawal of 1.0 ml. aliquots.

Buffer Solutions - The tris(hydroxymethyl)aminomethane-hydrochloric acid and ethylenediamine-hydrochloric acid buffers, 0.2 F with respect to the amine component, and of the desired pH, were prepared as described by Iselin and Niemann[51].

Aqueous Formaldehyde - Merck reagent grade formaldehyde, 36-38%, was adjusted to pH 8 with aqueous sodium hydroxide.

Procedure - The substrate, or substrate plus inhibitor, was placed in a 10 ml. volumetric flask, and approximately 7.5 ml. of distilled water added. Heat was applied if necessary to effect solution, 1.0 ml. of 0.2 F buffer then added, and the stoppered flask placed in a constant temperature water bath at 25.0 ± 0.1° C. Sufficient time was allowed for the contents of the flask to come to temperature, and then 1.0 ml of enzyme solution added at "zero time" minus 20 seconds. The solution was immediately made up to volume, the flask stoppered and inverted gently 10-12 times to ensure adequate

mixing, and then returned to the constant temperature bath. A 1.0 ml. aliquot was then withdrawn and delivered into a 10 ml. shell vial containing 1.0 ml. of formaldehyde solution. Further aliquots were then withdrawn at the desired times, and immediately added to formaldehyde, as above, to stop the hydrolytic reaction.

The above mixtures were immediately titrated potentiometrically with standard 0.01 N sodium hydroxide, by use of a Beckman Model G pH meter, equiped with a No. 270-6 calomel electrode and a No. 290-11 glass electrode. The alkali was added from a burette, graduated in 0.01 ml., equipped with a capillary tip of sufficient length to permit introduction of the reagent beneath the surface of the solution being titrated. Adequate stirring was achieved by rotation of the shell vial[51]. The end-point of the titration and extent of hydrolysis were determined by examination of the curve constructed for each titration[51]. Where the reaction was apparently zero-order with respect to the substrate concentration, the initial velocities were estimated from a plot of $([S]_o - [S])$ versus time; where the reaction was approximately first-order with respect to the substrate concentration the initial velocities were estimated from a plot of $\log ([S]_o - [S])$ versus time.

Blank determinations were made with all substrates and inhibitors, and with the enzymes. In no case was it found necessary to correct for non-enzymatic hydrolysis, or for

autolysis of chymotrypsin, (the instability of trypsin has been discussed elsewhere), the reaction periods being relatively short (30-60 min.). Frequent checks were also made to determine if the pH was being maintained constant.

The precision of the measurements reported in this study was identical with that obtained by Huang and Niemann[32].

N-Acetyl-L-Arginine $.2H_2O$ - This compound was first prepared by Bergmann and Zervas[56]. However, it was found that on duplication of their procedure, racemization was apt to occur. Therefore, various modifications were tried, and it is felt that the procedure given below is the most satisfactory.

L-Argininehydrochloride ($[\alpha]_D^{25^\circ} = +26.7 \pm 0.5^\circ$ (C = 1.7% in 6N HCl)), 50.0 g., and sodium carbonate, 40.0 g., were dissolved in 375 ml. of water. The solution was cooled in an ice-bath, and 25 ml. of 95% acetic anhydride was added dropwise and with constant shaking. More sodium carbonate was added, if necessary, to bring the final pH to approximately 8.0. After standing overnight in the ice-box, copious crystallization had occurred. The product was recrystallized twice from water. Yield 35 g., m.p. 258-260° dec.*, $[\alpha]_D^{25^\circ} = +9.4 \pm 0.15^\circ$ (C = 3.5% in water). Lit.[57], m.p. 269-270°, $[\alpha]_D^{26} = +7.72$.

Anal. calcd. for $C_8H_{16}O_3N_4 \cdot 2H_2O$ (252.3): C, 38.1; H, 7.9; N, 22.2. Found: C, 38.3; H, 7.7; N, 22.4.

---

* All melting points are corrected.

To test the optical purity of the N-acetyl-L-arginine, part of the material was hydrolyzed by refluxing for 6 hours in 6N HCl, and the rotation determined for the L-arginine thus obtained. $[\alpha]_D^{25^\circ}$ = + 27.0 ± 0.5° (C = 1.1% in 6N HCl). Lit. (58), $[\alpha]_D^{23^\circ}$ = + 26.9 (C = 1.7% in 6N HCl).

N-Acetyl-L-Arginine Methyl Ester Hydrochloride - N-Acetyl-L-Arginine was dissolved in methanol, and the solution saturated with dry HCl at 0° C. After 2 days in the ice box, the solution was evaporated at 30° *in vacuo*. The oil thus obtained could neither be crystallized, nor completely freed from excess HCl. An attempt to prepare the crystalline ethyl ester also failed.

N-Acetyl-L-Argininamide Hydrochloride - Dissolved the oily N-acetyl-L-argininemethyl ester in methanol, and saturated the solution with dry NH_3 at 0° C. After standing for 2 days in the ice box, the solution was evaporated at 30° *in vacuo*. The oil thus obtained was extremely hygroscopic, and contained much ammonium chloride. Precipitation from various alcohols finally gave an amorphous material that could be crystallized from abs. ethanol. The yield of crystalline products was low. M.p. 128-132°.

Anal. calcd. for $C_8H_{17}O_2N_5 \cdot HCl$(251.7): C, 38.2; H, 7.2; N, 27.8.

Found: C, 38.3; H, 7.4; N, 27.6.

L-Arginine Methyl Ester Dihydrochloride - The method of preparation was that of Dirr and Späth(59). To 5.0 g. of

L-arginine hydrochloride added 100 ml. of methanol. The suspension was saturated with dry HCl at 0° C, and the resulting solution placed in the ice-box for 2 days. Evaporation *in vacuo* gave an oil that crystallized on standing. Recrystallized twice from ethanol. Yield 3.5 g. m.p. 185-187° dec. Lit[59], m.p. 195° dec.

Benzoyl-L-Argininamide Hydrochloride·H_2O - This material was prepared by Dr. Rinderknecht, according to the procedure of Bergmann *et al.*[10] m.p. 120-125°, decomp. 260°

Anal. Calcd. for $C_{13}H_{19}O_2N_5 \cdot HCl \cdot H_2O$ (331.8): N, 21.2

Found: N, 21.2.

Acetyl-L-Tyrosine - This material was prepared as directed by duVigneaud and Meyer[57]. Colorless rods, m.p. 151-153°, $[\alpha]_D^{25°} = +47.4°$ (C= 2% in water). Lit.[57], m.p. 152-154°, $[\alpha]_D^{26°} = +47.5°$.

Anal. calcd. for $C_{11}H_{13}O_4N$(223): C, 59.2; H, 5.9; N, 6.3.

Found: C, 59.2; H, 5.8; N, 6.2.

Acetyl-L-Tyrosinamide - Acetyl-L-tyrosine was esterified in the usual manner with ethanolic-hydrogenchloride to give acetyl-L-tyrosine ethyl ester, m.p. 96-97°, $[\alpha]_D^{25°} = +24.7$ (C = 7% in ethanol), in an 80% yield[52]. This material was dissolved in methanol, the solution saturated with ammonia at 0°, the reaction mixture allowed to stand at 25° for 2 days, the solution evaporated to dryness *in vacuo*, and the residue recrystallized twice from aqueous-ethanol to give a 60% yield of the amide. m.p. 226-228°, $[\alpha]_D^{25°} = +49.7$

(C = 0.8% in water). Lit.[23], m.p. 222-224°.

Anal. calcd. for $C_{11}H_{14}O_3N_2$(222): C, 59.5; H, 6.4; N, 12.6.

Found: C, 59.5; H, 6.4; N, 12.6.

Acetyl-D-Tyrosine Ethyl Ester - This material was prepared by R. V. MacAllister[52]. m.p. 95-97°, $[\alpha]_D^{25°}$ = - 24.8° (C = 7% in ethanol).

Anal. calcd. for $C_{13}H_{17}O_4N$(251): C, 62.1; H, 6.8; N, 5.6.

Found: C, 62.2; H, 6.8; N, 5.6.

Acetyl-D-Tyrosinamide: - This material was prepared by R. V. MacAllister[52]. m.p. 225-226°, $[\alpha]_D^{25°}$ = - 49.4° (C = 0.9% in water).

Anal. calcd. for $C_{11}H_{14}O_3N_2$(222): C, 59.5; H, 6.4; N, 12.6.

Found: C, 59.7; H, 6.6; N, 12.5.

Nicotinyl-L-Tyrosinamide - This material was prepared by R. V. MacAllister[26]. m.p. 226-227°.

Anal. calcd. for $C_{15}H_{15}O_3N_3$(285): C, 63.2; H, 5.3; N, 14.7.

Found: C, 63.0; H, 5.6; N, 14.8.

Nicotinyl-DL-Tyrosinamide: - This material was prepared by R. V. MacAllister[26]. m.p. 224-226°.

Anal. calcd. for $C_{15}H_{15}O_3N_3$(285): C, 63.2; H, 5.3; N, 14.7.

Found: C, 63.4; H, 5.6; N, 14.5.

Bibliography

1. Northrop, J. H., and Kunitz, M., Science, 73, 262 (1931).

2. Northrop, J. H., Kunitz, M., and Herriott, R. M., Crystalline Enzymes, 2nd ed. Columbia University Press, New York (1948).

3. Kunitz, M., Enzymologia, 7, 1 (1939).

4. Kunitz, M., J. Gen. Physiol., 22, 207 (1939).

5. Neurath, H., and Schwert, G., Chem. Rev., 46, 69 (1950).

6. Kunitz, M., J. Gen. Physiol., 22, 447 (1939).

7. Bergmann, M., Fruton, J. S., and Pollok, H., Science, 85, 410 (1937).

8. Bergmann, M., and Fruton, J.S., J. Biol. Chem., 118, 405 (1937).

9. Bergmann, M., and Fruton, J. S., J. Biol. Chem., 124. 321 (1938).

10. Bergmann, M., Fruton, J.S., and Pollok, H., J. Biol. Chem., 127, 643 (1939).

11. Hofmann, K., and Bergmann, M., J. Biol. Chem., 130, 81 (1939).

12. Hofmann, K., and Bergmann, M., J. Biol. Chem., 138, 243 (1941).

13. Fruton, J. S., and Bergmann, M., J. Biol. Chem., 145, 253 (1942).

14. Bergmann, M., and Fruton, J.S., Adv. in Enzymol., 1, 63 (1941).

15. Bergmann, M., Adv. in Enzymol., 2, 49 (1942).

16. Brand, E., Ellenbogen, E., Erlanger, B., Plentl, A., and Polatnik, J., Abstracts of Papers, 117th Meeting, Am. Chem. Soc., Philadelphia, 1950, p. 56C

17. Iselin, B.M., Huang, H.T., MacAllister, R.V., and Niemann C. J. Am. Chem. Soc., 72, 1729 (1950).

18. Kaufman, S., and Neurath, H., Arch. Biochem., 21, 437 (1949).

19. Schwert, G. W., Neurath, H., Kaufman, S., and Snoke, J.E., J. Biol. Chem., 172, 221 (1948).

20. Snoke, J. E., and Neurath, H., Arch. Biochem., 21, 351 (1949).

21. Schwert, G. W., and Eisenberg, M. A., J. Biol. Chem., 179, 665 (1949).

22. Jansen, E. F., Fellows Nutting, M.D., Jang, R., and Balls, A. K., J. Biol. Chem., 179, 189 (1949).

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

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

25. Schwert, G. W., and Kaufman, S., J. Biol. Chem., 180, 517 (1949).

26. MacAllister, R. V., Thesis, Calif. Inst. of Technology (1949)

27. Huang, H. T., Unpublished Results.

28. Shine, H., Unpublished Results.

29. MacAllister, R. V., and Niemann, C., J. Am. Chem. Soc., 71, 3854 (1949).

30. Iselin, B. M., Huang, H. T., and Niemann, C., J. Biol. Chem., 183, 403 (1950).

31. MacAllister, R. V., Harmon, K. M., and Niemann, C., J. Biol. Chem., 177, 767 (1949).

32. Huang, H. T., and Niemann, C., J. Am. Chem. Soc., In Press.

33. Kaufman, S., and Neurath, H., Arch. Biochem., 21, 245 (1949).

34. Kaufman, S., and Neurath, H., J. Biol. Chem., 181, 623 (1949).

35. Michaelis, L., and Menten, M.L., Biochem. Z., 49, 333, (1913)

36. Van Slyke, D. D., and Cullen, G. E., J. Biol. Chem., 19, 141, 211 (1914).

37. Briggs, G. E., and Haldane, J. B. S., Biochem. J., 19, 338 (1925).

38. Haldane, J. B. S., "Enzymes", Longmans Green, London (1930).

39. Chance, B., J. Biol. Chem., 151, 553 (1943).

40. Straus, O.H., and Goldstein, A., J. Gen. Physiol., 26 559 (1943).

41. Goldstein, A., J. Gen. Physiol., 27, 529 (1944).

42. Elkins-Kaufman, E., and Neurath, H., J. Biol. Chem., 175, 893 (1948).

43. Lineweaver, H., and Burk, D., J. Am. Chem. Soc., 56, 658 (1934).

44. Harmon, K., and Niemann, C., J. Biol. Chem., 178, 743 (1949).

45. Franz, I.D., Jr., and Stephensen, M., J. Biol. Chem., 169, 359 (1947).

46. Butler, J.A.V., J. Am. Chem. Soc., 63, 2971 (1941).

47. Irving, G.W., Jr., Fruton, J.S., and Bergmann, M., J. Biol. Chem. 138, 231 (1941).

48. Harmon, K.M., Thesis, Calif. Inst. of Technology, (1948).

49. Kunitz, M., and Northrop, J.H., J. Gen. Physiol., 17, 591 (1933).

50. Jansen, E.F., Fellows Nutting, M.D., Jang, R., and Balls, A.K., J. Biol. Chem., 185, 209 (1950).

51. Iselin, B.M., and Niemann, C., J. Biol. Chem., 182, 821 (1950).

52. Thomas, D.W., MacAllister, R.B., and Niemann, C., J. Am. Chem. Soc., In Press.

53. Snoke, J.E., and Neurath, H., J. Biol. Chem., 181, 789 (1949)

54. McIlwain, H., Nature, 158, 898 (1946).

55. Pauling, L., Am. Scientist, 36, 51 (1948).

56. Bergmann, M., and Zervas, L., Biochem. Z. 203, 280 (1928).

57. du Vigneaud, V., and Meyer, C.E., J. Biol. Chem., 98, 295 (1932).

58. Schmidt, C.L.A., "The Chemistry of the Amino Acids and Proteins", Thomas, Springfield, (1944).

59. Dirr, K., and Späth, H., Z. physiol. Chem., 237, 121 (1935).

Propositions

1. The claim (1) that in 0.1 M hydrochloric acid solutions of glucose, iron (III) preferentially complexes with the -isomer of glucose does not seem justified from the data presented.

(1) Delaney, Kleinberg and Argersinger, J. Am. Chem. Soc., 72, 4277 (1950).

2. (a) In the mineral acid catalyzed hydrolysis of low molecular weight, unsubstituted amides, a maximum rate is obtained in the concentration range of 3-6 M acid. It is predicted that such a maximum would also be found in the acid-catalyzed hydrolysis of hydrazides.

(b) The statement of Laidler and Socquet (2) on p. 527 leads to the impression that the mineral acid-catalyzed hydrolysis of amides can be characterized in terms of equations (24) and (25). It is felt that equation (12) more accurately describes the observed maximum in such an acid-catalyzed hydrolysis.

(2) Laidler and Socquet, J. Phys. and Colloid Chem., 54, 519 (1950).

3. It is felt that heteroauxin (indole acetic acid) may not function as a plant hormone in the generally excepted sense, but rather as a catabolic inhibitor. Thus the effect of auxin a and of hetero auxin on growth may be very different.

4. Chymotrypsin is only known to catalyze the hydrolysis of substrates where the "specific" amino acid is of the L- configuration (3). Acetyl-L-tyrosylglycinamide is known to be hydrolyzed by chymotrypsin, giving acetyl-L-tyrosine and glycinamide. It would be worthwhile to investigate the possibility that acetylglycyl-D-tyrosinamide, or a similar compound, might be hydrolyzed to give acetylglycine and D-tyrosinamide.

(3) This Thesis, p. 29.

5. K_I gives a true measure of the affinity of an enzyme for an inhibitor (4), and while it is felt that K_S also is a true measure of the affinity of chymotrypsin for a substrate, this is not known with certainty. Since secondary and tertiary amides are not hydrolyzed by chymotrypsin, and since the amides of "specific" D-amino acids act as inhibitors, it might be possible to estimate the value of k_2/k_1 for an amide substrate by determining the inhibitor-enzyme dissociation constants for a series of substituted D- and L- "specific" amino acid amides.

(4) This Thesis, p. 63.

6. (a) Neurath and Schwert (5) state that substrates for trypsin must have terminal cationic groups on the "specific" amino acid side chains, and that the steric effect of the side chains is relatively unimportant. The evidence of Brand et al (6) and Hofmann and Bergmann (7) can better be interpreted to indicate that a cationic group is not necessary, but that the "specific" amino acid side chain must have a certain critical length.

(b) It is suggested that the following compound be tested as a trypsin substrate:

$$CH_3 - \overset{O}{\overset{\|}{C}} - (CH_2)_4 - CHNH_2 - CONH_2$$

(5) Neurath and Schwert, Chem. Revs., 46, 104, 107 (1950).
(6) Brand et al, Abstracts of Papers, 117th Meeting, Am. Chem. Soc., Philadelphia, 1950, p. 56 C.
(7) Hofmann and Bergmann, J. Biol. Chem., 130, 81 (1939).

7. ATP is present in blood in relatively high concentrations. Fibrinogen resembles myosin in many of its properties. It would be worthwhile to investigate the role of ATP in coagulation.

8. The theory of Riseman and Kirkwood (8), on the role of ATP in muscle contraction, should be extensively investigated using model systems.
(8) Riseman and Kirkwood, J. Am. Chem. Soc., 70, 2820 (1949).

9. The recent work of Jansen et al (9) indicates a very specific reaction between chymotrypsin and diisopropylfluorophosphate, the enzyme being phosphorylated near, or at the enzymatically active site of the molecule. This phosphorylating agent offers opportunities to study the nature of the enzymatically active group. Attempts to reverse the phosphorylation should be made.
(9) Jansen et al, J. Biol. Chem., 185, 209 (1950).

10. (a) The study of the following compounds as chymotrypsin substrates or inhibitors would give further evidence as to the mode of action of the enzyme.

(1) $\text{phenyl-}CH_2\text{-}CH(NHCO\ \text{phenyl})\text{-}CH_2\text{-}CO_2R$

(2) $\text{phenyl-}CH_2\text{-}CH(NHCO\ \text{phenyl})\text{-}CH = CH\text{-}CO_2R$

(3) $\text{phenyl-}CH(NHCO\ \text{phenyl})\text{-}CH_2\text{-}CO_2R$

(4) phenyl-CH-CH = CH-CO_2R
        |
        NHCO phenyl

(5) phenyl-CH_2-CH_2-CH-CO_2R
                  |
                  NHCO phenyl

(6) phenyl-CH_2-CH_2-C(=O)-CH_3

(7) phenyl-CH_2-CH_2-NO_2

11. It is proposed that a portion of the undergraduate student body be allowed to major in the Humanities.