I. THIOCARBAMATE STUDIES.

II. PRELIMINARY INVESTIGATIONS OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACYLATED AMINO ACID ESTERS.

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It has been a rewarding experience to have pursued my graduate research under the direction of Professor Carl Niemann. His willingness to discuss every problem coupled with an insistence on independent thought and action has fulfilled the concepts of research to the highest degree.

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This thesis is fondly dedicated to my wife, Harriet. Her untiring efforts to provide the material necessities and a suitable atmosphere for a comfortable home have contributed far more than words can express.

ABSTRACT

The known reactions of N,S-diphenylthiocarbamate were extended by studies of the effects of various reagents and conditions. The results are discussed in terms of previously suggested reaction mechanisms.

The mixed anhydrides between S-phenylthiocarbonyl-chloride and carboxylic acids were briefly investigated as preparative intermediates. Low yields of the expected products offset the utility of this method.

The non-reactivity of secondary amine S-phenylthiocarbamates was further demonstrated with N-pentamethylene-S-phenylthiocarbamate. The inert nature of this compound indicates that the effects are not due to steric hindrance.

A brief investigation of the reactions of S-benzylthio- and O-benzyl-N-phenylcarbamate suggest that they have little value as preparative intermediates as compared to the S- and O-phenyl derivatives.

Some S-phenylthiocarbamates of amino acid derivatives were prepared, and their use in a polymerization reaction and stepwise dipeptide synthesis is described. The results suggest that the various methods are not as elegant as indicated earlier.

A recently developed automatic titration instrument was employed in a preliminary study of the alpha-chymotrypsin catalyzed hydrolysis of some acylated amino acid esters. A useable system was developed, and some preliminary results

were obtained concerning techniques, data treatment, surface effects and steric effects in enzyme catalyzed reactions.

The kinetics of the alpha-chymotrypsin catalyzed hydrolysis of methyl hippurate were re-investigated. In addition, the enzyme-inhibitor dissociation constants of two competitive inhibitors were re-determined. The results are compared with values obtained earlier.

Benzoyl-L-valine methyl ester, a new substrate for this enzyme, was studied in the catalyzed reaction employing the above techniques. Additional refinements in technique and errors in this system are discussed.

A number of potential competitive inhibitors, the N-acetyl amino acid N'-methylamides, were synthesized and tested for inhibitory activity in the alpha-chymotrypsin catalyzed hydrolysis of methyl hippurate and benzoyl-L-valine methyl ester. The results indicate that, of the compounds studied, only those containing an aromatic residue have a measurable effect in the present systems.

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I. THIOCARBAMATE STUDIES

A. Introduction*

It was shown by Schuller and Niemann (1) that S-phenylthiocarbamates derived from primary amines readily react with primary and secondary amines under basic conditions to form di- and tri-substituted ureas, while S-phenylthiocarbamates derived from secondary amines are inert to these reagents. The first reaction was further examined by Crosby and Niemann (2) and proposed as a general method for the synthesis of substituted ureas. In addition, it was shown that the reaction of S-phenylthiocarbamates prepared from primary amines with certain hydrazides and amides give semicarbazides and acylureas respectively (2). Although most of the above reactions proceed in nearly quantitative yield at room temperature, the substitution of oxygen for sulfur in the carbamates necessitated the use of much higher reaction temperatures to attain satisfactory yields (2).

In addition to the above application, it was suggested earlier by Ehrensvärd (3) that the S-phenylthiocarbonyl group was a satisfactory blocking group for the amino moiety of amino acids. Although no experimental evidence was presented, it was further suggested by this author that

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*Portions of the work reported here were included in the paper of Crosby and Niemann (2).

the blocking group could be readily removed by treatment with lead acetate. That this was not the case, without undesirable products forming, was demonstrated by Lindenmann, Khan, and Hofmann (4) who found that S-phenylthiocarbamates of dipeptide esters on treatment with lead acetate, were converted essentially quantitatively to substituted hydantoins.

A more recent application of the S-phenylthiocarbamates of amino acids is due to Noguchi and co-workers (5) who have described the thermal polymerization of these derivatives and those of dipeptides in a manner which supposedly yields high molecular weight, linear polypeptides.

Because of the wide variety of possibilities for reaction studies offered by the above observations, it was considered that an examination of the reactions of thiocarbamates under conditions differing from those previously investigated (1-5), as well as with different types of reagents would be of value in broadening the knowledge concerning the chemical behavior of these compounds. In addition, the proposed formation of mixed anhydrides between carboxylic acids and S-phenylthiocarbonyl chloride (2) was briefly investigated as a preparative method.

S-Phenylthiocarbamates of primary amines not only react with amines and some amides as previously noted (1,2), but also undergo hydrolysis, base catalyzed decomposition

in non-aqueous media, and salt formation with a strong base under the latter conditions. It also was found that the lack of reactivity of the secondary amine thiocarbamates under the above conditions (1) could not be attributed to steric hindrance.

Extension of the reactions of S-phenylthiocarbamates to the systems S-benzylthio- and O-benzyl-carbamates were generally unsuccessful.

Attempted preparations of mono-S-phenylthiocarbamate hydrochlorides of several diamines, and their subsequent base catalyzed polymerization to polyureas, as well as the direct preparation of the polyureas from the acid chloride and the diamine generally led to products which were of a questionable nature. However, the polymerization (5) of the S-phenylthiocarbamate of glycine (4,5) appeared to proceed (in much diminished yield) as stated. In addition, the use of the S-phenylthiocarbonyl group (3-5) in the preparation of a number of S-phenylthiocarbonyl dipeptides was investigated.

B. The Preparation and Reactions of N,S-Diphenylthiocarbamate (2,6).

Although this compound may be obtained from thiophenol and phenylisocyanate (6), the present study was based on the applicability of the reaction of S-phenylthiocarbonyl chlo-

ride (7,8) with primary and secondary amines (1-8). While the reaction proceeded as stated (6), attempted recrystallization from hot ethanol-water solutions (6) led to mixtures of compounds with wide melting ranges. These were separated by ligroin extractions and identified as N,S-diphenylthiocarbamate and N,N'-diphenylurea.*

An extension of the above observation was the investigation of the effect of varying amounts of water on the course of the reactions of N,S-diphenylthiocarbamate with 2-aminopyridine as well as the effect of water on solutions of the thiocarbamate alone. In the first case addition of 9 V % water to an ethanol solution of the reactants plus triethylamine did not appear to change the composition of the product, while the introduction of 25 V % water to a dioxane solution of the same reaction mixture resulted in the isolation of sym-diphenylurea contaminated with the expected N-phenyl-N'-(2-pyridyl) urea. Repetition of this type of treatment employing dioxane solutions of the thiocarbamate alone plus a one mole excess of water (ca. 1.3 V %) in the presence and absence of triethylamine did not appear to alter the starting material. These and the previous observations (6) would suggest that the thiocarbamates are relatively inert in solutions containing low percentages of

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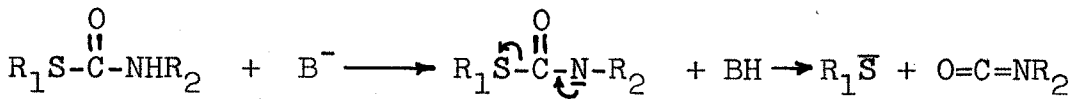
*This undoubtedly accounts for the report that recrystallization of S-phenylthiocarbamates from ethanol-water gave compounds that had unsatisfactory analyses (6).

water (cf. 2,6) at room temperatures, but that in the presence of large amounts of water or at elevated temperatures the side reactions accompanying hydrolysis lead to large amounts of the symmetrical ureas of the primary amine employed in preparing the S-phenylthiocarbamate.

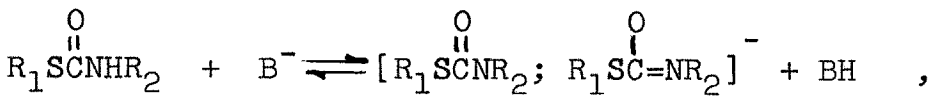
Although the above reaction might be postulated to be a general one, Riemschneider (9), in addition to proposing a new method for the preparation of some thiocarbamates, has recently noticed that while the treatment of S-n-butyl-N-cyclohexylthiocarbamate with boiling sodium hydroxide solutions leads to sym-dicyclohexylurea in 86% yield, the same treatment of S-phenyl-N-t-butylthiocarbamate gives a 77% yield of t-butylamine. While this fact invalidates the generality concerning the transformation of thiocarbamates of primary amines to the corresponding sym-ureas, it suggests an even more interesting possibility of further investigations of the steric factors operating in the reactions of these compounds. Further elaboration of these steric factors might well aid in a final elucidation of the mechanism(s) in operation during the reactions of these compounds.

The mechanism(s) of the reactions of thiocarbamates have been considered by several authors (1,2,4,5). While it has been suggested (4) that the thiocarbamate first loses a proton and undergoes a 1:2 elimination reaction

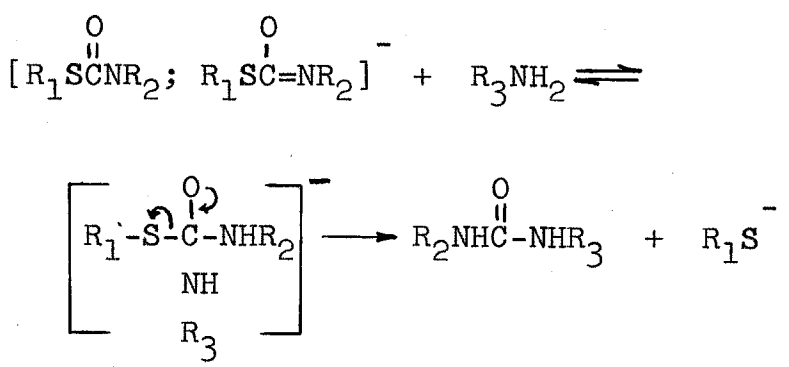
to form an isocyanate,



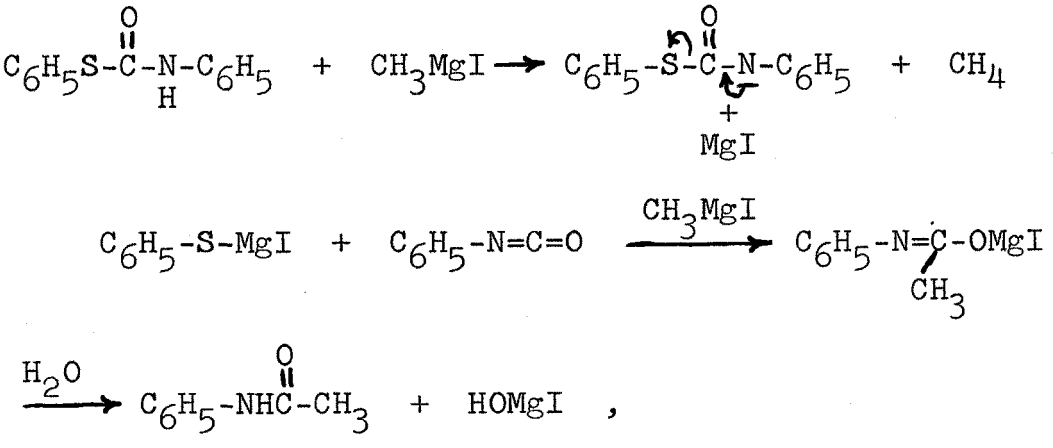
which then reacts with a primary or secondary amine to form di- or tri-substituted ureas, it has been shown (2) that the presence of an isocyanate cannot be demonstrated by the use of appropriate trapping agents or by carrying out the reaction in the presence of an excess of isopropanol or ethanol. These facts, plus the obvious base-dependence of the system, led these authors (1,2) to suggest that the reaction could be formulated as proceeding by the removal of a proton to form a resonance-stabilized intermediate ion, i.e.



which is then attacked by the primary or secondary amine with the concomitant elimination of the thiol anion and formation of the substituted urea, e.g.



In investigating the reactions of N,S-diphenylthiocarbamate it was apparent that the generation of the first intermediate and its tendency to rearrange to form either phenylisocyanate or to remain as the anion would be interesting to demonstrate. For this reason N,S-diphenylthiocarbamate was treated with two moles of methylmagnesium iodide. It was supposed that the reaction would proceed as follows in the first instance,



while in the second instance it did not seem likely that the intermediate charged species would be subject to attack by the Grignard reagent. The latter reaction appears to be predominant in that salt formation was observed (evolution of gas and separation of white solid), but treatment of the reaction mixture with dilute hydrochloric acid resulted in recovery of a high percentage of the starting material.

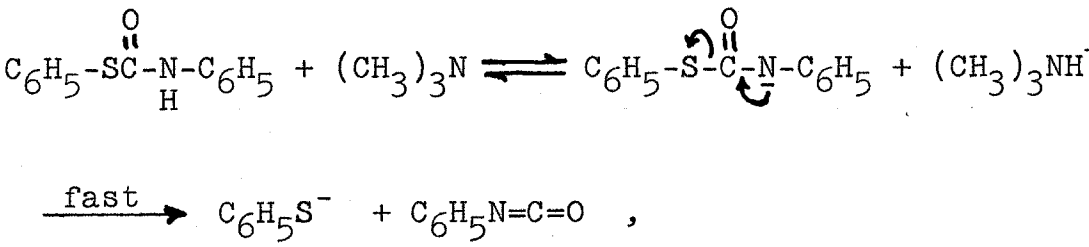
It is recognized, however, that this is not conclusive

evidence for the non-existence of an isocyanate intermediate since under the conditions employed (ca. 35°C.) the salt formed may not undergo the postulated (4) rearrangement. However, the foregoing facts and earlier observations (1,2) coupled with the observations of the behavior of N-t-butyl-S-phenylthiocarbamate (9) in basic solution and the extremely different behavior of t-butylisocyanate observed by Brauner (10) under similar conditions are strong evidence against the isocyanate mechanism. The latter author has shown that treatment of t-butylisocyanate with water or caustic potash yields only N,N'-di-t-butylurea, and none of the free t-butylamine (cf. page 6), and the urea so formed is identical to the urea formed by the treatment of t-butylisocyanate with t-butylamine.

Further information regarding the reactions of N,S-diphenylthiocarbamate was obtained by considering the method reported by Noguchi et al. (5) for the formation of amide bonds by heating the S-phenylthiocarbamates of amino acids in benzene, dioxane, or a mixture of either with pyridine. The present study demonstrates that on heating N,S-diphenylthiocarbamate with acetic acid in boiling dioxane the thiocarbamate is recovered unchanged. On the other hand, addition of triethylamine to the reaction system results in a high (79%) conversion of the thiocarbamate to sym-diphenylurea.

The above results may be compared to those of Naegeli and Tyabji (11) who found that the treatment of phenylisocyanate with acetic acid in boiling benzene solution yields approximately equal percentages of sym-diphenylurea and acetanilide. It is apparent that the two cases are not equivalent, however, due to the presence of triethylamine in one system and the evidence cannot be employed to prove or disprove the possibility of an isocyanate intermediate. The present results, however, are in agreement with the evidence that basic catalysts are required in all of the reactions of thiocarbamates that have been investigated (1-6).

It has been shown that the S-phenylthiocarbamates of primary amines do not react with isopropanol and ethanol under conditions which in the presence of primary amines convert them essentially quantitatively to di-substituted ureas (2,6). However, treatment of a solution of N,S-diphenylthiocarbamate in absolute ethanol with excess triethylamine for eighteen days at room temperature led to a mixture of low-melting solids. These were not separated or characterized, but a qualitative comparison of the rates of this reaction (cf. 2) with that of ethanol and phenylisocyanate in the presence of triethylamine (12) strongly suggests that an isocyanate is not rapidly formed, i.e.



$$\xrightarrow{\text{fast}} C_6H_5S^- + C_6H_5N{=}C{=}O \quad ,$$

for Baker and Gaunt (12) have clearly shown that the reaction of phenylisocyanate and ethanol readily occurs at 20°, and further, that it is accelerated by added triethylamine.

C. Mixed Anhydrides of S-Phenylthiocarbonic Acid and Carboxylic Acids

In view of the stoichiometry of the reaction of S-phenylthiocarbonyl chloride with DL-phenylalanine in the presence of excess aqueous sodium hydroxide, Crosby and Niemann (2) proposed that a mixed anhydride was formed by reaction of the amino acid carboxyl group with the acid chloride and was then decomposed by excess alkali. They also suggested that this mixed anhydride could be employed for the synthesis of derivatives of the carboxyl function. Two cases were examined for this possibility, using different reaction conditions.

The attempted formation of a mixed anhydride by the reaction of acetic acid and S-phenylthiocarbonyl chloride in the presence of triethylamine in an aqueous heterogeneous system followed by treatment with aniline resulted in

the formation of some N,S-diphenylthiocarbamate with no evidence of anilide formation. On the other hand, repetition of the above reaction in anhydrous dioxane gave a 44% yield of crude acetanilide. Essentially the same results were obtained when hippuric acid was substituted for acetic acid in the non-aqueous system.

These facts are in direct support of the proposal (2) regarding mixed anhydride formation, and they also tend to clear up the question (2) as to whether any carboxylic acid will catalyze the hydrolytic degradation of phenylthiocarbonyl chloride. However, in view of the low yields of purified products and the cost of the starting material it is doubtful that this method is of general preparative interest. In addition, little is known concerning the side reactions occurring in this system, but it is suggested that the highly nucleophelic thiophenate ion formed as a product may enter into these (cf. 2), and this may account for the low yields obtained.

D. Attempted Reactions of N-Pentamethylene-S-phenylthiocarbamate (cf. 2)

As it is conceivable that the lack of reactivity of N-methyl-N-phenyl-S-phenylthiocarbamate with primary and secondary amines (1,2) may be attributed to steric factors (2), a number of typical reactions of the primary amine thio-

carbamates were attempted with N-pentamethylene-S-phenyl-thiocarbamate.

This compound, prepared as described earlier (2,8), was recovered unchanged from extended treatment with excess diethylamine, excess aniline and triethylamine, and acetic acid plus triethylamine in boiling dioxane solutions. In addition, in the last case mentioned the starting material was recovered after standing in the reaction mixture plus water for nearly six months.

Thus, unlike the S-thiophenylcarbamates of primary amines (cf. 1,2, and above) the secondary amine compounds are extremely stable. In addition, the possibility that this stability is due to steric hindrance in the N-methyl-N-phenyl-S-phenylthiocarbamate (cf. 2) does not appear likely, since the same lack of reactivity was noted in the present case where steric factors were reduced. Further support of this view is derived from the report of Aubert and coworkers (13) who found that while the N-disubstituted thioureas of glycine were readily obtained by the aminolysis of N-dithiocarbethoxy glycine with secondary amines the opposite route of treating the N-disubstituted-S-ethyldithiocarbamates with glycine gave no reaction unless one of the N-substituents of the S-ethyldithiocarbamate was a hydrogen atom.

The above facts plus the arguments advanced earlier (1,2) clearly show that the reactivity of this general class

of compounds with primary and secondary amines and with hydrolytic reagents is generally confined to the primary amine thiocarbamates.

E. Reactions of S-Benzylthio- and O-Benzyl-N-phenylcarbamates

Crosby and Niemann (2) demonstrated that in the reactions of O,N-diphenylcarbamate and N-phenyl-(β-phenethyl)-thiocarbamate with primary amines it was necessary to employ elevated temperatures in order to obtain good yields of the ureas in the course of one hour. It was of interest to determine if the same conditions were necessary in the case of S-benzylthio- and O-benzyl-N-phenylcarbamates.

Although the preparation of S-benzylthiocarbonyl chloride has been described (14), without experimental detail, two attempts to synthesize this compound by methods employing phosgene and base catalysts (cf. 2,7) or employing phosgene and lead benzyl mercaptide (cf. 8) resulted in extremely low yields with high recovery of the mercaptan.

Reaction of the crude acid chloride with excess aniline gave a crystalline solid which on treatment at room temperature with aniline and triethylamine in ethanol solution was apparently converted to very impure N,N'-diphenylurea.

O-Benzyl-N-phenylcarbamate (15) was prepared from carbobenzoxychloride and aniline. Treatment of a dioxane solution of the carbamate with 2-aminopyridine and tri[illegible]

room temperature gave none of the expected urea. Heating the reaction mixture under refluxing conditions for three days resulted in a viscous oil that resisted all attempts at purification.

It appears, therefore, that while the S-phenylthio- and O-phenyl-carbamates of primary amines (cf. 2) can be employed for the facile preparation of substituted ureas, the extension of these reactions to other systems is of doubtful value as a preparative method.

F. Amino Acid S-Phenylthiocarbamates

The original interest of earlier workers (1,3,4) in the S-phenylthiocarbamates was based on their applications to amino acid chemistry, and the report by Noguchi and co-workers (5) that S-phenylthiocarbamates of amino acids could be polymerized in pyridine-benzene solution in good yield to give high molecular weight polypeptides represented another application of the reactions of these compounds. In view of the well-established reactivity of the thiocarbamates in basic media (1,2,4) as well as the results in the presence of acetic acid (cf. pg. 9), it was of interest to examine the Noguchi method (5).

The preparation of S-phenylthiocarbonyl glycine (4), and its subsequent polymerization were carried out as directed (5). The product, purified as described by the authors (5), did not fit the analysis for polyglycine and

contained traces of sulfur. Extensive purification by continuous solvent extraction or by steam distillation followed by solvent extraction gave products that were sulfur-free. Analysis, however, showed that these compounds contained ash, of unknown origin, and had low percentages of carbon and nitrogen. Although this suggested the presence of urea linkages, Professor R. M. Badger kindly examined the infra-red spectra of these preparations in the solid state and found them to be in excellent agreement with the spectra of authentic samples of polyglycine. These spectra are reproduced in Figure 1.

An attempted polymerization employing the above conditions was run with S-phenythiocarbonyl glycine ethyl ester. The starting material was not recovered, nor was any polymeric material formed. The oily products were found to contain a crystalline compound that was suspected to be the sym-urea (cf. reactions of N,S-diphenylthiocarbamate) of glycine ethyl ester described by Fischer (16). However, lack of agreement of the melting point observed with the published value (16) made this supposition unlikely, and further information as to the identity of this compound was not sought.

Several facts of interest in the formation of polypeptides from the S-phenylthiocarbamates of amino acids (5) have recently been reported by Weymouth (17). This author has carefully reevaluated the earlier work (5), and sug-

gests that in addition to the occurrence of extensive side reactions, the polymers obtained, in low yield, have molecular weights roughly ten-fold less than claimed (5).

Also, unlike the previous results obtained here and in earlier work (1,2), this author (17) has demonstrated that a secondary amine S-phenylthiocarbamate, i.e., sarcosine, undergoes polymerization to yield polysarcosine. While this does not agree with the known lack of reactivity generally ascribed to the thiocarbamates of secondary amines, it does definitely rule out the possibility of an isocyanate intermediate in this case, and suggests that other mechanisms (cf. 5) must be considered for the polymerization reaction.

In addition to the above brief study of the polymerization reaction, the use of the S-phenylthiocarbonyl group as a protecting group (3,4,5) in the attempted synthesis of a number of dipeptides was investigated. Although the literature (3,4,5) suggests that the use of this group supplies an easy route to these compounds, it has been the experience of the present author that such claims should be viewed with caution. Whereas many of the reactions studied proceed essentially as noted (4,5), the end products often present considerable problems in purification.

G. Experimental*

S-Phenylthiocarbonyl Chloride.--This compound was prepared from phosgene and thiophenol by the method of Rivier (7), and fractionally distilled to give a product b.p. 79.5-77.0°C./3.5-4.5 mm., N_D^{25} = 1.5812.** Yield 26%, based on the recovery of 27% of thiophenol.

An improved method is as follows: Cylinder phosgene washed with cottonseed oil and dried with concentrated sulfuric acid was passed into 700 ml. of toluene in a 3-l., three-necked flask with mechanical stirring and cooling in an ice-salt mixture until 224 g. (2.27 moles) had been collected. Thiophenol (243 g., 2.2 moles) was rapidly introduced through a dropping funnel, followed by the dropwise addition of 256 g. (2.53 moles) of redistilled triethylamine in 400 ml. of toluene. During the addition of the triethylamine solution, which required ca. 1.5 hours, the reaction mixture was cooled as above and stirred continuously. A light-yellow solid separated from the reaction mixture, and at the end of the addition period a heavy slurry resulted. Three hundred ml. of water and 500 ml. of 6 N hydrochloric acid were added and the solid dissolved. The

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*Analyses by Dr. A. Elek. All melting points uncorrected.

**Pure S-phenylthiocarbonyl chloride boils at 89.5-90.0°/5 mm., N_D^{25} 1.5801 (6).

phases were separated, the aqueous phase extracted with two 100-ml. portions of ether, the combined organic phases washed with 250 ml. of 1 M sodium carbonate solution, and dried over magnesium sulfate.

After removal of the toluene in vacuo at steam temperature, the light-orange residue was fractionally distilled through a ten inch Vigreux column at reduced pressure. There was essentially no fore-run and the yellow product* was collected over the range 75.0-78.0°/3.5-4.5 mm., N_D^{25} 1.5803.** Yield, 284 g., 75%, based on utilization of all of the thiophenol.

Attempted Synthesis of S-Benzylthiocarbonyl Chloride via the Reaction of Benzyl Mercaptan with Phosgene.--Treatment of an ice-bath cooled solution of 150 g. (1.52 moles) of phosgene and 183.5 g. (1.48 moles) of redistilled benzyl mercaptan in 700 ml. of toluene with 1500 ml. of 1 M sodium carbonate followed by aeration to remove excess phosgene gave on removal of the aqueous phase, drying and fractional distillation as above 143.0 g. (78%) of benzyl mercaptan and

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*Although the product should be colorless, attempts to remove the yellow color by very careful fractional distillation were unsuccessful. However, the color was not objectionable, and it was noted that it was immediately discharged on addition of the acid chloride to reaction systems containing bases.

**Pure S-phenylthiocarbonyl chloride boils at 89.5-90.0°/5 mm., N_D^{25} 1.5801 (6).

approximately 3 ml. of material, b.p. 101°/5 mm. S-benzylthiocarbonylchloride boils at 133°/18 mm. (14).

Attempted Synthesis of S-Benzylthiocarbonyl Chloride by the Reaction of Lead Benzyl Mercaptide with Phosgene.*--Lead benzyl mercaptide (18) (21.0 g., 0.046 mole) slurried in 20 ml. of toluene was added to ca. 20 g. (0.2 mole) of purified phosgene in 100 ml. of toluene with ice cooling and mechanical agitation. The brilliant yellow color of the mercaptide was rapidly discharged and a finely divided white solid separated. After the addition was completed, the heavy slurry was stirred at ice temperature for ca. 0.5 hour and then allowed to stand at room temperature overnight. Aeration of the reaction mixture with dry nitrogen to remove excess phosgene followed by removal of the solid by filtration and fractional distillation gave 4.5 ml. of fore-run b.p. 68.5-126.5°/16-17 mm. and 1.4 g. (8%) of product, b.p. 126.5-128.0°/15-17 mm. (cf. above).

N,S-Diphenylthiocarbamate.--This compound was prepared as directed by Crosby and Niemann (2). Recrystallization of the essentially quantitative yield of crude product from hot aqueous-ethanol (6) gave a 15% yield of crystalline prod-

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*This method is essentially that of Riemschneider and Lorenz (8) which has been used to prepare a number of analogous compounds.

uct m.p. 125-126.2°.* Addition of water to the filtrate near the boiling point gave a crystalline solid (3.4 g.) on cooling, m.p. 123°-199°. Repeated extraction of this solid with hot 60-70° ligroin and cooling gave 2.0 g. (17.5%) of product m.p. 126-127°.* The residue from the ligroin extractions was recrystallized from ethanol to yield 0.74 g. of sym-diphenyl urea m.p. 237.0-241.6°.

Repetition of the above procedure modified to include recrystallization of the crude, dry product from 2:1 ligroin-toluene gave a 94% yield of white needles, m.p. 125.6-127.6°.*

Attempted Reactions of N,S-Diphenylthiocarbamate with Amides (cf. 2).--N,S-Diphenylthiocarbamate (0.57 g. 0.0025 mole), benzamide (0.30 g., 0.0025 mole) and triethylamine (0.7 ml., 0.005 mole) were heated for 2.5 hours under refluxing conditions in 20 ml. of dry dioxane. After standing at room temperature for two hours the clear solution was poured into ice cold 1 N hydrochloric acid, the solid collected, washed with three 10-ml. portions of 2 N-hydrochloric acid and air dried. Repeated extraction of the crude solid with hot 60-70° ligroin and cooling of the extracts gave 0.26 g. (46% recovery) of N,S-diphenylthio-

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*Literature value (2) 123.3-124°.

carbamate, m.p. 122.3-124.5°. The ligroin insoluble residue had a melting point range from ca. 200-227° with decomposition.* Literature value for N-benzoyl-N'-phenylurea 210-211° with decomposition (2).

In a similar experiment employing malonamide a negligible amount of ligroin insoluble material was obtained. Recovered 0.22 g. (38.5%) of the thiocarbamate, m.p. 125.1-127.1°.

The Reaction of N,S-Diphenylthiocarbamate with 2-Aminopyridine in Aqueous-organic Media.--In a mixture of 15 ml. of dioxane and 5 ml. of water 1.14 g. (0.005 mole) of N,S-diphenylthiocarbamate was treated with 0.47 g. (0.005 mole) of 2-aminopyridine and 1.4 ml. of triethylamine. After standing overnight the solvents were evaporated at steam temperature under a stream of illuminating gas. The solid residue was triturated with 60-70° ligroin and air dried: yield 0.52 g. The crude solid was recrystallized from 22 ml. of 1:10 water-ethanol: m.p. 185-238.5° with most of solid melting in upper portion of range.

Treatment with warm 5% hydrochloric acid did not appear to dissolve an appreciable quantity of the solid. Decantation of the solvent followed by addition of excess 3 N sodium carbonate solution did not yield any of the expected

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*Crosby and Niemann (2) obtained an 80% yield of the urea when the reaction was carried out in a much more concentrated solution containing a large excess of triethylamine.

product, vide post.

An identical experiment in which a solvent mixture containing 2 ml. of water and 20 ml. of absolute ethanol was substituted for the aqueous-dioxane resulted in the formation of 0.42 g. of crude product. Recrystallization from 22 ml. of 1:10 water-ethanol gave white, fluffy needles, m.p. 183-185.1°. Literature value for N-phenyl-N'-(2-pyridyl)-urea (2) 185-186°.

Treatment of the above solid with warm 5% hydrochloric acid, followed by filtration and neutralization resulted in the separation of N-phenyl-N'-(2-pyridyl)urea.

Attempted Reactions of N,S-Diphenylthiocarbamate with Water.--To 1.14 g. (0.005 mole) of N,S-diphenylthiocarbamate in 15 ml. of dry dioxane was added 0.2 ml. (ca. 0.01 mole) of water, and the clear solution was allowed to stand overnight. Evaporation of the solvent, trituration of the solid residue with 60-70° ligroin and recrystallization of the solid from ethanol-water gave none of the expected N,N'-diphenylurea (cf. above).

A duplicate experiment containing, in addition, 1.4 ml. of triethylamine gave the same results.

The Treatment of N,S-Diphenylthiocarbamate with Methylmagnesium Iodide.--To the Grignard reagent prepared from 0.61 g. (0.025 mole) of magnesium turnings and 3.55 g. (0.025 mole) of redistilled methyl iodide in 20 ml. of ether

was added dropwise 2.29 g. (0.01 mole) of N,S-diphenylthiocarbamate in 30 ml. of ether. Very little heat was evolved, but a white solid separated and a gas escaped from the system through a bubble counter. After the addition the mixture was stirred at room temperature for 0.5 hour and under refluxing conditions for two hours. With cooling in ice 30 ml. of 1 N hydrochloric acid was added very slowly to the reaction mixture. The first drop initiated an extremely exothermic reaction and there was some loss from the system. When the addition was completed, the phases were separated, the organic phase dried over magnesium sulfate and the solvent evaporated. The solid residue, 1.80 g., 78.5%, had m.p. 124-126°, after trituration with hot 60-70° petroleum ether. Melting point of starting material: 125.6-127.6°.

Attempted Reaction of Acetic Acid with N,S-Diphenylthiocarbamate.--To 0.57 g. (0.0025 mole) of N,S-diphenylthiocarbamate dissolved in 20 ml. of dry dioxane was added 3 ml. (0.05 mole) of glacial acetic acid followed by heating under refluxing conditions for 2 hours. After standing overnight the clear, colorless solution was poured into 50 g. of ice in 50 ml. of water. The white solid was collected, washed with water and air dried. Yield, 0.53 g., 93% recovery. Recrystallization of a small sample from 60-70° ligroin gave long, white needles, m.p. 125.3-126.3°. Starting material, m.p. 125.6-127.6°.

Attempted Reaction of Acetic Acid with N,S-Diphenylthiocarbamate and Triethylamine.--The above procedure was repeated with the exception that 14 ml. (ca. 0.1 mole) of triethylamine was added. When worked up, the crude solid was not soluble in hot, 60-70° ligroin, and had m.p. 233.0-235.5°. Apparently impure sym-diphenylurea. Yield 0.21 g., 79.5%.

In a similar experiment containing equimolar quantities of all three compounds the system was attached to a bubble counter to observe any gas evolution. When heat was applied a slow evolution of gas was observed which increased as the system reached the boiling point. A steady stream of gas passed through the bubble counter for ca. 10 minutes and then the flow became intermittent. After ca. 1 hour at the boiling point of the solution the evolution of gas had essentially ceased.

The system was worked up as before and gave sym-diphenylurea, m.p. 239.5-241.5°, after one recrystallization from methanol.

The Reaction of N,S-Diphenylthiocarbamate with Absolute Ethanol (cf. 2).--In a tightly closed flask 2.3 g. (0.01 mole) of N,S-diphenylthiocarbamate and 2 ml. of triethylamine were dissolved in 20 ml. of absolute ethanol. After standing for eighteen days at room temperature the solvent was evaporated under a stream of illuminating gas. The solid residue was extremely soluble in warm 50-60° ligroin

and separated on cooling as an oil which solidified. The air dried product (odor of diphenyldisulfide) 1.9 g., had melting range 38-46°. One recrystallization from 50-60° ligroin gave crystals, m.p. 41-47°. A subsequent recrystallization from methanol-water gave crystals, m.p. 52-58°.

It was supposed that the product obtained was a mixture of diphenyldisulfide, m.p. 61° and O-ethyl-N-phenylcarbamate, m.p. 52°. However due to the similar solubility characteristics of these compounds further attempts to separate them were not made.

The Attempted Preparation of Acetanilide via the Mixed Anhydride Between S-Phenylthiocarbonic Acid and Acetic Acid.--S-Phenylthiocarbonyl chloride (1.58 g., 0.009 mole) was added over a fifteen minute period with vigorous stirring to 20 ml. of water containing 0.6 g. (0.01 mole) of glacial acetic acid and 1.01 g. (0.01 mole) of triethylamine. As the acid chloride tended to separate 15 ml. of absolute ethanol was added to promote mixing. After two hours at room temperature, 0.93 g. (0.01 mole) of aniline was added in a dropwise manner. The white solid that immediately formed was filtered out, washed with 2 N hydrochloric acid, water and air dried. Recrystallization from ethanol by the addition of water to the cold solution gave white needles, m.p. 126.5-127.7° (N,S-diphenylthiocarbamate has m.p. 125.6-127.6°). The yield was not determined.

When the above procedure was modified by employing dry dioxane as the solvent and cooling in an ice bath, a white solid rapidly separated from the reaction mixture as the acid chloride was added. After allowing the system to stir for one hour at room temperature 0.93 g. (0.01 mole) of aniline was slowly added. The mixture was stirred an additional hour, filtered, and the filtrate evaporated to dryness with exclusion of air. The residual yellow solid was washed with 3 N sodium carbonate solution, 5% hydrochloric acid, water, and was air dried. Yield 0.6 g. (44%).

Recrystallization of a small sample from 60-70° ligroin gave white needles, m.p. 112.9-113.9° (lit. value for acetanilide 113-114° (19)).

Hippurylanilide.--Hippuric acid (1.79 g., 0.01 mole) and triethylamine (1.01 g., 0.01 mole) were dissolved by warming in 25 ml. of dry dioxane. The solution was cooled in an ice bath and 1.73 g. (0.01 mole) of S-phenylthiocarbonyl chloride was added slowly with vigorous stirring. Due to the heavy slurry that formed the addition of 15 ml. of dioxane was necessary to promote stirring. After 15 minutes 0.93 g. (0.01 mole) of redistilled aniline was added portionwise to the reaction mixture. Stirring was continued for 1.5 hours at room temperature, and then the reaction mixture was poured into 105 ml. of ice-cold 0.3 N hydrochloric acid. The flocculent solid was removed and washed on the filter with 100 ml. of 2 N hydrochloric acid

and 200 ml. of water. After this treatment the residue had considerable odor of thiophenol which was removed by suspension with constant agitation for 15 minutes in 100 ml. of 10% sodium hydroxide solution. The resulting solid was washed with 2 N hydrochloric acid and with water and air dried.

The air dried solid still contained sulfur compounds (odor). Recrystallization from ethanol-water gave 0.43 g. (17%) of white needles, m.p. 211.5-212.5° (lit. value for hippurylanilide 208.5° (20)).

Preparation of N-Pentamethylene-S-phenylthiocarbamate.--This compound was prepared by the method (2) described for the synthesis of N,S-diphenylthiocarbamate. Recrystallization of the crude product from 60-70° ligroin gave a 79% yield of white crystals, m.p. 60-61.6°. Literature values 60.0-60.5° (2), 62° (8).

Attempted Reactions of N-Pentamethylene-S-phenylthiocarbamate with Various Reagents.--Two and two tenths grams (0.01 mole) of the thiocarbamate was dissolved in 15 ml. of dry toluene and allowed to stand overnight in the presence of 5.1 ml. (0.05 mole) of redistilled diethylamine. The clear solution was then heated on the steam bath under a reflux condenser for two hours. Removal of the solvent, washing with 2 N hydrochloric acid and air drying gave 2.2 g. (100%) of material, m.p. 60-61.5°.

Treatment of the same quantity of the thiocarbamate under the same conditions with 0.95 ml. (0.01 mole) of redistilled aniline and 2.4 ml. (0.02 mole) of triethylamine gave 2.26 g. (> 100%) of crude product, m.p. 59.5-61.0°. Recrystallization from 60-70° ligroin gave 1.7 g. (77%) of tan solid, m.p. 58.9-60.1°.

N-Pentamethylene-S-phenylthiocarbamate (1.1 g., 0.005 mole) was dissolved in 10 ml. of dry dioxane. Acetic acid (0.3 g., 0.005 mole) and triethylamine (0.5 g., 0.005 mole) were added and the solution was heated at the boiling point for two hours. The clear solution was allowed to stand at room temperature for eighteen days, after which it was poured into 50 ml. of water and set aside. After a period of approximately six months, the white solid was collected, washed with water and air dried. Recovered 0.96 g., 86%, m.p. 60-61.5°.

Attempted Preparation of N-Phenyl-S-benzylthiocarbamate and its Reaction with Aniline.--Five drops of the crude S-benzylthiocarbonyl chloride (p. 19 , method of Rivier (7)) was treated with 10 drops of aniline with the immediate formation of white solid. The system was well-mixed, washed with two 1-ml. portions of 5% hydrochloric acid, three 1-ml. portions of water and air dried. The residue was dissolved in hot 60-70° ligroin and separated as long, white needles on cooling. Rough m.p. 94.8-97.0°.

The above solid was dissolved in 1.5 ml. of absolute

ethanol and allowed to stand for one day in the presence of 10 drops of aniline and 10 drops of triethylamine. At the end of this period the characteristic stench of benzylmercaptan was noted. Addition of 2 ml. of water caused the separation of a white solid. The solution was warmed until clear and allowed to cool. The white needles that separated were washed twice with 0.5-ml. portions of 50% aqueous-ethanol and dried in vacuo. Melting range was 85-225° with most of the solid melting in high end of range. The solid was triturated with hot 60-70° ligroin, washed with 5% hydrochloric acid, water, and recrystallized from ethanol-water. Unfortunately this product was inadvertantly discarded, but the melting point behavior suggests that the crude solid was a mixture of the thiocarbamate and N,N'-diphenylurea.

The Attempted Reaction of O-Benzyl-N-phenylcarbamate with 2-Aminopyridine.--O-Benzyl-N-phenylcarbamate (m.p. 75.9-77.1°; lit. value 78° (15)) was prepared from carbobenzoxychloride and aniline in dioxane solution. The carbamate (0.57 g. 0.0025 mole) and 0.29 g. (0.0025 mole) of 2-aminopyridine were dissolved in 10 ml. of dry dioxane containing 0.7 ml. (0.005 mole) of triethylamine and allowed to stand overnight at room temperature. The solvent was removed and the residue treated with 5 ml. of 60-70° petroleum ether. All of the solid dissolved or melted so urea formation seemed unlikely. The ligroin was evaporated

and the solid residue was heated under refluxing conditions for three days in a mixture of 10 ml. of dioxane and 1 ml. of triethylamine. When the solvent was removed, a red-brown oil remained that did not yield solid products on treatment with petroleum ether, or on attempted recrystallizations from ethanol or ethanol-water mixtures.

N-(S-Phenylthiocarbonyl)-glycine Ethyl Ester.--This compound was best prepared from glycine ethyl ester by the method of Lindenmann and coworkers (4). Repeated attempts to employ triethylamine as a catalyst in the reaction of glycine ethyl ester hydrochloride with S-phenylthiocarbonyl chloride resulted in mixtures of compounds that were purified only with difficulty.

In one instance glycine ethyl ester prepared as directed (4) from 30 g. (0.22 mole) of the hydrochloride was added to 17.3 g. (0.1 mole) of S-phenylthiocarbonyl chloride in dry ether to yield 23.8 g. (99.6%) of crude product, m.p. 93.0-95.5° (lit. value (4) 104-106°). Recrystallization of a small sample from ethanol gave long prisms, m.p. 103.5-104.8°.

N-(S-Phenylthiocarbonyl)-glycine.--As this compound was much more easily purified than the ethyl ester, most of the preparations mentioned above were converted directly to the acid by the published method (4). Generally, to obtain the yields reported (4), it was found necessary to salt out the product.

In a typical experiment 20 g. (0.084 mole) of the crude ester was suspended in 60 ml. of a 1:1 solution of glacial acetic acid and concentrated hydrochloric acid. After heating under refluxing conditions for 10 minutes the clear solution was cooled at the tap and 200 ml. of water was slowly added. Some white solid separated. The addition of 70 g. of sodium chloride caused the separation of much more solid. The product was washed with water and air dried. Yield 15.7 g. (89%), m.p. 150-154°. (Lit. value 153-154° (4)). The compound was recrystallized from 70 ml. of ethyl-acetate plus 300 ml. of toluene to yield 12.2 g. of fine white needles, m.p. 155.0-157.5° with decomposition.

Polymerization of N-(S-Phenylthiocarbonyl)-glycine.--This preparation was carried out as described by Noguchi and coworkers (5). As there were some additional observations made in this case the details will be included.

The carbamate (2.1 g., 0.01 mole) was dissolved in 80 ml. of sodium-dried reagent grade benzene in an acid-washed and oven-dried Pyrex ampoule. Pyridine (8 ml., ca. 0.1 mole), reagent grade previously dried over potassium hydroxide pellets, was added, and after cooling in an ice-salt bath the ampoule was carefully sealed. The ampoule was placed in a chamber, and the vapor from boiling benzene was employed to maintain the required temperature. After about 2.5 hours the solution was turbid and some solid had separated. In two days considerable flocculent

solid was formed, and at the end of the prescribed six-day reaction period a heavy, yellow-brown precipitate was present in the reaction mixture. The ampoule was recooled in an ice-salt mixture and the tip carefully removed by scratching and heating. There was no apparent pressure in the system although it has been reported (5) that a stoichiometric amount of carbon dioxide is formed in this reaction.

The flocculent solid was separated, washed with four 20-ml. portions of benzene, four 15-ml. portions of ethanol and three 15-ml. portions of ethyl ether. After drying *in vacuo* the tan, mobile powder weighed 0.38 g. (67% assuming all the starting material was converted to polymer).

Anal. Calc. for $(C_2H_3ON)_n$ C,42.10; H,5.30; N,24.55; S,0.00
Found C,41.29; H,6.26; N,20.79; S,0.15.

Further purification of this material was carried out by two paths.

Approximately one half of the solid was steam distilled with 10 ml. of 1 *N* hydrochloric acid and much odor of sulfur containing compounds was noted. The residual solid was filtered out, washed with water, and with many small portions of ethanol. After further washings with ether the compound was dried *in vacuo* over phosphorous pentoxide.

Anal. Calcd. for $(C_2H_3ON)_n$ C,42.10; H,5.30; N,24.55

Found C,39.05; H,5.65; N,21.69 plus some ash.

The other half of the polymeric material was continuously extracted with dry benzene for sixty hours, washed with three 5-ml. portions of ethanol, three 5-ml. portions of ether, and dried *in vacuo* over phosphorous pentoxide.

Anal. Calcd. for $(C_2H_3ON)_n$ C,42.10; H,5.30; N,24.55

Found C,39.17; H,5.08; N,21.51 plus some ash.

The Reactions of N-(S-Phenylthiocarbonyl)-glycine Ethyl Ester under the Noguchi (5) Polymerization Conditions.--In an experiment carried out simultaneously with the preceding reaction 2.4 g. (0.01 mole) of the ester was treated in an identical manner. No solid was formed, but the reaction ampoule had considerable gas pressure (cf. above) when opened. The solvent was removed at reduced pressure and a mobile oil remained. Treatment with 20 ml. of 1:1 benzene-ligroin resulted in the formation of some light yellow crystals. As the remaining solution apparently contained much pyridine it was extracted with two 20-ml. portions of 1% hydrochloric acid. As a result of this treatment much white crystalline material separated from the organic phase. The solid was removed, washed once with ethyl ether and air dried, m.p. 124-124.5°.* Evaporation of the organic phase

*Fischer gives m.p. 146° for the symmetrical urea of glycine ethyl ester (16).

in vacuo gave additional white solid plus a yellow oil. The solid was washed free of the oil with petroleum ether and air dried, m.p. *ca.* 90°.

No further attempt was made to identify these compounds.

N-(S-Phenylthiocarbonyl)-L-leucine Methyl Ester.--This compound was prepared by the method employed for the glycine derivative (4), except that the ester was liberated from its hydrochloride with 50% potassium carbonate solution, and the reaction was carried out in ether solution.

From the ester prepared from 10.5 g. (0.053 mole) of L-leucine methyl ester hydrochloride and 4.15 g. (0.024 mole) of S-phenylthiocarbonyl chloride there was obtained 4.58 g. (64.3%) of colorless prisms, m.p. 59.5-61.5° after one recrystallization from hexane. (Noguchi (5) reports the ethyl ester to be an intractable oil.) Concentration of the mother liquors gave an additional 1.51 g. of product to bring the total yield to 85.3%.

N-(S-Phenylthiocarbonyl)-L-leucine.--This compound was obtained as an oil as described by Noguchi (5). Although he reported that this oil resisted all attempts to induce crystallization it was found that after drying at low pressure over P_2O_5 the oil could be solidified on treatment with n-hexane. However, on exposure to air the compound reverted to an intractable oil.

N-(S-Phenylthiocarbonyl)-L-leucyl-DL-alanine Methyl Ester.--The crude acid from the previous reaction was converted to the acid chloride with thionyl chloride by the method previously described for the preparation of N-(S-phenylthiocarbonyl)-DL-alanyl chloride (5). Treatment of an ether solution containing ca. 0.015 mole of the crude acid chloride with an ether solution of DL-alanine methyl ester prepared from 4.5 g. (0.032 mole) of the hydrochloride with cooling and swirling resulted in the formation of a white solid. This solid was removed by filtration, washed with ether and air dried. When washed with 1 N hydrochloride acid only 0.3 g. of solid remained undissolved. Recrystallization of the residue from ca. three volumes of methanol gave white needles, m.p. 173.0-174.5°.

The ether solution from above was extracted with 1 N hydrochloric acid, 1 F sodium bicarbonate solution and water. After drying over magnesium sulfate the solvent was removed in vacuo. The residual brown oil was partially induced to solidify from ethanol-hexane to yield 0.2 g. of tan powder, m.p. above 260° with decomposition.

From the ethanol-hexane filtrate on evaporation an oil was obtained that was induced to solidify by treatment with methanol, m.p. 59.0-60.5°.

None of the products above were obtained in either sufficient quantity or purity to warrant analysis. The results, however, emphasize the difficulties inherent in the use of

the S-phenylthiocarbonyl group in dipeptide synthesis (cf. page 17).

N-(S-Phenylthiocarbonyl)-glycyl Chloride.--Preparation of this compound according to the method of Lindenmann and coworkers (4) resulted in a 95% yield of crude product, m.p. 86-92° dec.; 83-85° (4); 87° (3).

It should be mentioned that this is an extremely labile compound. Heating causes extensive decomposition, and traces of moisture or alcohols transform the acid chloride into mixtures of inseparable solids.

The Attempted Reaction of N-(S-Phenylthiocarbonyl)-glycyl Chloride with L-Leucine and L-Proline Methyl Esters.--One and three-tenths g. (0.013 mole) of the crude acid chloride in 50 ml. of ice-cooled dry ether was treated in a dropwise manner with 75 ml. of an ether solution of L-leucine methyl ester liberated from 5.45 g. (0.030 mole) of the hydrochloride. During the addition the system was stirred mechanically and moisture was excluded. A white solid separated, and the resulting suspension was allowed to stand overnight at room temperature. The white solid was removed by filtration, washed with three 10-ml. portions of dry ether and air dried (2.06 g.). The filtrate and washings were combined, extracted with three 10-ml. portions of 1 N hydrochloric acid, and once with 10 ml. of water. After drying over magnesium sulfate, the solvent was removed at ca. 40° in vacuo. The viscous oil obtained

was triturated with 2 ml. of 1:1 toluene-hexane and the resulting solid (4.0 g., 90%) was washed with hexane and air dried. Recrystallization of the crude solid from a solvent composed of 30 ml. of toluene and 45 ml. of hexane gave 3.27 g. (74%) of white needles, m.p. 64.5-71.5°. This compound, judging by its odor, was contaminated with much thiophenol and its oxidation products. In line with the earlier experiences with these compounds no further attempt was made to purify it.

A similar preparation in which L-proline methyl ester was substituted for L-leucine methyl ester led to an oil that resisted all attempts of crystallization. It is recognized in this case that part of the difficulty may well have been due to the known (21) ease of transformation of proline esters to the corresponding diketopiperazine.

In closing it should be noted that in both of the above cases, conversion of the products to the free acid (cf. above and ref. 4) led to compounds with melting points of 155° and 150° respectively which are suspiciously near to the melting points observed for N-(S-phenylthiocarbonyl) glycine (cf. pg. 31). This would suggest that either this compound was not removed in purifying the N-(S-phenylthiocarbonyl)-dipeptide esters, or, that it is formed by hydrolysis of these compounds under the conditions employed for preparing the S-phenylthiocarbonyldipeptides.

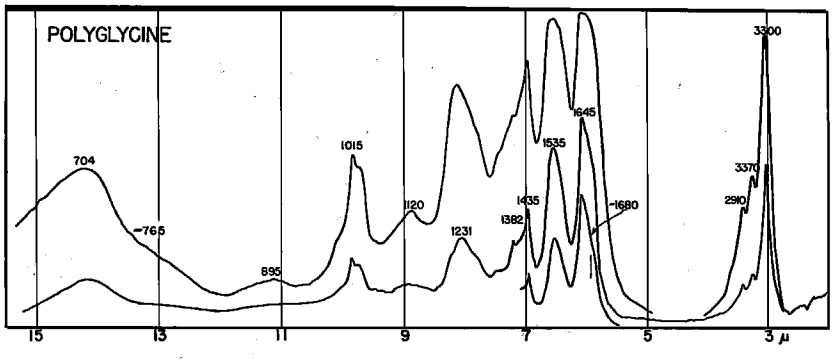

Figure 1.--The infra-red spectra of polyglycine prepared by the Noguchi (5) method. Samples in solid potassium bromide disks and spectra prepared by Professor R. M. Badger, at three different concentrations.

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PART II.

PRELIMINARY INVESTIGATIONS OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACYLATED AMINO ACID ESTERS

A. Introduction

Although the esterase activity of alpha-chymotrypsin has been known for some time (1), the detailed study of this activity has been somewhat limited by the available methods of analysis. In many instances (1-14), titrimetric procedures based on the intermittent addition of standard alkali to weakly buffered (1) or non-buffered solutions (15) have been used, while in one case titration after the addition of excess formaldehyde was the method employed (16). Other methods that have been proposed are: the formation of hydroxamides and colorometric estimation of the ferric complex (4); manometric determination of carbon dioxide liberated from bicarbonate buffers (17); and, spectrophotometric estimation of the products of hydrolysis (18). While these methods have provided much of the knowledge concerning the alpha-chymotrypsin catalyzed hydrolysis of esters, they appear to be tedious and in some cases only semiquantitative. The recently introduced recording titrimeter of Neilands and Cannon (19) permits the study of a large number of such reactions with considerable speed and accuracy. This device mechanically adds micro-amounts of standard alkali in an essentially continuous manner to maintain a predetermined, constant ($\pm$ 0.02 pH units) pH with automatic recording of time and volume of reagent delivered. A description of this instrument's functional parts is included in the

experimental section.

Many of the acylated amino acid esters which function as substrates in the alpha-chymotrypsin catalyzed reaction have been tabulated by Neurath and Schwert (2). In some of these cases the data are entirely qualitative, while in others the data, though quantitative, are limited to comparison with systems employing similar mixed solvents. The latter systems have been employed because of the low solubility of the substrates in water alone; however, the difficulties of comparing these results with those obtained from entirely aqueous systems have been pointed out (16).

The object of the present study was to employ the Neilands-Cannon (19) technique in the development of a method for the determination of the esterase activity of alpha-chymotrypsin in non-buffered, aqueous solutions at suitable substrate and enzyme concentrations. The ultimate goal was the use of such systems in the evaluation of the enzyme-inhibitor dissociation constants of a series of competitive inhibitors (See Part IV). The use of non-buffered solutions was permitted by the instrumentation. The second condition of reasonable substrate concentration in aqueous solution was limited by the solubility of the various compounds studied. The last restriction was determined by the rate of hydrolysis of these substrates and the physical limitations of the analytical method.

From these preliminary studies, in addition to developing a useable method, a number of interesting facts have

been ascertained. They are: evidence of surface effects at low enzyme concentrations; added evidence of the validity of the accepted mechanism of enzyme-catalyzed reactions; and, a new approach to the question of substrate susceptibility to hydrolysis based on a simple interpretation of the substrate structure.

B. The alpha-Chymotrypsin Catalyzed Hydrolysis of Acetyl-L-phenylalanine Glycolamide Ester

It has been shown that this compound is quite soluble in water, and is qualitatively a reasonable substrate for this enzyme system (20). The development of a suitable quantitative method was limited, however, by the rapid rate of hydrolysis of this compound and the concomitant difficulties encountered when the enzyme concentration was lowered to permit the determination of the rate.

While it is known that surface effects are not noticeable at enzyme concentrations of approximately 10^{-2} mg. protein nitrogen per ml. (21), the present results indicate that these effects do become important at approximately 10^{-4} mg. protein nitrogen per ml. The main difficulty appeared to be adsorption of the protein on the surfaces of various components of the reaction system as well as the vessels employed in preparing and delivering the enzyme solution. Application of "Dessicote" (22) to the components of the titration cell reduced these effects, but did not eliminate them. Attempts to clean the surfaces or inactivate residual

protein by treatment with 3 N hydrochloric acid wash were more successful. This technique allowed a measure of reproducibility not realized with the previous methods. The results of these studies are summarized in Table I.

Although the above developments appeared to lead to a satisfactory method for the determination of the rates of hydrolysis of this compound at low enzyme concentrations, it was felt that the uncertainty in this latter value would constantly invalidate the results of such experiments. In addition, the next step of treating all of the glassware in a highly specialized and controlled manner to remove this uncertainty was outside the limits of the present study. Consequently, this system was abandoned, and more reasonable substrates were sought where the limitations of enzyme concentration would not be critical.

C. The alpha-Chymotrypsin Catalyzed Hydrolysis of Some Selected Acylated Amino Acid Esters

Most of the previous work in this field had indicated the necessity of an aryl residue in either the amino acid side chain or the acyl function of substrates suitable for this enzyme (cf. 2). Such compounds, however, generally have low solubilities in water, but extremely high rates of hydrolysis (2). Thus, the approach to the problem was somewhat empirical based as much as possible on analogy with prior knowledge. Avoidance of the difficulty encountered in the preliminary investigations of the hydrolysis of the

glycolamide ester was the main factor in the selection of suitable substrates. As a general rule compounds which allowed the use of approximately 10^{-1} mg. protein nitrogen per ml. were sought. In addition, the dictates of solubility mentioned above were necessarily considered.

It had been noticed (23) that acetyl-D-tryptophan methyl ester was slowly hydrolyzed in the presence of alpha-chymotrypsin. However, a semi-quantitative attempt to employ this compound as a substrate at an initial concentration $[S]_o$* of 5×10^{-4} M and an enzyme concentration [E]* of approximately 0.03 mg. protein nitrogen per ml. resulted in essentially no hydrolysis in twenty minutes. Doubling of the enzyme concentration did not noticeably increase the rate.

The utilization of N-benzoyl-L-methionine ethyl ester as a substrate for alpha-chymotrypsin (2) suggested that the corresponding N-acetyl derivative in addition to being more soluble might prove to be suitable in this respect. In this case, however, an attempt to follow the rate of reaction at initial concentrations $[S]_o = 5 \times 10^{-3}$ M and [E] = 0.075 mg. protein nitrogen per ml. failed due to the rapidity of the reaction at this enzyme concentration. Essentially the same results were obtained when 16×10^{-3} M N-acetyl-L-leucine methyl ester or 4×10^{-3} M N-acetyl-S-benzyl-L-cysteine methyl ester was treated with alpha-

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*The symbols employed throughout this chapter are those defined by Huang and Niemann (24).

chymotrypsin at the above concentration.

While the preceding results did not appear to lead to a satisfactory solution to the problem of finding suitable substrate systems for use in the Neilands-Cannon (19) apparatus, the earlier observations of Huang and Niemann (16) on the system methyl hippurate-alpha-chymotrypsin suggested that this compound would fulfill the requirements as to solubility, rate of hydrolysis and reasonable enzyme concentrations. In a preliminary set of experiments this was found to be the case (cf. Table II, fig. 1), and many of the final techniques were developed through the use of this system.

D. Preliminary Studies of the System Methyl Hippurate-alpha-Chymotrypsin

As the previous results were mainly qualitative in nature, the manipulative techniques developed in those cases were somewhat crude. With the advent of a suitable substrate system, it soon became apparent that a number of additional factors required consideration in the development of a quantitative method. Previously, the factors of the careful measurement of substrate and enzyme concentrations, exclusion of carbon dioxide, instrument calibration, and general cleanliness had been observed. It was now necessary to consider the stability of enzyme solutions, an accurate method for the determination of zero time, and the non-enzymatic hydrolysis of the substrate.

In these and the earlier investigations the enzyme stock solutions were adjusted to approximately pH 8 before introduction into the titration cell. In some instances these stock solutions were maintained at this pH for several hours and invariably such preparations displayed lowered activity as catalysts. As the autolysis of alpha-chymotrypsin at pH 7.9-8.0 is well recognized (2) this apparently was the cause of the observed behavior. To eliminate this factor a separate apparatus was designed for the adjustment of an aliquot of the enzyme stock solution to the required pH just prior to its use (cf. Experimental).

The problem of the accurate determination of zero time was not an easy one. As noted above non-enzymatic hydrolysis of the substrate and autolysis of the enzyme occurred. Both of these processes liberated hydrogen ion while lowering the effective values of $[S]_o$ and [E]. Since the instrumentation employed (19) depends upon the liberation of hydrogen ion, or, more exactly, upon the value of the instantaneous pH in the reaction cell, the reactants must be quite close to the operating pH when mixed to avoid large zero time errors. Empirically it was found that the most reproducible results were obtained by an adjustment of the pH of the reactants less the enzyme to approximately 0.05 pH units above the operating value. The enzyme stock solution was then rapidly adjusted in the above mentioned apparatus with approximately 1 N sodium hydroxide solution to about 0.1 pH

unit above this pH, and the required amount of enzyme solution was quickly introduced into the reaction cell as both solutions approached the operating pH. Generally this technique allowed a determination of zero time for this system within plus or minus five seconds.

To reduce errors in weighing of substrate samples for each kinetic run it appeared reasonable to prepare stock substrate solutions and employ aliquots of these for the individual runs. While this, at first, appeared to be a satisfactory method, it was noted that the values of the kinetic constants obtained from a series of runs obtained by progressing from low to high substrate concentrations differed from those obtained when the inverse order was employed (Tables IV and VI; fig. 2). In addition, it was found that such data could be sensibly corrected to the same values for each concentration by applying an approximate correction for non-enzymatic hydrolysis of the substrate in the stock solutions (Table VII; fig. 3). On the basis of these facts it was decided that a procedure involving preparation of individual substrate stock solutions and their subsequent use within twenty to thirty minutes was capable of more accuracy than the approximate correction mentioned above. Consequently, this plan was adopted for the remainder of the studies.

In addition to the above factors it was noted that individual inhibitor stock solutions must be provided if the inhibitor requires extensive heating to effect its

solution. Again, this requirement is due to the non-enzymatic hydrolysis of the substrate and the concomitant error in the initial concentrations.

Until the achievement of a quantitative, reproducible system was realized, a consideration of the various methods of analysis of the data and assumption of a mechanism followed in the enzyme-catalyzed reaction was unnecessary. In their original study of the alpha-chymotrypsin catalyzed hydrolysis of methyl hippurate Huang and Niemann (16) discussed the possible mechanisms and developed the rate equations for this system. It was shown that for the case where the benzamido moiety of methyl hippurate may interact with both centers ρ_1 and ρ_2 of the catalytic site of the enzyme (25,26) it might be considered that methyl hippurate forms two intermediate complexes ES and ES'. The former complex ES is considered to be the result of the benzamido and ester functions combining with the respective centers ρ_1 and ρ_3 to form the usual enzyme-substrate complex which then undergoes hydrolysis, while interaction of the benzamido group with ρ_2 alone forms ES' which cannot undergo hydrolysis. This consideration leads to equations 1 and 2 for the case of low extent of hydrolysis.

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

$$E_f + S_f \underset{k_5}{\overset{k_4}{\rightleftharpoons}} ES' \qquad (2)$$

By definition $K_S = (k_2 + k_3)/k_1$ and $K_\sigma = K_5/k_4$. Assuming zone A conditions (27,28) and that $d[S]/dt \gg d[ES]/dt$ it has been shown that

$$[ES] = \frac{[E][S]}{K_S + [S]\,(1 + K_S/K_\sigma)} \qquad (3)$$

and

$$\frac{1}{v} = \frac{K_S}{V} \times \frac{1}{[S]} + \frac{1}{V}\,(1 + \frac{K_S}{K_\sigma}) \qquad (4)$$

By the usual plot of $1/v_o$ versus $1/[S]_o$ (29) the authors (16) arrived at values of the apparent intercept $1/V' = (1 + K_S/K_\sigma)/V$ and the apparent Michaelis constant $K_S' = K_S K_\sigma/(K_S+K_\sigma)$. From these the values $k_3' = 2.2 \times 10^{-3}$ M per min. per mg. protein nitrogen per ml. and $K_S' = 8.5 \times 10^{-3}$ M were obtained. These constants have recently been reviewed and revised by Foster and Niemann (30) and the preferred values are $k_3' = 2.2 \pm 0.2$ M per min. per mg. protein nitrogen per ml. and $K_S' = 6.5 \pm 0.5 \times 10^{-3}$ M.

In the present study the values $k_3' = 3.3 \times 10^{-3}$ M per min. per mg. protein nitrogen per ml. and $K_S' = 7.7 \times 10^{-3}$ M were obtained from estimation of the initial velocities (v_o) from the second two-minute interval of a series

of preliminary reactions and a plot of $1/v_o$ *versus* $1/[S]_o$ (29). (Table II, fig. 1).

The subjectivity of such procedures for the estimation of initial velocities has been considered and criticized (30). Of the numerous methods that circumvent such estimates, two of the most recent were employed in the evaluation of the primary data. The first, described by Booman and Niemann (31), for a general case, can be utilized for the present situation where interaction with the hydrolysis products can be neglected. In this plot advantage is taken of equation 5.

$$\left(\int_o^t [S]dt\right)/([S]_o-[S]_t) = (2\,K_S' + [S]_o)/2k_3'[E]) \; + \; ([S]_t/2k_3'[E]) \qquad (5)$$

from which it follows that a plot of $\left(\int_o^t [S]dt\right)/([S]_o-[S]_t)$ *versus* $[S]_t$ leads to a series of lines of slope $1/2k_3'[E]$ and ordinate intercept $(2K_S' + [S]_o)/2k_3'[E])$ for various values of $[S]_o$. It is also shown that the intersections of the experimental lines with the corresponding abscissa values of $[S]_o$ possess ordinate values of $[S]_o/v_o$. Therefore, in addition to other valuable data to be gained from this plot (cf. 31), one is in a position to determine initial velocities in a completely objective manner.

The second method of data evaluation was developed later by the same authors (32), and unlike the above method (31) requires no assumptions as to the mechanism of the enzyme-catalyzed reaction. The treatment of the primary data is entirely mathematical, and leads to values for the initial velocities plus an estimate of the variability of these values.

A comparison of these two methods has been made during the course of these preliminary studies. The initial velocities so obtained were employed in a least squares treatment of two separate $[S]_o/v_o$ *versus* $[S]_o$ plots (29) and gave for the first method (31): $k_3' = 3.37 \pm 0.04 \times 10^{-3}$ M per min. per mg. protein nitrogen per ml. and $K_S' = 8.5 \pm 0.2 \times 10^{-3}$ M; and for the second method (32): $k_3' = 3.37 \pm 0.08 \times 10^{-3}$ M per min. per mg. protein nitrogen per ml. and $K_S' = 8.3 \pm 0.4 \times 10^{-3}$ M (Table IV; fig. 2). While these values were those from data obtained from kinetic runs that had errors in the initial substrate concentrations due to the previously mentioned non-enzymatic hydrolysis of the substrate, and are not necessarily correct in an absolute sense, the fact remains that the constants are in excellent agreement. These facts add support to the mechanism assumed (31) in developing the so-called "Area plot" as well as to the essential value of the more recent "orthogonal polynomial" method (32).

As the latter method lends itself to a more rapid interpretation of the data and yields values of the variability

of the data, it was adopted for general use in the remainder of these studies.

With the above preliminary investigations completed, and most of the problems of the analytical method well defined the transition into the main study of the inhibition of the alpha-chymotrypsin catalyzed hydrolysis of esters would appear to be the next step. However, for reasons to be explained in Part IV of this thesis further preliminary work was necessary in order to obtain optically active N-acylated amino acid esters as substrates for this enzyme.

E. Preliminary Investigations of the Role of Hindered N-Acylated Amino Acid Esters in the alpha-Chymotrypsin Catalyzed Reaction

As discussed earlier, various approaches to the selection of suitable acylated amino acid esters for substrates in the enzyme-catalyzed hydrolysis were mainly based on analogy. And, the previous choices were not satisfactory in that the rates of their hydrolyses were too rapid to allow a determination of the rates with the analytical method being studied.

A consideration of the various factors influencing the rates of such reactions suggested that a suitable substrate would be one in which the labile segment, i.e., the carbonyl group, was as sterically hindered as possible. In addition the known influence of the acyl residue on the rates of alpha-chymotrypsin catalyzed hydrolyses of amino acid deriva-

tives is nicotinyl > benzoyl ≈ chloroacetyl > trifluoroacetyl > acetyl > formyl (30). Therefore, use of the least reactive case here in conjunction with steric hindrance might lower the rates of hydrolysis to reasonable values.

As pointed out by Newman (33), the most hindered case would be expected to be that in which the amino acid side chain contained a maximum number of hydrogen atoms with a "six number" of six. The best case in the naturally occurring amino acids is valine which has six such hydrogen atoms.

Based on the above ideas an investigation of some derivatives of L-valine as suitable substrates was undertaken. This proved to be a fruitful choice, and, also, tentatively provides some additional information as to the factors operating in alpha-chymotrypsin catalyzed reactions.

Crude N-formyl-L-valine isopropyl ester (the most extreme case investigated) at a concentration of approximately 20 x 10^{-3} M was treated with enzyme solutions first at 0.015 mg. protein nitrogen per ml. and then at 0.15 mg. protein nitrogen per ml. In both cases the rate of hydrolysis (if any) was so low as to suggest that the compound was not functioning as a substrate for the enzyme (Table VIII).

The next compound investigated was N-acetyl-L-valine methyl ester. This compound was noted to be very soluble in water, and at an initial concentration of 16 x 10^{-3} M and with enzyme concentrations as above it was hydrolyzed at a rate approximately one-tenth that of methyl hippurate at

the same concentration of enzyme and substrate (Table VIII).

As the influence of the benzoyl group in increasing the rates of hydrolysis is well known (30), N-benzoyl-L-valine methyl ester was the next choice. This compound, though quite insoluble in water, when studied at an initial concentration of 2×10^{-3} M, and with [E] = 0.15 mg. protein nitrogen per ml. was hydrolyzed at approximately one-third the rate of methyl hippurate at the same concentration (Table VIII).

While this last compound was the substrate employed in much of the later work, a further brief extension of the investigation of steric effects was made in an attempt to determine if Newman's "Rule of Six" (33) was of consequence in the alpha-chymotrypsin-catalyzed hydrolysis of esters.

N-Acetyl-L-isoleucine methyl ester, with a "six number" (33) of five was prepared and the hydrolysis in the presence of 0.15 mg. protein nitrogen per ml. was observed at $[S]_o$ values of approximately 16×10^{-3} M and 20×10^{-3} M. The rates were significantly more rapid than that of the hydrolysis of the corresponding L-valine derivative at the same concentrations (Table VIII).

Although it had been observed in the previous work that N-acetyl-L-leucine methyl ester was very rapidly hydrolyzed in the presence of 0.15 mg. protein nitrogen per ml., some work (34) in these laboratories on the hydrolysis of methyl hippurate at enzyme concentrations of 0.0015 mg. protein nitrogen per ml. suggested that reasonable reproducibility

and agreement with results at the higher enzyme concentrations could be realized. In view of these facts values of the initial velocities for the alpha-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-leucine methyl ester at two enzyme concentrations were obtained (Table VIII).

While the "six-number" in the three cases are 6, 5 and 1 respectively, the initial velocities converted to identical enzyme concentrations are in the approximate ratio 4:5:800. It is recognized that the data obtained are of a preliminary nature, and that there is, in addition, a large gap in the series. The idea, however, that there may be a direct correlation between the number of hydrogen atoms available for the pseudo-six membered ring formation (33) and the rate of hydrolysis is very suggestive, and might well provide one key to the design of future model substrates for enzyme-catalyzed reactions.

F. Experimental*

Analytical Procedures

As much of the preliminary work has been mentioned above, only the final procedures developed will be described.

Standard Sodium Hydroxide Solutions.--These were prepared as directed by Swift (35), stored in polyethylene

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*Analyses by Dr. A. Elek. All melting points are corrected except as noted. Boiled, distilled water protected with soda lime was used in all cases where carbon dioxide was to be avoided.

bottles with siphons and soda lime protection, and were individually standardized against Merck "Primary Standard" potassium acid phthalate.

Substrate Solutions.--The required amounts of substrate necessary to provide suitable concentrations in the individual stock solutions were accurately weighed into G. S. Pyrex volumetric flasks. About twenty minutes before the start of a run the individual substrate sample was dissolved in nearly the total volume of water by warming if necessary. The flask was then thermostated at 25 $\pm$ 0.1°.

Enzyme Solutions.--These were prepared just prior to use by weighing the required amount of enzyme directly into a tared G. S. Pyrex volumetric flask containing *ca.* 1 ml. of water. The solution was slowly diluted to the mark, gently inverted *ca.* five times, and thermostated. If a delay in use of the enzyme was anticipated, the solutions were stored at 4°. Generally, only enough enzyme stock solution was prepared for a set of four runs, and as these could be carried out in less than three hours, refrigeration of the enzyme solution was unnecessary.

Operation and Calibration of the pH-Stat-Titrimeter.--This instrument, a modification of the design of Neilands and Cannon (19), was built by M. D. Cannon, International Instruments Co., Canyon, California. Its operation for variable pH titrations has been described (20). The essen-

tial features for use in constant pH titrations are: an "Agla" micrometer syringe for delivering standard solutions driven by a variable speed, reversible motor that is controlled by a moving coil galvanometer (Contact Meter Relay) with manually-adjustable fixed contacts that permit the choice of the required pH; a Leeds and Northrup Model 7664-41 A.C.-operated pH meter with input from a Beckman No. 4990-29 glass electrode and No. 5970-29 calomel reference electrode and output across the coil of the Contact Meter Relay; a Leeds and Northrup Speedomax Type G Recorder whose pen response is electrically controlled by a bridge circuit in which one leg of the bridge is geared directly to the syringe drive motor; and, allied circuitry that allows the operator to zero the pen and calibrate the pen response, start, stop and reverse the syringe drive unit, control the syringe drive motor speed, and select the desired scale setting to obtain various pen responses for a certain volume of titrant delivered.

The following procedure was employed in preparing the instrument for analytical use. The main power switch was turned on for at least fifteen minutes prior to use to allow the components to reach operating temperature. Buffer (pH 7) was placed in a clean, dry reaction vessel (see later for a description of titration cell) and the pH meter standardized. The recorder was manually standardized,* and the

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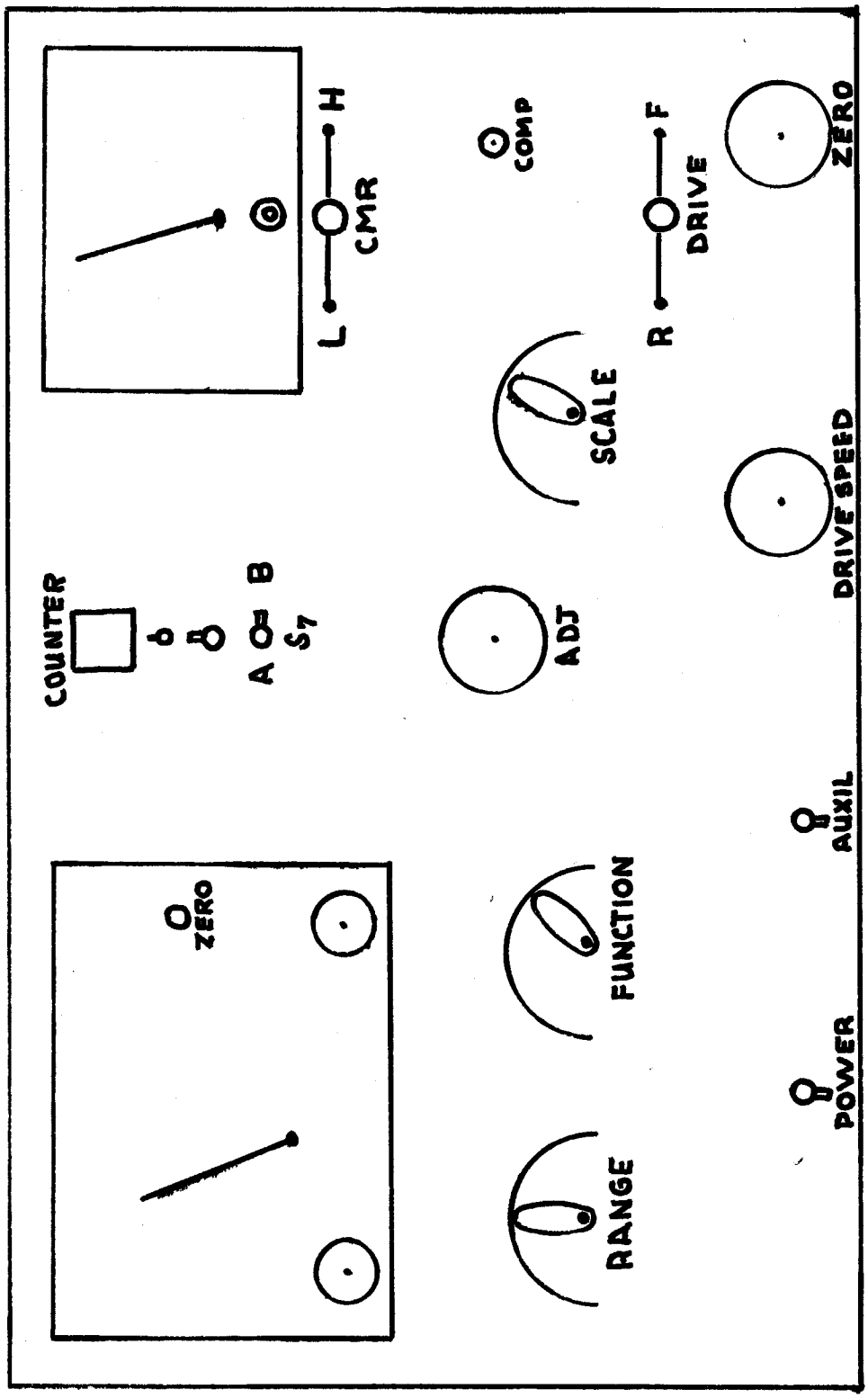
*This recorder is equipped with an automatic, gear-driven standardization system, but it performs this function every 48 minutes and then only when the chart drive motor is running.


ZERO
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S7
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CMR
COMP
SCALE
R
F
DRIVE
DRIVE SPEED
ZERO

pen zeroed. With the scale switch at position 10 the micrometer head was advanced exactly ten revolutions by placing the drive switch in the forward position which corresponds to a delivery of 0.100 ml. of titrant and should move the pen exactly two inches from its zero position. If this was not the case the pen position was corrected with the adjust potentiometer. Switching the scale selector to position 5 should move the pen exactly four inches from the zero position. If this response was not obtained the position of the pen was adjusted with the compensate potentiometer. The scale switch was then switched back and forth between positions 5 and 10 with the corresponding adjustments at each position until the desired pen response was achieved. After this calibration the pen was returned to zero by placing the drive switch in the reverse position and allowing the instrument to run to the reverse limit. Once the above calibration was performed, it was seldom necessary to recheck the instrument unless it was turned off.

A final step in the calibration was a check of the Contact Meter Relay (CMR). This was carried out by locking the zero knob on the pH meter and moving the pH indicator needle upscale by means of the standardization potentiometer. With the CMR switch in the low position and S_7 at position A the indicating pointer of the CMR will operate ("kick" upscale) whenever the pH, simulated manually in this case, drops below the pre-selected value. After the desired setting was achieved it was seldom necessary to re-

adjust this portion of the instrument, a daily check being sufficient.

Titration Cell.--In preliminary runs employing a cell design essentially that of Neilands and Cannon (19), it became apparent that the problems of cleaning and drying the cell between each experiment reduced the speed and, hence, the utility of the system. Accordingly, a new type of cell was designed. This combined the temperature control features of the above cell with an inner container that could be rapidly removed and replaced with a clean, dry vessel. With standardization in mind, the outer jacket was designed to hold 50-ml. Pyrex beakers with the lip cut off to provide approximately 40 ml. total capacity. The fit between the jacket and the beakers was as close as possible and a few drops of water were generally added to increase contact area and aid heat transfer.

With this arrangement and the full output of a Precision constant temperature bath at 25.0 $\pm$ 0.1° passing through the jacket it was found that the temperature within the cell, checked with a calibrated, precision thermometer, agreed exactly with the temperature of the constant temperature bath. However, as it was also noted that the equilibration time was approximately 10 minutes for a five degree temperature differential all solutions for use in this cell were maintained at 25.0 $\pm$ 0.1° until they were transferred to the titration cell.

The cell was covered at the top with a Lucite plate with a circular groove to provide alignment of the cell and the cover. Holes in the cover were provided to allow the introduction of the pair of Beckman electrodes, a nitrogen inlet tube, a small glass propeller (ca. 2 cm. in diameter) driven by a Palo-Meyers stirring motor, the hypodermic needle from the "Agla" syringe, and the reactants. The cover and all of the components mentioned were clamped firmly in position and the cell was supported in correct alignment by an air operated, elevating table provided by the manufacturer.

The stirrer, of low pitch to reduce cavitation, was designed to move the solution in a manner that reduced introduction of gas into the solution and at the same time to direct the flow against the glass electrode to provide maximum response. In this respect, the glass electrode and the hypodermic needle were positioned approximately diametrically opposite to one another across the stirrer. This system was very satisfactory, and no effect on the kinetic rates was observed except when the stirring speed was very low giving rise to poor mixing or excessively fast causing foaming of the solution.

The reaction system was maintained free of carbon dioxide by introducing dry nitrogen, previously passed through a bed of potassium hydroxide pellets and a distilled water bubbler, near the top of the cell. In this

way, air was swept out and evaporation errors minimized. No change in pH could be observed when the nitrogen flow system was in operation, but when the flow was turned off the system would quite rapidly titrate the carbon dioxide taken up by the slightly alkaline solutions.

Enzyme pH-Adjustment System.--As noted in the discussion enzyme solutions could not be maintained near pH 7.9 for extended periods without loss of activity. In addition, the problems of correct zero time required a constant knowledge of the pH of the enzyme solution prior to its introduction into the titration cell. For these reasons a separate, simple titration system was devised for the adjustment of the enzyme stock solution to the required pH.

A Lucite cover plate with holes positioning a pair of electrodes as above, a nitrogen inlet tube, a small glass propellor driven by a Precision stirring motor, and a hypodermic needle from a syringe held in a micrometer measuring device (36) was used in conjunction with a 10-ml. beaker and a separate Leeds and Northrup pH meter (as above). This system allowed the adjustment of small samples of the enzyme stock solution to the required pH, and, in addition, permitted continuous observation of this pH.

The Procedure for a Typical Kinetic Run.--The substrate stock solution prepared as above was diluted to the mark, gently inverted 10-12 times and the required volume pipetted

into the titration cell.* Standard sodium chloride solution (0.2 M) was pipetted in to give a final concentration of 0.02 M. Water, if necessary, was added to bring the total volume to within 1 ml. of the final volume. This solution was then adjusted to ca. pH 7.95 by the addition of standard sodium hydroxide solution from the previously filled and positioned "Agla" syringe. With all of the controls, except the drive switch in the operating position, ca. 2.5 ml. of the enzyme stock solution was rapidly adjusted in the described system to ca. pH 8 by the addition of very small amounts of 0.85 N sodium hydroxide solution. A weight-calibrated syringe in a two-stop holder (37) was then quickly rinsed twice with ca. 0.25-ml. portions of the enzyme solution. The pH of each system was continuously observed during this period (1-3 minutes) and as each approached the operating pH, i.e., 7.9, the syringe was quickly filled, the drive switch placed in the forward position and the enzyme aliquot (1 ml.) introduced beneath the surface of the solution in the titration cell. With this technique the titration either started immediately or within 3-5 seconds after the enzyme was introduced. The syringe-drive speed control was then adjusted to maintain the addition of standard base at just a slightly greater rate than that of the reaction. This provides a stepwise curve, but it avoids the possi-

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*In those cases requiring the addition of inhibitors, aliquots of inhibitor stock solutions made up in essentially the same manner as that described for substrate were also introduced into the cell at this time.

bility that the apparatus is falling behind the reaction (19). The reaction was then allowed to proceed for the required time (16-24 minutes) with occasional readjustment of the drive speed control as necessary. When the run was completed, the zero button on the pH meter was locked in, the drive switch was reversed,* the reaction mixture was removed and discarded, the electrodes and stirrer were rinsed and dried, the "Agla" syringe was refilled, and when a clean, dry cell was introduced the cycle could be repeated.

Analysis of the Primary Data.--The chart paper is graduated in tenths of inches in both dimensions and the chart speed may be varied to move either one-half or one inch per minute. These facts coupled with the calibration mentioned above allow the time and volume data to be taken directly from the charts. The volume information may be transformed directly into milliequivalents per milliliter by multiplication by a constant times the base concentration, or, as in these studies, may be employed to calculate initial velocities in terms of volume. The latter values can then be converted to milliequivalents per milliliter per min. (cf. Table III).

In the present studies the volume data were estimated to $\pm$ 0.5 x 10^{-3} ml. and rounded off to the nearest 1 x 10^{-3} ml. Later, improved techniques and increased sensitivity

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*In addition to the other features mentioned this instrument is provided with forward and reverse limit switches which automatically turn off all auxiliary circuits at either extreme of the syringe travel.

of the instrument (cf. Part IV, Experimental) permitted an estimate of the volume data, when necessary, to 0.2 x 10^{-3} ml. A chart speed of one-half inch per minute was generally employed in these studies, and the time readings could be estimated to less than ± 3 seconds.

Preparation of Substrates and Inhibitors.

Acetyl-L-phenylalanine Glycolamide Ester.--This compound was prepared by R. J. Kerr, m.p. 120.5-121.5°, $[\alpha]_D^{25.3}$ = + 2.2 ± 0.2° (C, 2.3% in ethanol). (20).

Anal. Calcd. for $C_{13}H_{16}O_5N_2$: C, 59.08; H,6.10; N,10.60
Found C, 59.12; H,6.05; N,10.61 (20).

Acetyl-D-tryptophan Methyl Ester.--The sample was prepared by H. T. Huang, m.p. 152.5°; $[\alpha]_D^{23}$= -12.0 ± 0.5° (C, 2% in methanol) (24).

Anal. Calcd. for $C_{13}H_{15}O_2N_3$ (245): C,63.7; H,6.2; N,17.2
Found C,63.6; H,6.2; N,17.1 (24)

Acetyl-L-methionine.--L-methionine (Schwarz Laboratories) was acetylated as described by du Vigneaud and Meyer (38). The crude product recrystallized once from water had m.p. 102.5-104.5°; m.p. 111.0-111.5° (38).

Acetyl-L-methionine Methyl Ester.--This compound was prepared by a modification of the method of Brenner and Huber (39) for the esterification of amino acids. It should be noted that a similar method for the esterification of

acylated amino acids has been recently described by Olechnowitz and Zimmerman (40), but the following method was developed in these laboratories prior to their report.

In a 3-necked, 100-ml. flask with a sealed stirrer, dropping funnel, and calcium chloride outlet to a gas trap, 20 ml. of absolute methanol was treated in a dropwise manner with 4.3 ml. (0.06 mole) of redistillled thionyl chloride with stirring and cooling in an ice-salt bath. When the addition was complete 9.5 g. (0.05 mole) of acetyl-L-methionine was added in several portions. A clear solution resulted. The cooling bath was removed, the reaction mixture was heated at ca. 40° for one hour, and then allowed to stand overnight. Removal of the solvent in vacuo at steam temperature gave a mobile oil that resisted crystallization. The oil was taken up in 100 ml. of chloroform. The solution was extracted with 10 ml. of 1 N hydrochloric acid, 20 ml. of 1 M sodium carbonate solution, 20 ml. of water, and the organic phase dried over magnesium sulfate. Removal of the solvent as above gave a clear oil that rapidly solidified on trituration with n-pentane. The resulting solid was recrystallized from a mixture of 100 ml. of ether and 20 ml. of n-pentane to give clusters of white needles, 6.17 g. (60%), after washing with two 10-ml. portions of 1:1 ether-pentane and drying in vacuo, m.p. 43.5-44.5°; $[\alpha]_D^{25} = -21.2°$ (C, 1% in ethanol).

Anal. Calcd. for $C_8H_{15}O_4NS$ (205.3): C, 46.80; H, 7.36
Found C, 47.03; H, 7.15

Acetyl-L-leucine Methyl Ester (41).--L-leucine was converted to the methyl ester hydrochloride as described by Brenner and Huber (39). The crude ester hydrochloride was acetylated with acetic anhydride in the presence of potassium carbonate in ethyl acetate-water by the method described by Huang and Niemann (24) for acetyl-L-tryptophan methyl ester.

From 5 g. (0.0275 mole) of crude ester hydrochloride in 40 ml. of 2.75 M potassium carbonate solution plus 50 ml. of ethyl acetate and 6 ml. (ca. 0.06 mole) of acetic anhydride there was obtained 4.2 g. of crude product after the usual purification steps (cf. above). This product was contaminated with acetic anhydride. Purification by treatment with moist chloroform-pyridine followed by extractions with dilute acid, dilute base and water gave 3.9 g. of oily solid after drying and removal of the solvent. The careful addition of excess n-pentane to a solution of this solid in ether gave white crystals, m.p. 43.0-44.5° (uncorr.). Karrer et al. (41) gave m.p. 74-75°.

This compound was quite hygroscopic and apparently contained some acetic acid. As extreme purity was not required for these preliminary studies, no attempt was made to further

purify this compound or to obtain its physical constants.*

N-Acetyl-S-benzyl-L-cysteine Methyl Ester.--S-Benzyl-L-cysteine prepared in 50% yield by the reduction of L-cystine with sodium metal in liquid ammonia and subsequent reaction with benzyl chloride as described by Wood and du Vigneaud (42) was converted to the methyl ester hydrochloride in 84% yield with thionyl chloride-methanol by the method of Brenner and Huber (39). Acetylation as above gave a 76% yield of fine white needles, m.p. 76.7-77.5°, after one recrystallization from absolute ether.

As above, other physical constants were not obtained.

Methyl Hippurate.--Matheson hippuric acid (0.1 mole) was esterified by the procedure described for the preparation of acetyl-L-methione methyl ester in 92% crude yield. The crude product was purified with difficulty by three recrystallizations from benzene and one from ether to give long, white needles, m.p. 82.0-83.0°. Yield, 35%.

Anal. Calcd. for $C_{10}H_{11}O_3N$ (193.2): C, 62.16; H,5.74; N,7.25

Found: C, 62.46; H,5.68; N,7.02

A second preparation of this compound employing 0.1 mole of once-recrystallized Matheson hippuric acid gave a much purer product. Only one recrystallization from ben-

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*It should be noted that most of these compounds were intermediates in the preparation of the N'-methylamides (see Part IV), and as such, high purity was not required.

zene and one from ether was necessary to obtain an analytical sample, m.p. 82.8-84.1°.

Anal. Calcd. for $C_{10}H_{11}O_3$ (193.2): C,62.16; H,5.74; N,7.25
Found: C,62.44; H,5.66; N,7.11

Huang and Niemann give m.p. 82-83° for this compound (16).

N-Acetyl-L-valine Methyl Ester (43).--L-valine was converted to the methyl ester hydrochloride in 97% yield by the thionyl chloride-methanol procedure (39). Acetylation of 0.81 g. (0.0048 mole) of the crude ester hydrochloride with 1 ml. (ca. 0.01 mole) of 97% acetic anhydride in the presence of 5 ml. of 4 M potassium carbonate solution and 10 ml. of ethyl acetate gave 0.19 g. (23%) of white needles, m.p. 63.0-64.1° (uncorr.), after the usual purification steps and one recrystallization from n-pentane. Reihlen and Knöpfle give m.p. 61.5° (43).

L-Valine Isopropyl Ester Hydrochloride.--An attempt to extend the system thionyl chloride chloride-methanol (39) to thionyl chloride-isopropanol was unsuccessful.

The crude compound was obtained by repeatedly saturating ice-cooled solutions of 2 g. (0.017 mole) of L-valine in 50 ml. portions of isopropanol with hydrogen chloride, heating under refluxing conditions and removing the solvent under reduced pressure until an oil remained. The crude product could be recrystallized from ether, but was used as obtained in the next preparation.

N-Formyl-L-valine Methyl Ester.--The method of du Vigneaud et al. (44) for the formylation of DL-cystine was modified for the preparation of this compound.

The ester (theoretically 0.017 mole), liberated from the hydrochloride with ammonia in chloroform, was treated with 25 ml. of 98-100% formic acid and 21 ml. of acetic anhydride at 60°. Immediate removal of excess reactants in vacuo at ca. 70° bath temperature gave a mobile oil that could be solidified at 70° and remained partially solid at 4° after drying over sodium hydroxide at 2 mm.

The crude product which contained acidic impurities (an aqueous solution had pH 3.8) was employed in the hydrolysis experiments. It was later found that the compound, after purification by acid and base extractions of a chloroform solution and evaporation of the solvent, could be crystallized from a concentrated solution in isopropyl ether by ice cooling. The crystalline solid reverted to an oil at room temperature.

Benzoyl-L-valine Methyl Ester.--This compound, described by Reihlen and Knöpfle (43), was twice prepared from the ester hydrochloride by treatment with benzoyl chloride in a manner similar to that described for the acetylation of L-tryptophan methyl ester (24).

Two grams (0.012 mole) of the ester hydrochloride was dissolved in 20 ml. of water containing 0.08 mole of potassium carbonate. Ethyl acetate (40 ml.) was introduced and

with rapid stirring 9.5 ml. (0.039 mole) of benzoyl chloride was added slowly from a dropping funnel. After stirring one hour at room temperature 2 ml. of pyridine was introduced. The mixture was stirred for 20 minutes, the phases were separated, the organic phase was extracted first with 25 ml. of 1 N hydrochloric acid, and then with 15 ml. of water, then dried over magnesium sulfate. The crude, oily solid obtained by evaporation of the solvent was twice recrystallized from n-hexane to yield 1.1 g. (39%) of white, silky needles, m.p. 110.5-111.0° after drying in vacuo: $[\alpha]_D^{25} = 42.9°$ (C, 0.4% in chloroform): literature values (43), m.p. 110.5°; $[\alpha]D = 44.6°$ (C, 0.4% in chloroform) calculated from $[M]_D = 105°$ (43).

Anal. Calcd. for $C_{13}H_{17}O_3N$ (235.3): C, 66.36; H, 7.28
Found: C, 66.92; H, 7.52

Repetition of this reaction with 4 g. (0.024 mole) of the ester hydrochloride gave 1.95 g. (35%) of needles, m.p. 110.5-111.5°; $[\alpha]_D^{25} = 46.0°$ (C, 0.4% in chloroform); mixed m.p. with first sample 110.2-111.0°.

Anal. Calcd. for $C_{13}H_{17}O_3N$ (235.3): C, 66.36; H, 7.28
Found: C, 66.80; H, 7.41.

As both samples of this compound analyzed high in both carbon and hydrogen, it was suggested by Dr. A. Elek that they contained traces of n-hexane. The second sample was dried in vacuo for 6 hours at 68° and resubmitted for analysis.

Anal. Calcd. for $C_{13}H_{17}O_3N$ (235.3): C, 66.36; H, 7.28
Found: C, 66.54; H, 7.44

Acetyl-L-isoleucine Methyl Ester.--The method of Reihlen and Knöpfle (43) for the acetylation of L-valine methyl ester hydrochloride was employed for the preparation of this compound. From 6.9 g. (0.038 mole) of the crude ester hydrochloride, prepared from L-isoleucine by the thionyl chloride-methanol method (39), 6.6 g. (0.08 mole) of anhydrous sodium acetate, 25 ml. (ca. 0.26 mole) of acetic anhydride, and three drops of pyridine, there was obtained 4.0 g. (56%) of crude solid when the reaction and purification were carried out as described (43). Repeated recrystallizations of the crude material from ether-hexane or ether-pentane resulted in the formation of well-formed crystals (melting from 44-56°) contaminated with a yellow oil. On treating 1 g. of the twice-recrystallized mixture with a solvent composed of 80 ml. of n-pentane and 20 ml. of ether at the boiling point the solid appeared to dissolve and the clear solution was decanted from the yellow oil. Cooling in ice and seeding caused the separation of colorless prisms. After five hours at 4° the solvent was decanted, and the mass of crystals was washed with three 10 ml. portions of cold n-pentane. The crystals had m.p. 54.0-55.0° (uncorr.) after drying in vacuo over paraffin. As before, other physical constants were not determined.

Table I.--Summary of Results of the alpha-Chymotrypsin Catalyzed Hydrolysis of Acetyl-L-phenylalanine Glycolamide Ester at pH 7.9 and 25.0°.

$[S]_o = 1 \times 10^{-3}$ M; [NaOH] = 0.009014 N; [NaCl] = 0.01 M

[E][a]	Volume titrant[b] / 10 minutes	Approximate[c] initial velocity
1.4	90	8.1[d]
14.0	420	37.8[d,e]
--	57	5.1[d,e]
14.0	7	$[S]_o = 0$[d,e]
--	21	1.0
--	21	1.0
7.0	305	13.7[e]
--	42	1.9[e]
--	26	1.2[f]
--	21	1.0[g]
--	37	1.7[g,h]
7.0	261	11.7[g,i]
7.0	210	9.4[g,j]
--	21	1.0[g]
--	16	0.7[g,k]
7.0	131	5.9[g,k,l]
7.0	282	12.7[g,k,l]
7.0	256	11.5[g,k,l]
7.0	352	15.8[g,k,m]
7.0	340	15.3[g,k,m]
7.0	348	15.7[g,k,m]

Table I.--continued

a. In units of 10^{-5} mg. protein nitrogen/ml. Based on analysis of 14.5% protein nitrogen: Armour lot #00592 (20). b. In units of 10^{-3} ml. c. In units of 10^{-6} M/min. d. Volume of reaction mixture 10 ml. All others 20 ml. e. Consecutive to previous experiment with aqueous washing between. f. Cell "Dessicoted," pretreated with a solution containing 7×10^{-5} mg. protein nitrogen/ml. and washed with water. g. All titration cell components "Dessicoted." h. Electrodes and stirrer pretreated with a solution containing 145×10^{-5} mg. protein nitrogen/ml. and washed with water. i. Enzyme solution adjusted to pH 8 30 minutes before run. j. Enzyme solution adjusted to pH 8 1.5 hours before run. k. Electrodes and stirrer pretreated with a solution containing 145×10^{-5} mg. protein nitrogen/ml., immersed in 3 N hydrochloric acid and washed with water. l. Individual enzyme stock solutions (145×10^{-5} mg. protein nitrogen/ml.) prepared by 1:10 dilution and adjusted to pH just prior to run. m. Enzyme samples taken from same stock solution (145×10^{-5} mg. protein nitrogen/ml.) and adjusted to pH just prior to run.

Table II.--Summary of Initial Velocities (v_o) Estimated from the Second One Minute Intervals in Preliminary Studies of the alpha-Chymotrypsin Catalyzed Hydrolysis of Methyl Hippurate.
$[E]^a$=0.14 mg. protein nitrogen/ml.; [NaOH]=7.285×10^{-2} M; [NaCl]=0.02 M.

Scale Reading[b] P_{t_2} at 2 min.	P_{t_1} at 1 min.	$P=P_{t_2}-P_{t_1}$ [b]	v_o [c]	$1/v_o$ [d]	$[S]_o$ [e]	$1/[S]_o$ [f]
99	52	47	3.42	2.92	20	50
93	48	45	3.28	3.05	20	50
95	48	47	3.42	2.92	20	50
92	46	46	3.35	2.98	20	50
72	39	33	2.40	4.16	8	125
75	41	34	2.48	4.04	8	125
70	37	33	2.40	4.16	8	125
85	45	40	2.91	3.43	12	83.3
89	50	39	2.84	3.52	12	83.3
89	50	39	2.84	3.52	12	83.3
82	43	39	2.84	3.52	12	83.3
95	52	43	3.13	3.19	16	62.5
97	53	44	3.20	3.12	16	62.5
45	22	23	1.68	5.97	4	250
49	27	22	1.60	6.24	4	250
106	55	51	3.72	2.69	24	41.6
108	57	51	3.72	2.69	24	41.6

$K_S' = 7.7 \times 10^{-3}$ M [g] $\qquad k_3' = 3.3 \times 10^{-3}$ g,h

a. Armour Lot #00592, 14.5% Protein nitrogen (20). b. In units of 10^{-3} ml. Corrected to $P_{t_o} = 0$. c. In units of 10^{-4} M/min. d. In units of 10^3 min/M. e. In units of 10^{-3} M. f. In units of M^{-1}. g. Calculated from slope and intercept of best line drawn by inspection (cf. fig. 1). h. In units of M/min. mg. protein nitrogen/ml.

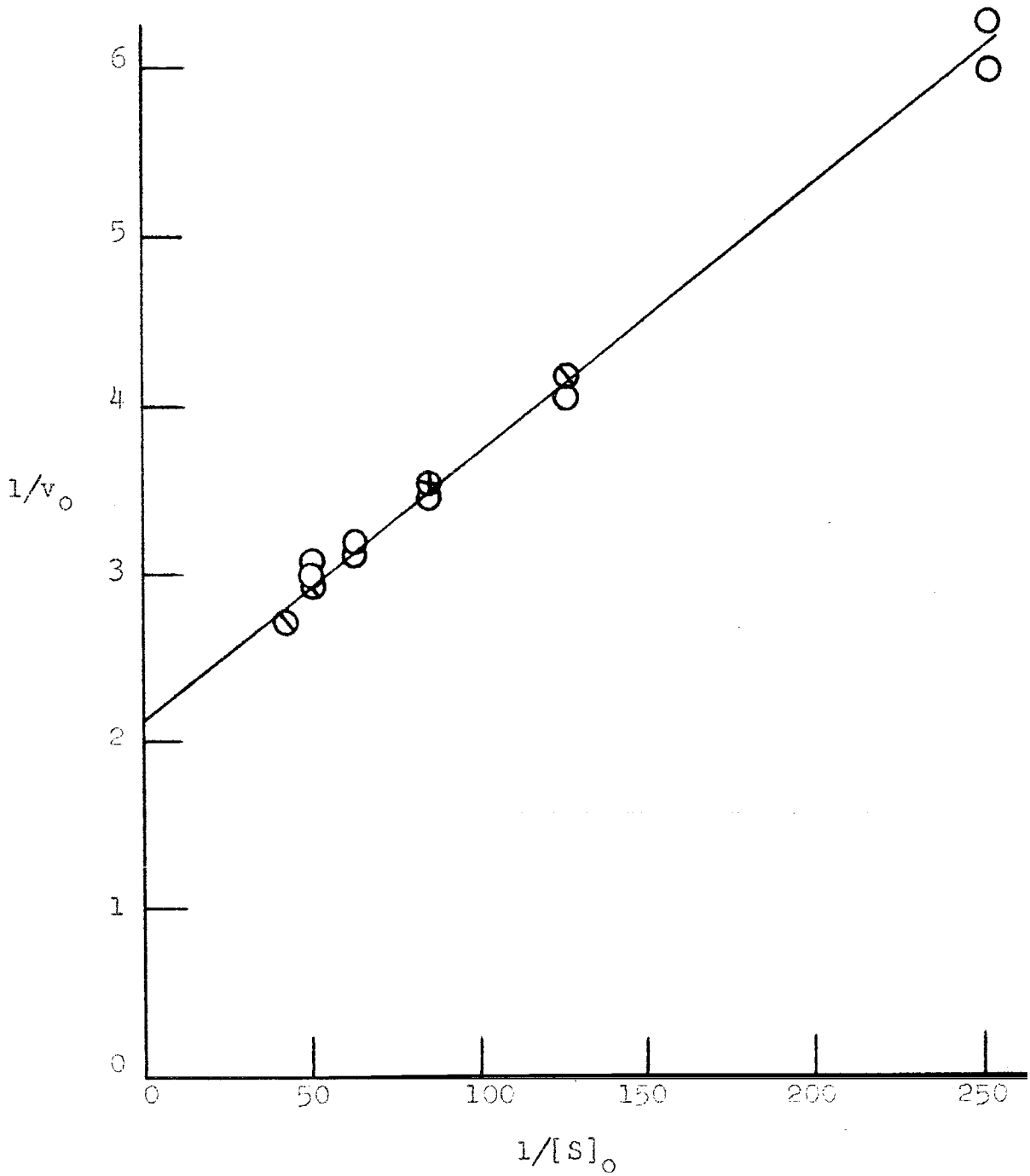

Figure 1. $1/v_o$ *versus* $1/[S]_o$ plot for the system alpha-chymotrypsin-methyl hippurate at pH 7.9 and 25°. $1/v_o$ in units of 10^3 min/M; $1/[S]_o$ in units of M^{-1}. ⊘ - duplicate point; ⊕ - triplicate point.

Table III.--Data for the alpha-Chymotrypsin Catalyzed Hydrolysis of Methyl Hippurate at pH 7.9 and 25.0°.

$[E]^a$ = 0.150 mg. protein nitrogen/ml; [NaOH] = 0.1609 N; [NaCl] = 0.02 M.

Run No.[b]	1-3-27	2-3-27	3-3-27	4-3-27	5-3-27	6-3-27	7-3-27	8-3-27	1-3-28	2-3-28	3-3-28	4-3-28
$[S]_o$[c]	20 S.R.[d]	16 S.R.	12 S.R.	8 S.R.	4 S.R.	4 S.R.	8 S.R.	12 S.R.	16 S.R.	20 S.R.	24 S.R.	24 S.R.
t[e]												
0	0	0	0	0	0	0	0	0	0	0	0	0
1	21	22	20	16	11	11	16	19	20	23	24	24
2	43	41	37	30	20	20	31	37	41	46	47	47
3	64	61	55	45	29	29	44	54	60	67	70	70
4	84	80	71	58	37	38	58	71	78	88	93	92
5	104	98	87	72	45	46	71	88	97	108	115	113
6	125	116	103	85	53	54	84	104	115	129	137	134
7	145	134	119	98	60	62	96	120	133	149	158	155
8	164	152	134	110	68	69	108	135	151	169	178	176
9	183	168	149	122	75	76	119	149	168	188	200	197
10	202	186	163	133	82	82	131	163	185	206	220	217
11	220	202	178	145	88	89	142	177	203	225	240	237
12	238	218	192	155	94	95	152	192	218	244	260	257
13	256	235	205	166	100	101	163	205	236	263	280	277
14	274	251	218	177	106	107	173	218	251	280	298	296

Table III.--continued

Run No.[b]	1-3-27	2-3-27	3-3-27	4-3-27	5-3-27	6-3-27	7-3-27	8-3-27	1-3-28	2-3-28	3-3-28	4-3-28
$[S]_o$[c]	20	16	12	8	4	4	8	12	16	20	24	24
	S.R.[d]	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.
15	292	266	232	186	112	113	182	232	267	298	318	315
16	308	282	245	197	117	118	192	245	283	316	338	335
17	325	297	258	206	122	124	202	257	298	333	356	353
18	343	312	270	215	127	128	211	270	313	350	375	371
19	360	326	282	224	132	133	220	282	328	367	393	389
20	376	341	294	232	137	138	228	293	343	383	412	408
21	392	355	306					305	358	400	430	425
22	408	370	318					316	372	416	448	442
23	424	384	329					327	386	432	465	460
24	439	397	340					338	399	447	482	477
v_o[f,g]	3.50	3.29	2.98	2.51	1.63	1.69	2.52	2.99	3.23	3.64	3.85	3.76
σv_o[f,g]	0.03	0.06	0.04	0.03	0.02	0.02	0.05	0.01	0.02	0.02	0.05	0.03
v_o'[f,h]	3.48	3.28	2.95	2.49	1.61	1.63	2.44	2.95	3.24	3.57	3.78	3.73

a. Armour Lot #90492, 15.0% protein nitrogen. b. Run number -month-date (1956). c. In units of 10^{-3} M. d. S.R. = "scale reading" in units of 10^{-3} ml. Corrected for S.R. = 0 at t_o. e. In units of minutes. f. In units of 10^{-4} M/min. g. Calculated by "orthogonal polynomial" method (32). h. Calculated from $[S]_o/v_o'$ obtained from "area plots" (31) (cf. Table IV).

Table IV.--Summary of the Data from Table III Comparing the Orthogonal Polynomial (32) and Area Plot (31) Methods of Obtaining Initial Velocities.

$[S]_o$[a]	P_m[b]	v_o[c]	σv_o[d]	$[S]_o/v_o$[e]	$[S]_o/v_o'$[e,f]	v_o'[c,g]
20	3	3.50	0.03	57.14	57.50	3.48
16	3	3.29	0.06	48.63	48.70	3.28
12	3	2.98	0.04	40.27	40.70	2.95
8	3	2.51	0.03	31.87	32.15	2.49
4	3	1.63	0.02	24.54	24.85	1.61
4	3	1.69	0.02	23.67	24.50	1.63
8	3	2.52	0.05	31.75	32.80	2.44
12	3	2.99	0.01	40.13	40.70	2.95
16	3	3.23	0.02	50.47	49.40	3.24
20	3	3.64	0.02	54.95	56.00	3.57
24	3	3.85	0.05	62.34	63.45	3.78
24	3	3.76	0.03	63.83	64.35	3.73

$K_S' = 8.3 \pm 0.4 \times 10^{-3}$ M[h] $\quad K_S' = 8.5 \pm 0.2 \times 10^{-3}$ M[h]

$k_3' = 3.37 \pm 0.08 \times 10^{-3}$ [h,i] $\quad k_3' = 3.37 \pm 0.04 \times 10^{-3}$ [h,i]

a. In units of 10^{-3} M. b. Order of polynomial employed in calculating v_o. c. In units of 10^{-4} M/min. calculated (32). d. $\pm$, in units of 10^{-4} M/min. Calculated (32). e. In units of minutes. f. Taken from "Area Plots" (31). g. Calculated from f. In units of 10^{-4} M/min. h. Calculated by least squares from the equation $[S]_o[E]/v_o = (K_S/k_3) + ([S]_o/k_3)$ (cf. fig. 2). i. In units of M/min. mg. protein nitrogen/ml.

Table V.--Data for the alpha-Chymotrypsin Catalyzed Hydrolysis of Methyl Hippurate at pH 7.9 and 25.0°.

$[E]^a$=0.150 mg. protein nitrogen/ml.; [NaOH]=0.1609 N; [NaCl] = 0.02 M

$[E]^a$ = 0.151 mg. protein nitrogen/ml.

Run No.[b]	1-4-4	3-4-4	4-4-4	5-4-4	6-4-4		2-4-4	2-4-5		1-4-5	3-4-5	4-4-5	5-4-5	6-4-5
$[S]_o$[c]	4	12	16	20	24		8	8		4	12	16	20	24
	S.R.[d]	S.R.	S.R.	S.R.	S.R.		S.R.	S.R.		S.R.	S.R.	S.R.	S.R.	S.R.
t[e]						t[e]			t[e]					
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	32	53	59	64	68	2	31	31	3	32	55	61	64	68
6	58	102	114	125	131	4	58	60	6	58	103	116	125	131
9	80	148	168	182	194	6	85	87	9	82	150	168	183	193
12	101	191	217	238	254	8	110	112	12	103	193	218	238	253
15	119	232	264	290	311	10	133	136	15	121	233	265	291	310
18	135	270	309	342	367	12	156	158	18	137	272	310	344	365
21	150	306	353	392	421	14	178	180	21	152	308	354	392	417
24	162	340	394	439	472	16	198	200	24	164	341	397	440	469
v_o[f]	1.74	2.92	3.26	3.51	3.66		2.50	2.59		1.76	2.97	3.34	3.51	3.67
σv_o[f]	0.04	0.03	0.03	0.04	0.05		0.06	0.02		0.03	0.04	0.02	0.03	0.04

a. Armour Lot #90492, 15.0% protein nitrogen. b. Run number -month-date (1956). c. In units of 10^{-3} M. d. S.R. = "scale reading" in units of 10^{-3} ml. Corrected for S.R. = 0 at t = 0. e. In units of minutes. f. In units of 10^{-4} M/min. Calculated by "orthogonal polynomial" method (32).

Table VI.--Summary of the Initial Velocities and Kinetic Constants from the Data of Table V.

$[S]_o$ [a]	P_m [b]	v_o [c]	σv_o [d]	$[S]_o/v_o$ [e]
4	3	1.74	0.04	22.99
8	3	2.50	0.06	32.00
12	3	2.92	0.03	41.10
16	3	3.26	0.03	49.08
20	3	3.51	0.04	56.98
24	3	3.66	0.05	65.57
4	3	1.76	0.03	22.73
8	3	2.59	0.02	30.89
12	3	2.97	0.04	40.40
16	3	3.34	0.02	47.90
20	3	3.51	0.03	56.98
24	3	3.67	0.04	65.40

$$K_S' = 6.86 \pm 0.16 \times 10^{-3}\ \underline{M}^{f}$$

$$k_3' = 3.13 \pm 0.03 \times 10^{-3}\ ^{f,g}$$

a. In units of 10^{-3} $\underline{M}$. b. Order of polynomial employed in calculating v_o (32). c. In units of 10^{-4} $\underline{M}$/min. d. $\pm$, in units of 10^{-4} $\underline{M}$/min. e. In units of minutes. f. Calculated by least squares from the equation $[S]_o[E]/v_o = (K_S/k_3) + ([S]_o/k_3)$ (cf. fig. 2). g. In units of $\underline{M}$/min. mg. protein nitrogen/ml.

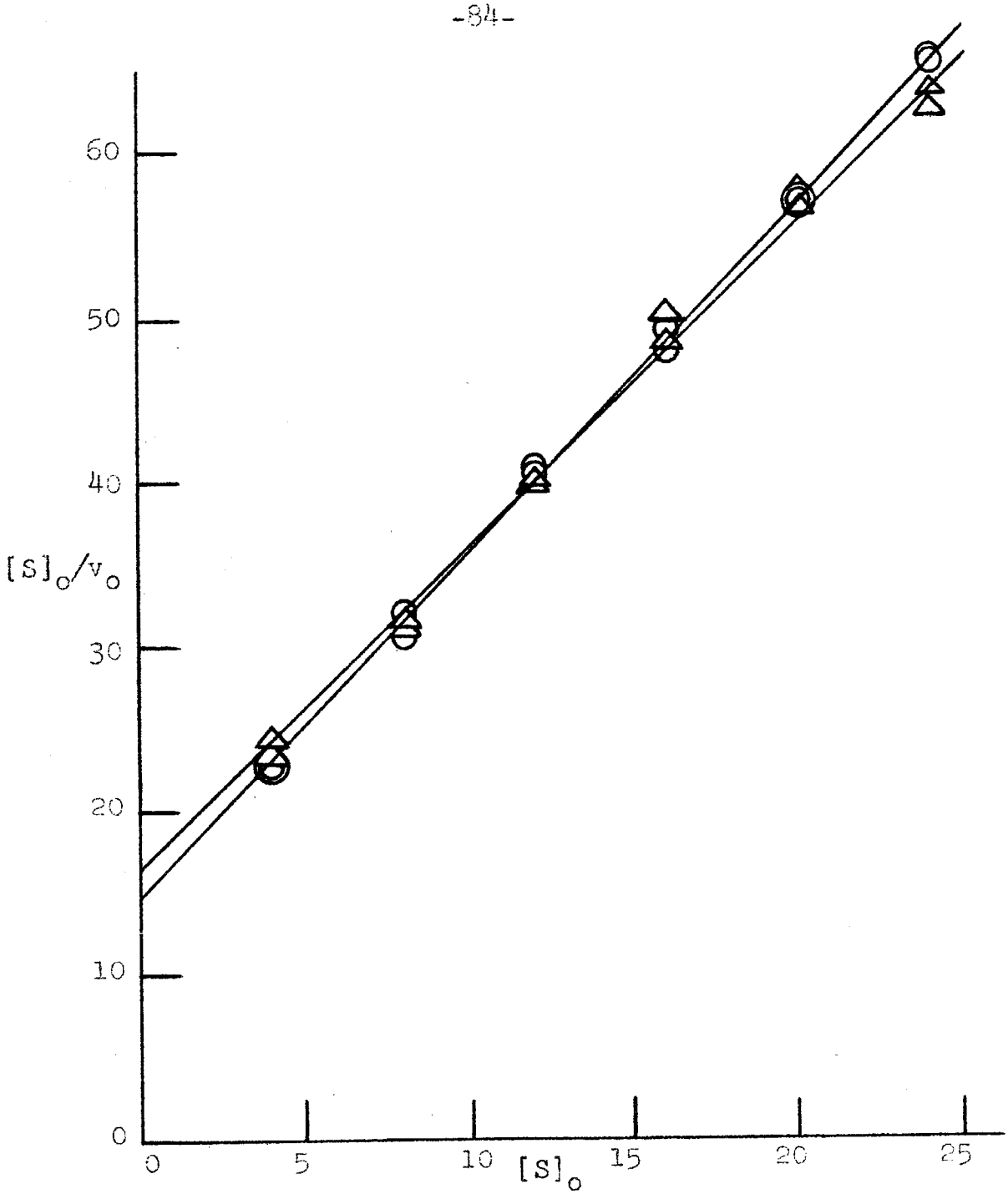

Figure 2. $[S]_o/v_o$ versus $[S]_o$ plot for the system alpha-chymotrypsin-methyl hippurate at pH 7.9 and 25°. △-data from Table IV and ○-data from Table VI showing effect of sequence of runs on intercepts due to non-enzymatic hydrolysis. Lines determined by least squares. $[S]_o/v_o$ in units of min. $[S]_o$ in units of 10^{-3} M.

Table VII.--Summary of the Rate Data for the alpha-Chymotrypsin Catalyzed Hydrolysis of Methyl Hippurate Corrected for Non-Enzymatic Hydrolysis[a]

Data from Table III				Data from Table V			
$t^{a,b}$	$[S]_o$[c,d]	$[S]_t$[a,c]	$[S]_t/v_o$[e]	$t^{a,b}$	$[S]_o$[c,d]	$[S]_t$[a,c]	$[S]_t/v_o$[e]
0.5	20.00	19.93	56.94	1.0	4.00	3.98	22.85
2.75	16.00	15.75	47.87	2.0	8.00	7.91	31.65
3.75	12.00	11.74	39.40	3.5	12.00	11.75	40.25
5.0	8.00	7.76	30.92	4.0	16.00	15.61	47.95
9.0	4.00	3.79	23.25	5.0	20.00	19.40	55.20
9.75	4.00	3.77	21.72	6.0	24.00	23.20	63.40
10.5	8.00	7.51	29.80	1.0	4.00	3.98	22.60
11.25	12.00	11.24	37.59	2.0	8.00	7.91	30.55
1.0	16.00	15.90	49.23	3.5	12.00	11.75	39.60
1.75	20.00	19.80	54.40	4.0	16.00	15.61	46.80
3.75[f]	24.00	23.48	60.99	5.0	20.00	19.40	55.25
4.5[f]	24.00	23.36	62.13	5.5	24.00	23.21	63.40

$K_S' = 6.8 \times 10^{-3}$ M[g]

$k_3' = 3.2 \times 10^{-3}$ g,h

a. Correction based on the value $[S]_t = [S]_o e^{1x10^{-4}min^{-1}t}$ and t estimated to the nearest quarter hour from the time substrate solution was made up. The constant $k = 1x10^{-4}min^{-1}$ was obtained from two determinations of the non-enzymatic hydrolysis at $[S]_o = 16 \times 10^{-3}$ M and 8×10^{-3} M at pH 7.9. b. In units of hours. c. In units of 10^{-3} M. d. Theoretical $[S]_o$ if no non-enzymatic hydrolysis. e. In units of minutes. f. Time uncertain. g. Calculated from slope and intercept of best line drawn by inspection (cf. fig. 3). h. In units of M/min. mg. protein nitrogen/ml.

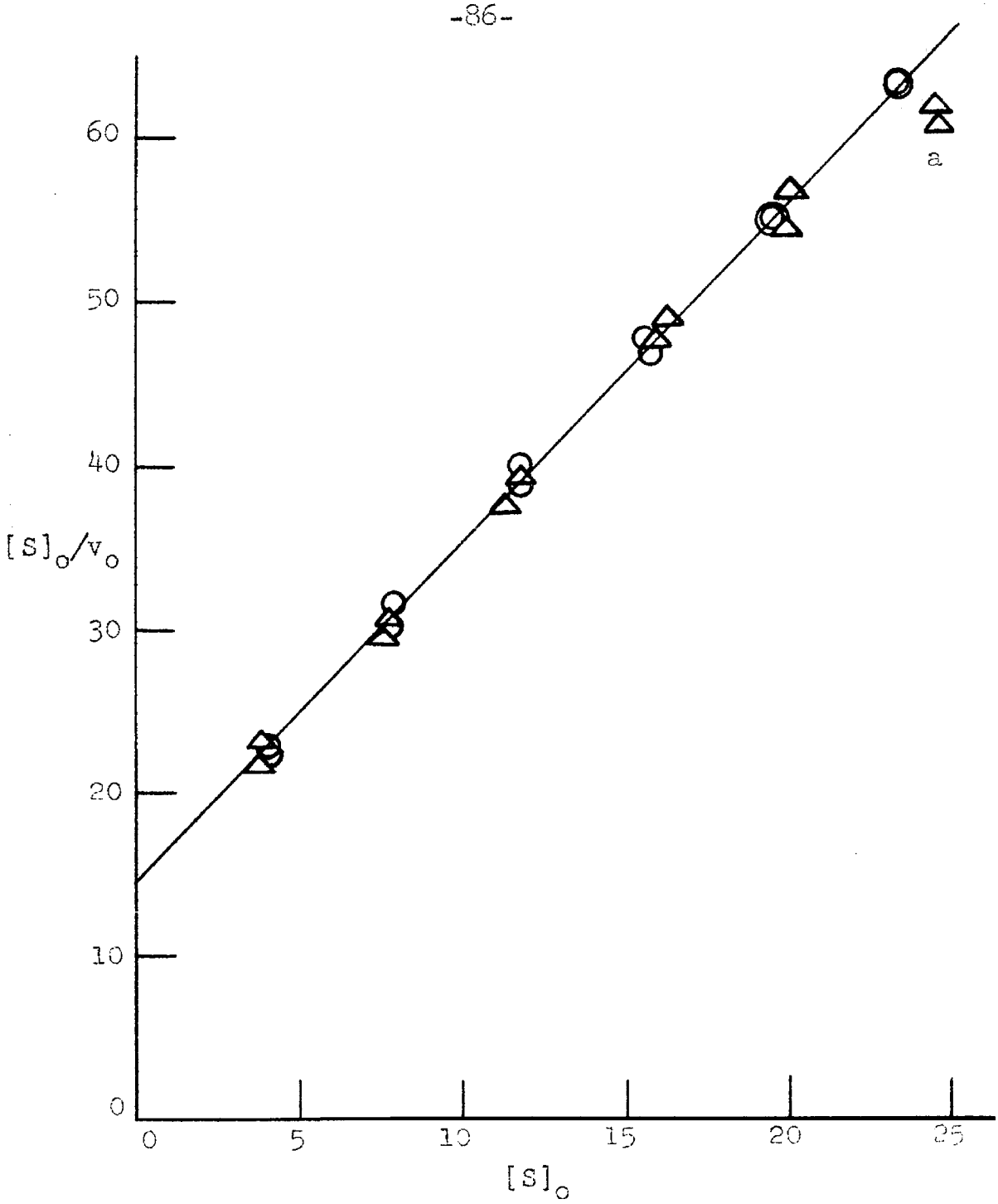

Figure 3. $[S]_o/v_o$ versus $[S]_o$ plot for the system alpha-chymotrypsin-methyl hippurate at pH 7.? and 25°. △-data from Table IV and ○-data from Table VI approximately corrected for non-enzymatic hydrolysis (cf. Table VII). $[S]_o/v_o$ in units of min. $[S]_o$ in units of 10^{-3} M. Line drawn by inspection.

a. Cf. footnote f Table VII.

Table VIII.--The alpha-Chymotrypsin Catalyzed Hydrolysis of Some Hindered Esters at pH 7.9 and 25° in 0.02 M Sodium Chloride Solution.

Compound	$[S]_o$ [a]	$[E]$ [b]	v_o [c]
Formyl-L-valine isopropyl ester	24	0.15	ca. 1
Acetyl-L-valine methyl ester	14	0.15	3.7
	21	0.15	4.4
	28	0.15	5.9
Acetyl-L-isoleucine methyl ester	17	0.15	5.1
	21	0.15	5.9
Acetyl-L-leucine methyl ester	18	0.0015	10
	18	0.0030	16
Benzoyl-L-valine methyl ester	1.0	0.15	2.2
	2.0	0.15	3.2
	3.0	0.15	4.0

a. In units of 10^{-3} M. b. In units of mg. protein nitrogen/ml. Armour Lot #90492, 15.0% protein nitrogen. c. In units of 10^{-5} M/min. Uncorrected for blanks. Estimated assuming linearity of rate curves from t = 2 minutes to t = 4 minutes.

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PART III

THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF METHYL HIPPURATE AND OF BENZOYL-L-VALINE METHYL ESTER

A. Introduction

The preliminary studies described in Part II demonstrated that methyl hippurate was a convenient substrate for use in the automatic titrimeter (1). As this instrument and the techniques developed were new in the field of enzyme kinetics, it was decided that a re-investigation of the alpha-chymotrypsin catalyzed hydrolysis of methyl hippurate (2) would serve several purposes. It would demonstrate whether the new method was capable of reproducing kinetic data obtained in an entirely different manner (cf. 2). In addition, this study would serve as a check on the kinetic constants originally obtained by Huang and Niemann (2) as well as the recalculated values of Foster and Niemann (3). Perhaps most important, it would provide confidence in a rapid method for the study of many other aspects of enzyme kinetics, such as, inhibition, metal ion effects, ionic strength effects and other effects, that are currently of interest in these laboratories.

For these reasons the kinetic study of the alpha-chymotrypsin catalyzed hydrolysis of methyl hippurate was repeated observing the precautions noted in Part II. In addition, as a further check on the reliability of the new techniques, the enzyme-inhibitor dissociation constants of acetyl-D-tryptophanamide and acetyl-D-phenylalanine methyl ester which had been obtained in the original study (2) were redetermined.

For reasons to be discussed more fully in Part IV it was also decided that an investigation of the kinetics of the alpha-chymotrypsin catalyzed hydrolysis of benzoyl-L-valine methyl ester was necessary. The preliminary studies in Part II had indicated that this compound would be a suitable substrate for use in conjunction with the analytical method available. The extension of these preliminary investigations and the results obtained are discussed in a later section of this chapter.

B. The System alpha-Chymotrypsin-Methyl Hippurate

The results to be discussed in this section were obtained from experiments performed observing the various precautions discussed in Part II. In addition, it should be mentioned that blank corrections due to apparent autolysis of the enzyme and non-enzymatic hydrolysis of the substrate were neglected. This appeared reasonable since the reaction times were not extended beyond twenty-four minutes and the initial rate of the combined blanks were generally less than the variability in the various initial velocities as determined by the Booman-Niemann calculation (4). Also, although the extents of hydrolysis were of the order of 30-66%, the possibility of product interaction with the enzyme was disregarded. Until further information is obtained the values of K_S' obtained must be considered as being composite in that they may contain a term due to

product-enzyme interaction (cf. 2).*

From duplicate experiments at initial substrate concentrations over the range 4 x 10^{-3} M to 24 x 10^{-3} M a set of data was obtained from which the initial velocities were evaluated by the method of Booman and Niemann (4). Taking advantage of equation 1

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

a least squares treatment of the initial velocities at various initial substrate concentrations gave the values K_S' = 6.57 ± 0.45 x 10^{-3} M and k_3' = 3.21 ± 0.09 x 10^{-3} M/min. mg. protein nitrogen/ml. The data for these determinations are listed in Tables I and II, and the $[S]_o[E]/v_o$ *versus* $[S]_o$ plot is shown in figure 1. Several months later in conjunction with the competitive inhibition studies discussed in Part IV another set of data was obtained. From triplicate experiments at initial substrate concentrations varied from 8 x 10^{-3} M to 20 x 10^{-3} M the values K_S' = 6.62 ± 0.86 x 10^{-3} M and k_3' = 2.90 ± 0.16 x 10^{-3} M/min. mg. protein nitrogen/ml. were obtained by the same evaluation procedures (cf. Tables III and IV; Fig. 2). Within experimental error the values of K_S' are in agreement. The values of k_3' do not overlap however, and possible causes may be considered. The

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*The terms employed in this section are those defined in Part II and ref. 13.

most likely explanation is a lowering of activity in the enzyme preparation. This would be expected to have no influence on the value of K_S' while lowering the value of k_3'. As only one ten gram sample of Armour Lot #90492 salt-free alpha-chymotrypsin was employed in these studies there should be no variability due to a change of enzyme preparations. In addition, the enzyme was continually stored at 4^o in a closed jar within a desiccator containing calcium chloride except for daily periods of about one hour when it was brought to room temperature for weighing. During the latter operation the preparation was exposed to the atmosphere only momentarily as portions were removed during the weighing operation. However, a check at about the same time as the later kinetic studies disclosed that the water content was 5.5%. This was determined from values of 15.8% protein nitrogen for enzyme dried to constant weight and 15.0% protein nitrogen for undried enzyme (the type employed in these studies). If it is assumed that in the initial studies the then newly-opened enzyme preparation was completely dry, the value for k_3' is then 3.04×10^{-3} M/min. mg. protein nitrogen/ml. rather than 3.21×10^{-3} M/min. mg. protein nitrogen/ml. Thus, without considering the possibility that there was any deterioration of this enzyme preparation with time, the difference in the k_3' values can be reasonably accounted for on the basis of water adsorption during manipulation.

In their original investigation, Huang and Niemann (2) obtained the values $K_S' = 8.5 \times 10^{-3}$ M and $k_3' = 2.2 \times 10^{-3}$ M/min. mg. protein nitrogen/ml. from a plot of $1/v_o$ versus $1/[S]_o$ (5). The substrate concentrations were varied over the range 5×10^{-3} M to 25×10^{-3} M and the initial velocities were determined by the best zero or first order plots of the data. Foster and Niemann (3) recently re-evaluated this data by the more objective Jennings-Niemann (6) method and obtained $K_S' = 6.5 \pm 0.5 \times 10^{-3}$ M and $k_3' = 2.2 \pm 0.2 \times 10^{-3}$ M/min. mg. protein nitrogen/ml. from a v_o versus $v_o/[S]_o$ plot (7).

As the present results for K_S' agree closely with the value obtained by Foster and Niemann (3) although the initial velocities were obtained by entirely different methods of treating the rate data, it would appear that the arguments advanced by the above authors (3) regarding the necessity for entirely objective methods of data analysis are correct. Also, the agreement tends to support the utility of both the "formal titration" method employed in the earlier investigation (2) as well as the present automatic titration technique.

The difference in the respective k_3' values, i.e., $2.2 \pm 0.2 \times 10^{-3}$ M/min. mg. protein nitrogen/ml. and $2.9 \pm 0.2 \times 10^{-3}$ M/min. mg. protein nitrogen/ml., is not significant if it is considered that the experiments were carried out by entirely different procedures employing different components to provide equivalent ionic strength,

i.e., 0.02 M tris-(hydroxymethyl)-aminomethane-hydrochloric acid in the first case and 0.02 M sodium chloride in the present case. In addition, the dependence of k_3 on the enzyme preparation employed is well-known, and uncertainties in this variable would also contribute to the lack of agreement in the values noted above.

As the above results established a measure of confidence in the analytical techniques developed, the inhibition of the alpha-chymotrypsin catalyzed hydrolysis of methyl hippurate by acetyl-D-tryptophanamide and by acetyl-D-phenylalanine methyl ester (2) was re-investigated.

In addition to equations 2 and 3

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

$$E_f + S_f \underset{k_5}{\overset{k_4}{\rightleftharpoons}} ES' \qquad (3)$$

which describe the reaction system for this substrate (2) equation 4 must be included for the case of competitive inhibition (2).

$$E_f + I \underset{k_7}{\overset{k_6}{\rightleftharpoons}} EI \qquad (4)$$

Defining $K_S = (k_2+k_3)/k_1$, $K_{S'} = k_5/k_4$ and $K_I = k_7/k_6$, and assuming zone A conditions (8,9) and that $d[S]/dt \gg d[ES]/dt$, $d[EI]/dt$ the rate expression is described by equation 5,

$$- \frac{d[S]}{dt} = \frac{k_3' [E][S]}{K_S' \left(1 + \frac{[I]}{K_I}\right) + [S]} \quad (5)$$ *

By defining another apparent constant $K_S'' = K_S' \left(1 + \frac{[I]}{K_I}\right)$ equation 5 is converted to equation 6

$$- \frac{d[S]}{dt} = \frac{k_3' [E][S]}{K_S'' + [S]} \quad (6)$$

which is the usual form of the rate expression. By determining the values of k_3' one is in the position to evaluate the type of inhibition, i.e., for competitive inhibition the value of k_3' should not change. From K_S'' and a prior knowledge of K_S' and [I] the value of K_I, the enzyme-inhibitor dissociation constant, may then be evaluated.

From a series of duplicate experiments with substrate concentrations varying from 8×10^{-3} M to 20×10^{-3} M and at a concentration [I] = 2×10^{-3} M of acetyl-D-tryptophanamide the values $k_3' = 3.09 \pm 0.21 \times 10^{-3}$ M/min. mg. protein nitrogen/ml. and $K_S'' = 10.2 \pm 1.2 \times 10^{-3}$ M were obtained from initial velocities determined by the Booman-Niemann method (4) and a least squares evaluation of the data employing equation 1. From the values of $K_S' = 6.57 \pm 0.45 \times 10^{-3}$ M, $K_S'' = 10.2 \pm 1.2 \times 10^{-3}$ M and [I] = 2×10^{-3} M a value $K_I = 3.7 \pm 1.4 \times 10^{-3}$ M was obtained.** The data and plot are

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*k_3' and K_S' are defined in Part II.

**The variability in the values of K_I obtained in the present studies was calculated from $dK_I = \left(\sum (\partial K_I)^2\right)^{\frac{1}{2}}$.

presented in Tables V and VI and figure 3.

In a similar series of triplicate experiments with initial substrate concentrations of 8, 12, 16 and 20 x 10^{-3} M and a 2 x 10^{-3} M concentration of the inhibitor acetyl-D-phenylalanine methyl ester the values $k_3' = 3.02 + 0.09$ x 10^{-3} M/min. mg. protein nitrogen/ml. and $K_S'' = 9.20 \pm 0.53$ x 10^{-3} M were determined as described above. From this K_S'' value at [I] = 2 x 10^{-3} M and $K_S' = 6.57 \pm 0.45$ x 10^{-3} M the value $K_I = 5.1 \pm 1.8$ x 10^{-3} M was obtained. The data are presented in Tables VII and VIII and the $[S]_o[E]/v_o$ versus $[S]_o$ plot in figure 4.

From a consideration of the respective k_3' values, i.e., 3.21 $\pm$ 0.09 x 10^{-3}, 3.09 $\pm$ 0.21 x 10^{-3} and 3.02 $\pm$ 0.09 x 10^{-3} all in units of M/min. mg. protein nitrogen/ml., in the order presented above, it is apparent that the inhibition is competitive within the limits of the present experimental error.

In their original study Huang and Niemann (2) obtained the values $K_I = 2.9$ x 10^{-3} M and 2.8 x 10^{-3} M for acetyl-D-tryptophanamide and acetyl-D-phenylalanine methyl ester respectively. The revised value for the first case is 2.4 $\pm$ 0.4 x 10^{-3} M (10) and for the second 2.6 $\pm$ 0.4 x 10^{-3} M (11). In addition, each of the latter values is supported by other independent data (cf. 10,11). The mean value of the inhibitor-enzyme dissociation constant for acetyl-D-tryptophanamide is $K_I = 2.3 \pm 0.4$ x 10^{-3} M based on re-evaluated data from fourteen independent determinations (10). The mean value

of this constant is not given for acetyl-D-phenylalanine methyl ester, but three independent determinations were re-evaluated to yield K_I = 2.0 ± 0.5 x 10^{-3} M, 2.6 ± 0.4 x 10^{-3} M and 2.2 ± 0.4 x 10^{-3} M (11).

Comparison of K_I Values Obtained for Acetyl-D-tryptophanamide vs. Methyl Hippurate

	K_I
Present study	3.7 ± 1.4 x 10^{-3} M
Huang and Niemann (2)	2.9 x 10^{-3} M
Foster, Shine and Niemann(10)	2.4 ± 0.4 x 10^{-3} M

Comparison of K_I Values Obtained for Acetyl-D-phenylalanine Methyl Ester vs. Methyl Hippurate

	K_I
Present study	5.1 ± 1.8 x 10^{-3} M
Huang and Niemann (2)	2.8 x 10^{-3} M
Foster and Niemann (11)	2.6 ± 0.4 x 10^{-3} M

In considering the apparent lack of agreement between the above values the factors that influence the magnitude and reliability of these constants should be examined.

Foster and Niemann (3) and Foster, Shine and Niemann(10) have discussed these problems and have shown that certain limits must be observed for enzyme and substrate concen-

trations if equation 7 is to be

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

employed for the simultaneous evaluation of k_3' and K_S'. The specific enzyme concentration $E_S' = [E]/K_S'$ must be below a maximum value of 0.1 or 0.6 for observations that are in error by $\pm$ 1% and $\pm$ 5% respectively (3). For an experimental error of $\pm$ 5% the specific substrate concentration $S_S' = [S]/K_S'$ must be between the limits 0.05 and 20 to allow the simultaneous evaluation of K_S and k_3 (3). By a consideration of equation 5 it is shown that if $I_I' = [I]/K_I$ has a value of 0.1 and there is an experimental error of $\pm$ 10% the term $K_S' (1+[I]/K_I)$ is equivalent to K_S' within experimental error (10). It is suggested that the values of I_I' must exceed 0.2 for K_I to be significant since the experimental error often may be $\pm$ 20% and, in addition, a value of I_I' of the order of 1.0 or greater is desirable if K_I is to be evaluated accurately (10).

In the present study the value of [E] was maintained at approximately 0.15 mg. protein nitrogen/ml. which corresponds to a concentration $[E] = 4.26 \times 10^{-5}$ M.* The value of $E_S' = [E]/K_S'$ was 6.5×10^{-3}, $E_I' = [E]/K_I$ was less than 1.2×10^{-3}, and $S_S' = [S]/K_S'$ varied between 1.2 and 3. How-

*Based on a molecular weight of 22,000 and a nitrogen content of 16% for monomeric alpha-chymotrypsin (cf. 3, 10, 11).

ever, while in the earlier study (10) the value of $I_I' = [I]/K_I$ was 0.83 for acetyl-D-tryptophanamide the present value of K_I yields $I_I' = 0.54$. In a similar manner the former value of I_I' for acetyl-D-phenylalanine methyl ester was found to be 0.77 (11) while the present value of K_I gives the result $I_I' = 0.4$. Therefore, based on the values of K_I determined in this study it must be recognized that the lack of agreement in the respective K_I values may well be attributed to the low I_I' values in the present experiments and the attendant error in evaluating K_I. Until further information concerning the value of these enzyme-inhibitor dissociation constants at higher values of I_I' is obtained, the former set of values (10,11) must be assumed to be the preferred ones.

C. The System alpha-Chymotrypsin-Benzoyl-L-valine Methyl Ester

It was indicated in Part II that the alpha-chymotrypsin catalyzed hydrolysis of benzoyl-L-valine methyl ester appeared to proceed at a rate suitable for evaluation in the Neilands-Cannon apparatus (1). While this was found to be the case, several factors that were unimportant in the investigations employing methyl hippurate assumed considerable importance in the present study. These were involved both with manipulative technique and the evaluation of the data. The approach to these problems is discussed below.

Methyl hippurate was generally employed in stock solu-

tions 40×10^{-3} M that could be made up by a few minutes warming at 50°, while benzoyl-L-valine methyl ester could only be maintained in supersaturated solutions at 25° at an upper limit of 5×10^{-3} M. In addition, to prepare such solutions it was necessary to heat the solutions to the boiling point. Fortunately, this compound appears to be very resistant to non-enzymatic hydrolysis: there was no drop in pH when a 4×10^{-3} M solution 0.02 M in sodium chloride was maintained at pH 7.9 and 25° for thirty minutes. However, the fact that crystals began to form in 5×10^{-3} M solutions maintained at 25° for one hour precluded the use of a single stock solution. Individual stock solutions were made up as before (cf. Part II, Experimental) and the remainders discarded after an aliquot was removed.

Another factor that assumed importance was the blank correction apparently due to autolysis of the enzyme. While this correction was negligible in the previous work it was necessary to apply such corrections in this case. For a typical experiment with $[S]_o = 4.05 \times 10^{-3}$ M the amount of 0.01964 N base required was 322×10^{-3} ml. in sixteen minutes at [E] = 0.15 mg. protein nitrogen/ml. and at a sodium chloride concentration of 0.02 M. In a similar experiment less the substrate the amount of 0.01964 N base required was 27.5×10^{-3} ml. This blank is in excess of 8.5% of the total base required for this substrate concentration. In addition, at lower substrate concentrations (1×10^{-3} M was the lower limit) the blank correction

amounted to an even larger percentage of the base required.

In the absence of information to the contrary, the blank correction was applied by subtracting the rate curve for the blank, point by point, from the experimental curves at the various substrate concentrations. This practice was also adopted in later inhibition studies described in Part IV. Some additional information and speculation concerning the nature of this blank will be discussed in that portion of the thesis.

Perhaps the most important difference between the system methyl hippurate-alpha-chymotrypsin and the present case was that of rate. In the range of substrate concentrations, 4×10^{-3} M to 24×10^{-3} M, employed in the former instance, slight variations due to zero time errors, voltage variation, pH meter instability and response and general experimental errors did not affect the results greatly. These effects were generally small compared to the initial velocities of 2 to 4×10^{-4} M/min. On the other hand, the system alpha-chymotrypsin-benzoyl-L-valine methyl ester was limited by substrate solubility to a maximum substrate concentration of 4×10^{-3} M and, empirically, due to the limitations of the analytical technique, to a minimum concentration of $[S]_o = 1 \times 10^{-3}$ M. For this range of substrate concentrations the initial velocities were on the order of $1.5 - 4 \times 10^{-5}$ M/min., and all of the above effects contributed considerably to the overall error.

While it was possible to minimize the manipulative

errors by refinements in experimental techniques, those errors that were dependent upon the stability and sensitivity of the analytical instrument could not be avoided. In extreme cases of variation of the rate curves from the usual, no attempt was made to employ the data for evaluation of the kinetic constants. Even with this selection, an examination of the data presented in Tables IX and X and figure 5 illustrate the lowered precision and reproducibility of this system as compared to that employing methyl hippurate.

It is felt, however, that the results presented represent the best values obtainable within the indicated experimental error and with the limitations of the analytical techniques.

From a series of sixteen experiments at different times over a two month period with substrate concentrations between 1×10^{-3} M and 4×10^{-3} M the values $k_3 = 0.55 \pm 0.04 \times 10^{-3}$ M/min. mg. protein nitrogen/ml. and $K_S = 4.2 \pm 0.4 \times 10^{-3}$ M were obtained by the evaluation procedures previously employed.* As before, interaction of the hydrolysis products with the enzyme was neglected, and the values of K_S so obtained are provisionally composite ones until further information is obtained. In this system, as in the

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*The symbols employed in this section are those defined by Huang and Niemann (13).

case of methyl hippurate, the value of [E] was approximately 4.3×10^{-5} M. The value of $E_S' = [E]/K_S$ was 10^{-2} which is below the required limit of 0.1 (3), and $S_S' = [S]/K_S$ varied between 0.24 and 0.97 which is above the minimum value 0.05 allowed for an experimental error of ± 5% (3). The data are presented in Tables IX and X and the plot of $[S]_o[E]/v_o$ versus $[S]_o$ in figure 5.

The pH-dependence of alpha-chymotrypsin catalyzed reactions is well known (cf. 3). Huang and Niemann (2) presented the dependence of the rate of hydrolysis of methyl hippurate on pH. Essentially the same type of behavior was noted in the present study. The data are presented in Table XI and the plot relating initial velocity and pH is shown in figure 6.

One point that had been tacitly ignored in the previous studies was the effect of dilution of the reaction mixture by the added standard base solution. By the consideration of a somewhat simplified, hypothetical system an idea of various effects due to this factor may be obtained. A system with $[S]_o = 5 \times 10^{-3}$ M, [E] = 0.15 mg. protein nitrogen/ml. and [NaCl] = 0.02 M in an initial volume of 10 ml. that requires the addition of 0.500 ml. (the total volume of the "Agla" syringe) of standard base to proceed to 50% hydrolysis was assumed. In this case the 5% dilution causes a 5% lowering of $[S]_t$ and $[E]_t$ below their respective values if no dilution is assumed. At the same time the titration of liberated carboxylic acid with standard

sodium hydroxide solution increases the ionic strength by 7%.

An examination of the data presented in Table IX shows that in every case the dilution due to addition of base was below 3.5%, and the extents of reaction did not exceed 25% hydrolysis. Also, as the experimental variability was generally high (cf. fig. 5), no attempt was made to correct for the dilution and the attendant effects noted above. This system, as well as all of the others discussed in this thesis, was treated as an ideal case, therefore the data presented have a small inherent error due to the effects of dilution and ionic strength change.

It is interesting to note that while the k_3 values in the usual units differ by a factor of approximately five for methyl hippurate and benzoyl-L-valine methyl ester, the respective values of $K_S' = 6.6 \times 10^{-3}$ M and $K_S = 4.2 \times 10^{-3}$ M are very similar. Apparently this similarity may be attributed to the benzoyl residue. Huang and Niemann(2) found that acetylglycine methyl ester was not a suitable substrate for alpha-chymotrypsin. The extent of hydrolysis was approximately two percent in twenty four hours. However, an extension (12) of the preliminary studies on the system alpha-chymotrypsin-acetyl-L-valine methyl ester described in Part II of this thesis have indicated that this compound has a K_S value on the order of 120×10^{-3} M and a k_3 value of approximately 0.2×10^{-3} M/min. mg. protein nitrogen/ml. Thus, by merely substituting the benzoyl

residue for the acetyl moiety the apparent affinity of the enzyme for the substrate is increased by a factor of nearly thirty.

In concluding this section it is suggested that although this compound lends itself to study with the present analytical techniques, the utility of the system is overshadowed by experimental difficulties. Further investigations in the directions indicated in Part II should provide more suitable systems for study with the automatic titration instrument (1).

D. Experimental

Analytical Procedures.--The techniques and instrumentation employed were described in Part II of this thesis. One modification was made in the studies employing benzoyl-L-valine methyl ester. As this compound was very insoluble in aqueous solution, the stock solutions were made up by heating the crystalline compound in nearly the required amount of water at the boiling point in a G.S. Pyrex volumetric flask. Solution was rapid at this temperature, but if any of the solid remained undissolved the compound rapidly crystallized when cooled to 25°. However, by carefully observing that all of the compound had dissolved, supersaturated solutions could be prepared from which crystals did not separate for approximately one hour at 25°. These solutions were always used within twenty to thirty minutes of the time that they were made up.

Substrates and Inhibitors.--The preparation of the methyl hippurate and the benzoyl-L-valine methyl ester used in these studies was described in Part II. The inhibitors are listed below.

Acetyl-D-tryptophanamide.--This compound was prepared by H. T. Huang. The following physical constants were given (13), m.p. 192-193°; $[\alpha]_D^{25}$ = -19° (C, 2% in methanol).

Anal. Calcd. for $C_{13}H_{15}O_2N_3$ (245): C,63.7; H,6.2; N,17.2

Found: C,63.6; H,6.2; N,17.1

Acetyl-D-phenylalanine Methyl Ester.--This compound was prepared by H. T. Huang. The following physical constants were given (14), m.p. 90-91°, $[\alpha]_D^{25}$ = -19° (C, 2% in methanol).

Anal. Calcd. for $C_{12}N_{15}O_3N$ (221): C,65.2; H,6.8; N,6.3

Found: C,65.2; H,6.8; N,6.4

Table I.--The alpha-Chymotrypsin Catalyzed Hydrolysis of Methyl Hippurate at pH 7.9 and 25°. $[E]^a$ = 0.150 mg. protein nitrogen/ml.; [NaOH] = 0.1609 N; [NaCl] = 0.02 M.

Run No.[b]	3-4-10	4-4-10		1-4-10	2-4-10	5-4-10	6-4-10	1-4-13	2-4-13	3-4-13	4-4-13	5-4-13	6-4-13
$[S]_o$[c]	8	8		4	4	12	12	16	16	20	20	24	24
	S.R.[d]	S.R.		S.R.	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.
t[e]			t[e]										
0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	32	32	3	32	32	57	54	64	62	65	65	67	70
4	61	60	6	58	59	107	104	121	119	127	126	131	137
6	88	87	9	82	82	153	150	175	173	185	184	193	199
8	114	112	12	102	103	198	195	226	223	240	238	253	258
10	138	136	15	121	121	239	236	275	272	294	292	310	315
12	161	158	18	138	138	278	276	320	319	345	344	365	371
14	182	180	21	152	152	315	313	365	363	395	394	419	425
16	203	200	24	165	165	350	348	407	404	442	441	470	477
H[f]	41	40		66	66	48	48	41	41	36	36	31	32
v_o[g]	2.63	2.60		1.84	1.86	3.09	2.96	3.48	3.50	3.64	3.67	3.62	3.88
δv_o[g,h]	0.03	0.04		0.05	0.03	0.05	0.03	0.04	0.06	0.04	0.04	0.10	0.03

a. Armour Lot #90492, 15% protein nitrogen. b. Run number -month-day (1956). c. In units of 10^{-3} M. d. S.R. = scale reading. Corrected to S.R. = 0 at t_o. In units of 10^{-3} ml. Estimated to $\pm$ 0.5 x 10^{-3} ml. and rounded off. e. In units of minutes. f. Approximate percent hydrolysis. g. Calculated by the orthogonal polynomial method (4). In units of 10^{-4} M/min. h. $\pm$.

Table II.--Summary of the Initial Velocities and Kinetic Constants Derived from the Data of Table I.

$[S]_o$ [a]	P_m [b]	v_o [c]	∂v_o [d]	$[S]_o[E]/v_o$ [e]
4	4	1.83	0.05	3.26
4	4	1.86	0.03	3.22
8	3	2.63	0.03	4.56
8	3	2.60	0.04	4.62
12	3	3.09	0.05	5.82
12	3	2.96	0.03	6.08
16	3	3.48	0.04	6.90
16	4	3.50	0.06	6.86
20	4	3.64	0.04	8.24
20	4	3.67	0.04	8.18
24	2	3.62	0.10	9.94
24	3	3.88	0.03	9.28

$$K_S' = 6.57 \pm 0.45 \times 10^{-3}\ \underline{M}\ [f]$$

$$k_3' = 3.21 \pm 0.09 \times 10^{-3}\ [f,g]$$

a. In units of 10^{-3} $\underline{M}$. b. Order of polynomial employed for calculation of v_o (4). c. In units of 10^{-4} $\underline{M}$/min. d. $\pm$, in units of 10^{-4} $\underline{M}$/min. e. In units of min. mg. protein nitrogen/ml. f. Evaluated by the method of least squares (cf. fig. 1). g. In units of $\underline{M}$/min. mg. protein nitrogen/ml.

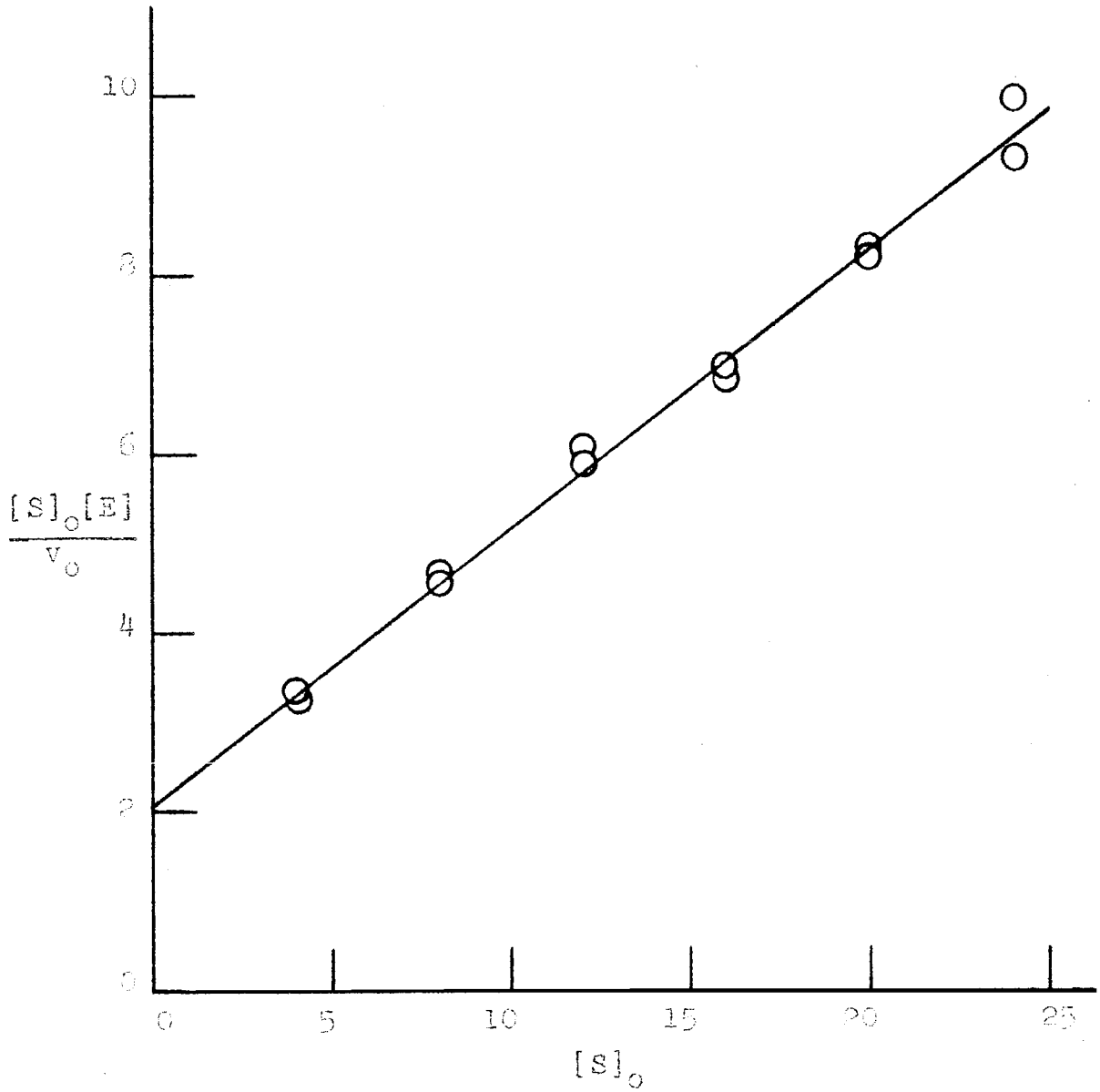

Figure 1. $[S]_o[E]/v_o$ versus $[S]_o$ plot for the alpha-chymotrypsin catalyzed hydrolysis of methyl hippurate at pH 7.6 and 25° in 0.02 M sodium chloride solution. Line determined by least squares. $[S]_o[E]/v_o$ in units of min. mg. protein nitrogen/ml. $[S]_o$ in units of 10^{-3} M. (cf. Table II).

Table III.--The alpha-Chymotrypsin Catalyzed Hydrolysis of Methyl Hippurate at pH 7.9 and 25°.
$[E]^a$ = 0.150 mg. protein nitrogen/ml.; [NaOH] = 0.1609 N; [NaCl] = 0.02 M

Run No.[b]	6-8-16	7-8-16	8-8-16	6-8-21	7-8-21	8-8-21		5-8-16	5-8-21	1-8-29*	2-8-29*	3-8-29*	4-8-29*
$[S]_o$[c]	11.99	16.00	19.93	11.94	15.86	19.99		7.99	7.99	8.06	11.80	15.74	19.96
	S.R.[d]	S.R.	S.R.	S.R.	S.R.	S.R.		S.R.	S.R.	S.R.	S.R.	S.R.	S.R.
t^e							t^e						
0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	51	55	60	48	54	58	2	29	28	35.5	42.5	45.0	47
6	97	107	116	91	105	114	4	55	53	67.0	80.5	86.0	92
9	140	156	170	134	152	167	6	79	77	97.0	117.5	127.0	135
12	180	203	220	173	197	217	8	102	100	124.5	154.0	165.0	178
15	218	248	270	210	241	266	10	123	122	151.5	188.0	203.0	218
18	255	291	318	245	282	313	12	145	142	177.0	220.0	239.5	258
21	290	332	364	279	322	359	14	165	162	201.0	251.0	274.5	296
24	324	373	408	310	361	404	16	184	180	224.0	281.5	309.0	335
H[f]	44	37	33	42	37	33		37	36	38	33	37	23
v_o[g]	2.88	3.04	3.36	2.61	2.98	3.22		2.50	2.20	2.57	2.86	3.11	3.31
σ_{v_o}[g,h]	0.04	0.02	0.05	0.03	0.02	0.01		0.05	0.01	0.04	0.02	0.04	0.03

a. Armour Lot #90492, 15% protein nitrogen. b. Run number -month-day (1956). c. In units of 10^{-3} M. d. S.R. = scale reading. Corrected to S.R. = 0 at t_o. In units of 10^{-3} ml. Estimated to $\pm$ 0.5 x 10^{-3} ml. and rounded off. e. In units of minutes. f. Approximate percent hydrolysis. g. Calculated by the orthogonal polynomial method (4). In units of 10^{-4} M/min. h. $\pm$.

* Base concentration 0.1380 N.

Table IV.--Summary of the Initial Velocities and Kinetic Constants Derived from the Data of Table III.

$[S]_o$ [a]	P_m [b]	v_o [c]	σv_o [d]	$[S]_o[E]/v_o$ [e]
7.99	4	2.50	0.05	4.79
7.99	2	2.20	0.01	5.45
8.06	4	2.57	0.04	4.70
11.99	4	2.88	0.04	6.24
11.94	3	2.61	0.03	6.86
11.80	2	2.86	0.02	6.19
16.00	3	3.04	0.02	7.89
15.86	3	2.98	0.02	7.98
15.74	3	3.11	0.04	7.59
19.93	4	3.35	0.05	8.90
19.99	3	3.22	0.01	9.31
19.96	3	3.31	0.03	9.05

$$K_S' = 6.61 \pm 0.86 \times 10^{-3}\ \underline{M}$$ [f]

$$k_3' = 2.90 \pm 0.16 \times 10^{-3}$$ [f,g]

a. In units of 10^{-3} M. b. Order of polynomial employed for calculation of v_o (4). c. In units of 10^{-4} M/min. d. $\pm$, in units of 10^{-4} M/min. e. In units of min. mg. protein nitrogen/ml. f. Evaluated by the method of least squares (cf. fig. 2). g. In units of M/min. mg. protein nitrogen/ml.

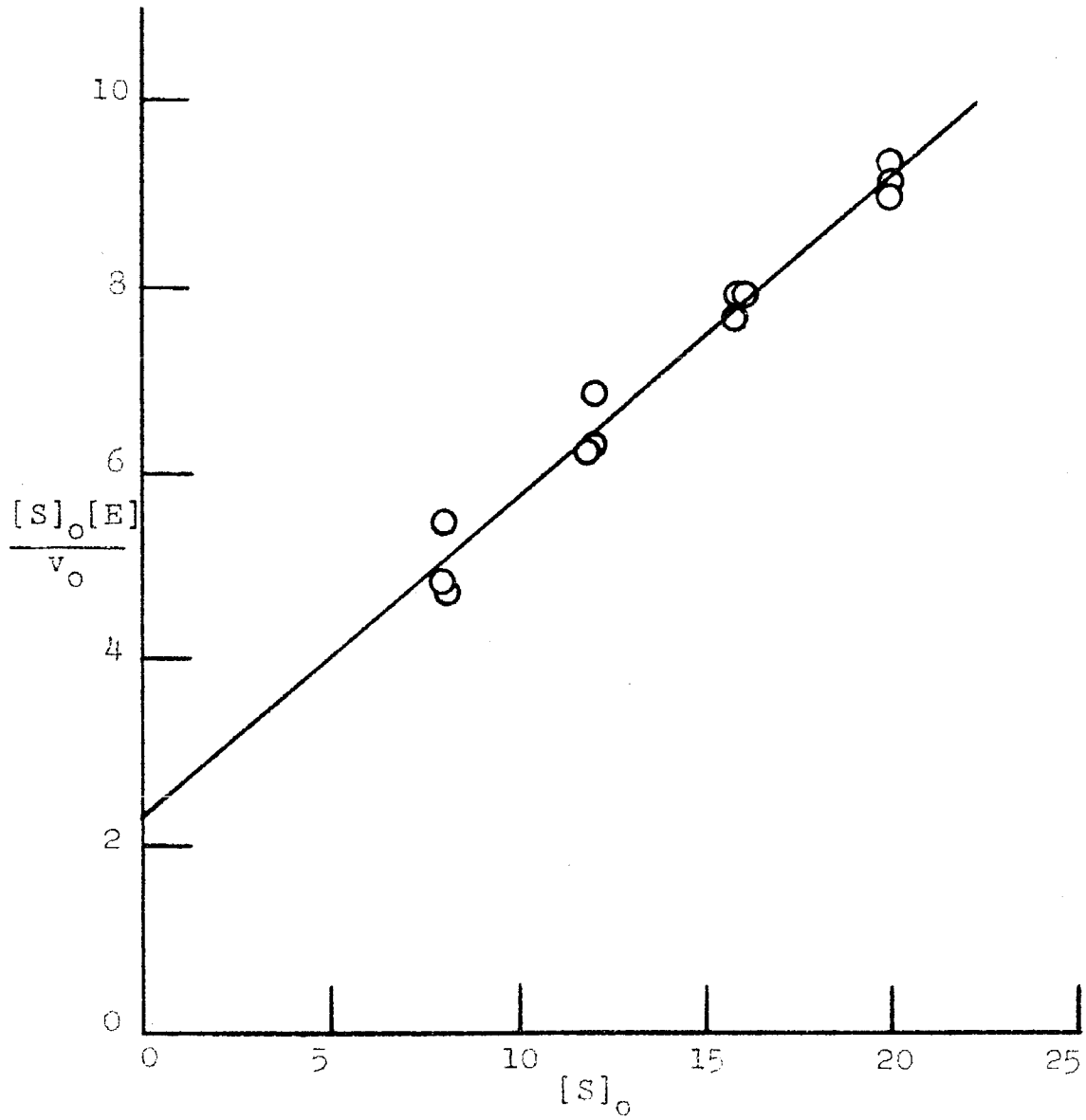

Figure 2. $[S]_o[E]/v_o$ versus $[S]_o$ plot for the alpha-chymotrypsin catalyzed hydrolysis of methyl hippurate at pH 7.9 and 25° in 0.02 M sodium chloride solution. Line determined by least squares. $[S]_o[E]/v_o$ in units of min. mg. protein nitrogen/ml. $[S]_o$ in units of 10^{-3} M (cf. Table IV).

Table V.--Inhibition of the alpha-Chymotrypsin Catalyzed Hydrolysis of Methyl Hippurate by Acetyl-D-tryptophanamide at pH 7.9 and 25° C. $[E]^a$ = 0.150 mg. protein nitrogen/ml.; [NaOH] = 0.1609 N; [NaCl] = 0.02 M. [I] = 2.0×10^{-3} M.

Run No.[b]	1-4-16	2-4-16	3-4-16	4-4-16	5-9-16	6-4-16	8-4-16		7-4-16
$[S]_o$[c]	8	8	12	12	16	16	20		20
	S.R.[d]	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.		S.R.
t[e]								t[e]	
0	0	0	0	0	0	0	0	0	0
3	37	36	45	46	50	50	57	2	40
6	70	69	89	87	96	98	110	4	76
9	102	99	128	126	142	144	162	6	110
12	131	127	165	163	185	187	210	8	144
15	158	154	200	198	227	228	259	10	177
18	184	179	233	231	266	268	305	12	208
21	207	202	263	263	304	305	349	14	240
24	230	224	293	292	340	342	390	16	270
H[f]	46	45	39	39	34	34	31		22
v_o[g]	2.03	2.04	2.55	2.58	2.68	2.78	3.04		3.20
σv_o[gh]	0.02	0.03	0.03	0.04	0.02	0.02	0.02		0.06

a. Armour Lot #90492, 15% protein nitrogen. b. Run number -month-day (1956). c. In units of 10^{-3} M. d. S.R. = scale reading. Corrected to S.R. = 0 at t_o. In units of 10^{-3} ml. Estimated to $\pm$ 0.5 x 10^{-3} ml. and rounded off. e. In units of minutes. f. Approximate percent hydrolysis. g. Calculated by the orthogonal polynomial method (4). In units of 10^{-4} M/min. h. $\pm$.

Table VI.--Summary of the Initial Velocities and Kinetic Constants Derived from the Data of Table V.

$[S]_o$ [a]	P_m [b]	v_o [c]	σv_o [d]	$[S]_o[E]/v_o$ [e]
8	3	2.03	0.02	5.91
8	4	2.04	0.03	5.88
12	3	2.55	0.03	7.06
12	4	2.58	0.04	6.98
16	2	2.68	0.02	8.96
16	3	2.78	0.02	8.63
20	2	3.04	0.02	9.87
20	3	3.20	0.06	9.38

$$k_3' = 3.09 \pm 0.21 \times 10^{-3}$$ [f,g]

$$K_S'' = 10.2 \pm 1.2 \times 10^{-3}\ \underline{M}$$ [f]

$$K_I = 3.7 \pm 1.4 \times 10^{-3}\ \underline{M}$$ [h]

a. In units of 10^{-3} $\underline{M}$. b. Order of polynomial employed for calculation of v_o (4). c. In units of 10^{-4} $\underline{M}$/min. d. $\pm$, in units of 10^{-4} $\underline{M}$/min. e. In units of min. mg. protein nitrogen/ml. f. Evaluated by the method of least squares (cf. fig. 3). g. In units of $\underline{M}$/min. mg. protein nitrogen/ml. h. Based on a value of $K_S' = 6.6 \pm 0.45 \times 10^{-3}$ $\underline{M}$.

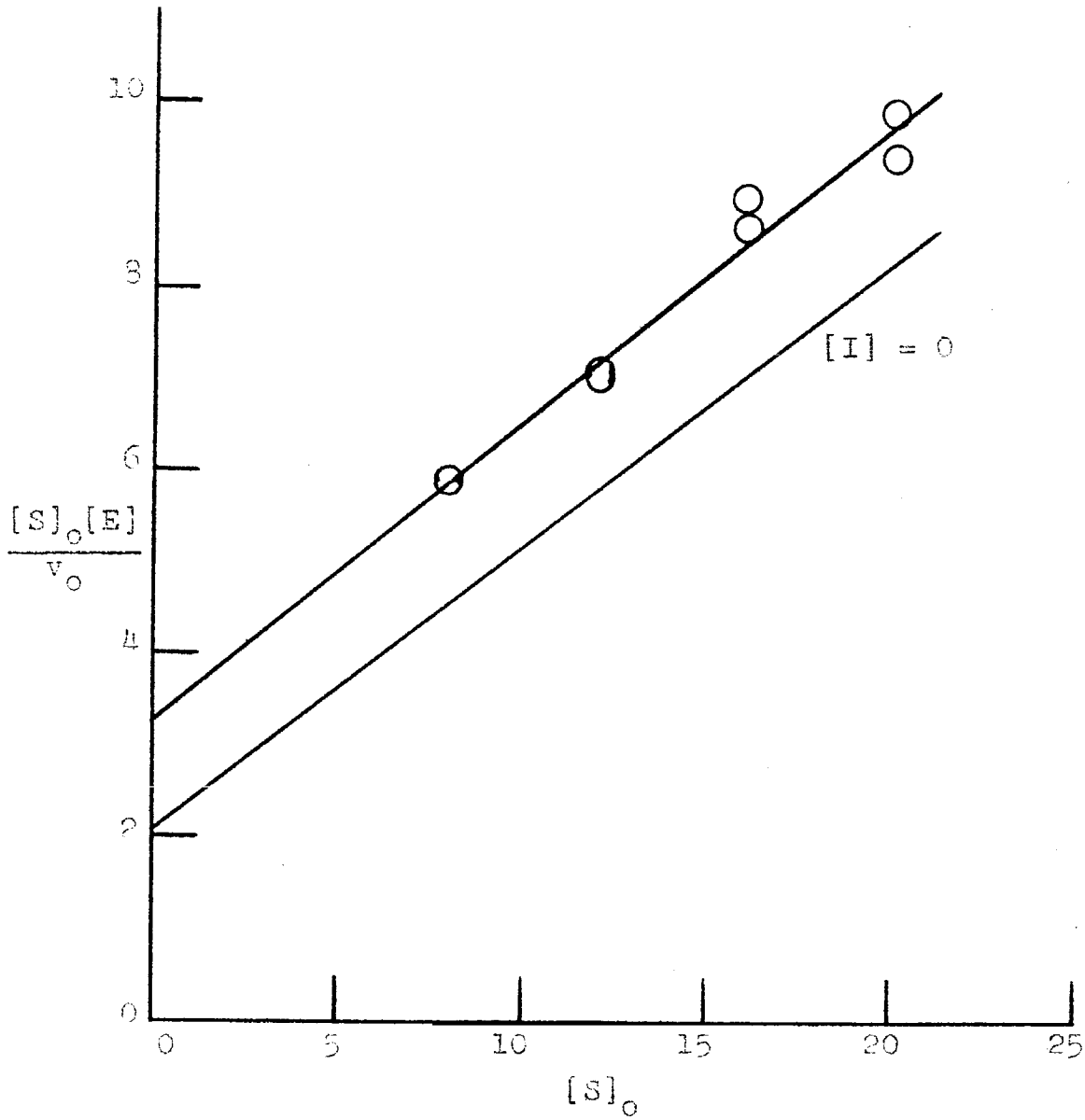

Figure 3.--$[S]_o[E]/v_o$ versus $[S]_o$ plot for the inhibition of the alpha-chymotrypsin catalyzed hydrolysis of methyl hippurate by 2×10^{-3} M acetyl-D-tryptophanamide at pH 7.9 and 25°. Line determined by least squares. $[S]_o[E]/v_o$ in units of min. mr. protein nitrogen/ml. $[S]_o$ in units of 10^{-3} M. (cf. Table VI).

Table VII.--Competitive Inhibition of the alpha-Chymotrypsin Catalyzed Hydrolysis of Methyl Hippurate by Acetyl-D-phenyl-alanine Methyl Ester at pH 7.9 and 25°.

$[E]^a$ = 0.150 mg. protein nitrogen/ml.; [NaOH] = 0.1609 N; [NaCl] = 0.02 M; [I] = 2×10^{-3} M.

Run No.[b]	1-4-18	2-4-18	3-4-18	4-4-18	5-4-18	6-4-18	7-4-18	8-4-18	1-4-23	2-4-23	3-4-23	4-4-23
$[S]_o$[c]	8	8	12	12	16	16	20	20	8	12	16	20
	S.R.[d]	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.
t[e]												
0	0	0	0	0	0	0	0	0	0	0	0	0
3	39	39	47	47	54	52	57	58	39	46	53	56
6	74	74	90	90	103	102	112	112	72	88	102	110
9	105	105	130	130	150	149	163	165	104	128	149	162
12	135	136	168	168	195	192	214	215	133	166	194	211
15	162	163	204	205	237	234	263	263	160	201	237	259
18	188	188	238	238	278	275	310	310	185	235	280	304
21	212	213	272	271	317	315	355	355	209	268	319	349
24	233	236	302	302	355	352	398	399	231	298	357	392
H[f]	47	48	40	40	36	35	32	32	46	40	36	32
v_o[g]	2.13	2.16	2.47	2.58	2.94	2.91	3.07	3.16	2.11	2.51	2.80	3.10
δv_o[gh]	0.04	0.03	0.07	0.03	0.02	0.04	0.01	0.02	0.03	0.02	0.02	0.02

a. Armour Lot #90492, 15% protein nitrogen. b. Run number -month-day (1956). c. In units of 10^{-3} M. d. S.R. = scale reading. Corrected for S.R. = 0 at t_o. In units of 10^{-3} ml. e. In units of minutes. f. Approximate percent hydrolysis. g. Calculated by the orthogonal polynomial method (4). In units of 10^{-4} M/min. h. ±.

Table VIII.--Summary of the Initial Velocities and Kinetic Constants Derived from the Data of Table VII.

$[S]_o$ [a]	P_m [b]	v_o [c]	δv_o [d]	$[S]_o[E]/v_o$ [e]
8	3	2.13	0.04	5.63
8	3	2.16	0.03	5.57
8	3	2.11	0.03	5.68
12	2	2.47	0.07	7.30
12	3	2.58	0.03	6.99
12	3	2.51	0.02	7.16
16	3	2.94	0.02	8.16
16	3	2.91	0.04	8.26
16	2	2.80	0.02	8.56
20	2	3.07	0.01	9.77
20	3	3.16	0.02	9.49
20	3	3.10	0.02	9.69

$k_3' = 3.02 \pm 0.09 \times 10^{-3}$ [f,g]

$K_S'' = 9.21 \pm 0.53 \times 10^{-3}$ M [f]

$K_I = 5.1 \pm 1.8 \times 10^{-3}$ M [h]

a. In units of 10^{-3} M. b. Order of polynomial employed in calculating v_o (4). c. In units of 10^{-4} M/min. d. $\pm$, in units of 10^{-4} M/min. e. In units of min. mg. protein nitrogen/ml. f. Evaluated by the method of least squares (cf. fig. 4). g. In units of M/min. mg. protein nitrogen/ml. h. Based on a value of $K_S' = 6.57 \pm 0.45 \times 10^{-3}$ M.

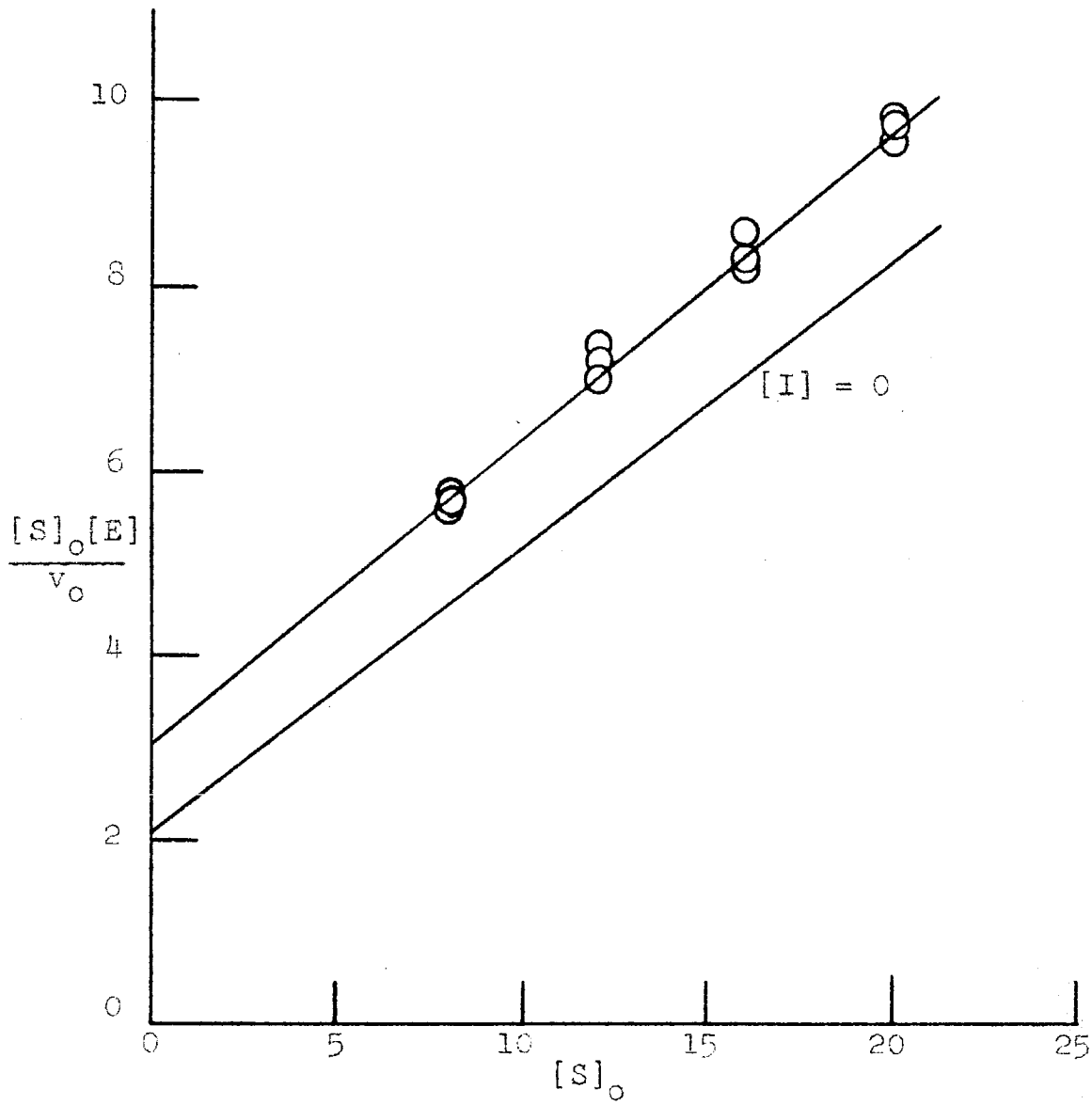

Figure 4.--$[S]_o[E]/v_o$ versus $[S]_o$ plot for the inhibition of the alpha-chymotrypsin catalyzed hydrolysis of methyl hippurate by 2 x 10^{-3} M acetyl-D-phenylalanine methyl ester at pH 7.9 and 25° in 0.02 M sodium chloride solution. Line determined by least squares. $[S]_o[E]/v_o$ in units of min. mr. protein nitrogen/ml. $[S]_o$ in units of 10^{-3} M. (cf. Table VIII).

Table IX.--The alpha-Chymotrypsin Catalyzed Hydrolysis of Benzoyl-L-valine Methyl Ester at pH 7.9 and 25°.
[NaOH] = 0.01964 N; [NaCl] = 0.02 M.

Run No.[a]	3-8-30	1-6-13	2-6-13	3-6-13	4-6-13	1-6-14	1-6-16	4-6-16	1-7-23	2-7-23	3-7-23	2-8-4	2-8-23	3-8-23	4-8-23	5-8-23	6-8-23
$[E]^b$	0.150	0.152	0.152	0.152	0.152	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.159	0.159	0.159	0.150	0.150
$[S]_o{}^c$	0 S.R.[d]	1.00 S.R.[de]	2.04 S.R.[de]	3.01 S.R.[de]	4.08 S.R.[de]	1.00 S.R.[de]	4.00 S.R.[de]	2.01 S.R.[de]	1.02 S.R.[de]	3.28 S.R.[de]	4.02 S.R.[de]	4.05 S.R.[de]	2.48 S.R.[de]	3.68 S.R.[de]	3.61 S.R.[de]	2.54 S.R.[de]	1.45 S.R.[de]
t^f																	
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	8.5	14.5	27.5	36.5	40.5	15.0	38.5	25.5	15.5	36.5	40.0	39.0	31.5	38.5	38.0	29.0	19.0
4	12.0	32.0	58.0	74.0	84.0	32.0	82.0	53.5	32.5	74.3	81.5	80.5	65.0	80.0	80.0	59.5	40.5
6	15.0	47.5	87.0	111.0	127.5	48.7	123.5	81.0	49.5	111.5	124.0	122.5	98.5	121.0	121.0	90.5	60.8
8	18.0	63.0	116.0	146.5	169.0	64.0	165.0	108.0	65.0	147.0	164.5	162.5	130.0	161.0	160.5	119.7	80.5
10	20.5	78.0	144.0	181.5	211.5	79.0	204.0	135.0	81.5	182.5	204.5	204.0	162.0	200.5	199.5	148.5	100.5
12	23.0	93.0	171.0	216.0	253.0	94.0	246.0	161.5	96.5	217.5	245.5	244.0	192.0	239.5	239.0	176.7	119.5
14	25.0	107.0	198.0	251.0	295.0	108.7	286.0	187.0	111.0	252.5	285.5	283.0	223.0	279.0	278.0	205.5	138.3
16	27.5	120.5	224.0	283.5	334.5	122.0	323.5	212.5	125.0	286.0	325.0	322.0	252.5	316.0	315.0	233.3	156.0
H^g	--	24	22	18	16	24	16	21	24	17	16	16	20	17	17	18	21
$v_o{}^h$	--	1.64	2.96	3.67	4.08	1.66	4.14	2.46	1.58	3.72	4.10	3.91	3.31	3.90	4.04	3.09	1.96
$\sigma v_o{}^{hi}$	--	0.03	0.03	0.06	0.08	0.03	0.06	0.07	0.04	0.02	0.03	0.07	0.03	0.05	0.04	0.03	0.05

a. Run number -month-day (1956). b. In units of mg. protein nitrogen/ml. Armour Lot 90492, 15% protein nitrogen. c. In units of 10^{-3} M. d. S.R. = scale reading. Corrected to S.R. = 0 at t_o. In units of 10^{-3} ml. Estimated error in reading $\pm$ 0.2 x 10^{-3} ml. e. Corrected for blank given in first column. f. In units of minutes. g. Approximate percent hydrolysis. h. Calculated by the orthogonal polynomial method (4). In units of 10^{-5} M/min. i. $\pm$.

Table X.--Summary of the Initial Velocities and Kinetic Constants Derived from the Data of Table IX.

$[S]_o$ [a]	P_m [b]	v_o [c]	σv_o [d]	$[S]_o[E]/v_o$ [e]
1.00	2	1.64	0.03	9.24
2.04	2	2.96	0.03	10.4
3.01	2	3.67	0.06	12.4
4.08	3	4.08	0.08	15.2
1.00	2	1.66	0.03	9.13
4.00	2	4.14	0.06	14.5
2.01	4	2.46	0.07	12.3
1.02	3	1.58	0.04	9.68
3.28	2	3.72	0.02	13.2
4.02	2	4.10	0.03	14.7
4.05	3	3.91	0.07	15.5
2.48	2	3.31	0.03	11.9
3.68	1	3.90	0.05	15.0
3.61	2	4.04	0.04	14.2
2.54	2	3.09	0.03	12.3
1.45	3	1.96	0.05	11.1

$$K_S = 4.2 \pm 0.4 \times 10^{-3}\ \underline{M}^{f}$$

$$k_3 = 0.55 \pm 0.04 \times 10^{-3}\ ^{f,g}$$

a. In units of 10^{-3} M. b. Order of polynomial employed for calculation of v_o (4). c. In units of 10^{-5} M/min. d. $\pm$, in units of 10^{-5} M/min. e. In units of min. mg. protein nitrogen/ml. f. Evaluated by the method of least squares (cf. fig. 5). g. In units of M/min. mg. protein nitrogen/ml.

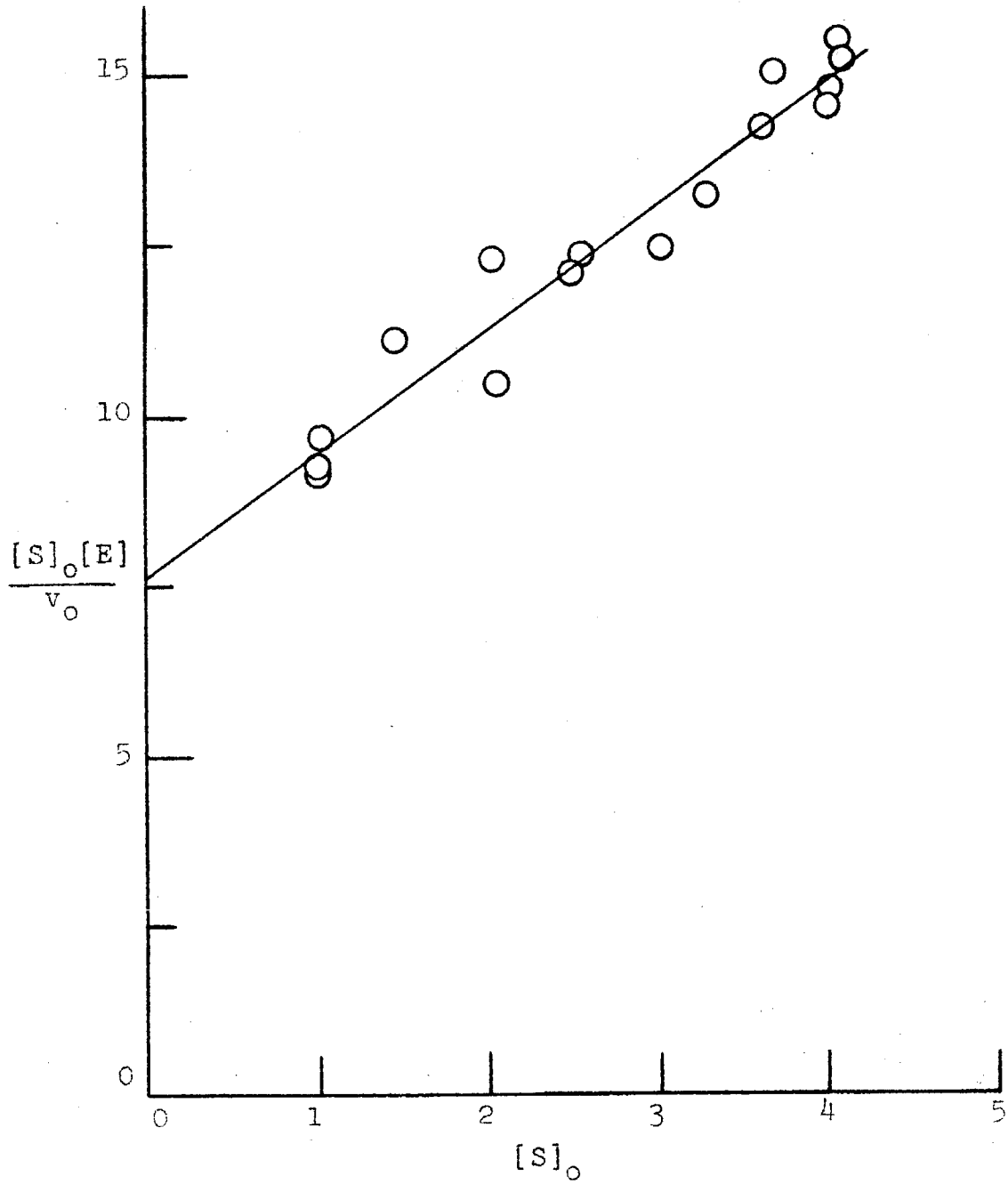

Figure 5.--$[S]_o[E]/v_o$ <u>versus</u> $[S]_o$ plot for the <u>alpha</u>-chymotrypsin catalyzed hydrolysis of benzoyl-L-valine methyl ester at pH 7.9 and 25° in 0.02 <u>M</u> sodium chloride solution. Line determined by least squares. $[S]_o[E]/v_o$ in units of min. mg. protein nitrogen/ml. $[S]_o$ in units of 10^{-3} <u>M</u>. (cf. Table X).

Table XI.--The alpha-Chymotrypsin Catalyzed Hydrolysis of Benzoyl-L-valine Methyl Ester at 25°: Dependence on pH. $[E]^a$ = 0.150 mg. protein nitrogen/ml.; [NaOH] = 0.01964 N; [NaCl] = 0.02 M.

Run No.[b]	1-7-25	2-7-25	3-7-25	1-6-16	4-7-25
$[S]_o$[c]	4.02	4.10	4.15	4.00	4.11
pH	7.5	7.7	7.8	7.9	8.1
	S.R.[d]	S.R.[d]	S.R.[d]	S.R.[d]	S.R.[d]
t[e]					
0	0	0	0	0	0
2	45	49	52	48	51
4	89	96	100	96	97
6	133	141	147	140	143
8	176	184	194	185	187
10	218	229	240	227	231
12	261	272	283	270	275
14	303	315	327	312	319
16	343	357	371	353	362
P_m[f]	2	3	3	3	3
v_o[g]	4.42	4.85	5.10	4.86	4.97
σv_o[gh]	0.02	0.08	0.08	0.03	0.07
$v_o/[S]_o$[i]	1.10	1.18	1.23	1.22	1.21

a. Armour Lot #90492, 15% protein nitrogen. b. Run number -month-day (1956). c. In units of 10^{-3} M. d. S.R. = scale reading. Corrected to S.R. = 0 at t_o. In units of 10^{-3} ml., $\pm$ 1 x 10^{-3} ml. Uncorrected for blanks. f. Order of polynomial employed for calculation of v_o (4). g. Calculated by orthogonal polynomial method. In units of 10^{-5} M/min. h. $\pm$. i. In units of 10^{-2} $min.^{-1}$.

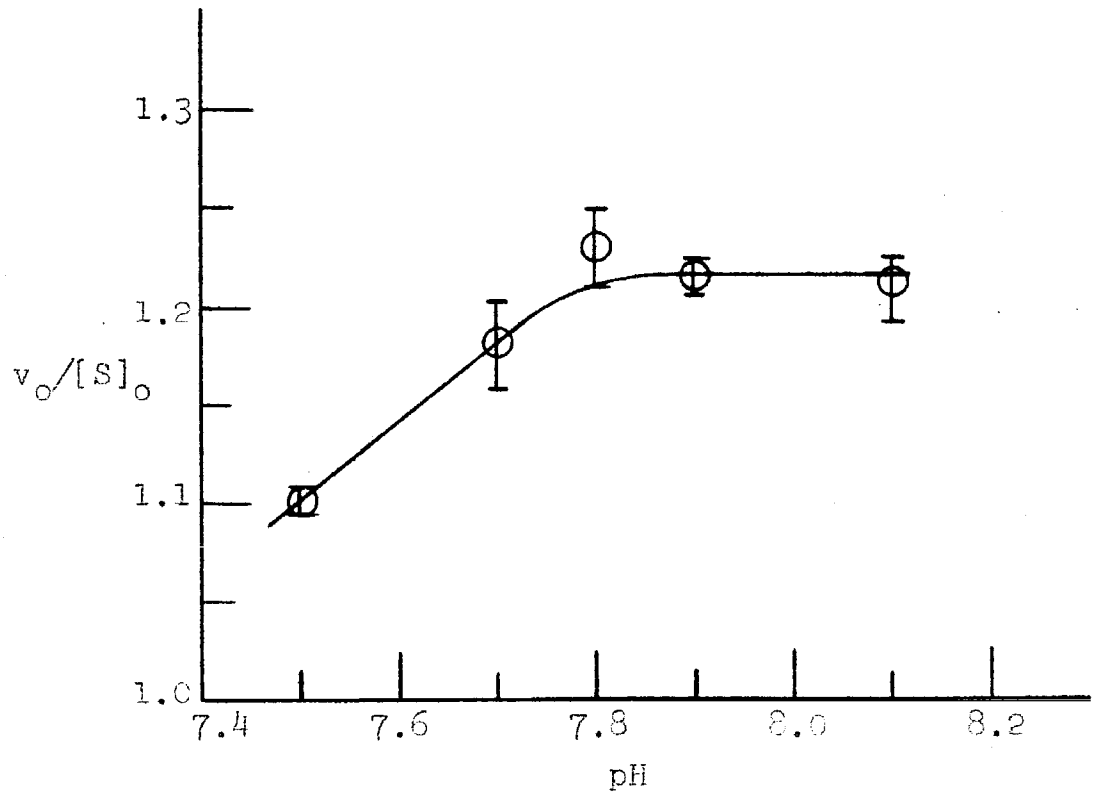

Figure 6.--Plot of $v_o/[S]_o$ versus pH for the system alpha-Chymotrypsin-benzoyl-L-valine methyl ester at 25° in 0.02 M sodium chloride solution. $v_o/[S]_o$ in units of 10^{-2} $min.^{-1}$ (cf. Table XI).

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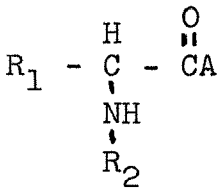
PART IV

THE ENZYME-INHIBITOR DISSOCIATION CONSTANTS OF SOME N-ACETYL AMINO ACID N'-METHYLAMIDES

A. Introduction

One approach to an understanding of the mode of action of the enzyme alpha-chymotrypsin has been a detailed analysis of the kinetics of hydrolysis of amino acid derivatives in its presence. Much of what was known in this area up to 1950 has been collected in a review by Neurath and Schwert (1), while the results of the more recent work of Niemann and various coworkers has been compiled by Foster and Niemann (2). Other recent general reviews that consider this field are those of Desnuelle (3), Schwert (4), Lindley (5), Fruton and Mycek (6) and Alberty (7). Although considerable information has been obtained concerning the allowed modifications of the groups R_2 and A in the generalized amino acid molecule



which may be made with retention of activity in the alpha-chymotrypsin catalyzed reactions, the extent of variation of R_1 has been somewhat limited (cf. 1,2). While it had been implied that aromaticity was a necessary requisite for specific substrates of this enzyme (1), Jennings and Niemann (8) demonstrated that acetyl-L-hexahydrophenylalaninamide was kinetically indistinguishable from acetyl-L-phenylalaninamide. Based on this past knowledge (1,2,8), it appeared reasonable to determine if other non-aromatic

L-amino acids possessed the necessary structural features that would allow them to function as specific substrates of alpha-chymotrypsin.

One approach to this problem, similar to the brief survey in Part II of this thesis, would be an examination of the kinetics of the enzyme-catalyzed hydrolysis of a series of suitable substrates, for example, the acylated-L-amino acid esters. However, the composite nature of the enzyme-substrate dissociation constant, K_S (see below), and the difficulties attendant in the interpretation of the significance of this value (cf. 2), coupled with the problems of experimental manipulation (cf. Part II), would preclude a detailed study of a large number of such systems.

A more reasonable approach is through the study of the effects of added competitive inhibitors to various systems of substrate-alpha-chymotrypsin. One advantage in this case is that a large number of compounds may be evaluated as inhibitors by employing one or more established substrate systems. Such investigations have supplied much of the knowledge concerning the structural requirements of compounds which interact reversibly with alpha-chymotrypsin. As before, the results of these studies have been reviewed (1) and compiled (9,10) by the various authors.

A system containing substrate, enzyme and competitive inhibitor neglecting interaction of the enzyme with hydrolysis products may be described by equations 1 and 2.

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

$$E_f + I_f \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EI \qquad (2)$$

By definition $K_S = \frac{k_2+k_3}{k_1}$, $K_I = k_5/k_4$, and, by application of the steady-state approximation, the rate expression is (10)

$$-d[S]/dt = k_3[E][S]/K_S(1+[I]/K_I) + [S] \qquad (3)$$

From a prior knowledge of K_S and [I] the value of K_I my be obtained. The results of such studies have the added advantage that the value of K_I may be considered to be a true equilibrium constant describing the affinity of the enzyme for the compound. However, in the case where the competitive inhibitor can function as a specific substrate of the enzyme, equation 2 must be replaced by equation 4, i.e.

$$E_f + S'_f \underset{k'_2}{\overset{k'_1}{\rightleftharpoons}} ES' \xrightarrow{k'_3} E_f + P'_{1f} + P'_{3f} \qquad (4)$$

Thus, the situation described by equations 1 and 4 is one of competitive hydrolysis of two substrates. Such systems have been considered by Foster and Niemann (11) and by Huang, Foster and Niemann (12). In this case the value of $K'_S = (k'_2 + k'_3)/k'_1$ is not a true equilibrium constant, for

its interpretation is subject to the same uncertainty as is that of K_S (cf. 2). For the particular instance, however, where $k_3' \rightarrow 0$, it is permissible to assume (13), for all practical purposes, a system described by equations 1 and 2, recognizing that the values of K_I so obtained only approximate a true equilibrium constant.

Previously studied competitive inhibitors derived from amino acids have mainly been confined to the uncharged derivatives of the D-enantiomorphs and the charged derivatives of the L-enantiomorphs (1,9,10). The one exception in the latter case is acetyl-L-tryptophanmethylamide which Huang and Niemann (13) found to be a competitive inhibitor in the system alpha-chymotrypsin-nicotinyl-L-tryptophanamide. This class of inhibitor was later extended to a series of acylated-L-tyrosinmethylamides by Lands (14). In these cases, although the compounds possess the L-configuration and thus can function as specific substrates of the enzyme, their low susceptibility to hydrolysis, i.e., $k_3' \doteq 0$, permits them to be considered as competitive inhibitors (13) subject to the conditions discussed above.

The present investigations were based on the above observations (13), and the previously stated need for information as to the effect of variation in the L-amino acid side chains on the binding of these molecules to the enzyme. The overall problem can be separated into two distinct sections. First: the synthesis of the necessary derivatives of the L-amino acids selected for study as

inhibitors against suitable substrates; and, second: the study of the inhibited hydrolysis reactions of these latter compounds.

B. The Synthesis of Some N-Acetyl-L-amino Acid N'-Methylamides

The seventeen L-amino acids (along with glycine) that were selected for this study are as follows: glycine, alanine, valine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan, proline, hydroxyproline, asparagine, glutamine, histidine, lysine, arginine, cystine, cysteine and methionine. The N-acetyl-N'-methylamides of some of these have been reported earlier. Huang and Niemann (13) prepared the derivatives of D- and L-tryptophan. Lands (14) prepared this derivative of L-tyrosine. Mizushima and coworkers (15-17) in a series of papers have described acetylglycinmethylamide (15) and acetyl-L-valinmethylamide (17) as well as the corresponding derivatives of DL-alanine (16), DL-leucine (15), DL-proline (16) and DL-norleucine (17).

Of the above eighteen amino acids chosen, all but glutamine were converted to various intermediates leading to the final products. Of these seventeen, asparagine, lysine, arginine, cystine, and cysteine were not converted to the desired N-acetyl-N'-methylamides. Out of the remaining twelve, the derivatives of valine and isoleucine resisted purification and were not obtained in an analytically pure form. The ten remaining N-acetyl amino acid N'-methyl-

amides were prepared and their physical constants determined. In addition, the corresponding derivatives of D-phenylalanine and D-tyrosine were prepared and characterized.

The two main reaction sequences selected for the preparation of these compounds were:

amino acid → amino acid ester hydrochloride → acylated amino acid ester → acylated amino acid methylamide (I)

amino acid → acylated amino acid → acylated amino acid ester → acylated amino acid methylamide (II)

In the cases of lysine and cysteine, protection of the ϵ-amino group in the former and of the sulfhydryl group in the latter was necessary prior to introduction into the reaction scheme.

The choice of sequence for a particular amino acid was generally guided by the extensive literature of these compounds (cf. 18), but, if possible, sequence I was selected to avoid the possibility of racemization in the first step of sequence II.

The first step of sequence I was carried out without exception by the elegant method of Brenner and Huber (19) employing thionyl chloride-methanol. A modification (cf. Part II) of this method was also employed for the second step of sequence II, except in the case of acetyl-L-hydroxyproline, and later, acetyl-L-asparagine where the reaction with diazomethane was the method employed.

Acetylations, both in step 2 of sequence I and step I of method II were generally carried out by the usual methods employing acetic anhydride in the presence of base or in glacial acetic acid. One somewhat neglected reaction, the treatment of amino acid ester hydrochlorides with excess acetyl chloride in benzene solution under refluxing conditions, first described by Curtius (20), was investigated for a number of such preparations. The yields were generally high, but the purity of the products was doubtful.

One additional method was suggested by the studies of Schwyzer and coworkers (21-23) on the use of cyanomethyl esters of acylated amino acids as acylating agents. These workers mentioned, however, that the reactivities of the simple carboxylic acids cyanomethyl esters in this reaction are unknown (21). Cyanomethyl acetate (24) was prepared and reacted under the prescribed conditions (23) with DL-tyrosine ethyl ester to yield acetyl-DL-tyrosine ethyl ester. Although this system was not exploited further, it appears to offer a mild acylation procedure for sensitive compounds.

In all cases the conversion of the acetylated amino acid esters to the corresponding methylamides was carried out by treatment with excess methylamine in methanol solution. Generally, no attempt was made to attain an extreme purity of the various intermediates since the end products were for the most part easily purified by recrystalliza-

tion. The compounds eventually employed in the inhibition studies and their physical constants are listed in Table I.

C. Inhibition Studies

As indicated earlier, Huang and Niemann (13) ascertained that the N-acetyl-N'-methylamides of D and L-tryptophan are competitive inhibitors in the system alpha-chymotrypsin-nicotinyl-L-tryptophanamide. Therefore, a reasonable starting point for the present study was a re-investigation of the inhibition of the alpha-chymotrypsin catalyzed hydrolysis of methyl hippurate by acetyl-L-tyrptophanmethylamide. If suitable results could be obtained in this system, the extension to the study of the other inhibitors could be made with some confidence.

The original intent was to investigate only those inhibitors with the L-configuration. For this reason, a series of duplicate experiments were performed as described in Part II with methyl hippurate concentrations ranging from 8×10^{-3} M to 20×10^{-3} M, an acetyl-L-tryptophanmethylamide concentration of 5×10^{-3} M and an enzyme concentration of 0.150 mg. protein nitrogen/ml. in 0.02 M sodium chloride solution at pH 7.9 and 25°. The initial velocities were evaluated by the method of Booman and Niemann (25). From these values at the various initial substrate concentrations and an $[S]_o[E]/v_o$ versus $[S]_o$ plot the values of $k_3 = 3.0 \times 10^{-3}$ M/min. mg. protein

nitrogen/ml. and $K_S' = K_S (1+[I]/K_I) = 7.6 \times 10^{-3}$ M (cf. equation 3) were obtained by a least squares analysis. For $[I] = 5 \times 10^{-3}$ M and $K_S = 6.6 \times 10^{-3}$ M this K_S' value yields $K_I = 33 \times 10^{-3}$ M. The variability in these preliminary results was not determined.*

This was an unexpected result. Huang and Niemann (13) had obtained the value $K_I = 4.8 \times 10^{-3}$ M and Foster and Niemann (10) revised this to be $6.5 \pm 1.5 \times 10^{-3}$ M. The present result is considerably different from either of these. That this was not an artifact due to some constant error was shown by a repetition of a single set of experiments as above at a later date. The data are presented in Tables II and III, and the $[S]_o[E]/v_o$ versus $[S]_o$ plot in fig. 1. A least squares analysis of the total data, omitting one point which was obviously aberrant gave the values $k_3 = 3.09 \pm 0.22 \times 10^{-3}$ M/min. mg. protein nitrogen/ml., and $K_S' = 8.0 \pm 1.3 \times 10^{-3}$ M. Employing $K_S = 6.6 \times 10^{-3}$ M the value of $K_I = 24 \times 10^{-3}$ M was obtained. Due to inhibitor solubility limitations the values of $I_I' = [I]/K_I$ in these cases are approximately 0.2, and the values of K_I obtained are only of the order of magnitude specified with high variability (cf. 9).

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*To avoid confusion in the later discussion only the symbols k_3, K_S and K_S' will be employed recognizing that the composite nature of k_3 and K_S for methyl hippurate are as discussed in Parts II and III.

As it was not clear why present value of K_I should differ by a factor of three to four from that obtained earlier (10), the various possibilities of error were examined. The inhibitor, prepared as directed (13), analyzed correctly and had physical constants that agreed quite closely with those of the earlier preparation. A mixed melting point of the two samples showed no depression. An examination of the blanks of enzyme and inhibitor and of inhibitor and substrate suggested that these effects were not responsible. The blank corrections were again less than the variability of the initial velocities. (*cf.* Part III, page 93).

In order to determine if this result was a real effect, or only one of methodology, an investigation of the inhibition of the alpha-chymotrypsin catalyzed hydrolysis of methyl hippurate by acetyl-D-tryptophanmethylamide was undertaken. From a series of eight experiments over the range of initial substrate concentrations $[S]_o = 8 \times 10^{-3}$ M to $[S]_o = 20 \times 10^{-3}$ M at an inhibitor concentration, $[I] = 5 \times 10^{-3}$ M, an enzyme concentration, [E] = 0.150 mg. protein nitrogen/ml., with other factors as before the values $k_3 = 3.06 \pm 0.18 \times 10^{-3}$ M/min. mg. protein nitrogen/ml. and $K_S' = 19.1 \pm 1.4 \times 10^{-3}$ M were obtained by the previously mentioned methods. The data and plot are presented in Tables IV and V and figure 2. This value of K_S' yields

$K_I = 2.5 \pm 0.4 \times 10^{-3}$ M.* Huang and Niemann (13) gave $K_I = 1.7 \times 10^{-3}$ M, and Foster and Niemann (10) re-evaluated this to be $1.8 \pm 0.3 \times 10^{-3}$ M. Thus, in the case of the D-enantiomorph there is agreement between the inhibitor-enzyme dissociation constants obtained in entirely different substrate systems.

In each of the above cases the respective values of k_3, i.e., 3.09 ± 0.22 and 3.06 ± 0.18 in units of 10^{-3} M/min. mg. protein nitrogen/ml. are in agreement within the specified limits with the value $k_3 = 3.21 \pm 0.09 \times 10^{-3}$ M/min. mg. protein nitrogen/ml. obtained for methyl hippurate alone (cf. Part III). It can be concluded that within the limits of the present experimental error the inhibition is competitive in both cases at the inhibitor concentrations employed.

This unexpected behavior of an inhibitor of the L-configuration coupled with the normal behavior of the corresponding D-enantiomorph suggested an interesting possibility. Foster, Shine and Niemann (9) tentatively concluded that there is reason to believe that the modes of combination of D- and L-enantiomorphs of amino acid derivatives with alpha-chymotrypsin are significantly different. This concept coupled with the idea of the bifunctional nature of methyl hippurate in alpha-chymotrypsin

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*As before (cf. Part III) the variability in K_I was calculated from $\delta K_I = (\Sigma(\partial K_I)^2)^{\frac{1}{2}}$.

catalyzed reactions (26,27) suggested that the present observations with a competitive inhibitor of the L-configuration might be an example of a general type of enzyme-bifunctional substrate-L-inhibitor interaction. To check this hypothesis it was decided to examine the inhibitor-enzyme dissociation constants of other pairs of D- and L-N-acetyl-N'-methylamides as determined in the system alpha-chymotrypsin-methyl hippurate and alpha-chymotrypsin-trifunctional L-amino acid derivatives. This investigation, and the possibility that other inhibitors of the L-configuration might give similar results in the methyl hippurate system, gave impetus to the various preliminary experiments described in Part II which were directed towards the discovery of acylated-L-amino acid esters suitable for use with the present analytical technique (28).

After the development of the system alpha-chymotrypsin-benzoyl-L-valine methyl ester (cf. Parts II and III) the inhibition constants of the N-acetyl-N'-methylamides of D- and L-tryptophan were re-investigated. Although, as indicated in Part III, this system is difficult to work with and is subject to greater experimental errors, the results are significant. The data obtained (Table XVI) from a series of twelve experiments over an initial substrate concentration range of 1.45×10^{-3} M to 3.51×10^{-3} M at a concentration of acetyl-D-tryptophanmethylamide of 0.5×10^{-3} M, an enzyme concentration of 0.150 mg. protein

nitrogen/ml. in 0.02 M sodium chloride solution at pH 7.9 and 25° were evaluated by the Booman-Niemann method (25). The initial velocities (Table XVII) at the respective substrate concentrations were employed to construct a plot of $[S]_o[E]/v_o$ versus $[S]_o$ (fig. 8). By a least squares analysis the values $k_3 = 0.53 \pm 0.03 \times 10^{-3}$ M/min. mg. protein nitrogen/ml. and $K_S' = 5.23 \pm 0.37 \times 10^{-3}$ M were obtained. The latter value at $[I] = 0.5 \times 10^{-3}$ M and $K_S = 4.2 \pm 0.4 \times 10^{-3}$ M for benzoyl-L-valine methyl ester (cf. Part III) yields $K_I = 2.1 \pm 1.2 \times 10^{-3}$ M. This value is in good agreement within the specified limits with those determined previously (cf. above and 10,13). The value $k_3 = 0.55 \pm 0.04 \times 10^{-3}$ M/min. mg. protein nitrogen/ml. was obtained for benzyl-L-valine methyl ester in Part III; therefore the respective values of k_3 are in agreement, within experimental error, and the inhibition is competitive.

A similar set of experiments (Tables XIV and XV, fig. 7) employing 5×10^{-3} M acetyl-L-tryptophanmethylamide as an inhibitor gave the values $k_3 = 0.60 \pm 0.08 \times 10^{-3}$ M/min. mg. protein nitrogen/ml. and $K_S' = 7.2 \pm 1.1 \times 10^{-3}$ M. From the latter value $K_I = 7.1 \pm 2.9 \times 10^{-3}$ M was obtained. As above, the inhibition is competitive. Recalling that the previous results with methyl hippurate gave $K_I = 24\text{-}33 \times 10^{-3}$ M, while Foster and Niemann reported $K_I = 6.5 \pm 1.5 \times 10^{-3}$ M, it is apparent that there is an appreciable difference between the inhibition by acetyl-L-tryptophanmethylamide of the alpha-chymotrypsin catalyzed

hydrolysis of a least two acylated-L-amino acid derivatives as compared to that of methyl hippurate.

The other D and L pairs of inhibitors selected for this particular segment of these studies were those of phenylalanine and tyrosine. Necessarily, as this was a somewhat exploratory investigation, a large number of observations could not be made. In addition, the limitations of solubility and a lack of prior knowledge of the inhibition constants of these inhibitors did not always permit the selection of a value of [I] that would yield a reasonable value of I'_I. The limitations of this latter quantity and its influence on the error in K_I were discussed in Part III (cf. 9).

The data for the investigations with methyl hippurate and the four N-acetyl-N'-methylamides derived from D- and L-phenylalanine and D- and L-tyrosine are presented in Tables VI-XIII and figures 3-6. The information for the corresponding studies with benzyl-L-valine methyl ester is contained in Tables XVIII-XXV and figures 9-12. A summary of the results of all of these studies with each substrate is presented in Table XXVI.

Although these results are somewhat tentative, and are not very accurate due to the factors discussed above, the results allow a reasonable estimate of the magnitudes of the various inhibition constants. An examination of Table XXVI indicates that where sufficient information is available the inhibition is competitive at each inhibitor

concentration employed, i.e., the respective values of k_3 are equivalent within the stated error for each substrate system. In addition, it is apparent that for all cases, except acetyl-L-tryptophanmethylamide, the inhibition constants obtained are in reasonable agreement within the present limits of error when determined from results derived from experiments with either substrate system. Thus it would appear that until further information is obtained the possibility of a general type of bifunctional substrate-enzyme-L-inhibitor interaction must be discarded.

The present limited information does not permit an adequate explanation of the behavior observed in the methyl hippurate-acetyl-L-tryptophanmethylamide system. The results suggest, however, that further investigations with bifunctional substrates and trifunctional inhibitors, or perhaps, specifically, inhibitors containing the indolyl residue, may provide additional knowledge regarding the interaction of these compounds with the catalytic site of the enzyme. A similar approach enabled Huang and Niemann (27) to advance an explanation as to why indole, a monofunctional inhibitor, was a competitive inhibitor in the system nicotinyl-L-tryptophanamide-alpha-chymotrypsin, but was a non-competitive inhibitor in the system methyl hippurate-alpha-chymotrypsin.

Recognizing that the possibility might exist that the results obtained from the studies of the other N-acetyl-N'-methylamides would be influenced by the use of methyl

hippurate, the additional compounds described earlier were all evaluated in the system alpha-chymotrypsin-benzoyl-L-valine methyl ester.

One interesting result of the studies concerning the inhibition of the catalyzed hydrolysis of benzoyl-L-valine methyl ester was obtained from the experiments measuring the extent of "hydrolysis," i.e., base uptake, in the absence of the substrate. It was mentioned in Part III that blank corrections were necessary in this system. In one particular inhibition study, that of acetyl-D-tryptophan-methylamide, some preliminary experiments and a blank were performed at an inhibitor concentration of 3×10^{-3} M. As this particular compound is an excellent inhibitor ($K_I \doteq 2 \times 10^{-3}$ M) it was later necessary to reduce the inhibitor concentration to 0.5×10^{-3} M to allow the reaction to proceed at a rate suitable for measurement. A comparison of the blanks at these two inhibitor concentrations shows that the rate, i.e., base uptake, increases as the inhibitor concentration decreases. In addition, each of these blanks takes up base at a lower rate than a sample containing only enzyme in 0.02 M sodium chloride solution. These data are presented in each table for the particular compound under study, and have been compiled in Table XXIX.

None of the other inhibitors studied show a decrease in the blank correction similar to the example mentioned above. Derivatives of tyrosine and histidine show an apparent effect, but these are undoubtedly due to the buffer-

ing capacity of the phenol and imidazole groups, for these effects were noted as well in the presence of substrate. These compounds caused sluggish response of the pH indicating device.

The source of this enzyme blank is not evident. Hirs (29) has shown that certain components of alpha-chymotrypsin which lack enzymatic activity can be separated by chromatographic techniques. While it may be that the blank, i.e. base uptake, is due to the enzyme catalyzed hydrolysis of these impurities, the well known (1) autolysis of the enzyme itself may be the source of the blank. In any event, the present results suggest that this process is no different than the enzyme catalyzed hydrolysis of simple model substrates in that it can apparently be inhibited by at least one specific inhibitor for the latter class of compounds.

It was anticipated from earlier results (1,26) that the inhibition constants of the various N-acetyl-N'-methylamides not having aromatic character would be large. Fortunately, this class of compound is generally quite soluble in aqueous solution; therefore, high inhibitor concentrations could be achieved. To reduce the number of experiments performed the members of the whole group were evaluated individually at an approximate initial substrate concentration of 3.5×10^{-3} M, an inhibitor concentration of about 50×10^{-3} M and an enzyme concentration of 0.15 mg. protein nitrogen/ml. in 0.02 M sodium chloride solution

at pH 7.9 and 25°. By this preliminary survey method it was anticipated that those compounds which acted as inhibitors could be detected and be more extensively studied. The use of inhibitor concentrations on the order of 50 x 10^{-3} M would allow the determination of inhibition constants roughly of the magnitude of K_I = 250 x 10^{-3} M. This is based on the suggested (9) value $I'_I = [I]/K_I$ = 0.2 as the lowest allowable limit for an experimental error of ± 20%.

The results of these preliminary experiments and of a set of duplicates performed at a later date are presented in Tables XXVII and XXVIII and figure 13. In the figure the solid line represents the line obtained from a least squares analysis of the data of experiments performed with the substrate alone, and the dotted lines are the extremes of the scatter in this data (cf. Part III, fig. 5). It is apparent that none of the non-aromatic N-acetyl-L-amino acid N'-methylamides are inhibitors of consequence within the limits of the present method.

Based on the above observations, the original intent of determining the effect of the non-aromatic side chains of L-amino acids on the affinity of alpha-chymotrypsin for a series of similar derivatives of these compounds has not been realized.

A possible solution to the problem of determining the magnitude of the effect of the changes in side chain might be found if the acetyl group was replaced by a different

acyl moiety (cf. Proposition 1).

On the basis of the present information it can be concluded that the particular non-aromatic compounds listed in Table I are not bound at the catalytic site of this enzyme to any great degree. That this is only a matter of degree, however, is clearly illustrated by a consideration of the derivatives of L-leucine. In Part II, it was noted that the hydrolysis of acetyl-L-leucine methyl ester was extremely rapid at enzyme concentrations of 0.15 mg. protein nitrogen/ml. In the present case an even higher concentration of acetyl-L-leucine-methylamide apparently displays a complete lack of affinity for this enzyme at the same protein nitrogen concentration.

With this example and the other results described previously, it is apparent that the specificity of alpha-chymotrypsin is only partially understood. Further investigations, perhaps some of them in the directions indicated in this thesis, should lead to a broader knowledge of these aspects of enzyme action.

D. Experimental*

Analytical Procedures.--The techniques and instrumentation employed were described in Part II. The necessary modifications for the use of benzoyl-L-valine methyl ester were described in part III. It should be mentioned that in

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*All analyses by Dr. A. Elek. All melting points are corrected except as noted.

the latter stages of this work Dr. M. D. Cannon supplied a more sensitive Contact Meter Relay (CMR) for use in conjunction with the automatic titrimeter (28). This modification allowed a measurable decrease in the variation of pH during any given reaction, and a concomitant decrease in the stepwise nature of the rate curves.

Substrates and Inhibitors.--The preparation of the methyl hippurate and of benzoyl-L-valine methyl ester was described in Part II.

The preparations of the various intermediates and of the N-acetyl-N'-methylamides are given below. The latter compounds employed in the inhibition studies are collected in Table I with pertinent physical data.

Acetylglycinmethylamide (15).--Matheson glycine ethyl ester hydrochloride (22.5 g., 0.16 mole) was acetylated with 19 ml. (ca. 0.20 mole) of 97% acetic anhydride in a mixture of 450 ml. of 1 M sodium bicarbonate solution and 200 ml. of ethyl acetate. After saturating the aqueous phase with sodium chloride 10.4 g. (44.8%) of a white crystalline product, m.p. 45.5-47.0°, was obtained by drying and evaporating the ethyl acetate extracts. Ten grams (0.069 mole) of the solid was dissolved in 100 ml. of methanol and with cooling in ice 46 g. (1.48 moles) of methylamine was passed in. After standing overnight at room temperature the solvent was removed in vacuo under nitrogen on the steam cone to yield 8.1 g. (90%) of crude

solid. This compound was twice recrystallized from a mixed solvent of 400 ml. of ethyl acetate plus 25 ml. of ethanol and once from 400 ml. of ethyl acetate: m.p. 157.5-158.0°. Lit. m.p. 158° (15).

Anal. Calcd. for $C_5H_{10}O_2N_2$ (130.2): C,46.14; H,7.75; N,21.53
Found: C,46.02; H,7.74; N,21.47.

L-alanine Methyl Ester Hydrochloride.--This compound was twice prepared in 97.5% crude yield by the method of Brenner and Huber (19). As a typical example of the procedure employed in many cases the details will be included.

In a 3-necked flask with sealed stirrer, dropping funnel and calcium chloride outlet tube connected to a gas trap, 18 ml. (ca. 0.45 mole) of reagent methanol was treated in a dropwise manner with 4.5 ml. (0.062 mole) of redistilled thionyl chloride. During the addition the solution was stirred and cooled in an ice-salt mixture. Under the same conditions 5.0 g. (0.056 mole) of L-alanine was then introduced in ca. five equal portions. The resulting slurry was allowed to come to room temperature and a clear solution resulted. This was heated at 40° for 0.5 hour and then left overnight. Removal of the solvent at reduced pressure gave a viscous oil which solidified on scratching. The white solid was pulverized and dried in vacuo. Yield 7.62 g. (97.5%), m.p. 109.0-111.5°.

Acetyl-L-alanine Methyl Ester.--The procedure of Curtius (20) for the preparation of this compound by heating the ester hydrochloride under refluxing conditions with excess acetyl chloride in benzene solution led only to an oil that resisted attempts at crystallization. Conversion of this oil to the corresponding methylamide gave a product of dubious composition. Repeated recrystallizations of the solid from ethanol-ethylacetate mixtures or toluene in an attempt to achieve a constant melting point and rotation were unsuccessful.

This compound, a low melting solid, was prepared in 86% crude yield by the procedure of Reihlen and Knöpfle (30) for the acetylation of L-valine methyl ester hydrochloride. The essential details were given in Part II where the method was employed for the preparation of acetyl-L-isoleucine methyl ester.

Acetyl-L-alaninmethylamide.--The impure acylated ester (6.6 g. 0.046 mole) was converted to the methylamide in 95% crude yield by the procedure given for the preparation of the corresponding glycine derivative. Three grams of the resulting solid was twice recrystallized from 200 ml. portions of toluene to give 2.3 g. of white needles with constant melting point and specific rotation: m.p. 181.2-182.0° (sealed tube, compound sublimes rapidly at ca. 170°); $[\alpha]_D^{25} = -51.1^\circ$ (C, 2% in ethanol.)

Anal. Calcd. for $C_6H_{12}O_2N_2$ (144.2): C,49.98; H,8.39; N,19.43
Found: C,50.06; H,8.56; N,19.43

L-Valine Methyl Ester Hydrochloride.--This compound was prepared in 97% crude yield by the thionyl chloride-methanol method (19).

Acetyl-L-valine Methyl Ester (30).--A successful preparation of this compound was described in Part III. Earlier, treatment of 6.0 g. (0.036 mole) of the ester hydrochloride with excess acetyl chloride for ca. 15 hours with heating under refluxing conditions in benzene solution led to an oil after evaporation of the solvents. The oil was dissolved in 50 ml. of chloroform, the solution was extracted with 10 ml. of 1 N hydrochloric acid, dried over magnesium sulfate and the solvent removed in vacuo. The resulting oil slowly solidified when cooled in ice, but attempts at recrystallization from ligroin or mixtures of ligroin-toluene or ligroin-benzene failed. The crude material was employed below.

Acetyl-L-valinmethylamide (17).--Conversion of the impure acylated ester to the methylamide as before gave a white solid (4.5 g., 73% based on the ester hydrochloride). Many attempts at purification with numerous solvent systems failed. Although the previous workers (17) prescribed ethyl acetate as a suitable solvent for recrystallization, it was repeatedly observed that this compound was only

sparingly soluble in ca. 200 volumes of this solvent at the boiling point and separated as a cottony mass on cooling. The analysis of a sample, m.p. 249.5-252.0° (sealed tube; the compound sublimes rapidly > 200°) that had been repeatedly recrystallized and appeared to have a constant rotation, $[\alpha]_D^{25} = -39°$ (C, 1% in ethanol), was unsatisfactory. Lit. m.p. > 250°; $[\alpha]_D^{13.6} = -40.5°$ (C, 1.7 in water) (17).

L-Leucine Methyl Ester Hydrochloride (19).--This compound was twice prepared in crude yields greater than 97% by the procedure of Brenner and Huber (19).

Acetyl-L-leucine Methyl Ester (31).--The preparation of this compound by the method of Huang and Niemann (32) was described in Part II.

A previous preparation was carried out by heating 18 g. (0.099 mole) of the crude ester hydrochloride under refluxing conditions for four hours in the presence of excess acetyl chloride in benzene solution. The resulting clear solution was extracted with three 35-ml. portions of 1 N hydrochloric acid and dried over magnesium sulfate. Removal of the solvent in vacuo gave a mobile oil. The crude material was employed below.

Acetyl-L-leucinmethylamide.--The impure acetyl ester was treated with methylamine in methanol solution as before. The crude product, m.p. 154-160° (12.9 g. 70% based on ester hydrochloride) was repeatedly recrystallized with

considerable loss from ethyl acetate and from toluene-hexane mixtures to constant melting point and rotation: m.p. 165.3-166.8°; $[\alpha]_D^{25}$ = -33.9° (C, 1% in water).

Anal. Calcd. for $C_9H_{18}O_2N_2$ (186.3): C,58.03; H,9.74; N,15.04

Found: C,58.08; H,9.84; N,15.09

L-Isoleucine Methyl Ester Hydrochloride.--This compound was prepared in 91% crude yield by the thionyl chloride-methanol method (19).

Acetyl-L-isoleucine Methyl Ester.--The preparation of this compound was described in Part II. An earlier application of the same method (30) to 6.1 g. (0.034 mole) of the crude ester hydrochloride resulted in ca. 4.3 g. (68%) of an oil. No attempt was made to purify this material.

Acetyl-L-isoleucinmethylamide.--Treatment of the crude material obtained above with methanolic methylamine solution in a manner similar to that described for the corresponding glycine derivative gave a white solid. The solubility characteristics of this material were very similar to those noted for the corresponding derivative of L-valine. The material, separating as a flocculent mass, was recrystallized several times from ethyl acetate. The melting ranges of such preparations were about 10°, e.g., 210-222°, 220-229°. Consequently, no further attempts to purify this material were made.

Acetyl-L-phenylalanine Methyl Ester.--L-phenylalanine methyl ester hydrochloride (prepared by P. E. Peterson (33) (5.0 g., 0.023 mole) was acylated by the method of Reihlen and Knöpfle (30) to yield 4.76 g. (93%) of crude product, m.p. 88.0-90.0°; $[\alpha]_D^{25}$ = +17.2° (C, 2% in methanol). Lit. m.p. 89-90°; $[\alpha]_D^{25}$ = +19.5° (C, 2% in methanol) (12).

Acetyl-L-phenylalaninmethylamide.--The solid from above was converted to the methylamide as before in 95% yield. This material was recrystallized once from ethyl acetate (flocculent mass) and twice from water as long, white needles to constant m.p. 207.3-208.6° and $[\alpha]_D^{25}$ = +19.4° (C, 1% in ethanol).

Anal. Calcd. for $C_{12}H_{16}O_2N_2$ (220.3): C,65.43; H,7.32; N,12.72
Found: C,65.39; H,7.31; N,12.79

Acetyl-L-tyrosine (34).--L-Tyrosine (15 g., 0.083 mole) was acetylated with acetic anhydride (20 ml., 0.2 mole) in the presence of 245 ml. of 2 N sodium hydroxide solution as directed by du Vigneaud and Meyer (34) to yield 8.2 g. (44%) of product, m.p. 147.5-150.5°, after one recrystallization from water. Lit. m.p. 152-154° (34).

Acetyl-L-tyrosine Methyl Ester (35).--To an ice-salt cooled solution of 3.7 ml. (0.052 mole) of thionyl chloride in 40 ml. of absolute methanol prepared in the usual manner, 8.2 g. (0.037 mole) of acetyl-L-tyrosine was added in ca. four equal portions. After warming to 40° for an hour,

the clear solution was allowed to stand overnight at room temperature. Removal of the solvent and trituration of the resulting glass with sodium carbonate solution gave a white solid. This was collected, washed repeatedly with water and air dried. The solid was recrystallized from 50 ml. of ethyl acetate to yield 8.14 g. (93%) of crystalline material, m.p. 135.0-139.0° after drying in vacuo over calcium chloride. Lit. m.p. 136-137° (35).

Acetyl-L-tyrosinmethylamide (14).--The above compound (7.1 g., 0.03 mole) was converted to the methylamide as before. The crude solid was recrystallized from a mixture of 100 ml. of ethyl acetate and 25 ml. of methanol to yield 4.3 g. (61%) of well-formed bypyramids, m.p. 192.2-193.3°; $[\alpha]_D^{25}$ = +44.2° (C, 1% in water). Lands (14) gave m.p. 191-192°.

Anal. Calcd. for $C_{12}H_{16}O_3N_2$ (236.3): C,61.00; H,6.83; N,11.86
Found: C,61.04; H,6.89; N,11.95

L-Tryptophan Methyl Ester Hydrochloride.--L-Tryptophan was converted to the methyl ester hydrochloride in 90% crude yield by the method of Brenner and Huber (19).

Acetyl-L-tryptophan Methyl Ester (32).--The acetylation of 10 g. (0.039 mole) of the methyl ester hydrochloride with acetic anhydride in the presence of aqueous potassium carbonate solution was carried out as directed by Huang and Niemann (32). Recrystallization of the crude solid from

ethyl acetate gave a 90% yield (9.2 g.) of fine, white needles, m.p. 152.5-154.4°. Lit. m.p. 152.5° (32).

Acetyl-L-tryptophanmethylamide (13).--The acetyl ester was converted to the methylamide by the usual procedure. The resulting solid was repeatedly recrystallized from water to constant melting point and specific rotation: m.p. 187.1-189.0°; $[\alpha]_D^{25}$ = +22.9° (C, 1% in ethanol). Lit. m.p. 183-184°; $[\alpha]_D^{25}$ = +20° (C, 1% in methanol) (13).

Anal. Calcd. for $C_{14}H_{17}O_2N_3$ (259.3): C,64.84; H,6.61; N,16.21

Found: C,64.95; H,6.69; N,16.14

A recheck of the melting point of the latter preparation (13) gave m.p. 187.0-188.5°. A mixed melting point of the two preparations was 187.5-189.0°.

Acetyl-L-proline (34).--Ten grams (0.087 mole) of L-proline was acetylated with acetic anhydride in the presence of 2 N sodium hydroxide as directed by du Vigneaud and Meyer (34). The crude product was recrystallized from water to yield 7.4 g. (54%) of acetyl-L-proline, m.p. 114-116°, after drying to constant weight in vacuo over phosphorous pentoxide. Lit. m.p. 116-117° (34).

Acetyl-L-proline Methyl Ester.--The crude compound was obtained in 78% yield as an oil after treatment with thionyl chloride-methanol in the manner described in Part II for the preparation of acetyl-L-methionine methyl ester. The impure product was employed below.

Acetyl-L-prolinmethylamide.--This compound was prepared in the usual manner by treatment of the acetyl ester with a methanolic solution of methylamine. The crude solid was recrystallized three times from toluene-hexane and three times from ethyl acetate to yield large, colorless bipyramids, m.p. 104.2-105.5°; $[\alpha]_D^{25} = -87.5°$ (C, 1% in ethanol).

Anal. Calcd. for $C_8H_{14}O_2N_2$ (170.2): C,56.45; H,8.29; N,16.46
Found: C,56.31; H,8.27; N,16.43

Acetyl-L-hydroxyproline (36).--L-Hydroxyproline (10 g., 0.076 mole) was stirred two hours at room temperature in 150 ml. of glacial acetic acid containing 10.5 ml. (ca. 0.11 mole) of 97% acetic anhydride as directed by Kolb and Toennies (36). A trace of undissolved solid was removed by filtration and the solution evaporated in vacuo at 100°. The residual oil was triturated with 75 ml. of dry ether to yield a white solid. The solid was collected, resuspended in 75 ml. of ether, filtered, washed with three 25-ml. portions of ether to remove residual acetic acid and dried in vacuo over calcium chloride and sodium hydroxide. Further purification was not attempted.

Acetyl-L-hydroxyproline Methyl Ester (37).--This compound was obtained as an oil by the addition of excess ethereal diazomethane solution to 3.5 g. (0.02 mole) of the crude acetyl-L-hydroxyproline suspended in 75 ml. of ice-cooled, anhydrous dioxane and removal of the solvents in vacuo. The procedure

is that of Neuberger (37) who obtained a crystalline compound, m.p. 78°, after a careful recrystallization from ethanol-ether solution.

Acetyl-L-hydroxyprolinmethylamide.--The above oil was treated with excess methylamine in methanol as before to yield 4 g. of a white, oily solid (crude yield ca. 100% based on acetyl-L-hydroxyproline). This solid was recrystallized from a solvent composed of 9 ml. of ethanol and 18 ml. of ethyl acetate to yield clusters of hexagonal rods. The air-dried material appeared to be solvated as it began to sinter at ca. 130°. After drying in vacuo at 68° the solid had m.p. 167.0-169.8°. Another recrystallization and drying as before gave similar crystals, m.p. 167.0-168.5°; $[\alpha]_D^{25} = -60.9°$ (C, 1% in ethanol).

Anal. Calcd. for $C_8H_{14}O_3N_2$ (186.2): C,51.60; H,7.58; N,15.04

Found: C,51.69; H,7.57; N,15.10

Acetyl-L-asparagine (38).--This compound was obtained in 34% crude yield, m.p. 160-165°, by the method given by Karrer and Schlosser (38) who found m.p. 165°.

Acetyl-L-asparagine Methyl Ester I (38).--Treatment of 1 g. of acetyl-L-asparagine with thionyl chloride-methanol in the manner described for the preparation of acetyl-L-methionine methyl ester (Part II) resulted in the separation of a white solid, m.p. $>$ 310°. The solid was collected and the filtrate evaporated to yield an oil (cf. 38).

Acetyl-L-aspartic acid N,N'-di-(methylamide)I.--The oil from the above preparation was treated with excess methylamine in methanol solution. Globular masses of microscopic needles m.p. 249-250° (dec.), separated from the reaction mixture. The solid was recrystallized from methanol and had m.p. 249.0-249.5° (dec.) after drying in vacuo over phosphorous pentoxide.

Anal. Calcd. for $C_7H_{13}O_3N_3$(187.2): C,44.91; H,7.00; N,22.45
Found: C,47.81; H,7.43; N,20.82
Calcd. for $C_8H_{15}O_3N_3$(201.2): C,47.75; H,7.51; N,20.88
(see below)

As the latter calculated analysis illustrates, the compound is the N,N'-di-(methylamide) of acetyl-L-aspartic acid.

A check of the esterification procedure was run by treating acetyl-DL-phenylalaninamide (33), m.p. 163-166° with thionyl chloride-methanol as above. Again, a white solid m.p. $>$ 320° that evolved ammonia on warming in sodium carbonate solution separated from the methanol solution. Evaporation of the supernatant liquid gave an oil that was crystallized from ether-hexane, m.p. 64.5-66° (uncorr.). Lit. m.p. 60-61° (39) for acetyl-DL-phenylalanine methyl ester. Apparently this mild treatment partially converts acylated amino acid amides to methyl esters and ammonium chloride.

Acetyl-L-asparagine Methyl Ester II (38).--Treatment of acetyl-L-asparagine with excess ethereal diazomethane

solution was carried out as directed by Karrer and Schlosser (38). The crude oil (cf. 38) was used directly in the next preparation.

Acetyl-L-aspartic acid N,N'-di-(methylamide).II.--In this preparation, as before, a white solid separated from the methanolic methylamine solution. This was collected, washed free of methylamine with methanol and dried in vacuo, m.p. 239.5-240.0° (dec.). Recrystallization from 98% alcohol-2% water gave a white solid, m.p. 240.5-241.0° (dec.)

Anal. Calcd. for $C_7H_{13}O_3N_3$ (187.2): C,44.91; H,7.00; N,22.45

Found: C,48.10; H,7.48; N,20.92

Calcd. for $C_8H_{15}O_3N_3$ (201.2) C,47.75; H,7.51; N,20.88

(see below)

Again, the N,N'-di-(methylamide) of acetyl-L-aspartic acid appeared to be the product. Further attempts to clarify this situation were not made. It should be mentioned, however, that a system similar to the latter sequence of reactions was employed by Karrer and Schlosser to prepare acetyl-L-asparaginamide (38).

Acetyl-L-histidine-monohydrate (40).--Anhydrous L-histidine m.p. 284-286° (dec.) prepared from the hydrochloride according to Pyman (41) who reported m.p. 287° was acetylated by the method of Bergmann and Zervas (40). Treatment of 5 g. (0.032 mole) of the free amino acid with 3.2 ml. (0.033 mole) of 97% acetic anhydride in 25 ml. of acetic acid under refluxing conditions for two hours, dilution with 50 ml. of

water and repeated distillations with 25 ml. portions of water in vacuo gave a glassy solid. This was dissolved in 25 ml. of hot water and 75 ml. of acetone was added. An oil separated that was induced to crystallize by cooling and agitation. The solid was collected, washed with 7:3 acetone-water and air dried. Yield 5.8 g. (84.5%), m.p. 160-164°. Lit. m.p. 169° (40). A small sample was recrystallized from aqueous-acetone, m.p. 164.5-167.0° (dec.); $[\alpha]_D^{25}$ = +41.9° (C, 4% in water). Lit. $[\alpha]_D^{20}$ = +44.7° (in water) (40).

Acetyl-L-histidine Methyl Ester.--This was obtained as an oil from 4.2 g. (0.02 mole) of acetyl-L-histidine-monohydrate by the procedure outlined in Part II for the esterification of acetyl-L-methionine. In this case additional thionyl chloride was employed to react with the water of hydration. The crude product was used below.

Acetyl-L-histidinmethylamide.--After allowing the acetylated ester to stand overnight in methanol containing excess methylamine, considerable white solid had separated. This was collected, washed repeatedly with portions of methanol and air dried. Yield, 2.48 g. (60% for two steps from acetyl-L-histidine-monohydrate). This solid was twice recrystallized with considerable loss from methanol to yield 0.9 g. of fine, white needles, m.p. 243.0-244.5°(dec.); $[\alpha]_D^{25}$ = +4.5° (C, 1% in water).

Anal. Calcd. for $C_9H_{14}O_2N_4$ (210.2): C,51.42; H,6.71; N,26.65
Found: C,51.54; H,6.70; N,26.52

Acetyl-L-arginine dihydrate (40,42).--This compound was prepared from L-arginine hydrochloride in 66% yield by treatment with acetic anhydride in aqueous potassium carbonate as described by Thomas (42). The compound appeared to dehydrate at ca. 120° and melted at 263-265° with decomposition. Lit. m.p. 270° (dec.) (40).

Acetyl-L-arginine Methyl Ester Hydrochloride (42).--This compound prepared from anhydrous acetyl-L-arginine by the thionyl chloride-methanol method was obtained as an oil which was very hygroscopic and resisted crystallization (cf. 42).

Acetyl-L-argininmethylamide Hydrochloride.--Treatment of the above oil with a methanol solution of methylamine in the usual manner gave an oil that slowly changed to a glass-like solid. Repeated attempts at recrystallization from various solvents gave negative results. Treatment of a 1 g. sample of the amorphous solid in 10 ml. of water with 1.1 g. of picric acid in 85 ml. of warm water gave 1.4 g. (damp weight) of fine, yellow needles on cooling. A small sample recrystallized from hot water had m.p. 179.0-181.5°. Decomposition of the picrate with barium hydroxide solution, filtration to remove barium picrate, acidification to pH 7 with 6 N sulfuric acid, filtration to remove barium sulfate

and removal of the solvent *in vacuo* gave an oil. Further attempts to purify this compound were not made.

α-N-Acetyl-ε-N-carbobenzoxy-L-lysine Methyl Ester.--ε-N-Carbobenzoxy-L-lysine methyl ester hydrochloride (2.3 g., 0.0068 mole) prepared as directed by Bergmann and coworkers (43) was rapidly stirred with 2 ml. (*ca.* 0.02 mole) of acetic anhydride in a mixture of 25 ml. of ethyl acetate and 10 ml. of 4 M potassium carbonate solution. After four hours the phases were separated. The organic phase was extracted with 7 ml. of 1 N hydrochloric acid, then with 5 ml. of water and was dried over magnesium sulfate. Evaporation of the solvent *in vacuo* at steam temperature gave an oil, 2.2 g. (96%), that resisted crystallization.

α-N-Acetyl-ε-N-carbobenzoxy-L-lysinmethylamide.--The crude product from above was treated with excess methylamine in methanol solution. After two days at room temperature, removal of the solvent at reduced pressure gave a white solid. This solid could be recrystallized (separating as a flocculent mass) from a large volume of ethyl acetate or from *ca.* two volumes of water. Anticipating that the same difficulties as those experienced with the arginine derivative would apply in this case, further investigations on this compound were abandoned.

Derivatives of Cysteine and Cystine.--The extent of the work on cysteine was described in Part II. Acetyl-S-benzyl-L-cysteine methyl ester was not converted to the methylamide.

In the case of cystine, preparation of N,N'-diacetyl-L-cystine di-(methyl ester) by the method of Pirie (44) gave after considerable difficulty a 47% yield of crystalline solid, m.p. 128-130°, after recrystallization from ethyl acetate-hexane solution. Lit. m.p. 128-129° (44). Conversion of this compound to the di-(methylamide) in the usual manner gave an oily solid. Various attempts at purification resulted in apparent decomposition (evolution of H_2S) and intractable mixtures.

Acetyl-L-methioninmethylamide.--Five grams (0.024 mole) of acetyl-L-methionine methyl ester (Part II) was treated with 28.5 g. (0.92 mole) of methylamine in 100 ml. of absolute methanol. After two days at room temperature the solvent was removed in vacuo at steam temperature. The resulting crude solid was recrystallized (cottony mass) twice from ethyl acetate to constant melting point and specific rotation. Yield 3.0 g. (60%); m.p. 180.9-181.5° (sublimes $>$ 160°); $[\alpha]_D^{25}$ = -11.4° (C, 1% in ethanol).

Anal. Calcd. for $C_8H_{16}O_2N_2S$ (204.3): C,47.03; H,7.89; N,13.71

Found: C,46.83; H,7.95; N,13.46

Acetyl-D-phenylalaninmethylamide.--Acetyl-D-phenylalanine methyl ester (2.2 g., 0.01 mole), prepared by H. T.

Huang (12), was treated with methanolic methylamine solution in the usual manner. The resulting solid was recrystallized twice from water to constant melting point and specific rotation. The long, white needles had m.p. 205.5-207.0°; $[\alpha]_D^{25}$ = -21.0°(C, 1% in ethanol).

Anal. Calcd. for $C_{12}H_{16}O_2N_2$ (220.3): C,65.43; H,7.32; N,12.72
Found: C,65.44; H,7.47; N,12.67

Acetyl-D-tyrosinmethylamide.--Acetyl-D-tyrosine ethyl ester (45) (1 g., 0.004 mole) was treated with a six-fold excess of methylamine in methanol solution. Removal of the solvent *in vacuo* after two days at room temperature gave a white solid. Recrystallization from 3 ml. of methanol plus 10 ml. of ethyl acetate gave 0.45 g. (47.5%) of fine white needles, m.p. 185.5-186.0°, which partially resolidified to prisms, m.p. 192.0-192.5° (cf. L-compound page 155). A further recrystallization as above did not alter the melting point or the specific rotation. $[\alpha]_D^{25}$ = -42.4°(C, 1% in water).

Anal. Calcd. for $C_{12}H_{16}O_3N_2$ (236.3): C,61.00; H,6.83; N,11.86
Found: C,60.98; H,6.88; N,11.79

Acetyl-D-tryptophanmethylamide.--The sample was prepared by H. T. Huang. The following physical constants were reported (13): m.p. 185-186°; $[\alpha]_D^{25}$ = -20 ± 1° (C, 1% in methanol).

Anal. Calcd. for $C_{14}H_{17}O_2N_3$ (259): C,64.9; H,6.6; N,16.2
Found: C,65.0; H,6.7; N,16.1.

The Acetylation of DL-Tyrosine Ethyl Ester with Cyanomethyl Acetate.--(Hood!). Cyanomethyl acetate (1.98 g., 0.02 mole) b.p. 76.5-80.5°/20 mm., N_D^{25} = 1.4053, prepared from chloroacetonitrile and anhydrous potassium acetate as directed by Henry (24) who reported N_D^{20} = 1.4107, was heated under refluxing conditions for two hours with 5 g. (0.024 mole) of DL-tyrosine ethyl ester, m.p. 106.5-108°, in 30 ml. of reagent ethyl acetate. When cooled, much solid that was soluble in dilute hydrochloric acid separated from the solution. An additional 0.5 g. of cyanomethyl acetate was introduced plus 1 drop of glacial acetic acid (cf. 23); the mixture was again heated for one hour at the boiling point, and then left at room temperature. The solution remained clear. After six days, agitation caused the separation of much white solid. This was collected, washed with ethyl acetate, then with 1 N hydrochloric acid and with water and air dried. Yield 3.3 g. (54%), m.p. 131.5-132.3°. Niemann and McCasland (46) report m.p. 133-134° for acetyl-DL-tyrosine ethyl ester.

Table I.--Properties of the N-Acetyl Amino Acid N'-Methylamides.

Amino Acid	m.p.[a]	$[\alpha]_D^{25}$
Glycine	157.5-158.0	--
L-Alanine	181.2-182.0	-51.1[b]
L-Leucine	165.3-166.8	-33.9[c]
L-Phenylalanine	207.1-208.5	+19.4[d]
D-Phenylalanine	205.5-207.0	-21.0[d]
L-Tyrosine	192.2-193.3	+44.2[c]
D-Tyrosine	184.5-186.0 (192.0-192.5)	-42.4[c]
L-Tryptophan	187.1-189.0	+22.9[d]
D-Tryptophan	185.0-186.0	-20[e] (13)
L-Proline	104.5-105.0	-87.5[d]
L-Hydroxyproline	167.0-168.5	-60.9[d]
L-Histidine	243.0-244.5 dec.	+ 4.5[c]
L-Methionine	180.9-181.5	-11.4[d]

a. Corrected, °C. b. (C, 2% in ethanol). c. (C, 1% in water). d. (C, 1% in ethanol). e. (C, 0.5% in methanol).

Table II.--Inhibition of the alpha-Chymotrypsin Catalyzed Hydrolysis of Methyl Hippurate by Acetyl-L-tryptophanmethylamide at pH 7.9 and 25°.
$[E]^a$ = 0.150 mg. protein nitrogen/ml.; [NaOH] = 0.1609 N; [NaCl] = 0.02 M; [I] = 5.0×10^{-3} M.

Run No.[b]	1-5-3	2-5-3	1-5-11		3-5-3	4-5-3	2-5-11	5-5-3	6-5-3	3-5-11	7-5-3	8-5-3	4-5-11
$[S]_o$[c]	8	8	8		12	12	12	16	16	16	20	20	20
	S.R.[d]	S.R.	S.R.		S.R.	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.
t[e]				t[e]									
0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	30	28	30	3	48	50	50	55	55	58	60	60	61
4	56	54	55	6	95	95	98	107	107	110	116	118	121
6	79	79	79	9	137	138	140	156	155	160	170	173	174
8	103	102	100	12	177	178	182	204	201	208	222	225	226
10	125	124	122	15	215	216	220	248	245	253	272	275	277
12	146	146	143	18	250	252	255	291	288	297	320	323	326
14	166	166	162	21	283	285	290	332	328	338	366	370	374
16	186	185	181	24	315	317	323	372	367	379	411	415	420
H[f]	37	37	36		42	42	43	37	37	39	33	33	34
v_o[g]	2.41	2.30	2.60		2.71	2.72	2.82	3.03	3.03	3.14	3.26	3.34	3.38
σv_o[gh]	0.06	0.03	0.09		0.03	0.02	0.04	0.02	0.03	0.03	0.02	0.01	0.03

a. Armour lot #90492, 15.0% protein nitrogen. b. Run number -month-day (1956). c. In units of 10^{-3} M. d. S.R. = scale reading. Corrected to S.R. = 0 at t_o. In units of 10^{-3} ml. Estimated to $\pm$ 0.5 x 10^{-3} ml. and rounded off. e. In units of minutes. f. Approximate percent hydrolysis. g. Calculated by orthogonal polynomial method (25). In units of 10^{-4} M/min. h. $\pm$.

Table III.--Summary of the Initial Velocities and Kinetic Constants Derived from the Data of Table II.

	$[S]_o$ [a]	P_m [b]	v_o [c]	σv_o [d]	$[S]_o[E]/v_o$ [e]
	8	3	2.41	0.06	4.98
	8	3	2.30	0.03	5.22
*	8	4	2.60	0.09	4.62
	12	3	2.71	0.03	6.64
	12	3	2.72	0.02	6.62
	12	3	2.82	0.04	6.39
	16	3	3.03	0.02	7.92
	16	3	3.03	0.03	7.92
	16	3	3.14	0.03	7.65
	20	3	3.26	0.02	9.21
	20	3	3.34	0.01	8.98
	20	3	3.38	0.03	8.88

$$k_3 = 3.09 \pm 0.22 \times 10^{-3}$$ [f,g]

$$K_S' = 8.0 \pm 1.3 \times 10^{-3}\ \underline{M}$$ [f]

$$K_I = 24 \times 10^{-3}\ \underline{M}$$ [h]

a. In units of 10^{-3} $\underline{M}$. b. Order of polynomial employed for calculation of v_o (25). c. In units of 10^{-4} $\underline{M}$/min. d. $\pm$, in units of 10^{-4} $\underline{M}$/min. e. In units of min. mg. protein nitrogen/ml. f. Evaluated by the method of least squares (cf. fig. 1). g. In units of $\underline{M}$/min. mg. protein nitrogen/ml. h. Based on the value $K_S = 6.6 \times 10^{-3}$ $\underline{M}$. Order of magnitude only.

* Not included in least squares analysis.

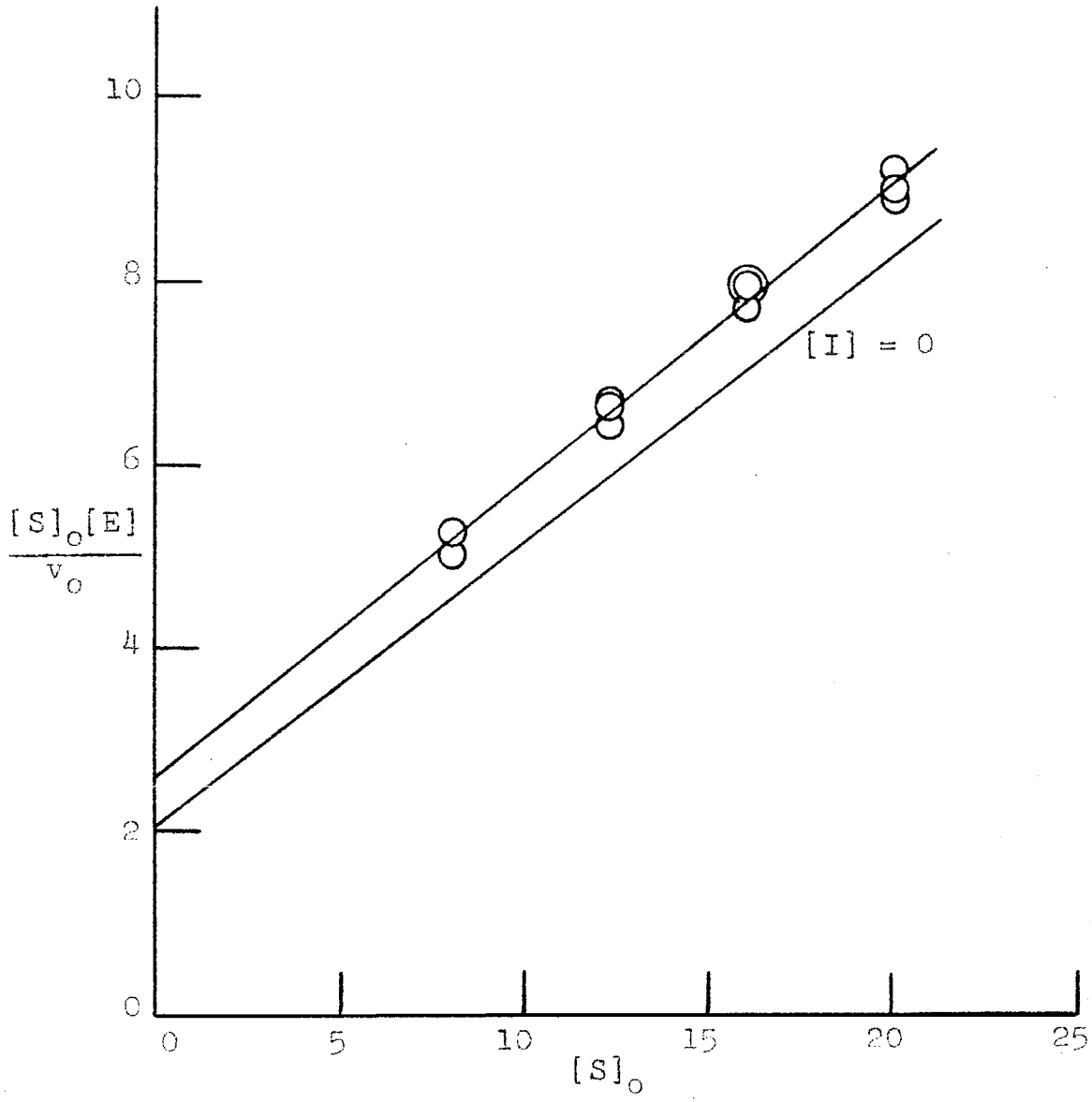

Figure 1.--$[S]_o[E]/v_o$ versus $[S]_o$ plot for the inhibition of the alpha-chymotrypsin catalyzed hydrolysis of methyl hippurate by 5.0 x 10^{-3} M acetyl-L-tryptophanmethylamide at pH 7.9 and 25° in 0.02 M sodium chloride solution. Line determined by least squares. $[S]_o[E]/v_o$ in units of min. mμ. protein nitrogen/ml. $[S]_o$ in units of 10^{-3} M. (Cf. Table III).

Table IV.--Inhibition of the alpha-Chymotrypsin Catalyzed Hydrolysis of Methyl Hippurate by Acetyl-D-tryptophanmethylamide at pH 7.9 and 25°. $[E]^a$ = 0.150 mg. protein nitrogen/ml.; [NaOH]= 0.1609 N; [NaCl] = 0.02 M; [I] = 5.0×10^{-3} M.

Run No.[b]	1-5-15	8-5-15	2-5-15	7-5-15	3-5-15	6-5-15	4-5-15	5-5-15
$[S]_o$[c]	8	8	12	12	16	16	20	20
	S.R.[d]	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.	S.R.
t[e]								
0	0	0	0	0	0	0	0	0
3	25	25	32	32	38	39	44	44
6	47	47	62	62	76	75	85	85
9	69	69	90	91	112	111	124	125
12	88	89	118	118	145	144	161	163
15	106	108	144	145	178	176	198	200
18	124	126	169	170	209	207	234	235
21	141	144	193	194	240	238	269	270
24	157	161	217	217	270	266	301	304
H[f]	32	32	29	29	27	27	24	24
v_o[g]	1.38	1.36	1.75	1.74	2.15	2.07	2.30	2.40
σv_o[gh]	0.02	0.02	0.02	0.02	0.03	0.01	0.02	0.02

a. Armour lot #90492, 15.0% protein nitrogen. b. Run number -month -day (1956). c. In units of 10^{-3} M. d. S.R. = scale reading. Corrected to S.R. = 0 at t_o. In units of 10^{-3} ml. Estimated to $\pm$ 0.5 x 10^{-3} ml. and rounded off. e. In units of minutes. f. Approximate percent hydrolysis. g. Calculated by orthogonal polynomial method (25). In units of 10^{-4} M/min. h. $\pm$.

Table V.--Summary of the Initial Velocities and Kinetic Constants Derived from the Data of Table IV.

$[S]_o$ [a]	P_m [b]	v_o [c]	σv_o [d]	$[S]_o[E]/v_o$ [e]
8	3	1.38	0.02	8.70
8	3	1.36	0.02	8.82
12	3	1.75	0.02	10.29
12	3	1.74	0.02	10.34
16	3	2.15	0.03	11.11
16	2	2.07	0.01	11.59
20	2	2.30	0.02	13.04
20	3	2.40	0.02	12.50

$k_3 = 3.06 \pm 0.18 \times 10^{-3}$ [f,g]

$K_S' = 19.1 \pm 1.4 \times 10^{-3}$ $\underline{M}$ [f]

$K_I = 2.6 \pm 0.4 \times 10^{-3}$ $\underline{M}$ [h]

a. In units of 10^{-3} $\underline{M}$. b. Order of polynomial employed for calculation of v_o (25). c. In units of 10^{-4} $\underline{M}$/min. d. $\pm$, in units of 10^{-4} $\underline{M}$/min. e. In units of min. mg. protein nitrogen/ml. f. Evaluated by the method of least squares (cf. fig. 2). g. In units of $\underline{M}$/min. mg. protein nitrogen/ml. h. Based on the value $K_S = 6.6 \pm 0.45 \times 10^{-3}$ $\underline{M}$.

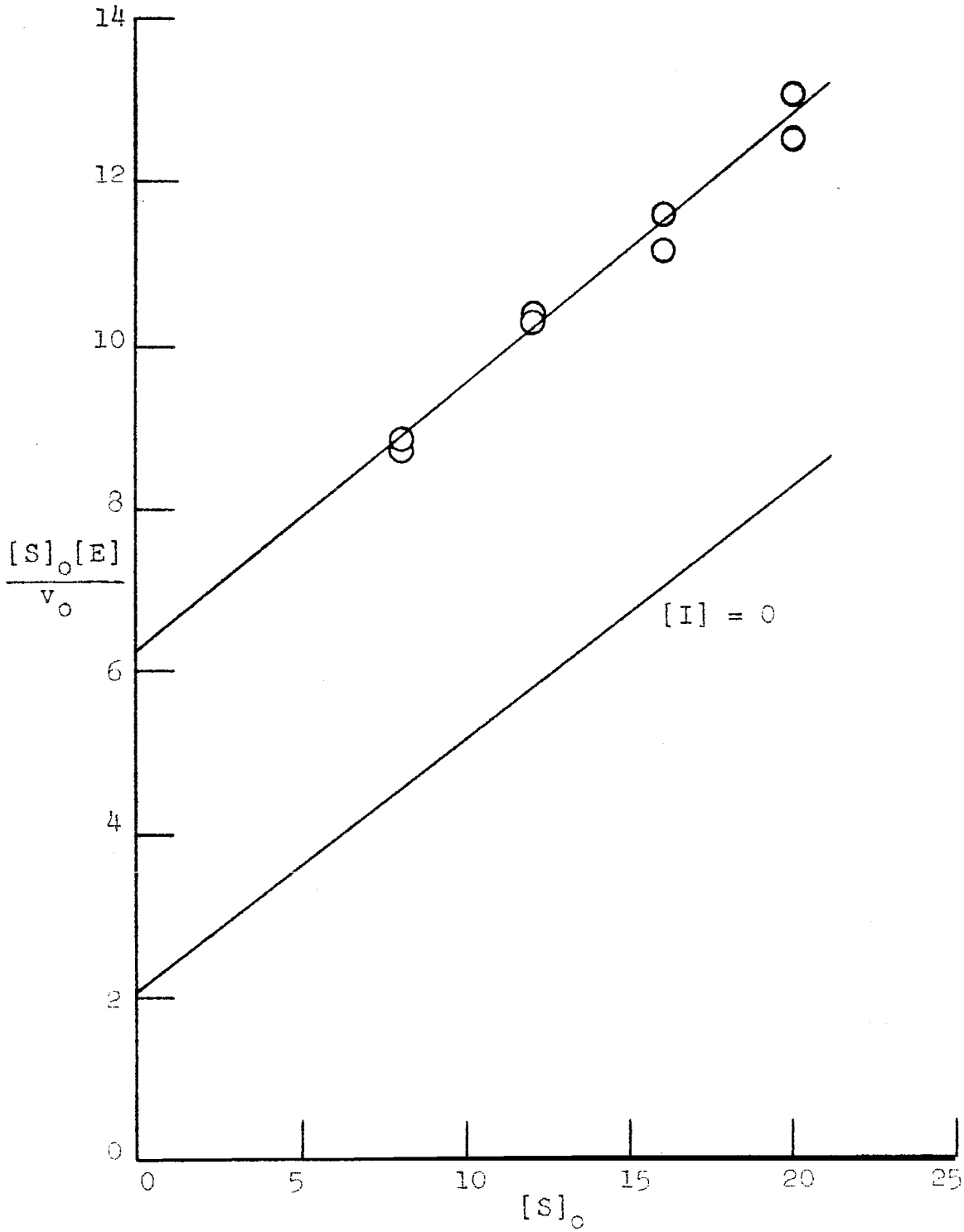

Figure 2.--$[S]_o[E]/v_o$ versus $[S]_o$ plot for the inhibition of the alpha-chymotrypsin catalyzed hydrolysis of methyl hippurate by 5.0×10^{-3} M acetyl-D-tryptophanmethylamide at pH 7.9 and 25° in 0.02 M sodium chloride solution. Line determined by least squares. $[S]_o[E]/v_o$ in units of min. mg. protein nitrogen/ml. $[S]_o$ in units of 10^{-3} M. (Cf. Table V).

Table VI.--Inhibition of the alpha-Chymotrypsin Catalyzed Hydrolysis of Methyl Hippurate by Acetyl-L-phenylalaninmethylamide at pH 7.9 and 25°.
$[E]^a$ = 0.150 mg. protein nitrogen/ml.; [NaOH] = 0.1609 N; [NaCl] = 0.02 M; [I] = 6.0×10^{-3} M.

Run No.[b]	5-8-27	6-8-27	7-8-27	8-8-27
$[S]_o$[c]	7.97	12.01	15.98	20.01
	S.R.[d]	S.R.	S.R.	S.R.
t[e]				
0	0	0	0	0
2	28	32	35	39
4	52	62	69	75
6	76	92	102	112
8	98	119	135	146
10	119	146	165	180
12	140	172	195	212
14	159	196	225	244
16	178	220	253	276
H[f]	36	30	25	22
v_o[g]	2.24	2.58	2.86	3.18
δv_o[gh]	0.04	0.02	0.02	0.04

a. Armour lot #90492, 15.0% protein nitrogen. b. Run number-month-day (1956). c. In units of 10^{-3} M. d. S.R. = scale reading. Corrected to S.R. = 0 at t_o. In units of 10^{-3} ml. Estimated to $\pm$ 0.5 x 10^{-3} ml. and rounded off. e. In units of minutes. f. Approximate percent hydrolysis. g. Calculated by orthogonal polynomial method (25). In units of 10^{-4} M/min. h. $\pm$.

Table VII.--Summary of Initial Velocities and Kinetic Constants Derived from the Data of Table VI.

$[S]_o$ [a]	P_m [b]	v_o [c]	dv_o [d]	$[S]_o[E]/v_o$ [e]
7.97	3	2.24	0.04	5.34
12.01	2	2.58	0.02	6.98
15.98	2	2.86	0.02	8.37
20.01	3	3.18	0.04	9.44

$$k_3 = 2.95 \pm 0.30 \times 10^{-3}$$ [f,g]

$$K_S' = 8.2 \pm 1.7 \times 10^{-3}\ \underline{M}$$ [f]

$$K_I = 25 \times 10^{-3}\ \underline{M}^h$$

a. In units of 10^{-3} $\underline{M}$. b. Order of polynomial employed for calculation of v_o (25). c. In units of 10^{-4} $\underline{M}$/min. d. $\pm$, in units of 10^{-4} $\underline{M}$/min. e. In units of min. mg. protein nitrogen/ml. f. Evaluated by the method of least squares (cf. fig. 3). g. In units of $\underline{M}$/min. mg. protein nitrogen/ml. h. Based on the value $K_S = 6.6 \times 10^{-3}$ $\underline{M}$. Order of magnitude only.

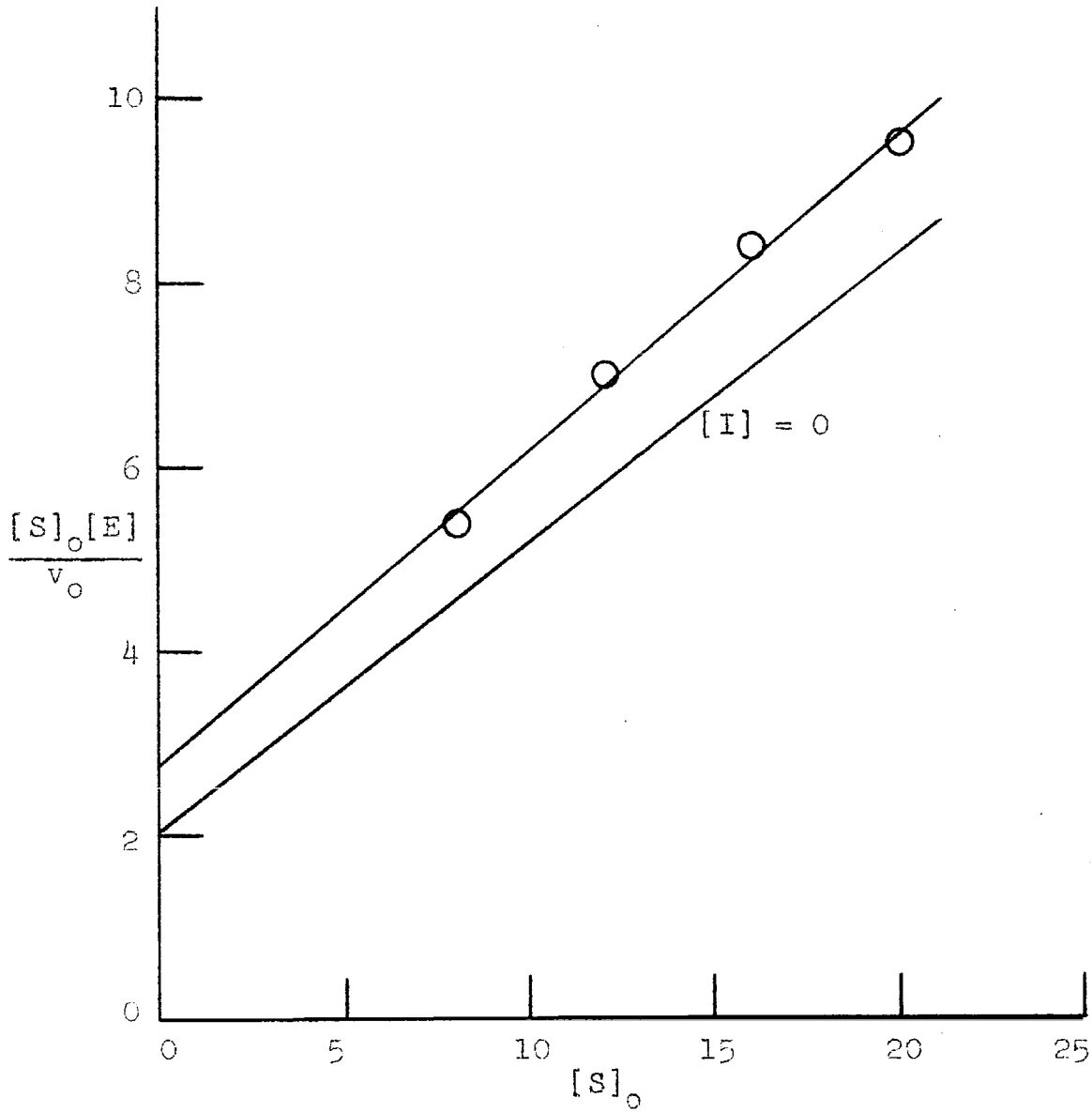

Figure 3.--$[S]_o[E]/v_o$ versus $[S]_o$ plot for the inhibition of the alpha-chymotrypsin catalyzed hydrolysis of methyl hippurate by 6.0×10^{-3} M acetyl-L-phenylalanin-methylamide at pH 7.9 and 25° in 0.02 M sodium chloride solution. Line determined by least squares. $[S]_o[E]/v_o$ in units of min. mg. protein nitrogen/ml. $[S]_o$ in units of 10^{-3} M. (cf. Table VII).

Table VIII.--Inhibition of the alpha-Chymotrypsin Catalyzed Hydrolysis of Methyl Hippurate by Acetyl-D-phenylalaninmethylamide at pH 7.9 and 25°.
[I] = 4.0×10^{-3} M; [NaOH] = 0.1380 N; [NaCl] = 0.02 M.

Run No.[b]	4-9-4	5-9-4	5-8-28	6-8-28	7-8-28	8-8-28
[E][a]	0.150	0.150	0.153	0.153	0.153	0.153
$[S]_o$[c]	8.22 S.R.*	19.96 S.R.*	8.15 S.R.[d]	11.93 S.R.[d]	15.98 S.R.[d]	20.08 S.R.[d]
t[e]						
0	0	3	0	0	0	0
2	307	453	30	36	42	46
4	595	890	58	70	82	90
6	868	1310	84	104	120	132
8	1125	1720	109	135	155	173
10	1375	2120	133	166	192	213
12	1612	2505	156	196	227	251
14	1840	2885	178	224	261	290
16	2060	3260	199	250	294	328
H[f]	35	22	34	29	25	33
v_o[g]	2.11	3.17	2.16	2.52	2.94	3.25
$\pm v_o$[gh]	0.01	0.02	0.02	0.01	0.06	0.02

a. Armour lot #90492, 15.0% protein nitrogen. b. Run number-month-day (1956). c. In units of 10^{-3} M. d. S.R. = scale reading. Corrected to S.R. = 0 at t_o. In units of 10^{-3} ml. Estimated to $\pm$ 0.5 x 10^{-3} ml. and rounded off. e. In units of minutes. f. Approximate percent hydrolysis. g. Calculated by orthogonal polynomial method (25). In units of 10^{-4} M/min. h. $\pm$.

* S.R. = scale reading. In units of 10^{-4} ml. Estimated error $\pm$ 2 x 10^{-4} ml.

Table IX.--Summary of the Initial Velocities and Kinetic Constants Derived from the Data of Table VIII.

$[S]_o$ [a]	P_m [b]	v_o [c]	σv_o [d]	$[S]_o[E]/v_o$ [e]
8.22	3	2.11	0.01	5.84
8.15	4	2.16	0.02	5.77
11.93	2	2.52	0.01	7.24
15.98	3	2.94	0.06	8.32
19.96	3	3.17	0.02	9.44
20.08	3	3.25	0.02	9.45

$k_3 = 3.28 \pm 0.11 \times 10^{-3}$ f,g

$K_S' = 11.11 \pm 0.62 \times 10^{-3}$ M[f]

$K_I = 5.9 \pm 1.3 \times 10^{-3}$ M[h]

a. In units of 10^{-3} M. b. Order of polynomial employed for calculation of v_o (25). c. In units of 10^{-4} M/min. d. $\pm$, in units of 10^{-4} M/min. e. In units of min. mg. protein nitrogen/ml. f. Evaluated by the method of least squares (cf. fig. 4). g. In units of M/min. mg. protein nitrogen/ml. h. Based on the value $K_S = 6.6 \pm 0.45 \times 10^{-3}$ M.

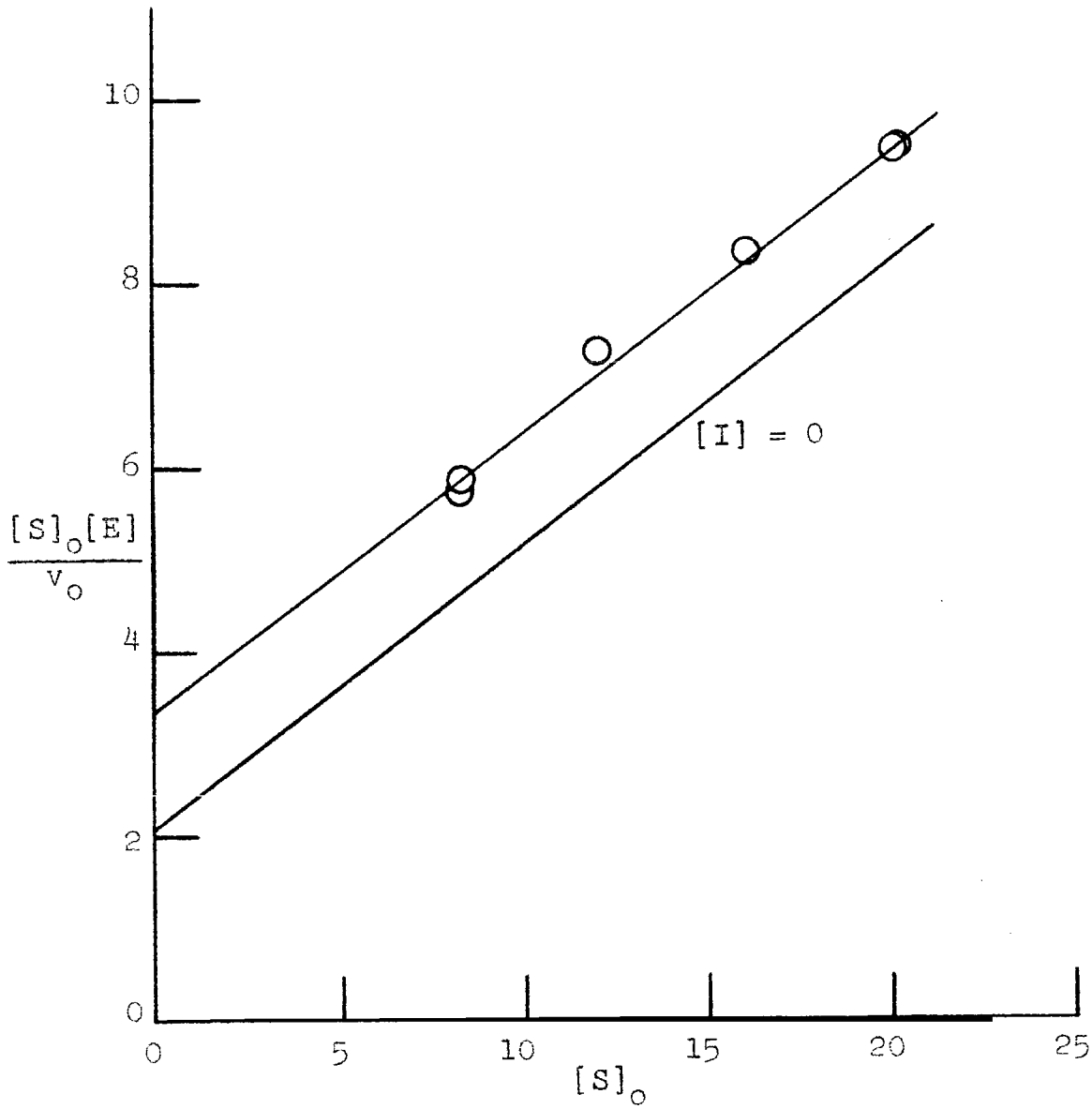

Figure 4.--$[S]_o[E]/v_o$ versus $[S]_o$ plot for the inhibition of the alpha-chymotrypsin catalyzed hydrolysis of methyl hippurate by 4.0×10^{-3} M acetyl-D-phenylalaninmethylamide at pH 7.9 and 25° in 0.02 M sodium chloride solution. Line determined by least squares. $[S]_o[E]/v_o$ in units of min. mg. protein nitrogen/ml. $[S]_o$ in units of 10^{-3} M. (cf. Table IX).

Table X.--Inhibition of the alpha-Chymotrypsin Catalyzed Hydrolysis of Methyl Hippurate by Acetyl-L-tyrosinmethylamide at pH 7.9 and 25°.
$[E]^{a}$ = 0.150 mg. protein nitrogen/ml.; [NaOH] = 0.1609 N; [NaCl] = 0.02 M; [I] = 20 x 10^{-3} M.

Run No.[b]	1-8-16	1-8-21		2-8-16	2-8-21	3-8-16	3-8-21	4-8-16	4-8-21
$[S]_o$[c]	7.99	8.10		11.93	11.91	15.92	15.88	20.03	20.12
	S.R.[d]	S.R.		S.R.	S.R.	S.R.	S.R.	S.R.	S.R.
t[e]			t[e]						
0	0	0	0	0	0	0	0	0	0
2	26	27	3	45	46	54	51	60	57
4	50	50	6	87	89	104	100	112	110
6	73	73	9	126	129	150	145	164	160
8	94	94	12	163	167	195	188	214	210
10	114	115	15	198	202	239	230	260	256
12	133	135	18	231	236	280	270	305	302
14	152	154	21	263	269	320	309	350	347
16	170	172	24	293	300	358	346	392	390
H[f]	34	34		40	40	36	35	32	31
v_o[g]	2.18	2.26		2.49	2.56	3.03	2.82	3.20	3.10
$\pm v_o$[gh]	0.02	0.02		0.02	0.01	0.07	0.02	0.06	0.03

a. Armour lot #90492, 15.0% protein nitrogen. b. Run number -month-day (1956). c. In units of 10^{-3} M. d. S.R. = scale reading. Corrected to S.R. = 0 at t_o. In units of 10^{-3} ml. Estimated to $\pm$ 0.5 x 10^{-3} ml. and rounded off. e. In units of minutes. f. Approximate percent hydrolysis. g. Calculated by orthogonal polynomial method (25). In units of 10^{-4} M/min. h. $\pm$.

Table XI.--Summary of the Initial Velocities and Kinetic Constants Derived from the Data of Table X.

$[S]_o$ [a]	P_m [b]	v_o [c]	dv_o [d]	$[S]_o[E]/v_o$ [e]
7.99	3	2.18	0.02	5.50
8.10	4	2.26	0.02	5.38
11.93	3	2.49	0.02	7.19
11.91	3	2.56	0.01	6.98
15.92	4	3.03	0.07	7.88
15.88	3	2.82	0.02	8.45
20.03	3	3.20	0.06	9.39
20.12	3	3.10	0.03	9.74

$k_3 = 2.98 \pm 0.18 \times 10^{-3}$ [f,g]

$K_S' = 8.6 \pm 1.0 \times 10^{-3}$ M [f]

$K_I = 66 \pm 38 \times 10^{-3}$ M [h]

a. In units of 10^{-3} M. b. Order of polynomial employed for calculation of v_o (25). c. In units of 10^{-4} M/min. d. $\pm$, in units of 10^{-4} M/min. e. In units of min. mg. protein nitrogen/ml. f. Evaluated by the method of least squares (cf. fig. 5). g. In units of M/min. mg. protein nitrogen/ml. h. Based on the value $K_S = 6.6 \pm 0.45 \times 10^{-3}$ M. Order of magnitude only.

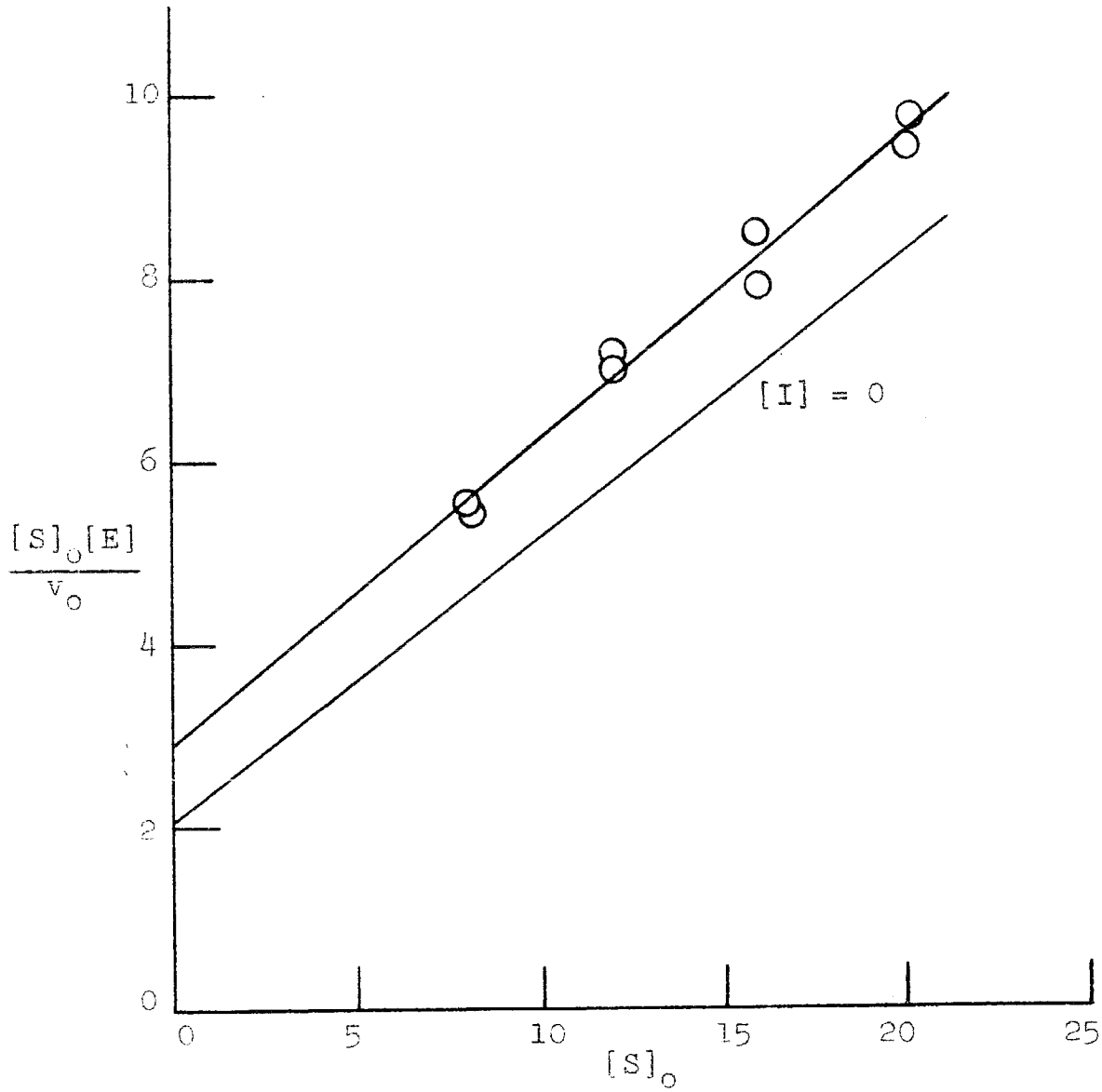

Figure 5.--$[S]_o[E]/v_o$ versus $[S]_o$ plot for the inhibition of the alpha-chymotrypsin catalyzed hydrolysis of methyl hippurate by 20 x 10^{-3} M acetyl-L-tyrosinmethylamide at pH 7.9 and 25° in 0.02 M sodium chloride solution. Line determined by least squares. $[S]_o[E]/v_o$ in units of min. mg. protein nitrogen/ml. $[S]_o$ in units of 10^{-3} M. (Cf. Table XI).

Table XII.--Inhibition of the alpha-Chymotrypsin Catalyzed Hydrolysis of Methyl Hippurate by Acetyl-D-tyrosinmethylamide at pH 7.9 and 25°.

$[E]^a$ = 0.150 mg. protein nitrogen/ml.; [NaOH] = 0.1380 N; [NaCl] = 0.02 M; [I] = 5.0×10^{-3} M.

Run No.[b]	5-8-29	6-8-29	7-8-29	8-8-29
$[S]_o$[c]	7.87	11.81	15.80	19.96
	S.R.[d]	S.R.	S.R.	S.R.
t[e]				
0	0	0	0	0
2	29	35	40	44.5
4	56	67	78	86
6	81	99	115	126.5
8	105	129	150	165
10	127.5	158	185	203
12	149.5	185	218	239.5
14	170	213	250	275.5
16	191	239	281	311
H[f]	34	28	25	22
v_o[g]	2.07	2.45	2.76	3.11
δv_o[gh]	0.02	0.03	0.01	0.02

a. Armour lot #90492, 15.0% protein nitrogen. b. Run number-month-day (1956). c. In units of 10^{-3} M. d. S.R. = scale reading. Corrected to S.R. = 0 at t_o. In units of 10^{-3} ml. Estimated to $\pm$ 0.5 x 10^{-3} ml. e. In units of minutes. f. Approximate percent hydrolysis. g. Calculated by orthogonal polynomial method (25). In units of 10^{-4} M/min. h. $\pm$.

Table XIII.--Summary of the Initial Velocities and Kinetic Constants Derived from the Data of Table XII.

$[S]_o$ [a]	P_m [b]	v_o [c]	v_o [d]	$[S]_o[E]/v_o$ [e]
7.87	3	2.07	0.02	5.70
11.81	3	2.45	0.03	7.23
15.80	2	2.76	0.01	8.59
19.96	3	3.11	0.02	9.63

$$k_3 = 3.07 \times 10^{-3}$$ [f,g]

$$K_S' = 10 \times 10^{-3}\ \underline{M}$$ [f]

$$K_I = 9.7 \times 10^{-3}\ \underline{M}$$ [h]

a. In units of 10^{-3} M. b. Order of polynomial employed for calculation of v_o (25). c. In units of 10^{-4} M/min. d. $\pm$, in units of 10^{-4} M/min. e. In units of min. mg. protein nitrogen/ml. f. Evaluated by the method of least squares (cf. fig. 6). g. In units of M/min. mg. protein nitrogen/ml. h. Based on the value $K_S = 6.6 \times 10^{-3}$ M. Order of magnitude only.

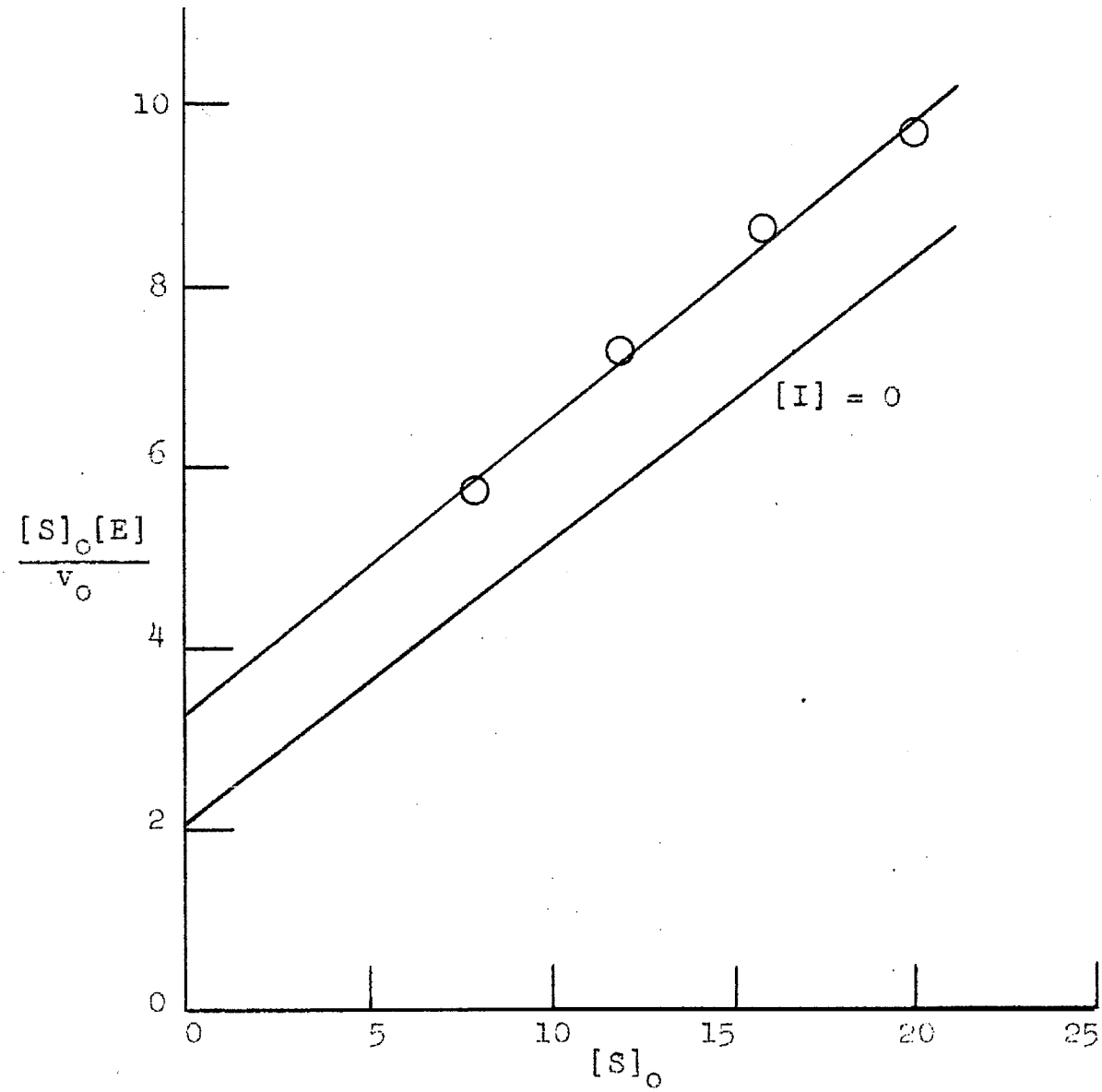

Figure 6.--$[S]_o[E]/v_o$ versus $[S]_o$ plot for the inhibition of the alpha-chymotrypsin catalyzed hydrolysis of methyl hippurate by 5.0×10^{-3} M acetyl-D-tyrosinmethylamide at pH 7.9 and 25° in 0.02 M sodium chloride solution. Line determined by least squares. $[S]_o[E]/v_o$ in units of min. mg. protein nitrogen/ml. $[S]_o$ in units of 10^{-3} M. (Cf. Table XIII).

Table XIV.--Inhibition of the alpha-Chymotrypsin Catalyzed Hydrolysis of Benzoyl-L-valine Methyl Ester by Acetyl-L-tryptophanmethylamide at pH 7.9 and 25°.
$[E]^a$ = 0.150 mg. protein nitrogen/ml.; [NaOH] = 0.01964 N; [NaCl] = 0.02 M; [I] = 5.0×10^{-3} M.

Run No.[b]	8-8-30	1-6-18	2-6-18	1-6-20	2-6-20	3-6-20	1-8-9	2-8-9	3-8-9	6-8-9	7-8-9
$[S]_o$[c]	0	2.56	2.03	2.64	2.02	1.00	2.97	1.99	1.49	0.99	3.08
	S.R.[d]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]
t[f]											
0	0	15	18	22	10	13	20	13	7	10	21
2	62	250	213	271	202	118	280	196	153	108	273
4	95	505	435	523	410	233	563	395	309	224	537
6	120	752	645	780	618	350	840	595	466	344	810
8	145	996	855	1022	825	462	1113	795	623	455	1077
10	165	1245	1068	1271	1027	573	1385	989	773	568	1339
12	187	1481	1269	1513	1223	674	1647	1180	922	675	1603
14	200	1735	1480	1758	1422	786	1912	1370	1070	780	1860
16	215	1970	1677	1998	1612	892	2170	1560	1217	882	2125
H[g]	--	15	16	15	16	18	14	15	16	18	14
v_o[h]	--	2.41	2.05	2.50	2.04	1.13	2.65	1.91	1.48	1.08	2.59
δv_o[i]	--	0.07	0.01	0.01	0.03	0.02	0.05	0.01	0.03	0.01	0.01

a. Armour lot #90492, 15.0% protein nitrogen. b. Run number -month-day (1956). c. In units of 10^{-3} M. d. S.R. = scale reading in units of 10^{-4} ml. Estimated error ± 2×10^{-4} ml. e. Corrected for blank given in first column. f. In units of minutes. g. Approximate percent hydrolysis. h. Calculated by the orthogonal polynomial method (25). In units of 10^{-5} M/min. i. ±.

Table XV.--Summary of the Initial Velocities and Kinetic Constants Derived from the Data of Table XIV.

$[S]_o$ [a]	P_m [b]	v_o [c]	σv_o [d]	$[S]_o[E]/v_o$ [e]
2.56	1	2.41	0.07	15.9
2.03	1	2.05	0.01	14.8
2.64	2	2.50	0.01	15.8
2.02	2	2.04	0.03	14.8
1.00	2	1.13	0.02	13.3
2.97	3	2.65	0.05	16.8
1.99	1	1.91	0.01	15.6
1.49	3	1.48	0.03	15.1
0.99	1	1.08	0.01	13.8
3.08	1	2.59	0.01	17.9

$$k_3 = 0.60 \pm 0.08 \times 10^{-3}$$ f,g

$$K_S' = 7.2 \pm 1.1 \times 10^{-3}\ \underline{M}$$ f

$$K_I = 7.1 \pm 2.9 \times 10^{-3}\ \underline{M}$$ h

a. In units of 10^{-3} $\underline{M}$. b. Order of polynomial employed for calculation of v_o (25). c. In units of 10^{-5} $\underline{M}$/min. d. $\pm$, in units of 10^{-5} $\underline{M}$/min. e. In units of min. mg. protein nitrogen/ml. f. Evaluated by the method of least squares (cf. fig. 7). g. In units of $\underline{M}$/min. mg. protein nitrogen/ml. h. Based on the value $K_S = 4.2 \pm 0.4 \times 10^{-3}$ $\underline{M}$.

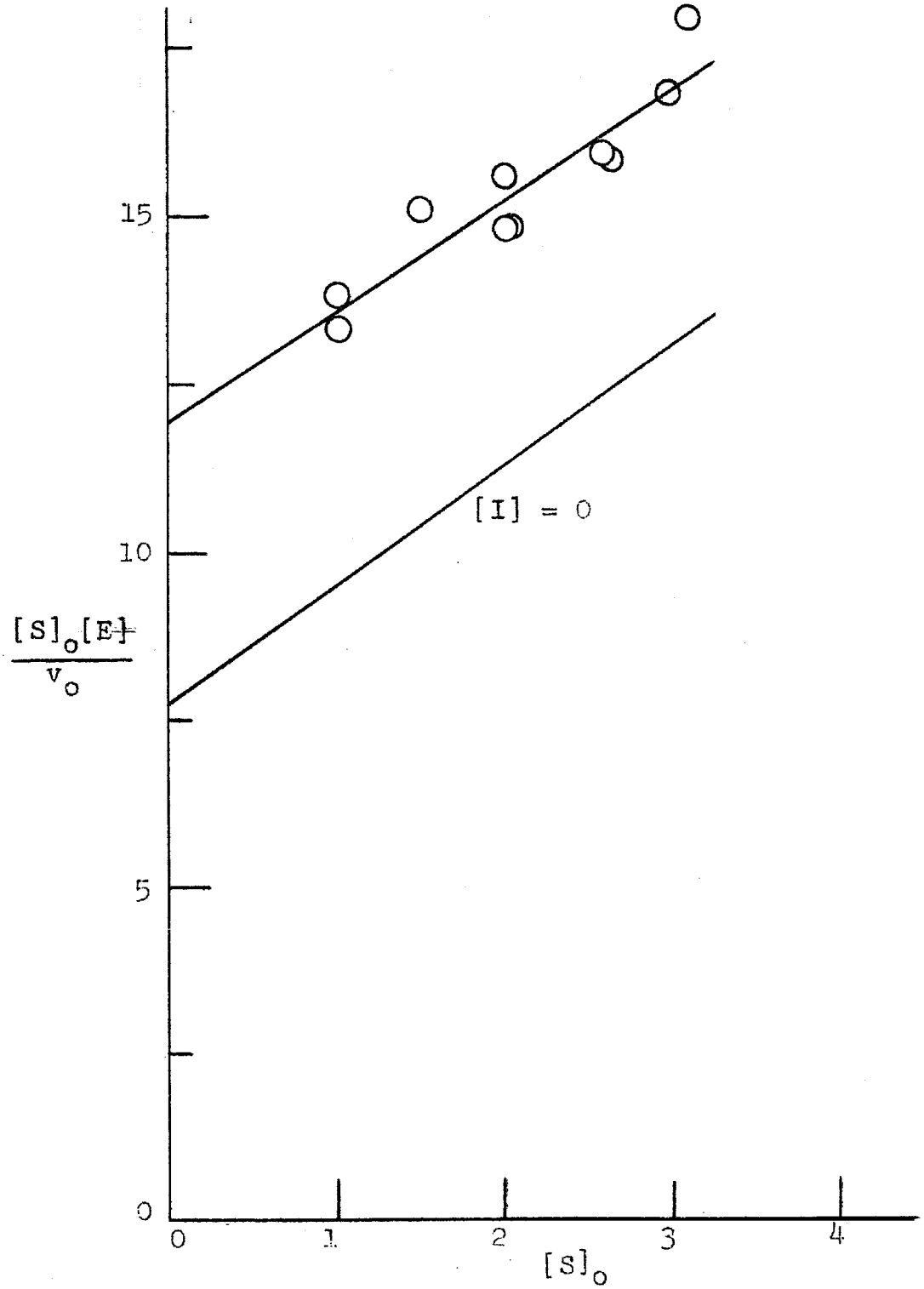

Figure 7.--$[S]_o[E]/v_o$ versus $[S]_o$ plot for the inhibition of the alpha-chymotrypsin catalyzed hydrolysis of benzoyl-L-valine methyl ester by 5.5×10^{-3} M acetyl-L-tryptophanmethylamide at pH 7.9 and 25° in 0.02 M sodium chloride solution. Line determined by least squares. $[S]_o[E]/v_o$ in units of min. mg. protein nitrogen/ml. $[S]_o$ in units of 10^{-3} M. (Cf. Table XV).

Table XVI.--Inhibition of the alpha-Chymotrypsin Catalyzed Hydrolysis of Benzoyl-L-valine Methyl Ester by Acetyl-D-tryptophanmethylamide at pH 7.9 and 25°.

$[E]^a$ = 0.150 mg. protein nitrogen/ml.; [NaOH] = 0.01964 N; [NaCl] = 0.02 M; [I] = 0.50 x 10^{-3} M.

Run No.[b]	1-8-30	1-8-2	2-8-2	3-8-2	4-8-2	5-8-2	6-8-2	1-8-22	2-8-22	3-8-22	4-8-22	6-8-22	7-8-22
$[S]_o$[c]	0	3.51	2.48	1.48	1.45	2.51	3.61	1.46	2.58	3.41	3.29	2.56	1.57
	S.R.[d]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]
t[f]													
0	0	15	15	18	17	4	8	0	0	0	0	0	0
2	60	350	280	183	203	270	353	175	276	319	307	254	181
4	100	685	545	355	375	530	700	340	535	634	617	510	360
6	130	1020	810	535	550	795	1055	510	805	959	927	767	536
8	153	1350	1080	715	725	1058	1400	680	1065	1279	1247	1019	713
10	170	1685	1340	890	900	1320	1740	845	1329	1599	1550	1266	890
12	190	2010	1595	1060	1064	1570	2075	1005	1577	1904	1852	1507	1063
14	210	2330	1845	1227	1230	1822	2405	1160	1831	2209	2162	1746	1228
16	228	2650	2090	1385	1385	2067	2735	1312	2078	2499	2450	1979	1385
H[g]	--	15	17	18	18	16	15	18	16	14	15	15	17
v_o[h]	--	3.28	2.55	1.76	1.80	2.58	3.31	1.73	2.68	3.08	3.00	2.52	1.80
σv_o[hi]	--	0.02	0.02	0.03	0.02	0.03	0.04	0.01	0.02	0.02	0.04	0.02	0.02

a. Armour lot #90492, 15.0% protein nitrogen. b. Run number -month-day (1956). c. In units of 10^{-3} M. d. S.R. = scale reading in units of 10^{-4} ml. Estimated error ± 2 x 10^{-4} ml. e. Corrected for blank given in first column. f. In units of minutes. g. Approximate percent hydrolysis. h. Calculated by the orthogonal polynomial method (25). In units of 10^{-5} M/min. i. ±.

Table XVII.--Summary of the Initial Velocities and Kinetic Constants Derived from the Data of Table XVI.

$[S]_o$ [a]	P_m [b]	v_o [c]	σv_o [d]	$[S]_o[E]/v_o$ [e]
3.51	3	3.28	0.02	16.0
2.48	4	2.55	0.02	14.6
1.48	2	1.76	0.03	12.6
1.45	2	1.80	0.02	12.1
2.51	3	2.58	0.03	14.6
3.61	4	3.31	0.04	16.4
1.46	2	1.73	0.01	12.7
2.58	2	2.68	0.02	14.5
3.41	3	3.08	0.02	16.6
3.29	3	3.00	0.04	16.4
2.56	3	2.52	0.02	15.2
1.57	4	1.80	0.02	13.1

$$k_3 = 0.53 \pm 0.03 \times 10^{-3}$$ [f,g]

$$K_S' = 5.23 \pm 0.37 \times 10^{-3}\ \underline{M}$$ [f]

$$K_I = 2.1 \pm 1.2 \times 10^{-3}\ \underline{M}$$ [h]

a. In units of 10^{-3} $\underline{M}$. b. Order of polynomial employed for calculation of v_o (25). c. In units of 10^{-5} $\underline{M}$/min. d. $\pm$, in units of 10^{-5} $\underline{M}$/min. e. In units of min. mg. protein nitrogen/ml. f. Evaluated by the method of least squares (cf. fig. 8). g. In units of $\underline{M}$/min. mg. protein nitrogen/ml. h. Based on the value $K_S = 4.2 \pm 0.4 \times 10^{-3}$ $\underline{M}$.

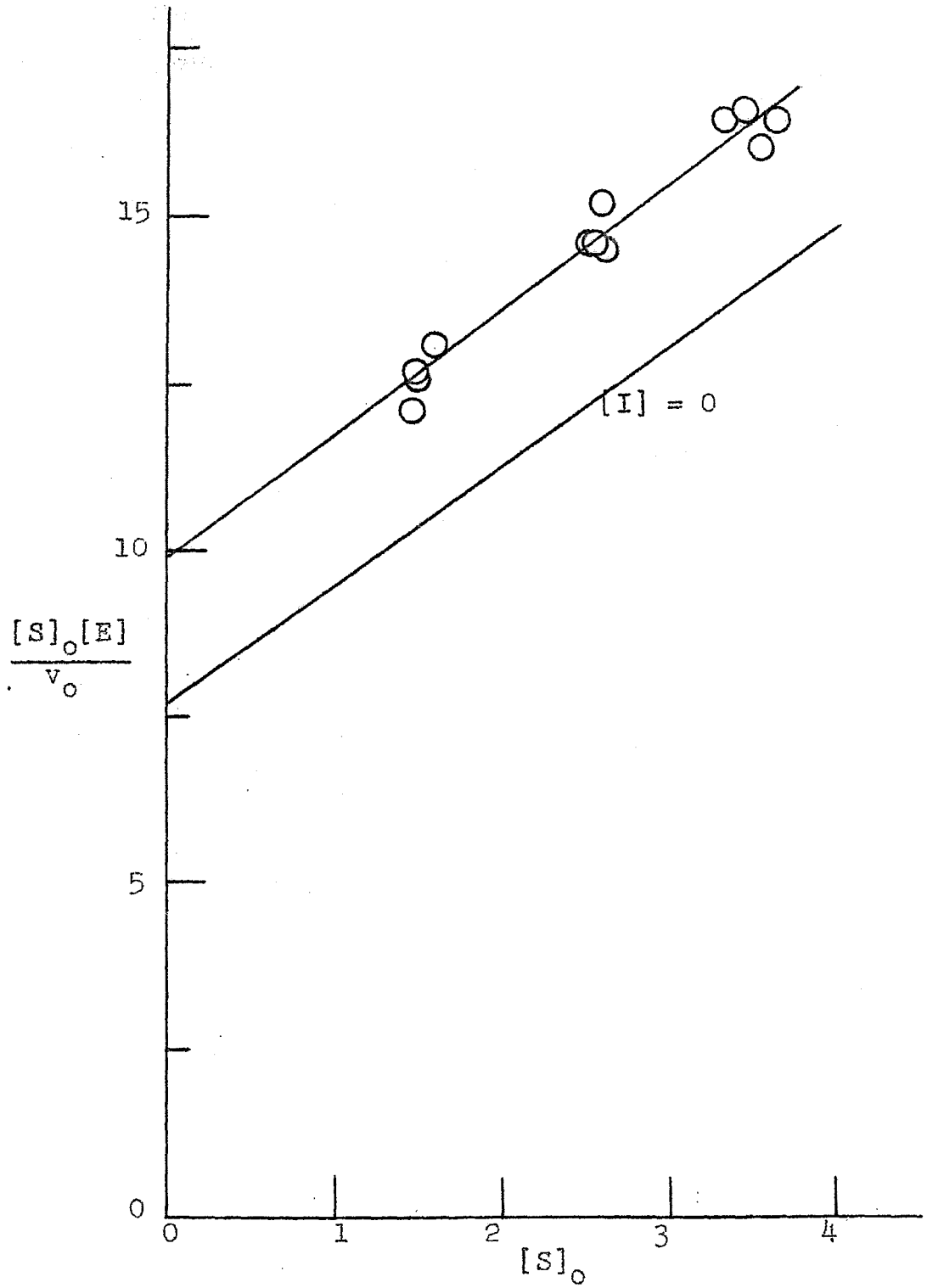

Figure 8.--$[S]_o[E]/v_o$ versus $[S]_o$ plot for the inhibition of the alpha-chymotrypsin catalyzed hydrolysis of benzoyl-L-valine methyl ester by 0.50×10^{-3} M acetyl-D-tryptophanmethylamide at pH 7.9 and 25° in 0.02 M sodium chloride solution. Line determined by least squares. $[S]_o[E]/v_o$ in units of min. mg. protein nitrogen/ml. $[S]_o$ in units of 10^{-3} M. (Cf. Table XVII).

Table XVIII.--Inhibition of the alpha-Chymotrypsin Catalyzed Hydrolysis of Benzoyl-L-valine Methyl Ester by Acetyl-L-phenylalaninmethylamide at pH 7.9 and 25°.
$[E]^a$ = 0.150 mg. protein nitrogen/ml.; [NaOH] = 0.01964 N; [NaCl] = 0.02 M; [I] = 6.0×10^{-3} M.

Run No.[b]	7-8-30	1-8-15	2-8-15	3-8-15	4-8-15	1-8-20	2-8-20	3-8-20
$[S]_o$[c]	--	0.97	1.39	1.87	2.48	1.00	1.41	1.93
	S.R.[d]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]
t[f]								
0	0	0	0	8	0	5	12	0
2	75	133	177	248	285	130	185	217
4	118	269	362	492	572	274	372	450
6	150	406	548	730	850	415	558	682
8	180	542	725	958	1130	550	740	909
10	205	674	905	1190	1405	685	920	1133
12	227	806	1078	1413	1678	813	1096	1345
14	250	932	1253	1629	1945	939	1270	1562
16	267	1061	1418	1850	2213	1067	1445	1753
H[g]	--	21	20	19	18	21	20	18
v_o[h]	--	1.36	1.84	2.42	2.86	1.39	1.83	2.17
σv_o[hi]	--	0.01	0.02	0.02	0.01	0.02	0.02	0.04

a. Armour lot #90492, 15.0% protein nitrogen. b. Run number- month-day (1956). c. In units of 10^{-3} M. d. S.R. = scale reading in units of 10^{-4} ml. Estimated error $\pm$ 2 x 10^{-4} ml. e. Corrected for blank given in first column. f. In units of minutes. g. Approximate percent hydrolysis. h. Calculated by the orthogonal polynomial method (25). In units of 10^{-5} M/min. i. $\pm$.

Table XIX.--Summary of the Initial Velocities and Kinetic Constants Derived from the Data of Table XVIII.

$[S]_o$ [a]	P_m [b]	v_o [c]	σv_o [d]	$[S]_o[E]/v_o$ [e]
0.97	2	1.36	0.01	10.7
1.39	2	1.84	0.02	11.3
1.87	2	2.42	0.02	11.6
2.48	2	2.86	0.01	13.0
1.00	2	1.39	0.02	10.8
1.41	2	1.83	0.02	11.6
1.93	3	2.17	0.04	13.3

$$k_3 = 0.61 \pm 0.15 \times 10^{-3}$$ [f,g]

$$K_S' = 5.6 \pm 1.4 \times 10^{-3}\ \underline{M}$$ [f]

$$K_I = 19 \times 10^{-3}\ \underline{M}$$ [h]

a. In units of 10^{-3} $\underline{M}$. b. Order of polynomial employed for calculation of v_o (25). c. In units of 10^{-5} $\underline{M}$/min. d. $\pm$, in units of 10^{-5} $\underline{M}$/min. e. In units of min. mg. protein nitrogen/ml. f. Evaluated by the method of least squares (cf. fig. 9). g. In units of $\underline{M}$/min. mg. protein nitrogen/ml. h. Based on the value $K_S = 4.2 \pm 0.4 \times 10^{-3}$ $\underline{M}$. Order of magnitude only.

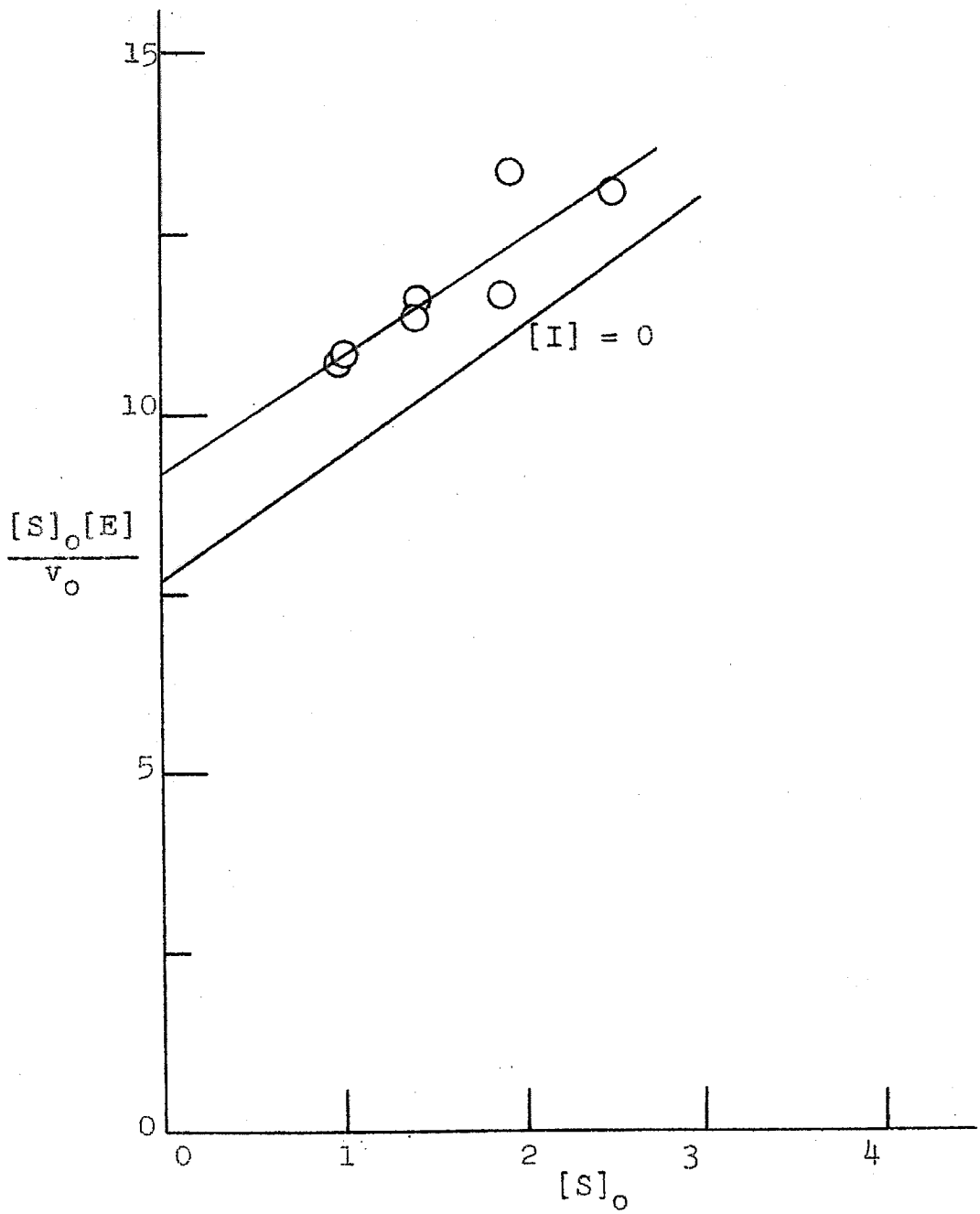

Figure 9.--$[S]_o[E]/v_o$ versus $[S]_o$ plot for the inhibition of the alpha-chymotrypsin catalyzed hydrolysis of benzoyl-L-valine methyl ester by 6.0×10^{-3} M acetyl-L-phenylalaninmethylamide at pH 7.9 and 25° in 0.02 M sodium chloride solution. Line determined by least squares. $[S]_o[E]/v_o$ in units of min. mr. protein nitrogen/ml. $[S]_o$ in units of 10^{-3} M (Cf. Table XIX).

Table XX.--Inhibition of the alpha-Chymotrypsin Catalyzed Hydrolysis of Benzoyl-L-valine Methyl Ester by Acetyl-D-phenylalaninmethylamide at pH 7.9 and 25°.

$[E]^a$ = 0.150 mg. protein nitrogen/ml.; [NaOH] = 0.01964 N; [NaCl] = 0.02 M; [I] = 4.0×10^{-3} M.

Run No.[b]	6-8-30	1-8-24	2-8-24	3-8-24	1-9-4	2-9-4
$[S]_o$[c]	0	2.94	2.47	1.97	2.98	2.01
	S.R.[d]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]	S.R.[de]
t[f]						
0	0	0	0	0	20	15
2	55	278	248	203	291	226
4	88	552	495	408	572	437
6	115	835	738	615	848	642
8	140	1098	971	817	1115	847
10	160	1365	1211	1016	1390	1048
12	178	1630	1445	1214	1658	1249
14	195	1893	1678	1409	1927	1445
16	210	2140	1906	1605	2185	1638
H[g]	--	14	15	16	14	16
v_o[h]	--	2.74	2.44	2.04	2.74	2.09
σv_o[hi]	--	0.02	0.01	0.03	0.01	0.01

a. Armour lot #90492, 15.0% protein nitrogen. b. Run number-month-day (1956). c. In units of 10^{-3} M. d. S.R. = scale reading in units of 10^{-4} ml. Estimated error ± 2×10^{-4} ml. e. Corrected for blank given in first column. f. In units of minutes. g. Approximate percent hydrolysis. h. Calculated by the orthogonal polynomial method (25). In units of 10^{-5} M/min. i. ±.

Table XXI.--Summary of the Initial Velocities and Kinetic Constants Derived from the Data of Table XX.

$[S]_o$ [a]	P_m [b]	v_o [c]	σv_o [d]	$[S]_o[E]/v_o$ [e]
2.94	3	2.74	0.02	16.1
2.47	2	2.44	0.01	15.2
1.97	2	2.04	0.03	14.5
2.98	2	2.74	0.01	16.3
2.01	2	2.09	0.01	14.4

$$k_3 = 0.55 \pm 0.04 \times 10^{-3}$$ [f,g]

$$K_S' = 6.0 \pm 0.4 \times 10^{-3}\ \underline{M}$$ [f]

$$K_I = 9.6 \pm 3.5 \times 10^{-3}\ \underline{M}$$ [h]

a. In units of 10^{-3} M. b. Order of polynomial employed for calculation of v_o (25). c. In units of 10^{-5} M/min. d. $\pm$, in units of 10^{-5} M/min. e. In units of min. mg. protein nitrogen/ml. f. Evaluated by the method of least squares (cf. fig. 10). g. In units of M/min. mg. protein nitrogen/ml. h. Based on the value $K_S = 4.2 \pm 0.4 \times 10^{-3}$ M.

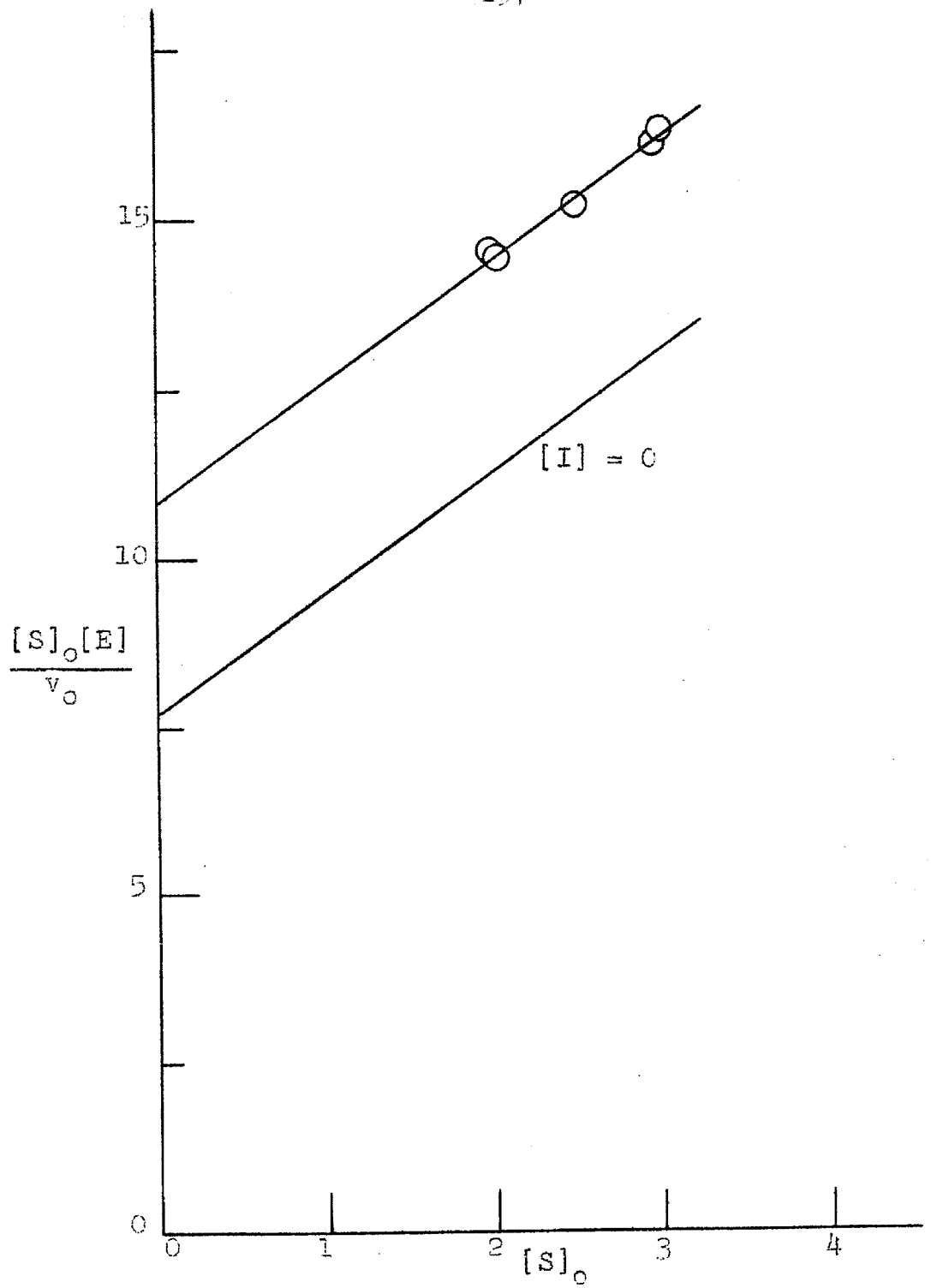

Figure 19.--$[S]_o[E]/v_o$ versus $[S]_o$ plot for the inhibition of the alpha-chymotrypsin catalyzed hydrolysis of benzoyl-L-valine methyl ester by 4.0×10^{-3} M acetyl-D-phenylalaninmethylamide at pH 7.9 and 25° in 0.02 M sodium chloride solution. Line determined by least squares. $[S]_o[E]/v_o$ in units of min. mg. protein nitrogen/ml. $[S]_o$ in units of 10^{-3} M. (Cf. Table XXI).

Table XXII.--Inhibition of the alpha-Chymotrypsin Catalyzed Hydrolysis of Benzoyl-L-valine Methyl Ester by Acetyl-L-tyrosin-methylamide at pH 7.9 and 25°.
[I] = 10 x 10^{-3} M; [NaOH] = 0.01964 N; [NaCl] = 0.02 M.

Run No.[b]	1-7-24	2-7-24	3-7-24	4-7-24	1-7-26	2-7-26	3-7-26
[E][a]	0.158	0.158	0.158	0.158	0.150	0.150	0.150
$[S]_o$[c]	0 S.R.[d]	1.48 S.R.[de]	2.44 S.R.[de]	3.51 S.R.[de]	1.99 S.R.[de]	2.48 S.R.[de]	2.93 S.R.[de]
t[f]							
0	0	0	0	0	0	0	0
2	6	22	32	35	27	27	30
4	10	42	60	74	51	55	63
6	13	61	89	112	75	84	95
8	16	79	117	144	99	113	125
10	19	99	145	179	123	141	155
12	22	116	173	215	146	168	184
14	24	135	201	251	169	196	213
16	27	152	225	285	191	223	241
H[g]	--	20	18	16	19	18	16
v_o[h]	--	2.03	2.98	3.63	2.51	2.81	3.21
σv_o[hi]	--	0.04	0.04	0.07	0.03	0.04	0.04

a. In units of mg. protein nitrogen/ml. Armour lot #90492, 15.0% protein nitrogen. b. Run number-month-day (1956). c. In units of 10^{-3} M. d. S. R. = scale reading in units of 10^{-3} ml. Estimated to ± 0.5 x 10^{-3} ml. and rounded off to nearest 1 x 10^{-3} ml. e. Corrected for blank given in first column. f. In units of minutes. g. Approximate percent hydrolysis. h. Calculated by the orthogonal polynomial method (25). In units of 10^{-5} M/min. i. ±.

Table XXIII.--Summary of the Initial Velocities and Kinetic Constants Derived from the Data of Table XXII.

$[S]_o$ [a]	P_m [b]	v_o [c]	σv_o [d]	$[S]_o[E]/v_o$ [e]
1.48	2	2.03	0.04	11.6
2.44	2	2.98	0.04	12.9
3.51	2	3.63	0.07	15.3
1.99	2	2.51	0.03	11.9
2.48	2	2.81	0.04	13.2
2.93	2	3.21	0.04	13.7

$$k_3 = 0.54 \pm 0.06 \times 10^{-3}$$ [f,g]

$$K_S' = 4.59 \pm 0.55 \times 10^{-3}\ \underline{M}$$ [f]

$$K_I = 105 \times 10^{-3}\ \underline{M}$$ [h]

a. In units of 10^{-3} $\underline{M}$. b. Order of polynomial employed for calculation of v_o (25). c. In units of 10^{-5} $\underline{M}$/min. d. $\pm$, in units of 10^{-5} $\underline{M}$/min. e. In units of min. mg. protein nitrogen/ml. f. Evaluated by the method of least squares (cf. fig. 11). g. In units of $\underline{M}$/min. mg. protein nitrogen/ml. h. Based on the value $K_S = 4.2 \pm 0.4 \times 10^{-3}$ $\underline{M}$. Order of magnitude only.

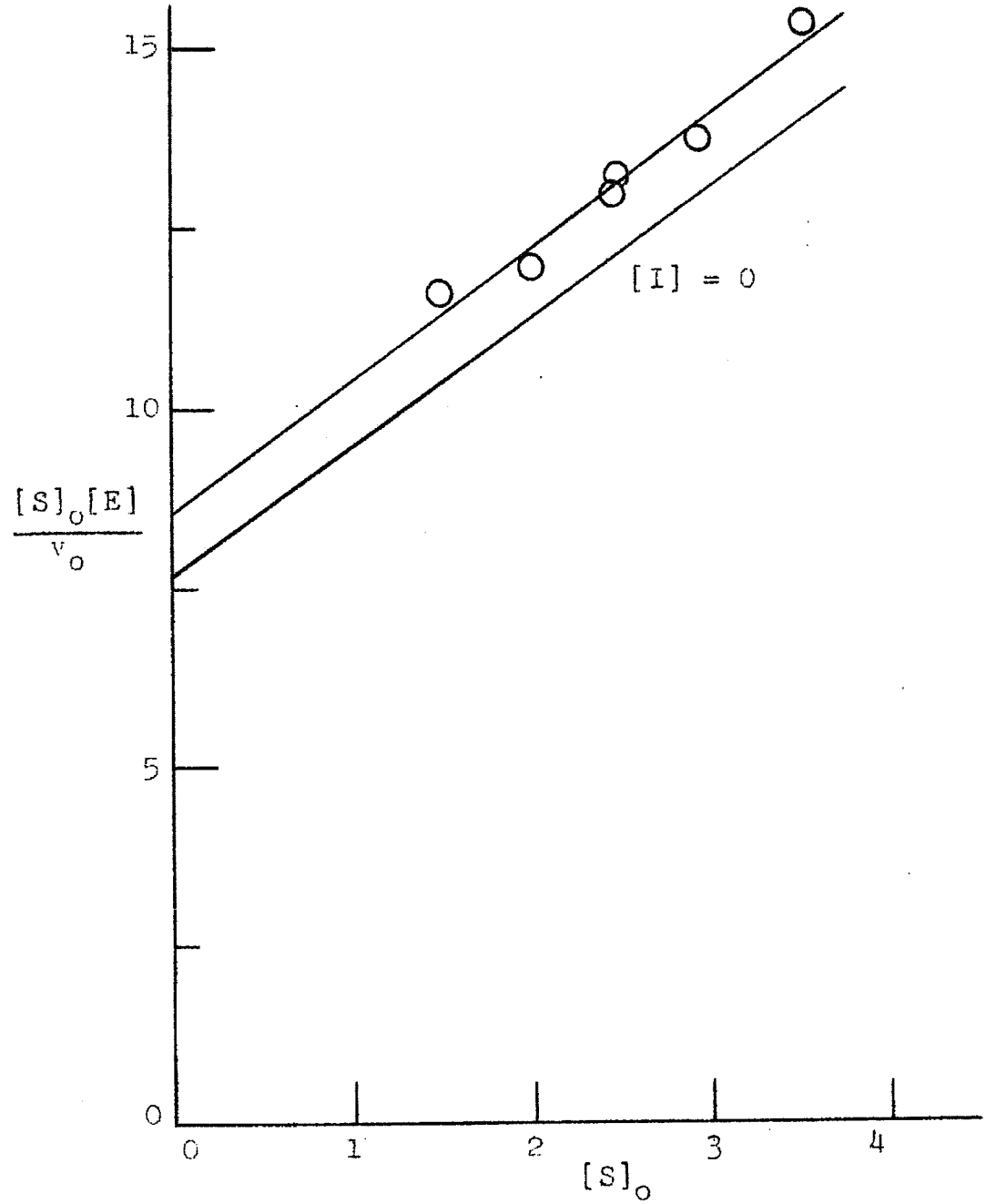

Figure 11.--$[S]_o[E]/v_c$ versus $[E]_o$ plot for the inhibition of the alpha-chymotrypsin catalyzed hydrolysis of benzoyl-L-valine methyl ester by 10 x 10^{-3} M acetyl-L-tyrosinmethylamide at pH 7.8 and 25° in 0.02 M sodium chloride solution. Line determined by least squares. $[S]_o[E]/v_o$ in units of min. mg. protein nitrogen/ml. $[S]_o$ in units of 10^{-3} M. (Cf. Table XXIII).

Table XXIV.--Inhibition of the alpha-Chymotrypsin Catalyzed Hydrolysis of Benzoyl-L-valine Methyl Ester by Acetyl-D-tyrosinmethylamide at pH 7.9 and 25°.

$[E]^a$ = 0.150 mg. protein nitrogen/ml.; [NaOH] = 0.01964 N; [NaCl] = 0.02 M; [I] = 5.0×10^{-3} M.

Run No.[b]	4-8-28	1-8-28	2-8-28	3-8-28
$[S]_o$[c]	0 S.R.[d]	3.46 S.R.[de]	3.03 S.R.[de]	2.52 S.R.[de]
t[f]				
0	0	0	0	0
2	40	310	300	250
4	70	620	585	500
6	90	925	875	750
8	110	1225	1155	995
10	130	1520	1430	1230
12	150	1810	1695	1470
14	165	2100	1955	1705
16	180	2380	2205	1930
H[g]	--	14	13	15
v_o[h]	--	3.09	2.96	2.51
σv_o[hi]	--	0.04	0.01	0.01

a. Armour lot #90492, 15.0% protein nitrogen. b. Run number-month-day (1956). c. In units of 10^{-3} M. d. S.R. = scale reading in units of 10^{-4} ml. Estimated error ± 2 x 10^{-4} ml. e. Corrected for blank given in first column. f. In units of minutes. g. Approximate percent hydrolysis. h. Calculated by the orthogonal polynomial method (25). In units of 10^{-5} M/min. i. ±.

Table XXV.--Summary of the Initial Velocities and Kinetic Constants Estimated from the Data of Table XXIV.

$[S]_o$ [a]	P_m [b]	v_o [c]	σv_o [d]	$[S]_o[E]/v_o$ [e]
3.46	2	3.09	0.04	16.8
3.03	2	2.96	0.01	15.4
2.52	2	2.51	0.01	15.1

$$K_S' = 5.6 \times 10^{-3}\ \underline{M}^f$$

$$K_I = 15 \times 10^{-3}\ \underline{M}^g$$

a. In units of 10^{-3} $\underline{M}$. b. Order of polynomial employed for calculation of v_o (25). c. In units of 10^{-5} $\underline{M}$/min. d. $\pm$, in units of 10^{-5} $\underline{M}$/min. e. In units of min. mg. protein nitrogen/ml. f. Estimated from intercept (fig. 12) of best line drawn with slope equivalent to that of line from substrate alone (cf. Part III, fig. 5) i.e., tacitly assuming competitive inhibition. g. Based on $K_S = 4.2 \pm 0.4 \times 10^{-3}$ $\underline{M}$. Order of magnitude only.

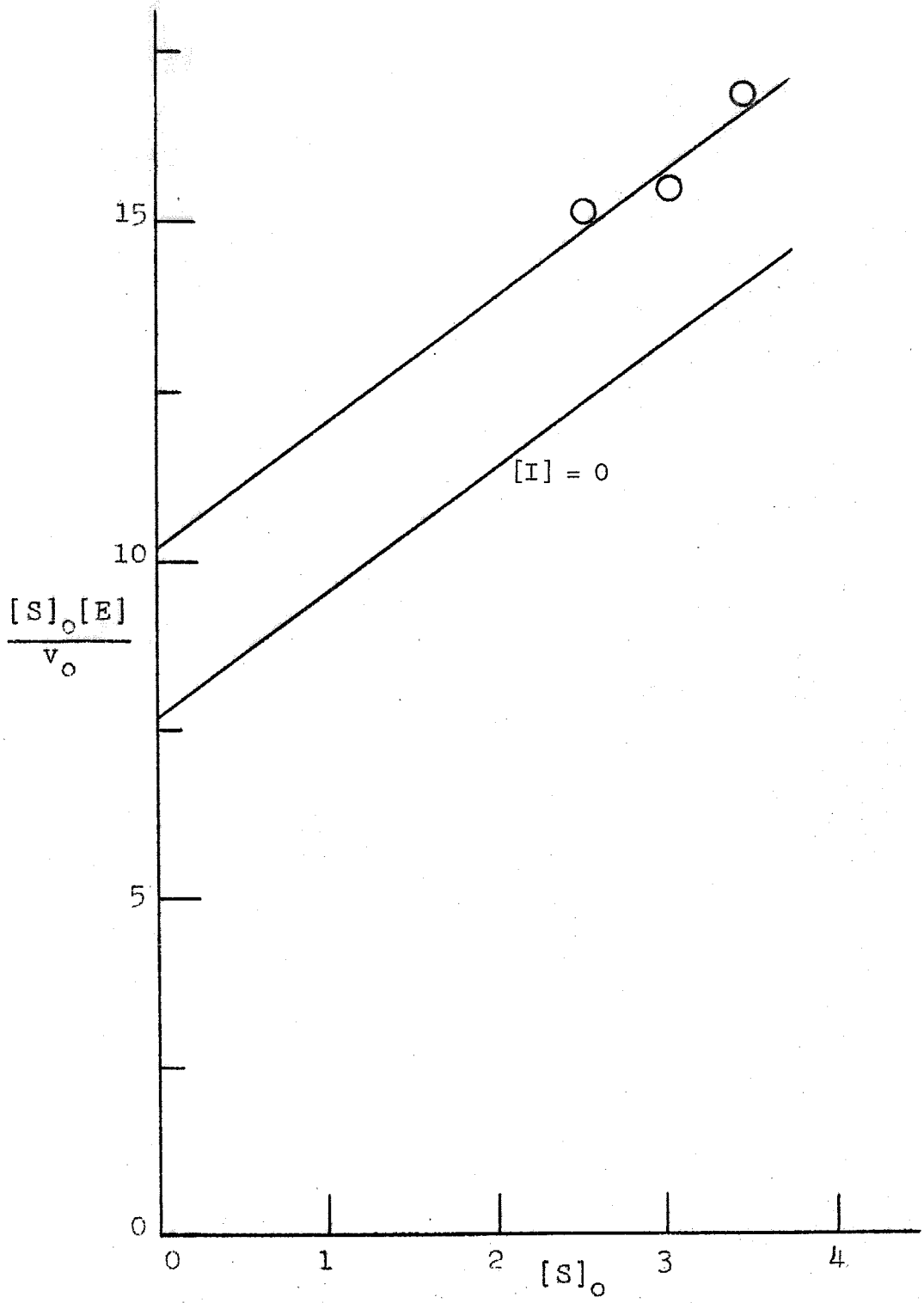

Figure 12.--$[S]_o[E]/v_o$ versus $[S]_o$ plot for the inhibition of the alpha-chymotrypsin catalyzed hydrolysis of benzoy-L-valine methyl ester by 5.0×10^{-3} M acetyl-D-tyrosinmethylamide at pH 7.9 and 25° in 0.02 M sodium chloride solution. Line drawn by inspection assuming competitive inhibition. $[S]_o[E]/v_o$ in units of min. mg. protein nitrogen/ml. $[S]_o$ in units of 10^{-3} M. (Cf. Table XXV).

Table XXVI.--Summary of the Kinetic Constants for the Inhibition of the alpha-Chymotrypsin Catalyzed Hydrolysis of Methyl Hippurate and of Benzoyl-L-valine Methyl Ester by Three Enantiomorphic Pairs of Competitive Inhibitors.[a]

Inhibitor[b]	k_3[c]	σk_3[d]	K_S[e]	σK_S[de]	[I][f]	I_I'[g]	K_I[f]	σK_I[h]
Substrate: Methyl Hippurate								
--	3.21	0.09	6.57	0.45	--	--	--	--
	2.90	0.16	6.61	0.85	--	--	--	--
L-Tryptophan	3.09	0.22	8.0	1.3	5.0	0.21	24[i]	
D-Tryptophan	3.06	0.18	19.0	1.4	5.0	1.9	2.6	0.4
L-Phenylalanine	2.95	0.30	8.2	1.7	6.0	0.24	25[i]	--
D-Phenylalanine	3.28	0.11	11.11	0.62	4.0	0.68	5.9	1.3
L-Tyrosine	2.98	0.18	8.6	1.0	20	0.30	66	38
D-Tyrosine	3.07	--	10.0	--	5.0	0.52	9.7[j]	--
Substrate: Benzoyl-L-valine Methyl Ester								
--	0.55	0.04	4.24	0.35	--	--	--	--
L-Tryptophan	0.60	0.08	7.2	1.1	5.0	0.70	7.1	2.9
D-Tryptophan	0.53	0.03	5.23	0.37	0.50	0.24	2.1	1.2
L-Phenylalanine	0.61	0.15	5.6	1.4	6.0	0.32	19[i]	--
D-Phenylalanine	0.55	0.04	6.01	0.42	4.0	0.42	9.6	3.5
L-Tyrosine	0.54	0.06	4.59	0.55	10	0.095	105[i]	--
D-Tyrosine	--		--		5.0	0.33	15[j]	--

a. In aqueous 0.02 M sodium chloride solution at pH 7.9 and 25°. b. N-Acetyl-N'-methylamide. c. In units of 10^{-3} M/min. mg. protein nitrogen/ml. d. ±. e. Value given is that of K_S' when inhibitor is present. In units of 10^{-3} M. f. In units of 10^{-3} M. g. $I_I' = [I]/K_I$ (cf. ref. 9 and text for discussion of the significance of this value). h. In units of 10^{-3} M. Calculated where indicated from $\partial K_I = (\Sigma(\partial K_I)^2)^{1/2}$. i. Order of magnitude only. Value uncertain due to low value of I_I' and high $\sigma K_S'$. j. Approximate value due to low number of measurements.

Table XXVII.--The alpha-Chymotrypsin Catalyzed Hydrolysis of Benzoyl-L-valine Methyl Ester in the Presence of Some N-Acetyl-L-amino Acid N'-Methylamides at pH 7.9 and 25°.

[NaOH] = 0.01964 N; [NaCl] = 0.02 M.

Run No.[a]	1-8-6	1-9-1	2-8-6	2-9-1	1-8-7	3-9-1	2-8-7	4-9-1
$[E]$[b]	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.150
$[S]_o$[c]	3.69	3.53	3.51	3.47	3.54	3.54	3.53	3.47
Added Compound[d]	glycine		L-alanine		L-leucine		L-proline	
$[I]$[e]	50.4	50.3	49.8	50.1	50.0	50.1	50.0	49.9
	S.R.[f]	S.R.[f]	S.R.[f]	S.R.[f]	S.R.[f]	S.R.[f]	S.R.[f]	S.R.[f]
t[g]								
0	38	0	20	28	20	28	18	30
2	435	386	400	422	394	414	413	423
4	833	758	790	810	775	788	820	825
6	1240	1140	1172	1189	1174	1172	1210	1209
8	1634	1506	1565	1577	1555	1542	1598	1584
10	2032	1872	1947	1952	1923	1905	1982	1954
12	2318	2237	2320	2330	2305	2269	2357	2325
14	2807	2602	2691	2703	2679	2642	2735	2692
16	3190	2957	3054	3064	3044	2999	3109	3057
H[h]	17	16	17	17	17	17	17	17
v_o[i]	3.84	3.76	3.74	3.87	3.82	3.77	3.97	4.00
σ_{v_o}[ij]	0.04	0.02	0.07	0.02	0.03	0.02	0.02	0.04

Table XXVII.--continued

Run No.[a]	3-8-14	1-9-3	4-8-14	2-9-3	5-8-14	3-9-3
$[E]$[b]	0.170	0.150	0.170	0.150	0.150	0.150
$[S]_o$[c]	3.54	3.43	3.48	3.47	3.56	3.49
Added Compound[d]	L-hydroxyproline		L-histidine		L-methionine	
$[I]$[e]	50.0	50.2	49.9	49.9	50.2	50.3
	S.R.[f]	S.R.[f]	S.R.[f]	S.R.[f]	S.R.[f]	S.R.[f]
t[g]						
0	22	30	5	0	60	0
2	452	410	448	425	535	395
4	869	787	855	805	995	765
6	1284	1160	1270	1183	1454	1147
8	1697	1528	1690	1540	1912	1517
10	2115	1898	2103	1898	2355	1890
12	2518	2263	2509	2251	2771	2256
14	2917	2623	2905	2608	3210	2623
16	3313	2975	3290	2950	3642	2992
H[h]	18	17	19	17	20	17
v_o[i]	4.04	3.75	4.04	4.15	4.39	3.83
σ_{v_o}[j]	0.03	0.01	0.03	0.07	0.33	0.02

a. Run number-month-day (1956). b. Armour lot #90492, 15.0% protein nitrogen. In units of mg. protein nitrogen/ml. c. In units of 10^{-3} M. d. N-Acetyl-N'-methylamide of amino acid indicated. e. Concentration of added compound in units of 10^{-3} M. f. S.R. = scale reading. In units of 10^{-4} ml., estimated to $\pm$ 2 x 10^{-4} ml. Corrected for the respective blanks given in Table XXIX. g. In units of minutes. h. Approximate percent hydrolysis. i. Calculated by orthogonal polynomial method (25). In units of 10^{-5} M/min. j. $\pm$.

Table XXVIII.--Summary of the Initial Velocities From the Data of Table XXVII.

Added Compound[a]	$[S]_o$[b]	P_m[c]	v_o[d]	σv_o[e]	$[S]_o[E]/v_o$[f]
Glycine	3.69	1	3.84	0.04	14.4
	3.53	2	3.76	0.02	14.1
L-Alanine	3.51	3	3.74	0.07	14.1
	3.47	2	3.87	0.02	13.5
L-Leucine	3.54	2	3.82	0.03	13.9
	3.54	2	3.77	0.02	14.1
L-Proline	3.53	2	3.97	0.02	13.3
	3.47	3	4.00	0.04	13.0
L-Hydroxyproline	3.54	1	4.04	0.03	14.9
	3.43	2	3.75	0.01	13.7
L-Histidine	3.48	1	4.04	0.03	14.6
	3.47	3	4.15	0.07	12.5
L-Methionine	3.56	1	4.39	0.33	12.2
	3.49	2	3.83	0.03	13.7

a. N-Acetyl-N'-methylamide. b. In units of 10^{-3} M. c. Order of polynomial employed for calculation of v_o (25). d. In units of 10^{-5} M/min. e. $\pm$, in units of 10^{-5} M/min. f. In units of min. mg. protein nitrogen/ml. (Cf. fig. 13).

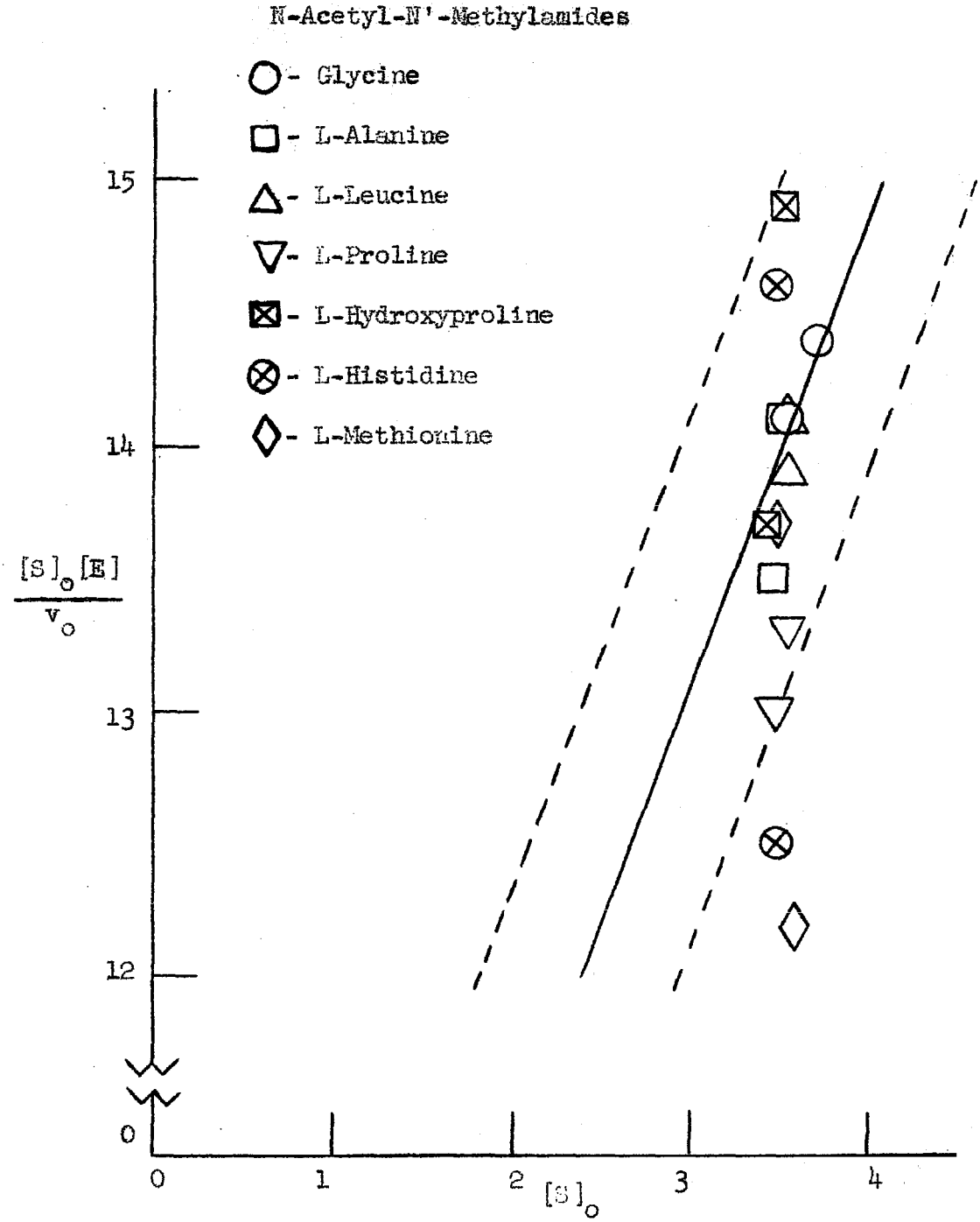

Figure 13.--$[S]_o[E]/v_o$ versus $[S]_o$ plot of the alpha-chymotrypsin catalyzed hydrolysis of benzoyl-L-valine methyl ester in the presence of some N-acetyl-L-amino acid N'-methylamides at pH 7.9 and 25° in 0.02 M sodium chloride solution. ——— Line determined by least squares for substrate alone (cf. Part III, fig. 5); ------ extremes of variability in data for substrate alone. Vertical scale expanded for clarity. $[S]_o[E]/v_o$ in units of min. mg. protein nitrogen/ml. $[S]_o$ in units of 10^{-3} M. (Cf. Table XXVIII).

Table XXIX. Base Uptake (Blanks) for alpha-Chymotrypsin Alone and in the Presence of some N-Acetyl-N'-methylamides at pH 7.9 and 25°.

[NaOH] = 0.01964 N; [NaCl] = 0.02 M

Run No.[a]	3-8-30	8-8-30	1-8-30	2-8-30	7-8-30	6-8-30	5-8-30	4-8-28
[E][b]	0.153	0.150	0.153	0.153	0.150	0.150	0.150	0.150
Added Compound[c]	---	L-Trypto-phan	D-Tryptophan		L-Phenyl-alanine	D-Phenyl-alanine	L-Tyro-sine	D-Tyro sine
[I][d]	---	5.0	0.50	3.0	6.0	4.0	10	5.0
	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]
t[f]								
0	0	0	0	0	0	0	0	0
2	85	62	60	40	75	55	65	40
4	120	95	100	65	118	88	103	70
6	150	120	130	85	150	115	130	90
8	180	145	153	100	180	140	155	110
10	205	165	170	110	205	160	180	130
12	230	183	190	120	227	178	200	150
14	250	200	210	130	250	195	215	165
16	275	215	228	140	267	210	230	180

Table XXIX.--continued

Run No.[a]	2-9-1	4-9-1	6-9-1	8-9-1	2-9-3	2-8-14	6-9-3
[E][b]	0.150	0.150	0.150	0.150	0.150	0.170	0.150
Added Compound[c]	Glycine	L-Alanine	L-Leucine	L-Proline	L-Hydroxy-proline	L-Histi-dine	L-Methio-nine
[I][d]	50.3	50.1	50.1	49.9	50.2	49.9	50.3
	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]
t[f]							
0	0	0	0	0	0	0	0
2	65	60	66	65	70	57	65
4	112	105	117	95	113	103	105
6	150	140	148	135	148	140	138
8	182	168	178	167	175	172	168
10	208	193	207	198	200	202	195
12	234	215	233	225	222	229	219
14	258	237	256	250	245	257	240
16	283	256	276	271	265	280	258

a. Run number-month-day (1956). b. Armour lot #90492, 15% protein nitrogen. In units of mg. protein nitrogen/ml. c. N-Acetyl-N'-methylamide. d. Concentration of added compound in units of 10^{-3} M. e. S.R. = scale reading. In units of 10^{-4} ml. Estimated to $\pm$ 2 x 10^{-4} ml. Best result of duplicate determinations. f. In units of minutes.

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PART V

PUBLICATIONS

The Evaluation of the Kinetic Constants of Enzyme-Catalyzed Reactions by the Method of Foster and Niemann[1]

By Thomas H. Applewhite and Carl Niemann[2]

Received April 4, 1955

In a recent communication from these laboratories Foster and Niemann,[3,4] in extending an earlier treatment of Walker and Schmidt,[5] described a procedure for the graphical evaluation of the kinetic constants of enzyme catalyzed reactions whose rates, in so far as they are dependent upon the concentration of enzyme and specific substrate, are described by equation 1

$$k_3[E]t = K_S\left(1 + [S]_0\sum_{j=1}^{n} 1/K_{P_j}\right)\ln [S]_0/[S]_t + \left(1 - K_S\sum_{j=1}^{n} 1/K_{P_j}\right)([S]_0 - [S]_t) \quad (1)$$

This procedure, which was developed for the case where $\sum_{j=1}^{n} 1/K_{P_j} = 1/K_P$, is based upon the fact that in a plot of $([S]_0 - [S]_t)/t$ *vs.* $(\ln[S]_0/[S]_t)/t$ *lines of slope* $[S]_0$ *drawn through the origin will intersect* those of slope $-K_S(K_P + [S]_0)/(K_P - K_S)$ at points which define, in terms of the ordinate $([S]_0 - [S]_t)/t$, the corresponding initial velocities. As this fact was not clearly established, nor clearly stated, in the earlier communication[3] we shall in this communication offer proof of its validity.

If equation 1 for the case where $\sum_{j=1}^{n} 1/K_{P_j} = 1/K_P$ is transformed into the usual slope-intercept form one obtains equation 2

$$([S]_0 - [S]_t)/t = k_3[E]K_P/(K_P - K_S) - K_S(K_P + [S]_0)(\ln[S]_0/[S]_t)/t(K_P - K_S) \quad (2)$$

It will be seen from equation 2 that with a plot of $([S]_0 - [S]_t)/t$ *vs.* $(\ln[S]_0/[S]_t)/t$ one will obtain, for various values of $[S]_0$ and t, a series of lines of slope $-K_S(K_P + [S]_0)/(K_P - K_S)$. For each of these lines there is a point corresponding to $t = 0$ and this point may be located by examination of the limits of the two parameters $([S]_0 - [S]_t)/t$ and $(\ln[S]_0/[S]_t)/t$ as $t \to 0$. The limit of $([S]_0 - [S]_t)/t$ as $t \to 0$ is clearly $-d[S]/dt$ and that of $(\ln[S]_0/[S]_t)/t$ as $t \to 0$ is $(-d[S]/dt)\,(1/[S]_0)$. Therefore, for the condition that $t = 0$ lines of slope $[S]_0$ drawn through the origin of a $([S]_0 - [S]_t)/t$ *vs.* $(\ln[S]_0/[S]_t)/t$ plot will intersect the lines of slope $-K_S(K_P + [S]_0)/(K_P - K_S)$ at points corresponding to $t = 0$. It is emphasized that the relation $([S]_0 - [S]_t)/t = [S]_0(\ln[S]_0/[S]_t)/t$ defines a point for the condition that $t = 0$ and does not describe the relation between $([S]_0 - [S]_t)/t$ and $(\ln[S]_0/[S]_t)/t$ for other values of t.

It has been noted above that the parameters of the points corresponding to $t = 0$, *i.e.*, the points of intersection of the lines of slope $[S]_0$ with those of slope $-K_S(K_P + [S]_0)/(K_P - K_S)$, are, respectively, $-d[S]/dt$ for the ordinate and $(-d[S]/dt)\cdot(1/[S]_0)$ for the abscissa. Since by definition $-d[S]/dt = v_0$ *and* $(-d[S]/dt)(1/[S]_0) = v_0/[S]_0$ *it* follows that a line drawn through the points of intersection corresponding to $t = 0$ for various values of $[S]_0$ will possess the same characteristics as one obtained by a plot of v_0 *vs.* $v_0/[S]_0$,[6] *i.e.*, will have a slope of $-K_S$, an ordinate intercept of $k_3[E]$ and an abscissa intercept of $k_3[E]/K_S$. That the initial velocities, *i.e.*, the values of v_0, are defined in terms of the ordinate, *i.e.*, $([S]_0 - [S]_t)/t$, is evident when the terms in K_P in equation 2 are eliminated through the use of the relation $([S]_0 - [S]_t)/t = [S]_0(\ln[S]_0/[S]_t)/t$ to give equation 3 which is singular for the condition that $t = 0$.

$$([S]_0 - [S]_t)/t = k_3[E][S_0]/(K_S + [S]_0) = v_0 \quad (3)$$

(1) Supported in part by a grant from Eli Lilly and Co.

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

(3) R. J. Foster and C. Niemann, *Proc. Natl. Acad. Sci.*, **39**, 999 (1953).

(4) Attention is called to three typographical errors in ref. 3: on p. 1000 the left hand member of equation 4 should read "$k_3[E]t$" instead of "$k_3[E]_t$," on pp. 1000 and 1002 the equation describing the slopes of the $([S]_0 - [S]_t)/t$ *vs.* $(\ln[S]_0/[S]_t)/t$ plots should read "$-K_S(K_P + [S]_0)/(K_P - K_S)$" instead of "$-K_S(K_P + [S]_0)/(K_S - K_P)$" and in Fig. 1, the slopes of the lines drawn through the hypothetical experimental points should read "$-K_S(K_P + [S]_0)/(K_P - K_S)$" instead of "$K_S(K_P + [S]_0)/(K_P - K_S)$."

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Contribution No. 1983 from the
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AUXIN-INDUCED WATER UPTAKE BY AVENA COLEOPTILE SECTIONS[1,2]

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It is known that auxin induces the uptake of water by plant tissues. Three principal suggestions have been made concerning the mechanism of such auxin-induced net water uptake. The first proposes that auxin in some manner plasticizes the cell wall. The second suggests that auxin brings about active synthesis of cell wall material. These two mechanisms suppose osmotic entry of water into the cell in response to lowered wall pressure. The third is that auxin brings about a non-osmotic transport of water into the tissue. Thimann (22) has grouped these into two hypotheses in the form of models. One model visualizes a pump (active transport) and the other a piston arrangement (cell wall pressure reduction). Such active transport has been rigorously defined by Rosenberg (21) as the movement of a substance against a chemical potential or concentration gradient, i.e., an uptake of water against a diffusion gradient as defined by Levitt (16). Active transport of water would consist then of water movement against an osmotic gradient. A fourth possibility in principle would be that an auxin-induced increase in the concentration of osmotically active solutes might occur within the cell.

The cells of plant tissues are surrounded by relatively rigid cell walls and one must discuss water movement in terms of diffusion pressure gradients rather than in terms of osmotic pressure. In the terminology proposed by Meyer and Anderson (19) and by Crafts et al (10), DPD = OP − WP where DPD = diffusion pressure deficit, OP = osmotic pressure of cell contents, and WP = wall pressure. The DPD gradient from an external solution to the inside of the tissue can then be expressed as Δ DPD = $DPD_i - DPD_e$ ($DPD_e = OP_e$) where i = internal and e = external. If Δ DPD is negative, the gradient is outward; if Δ DPD is positive, the gradient is inward. To demonstrate a net active water uptake it is necessary to show that water uptake occurs while the purely osmotic Δ DPD is negative. When the external solution is hypertonic, wall pressure becomes zero and $DPD_i = OP_i$. The net uptake of water from hypertonic solution would then involve movement against an osmotic gradient. It will be shown below that cells of Avena coleoptile sections are essentially in diffusion pressure equilibrium with the external solution over a wide range of external solute concentrations both in the presence and in the absence of auxin. There is no detectable net uptake of water from hypertonic solution by this tissue. It is concluded that water uptake by Avena coleoptile sections both in the presence and absence of auxin is a purely osmotic phenomenon.

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[2] Report of work supported in part by the Herman Frasch Foundation.
[3] Postdoctoral Fellow, National Science Foundation.

MATERIALS AND METHODS

The material used in this work consisted of 5-mm sections cut 2 mm below the apex of Avena (variety Siegeshafer) coleoptiles. The Avena seedlings were grown in vermiculite contained in stainless steel trays and were watered with distilled water. They were harvested when 96 hours old. Only those coleoptiles 30 ± 2.5 mm in length were used. Each part of each experiment was done using 20 sections floated on 20 ml of solution.

Changes in water content of the sections were measured as changes in section length. Net water uptake by coleoptile sections is attended by only small changes in section diameter. Kelly (12) has shown that under conditions similar to those used in elongation studies water uptake in the presence of auxin is linear with time. Measurements of section length were made under a dissecting microscope with a decimillimeter stage micrometer. The variability in elongation rates of sections under the present conditions has been discussed by McRae and Bonner (18).

Three general basal media were used: 1) potassium maleate buffer (0.0025 M, pH 4.8) with or without potassium indoleacetate (5 mg/l); 2) potassium maleate buffer, sucrose (0.09 M), $MnSO_4$ (100 mg/l), arginine (100 mg/l) and with or without potassium indoleacetate (5 mg/l); 3) redistilled water with or without indoleacetic acid (IAA) (5 mg/l, pH 5.0 to 5.5), potassium-free. All solutions were made up with redistilled water.

In the following report, the terms hypotonic and hypertonic are used in referring to solutions external to the cell. These terms are defined as follows: hypertonic = solution whose OP is greater than OP_i; hypotonic = solution whose OP is less than OP_i. It will be shown below that OP_i for freshly cut Avena coleoptile sections under the present conditions is equivalent to approximately 0.4 M mannitol.

EXPERIMENTAL RESULTS

Data on the time course of elongation of sections in media of two different osmotic concentrations are given in figure 1. In these experiments, medium 1 containing buffer alone was used. To this IAA was added as indicated. Mannitol was used as the osmotically active solute. The sections were allowed one hour to come to osmotic equilibrium with the solution before addition of auxin. It is evident that sections do not elongate in response to auxin in an external osmotic concentration of 0.5 M. The use of elevated temperatures and of forced aeration does not increase elongation under conditions of high mannitol concentration.

The respiratory rate of Avena coleoptile sections is depressed in the presence of increasing concentration of an external solute, as is shown in figure 2.

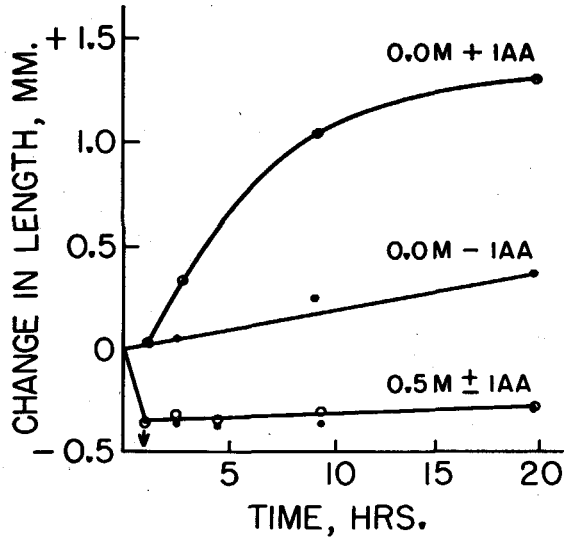

FIG. 1. Effect of external osmotic concentration on elongation of Avena coleoptile sections as a function of time. 0.0025 M potassium maleate, pH 4.8 with or without 0.5 M mannitol, and with or without 5 mg/l IAA. IAA added at arrow.

The rates of oxygen uptake were measured over a period of 3 hours. The presence of IAA increases rate of respiration at low external osmotic concentrations, as has been earlier reported by Commoner and Thimann (9), Bonner (5) and others. The auxin-induced increment in respiration diminishes with increasing external osmotic concentration, and disappears at an OP_e of 0.4 M in which elongation is reduced to a very low value. These results parallel those obtained with Jerusalem artichoke storage tissue in which the respiratory increment induced by IAA disappears as OP_e is increased (6).

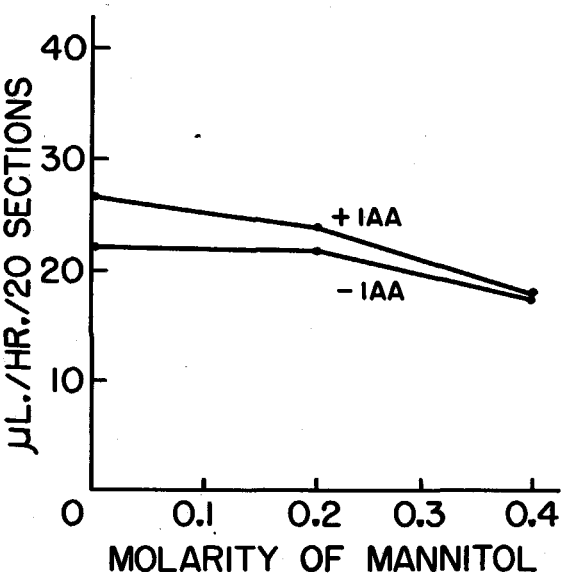

FIG. 2. Effect of external osmotic concentration and of IAA on oxygen uptake of Avena coleoptile sections. 0.0025 M potassium maleate buffer, pH 4.8, 5 mg/l IAA.

It is known that the response of coleoptile sections to IAA is prolonged in time by the addition of sucrose or certain other substances to the media. Figure 3 gives data on the time course of the elongation response to IAA in the more complete basal medium containing sucrose, manganese and arginine, and containing added mannitol to produce varying osmotic pressures. The sections were first equilibrated in the medium for one hour, after which auxin was added. In the basal medium alone ($OP_e = 0.09$ M) elongation starts at once when IAA is added. At

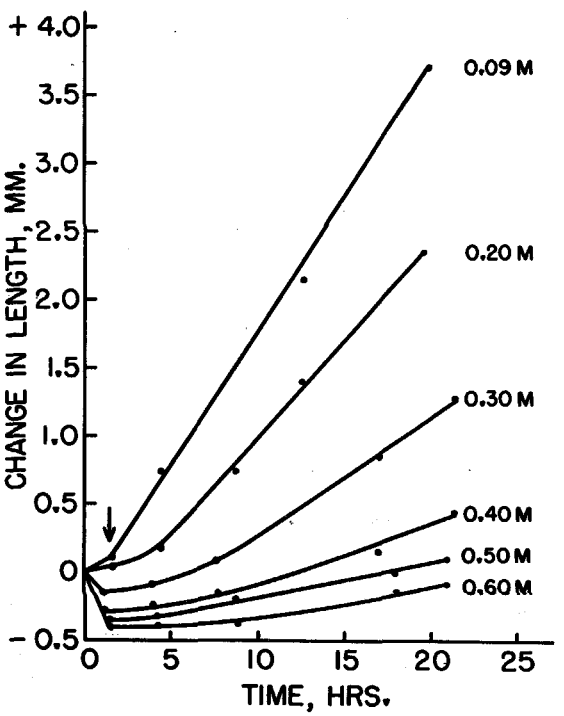

FIG. 3. Effect of external osmotic concentration on elongation of Avena coleoptile sections as a function of time. 0.09 M sucrose plus various concentrations of mannitol, 100 mg/l arginine, 100 mg/l $MnSO_4$, 0.0025 M potassium maleate, pH 4.8. IAA (5 mg/l) added at arrow.

higher values of OP_e, a lag period in attainment of steady state elongation is evident. The length of this lag period is greater the larger the value of OP_e. The final steady state elongation rates also decrease regularly as OP_e is increased. Figure 4 summarizes data on the elongation of sections in the presence or absence of IAA and in media of varying osmotic concentrations. The effects of increasing external osmotic concentration on endogenous elongation (elongation in the absence of added IAA) do not parallel effects on the auxin-induced elongation of these sections. The endogenous elongation exhibits the initial non-linear decline with increasing OP_e at a lower concentration than does elongation in response to added IAA. The two coincide at an external osmotic con-

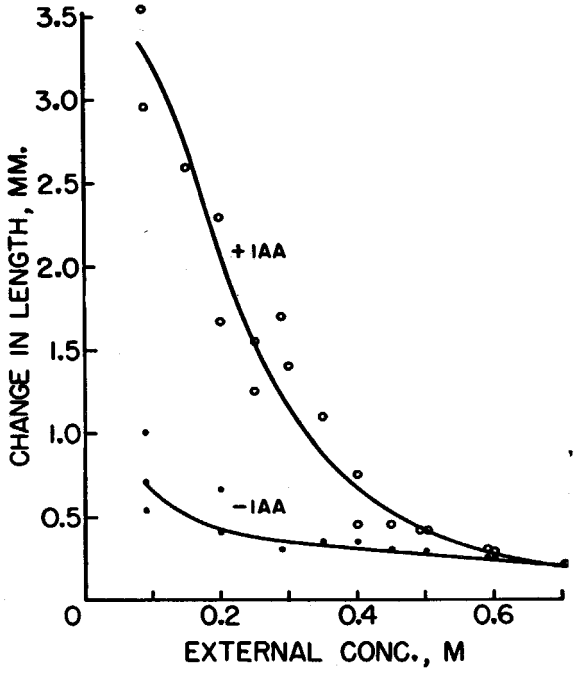

FIG. 4. Effect of external osmotic concentration and of auxin on total elongation of Avena coleoptile sections. Incubation time 20 hrs. 0.09 M sucrose plus various concentrations of mannitol, 100 mg/l arginine, 100 mg/l $MnSO_4$, 0.0025 M potassium maleate, pH 4.8, with or without 5 mg/l IAA.

centration of approximately 0.7 M. The cells of sections placed in solutions more concentrated than approximately 0.4 M are rapidly plasmolyzed, as will be discussed in detail below. After a 20-hour incubation period, however, deplasmolysis has occurred. This is true, for example, of sections placed in solutions as concentrated as 0.6 M. Elongation has then occurred in the presence of solutions initially hypertonic to the 0.4 M osmotic concentration of the initial sections.

Burström (7) has shown that the irreversible extension of wheat roots is independent of OP_e in the hypotonic region. This would suggest that the cell elongation of such roots is not due to a mere passive stretching of the wall and favors some hypothesis relating active wall synthesis to cell enlargement. In order to determine if a similar situation obtains for the Avena coleoptile, sections were placed in plasmolyzing solutions for one hour after 20 hours of elongation. The difference between initial plasmolyzed length (plasmolyzed at zero hours) and final plasmolyzed length (plasmolyzed at 20 hours) is taken as the irreversible elongation in accordance with Burström's definition. The data for these experiments are given in figure 5. The irreversible component, like the total elongation of Avena coleoptile sections, is an inverse function of OP_e. The Avena coleoptile is not, therefore, similar in behavior to Burström's wheat roots as far as irreversible extension is concerned. In the case of the Avena coleoptile, the greater the turgor pressure the greater the elongation rate. This implies that something more than intimate contact of cytoplasm with the cell wall is essential to increase in coleoptile length.

Osmotic Quantities during Elongation

In order to ascertain to what extent purely osmotic considerations govern auxin-induced water uptake in the Avena coleoptile it is necessary to have measurements of DPD and of OP_i during the elongation process. The simplified method of Ursprung (25) was used for determination of the DPD_i of Avena coleoptile tissue. The method consists in measuring the lengths of a group of sections which are then placed in a graded series of mannitol concentrations. The sections are held submerged in stainless steel baskets and argon bubbled through the solutions to maintain anaerobic conditions. A separate group of sections is used for each mannitol concentration. After one to two hours, the sections are removed and their lengths quickly measured. That group which shows no change in length is said to have been in a solution whose $OP = DPD_i$. The maintenance of anaerobic conditions during the DPD determination insures to a high degree that metabolic processes such as are essential to IAA induced net water uptake are kept at a minimum and that any such metabolic components of DPD are not measured.

According to classical osmotic theory, the simplified method of Ursprung should also be capable of yielding values of OP_i for the tissue measured. The cytoplasm should pull away from the cell wall at incipient plasmolysis and as OP_e is further increased, the cytoplasm should continue to contract without

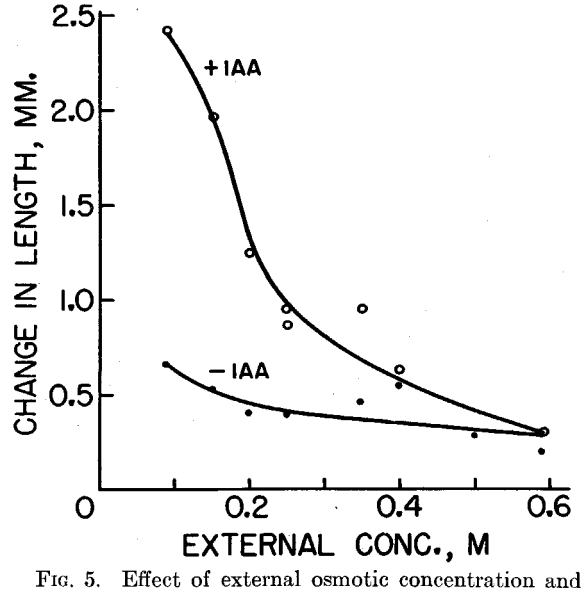

FIG. 5. Effect of external osmotic concentration and auxin on irreversible elongation of Avena coeloptile sections. Incubation time 20 hrs followed by plasmolysis in 1 M mannitol. Same experimental conditions as figure 4.

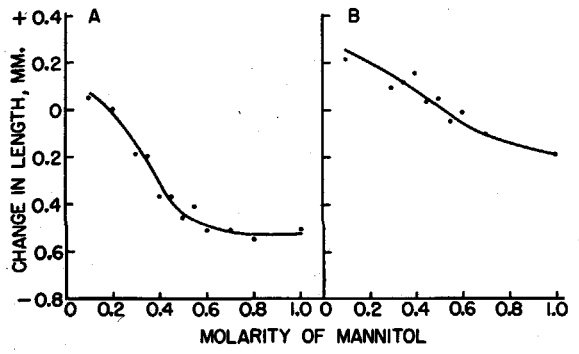

FIG. 6 A. Change in length of Avena coleoptile sections as a function of external mannitol concentration. Incubation for 1 to 2 hours under anaerobic conditions. Freshly cut sections.

FIG. 6 B. Change in length of Avena coleoptile sections as a function of external mannitol concentration. Incubation for 1 to 2 hrs under anaerobic conditions. Sections previously grown 20 hrs in complete medium of osmotic concentration 0.5 M (mannitol + sucrose) solution and containing 5 mg/l IAA.

further cell shrinkage. Hence, the curve which relates tissue length (or change in length) to OP_e should show a sharp inflection at the OP_e of incipient plasmolysis, and should become a straight line of zero slope for higher values of OP_e. Ketellapper (13) has previously attempted to determine the osmotic concentration of Avena section tissue in this way. His data appear to follow closely the expectations of classical osmotic lore. This is not true in the present experiments. Figure 6 A presents data on the length of freshly excised Avena sections after one hour of anaerobic incubation in media of varying OP_e. The length of the sections decreases with increasing OP_e up to an external concentration of approximately 0.6 M. Although there is an inflection in the curve between 0.4 and 0.6 M, there is no sharp change in slope. OP_i apparently lies between 0.4 and 0.6 M but cannot be determined more precisely by this method. The same is true for tissue which has been previously incubated in a medium of high OP_e. Figure 6 B gives data on tissue incubated anaerobically for one hour in media of varying OP_e after 20 hours in solution containing IAA in 0.5 M solution (mannitol plus sucrose). There is no evident inflection in the curve of figure 6 B. It is necessary to conclude, therefore, that OP_i cannot be measured in this way for Avena coleoptile section tissue. This method is, however, satisfactory for DPD determinations, and yields a precision of determination of approximately $\pm$ 0.05 M. Table I gives DPD values for sections after varying pretreatment. The data of table I show that DPD_i as measured by the short term anaerobic incubation method is always equal to the OP_e of the external solution in which the section has previously been incubated. Equilibration of tissue DPD with external OP appears to take place within one hour over a wide range of OP_e values. Equilibration of DPD_i and OP_e is maintained through a 20-hour period both in the presence and absence of added auxin. There is no evidence in these data for any component of DPD_i dependent on aerobic metabolism and measurable by the present method. This important point was further studied by experiments in which argon was bubbled through solutions in which sections had previously been elongating under the influence of auxin. The change of section length with time in argon for

TABLE I

DIFFUSION PRESSURE DEFICIT (DPD) OF AVENA COLEOPTILE SECTIONS AFTER INCUBATION FOR 1 HR AND 20 HRS IN MEDIA OF DIFFERENT OSMOTIC CONCENTRATIONS

EXTERNAL CONC, M	DPD OF SECTION TISSUE, M		
	AFTER 1 HR	AFTER 20 HRS	
		+IAA	-IAA
0.25	0.25	0.27	0.28
0.35	0.36	0.39	0.40
0.40	0.37	0.41	0.46
0.50	0.47	0.52	0.45
0.59	0.60	0.54	0.56

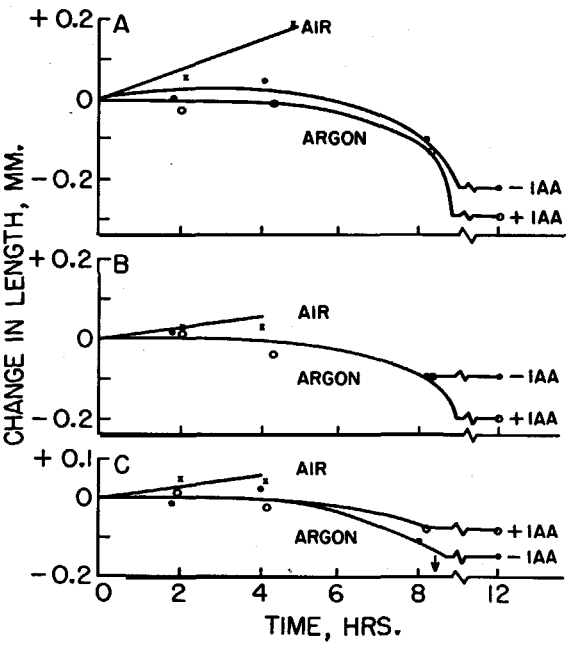

FIG. 7 A. Effect of anaerobic conditions (argon) on change in length of Avena coleoptile sections in 0.4 M solution (0.0025 M potassium maleate, pH 4.8, 100 mg/l arginine, 100 mg/l $MnSO_4$, 0.09 M sucrose, mannitol, with or without 5 mg/l IAA) following 20 hrs in same solution under aerobic conditions. Sections transferred to distilled water at arrow indicated in figure 7 C.

FIG. 7 B. Effect of anaerobic conditions on change in length of sections in 0.5 M solution. Conditions same as in figure 7 A except for mannitol concentration.

FIG. 7 C. Effect of anaerobic conditions on change in length of sections in 0.6 M solution. Conditions same as in figure 7 A except for mannitol concentrations.

sections in solutions of several different values of OP_e are plotted in figure 7 A, B and C. No change in section length occurs during the first four hours after conditions are made anaerobic. After 4 hours the sections begin to shrink. This is because the tissue is killed by the prolonged anaerobic incubation as shown by microscopic examination and lack of section expansion after transfer to distilled water. Two hours of anaerobiosis are, however, evidently without deleterious effects. These data tend to confirm the conclusion that the tissue is in DPD equilibrium with the external solution at all times, and that IAA-induced elongation occurs in the absence of any apparent DPD gradient over the entire range of external osmotic concentrations used. Since water enters the cell under the influence of auxin it must do so under the influence of a positive DPD gradient. This is apparently small and not detectable by the present methods of measurement.

When sections are placed in hypertonic solution, their cells are rapidly plasmolyzed. The cells of such sections subsequently deplasmolyze under aerobic conditions. One might, therefore, conclude that absorption of solutes or production of solutes within the tissue has taken place. According to this view, the sections deplasmolyze because OP_i is increased and the solution is no longer hypertonic. At the same time, wall pressure increases above zero so that DPD remains constant while OP_i increases. Le Gallais (14) has shown that the OP of sap expressed from Avena coleoptile section tissue increases proportionally to external osmotic concentration regardless of solute used and both in the presence and absence of auxin. Le Gallais' work was done with the cryoscopic method of osmotic pressure determination. His results suggest, however, that the cryoscopic values for OP_i are erroneously high because the sap expressed includes plasmolytic solution which has entered the tissue without entering the vacuole. The cryoscopic technique is not, therefore, useful for the present purpose. That an increase in OP_i does take place during incubation of sections in initially hypertonic solutions can however be shown by the plasmolytic method of OP determination. This method consists in the examination of coleoptile sections from the DPD determination solutions under the high power of the microscope. Cells are counted through the median region of the layer of cells just below the outer epidermis. OP_i is taken as equal to the OP_e of that solution in which sections have 50 % of their cells plasmolyzed.

The initial OP_i of freshly cut Avena coleoptile cells was found to be 0.42 M. Sections incubated for 20 hours yield various values of OP_i, depending on the solution used for incubation. Results of typical experiments are summarized in table II. In the first place, sections incubated in water alone (no solute) decrease in OP_i if IAA is present. This effect is apparently due to dilution of cell contents by the water taken up under the influence of auxin as has been previously described. Sections in mannitol alone show small increases in OP_i over the 20-hour period. Since some elongation occurs in mannitol, a small amount of mannitol must have entered the tissue. The addition of sucrose to the medium, however, permits very large increases in OP_i to take place. This is true both for solutions which are initially hypotonic and for those which are initially hypertonic (with added mannitol). The osmotic pressure increase in mannitol plus sucrose is larger than that in sucrose alone in the presence of IAA. This is undoubtedly due to the greater dilution of the cell contents in the latter case as pointed out by Hackett (11) for potato tissue. The presence of sucrose in the medium evidently permits the tissue to make an extensive adjustment of OP_i.

TABLE II

INTERNAL OSMOTIC PRESSURE OF AVENA COLEOPTILE SECTIONS AFTER INCUBATION IN VARIOUS SOLUTIONS

TREATMENT	INTERNAL OSMOTIC PRESSURE (OP_i), M	
	+IAA	-IAA
Redistilled H_2O	0.32	0.42
0.0813 M Sucrose	0.53	0.68
0.59 M Mannitol	0.44	0.45
0.5 M Mannitol + 0.0813 M sucrose	0.69	0.69
0.09 M Mannitol	0.43	0.47
0.09 M Ethylene glycol	0.42	0.52
0.045 M NaCl	0.45	0.60

Incubation: 10 hrs for H_2O treatment, 20 hrs for other treatments. Initial $OP_i = 0.42$ M.

The data of figure 8 further concern the question of the role of sucrose in the elongation of Avena coleoptile sections. Sections were incubated for 29 hours in initially hypertonic solutions made up of mannitol or sucrose and mannitol, and with or without IAA. The sections were initially plasmolyzed by the hypertonic solutions in all cases. Deplasmolysis only occurred with sections incubated in the presence of sucrose. Sucrose must therefore contribute to the

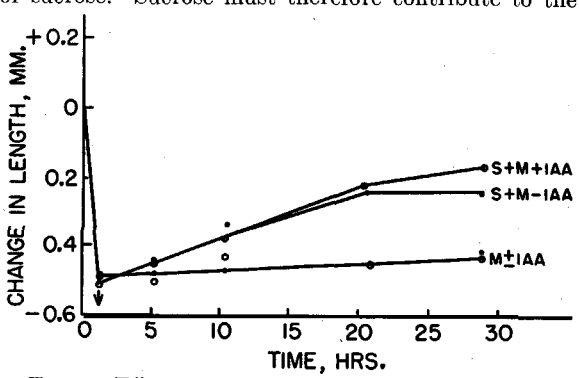

FIG. 8. Effect of 0.0813 M sucrose on change in length of Avena coleoptile sections in initially hypertonic solution containing mannitol in concentrations such that total molarity is 0.59 M. IAA added at arrow. 5 mg/l K-free IAA, no buffer. Final pH 5.5.

increase in OP_i of the tissue. Sections which have deplasmolyzed elongate more slowly than those which have never been plasmolyzed in agreement with the similar findings of Ketellapper (13). Plasmolysis itself apparently causes some irreversible damage.

It has been shown above that the presence of a small amount of sucrose in the medium permits sections to deplasmolyze in an otherwise hypertonic concentration of mannitol. The presence of sucrose similarly maintains OP_i and rate of water uptake in the presence of auxin. The following type of experiment was done to find out whether sucrose pretreatment can maintain subsequent elongation rate in hypotonic mannitol solution. Sections were pretreated in 0.09 M sucrose with IAA for 4 hours and were then transferred to 0.09 M mannitol and IAA for periods up to 20 hours. The results are given in figure 9.

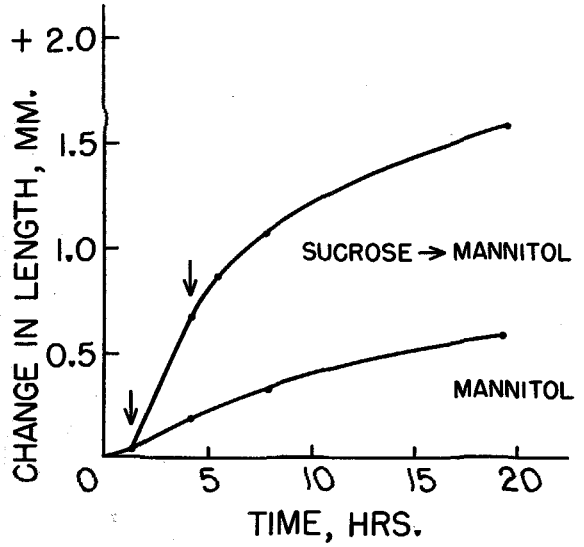

FIG. 9. Effect of 0.0813 M sucrose pretreatment on subsequent elongation of Avena coleoptile sections in 0.09 M mannitol. IAA added at first arrow. Transfer to mannitol at second arrow. 5 mg/l K-free IAA, no buffer; final pH 5.5.

The elongation rate with the latter solution drops off rapidly with time. The initial elongation rate in sucrose is greater than that in an equi-osmolar concentration of mannitol. The initial rate following transfer from sucrose to mannitol is greater than the initial rate for sections maintained in mannitol alone, but the effect does not persist. Apparently the continuing presence of sucrose is required for maintenance of elongation rate. This is consistent with the idea first presented by Thimann and Schneider (24) that sucrose acts in part to maintain OP_i at a constant high level during auxin-induced elongation.

It is of interest to know what other solutes can replace sucrose in its role of maintaining elongation rate and, apparently, OP_i. A variety of permeating substances were used, each in a concentration of 0.01 M. The results, presented as progress curves of elongation, are given in figure 10. Glucose is nearly

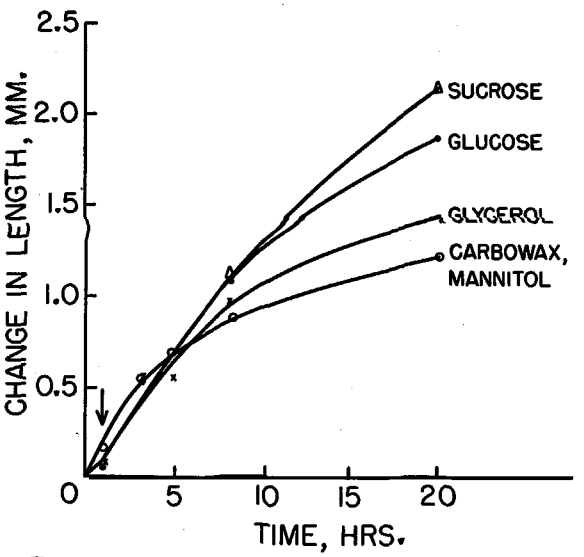

FIG. 10. Change in length of Avena coleoptile sections as a function of time and in the presence of several different solutes. Each solute given at 0.01 M concentration. IAA added at arrow. No buffer, 5 mg/l K-free IAA, final pH 5.5.

as effective as sucrose. Glycerol is but little superior to mannitol in the support of continued elongation rate. Table III gives further data on the elongation rates of sections in solutions containing various solutes, all tested at a higher concentration, 0.09 M (0.045 M for KCl and NaCl). At this concentration, sucrose and glucose are considerably more effective than any of the other solutes tested. It may be seen that the substances used form a series of varying *effectiveness in replacing sucrose or glucose*. Even an apparently non-metabolizable substance such as ethylene glycol is one third as effective as sucrose. NaCl and KCl possess an intermediate status.

TABLE III

EFFECT OF VARIOUS SOLUTES ON IAA-INDUCED ELONGATION OF AVENA COLEOPTILE SECTIONS

SOLUTE	ELONGATION, MM
Sucrose (0.0813 M)	2.86
D-Glucose	2.79
NaCl (0.045 M)	2.23
KCl (0.045 M)	1.75
D-Raffinose	1.16
Glycerol	1.01
Ethylene glycol	0.90
D-Ribose	0.87
Urea	0.77
L-Rhamnose	0.70
D-Xylose	0.61
L-Sorbose	0.55
L-Arabinose	0.23
D-Arabinose	0.22

Solutions 0.09 M except where noted. Incubation 20 hrs. 5 mg/l IAA throughout. Media unbuffered pH 5.4 to 6.1 for all solutions except that containing urea in which pH was 7.0. Elongation above mannitol control.

The absolute elongation of sections in 0.09 M solution is depressed as compared to that in 0.01 M solution for all solutes tested except sucrose and glucose. These two substances are more effective in increasing elongation rate in the 0.09 M concentration than at 0.01 M. This is undoubtedly due in part to the fact that sucrose and glucose are metabolizable as shown by Bonner (4). In addition, however, sections incubated in IAA sucrose-containing solutions show an increase in OP_i as compared to similar sections incubated in mannitol or in glycol, a readily absorbed solute (table II). In the absence of IAA, OP_i is increased somewhat above initial values. The relatively large increases in OP_i in the presence of sucrose may be due to the formation by the tissue of smaller molecules from the large sucrose molecules taken in. In any case, the osmotic readjustment caused by sucrose appears to be independent of the presence or absence of IAA (table II).

It has been noted above that when Avena coleoptile sections are placed in solutions of high external osmotic concentration there is a lag period in the attainment of steady state auxin-induced elongation. The experiments outlined above have suggested that this lag period may represent the time needed by the cells of the tissue to accumulate solutes and to increase in OP_i. The following experiments were carried out to further characterize the effects of different external solutes on the lag period. Sections were placed in hypotonic solution, 0.2 M, containing mannitol alone or mannitol with a small amount of sucrose or NaCl. All solutions contained IAA. The sections were left in these solutions for four hours to permit any osmotic readjustment to occur. The sections were then transferred to an equi-osmolar solution of mannitol, sucrose and IAA. The data of figure 11 show that sections pretreated for four hours in mannitol alone show a lag period in attainment of steady state elongation when transferred to mannitol, sucrose and IAA. If the pretreatment included sucrose or KCl in addition to mannitol the sections start elongating immediately and rapidly attain the final steady state rate. Apparently, the lag period does represent a period of readjustment of internal osmotic concentration. The length of the lag period is dependent on the presence in the external solution of readily absorbable solutes. It may be noted that NaCl behaves as does KCl in permitting shortening of the lag period.

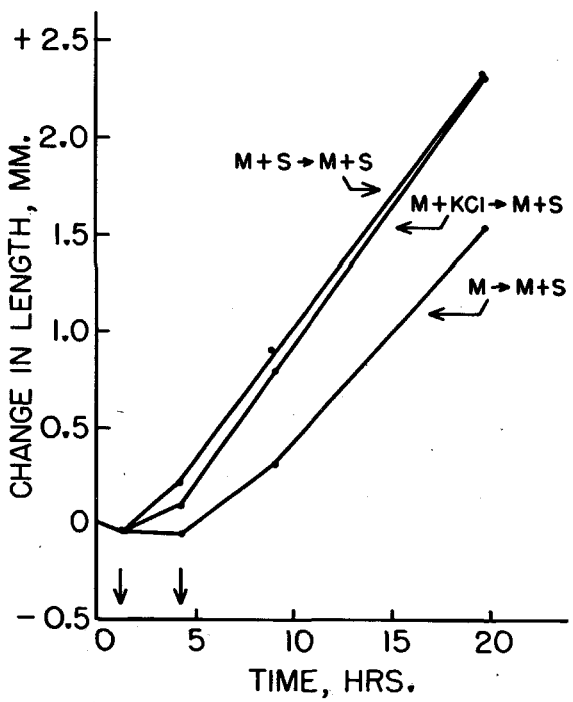

FIG. 11. Effect of pretreatment with mannitol alone or mannitol in the presence of KCl or sucrose on subsequent elongation of Avena coleoptile sections in mannitol and sucrose. Total solute concentration 0.2 M. IAA added at first arrow. Transfer to mannitol and sucrose at second arrow. 5 mg/l K-free IAA, no buffer, final pH 5.0–5.2.

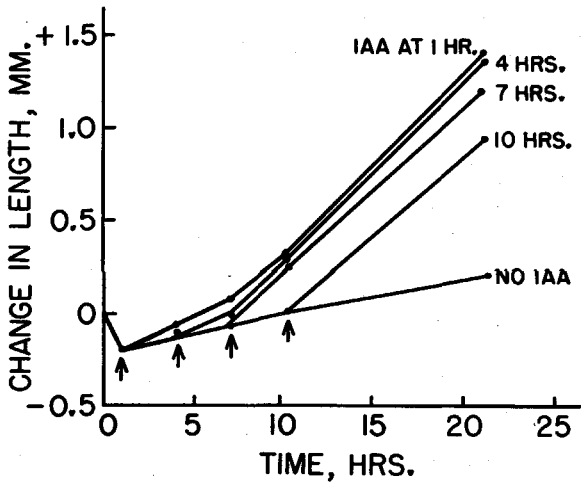

FIG. 12. Effect of time of addition of IAA on subsequent elongation of Avena coleoptile sections in solution of 0.3 M total concentration. IAA added at arrows. 0.0813 M sucrose, 0.21 M mannitol, 0.0025 M potassium maleate, pH 4.8, 5 mg/l IAA.

The osmotic regulation which takes place during the lag period in solutions of moderate osmotic concentration is not dependent upon the presence of auxin in the external medium. In order to test the effect of auxin on the lag process, sections were incubated in 0.3 M solutions containing mannitol and sucrose. Auxin was added at various times and the elongation rate after addition of auxin was followed. The data are presented in figure 12. It is apparent that the lag period extends for about 7 hours and that its duration is independent of the presence or absence of auxin in the external solution.

Discussion

An important conclusion of the present work is that the auxin-induced uptake of water by Avena coleoptile sections follows osmotic principles. It is of interest to consider these results in relation to earlier reports of metabolically maintained DPD gradients.

In order to determine whether a tissue is able to accumulate water against a diffusion pressure gradient it is necessary to possess information concerning the osmotic pressure (OP_i) of the tissue. The simplified method of Ursprung (25) has been shown above to be inapplicable because of the absence of a clear-cut inflection in the volume-OP_e relation as OP_e is increased. Coleoptile tissue as well as other tissues discussed by Crafts et al (10) continue to shrink as OP_e is increased above OP_i. The cryoscopic technique when applied to tissue sections which have been in contact with plasmolyzing solutions is attended by serious errors in assessment of OP_i as has been shown by Le Gallais (14). In the case of plasmolyzed tissues, for example, the cryoscopic method yields values of apparent OP_i which are too high. The plasmolytic method, however, can be used for OP_i determinations even with tissues which have been incubated in initially hypertonic solutions.

Bogen (2) has shown that leaf tissues deplasmolyze under aerobic conditions in sucrose solutions in which they are initially plasmolyzed. A similar result is reported here. In the present case it has been shown that sucrose penetrates the Avena coleoptile sufficiently rapidly to permit of deplasmolysis in less than 20 hours and that the deplasmolysis is an osmotic one. Bogen's experiment would appear to be susceptible to a similar interpretation. It has been reported by Bonner et al (6) that Jerusalem artichoke discs can take up water under the influence of auxin from solutions which are initially hypertonic. Although the determinations of initial OP_i are difficult with this tissue they can be made and the values reported earlier have been confirmed. Determinations of OP_i after a period of water uptake are still more difficult to perform with Jerusalem artichoke tissue and are more questionable. Burström, (8) who used cryoscopic techniques, found that OP_i of such tissue increases with time in initially hypertonic solutions. The cryoscopic technique requires an elaborate correction if it is to be used under these conditions. Nonetheless, it is probable that the OP_i of Jerusalem artichoke increases with time in hypertonic solutions as shown in the present paper for Avena. All of the experiments of Bonner et al were done in the presence of an absorbable solute, namely potassium phosphate buffer. The water loss which takes place when Jerusalem artichokes in initially hypertonic solution (0.2 M mannitol) are transferred to anaerobic conditions may be due to progressive tissue damage as it is with Avena coleoptile sections.

The most nearly valid evidence, at least as a first approximation, of water uptake against a diffusion pressure gradient in higher plants is contained in the work of van Overbeek (20). It was found by van Overbeek that the exudate of an excised tomato root-shoot system may have an osmotic concentration 1 atmosphere less than that of the solution bathing the root. Arisz et al (1) using more refined techniques of measurement reduced this difference to 0.5 atmosphere or less. The latter workers propose that the lower OP of the exudate is due to salt absorption from the sap between the root and the stump as well as to dilution by water from tissues adjacent to the xylem. Furthermore, if the zone of water absorption exceeds the zone of salt absorption, dilution of the sap would occur.

Neither the present work, nor other work reported on water, clearly support the concept of active uptake as defined by Rosenberg (21) and Levitt (16). It is clear however that the performance of auxin-induced water uptake is attended by an increase in respiratory rate and that the auxin-induced increment is abolished by external solute concentrations which abolish net water uptake. The increase in respiration caused by auxin does not then have to do with water accumulation against a water concentration gradient but would appear rather to be related in some way to the primary act involved in cell elongation.

It has long been known that sugars (3) or potassium salts (24) act as co-factors in auxin-induced elongation of coleoptile sections. The role of these substances is in part an osmotic one. They contribute to the maintenance of OP_i. The presence of sucrose in the medium extends the period over which elongation is linear with time. This effect cannot be achieved by sucrose pretreatment as shown by experiments in which sections decrease in elongation rate after transfer to sucrose-free solutions. The effect of sucrose on rate of water uptake may be due in part to the utilization of the material in respiration and in the support of cellular syntheses. In addition however sucrose clearly provides internal osmotically active material both as intact sucrose molecules and perhaps as metabolically produced smaller molecules. The lesser effectiveness of salts and especially of other sugars is presumably due to their being less effective as food material and less abundantly converted to smaller molecules.

It has been concluded that in the Avena coleoptile DPD_i is at all times essentially in equilibrium with OP_e and that by the methods presently available no detectable differences between these two quantities can be found. Within the limits of measurement of DPD ($\pm$ 0.05 M) no movement of water against a DPD gradient appears to take place with Avena coleoptile sections. When non-absorbable solute is used to constitute a hypertonic solution, no elongation of the tissue takes place. It is therefore highly unlikely that active water uptake is a factor in auxin-induced elongation in this tissue. This agrees with the conclusions reached earlier with other tissues by Levitt (15, 17) and by Thimann (23). Adjustments

of osmotic concentration take place in the coleoptile section provided that an absorbable solute is present in the medium. These osmotic adjustments are however independent of auxin and occur in the absence as well as in the presence of added growth substance.

Since auxin-induced water uptake is a purely osmotic phenomenon in the Avena coleoptile and since auxin does not appear to directly influence internal osmotic concentration, it may be concluded that auxin must in some way decrease cell wall pressure. The fact that the irreversible component of the total elongation behaves as an inverse function of OP_e in the hypotonic region is consistent with the hypothesis that cell wall plasticization rather than cell wall synthesis is a primary cell wall effect. This is in contrast to the state of affairs with wheat roots (7) in which irreversible extension is independent of OP_e in the hypotonic region as reported by Burström. In any case it would appear that a detailed investigation of cell wall metabolism as related to the presence or absence of auxin may be required for the further elucidation of the mechanism of auxin action.

Summary

Avena coleoptile sections are in apparent diffusion pressure equilibrium with the external solution at all times, i.e., there is no detectable aerobic metabolic component of DPD in either hypertonic or hypotonic solution.

Both total elongation and irreversible elongation are inverse functions of external osmotic concentration in the hypotonic region.

Tissue placed in initially hypertonic solution deplasmolyzes and grows if a sufficient concentration of an absorbable solute such as sucrose is present in the external solution. It has been shown that the role of the permeating solutes is to cause an increase in internal osmotic concentration of the tissue.

Tissue in hypotonic solution maintains or increases in internal osmotic concentration as elongation occurs, provided that absorbable solute is present in the external solution. Tissue maintained in distilled water exhibits a decrease in internal osmotic concentration as elongation occurs.

When Avena coleoptile sections are placed in slightly hypotonic or in hypertonic solution there is a lag period in the attainment of steady state elongation rate. The duration and intensity of this lag period increases with external osmotic concentration. The length of the lag period is independent of the presence of auxin and is determined by the time required by the section to make an osmotic readjustment to the external solution by absorption of solutes.

Auxin-induced water uptake in Avena coleoptile sections does not appear to be due to the effect of auxin on maintenance of a metabolically controlled component of internal diffusion pressure deficit or to effects on the osmotic concentration of the tissue. The present evidence points to effects of auxin on the cell wall as an important factor in auxin-induced cell elongation.

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PART VI

PROPOSITIONS

PROPOSITIONS

1. The apparent affinity of alpha-chymotrypsin for inhibitors of catalyzed reactions appears to depend mainly on the presence of an aromatic residue in the inhibitor molecule (1). It is proposed that this affinity will be modified by the non-aromatic portion of the molecule, and the use of the N-nicotinyl- or N-benzoyl-L-amino acid N'-methylamides will permit an estimate of the effects of the non-aromatic side chains on the binding of these molecules by the enzyme.

2. N-Substituted mono-, di- and triamides of phosphoric acid have been shown to react with carboxylic acids to yield N-substituted carboxylic acid amides (2). It is proposed that the recently described triamidophosphate (3) will be a useful reagent for the direct preparation of carboxylic acid amides from the acids.

3. Two different mechanisms (cf. 4) have been advanced for the formation of hydrazines by the reaction of chloramine with ammonia, primary, secondary and tertiary amines. The first suggests that the added base produces the reactive species from chloramine, while the second considers the reaction to be a bimolecular displacement reaction. It is proposed that these differences could be distinguished by a study of the reactions between suitable alkyl amines and alkyl-

chloramines. In addition, this would provide a synthetic route to 1,2-disubstituted hydrazines.

4. It has recently been suggested that the acylation of alpha-chymotrypsin with p-nitrophenylacetate is a type of reaction that can be extended to describe the behavior of this enzyme in all of its hydrolytic reactions (5). It is proposed that the highly active esters such as the p-nitrophenylacetates are not cleaved in a reaction similar to that of the usual acylated amino acid derivatives, and, further, that the hydrolysis of p-nitrophenyl esters of acylated amino acids will not display the stereospecificity associated with the usual chymotryptic activity.

5. While the base dependence of the reactions of the primary amine S-phenylthiocarbamates with amines has been firmly established, the rate dependence on base concentration has not been determined (6,7). A definite solution to this problem could be realized by a spectrophotometric study of the base catalyzed reaction of S-(p-nitrophenyl)-N-phenylthiocarbamate with amines.

6. Amino acids labeled with C^{14} in the 1 or 2 position have been shown to display an isotope effect in ion-exchange chromatography (8). It is proposed that such

effects will also be noted by a time study of enzymatic synthetic reactions, and further, that such effects may assume statistical importance in incorporation studies of such labeled compounds in biological systems.

7. It has recently been reported that the direct acylation of 3-picoline in the presence of potassium amide can be carried out employing esters of heterocyclic or aromatic acids, but that the reaction fails with aliphatic esters (9). It is proposed that this is due to the greater basicity of the 3-picoline anion as compared to the aliphatic ester anions coupled with a greater reactivity of the latter to form acetoacetic ester type compounds. These effects could be overcome by the use of aliphatic esters that do not contain alpha-hydrogen atoms.

8. It is proposed that the reaction of thionyl chloride-methanol solutions with carboxylic acid amides to yield the esters and ammonium chloride under mild conditions (1) provides a method for the rapid determination of total asparagine and glutamine in polypeptides.

9. It has been suggested that the two exceptions to the "ortho" rule (10) i.e., 2,4-dichloro-6-fluoro- and

2,4-dibromo-6-fluorophenoxyacetic acids, owe their growth promoting activity to conversion to the active 2,4-compounds or to a lack of steric hindrance by the small ortho-fluorine atom (11). It is proposed that:

a. These compounds be critically re-evaluated as auxins.
b. If they are active the latter reason above is the more likely.
c. If they are active, the 2,4-difluoro-6-chloro analog should be even more active.

10. 2,4-Dichlorophenoxyacetic acid pretreatment of sections of bean seedlings inhibits polar transport of indoleacetic acid (12). It is proposed that the following series of experiments would provide valuable information regarding this process:

a. Equilibration of the pretreated sections with buffered solutions followed by transport tests.
b. Equilibration of the pretreated sections with indoleacetic acid solutions followed by transport tests.
c. Progressive decapitation followed by transport tests.

11. A simple change in the wiring of the ceiling lights in 22 Gates could be easily accomplished. It is proposed that everyone in attendance would benefit if the last row of lights was never extinguished during general chemistry seminars.

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