S T U D I E S I N C A R B O H Y D R A T E
A N D A M I N O A C I D C H E M I S T R Y

Thesis by

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In Partial Fulfillment of the Requirements

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Acknowledgement

It is a pleasure to acknowledge gratefully the guidance and encouragement which I received from Professor Carl G. Niemann during the course of my graduate studies and research.

I am grateful to Professor H. J. Lucas for his interest in my work, and for many helpful discussions.

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I wish also to express my appreciation and gratitude to my wife, Alice, for her help and kind, patient understanding.

Abstract

The optimum conditions for the colorimetric determination of hexoses by reaction with carbazole in hot sulfuric acid solution have been determined, and a convenient procedure for the analysis is described.

The inhibition of the chymotrypsin catalyzed hydrolysis of N-acetyl-L-tyrosylglycinamide by equimolar quantities of its D antipode has been observed to be a function of the concentration of the DL mixture. The general features of the above system have been described, and an explanation has been advanced relative to the lack of antipodal inhibition obtaining in the case of N-benzoyl-DL-tyrosylglycinamide.

Several new tyrosine derivatives have been synthesized which have been used to show that the rate of the chymotrypsin catalyzed hydrolysis of N-acyl-L-tyrosinamides is critically dependent upon the nature of the acyl group. The pertinent equilibrium and rate constants have been determined.

The competitive nature of the inhibitory effect of N-acetyl-D-tyrosinamide on the enzymatic hydrolysis of N-acetyl-L-tyrosinamide has been demonstrated. An inhibitory effect by the corresponding D ethyl ester is described.

A rapid and convenient method for the resolution of DL tyrosine has been devised.

The hydrolysis of N-acetyl-L-tyrosinhydrazide by chymotrypsin has been demonstrated.

Table of Contents

PROPOSITIONS

(Submitted by - R. V. MacAllister)

1. It is common engineering practice to remove as much water as possible from suspensions by filteration before passing the material through a relatively costly drying process. Often the amount of water removed by filtration falls far short of what one could hope for on the basis of void volumes. I have observed that water can be filtered out of starch suspensions much more nearly completely if a relatively small amount of surface active material is added to the suspensions. I propose that this effect be studied in detail to determine the optimum conditions and range of its applicability.

2. It has been observed that high molecular weight petroleum fractions are carried through natural gas lines when the pressure in the line is above the critical value of the more volatile constitutents. I propose that this effect be applied to the "distillation" of high molecular weight substances for their purification and possibly as an analytical tool.

3. Starch has been used extensively as an adsorbent in the chromatographic analysis of materials such as protein hydrolysates. The adsorption capacity of starch is, in

PROPOSITIONS (Cont.)

many cases, associated with the unbranched fraction of the starch. I propose that the unbranched fraction be crystallized and evaluated as an adsorbent in chromatographic analysis of this type.

4. The Michaelis Menten theory of enzyme activity postulates a substrate-enzyme complex as an intermediate. Kinetic studies indicate that competitive inhibitors form similar complexes which have equilibrium constants of about the same order of magnitude as that of the substrate-enzyme complex. I propose that a critical test of this hypothesis could be carried out by osmotic pressure measurements of the enzyme in the presence and absence of the inhibitor.

cf. Keilin, D., and Mann, T., Proc. Royal Soc., London, B122, 119, (1937)

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

5. It is often necessary to eliminate enzyme activity in food products. The usual heat treatment often causes "off" flavors and colors to develop. I propose that the use of specific inhibitors be investigated and applied in such cases.

PROPOSITIONS (Cont.)

6. I propose that an enzyme be removed from solution in a specific manner by:

   A. Suspending an excess of a finely divided, insoluble inhibitor in the enzyme solution to function as a specific adsorbent.

   B. Adding the enzyme to a highly supersaturated solution of the inhibitor, and then effecting a rapid crystallization of the inhibitor.

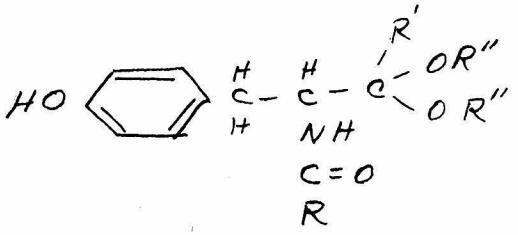
7. I propose that the treatment of amines or amino acid derivatives by carbon monoxide be studied as a method of formylation.

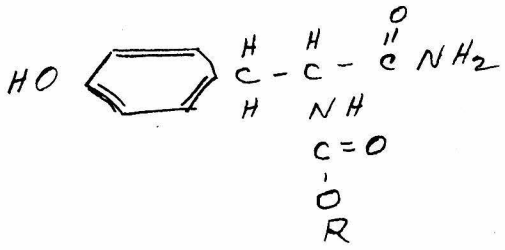
8. I propose that polymers of "D"-tyrosine be prepared and tested for specific adsorbent and proteolytic activity.

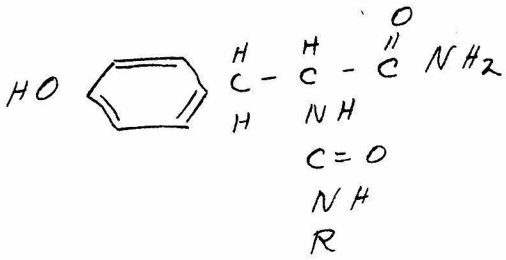
9. I propose that the Kolbe type reaction be investigated, as a possible method, for the preparation of cyclobutadiene.

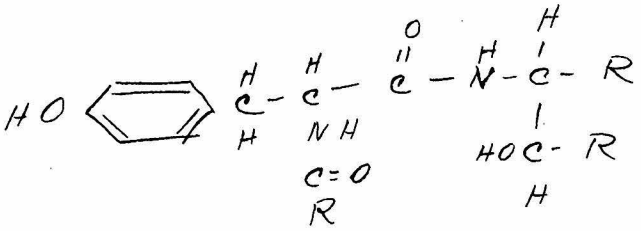
10. I propose that the effect of variation in the nature of the secondary peptide group of a chymotrypsin substrate is related to the negative charge on the carbonyl oxygen and that this effect might be significant in the anomolous synthetic action of popain.

11. I propose that the following types of compounds be prepared and investigated on the basis of susceptibility to chymotrypsin activity.









Part I

The Colorimetric Determination of Hexoses With Carbazole

By George Holzman, Robert V. MacAllister,
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* Contribution No. 1137

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THE COLORIMETRIC DETERMINATION OF HEXOSES WITH CARBAZOLE

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

(Received for publication, July 16, 1947)

The difficulty of applying classical procedures to the qualitative and quantitative determination of carbohydrate in certain biological materials has led to the development of colorimetric methods in which the carbohydrate-containing material is treated with strong mineral acids, causing the formation of substances which will react with compounds such as diphenylamine, resorcinol, orcinol, indole, carbazole, etc., to give distinctively colored products. The rate at which these colored products are formed (1) and the nature of their absorption spectra are frequently sufficiently distinctive to allow their use in differentiating the various sugars.

One of the most widely used of the above colorimetric methods is the carbazole-sulfuric acid method first described by Dische (2–4) and further developed by Gurin and Hood (5, 6) for the identification and estimation of hexoses and pentoses. The latter procedure was used by Seibert and Atno (7) for the analysis of the polysaccharides present in serum and by Knight (8) for the identification of the sugars present in influenza virus. Dische (9) has recently described a modification of the carbazole method for the analysis of uronic acids.

Although the carbazole-sulfuric acid method has been used extensively, no systematic study of the variables influencing this method appears to have been reported. Difficulties encountered in the quantitative application of the carbazole method have been commented upon (7) and indeed the significance of the carbazole reaction, or other color tests, for the qualitative identification of sugars has been questioned (10). Recently in the course of a study of the polysaccharide fractions from hog gastric mucin, we have had occasion to investigate the more important variables associated with the carbazole reaction as applied to the determination of hexoses. In the course of this study the optimum conditions for the quantitative determination of hexose were determined and certain aspects of the qualitative identification of hexoses were examined.

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† Contribution No. 1137.

EXPERIMENTAL

Reagents—Eastman White Label carbazole was precipitated three times from a concentrated sulfuric acid solution by dilution with cold water, and the dried product recrystallized from toluene. The sugars were recrystallized from aqueous ethanol according to conventional methods (11). Technical furfural was fractionally distilled and the fraction boiling at 80–81° and 50 mm. reserved for use. In order to obtain lower blanks, C.P. sulfuric acid was refluxed with potassium persulfate (20 mg. per liter) until a negative test for oxidizing agents was obtained with starch-iodide.

Apparatus—A Klett colorimeter, green filter No. 54, was used for all colorimetric analyses. Duplicate or triplicate analyses were always performed and the results reported are average values. A Beckman model DU spectrophotometer, equipped with a 1 cm. cell, was used in determining spectral absorption. Intensities were measured at 10 mμ intervals except

TABLE I

Effect of Sulfuric Acid Concentration

Concentrated H_2SO_4 added	Klett value*
per cent by weight	
80	215
82	230
84	230
86	220
89	180

* Corrected for blank on 1 ml. of water.

in the region of maxima and minima where the interval was reduced to 2 to 5 mμ.

Effect of Sulfuric Acid Concentration—With the amount of carbazole set at 1.5 mg., the quantity of hexose at 100 γ of glucose per ml., and 10 minutes for the time of heating, variation of the sulfuric acid concentration gave rise to the values shown in Table I. Similar results were obtained with galactose.

Effect of Carbazole Concentration—With the concentration of the added sulfuric acid maintained at 84 per cent (by weight), the amount of hexose at 100 γ of glucose per ml., and the time of heating at 10 minutes, variation of the carbazole concentration between the limits of 1.5 and 4.5 mg. gave rise to the values presented in Table II. Similar results were obtained with galactose.

Effect of Time of Heating—Test solutions containing 9 ml. of 84 per cent

sulfuric acid, 3 mg. of carbazole, and 100 γ of glucose in 1 ml. of water were heated in a boiling water bath for varying periods. It was found that 80 per cent of the maximum color intensity was attained after heating for $7\frac{1}{2}$ minutes, 99 per cent after 10 minutes, 100 per cent after 15 minutes, and 97 per cent after 20 minutes. As in other experiments galactose gave similar results.

TABLE II

Effect of Carbazole Concentration

Carbazole present	Klett value*
mg.	
1.5	240
2.5	316
3.0	363
3.5	402
4.5	438

* Corrected for blank on 1 ml. of water.

TABLE III

Determination of Glucose with Modified Procedure

Glucose	Klett value*	Average deviation, Klett units
γ		
140	682	6
120	607	12
100	527	11
80	425	10
60	349	3
40	272	4
20	185	7
10	144	4
5	129	5

* Average of six separate determinations.

Modified Procedure[1]—A reagent was prepared by adding 10 ml. of a 1.0 per cent solution of carbazole in absolute ethanol to 300 ml. of 84 per cent sulfuric acid. 9 ml. portions of this reagent were chilled in an ice bath, 1 ml. of the hexose solution poured onto the reagent, and the solutions

[1] The procedure of Gurin and Hood (5) consists of heating 1.5 mg. of carbazole (0.3 ml. of a 0.5 per cent solution in ethanol), 1 ml. of hexose solution, and 9 ml. of 89 per cent sulfuric acid (8:1 concentrated sulfuric acid and water) for 10 minutes in a boiling water bath.

thoroughly mixed and heated in a boiling water bath for 15 minutes. After cooling in an ice bath, the intensity of the color produced was determined in the Klett colorimeter. Typical results, obtained with glucose, are given in Table III.

DISCUSSION

It is seen from Table I that the Klett values are particularly dependent upon the acid concentration and that maximum color intensity is obtained with 82 to 84 per cent added sulfuric acid. It was shown, by means of extinction curves, that the differences noted in Table I were due to differences in intensity alone. While extinction values varied widely with sulfuric acid concentration, the position of the maxima lay between 540 and 550 mμ in every instance. However, it should be pointed out that qualitative observations of Dische (2) suggest that significant changes in respect to the position of the maxima may occur also if the sulfuric acid concentration is varied widely.

The dependence of the Klett values upon the carbazole concentration (Table II) emphasizes the necessity of precision in adding the carbazole to the reaction mixture. An error of 3 per cent in the addition of 1.5 mg. of carbazole (0.3 ml. of a 0.5 per cent solution in ethanol) would cause a corresponding variation of about 4 units in Klett values. It is obvious that the addition of small volumes of a carbazole solution is undesirable, especially since the solvent is ordinarily absolute ethanol, which is difficult to pipette accurately. While large amounts of carbazole undoubtedly increase the sensitivity of the procedure, especially since the blank values are practically constant over the range studied, the low solubility of carbazole in the diluted sulfuric acid limits the upper concentration.

In contrast to other variables the time of heating is not particularly critical, provided the time is not less than 10 minutes or more than 20 minutes. A period of heating of 15 minutes, with 84 per cent sulfuric acid, appears to be a reasonable choice.

In order to simplify the procedure of Gurin and Hood (5) the carbazole was dissolved in a relatively large quantity of 84 per cent sulfuric acid and aliquots of this reagent were used for analysis. This reagent simplified the procedure for routine analysis as well as eliminated errors arising from the addition of carbazole to individual tubes. The reagent is prepared by mixing an ethanolic solution of carbazole with 84 per cent sulfuric acid, because solid carbazole dissolves very slowly in sulfuric acid of this concentration. Although the reagent is known to be stable for at least 6 hours, occasionally a green color has appeared after standing for more than 24 hours. It is recommended that the reagent be prepared daily, as was suggested by Seibert and Atno (7).

Nitrate and ferric iron are presumed to interfere in the carbazole procedure of Gurin and Hood (5). No interference from these constituents, or from nitrite, was observed with the modified procedure. The same Klett values were obtained, within the limits of precision discussed below, in the presence or absence of 5 γ of sodium nitrate, sodium nitrite, or ferric chloride when the amount of hexose present was 100 γ of glucose. Sodium

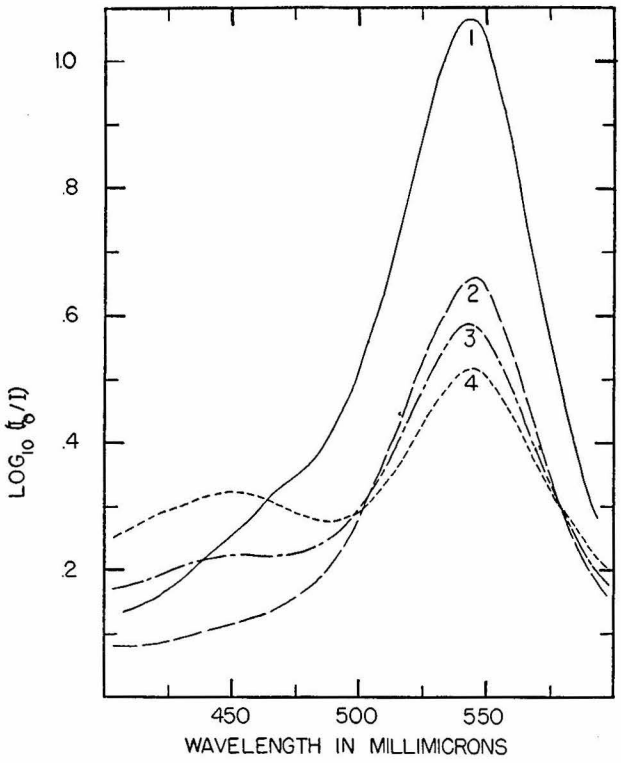

FIG. 1. Absorption curves for colors obtained with various sugars on reaction with carbazole. Curve 1, 100 γ of fructose; Curve 2, 100 γ of glucose; Curve 3, 100 γ of galactose; Curve 4, 100 γ of mannose.

nitrite imparted a faint green color, as did ferric chloride at higher concentrations, to the cold carbazole-sulfuric acid solution. However, this color generally disappears on heating.

While the modified procedure possesses the advantages of convenience and reliability, the precision would appear to be no greater than that of the original when the latter is applied with extreme care. The modified procedure has a precision of 2 to 5 per cent (Table III) in the range of 50 to 150

γ of glucose. The relatively low precision is still unexplained, although the complexity of the reactions occurring in sulfuric acid, revealed by the extinction curves discussed below, suggests that experimental conditions may not still be sufficiently reproducible.

The extinction curves for the carbazole-hexose colored products obtained by the modified procedure are shown in Fig. 1. The curves resemble qualitatively those obtained previously (7, 8). However, the color ob-

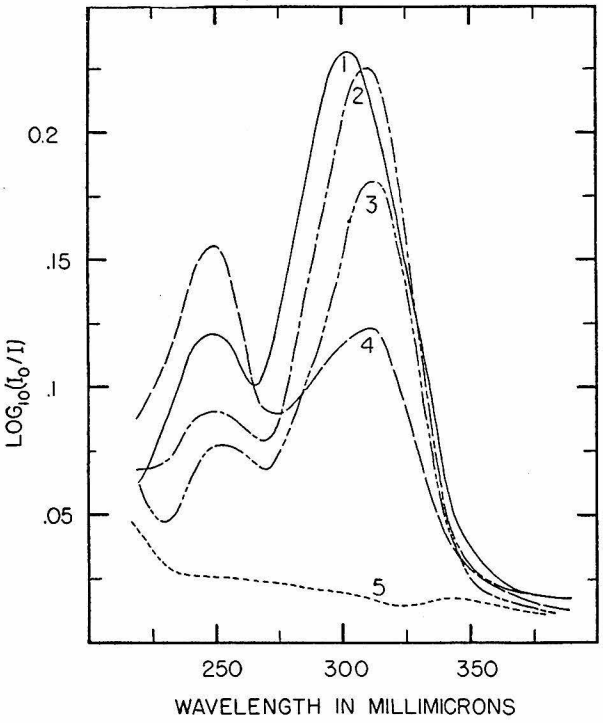

FIG. 2. Absorption curves for various sugars heated in sulfuric acid solution. Curve 1, 100 γ of galactose; Curve 2, 100 γ of glucose; Curve 3, 100 γ of fructose; Curve 4, 100 γ of mannose; Curve 5, 100 γ of N-acetylglucosamine.

tained with mannose does not appear to be as markedly different from that of the other hexoses as has been observed by others (7, 8). It would appear that precise control of the sulfuric acid concentration is of utmost importance, not only for quantitative procedures, but also in the qualitative interpretation of absorption spectra. Owing to the similarity of the curves for glucose, fructose, mannose, and galactose, the low precision of the carbazole method, and because of possible interferences from other

types of compounds (5), the qualitative identification of sugars by means of the modified procedure would appear to be dubious.

Relevant to the problem of spectral identification of sugars are the extinction curves in the ultraviolet region that have been obtained for sugars in sulfuric acid solution in the absence of carbazole. Fig. 2 shows curves obtained for 100 γ of sugar in 1 ml. of water and 9 ml. of 84 per cent sulfuric acid solution after heating 15 minutes on the water bath. The hex-

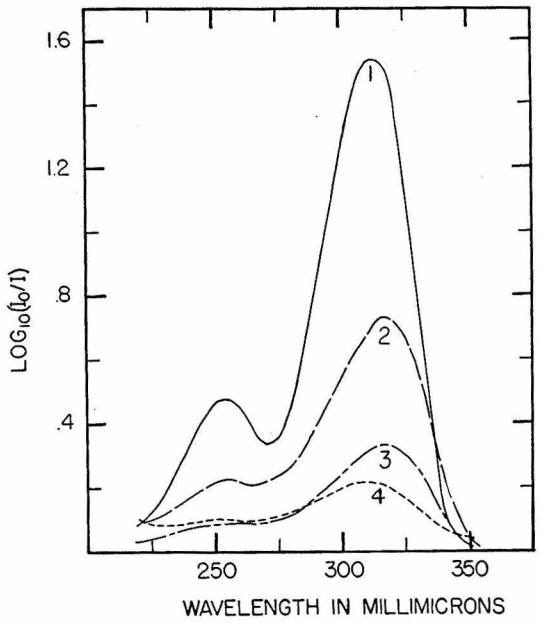

FIG. 3. Absorption curves for furfural and various sugars in sulfuric acid solution. Curve 1, 100 γ of furfural; Curve 2, 100 γ of fructose after standing 40 minutes; Curve 3, 100 γ of fructose after standing 5 minutes; Curve 4, 100 γ of fructose after standing 40 minutes and then heating 15 minutes in a water bath. Curves for galactose, mannose, glucose, and N-acetylglucosamine are not shown, since the densities are below 0.03 throughout the wave-length range.

oses studied exhibit maxima at about 250 and 320 mμ. Fructose, glucose, and galactose show similar curves, while mannose differs in having less absorption at 320 mμ than at 250 mμ. The positions of the maxima correspond closely to those observed for furfural under the same conditions; the apparent conversion of the hexoses to a furfural derivative would appear to be only 10 to 20 per cent as judged from the spectra. While the mechanism of the carbazole reaction and similar color tests is not clearly

understood, presumably the formation of aldehyde intermediates is important (2, 9). It is significant that N-acetylglucosamine, which does not give a color test with carbazole, also shows no specific absorption in the ultraviolet in sulfuric acid solution.

The relative heights of the maxima in the ultraviolet are not correlated with the intensity of the colors produced in the carbazole reaction, a fact which might argue for the unimportance of the compounds showing ultraviolet absorption in the subsequent color reaction. However, extinction curves of unheated sulfuric acid solutions of the hexoses (Fig. 3) suggest a complicating feature. The ultraviolet spectra were found to be sensitive to time of heating, and the extinction values increased and then decreased on heating for successively longer periods. Glucose, fructose, mannose, and N-acetylglucosamine show no appreciable absorption in the cold, while fructose is converted rapidly to an intermediate showing specific absorption in the region 250 to 320 mμ; maximum absorption is reached after about 40 minutes at 25°. When the fructose solution is then heated, the specific absorption decreases markedly, indicating other reactions leading to decomposition. Fructose also shows characteristic behavior in the carbazole reaction in that a color appears several times faster and with greater intensity than for any of the other hexoses. It is apparent that the marked difference in behavior of fructose from other sugars in cold sulfuric acid could readily be adapted to its detection under suitable conditions. Since it is known that heated acid solutions of the hexoses will not react appreciably with carbazole in the cold (9), the carbazole reaction would appear to consist of at least two series of reactions, (1) the conversion of hexoses to intermediates showing specific ultraviolet absorption and the simultaneous decomposition of these intermediates in hot acid solution, and (2) the reaction of some or all of the products with carbazole in hot acid solution to yield a stable visible color.

SUMMARY

The optimum conditions for the colorimetric estimation of hexoses by reaction with carbazole in hot sulfuric acid solution have been determined and a convenient procedure, giving results with a precision of 2 to 5 per cent in the range of 50 to 150 γ of glucose, is described. The colors obtained with glucose, galactose, fructose, and mannose are not sufficiently distinctive to allow their ready differentiation and identification by spectral measurements. The significance of the ultraviolet spectra of heated and unheated sulfuric acid solutions of hexoses to the problem of estimation and identification of hexoses is discussed.

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Part II

The Hydrolysis of N-Benzoyl-DL-Tyrosylglycinamide, N-Acetyl-L-Tyrosylglycinamide, and N-Acetyl-DL-Tyrosylglycinamide by Chymotrypsin

By Robert V. MacAllister, Kent M. Harmon, and Carl Niemann

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THE HYDROLYSIS OF N-BENZOYL-DL-TYROSYLGLYCINAMIDE, N-ACETYL-L-TYROSYLGLYCINAMIDE, AND N-ACETYL-DL-TYROSYLGLYCINAMIDE BY CHYMOTRYPSIN

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(Received for publication, August 16, 1948)

Contrary to the report of Bergmann and Fruton (1) the L peptide present in aqueous solutions of N-benzoyl-DL-tyrosylglycinamide, prepared by two independent methods, is rapidly hydrolyzed by crystalline chymotrypsin. At 40° and pH 7.8 in a 0.00344 M solution[1] of the DL mixture the L component is hydrolyzed as rapidly as in a 0.00172 M solution of the L peptide (Table I). Because of the relative insolubility of N-benzoyl-DL-tyrosylglycinamide, it was not possible to study the reaction at higher substrate concentrations, and attention was directed to the much more soluble N-acetyl-DL-tyrosylglycinamide.

The cryoscopic properties of N-acetyl-DL-tyrosylglycinamide in aqueous solution were determined over a concentration range varying from near saturation to approximately one-third of that value, and no indication was obtained suggestive of extensive interaction between the D and L peptides. Thus it can be concluded that in aqueous solutions of the DL peptide one is confronted with a simple mixture of the D and L peptides.

The chymotrypsin-catalyzed hydrolysis of the L peptide present in aqueous solutions of N-acetyl-DL-tyrosylglycinamide was studied, and at pH 7.8 and either 25° or 40° the rate of hydrolysis of the L component present in a 0.10 M solution of the DL peptide was found to be approximately one-half of the rate observed with a 0.050 M solution of the L peptide. At 40° in a solution 0.00172 M with respect to the L component the rates of hydrolysis were found to be identical, within experimental error, for solutions of the L and DL peptides. These data, summarized in Table II, clearly indicate that inhibition of hydrolysis of the L peptide by the D peptide is a function of enzyme-substrate concentrations and that the process is one involving competitive interaction between the D and L peptides and the enzyme. Thus under conditions of low substrate and inhibitor concentration the enzyme is unsaturated in the Michaelis sense and sufficient

* National Research Council Predoctoral Fellow.

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[1] A molar solution is defined for the purposes of this investigation as one containing 1 gm. formula weight per liter of solution.

reactive sites are available to permit optimum or near optimum hydrolysis of the L peptide, the presence of an equimolar quantity of the D peptide being without demonstrable effect. In solutions of high substrate and inhibitor concentration the enzyme can be considered to be operating at or near the limiting rate, and under these conditions interaction between the enzyme and the D peptide can cause substantial inhibition of the rate of hydrolysis of the L peptide. A more precise definition of the above system must await the accumulation of additional data.

Further study of the system involving N-benzoyl-DL-tyrosylglycinamide does not appear attractive, since it is clear that the limited solubility of this peptide and the relatively rapid rate of hydrolysis of the L component by chymotrypsin would prevent the attainment of conditions requisite for

TABLE I

Hydrolysis of N-Benzoyltyrosylglycinamide at 40° and pH 7.8

The DL substrate = 0.00344 M; the L substrate = 0.00172 M.

E_0, mg. protein N per ml.	Time of reaction	$S_0 - S$		First order rate constant, k		Proteolytic coefficient, C	
		DL	L	DL	L	DL	L
	min.	*mM per ml.* $\times 10^3$	*mM per ml.* $\times 10^3$				
0.0154*	10	0.66	0.70	0.049	0.052	3.2	3.4
	20	1.18	1.22	0.058	0.062	3.7	4.0
0.0154†	10	0.71	0.65	0.053	0.047	3.5	3.1
	20	1.24	1.15	0.063	0.055	4.1	3.6
	30	1.60	1.38				

* Crystalline chymotrypsin preparation from Armour.

† Crystalline chymotrypsin preparation from Lehn and Fink.

the demonstration of inhibition by the D antipode. Practically there can be no objection to the use of N-benzoyl-DL-tyrosylglycinamide, which is much more readily available than its L component, as a test substrate for chymotrypsin-like activity.

In the hydrolysis experiments it was observed that a plot of the log of the substrate concentration *versus* time was generally linear, indicating that the reaction was approximately first order with respect to substrate. Rate constants and so called proteolytic coefficients (2) calculated by the commonly used first order rate expression are given in Tables I and II. Comparison of the proteolytic coefficients, which should be considered as approximate values because of possible complications arising from the comparatively slow hydrolysis of the terminal amide bond (3), shows that under comparable conditions N-benzoyl-L-tyrosylglycinamide is hydrolyzed by chymotrypsin much more rapidly than is the N-acetyl-L-peptide amide.

The kinetics of the hydrolysis of N-acetyl-L-tyrosylglycinamide by chymotrypsin at 25° and pH 7.8 were investigated and, assuming that hydrolytic cleavage of the terminal amide bond was so slow as to be ignored, it was found as in the chymotrypsin-catalyzed hydrolysis of N-benzoyl-L-tyrosinamide and ester (4) that the reaction was apparently first order at all initial substrate concentrations but that the first order rate constant (k) increased with decreasing initial substrate concentrations. Two possible explanations for this behavior may be advanced. If inhibition by the

TABLE II

Hydrolysis of N-Acetyltyrosylglycinamide pH 7.8

Temperature	E_0*	S_0†	Time of reaction	$S_0 - S$, mM per ml. × 10^3		First order rate constant, k		Proteolytic coefficient, C	
				DL	L	DL	L	DL	L
°C.			*min.*						
40	0.0154	0.00172	30	0.29	0.36	0.0061	0.0079	0.39	0.51
			60	0.60	0.64	0.0071	0.0077	0.46	0.50
40	0.031	0.00172	20	0.45	0.42	0.015	0.014	0.45	0.45
			40	0.63	0.70	0.011	0.013	0.37	0.42
			60	0.90	0.85	0.012	0.011	0.40	0.36
40	0.060	0.05	20	7.4	12.8	0.0079	0.015	0.13	0.24
			40	13.5	22.0	0.0077	0.015	0.13	0.24
25	0.075	0.05	20	3.9	7.5	0.0041	0.0081	0.054	0.11
			40	8.1	14.2	0.0044	0.0083	0.058	0.11
			60		20.0		0.0085		0.11
			80	13.7		0.0040		0.054	
25	0.30	0.05	10	7.6	14.1	0.0165	0.0329	0.055	0.11
			20	12.6	24.8	0.0146	0.0342	0.049	0.11
			30		32.2		0.0342		0.11
			40	24.4		0.0167		0.056	

* Mg. of protein N per ml.; crystalline chymotrypsin preparation from Lehn and Fink.

† Concentration of L form of substrate.

hydrolysis products occurs, the course of the reaction may be described by the expression

$$kEt = 2.3\left[K_m + \frac{1}{n}(S_0 + I)\right]\log\frac{S_0}{S} - \left(\frac{1-n}{n}\right)(S_0 - S)$$

where K_m = the Michaelis constant, n = the ratio of the inhibition constant to K_m, I = the inhibitor concentration, S_0 = the initial substrate concentration, S = the substrate concentration at time t, and E = the enzyme concentration. In the absence of inhibition by the hydrolysis products the rate law may be expressed by the integrated Michaelis-Menten equation (4)

$$kEt = 2.3K_m \log \frac{S_0}{S} + (S_0 - S)$$

The available data appear to fit either equation equally well and in the absence of data relative to possible inhibition by the hydrolysis products the simpler integrated Michaelis-Menten equation has been provisionally adopted for comparative purposes. In Table III are tabulated the experimental values of $(S_0 - S)$ and those calculated from the integrated Michaelis-Menten equation with the best experimental values of $K_m = 0.03$ M and $k = 0.0089$ mM per minute per mg. of enzyme nitrogen. The agreement between experimental and calculated values of $(S_0 - S)$ is within experimental error.

TABLE III

Comparison of Experimental and Calculated Results

Temperature 25°, $K_m = 0.03$ M, $k = 0.0089$ mM per minute per mg. of enzyme nitrogen, $E_0 = 0.15$ mg. of protein N per ml.

S_0	Time of reaction	Per cent hydrolysis	$S_0 - S$ (observed)	$S_0 - S$ (calculated)
mM per ml.	*min.*		*mM per ml.* $\times 10^3$	*mM per ml.* $\times 10^3$
0.05	10	14.9	7.4	8.1
	20	27.6	13.8	15.7
	40	48.6	24.3	27.3
0.03	10	16.9	5.1	6.3
	20	37.1	11.1	11.6
	40	65.2	19.6	20.0
0.02	10	26.1	5.2	4.9
	20	45.2	9.0	8.9
	30	59.4	11.9	12.4
0.0083	10	31.1	2.6	2.5
	20	53.2	4.4	4.3
	30	68.7	5.7	5.7
0.0050	10	31.0	1.6	1.6
	20	53.3	2.7	2.7
	30	70.6	3.5	3.5

In view of the magnitude of K_m it appears likely that the extent of inhibition of hydrolysis of N-acetyl-L-tyrosylglycinamide by an equimolar quantity of the D antipode observed in solutions 0.10 M with respect to the DL peptide is at or near the limiting value.

EXPERIMENTAL

*N-Benzoyl-*DL*-tyrosylglycinamide*—According to the procedure of Bergmann and Fruton (1), *p*-hydroxybenzaldehyde was condensed with hippuric acid, the azlactone allowed to react with glycine ester, and the dehydro-

peptide ester hydrogenated and ammoniated to give N-benzoyl-DL-tyrosylglycinamide trihydrate after recrystallization from aqueous ethanol.

Analysis—$C_{18}H_{19}O_4N_3 \cdot 3H_2O$. Calculated. C 54.7, H 6.4, N 10.6
Found. " 54.8, " 6.4, " 10.5

L-Tyrosine was converted into O,N-diacetyl-DL-tyrosine (5), the acetyl groups removed, and the DL-amino acid benzoylated with benzoyl chloride and sodium hydroxide to give O,N-dibenzoyl-DL-tyrosine, m.p. 215–217° (corrected) after recrystallization from aqueous ethanol.

Analysis—$C_{23}H_{19}O_5N$. Calculated, N 3.6; found, N 3.7

A solution of 10 gm. of O,N-dibenzoyl-DL-tyrosine in 200 ml. of absolute ethanol was saturated with dry hydrogen chloride at 0°, the solution refluxed for 10 hours, the reaction mixture evaporated *in vacuo* to a thick syrup, the latter triturated with aqueous sodium bicarbonate, and the solid that formed recrystallized from aqueous ethanol to give 4.5 gm. of N-benzoyl-DL-tyrosine ethyl ester, m.p. 123–124° (corrected).

Analysis—$C_{18}H_{19}O_4N$. Calculated, N 4.5; found, N 4.3

A solution of 8 gm. of N-benzoyl-DL-tyrosine ethyl ester in 20 ml. of absolute ethanol was slowly added to a refluxing solution of 40 ml. of 85 per cent hydrazine hydrate in 10 ml. of absolute ethanol, the reaction mixture refluxed for an additional 4 hours, chilled, and the recovered hydrazide washed with warm ethanol. Yield, 6.4 gm. of a product melting at 230–231° (corrected).

Analysis—$C_{16}H_{17}O_3N_3$. Calculated. C 64.2, H 5.7, N 14.0
Found. " 64.4, " 5.5, " 13.9

A solution containing 2.5 gm. of N-benzoyl-DL-tyrosinhydrazide, 5 ml. of concentrated hydrochloric acid, and 2.5 ml. of glacial acetic acid in 50 ml. of water was chilled to −10°, 10 ml. of a 7.5 per cent solution of sodium nitrite added with vigorous stirring, and the azide recovered, washed with cold water, dissolved in ethyl acetate, washed with aqueous sodium bicarbonate and water, and added to an ethereal solution of glycine ethyl ester prepared from 5 gm. of the hydrochloride. The reaction mixture was allowed to stand at 25° for 48 hours, then extracted with dilute hydrochloric acid and with water, the solvents removed, and the syrup triturated with a small amount of water. Recrystallization of the product from aqueous ethanol gave 2.0 gm. of N-benzoyl-DL-tyrosylglycine ethyl ester, m.p. 156–158° (corrected).

Analysis—$C_{20}H_{22}O_5N_2$. Calculated. C 64.9, H 6.0, N 7.6
Found. " 64.7, " 6.3, " 7.8

Ammonolysis of the above ester, in methanol solution, gave 1.8 gm. of N-benzoyl-DL-tyrosylglycinamide after recrystallization from aqueous ethanol.

Analysis—$C_{18}H_{19}O_4N_3 \cdot 3H_2O$. Calculated. C 54.7, H 6.4, N 10.6
Found. " 54.9, " 6.6, " 10.5

N-Benzoyl-L-tyrosylglycinamide—This acyldipeptide amide was prepared according to the directions of Bergmann and Fruton (1). The product was obtained as fine needles, m.p. 218–219° (decomposition).

N-Acetyl-DL-tyrosylglycinamide—A solution of 25 gm. of N-acetyl-DL-tyrosine ethyl ester (6) in 60 ml. of absolute ethanol was slowly added to a refluxing solution of 12.5 gm. of 85 per cent hydrazine hydrate in 20 ml. of absolute ethanol, and the reaction mixture refluxed for an additional 2 hours, chilled, the solid recovered, washed with ethanol, and dried to give 21 gm. of N-acetyl-DL-tyrosinhydrazide, m.p. 227–227.5° (corrected).

Analysis—$C_{11}H_{15}O_3N_3$. Calculated. C 55.6, H 6.4, N 17.7
Found. " 55.5, " 6.1, " 17.7

A solution of 1.5 gm. of sodium nitrite in 10 ml. of water was added to a well stirred solution, maintained at −5°, containing 4.0 gm. of N-acetyl-DL-tyrosinhydrazide, 10 ml. of concentrated hydrochloric acid, and 5 ml. of glacial acetic acid in 50 ml. of water. The azide was recovered, washed with cold water, taken up in ethyl acetate, and the latter solution washed with aqueous sodium bicarbonate and water, and added to an ethereal solution of glycine ethyl ester prepared from 10 gm. of the hydrochloride. The reaction mixture was washed with dilute hydrochloric acid and with water, and the solvents removed from the dried non-aqueous phase to give 3.5 gm. of N-acetyl-DL-tyrosylglycine ethyl ester, m.p. 135–136° (corrected), after recrystallization from aqueous ethanol.

Analysis—$C_{15}H_{20}O_5N_2$. Calculated. C 58.5, H 6.6, N 9.1
Found. " 58.4, " 6.5, " 9.0

Ammonolysis of 2.0 gm. of the above ester, in methanol solution, gave 1.2 gm. of N-acetyl-DL-tyrosylglycinamide, m.p. 205–206.5° (corrected) after recrystallization from aqueous ethanol.

Analysis—$C_{13}H_{17}O_4N_3$. Calculated. C 55.9, H 6.1, N 15.1
Found. " 56.1, " 6.2, " 15.3

N-Acetyl-L-tyrosylglycinamide—A solution of 37 gm. of N-acetyl-L-tyrosine (5) in 500 ml. of absolute ethanol was saturated with dry hydrogen chloride at 0°, the solution refluxed for 4 hours, the solution concentrated *in vacuo* to a thick syrup, the pH adjusted to 7.5 with aqueous sodium

carbonate, and the crude product recrystallized from aqueous ethanol to give 25 gm. of N-acetyl-L-tyrosine ethyl ester, m.p. 96–97° (corrected).

Analysis—$C_{13}H_{17}O_4N$. Calculated. C 62.1, H 6.8, N 5.6
Found. " 61.8, " 6.9, " 5.3

According to the procedure used for the DL peptide, 22 gm. of the above ester gave 17 gm. of N-acetyl-L-tyrosinhydrazide, m.p. 235.5–236° (corrected).

Analysis—$C_{11}H_{15}O_3N_3$. Calculated. C 55.6, H 6.4, N 17.7
Found. " 55.4, " 6.5, " 17.8

N-Acetyl-L-tyrosinhydrazide was converted into N-acetyl-L-tyrosylglycine ethyl ester, m.p. 133–135° (corrected) in a manner similar to that described for the preparation of the corresponding DL peptide. The yields varied between 55 and 75 per cent of the theoretical.

TABLE IV

Molecular Weight of N-Acetyl-DL-Tyrosylglycinamide in Aqueous Solution

Peptide in 1000 gm. water	Freezing point depression	Mol. wt.
gm.	°C.	
21.40	0.137	290
21.00	0.137	284
10.50	0.070	278
10.30	0.068	281
6.17	0.038	301

Analysis—$C_{15}H_{20}O_5N_2$. Calculated. C 58.5, H 6.6, N 9.1
Found. " 58.6, " 6.9, " 9.4

Ammonolysis of the above ester gave 78 per cent of N-acetyl-L-tyrosylglycinamide, m.p. 225–226° (corrected).

Analysis—$C_{13}H_{17}O_4N_3$. Calculated. C 55.9, H 6.2, N 15.1
Found. " 55.9, " 5.9, " 15.0
$[\alpha]_D^{24} = +35.0°$ (4% in 50% aqueous acetone)

Cryoscopic Measurements—The technique described by Beckmann (7) was used for the determination of the freezing points of aqueous solutions of N-acetyl-DL-tyrosylglycinamide. The molecular weights given in Table IV are to be compared with the theoretical value of 279, assuming ideal solution behavior.

Enzymatic Studies—Preparations of crystalline chymotrypsin obtained from Lehn and Fink or Armour were used in these studies and were found to give comparable results. The reaction mixtures were 0.015 M in phosphate, adjusted to pH 7.8. The extent of hydrolysis was determined by a formol

titration to pH 8.1 with a Beckman model G pH meter for indication of the end-point. In general 0.5 ml. aliquots of the 0.05 M substrate solutions were titrated with 0.01 M sodium hydroxide after the addition of 0.5 ml. of 35 per cent aqueous formaldehyde which had been adjusted to pH 8.0 by the addition of basic magnesium carbonate. With the 0.0017 M substrate solutions 2.0 ml. aliquots were titrated.

SUMMARY

The inhibition of the chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tyrosylglycinamide by equimolar quantities of its D antipode has been observed to be a function of the relative concentration of the DL mixture and enzyme. The general features of the above system have been described, and an explanation has been given relative to the lack of antipodal inhibition obtaining in the case of N-benzoyl-DL-tyrosylglycinamide.

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Part III

The Hydrolysis of Various N-Acylated Tyrosinamides by Chymotrypsin

DISCUSSION

The Hydrolysis of Various N-Acylated Tyrosinamides by Chymotrypsin

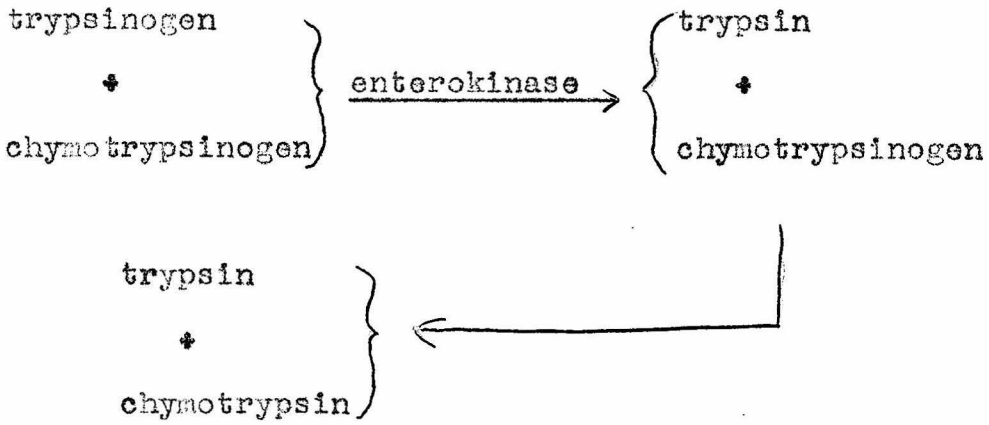
Chymotrypsin is a member of the large group of "proteases" -enzymes which catalyze the hydrolysis of proteins and protein hydrolytic products.

In the normal digestive process, a partial breakdown of proteins is effected in the stomach by the enzyme, pepsin. The degradation of the protein is continued in the small intestine. As early as 1836 it was noted that some principle, called trypsin, generated by the pancreas is responsible for this further hydrolysis. It was also found that fresh pancreatic juice had no action on proteins or protein hydrolysis products, but that the juice must be activated by a material called enterokinase which exists in the lining of the intestine. (The pancreatic juice can also be self activated slowly by standing at pH 6.0) Vernon (1) showed that the proteolytic power of pancreatic juice is activated much more rapidly by freshly activated trypsin than by enterokinase. This fact, and the observation that the activity of the pancreatic juice as measured by milk curd formation and by casein hydrolysis did not show a parallelism in inactivation experiments, lead to the idea that two or more proteolytic enzymes were present in the pancreatic juice.

In 1932, Northrup and Kunitz (2) obtained crystalline trypsin from active pancreatic juice. While looking for a precursor in the inactive juice, they isolated an enzymatically inactive crystalline protein which was later called chymotrypsinogen. This protein is not activated by enterokinose, but it is activated by trypsin to give a new enzyme, also obtained in the crystalline condition, which was named chymotrypsin.

Chymotrypsin was found to be quite distinct from trypsin, especially on the basis of the types of protein linkages which are susceptible to its action.

The current concept of the interrelationships involved in the trypsin-chymotrypsin system might be outlined as follows.



The change of chymotrypsinogen to chymotrypsin takes place best at pH 7 to 8. The active enzyme contains 6 more NH_2 groups than the zymogen, but the process does not involve the formation of split products.

Until 1935, but very little was known about the specificity of proteinase activity on genuine proteins. It was only known that acidic and basic groups are formed in equivalent amounts. No synthetic substrates had been shown to be hydrolysed by a proteinase, and no peptidase was known which would hydrolyse real protein. These factors lead to the possibility that unknown types of linkages exist in protein. The work of Bergmann and associates in 1935 (3) in proving that a number of synthetic peptides are hydrolyzable by papain was, accordingly, a marked contribution to the field of enzyme and protein chemistry. This discovery opened up a new approach to the systematic study of proteinase specificity. Such studies were carried out on chymotrypsin, and in a series of papers from the Bergmann group (4,5,6), a number of synthetic substrates, readily hydrolyzed by chymotrypsin, were described. It was apparent from these studies that chymotrypsin has at least two distinct specificities in that it acted both as an endo peptidase and as an amidase at linkages involving the carbonyl groups of N-acylated tyrosine or phenylalanine. More recently (7,8,9,10,11) it has been found that chymotrypsin has an esterase activity which seems to involve the same reactive sites as the amidase activity (12,13). It has also been established that N-acylated-methionine and tryptophane esters are hydrolyzed by chymotrypsin (11).

It had been noted (14) that there is a marked difference in the rates at which N-benzoyl and N-acetyl-L-tyrosyl glycinamides are hydrolyzed by chymotrypsin. The same effect was observed (7) in comparing the rates of hydrolysis of N-acetyl and N-benzoyl tyrosine esters and amides.

It seemed desirable to study the effect of a more extended variation in the type of amino nitrogen substitution in a chymotrypsin substrate, and to investigate in more detail the factors involved in the inhibition of the hydrolysis of an L-substrate by the corresponding D-isomer. To this end a series of -L-, and DL-tyrosinamides were prepared in which the amino nitrogen was acylated by acetyl, formyl, benzoyl, iso-nicotinyl, nicotinyl, and picolinyl groups.

N-acetyl-D-tyrosine ethyl ester and amides were prepared in order to extend the inhibition experiments to high concentrations of the D isomers. For this purpose a rapid and convenient method for the resolution of -DL- tyrosine was devised. This procedure is described in the section "Preparation of Substrates".

It was found that the course of the hydrolysis of the acetyl, formyl, benzoyl, iso nicotinyl, and nicotinyl-L-tryosinamides followed the Michaelis Menten equation (15),

$$-\frac{ds}{dt} = v = \frac{k'es}{K_m + s} = \frac{Vs}{K_m + s} \qquad (1)$$

where K_m is the enzyme-substrate dissociation constant, k' is the specific rate constant, e is enzyme concentration, and s is the substrate concentration. V corresponds to the value of v when $s \gg K_m$.

In evaluating K_m and k' (or V), it is convenient to use the Lineweaver and Burk (16) procedure of plotting the reciprocals of initial velocities against the reciprocals of initial substrate concentrations, for upon rearranging equation (1) a linear relationship between the reciprocals obtains.

$$\frac{1}{v} = \frac{K_m}{V} \cdot \frac{1}{s} + \frac{1}{V} \qquad (2)$$

In considering the hydrolysis of N-acetyl-L-tyrosinamide, by referring to figures (1) and (2), it is apparent that at the lower initial substrate concentrations, the hydrolysis experiments follow first order kinetics, whereas at the higher concentrations, the reaction initially follows zero order. In order to determine the initial velocity of the lower concentrations experiments, the log plot can be used most conveniently, whereas at the higher concentrations, the concentration vs time plot is more convenient.

Figure (3) is a plot of equation (2) applied to the hydrolysis of N-acetyl-L-tyrosinamide, from which the values of V and K_m can be calculated.

If the Michaelis-Menten equation is integrated we have the relationship,

$$F(s) = 2.3\ K_m\ \text{Log}\ \frac{s_o}{s} + (s_o - s) = k'et \qquad (3)$$

A plot of F(s) against t should be linear and should afford a more rigorous test of the applicability of the Michaelis-Menten equation because every point on every hydrolysis curve should fit on a single plot. Referring to figure (4), is is apparent that such is the case, and the data for the hydrolysis of N-acetyl-L-tyrosinamide fits the integrated Michaelis-Menten equation very well over a large range of substrate concentrations.

The same methods of analysis were applied to the data obtained for the other substrates. From the plot of the integrated Michaelis-Menten function vs time, (Figures 5 to 17), it is apparent that all of these substrates follow the equation quite well.

The N-picolinyl-L-tyrosinamide has such a low solubility that the hydrolysis could be carried out at but one, (the maximum), concentration. The data obtained are inconclusive. The K_m and V values of these various N-acylated tyrosinamides are tabulated below:

Acyl Group	K_m	V
Formyl	11.2	.39
Acetyl	28.2	2.4
Benzoyl	1.9	4.0
Iso Nicotinyl	8.4	6.2
Nicotinyl	13.1	6.1

It is apparent that V, which presumably is a measure of the rate of activation of the enzyme-substrate complex, is of the same order of magnitude for the acetyl, benzoyl, iso-nicotinyl and nicotinyl substrates. The observed differences in the overall rates of hydrolysis can be attributed to the variation of the K_m value which is more nearly a measure of the attraction of the enzyme for the substrate.

However, it must be borne in mind that the true equilibrium constant of the enzyme + substrate $\rightleftharpoons$ complex reaction is inherently indeterminate as measured kinetically, because K_m necessarily involves three reaction rate constants. For this reason, the evaluation of the specific group effect on the affinity of the enzyme for a substrate might well be treated more adequately on the basis of specific inhibition experiments.

The development of the nicotinyl and iso nicotinyl derivatives has provided readily prepared substrates which have several advantages.

1. They are hydrolysed at a rate comparable with benzoyl derivatives, and are considerably more soluble in water.

2. The N-nicotinyl-Dl-tyrosinamide is also soluble and provides a convenient substrate for "D" inhibition studies. (The N-benzoyl-DL-tyrosinamide is extremely insoluble.)

N-nicotinyl- derivatives of phenylalanine, and tryptophane have been prepared by Drs. Islen and Hyang of this laboratory and have been found to be convenient enzyme hydrolysis substrates also.

These developments are the subject of an article which will be published soon.

N-nicotinyl-L-tyrosinhydrazide was found to be hydrolyzable by chymotrypsin. Referring to table (VII) and to figures (20) it can be seen that the reaction follows first order kinetics. (This observation is somewhat in conflict with the results obtained by Neurath et al. (8).) The list of linkages hydrolyzable by chymotrypsin must now include peptides, amides, esters, and hydrazides.

If an enzyme catalyzed reaction follows the Michaelis-Menten scheme, it can be shown (16) that the effect of an inhibitor, which competes reversibly with the substrate for

active sites on the enzyme, can be expressed in an equation of the form:

$$\frac{1}{v} = (1 + \frac{i}{K_I}) \cdot \frac{K_m}{V} \cdot \frac{1}{s} + \frac{1}{V} \qquad (4)$$

where i is the concentration of the inhibitor and K_I is the ratio:

$$\frac{(\text{inhibitor})(\text{enzyme})}{(\text{inhibitor-enzyme-complex})}$$

A series of measurements at various substrate and inhibitor concentrations will enable one to evaluate K_I, V, and K_m from a plot of the reciprocals of the velocity vs. the reciprocal of the substrate concentration.

A series of hydrolysis experiments were carried out using N-acetyl-L-tyrosinamide as the substrate and N-acetyl-D-tyrosinamide as the inhibitor. Referring to tables (VIII) to (XIII) and figures (26) and (27) it is apparent that this is an example of competitive inhibition.

By integrating equation (3) we have

$$F(s) = 2.3(1 + \frac{i}{K_I}) K_m \text{ Log } \frac{s_o}{s} + (s_o - s) = Vt \qquad (5)$$

If the F(s) is plotted against time, a linear relationship between F(s) and t should be observed, and all the experimentally determined values for every hydrolysis experiment could be placed on one plot. For convenience, each inhibitor concentration has been used to make such a curve.

Referring to figures (21) to (25) it is apparent that the data fit into a linear relationship well. The small variation in K_I probably is not significant. In accordance with our attitude that the inhibitor equilibrium constant might be most significant in relating structure to affinity, an inhibition experiment was carried out using N-acetyl-L-tyrosinamide as the substrate, and N-acetyl-D-tyrosine ethyl ester as the inhibitor. Considering that this is a competitive type of inhibition, the data were treated in the same manner as in the N-acetyl-D-tyrosinamide inhibition experiments. The results of these experiments (see table (XIII) and figures (28) to (30).) lead to a K_I value of 5.20. The relative values of 5.20 and 11.0 (the average K_I for the D amide) are probably a more true indication of the relative affinities of the enzyme for the substrate as influenced by that part of the substrate molecule, than are the K_m values of the L-ester and amide.

Further experiments were carried out, using N-nicotinyl-DL-tyrosinamide to determine the effect of the -D-isomer as an inhibitor. Similar experiments were performed using N-iso-nicotinyl-DL-tyrosinamide.

These experiments were not extensive but they have indicated that the K_I value of the N-iso-nicotinyl-D-tyrosinamide is about 2 whereas the K_I value of the N-nicotinyl-D-tyrosinamide is about 10.

These observations lend support to the view that an amino N near the secondary peptide linkage tends to decrease the affinity of the enzyme for the substrate. It has been reported (10) that glycyl tyrosinamide has an exceptionally high K_m value which is consistent with this view. It must be noted, however, that the basic strength of the pyridyl nitrogen is low - ($K_B = 3.55 \cdot 10^{-11}$ (17)) and so the effect can hardly be ascribed to approach of a positive charge as has been suggested in the case of the glycyl peptide.

EXPERIMENTAL

Enzyme Preparation and Methods

A crystalline chymotrypsin preparation procured from Armour and Company of Chicago, Illinois (lot number 70902) was used in all of these experiments. This preparation was found to contain 47.0 per cent of protein (precipitable by trichloroacetic acid) or 7.51 per cent of protein nitrogen. The non-protein material is $MgSO_4$. The dry enzyme was weighed out for each individual hydrolysis experiment. This practice was adopted in order to avoid any complications which might arise as a result of changing degree of activity in a stock solution of the enzyme.

In carrying out a hydrolysis experiment, the substrate is dissolved in 6 ml of water and 2 ml of a stock buffer solution (.100 formal ethylene diamine adjusted to pH 8.00 with HCl). The temperature is brought to 25°C, and the enzyme, dissolved in 1 ml of water, is added. The volume of the system is made exactly to 10.00 ml by adding water. The solution is mixed thoroughly, and samples are removed for analysis at frequent intervals of time. Zero time corresponds to the instant the enzyme solution makes contact with the substrate solution.

To determine the extent of hydrolysis at any given time, the sample (1.00 ml) is mixed with 1.00 ml of 35 per cent neutral formaldehyde, and then titrated with .01000 N-sodium hydroxide to a pH of 8.00. This titration is carried out electrometrically on a Beckman model G glass electrode pH meter.

It is possible to remove the first sample and to mix it with the formaldehyde solution within 60 to 70 seconds after zero time. This value is used as the blank unless it is apparent that more than 1 per cent hydrolysis has occurred within 1 minute. In such cases, the blank value is determined by a method of successive approximations.

Control experiments were carried out on all substrates and on the enzyme. It was found that there is very little if any increase in titrable acidity in either the substrate or enzyme over a two hour period, which was the normal period of hydrolysis studied.

Preparation of Substrates

L-Tyrosine Ethyl Ester

100 gm L-tyrosine (Lemke) was suspended in 500 ml of absolute alcohol. Dry HCl was passed into the suspension until all of the solid dissolved. The solution was refluxed for 24 hours. The solution was then filtered and concentrated under vacuum. The ester hydrochloride, which crystallized out, was filtered and dissolved in a minimum of cold water. A concentrated aqueous solution containing 70 gm of K_2CO_3 was added to the cold solution. The tyrosine ethyl ester crystallized out. It was removed by filtration, then purified by two recrystallizations from ethyl acetate. The yield was 65 gm.

Melting Point: 105-106°C (Corr.)

The melting point recorded in the literature (18) is 108-109. Repeated recrystallizations failed to raise the melting point of our product from 105-106.

L-Tyrosine Amide

50.0 gm L-tyrosine ethyl ester was dissolved in 300 ml methyl alcohol (99%). After cooling this solution to 0°C, it was saturated with NH_3. The resulting solution was held at room temperature for 2 days. The solution was then concentrated under vacuum to a syrup which crystallized upon stirring. The solid was removed from the mother liquor by filtration, then recrystallized from an alcohol-water mixture.

Yield: 30 gm.

Melting Point: 155-156 (Corr.)

This melting point agrees with the literature values (19).

DL-Tyrosine

The method of du Vigneaud and Meyer (20) was used to racemize L-tyrosine. The acetylated product was not isolated, but was immediately converted to DL-tyrosine. 100 gm. L-tyrosine (Lemke) was dissolved in 560 ml. water and 416 ml. 2.7 N-sodium hydroxide. This solution was agitated vigorously in an ice bath while 400 ml. of acetic anhydride was added slowly. The solution was then held at 60° for 20 hours.

The solution was made approximately 3 N in hydrogen chloride and refluxed for 20 hours. It was then filtered and neutralized to pH 5.6 with sodium carbonate. The solid obtained was filtered, washed thoroughly with water, and dried. The product had zero rotation.

DL-Tyrosine Ethyl Ester

20 gm. of DL-tyrosine was suspended in 200 ml. of ethyl alcohol. Dry HCl was passed into the suspension until all of the solid dissolved. The solution was then refluxed for 20 hours, and then it was evaporated under vacuum to dryness. The solid was dissolved in a minimum of cold water. K_2CO_3 was added to raise the pH to 8.0. The solid which precipitated was recrystallized twice from ethyl acetate.

Yield: 16.0 gm.

Melting Point: 110-111 (Corr.)

DL-Tyrosinamide

A solution of 16.0 gm. of DL-tyrosine ethyl ester in 150 ml. of methanol was saturated with NH_3 at 0°. The solution allowed to stand at room temperature for 2 days. The solution was then evaporated under vacuum to dryness. The solid residue was recrystallized twice from an alcohol-water mixture.

Yield: 12 gm.

Melting Point: 163-164.5 (Corr.)

N-Formyl-L-Tyrosinamide

The method which du Vigneaud and Loving (21) used to formylate cystine was adapted to the formylation of L-tyrosinamide. 2.50 gm. of L-tyrosinamide was dissolved in 45 ml. of 80% formic acid. The solution was heated to 60°. Then 17 ml. of acetic anhydride was added to the well agitated solution at such a rate that the temperature was maintained at 60°. The solution was allowed to stand at room temperature for 12 hours, then it was concentrated under vacuum to a syrup which crystallized from water.

Yield: 1.8 gm.

Melting Point: 184-186 (Corr.)

Analysis: ($C_{10}H_{12}O_3N_2$),

Calculated: C = 57.7, H = 5.82, N = 13.45

Found: C = 58.0, H = 6.08, N = 13.11

N-Benzoyl-L-tyrosine

12 gm. of L-tyrosine was dissolved in 90 ml. of 2N sodium hydroxide. The solution was cooled in an ice bath. Then 16 ml. of benzoyl chloride and 100 ml. of 2.7N sodium hydroxide were added in small increments to the well agitated solution. 25 ml. of concentrated HCl was added to the solution. The solid which precipitated was filtered out and dried. It was treated twice with warm ligroin (60-70) to remove benzoic acid, then recrystallized from ethanol.

Yield: 18 gm.

Melting Point: 164-165 (Corr.)

(The melting point reported in the literature (22) is 165-166.)

N-Benzoyl-L-Tyrosine Ethyl Ester

16 gm. of N-benzoyl-L-tyrosine was dissolved in 300 ml. ethanol. Dry HCl was passed into the solution until it was saturated (at 40°). The solution was then refluxed for 20 hours. The solution was evaporated under vacuum to a heavy syrup which crystallized upon stirring. The solid was recrystallized from ethyl acetate.

Yield: 12 gm.

Melting Point: 120-121 (Corr.)

This melting point agrees with the value reported by Bergman and Fruton (23).

N-Benzoyl-L-Tyrosinamide

10 gm. of N-benzoyl-L-tyrosine ethyl ester was dissolved in 200 ml. of methanol. The solution was then saturated with NH_3 at 0°, and then allowed to stand at room temperature for 2 days. The solution was then evaporated to dryness under vacuum, and the residue was recrystallized from a water-alcohol mixture.

Yield: 8.0 gm.

Melting Point: 210-211 (Corr.)

This value agrees with that reported by Kaufman et al. (24)

Analysis: ($C_{16}H_{16}O_3N_2$

Calculated: C 67.6, H 5.68, N 9.86

Found: C 67.7, H 5.78, N 9.80

N-Benzoyl-DL-Tyrosinamide

N-benzoyl-DL-tyrosinamide was prepared from DL-tyrosine by the same series of reactions which were used in preparing N-benzoyl-L-tyrosinamide from L-tyrosine.

Melting Point: 242-244 (Corr.)

Bergman and Fruton (25) reported a melting point of 238 for this compound.

Analysis: ($C_{16}H_{16}O_3N_2$)

Calculated: C 67.6, H 5.68, N 9.86

Found: C 67.5, H 5.61, N 9.80

N-Acetyl-L-Tyrosinamide

2.00 gm. of N-acetyl-L-tyrosine ethyl ester was dissolved in 50 ml. of methanol. The solution was saturated with NH_3 at 0°, then allowed to stand at room temperature in a stoppered flask for 2 days. The solution was then evaporated to dryness under vacuum. The residue was recrystallized twice from an alcohol-water mixture.

Melting Point: 226-228 (Corr.)

Analysis: ($C_{11}H_{14}O_3N_2$)

Calculated: C 59.5, H 6.37, N 12.6

Found: C 59.5, H 6.43 N 12.6

N-Acetyl-DL-Tyrosinamide

2.00 gm. of N-acetyl-DL-tyrosine ethyl ester (8), was dissolved in 50 ml. of methanol. This solution was saturated at 0° with NH_3, then allowed to stand at room temperature for 2 days. The solution was then evaporated to dryness under vacuum. The residue was recrystallized twice from an alcohol-water mixture.

Melting Point: 197-198 (Corr.)

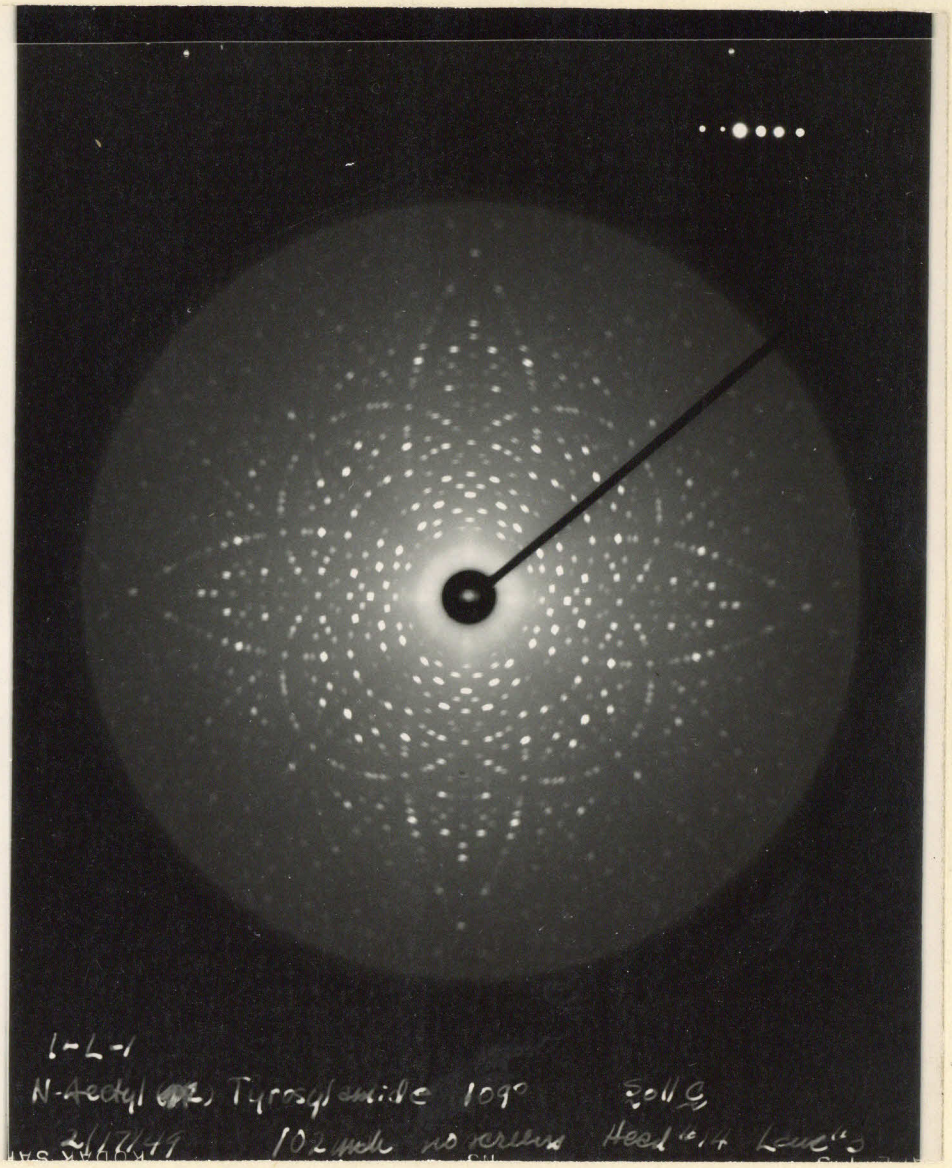
Analysis: ($C_{11}H_{14}O_3N_2$)

Calculated: C 59.5, H 6.37, N 12.6

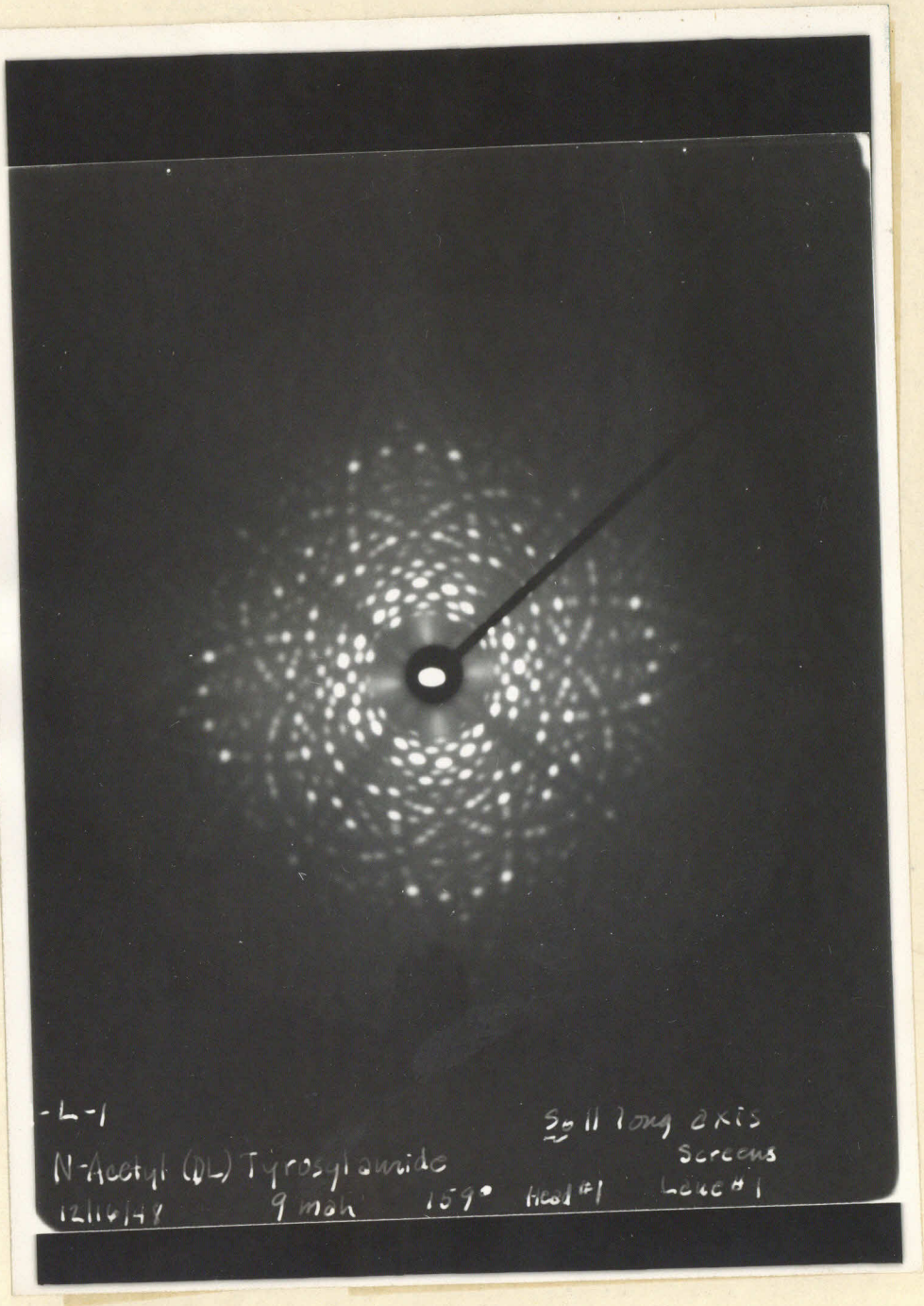
Found: C 59.4, H 6.40, N 12.5

By careful control of the conditions of crystallization, it was possible to prepare large, well-defined, single crystals of the L and DL acetyl tyrosinamides. Single crystals of each of these preparations were used by Drs. Corey and Carpenter for an X-ray diffraction study. The patterns obtained from these crystals were identical. Further investigation showed that the D and L isomers crystallize separately from a solution containing equimolar quantities of L and D. The pure L compound melts at 226-228, and the DL mixture, (powdered for introduction into the capillary tube), melts at 197-198. The melting point of individual crystals from the carefully crystallized DL mixture is 226-228. If a mixed melting point is carried out with a single crystal isolated from the DL mixture, and a known L crystal, the observed value is either 196 or 226, depending on whether a D or an L crystal happened to be selected. These observations were of help in determining the course of the resolution experiments reported elsewhere in this thesis.

The X-ray work will appear in an article in the Cryst. Acta.



N-ACETYL-L-TYROSINAMIDE



N-ACETYL-DL-TYROSINAMIDE

N-Picolinyl-Tyrosinamide

A. Picolinyl Azide.--5.3 gm. of picolinhydrazide was dissolved in 50 ml. of cold 1 N HCl. The solution was agitated thoroughly while 10 ml. of a 30 per cent solution of sodium nitrite was slowly added. 5.0 gm. of $NaHCO_3$ was added, then the mixture was extracted with ethyl acetate. The ethyl acetate solution was evaporated under vacuum to produce a mass of crystals of the azide.

B. Reaction of Azide with Tyrosinamide.--1.5 gm. of the azide prepared as above was added to a solution of 2.00 gm. of L-tyrosinamide in 8 ml. of pyridine. After 12 hours at room temperature, a solid had crystallized out. This was recrystallized from glacial acetic acid.

Melting point: 242-243 (Corr.)

Analysis: ($C_{15}H_{15}O_3N_3$)

Calculated: C 63.2, H 5.31, N 14.73

Found: C 63.3, H 5.42, N 14.86

N-Picolinyl-DL-Tyrosinamide

N-picolinyl-DL-tyrosinamide was obtained from DL-tyrosinamide by the same procedure outlined above for the L isomer.

Melting Point: 249-250 (Corr.)

Analysis: ($C_{15}H_{15}O_3N_3$)

Calculated: C 63.2, H 5.31, N 14.73

Found: C 63.2, H 5.44, N 14.72

Nicotinyl Azide

10.6 gm. of nicotinhydrazide was dissolved in 120 ml. of 1 N HCl. 20 ml. of a 30 per cent solution of sodium nitrite was added slowly to the well agitated solution which was immersed in an ice bath. 10 gm. of sodium bicarbonate was added to the solution, which was then extracted with ethyl acetate. The ethyl acetate solution was washed with water, then evaporated under vacuum. Large crystals of the azide formed. The azide explodes violently when heated above the melting point. The azide has a marked irritating effect on the skin.

Yield: 9.0 gm.

Melting Point: 50

Analysis: ($C_6H_4O N_4$)

Calculated: C 48.5, H 2.72, N 37.8

Found: C 48.8, H 2.91, N 37.7

N-Nicotinyl-L-Tyrosine Ethyl Ester

4 gm. of L-tyrosine ethyl ester was dissolved in 20 ml. of ethyl acetate. 2.5 gm. of nicotinyl azide was added, and the mixture allowed to stand at room temperature for 20 hours. The solution was then extracted with dilute HCl until the extract had a pH of 5.0. The ethyl acetate solution was then evaporated to a syrup which soon crystallized. The solid was recrystallized from ethyl acetate.

Yield: 2.5 gm.

Melting Point: 147-149 (Corr)

Analysis: ($C_{17}H_{18}O_4N_2$)

Calculated: C 65.0, H 5.79, N 8.91

Found: C 65.2, H 6.00, N 8.88

N-Nicotinyl-L-Tyrosine Hydrazide

6.00 gm. of N-nicotinyl-L-tyrosine ethyl ester was dissolved in 20 ml. of ethanol. 1.00 gm. of hydrazine was added, and after 12 hours the hydrazide had crystallized out. The solid was recovered by filtration and recrystallized from ethanol.

Melting Point: 242-243 (Corr.)

Analysis: ($C_{15}H_{16}O_3N_4$)

Calculated: C 60.0, H 5.38, N 18.65

Found: C 59.9, H 5.30, N 18.63

N-Nicotinyl-L-Tyrosine

.50 gm. of L-tyrosine was dissolved in 2 ml. of 2.7N sodium hydroxide. .45 gm. of nicotinyl azide was added to the solution, and the mixture was shaken vigorously for 20 minutes. The pH was lowered to 4.5 with dilute HCl. The solid was filtered out and washed with cold water. The product was then recrystallized from water.

Melting Point: 238-239 (Corr.)

Analysis: ($C_{15}H_{14}O_4N_2$)

Calculated: C 63.0, H 4.94, N 9.80

Found: C 63.2, H 5.22, N 9.54

N-Nicotinyl-L-Tyrosinamide

2.00 gm. of L-tyrosinamide was dissolved in 8 ml. pyridine. 1.5 gm. of nicotinyl azide was added to the solution. After 12 hours at room temperature, the amide crystallized out. It was purified by recrystallizing from water twice.

Melting Point: 226-227 (Corr.)

Analysis: ($C_{15}H_{15}O_3N_3$)

Calculated: C 63.2, H 5.31, N 14.73

Found: C 63.0, H 5.56, N 14.80

N-Nicotinyl-DL-Tyrosinamide

The same procedure used for preparing N-nicotinyl-L-tyrosinamide was used to form N-nicotinyl-DL-tyrosinamide from DL-tyrosinamide and nicotinyl azide.

Melting Point: 224-226 (Corr.)

(A mixed melting point with the L-isomer was 216-219)

Analysis: ($C_{15}H_{15}O_3N_3$

Calculated: C 63.2, H 5.31, N 14.73

Found: C 63.4, H 5.58, N 14.53

Iso Nicotinic acid

200 ml. of gamma picoline was distilled through a 60 cm. helix-packed column, and the fraction boiling at 142.7-142.9° was collected. 50 gm. of the purified gamma picoline was dissolved in 2500 ml. of water continaing 90 gm. $KMnO_4$, and refluxed for 3 hours. 90 gm. more of $KMnO_4$ was added and the

heating continued for 3 hours. The MnO_2 was filtered out and the filtrate was concentrated under vacuum to a volume of 150 ml. The pH was lowered to 4.0 with HCl. The acid which precipitated out was removed by filtration and dried.

Melting Point: 318-321 (Corr.)

The literature value for the melting point is 323-326 (26).

Hydrazide of Iso Nicotinic Acid

30 gm. of iso nicotinic acid was suspended in 250 ml. of ethanol. HCl was passed into the solution until it was saturated at 40°. The mixture was then refluxed for 24 hours. The solution was filtered and evaporated under vacuum to dryness. The solid was dissolved in water and K_2CO_3 added until the pH was 8.0. The mixture was then extracted with ether. The ether was stripped off, and the residual ester was dissolved in ethanol, (100 ml.), and mixed with 6.4 gm. of hydrazine. After 20 hours, the hydrazide crystallized from the solution. The solid was filtered out and recrystallized from ethanol.

Melting Point: 172-173 (Corr.)

Analysis: ($C_6H_7O N_3$)

Calculated: C 52.5, H 5.14, N 30.7

Found: C 52.8, H 5.41, N 30.8

Iso Nicotinyl Azide

5.2 gm. of iso-nicotinhydrazide was dissolved in 60 ml. of 1 N HCl and immersed in an ice bath. 10 ml. of a 30 per cent

solution of sodium nitrite was added slowly to the well agitated solution. 5.00 gm. of sodium bicarbonate was added, and the mixture was then extracted with ethyl acetate. The ethyl acetate solution of the azide was evaporated under vacuum to a syrup which crystallized upon cooling to -20°.

N-Iso Nicotinyl-L-Tyrosinamide

3.5 gm. of L-tyrosinamide was dissolved in 10 ml. of pyridine. 2.5 gm. of iso nicotinyl azide was added. After 20 hours at room temperature, a crystalline solid formed. The solid was filtered out, and recrystallized from water.

Melting Point: 231-232 (Corr.)

Analysis: ($C_{15}H_{15}O_3N_3$)

Calculated: C 63.2, H 5.31, N 14.73

Found: C 63.3, H 5.48, N 14.84

N-Iso Nicotinyl-DL-Tyrosinamide

The procedure outline above for L-tyrosinamide was applied to DL-tyrosinamide.

Melting Point: 242-243 (Corr.)

Analysis: ($C_{15}H_{15}O_3N_3$)

Calculated: C 63.2, H 5.31, N 14.73

Found: C 63.3, H 5.53, N 14.56

N-Nicotinyl-L-Tyrosylglycine Ethyl Ester

2.00 gm. of nicotinyl azide was added to an ether solution containing 2.00 gm. of glycine ethyl ester. After 20 hours, the solution was extracted with dilute HCl, then washed with

water. The ether solution was then evaporated under vacuum to dryness. The solid residue was recrystallized from water.

Melting Point: 179-181 (Corr.)

Analysis ($C_{19}H_{21}O_5N_3$)

Calculated: C 61.5, H 5.70, N 11.31

Found: C 61.4, H 5.92, N 11.21

Nicotinyl Ethyl Ester

25 gm. of nicotinic acid was suspended in 500 ml. of ethanol. Dry HCl was passed into the mixture until it was saturated at 40°C. The mixture was then refluxed for 20 hours. The solution was evaporated to a syrup. Upon adding water and K_2CO_3, the ester separated as an oil. The ester was taken up in ether, washed with water, and dried over $CaSO_4$. The ether was stripped off and the ester distilled at 4-5 mm. Hg pressure at 81-82.

Yield: 19.7 gm.

Ethyl Nipecotate

10 gm. of ethyl nicotinate was dissolved in 50 ml. of methyl cyclohexane. 2.0 gm. of Raney nickel was added, and the mixture treated with H_2 at 2100 psig at 170°C. for 2 hours. The reaction mixture was filtered, and the methyl cyclohexane stripped off.

Nipecotyl Hydrazide

The ester prepared above was dissolved in 20 ml. ethanol,

and 6.0 gm. hydrazine added. After 20 hours, the solution was evaporated under vacuum to a syrup which crystallized upon stirring. The solid was separated and recrystallized from ethyl acetate.

Melting Point: 123-125 (Corr.)

Analysis: ($C_6H_{13}ON_3$)

Calculated: C 50.4, H 9.15, N 29.4

Found: C 50.5, H 9.28, N 29.4

N-Nipecotyl-L-tyrosine Ethyl Ester

5.00 gm. of N-nicotinyl-L-tyrosine ethyl ester was dissolved in 190 ml. methylcyclohexane and 60 ml. ethanol. 2.3 gm. of Raney nickel was added, and the mixture treated with H_2 at 2100 psig and 150°C for 2 hours. The reaction mixture was then filtered and evaporated under vacuum to a syrup which crystallized upon adding ether. The solid was then recrystallized from a mixture of ethyl acetate and methanol.

Melting Point: 153-154.5 (Corr.)

Analysis: ($C_{17}H_{24}O_4N_2$)

Calculated: C 63.8, H 7.57, N 8.75

Found: C 63.9, H 7.67, N 8.87

N-Acetyl-D-Tyrosine Ethyl Ester

16 gm. of N-acetyl-DL-tyrosine ethyl ester was dissolved in 160 ml. methanol and 720 ml. water. The pH was adjusted to 8.00 with sodium hydroxide. 100 mg. of chymotrypsin was added, and 1 N sodium hydroxide added as needed to maintain

the pH at 8.0. The hydrolysis was extremely rapid, and in 10 minutes, all of the L-ester was hydrolyzed. No further hydrolysis occured over a 30 minute period. The hydrolysate was evaporated under vacuum to a volume of 150 ml. The D-ester crystallized out upon standing. The melting point agrees with that of a previously prepared N-acetyl-L-tyrosine ethyl ester.

Yield: 7.00 gm.

Analysis: ($C_{15}H_{17}O_4N$)

Calculated: C 62.1, H 6.82, N 5.58

Found: C 61.9, H 7.02, N 5.37

N-Acetyl-D-Tyrosinamide

3.5 gm. of N-acetyl-D-tyrosine ethyl ester was dissolved in 100 ml. of methanol. The solution was saturated with NH_3 at 0°C. The solution was held at room temperature for 2 days. The solution was evaporated under vacuum to dryness. The residue was recrystallized twice from a water-alcohol mixture. A solution of this product was treated with chymotrypsin at pH 8.00 for 2 hours. No hydrolysis occurred.

Melting Point: 225-226 (Corr.)

This value agrees with the melting point of the L-isomer. A mixed melting point with the L-isomer is 196-197 which agrees with an authentic DL-compound.

Analysis: ($C_{11}H_{14}O_{13}N_2$)

Calculated: C 59.5, H 6.37, N 12.6

Found: C 59.7, H 6.64, N 12.5

Results

In the following pages of tabulated data and graphs, the symbols employed are defined in the following manner.

t = time in minutes

e = enzyme concentration $\frac{\text{(mg. enzyme N)}}{\text{(ml.)}}$

s = concentration of substrate $\frac{\text{(micromoles)}}{\text{(ml.)}}$

s_o = initial substrate concentration $\frac{\text{(micromoles)}}{\text{(ml.)}}$

$\frac{1}{s}^*$ = $\frac{1}{\text{concentration of substrate}}$ $\frac{1}{\frac{\text{(millimoles)}}{\text{(ml.)}}}$

$\frac{1}{v}^*$ = $\frac{1}{\text{velocity of substrate hydrolysis}}$ $\frac{1}{\frac{\text{(millimoles)}}{\text{(min.)}\quad\text{(ml.)}}}$

V = maximum velocity $\frac{\text{(micromoles)}}{\text{(min.)}\quad\text{(ml.)}}$

K_m = equilibrium constant of the reaction:

enzyme-substrate complex $\rightleftharpoons$ enzyme + substrate

$\frac{\text{(micromoles)}}{\text{(ml.)}}$

K_I = equilibrium constant of the reaction:

enzyme-inhibitor complex $\rightleftharpoons$ inhibitor + enzyme

$\frac{\text{(micromoles)}}{\text{(ml.)}}$

$$F(s) = 2.3\ K_m\ \text{Log}\ \frac{s_o}{s} + (s_o - s) \qquad \frac{\text{(micromoles)}}{\text{(ml.)}}$$

$$F(s)^* = 2.3\ \left(1 + \frac{i}{K_I}\right)\ K_m\ \text{Log}\ \frac{s_o}{s} + (s_o - s) \qquad \frac{\text{(micromoles)}}{\text{(ml.)}}$$

Table I

The Hydrolysis of N-Acetyl-L-Tyrosinamide
by Chymotrypsin

$K_m = 28.2 \frac{(\text{micromoles})}{(\text{ml})}$

$e = .150 \frac{(\text{mg. enzyme N})}{(\text{ml})}$

s_o	t	s	Log s	s_o-s	2.3 K_m Log$\frac{S_o}{s}$	F(s)
5.00	1.6	4.90	.690	.10	.59	.69
	11.8	4.36	.640	.64	3.9	4.54
	30.0	3.55	.550	1.45	9.7	11.15
	43.5	3.05	.484	1.95	14.0	15.95
	67.5	2.25	.352	2.75	22.5	25.25
	88.0	1.75	.243	3.25	29.6	32.85
10.0	1.8	9.80	.992	.20	.5	.7
	10.1	9.10	.958	.90	2.7	3.6
	20.4	8.20	.913	1.8	5.7	7.5
	40.4	6.54	.816	3.5	12.0	15.5
	102.0	3.32	.522	6.7	31.1	37.8
20.0	7.3	18.9	1.277	1.1	1.6	2.7
	15.0	17.9	1.240	2.1	3.2	5.3
	31.0	15.4	1.189	4.6	7.4	12.0
	47.0	13.5	1.127	6.5	11.3	17.8
	71.0	11.0	1.042	9.0	16.9	25.9
	100.0	8.56	.932	11.5	24.0	36.5
40.0	15.0	36.5	1.562	3.5	2.6	6.1
	22.7	35.3	1.548	4.7	3.6	8.3
	34.0	33.3	1.522	6.7	5.2	11.9
	48.7	30.1	1.479	9.9	8.1	18.0
	69.4	26.6	1.425	13.4	11.6	25.0
	102.0	21.4	1.331	18.6	17.7	36.3

Fig.-1

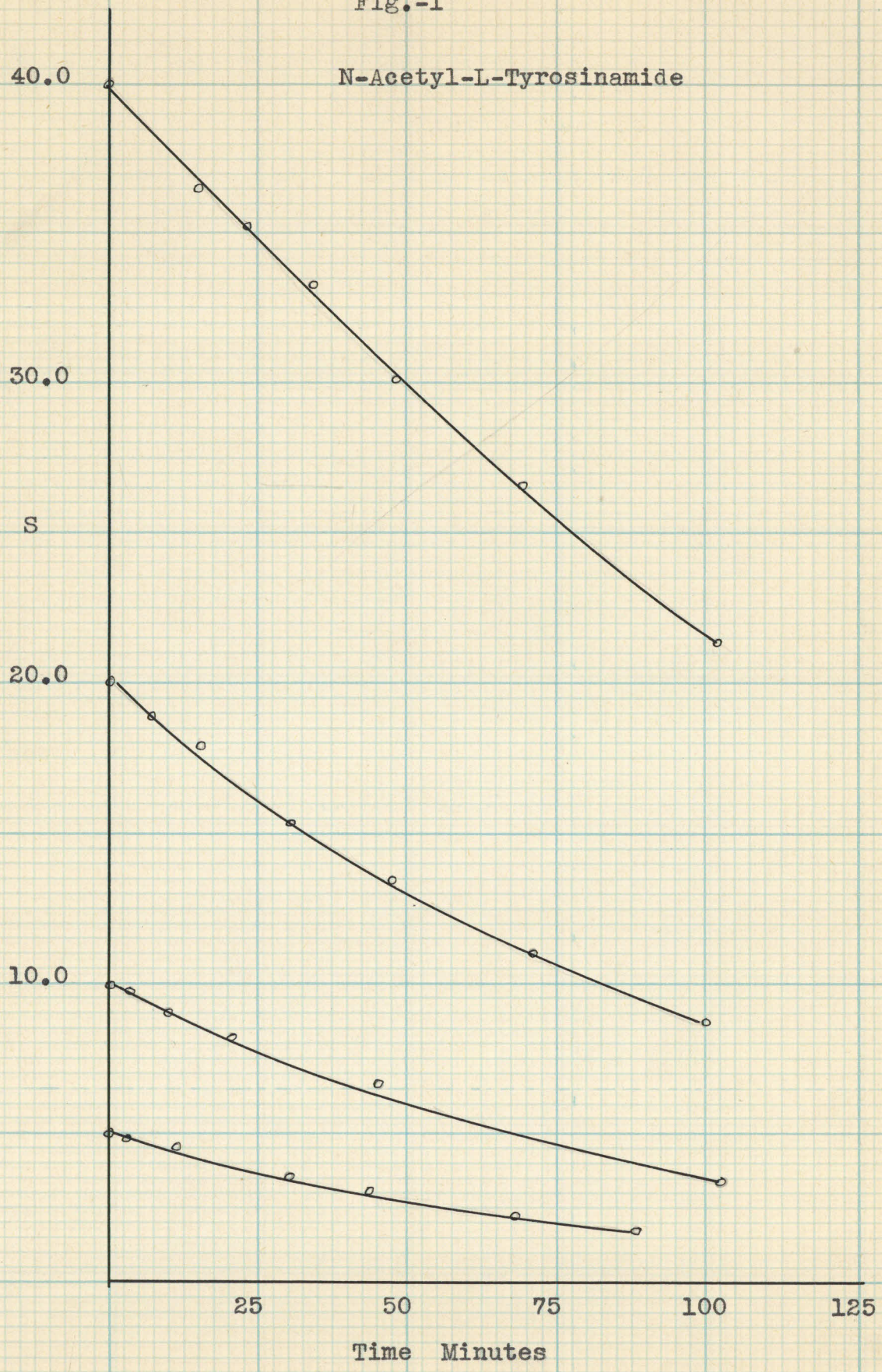

N-Acetyl-L-Tyrosinamide
40.0
30.0
S
20.0
10.0
25
50
75
100
125
Time Minutes

Fig.-2

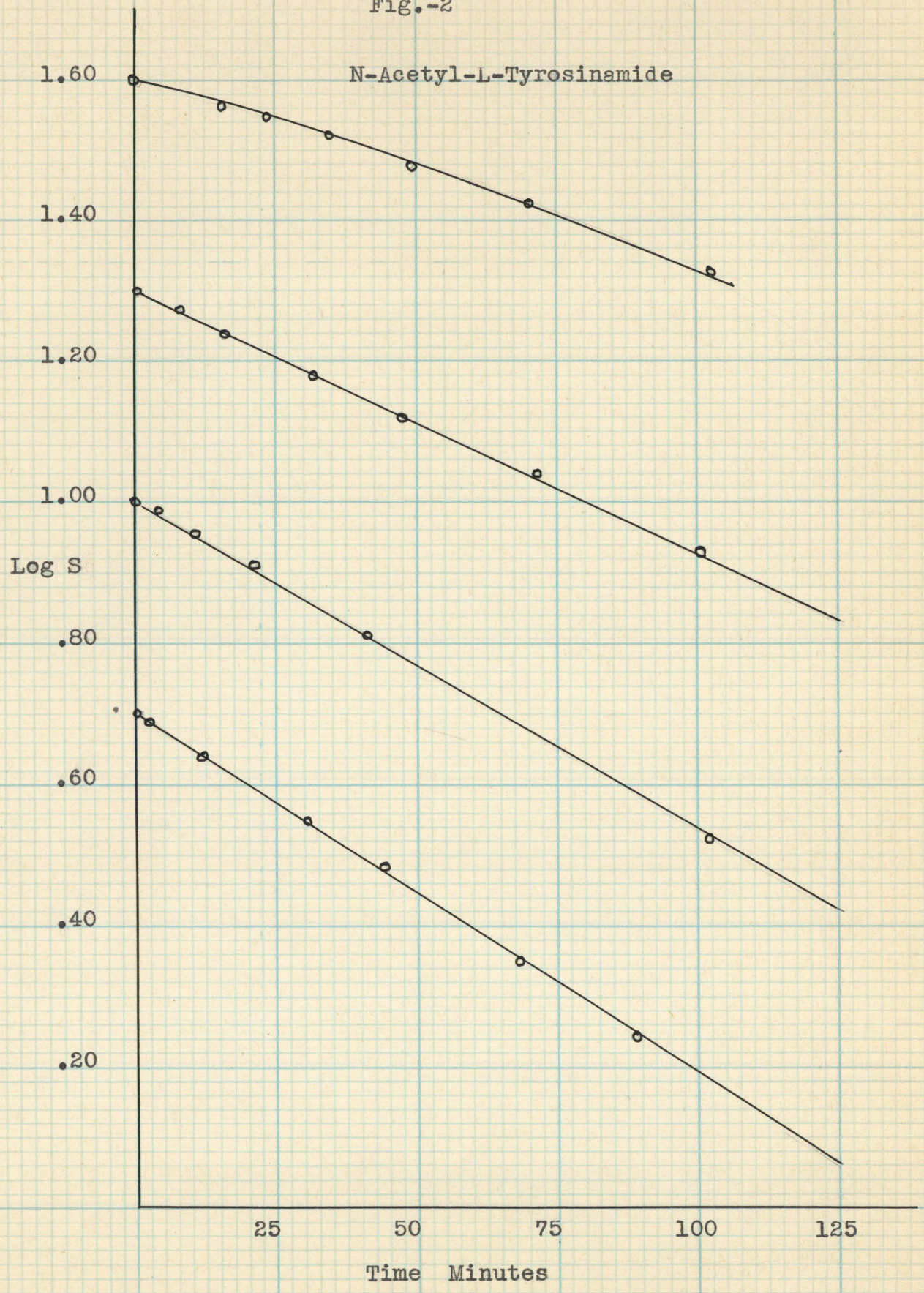

N-Acetyl-L-Tyrosinamide
1.60
1.40
1.20
1.00
Log S
.80
.60
.40
.20
25
50
75
100
125
Time Minutes

Fig.-3

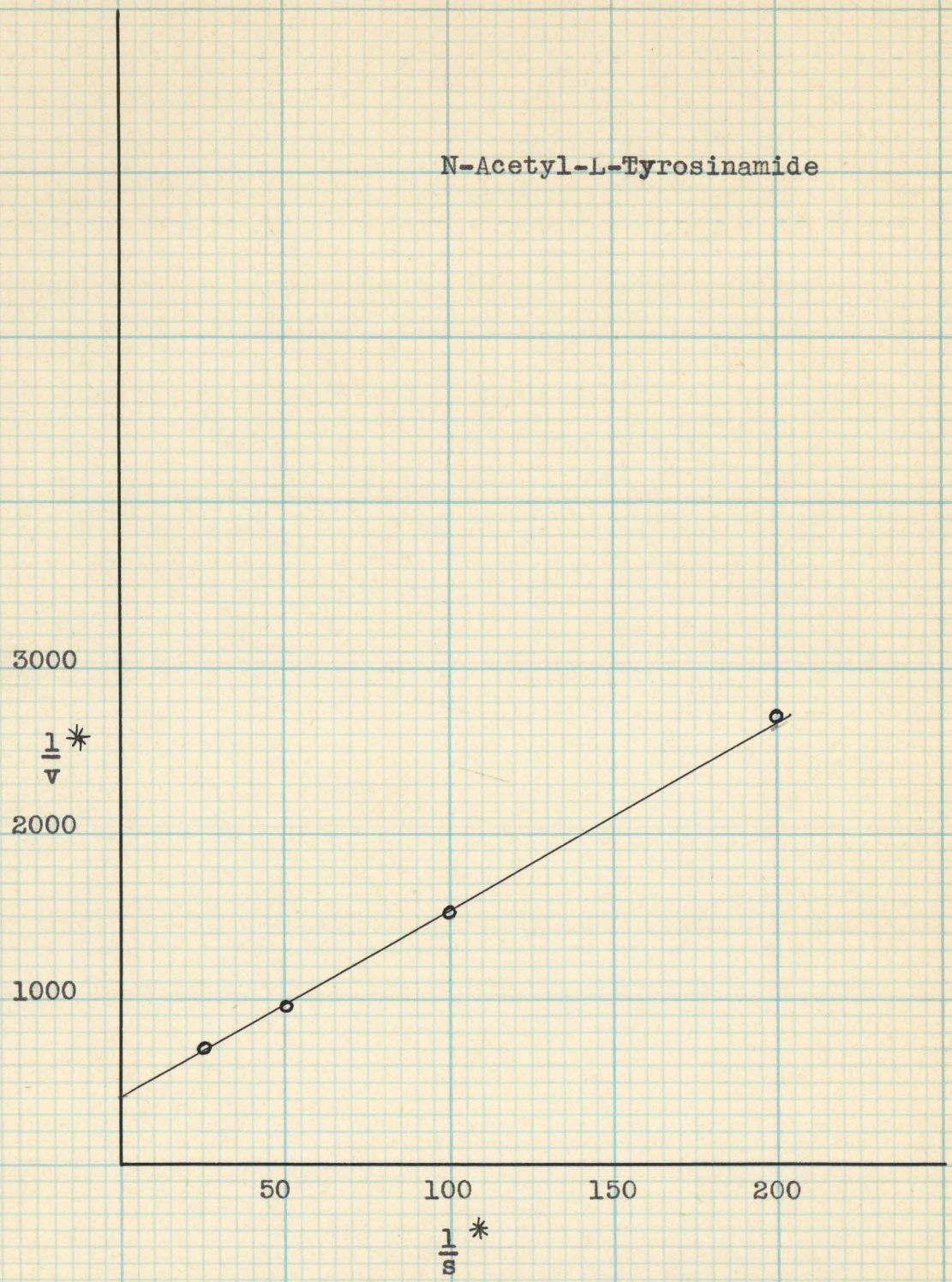

N-Acetyl-L-Tyrosinamide
3000
$\frac{1}{v}^*$
2000
1000
50
100
150
200
$\frac{1}{s}^*$

Fig.-4

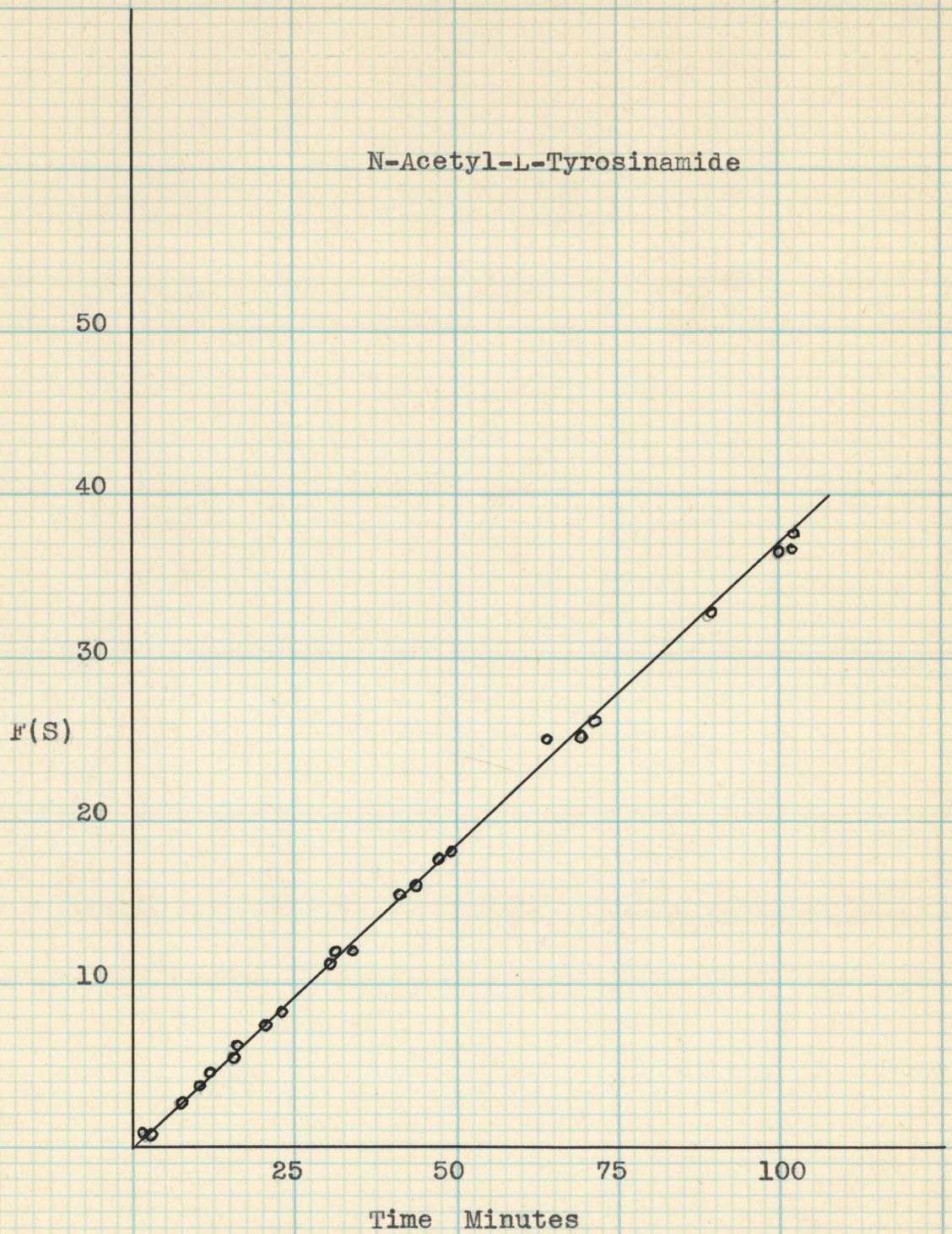

N-Acetyl-L-Tyrosinamide
50
40
30
F(S)
20
10
25
50
75
100
Time Minutes

Table II

The Hydrolysis of N-Formyl-L-Tyrosinamide
by Chymotrypsin

$K_m = 11.2 \frac{(\text{micromoles})}{(\text{ml})}$

$e = .150 \frac{(\text{mg. enzyme N})}{(\text{ml})}$

s_o	t	s	Log s	$s_o - s$	2.3 K_m Log $\frac{s_o}{s}$	F(s)
10.0	10.0	9.76	.990	.24	.28	.5
	21.0	9.64	.985	.36	.39	.8
	42.0	8.87	.948	1.13	1.34	2.5
	81.0	7.79	.892	2.21	2.78	5.0
	116.0	6.95	.842	3.05	4.07	7.1
20.0	9.2	19.9	1.299	.1	.05	.2
	21.3	19.1	1.282	.9	.52	1.4
	31.8	18.8	1.274	1.2	.70	1.9
	50.2	18.1	1.258	1.9	1.11	3.0
	78.0	17.0	1.231	3.0	1.83	4.8
	115.0	15.7	1.196	4.3	2.74	7.0
40.0	32.0	39.0	1.592	1.0	.28	1.3
	53.0	37.9	1.579	2.1	.62	2.7
	85.0	36.1	1.558	3.9	1.16	5.1
	131.0	34.1	1.533	5.9	1.78	7.7

Fig.-5

N-Formyl-L-Tyrosinamide

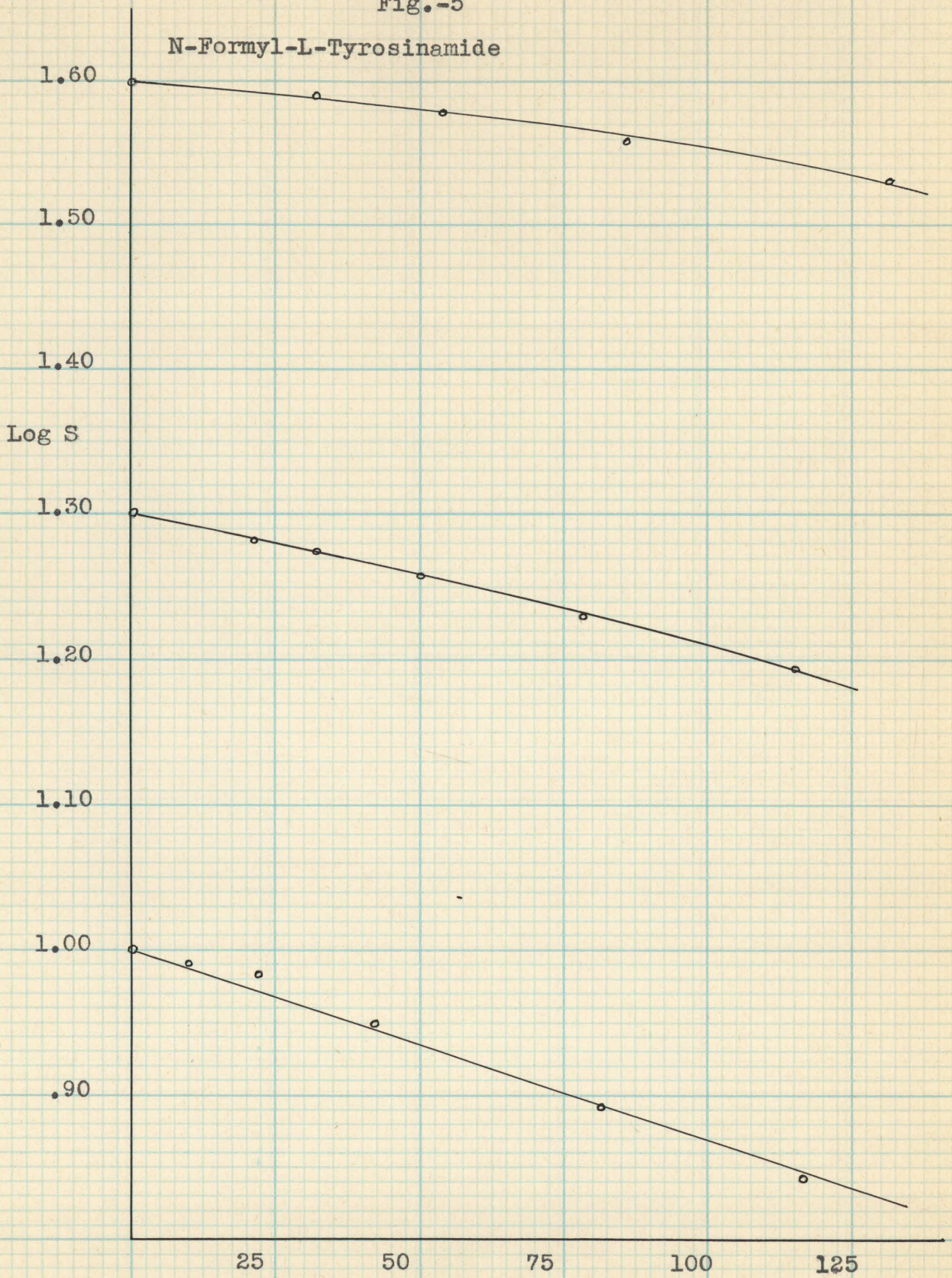


Fig.-6

N-Formyl-L-Tyrosinamide

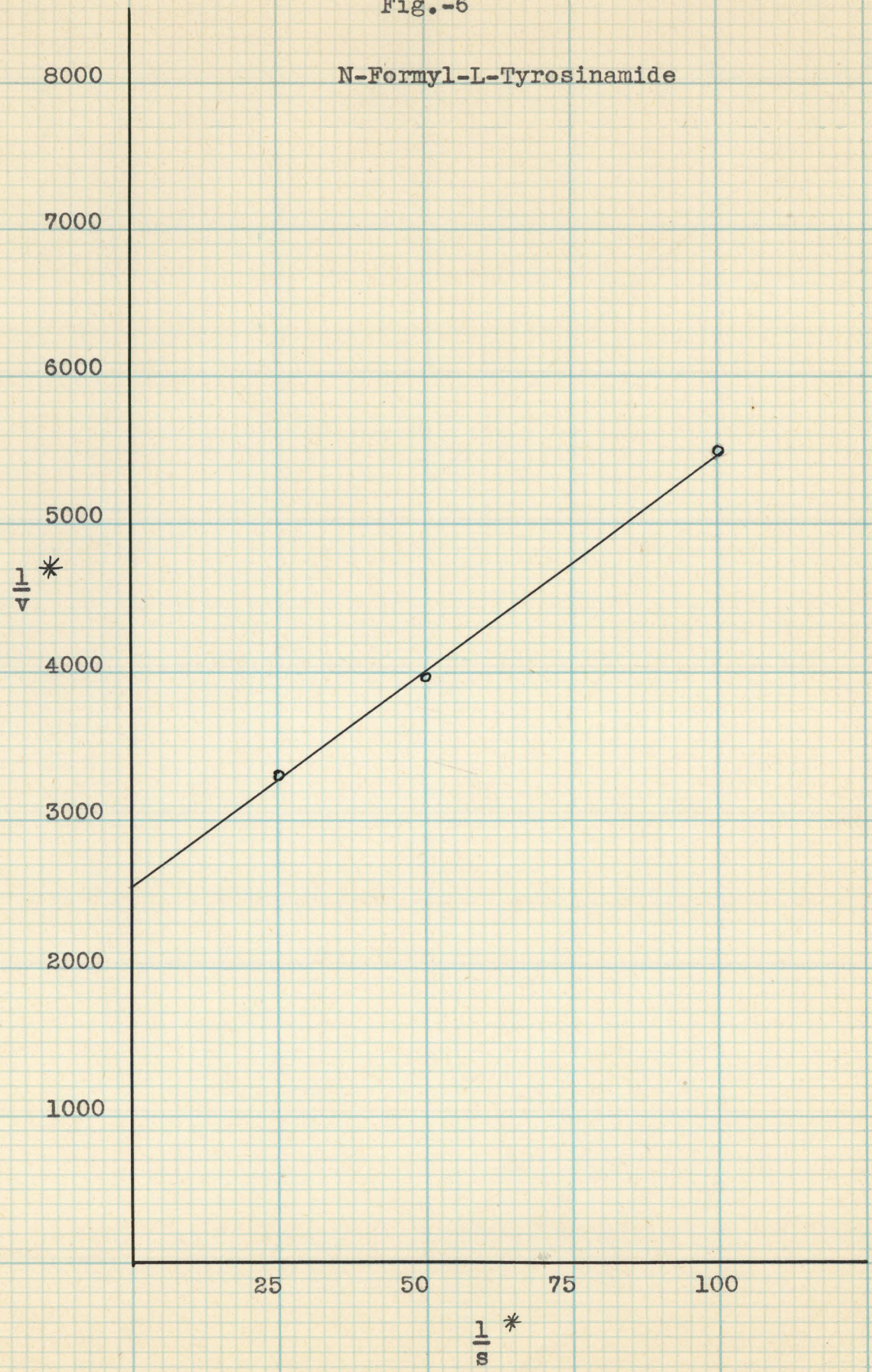


Fig.-7

N-Formyl-L-Tyrosinamide

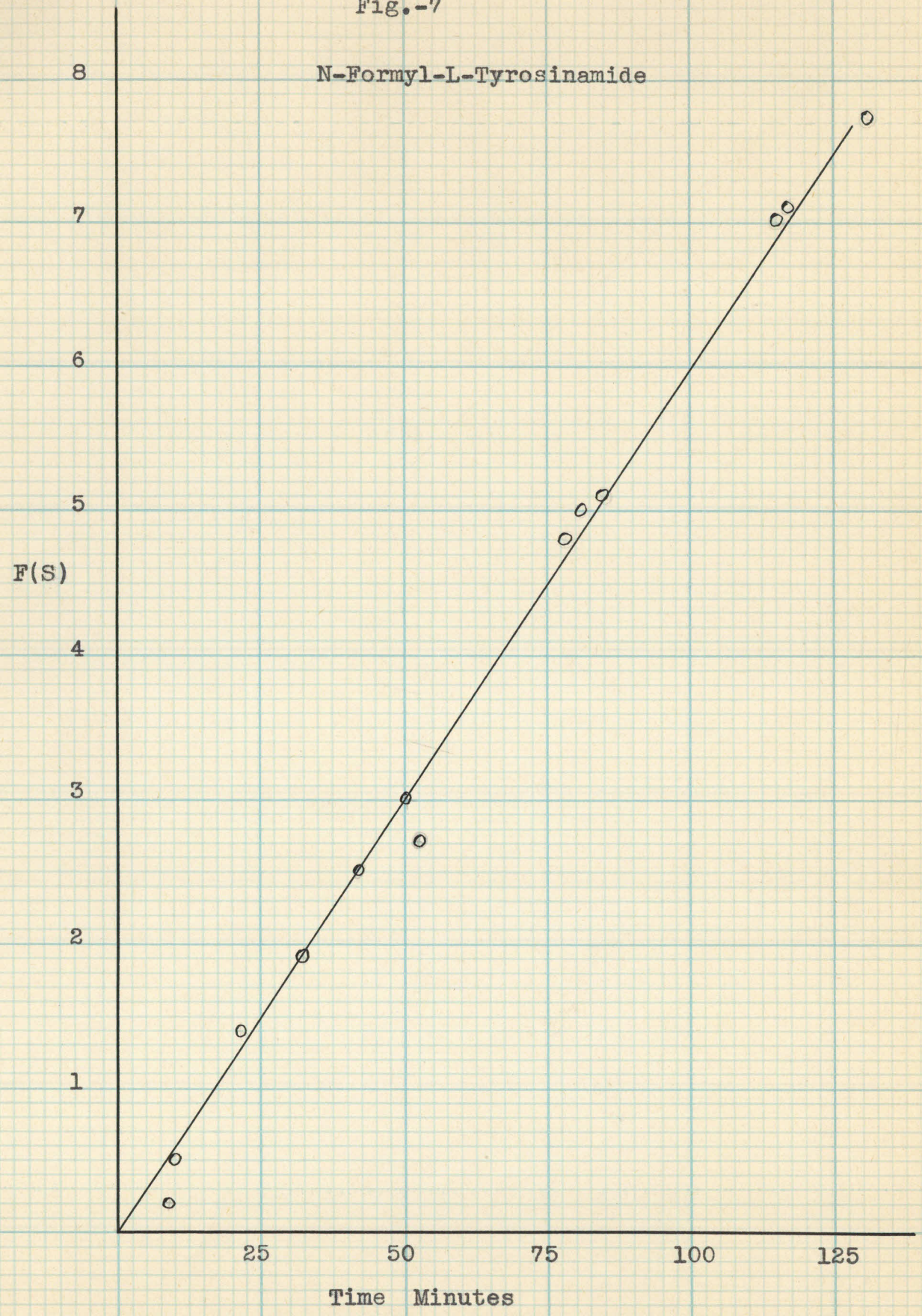


Table III

The Hydrolysis of N-Benzoyl-L-Tyrosinamide
by Chymotrypsin

$K_m = 1.9 \frac{(\text{micromoles})}{(\text{ml})}$

$e = .075 \frac{(\text{mg. enzyme N})}{(\text{ml})}$

s_o	t	s	Log s	s_o-s	2.3 K_m Log $\frac{s_o}{s}$	F(s)
2.50	1.1	2.32	.366	.68	.15	.8
	10.3	1.12	.050	1.38	1.52	2.9
	20.0	.52	- .284	1.98	3.0	5.0
	30.2	.11	- .958	2.39	5.9	8.3
5.00	1.2	4.62	.666	.38	.15	.5
	5.9	3.53	.548	1.47	.66	2.1
	11.2	2.58	.412	2.42	1.25	3.7
	20.2	1.38	.141	3.62	2.44	6.1
10.0	1.3	9.65	.985	.35	.07	.4
	8.0	8.00	.904	2.0	.42	2.4
	15.7	6.20	.793	3.8	.90	4.7
	27.0	3.54	.550	6.5	1.96	8.5
	41.0	1.85	.268	8.1	3.19	11.3

Fig.-8

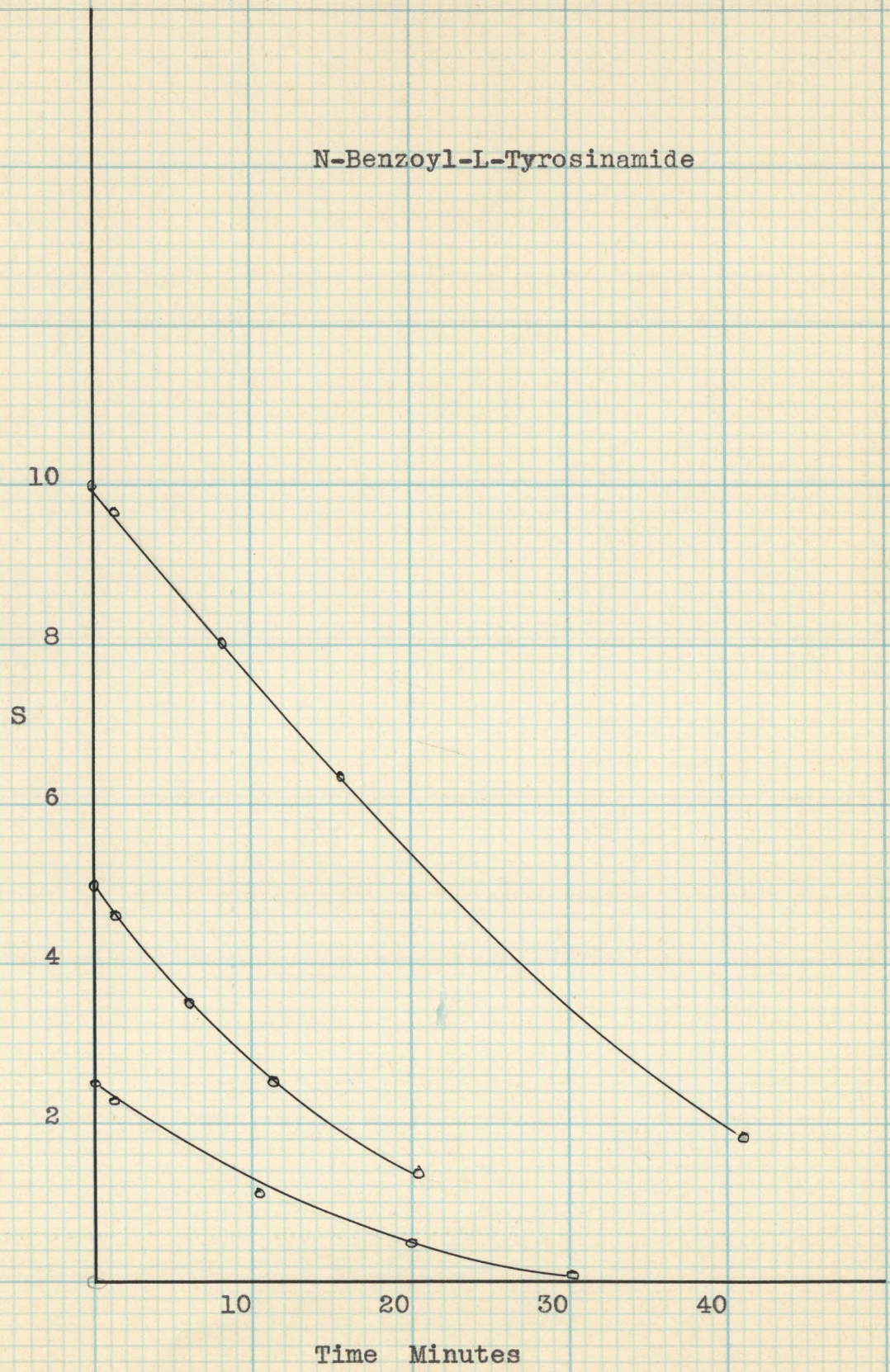


Fig.-9

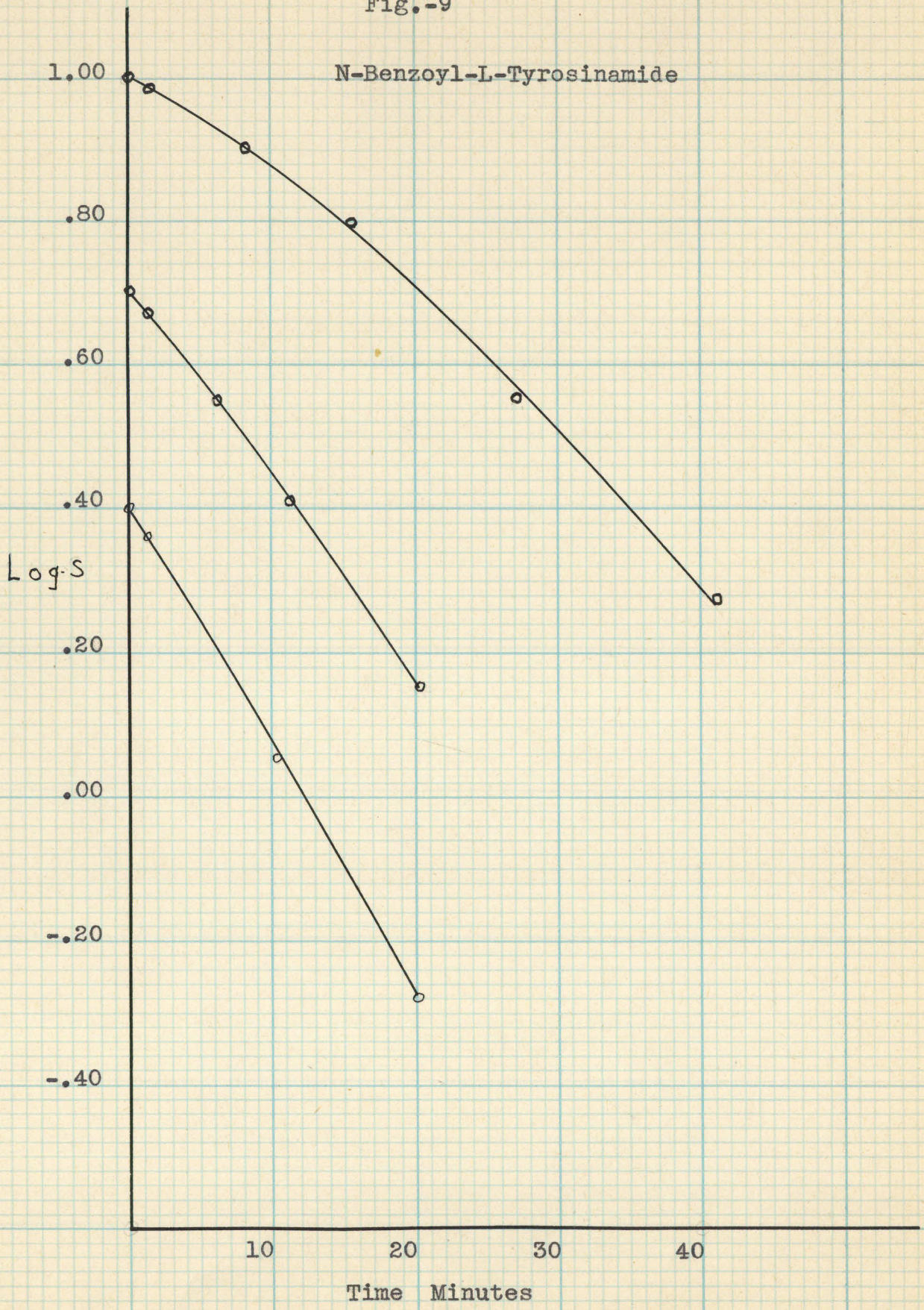

N-Benzoyl-L-Tyrosinamide
1.00
.80
.60
.40
.20
.00
-.20
-.40
Log S
10
20
30
40
Time Minutes

Fig.-10

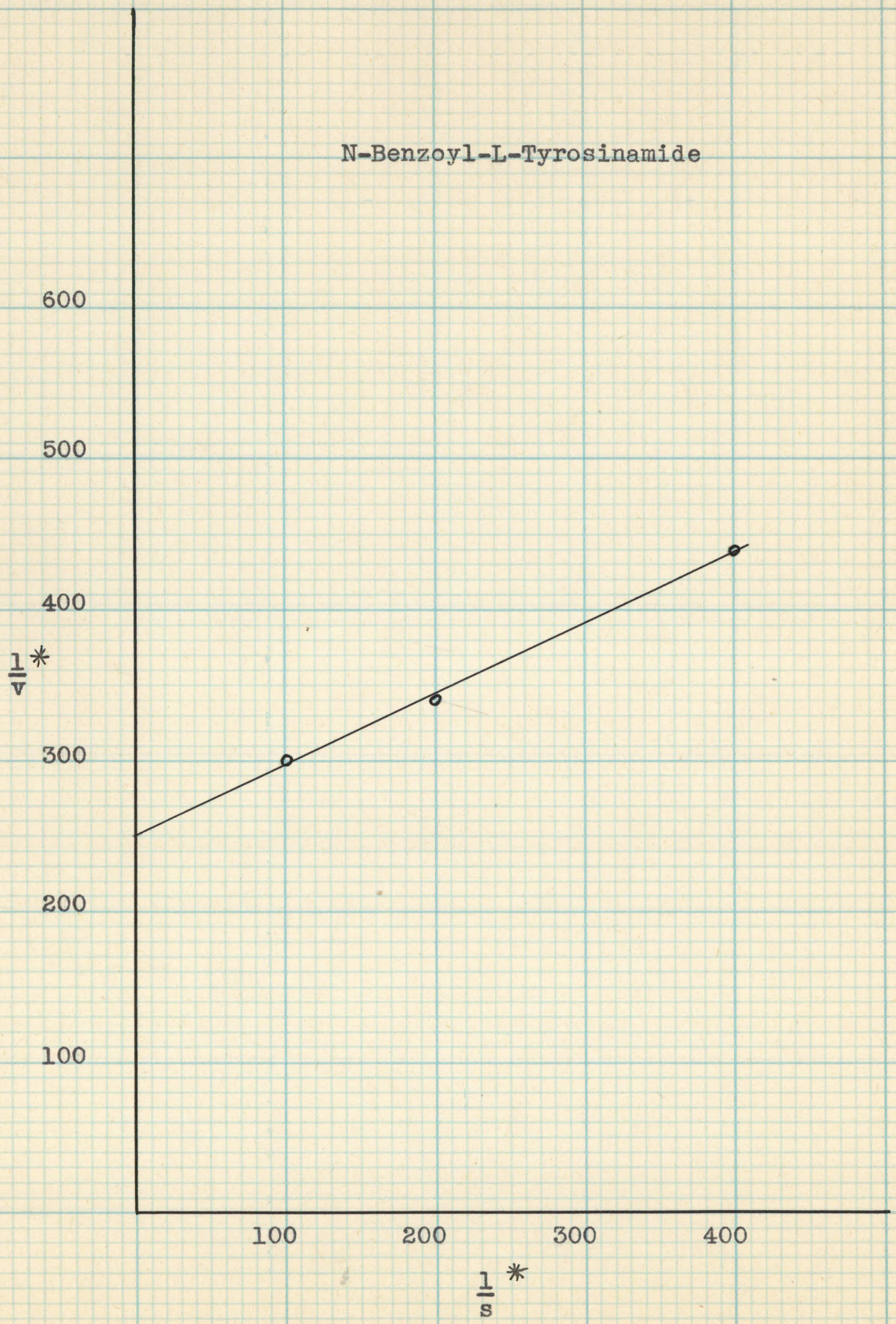

N-Benzoyl-L-Tyrosinamide
600
500
400
300
200
100
$\frac{1}{v}^*$
100
200
300
400
$\frac{1}{s}^*$

Fig.-11

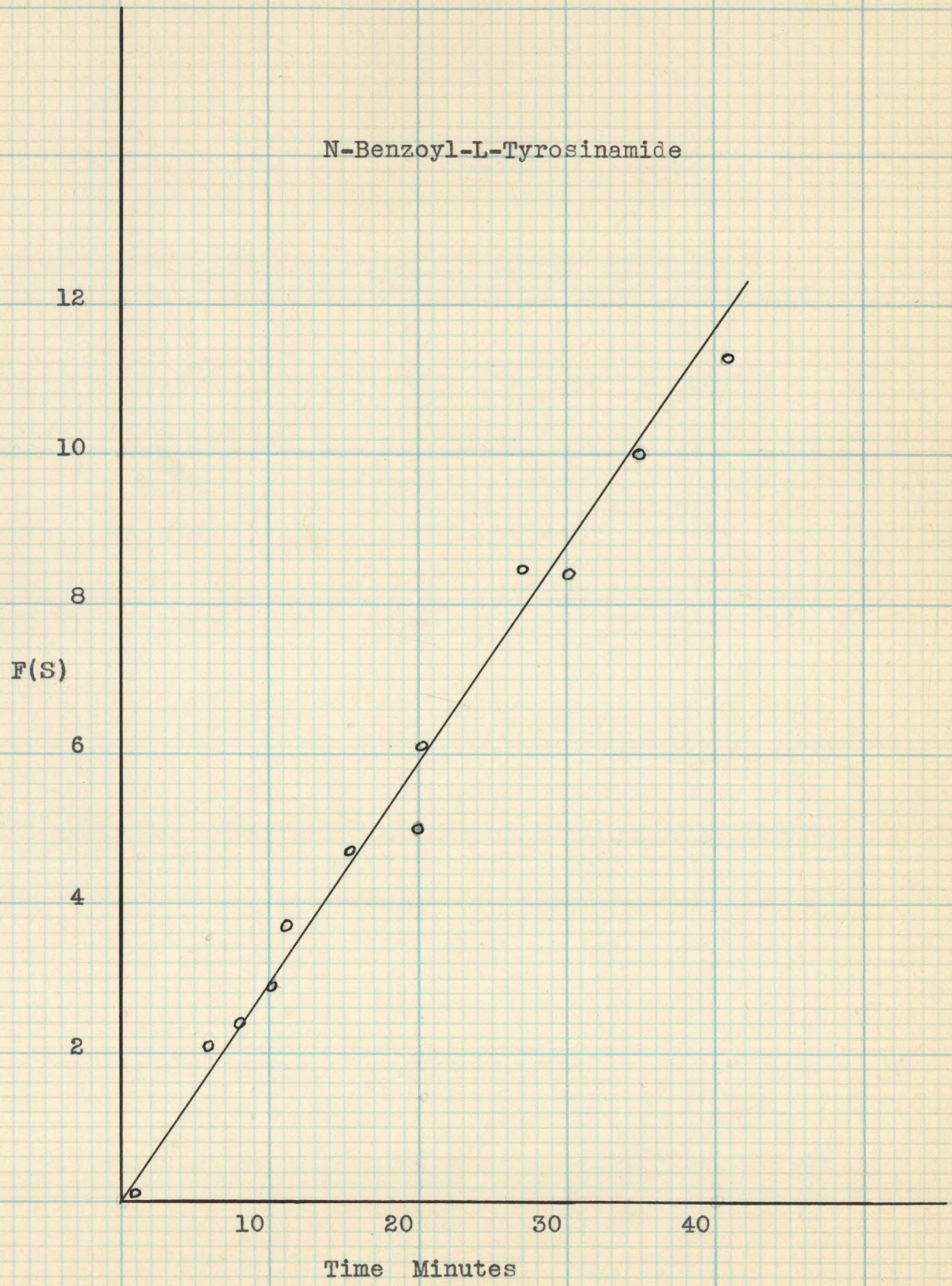

N-Benzoyl-L-Tyrosinamide
12
10
8
F(S)
6
4
2
10
20
30
40
Time Minutes

Table IV

The Hydrolysis of N-Iso Nicotinyl-L-Tyrosinamide by Chymotrypsin

$K_m = 8.40 \frac{(\text{micromoles})}{(\text{ml})}$

$e = .075 \frac{(\text{mg. enzyme N})}{(\text{ml})}$

s_o	t	s	Log s	$s_o - s$	$2.3 K_m \text{ Log } \frac{S_o}{s}$	F(s)
2.50	1.2	2.40	.380	.10	.33	.43
	5.1	2.05	.312	.45	1.68	2.13
	10.1	1.66	.221	.84	3.44	4.2
	20.0	1.11	.046	1.39	6.83	8.2
	30.0	.66	- .180	1.84	11.2	13.0
	39.2	.41	- .381	2.09	15.1	17.2
5.00	1.3	4.72	.675	.28	.46	.74
	6.5	3.84	.585	1.16	2.22	3.38
	13.0	2.99	.476	2.01	4.34	6.35
	19.7	2.29	.359	2.71	6.55	9.26
	30.2	1.57	.195	3.43	9.72	13.15
	40.0	1.08	.036	3.92	13.1	17.0
	52.3	.44	- .352	4.56	20.4	25.0
	74.0	.17	- .782	4.83	28.4	33.2
20.0	1.5	19.5	1.291	.5	.19	.7
	8.2	17.5	1.244	2.5	1.12	3.6
	15.8	15.3	1.187	4.7	2.26	7.0
	21.7	13.7	1.138	6.3	3.18	9.5
	33.5	10.8	1.036	9.2	5.1	14.3
	48.0	8.0	.902	12.0	7.7	19.7
	64.5	4.6	.661	15.4	12.3	27.7

N-Iso Nicotinyl-L-Tyrosinamide

Fig.-12

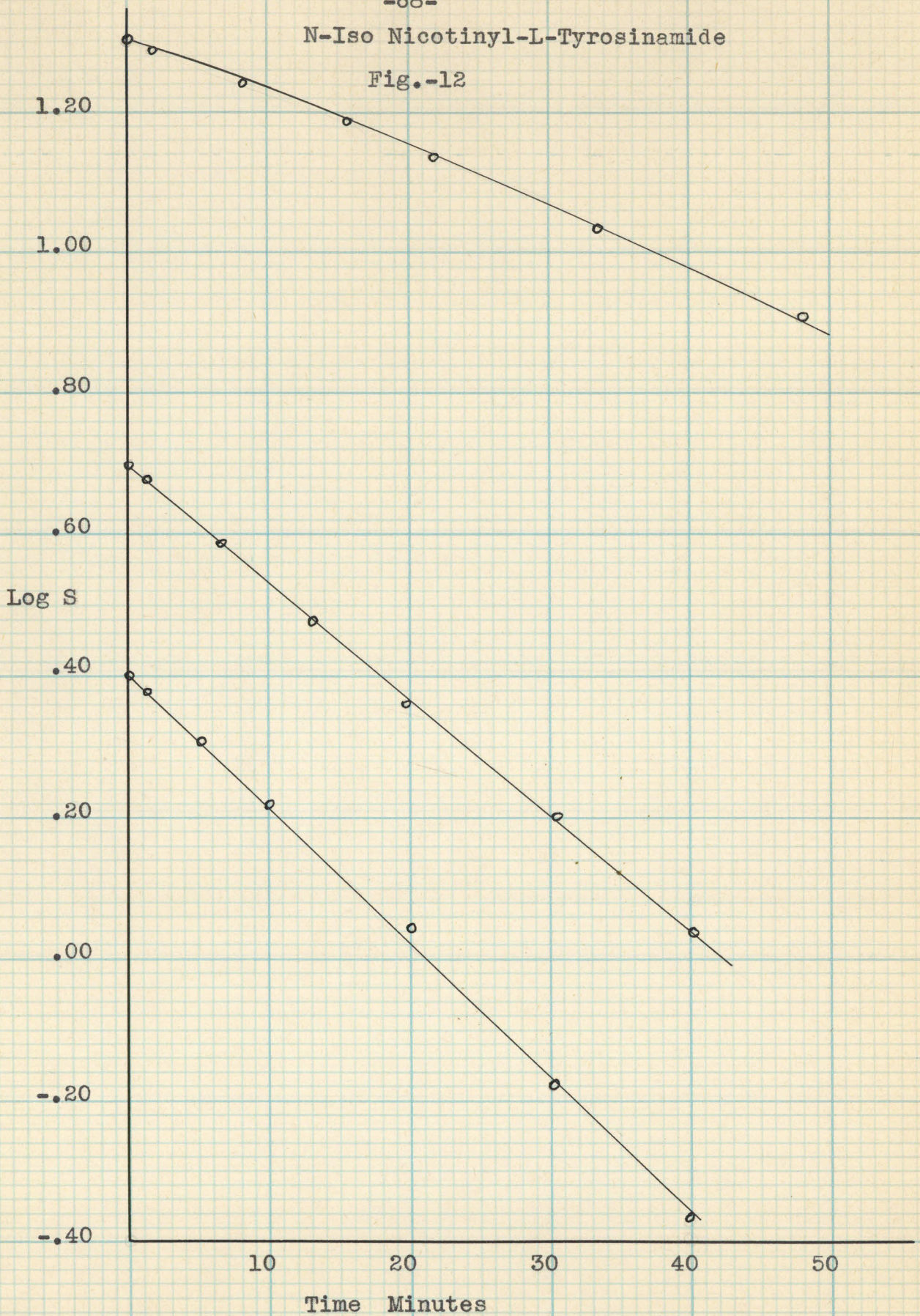


Fig.-13

N-Iso Nicotinyl-L-Tyrosinamide

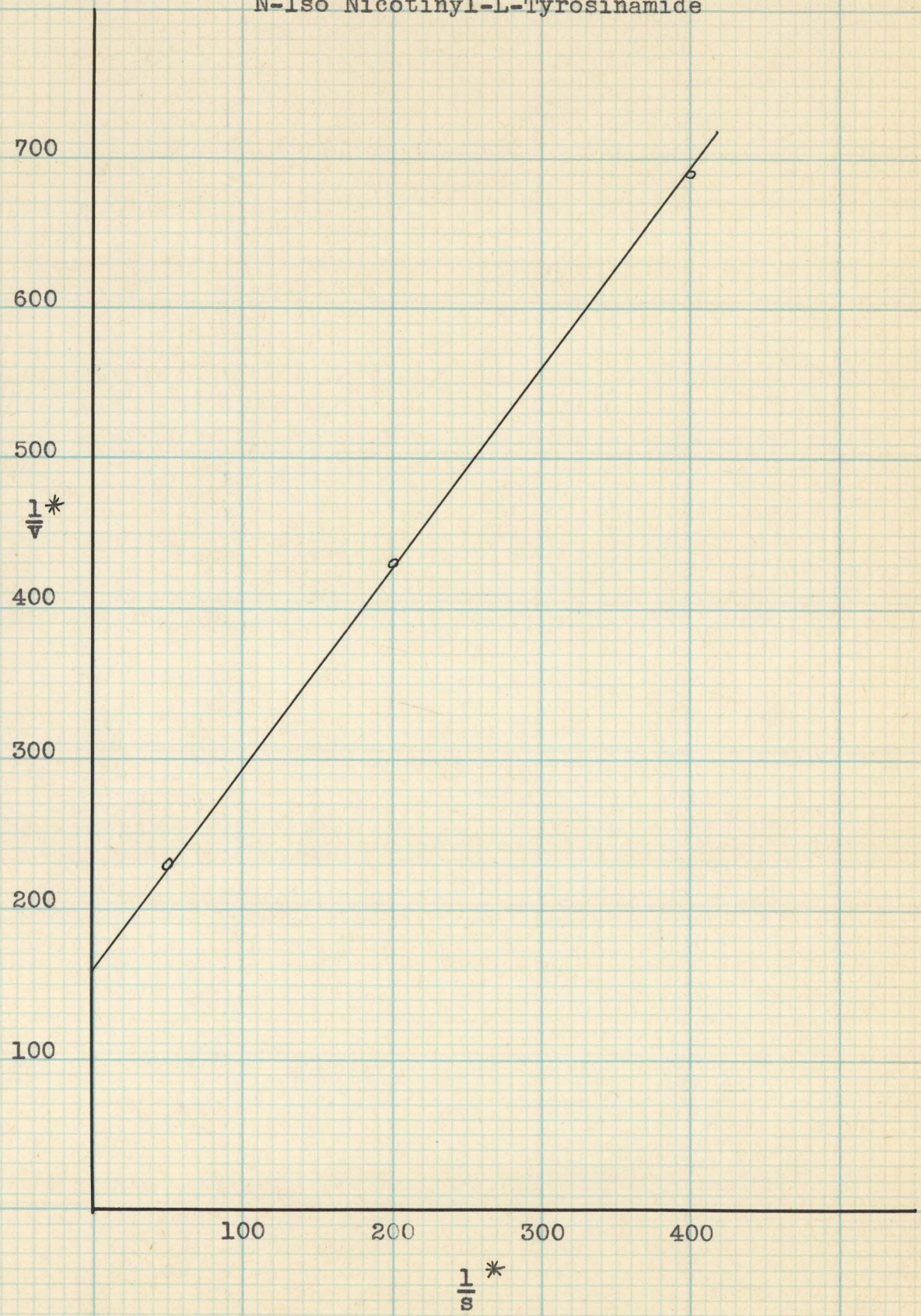


Fig.-14

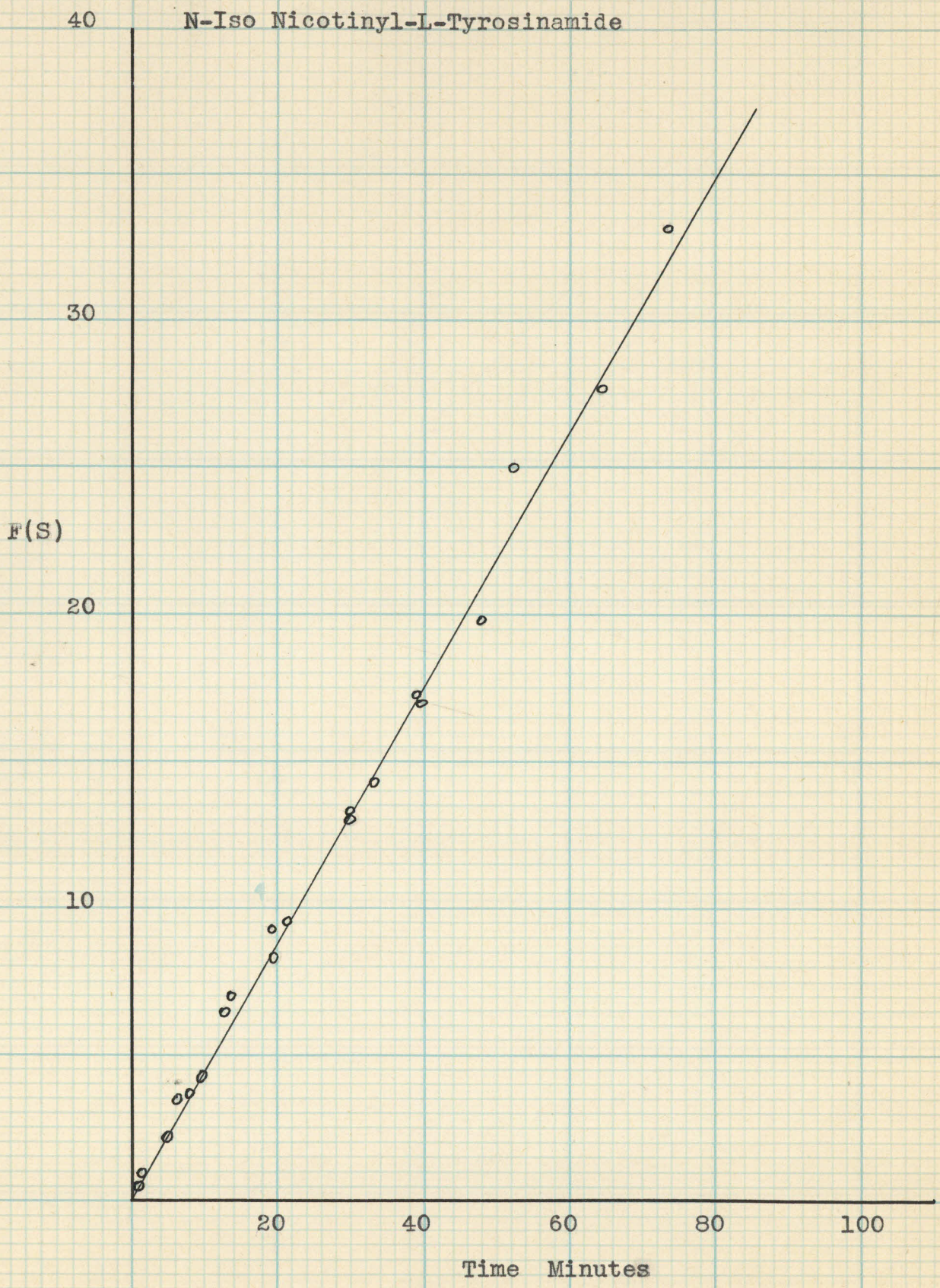

N-Iso Nicotinyl-L-Tyrosinamide
40
30
20
10
F(S)
20
40
60
80
100
Time Minutes

Table V

The Hydrolysis of N-Nicotinyl-L-Tyrosinamide by Chymotrypsin

$K_m = 13.1 \frac{(\text{micromoles})}{(\text{ml.})}$

e	s_o	t	s	Log s	s_o-s	2.3 K_m Log $\frac{s_o}{s}$	F(s)
.075	5.00	1.2	4.95	.695	.05	.15	.2
		6.3	4.25	.629	.25	2.14	2.4
		11.0	4.05	.607	.95	2.77	3.7
		20.1	3.10	.492	1.90	6.26	8.2
		30.4	2.37	.375	2.63	9.78	12.4
.075	20.0	1.3	19.6	1.293	.4	.27	.7
		8.1	17.8	1.251	2.2	1.56	3.8
		14.4	16.1	1.207	3.9	2.86	6.8
		22.0	14.0	1.146	6.0	4.67	10.7
		33.0	11.1	1.046	8.9	7.70	16.6
		48.7	8.1	.909	11.9	11.8	23.7
.150	10.0	1.0	9.80	.992	.2		
		20.0	4.80	.681	5.2		
		40.0	3.30	.518	6.7		
		61.0	1.60	.204	8.4		

Fig.-15

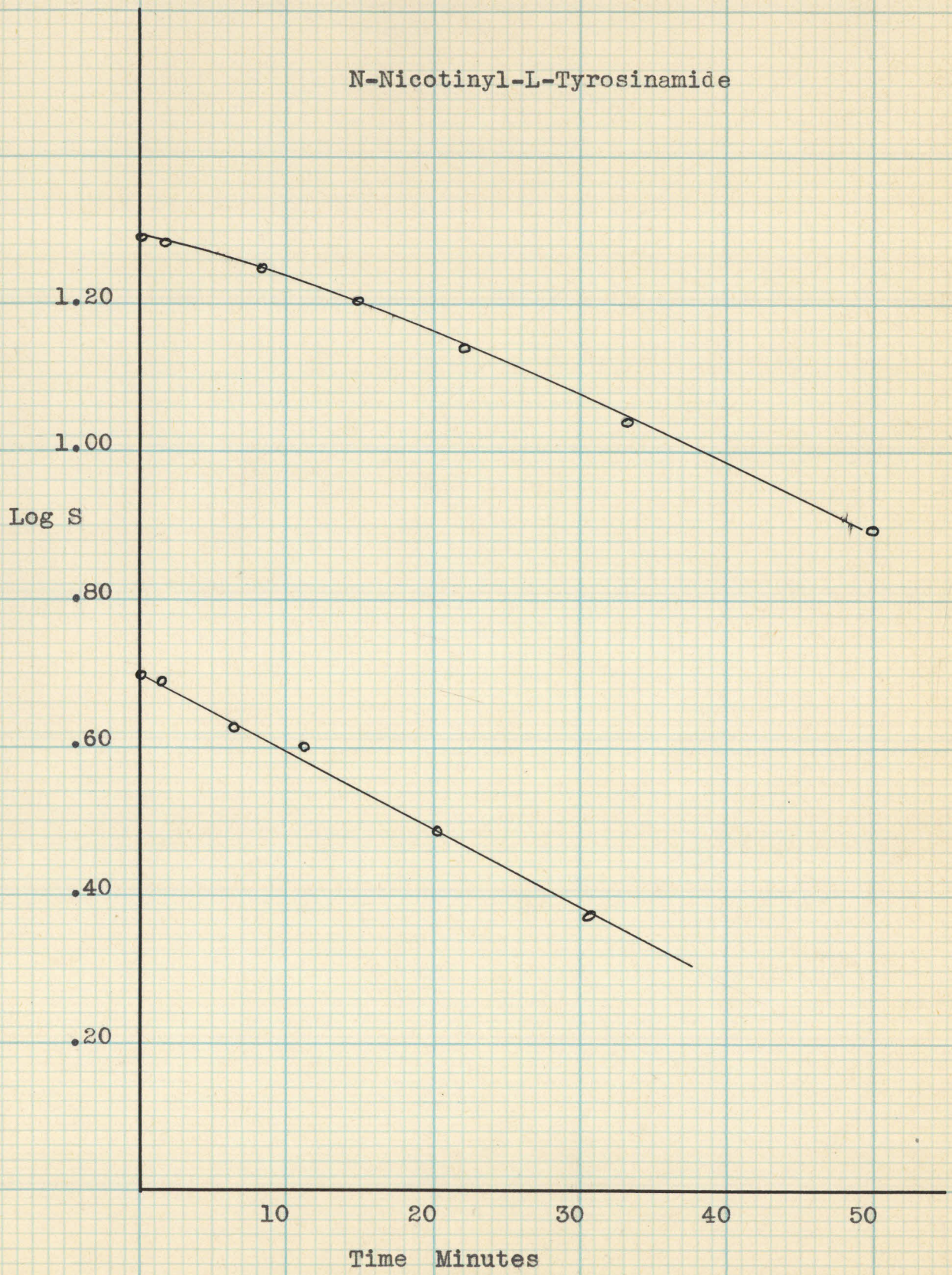

N-Nicotinyl-L-Tyrosinamide
1.20
1.00
Log S
.80
.60
.40
.20
10
20
30
40
50
Time Minutes

Fig.-16

N-Nicotinyl-L-Tyrosinamide

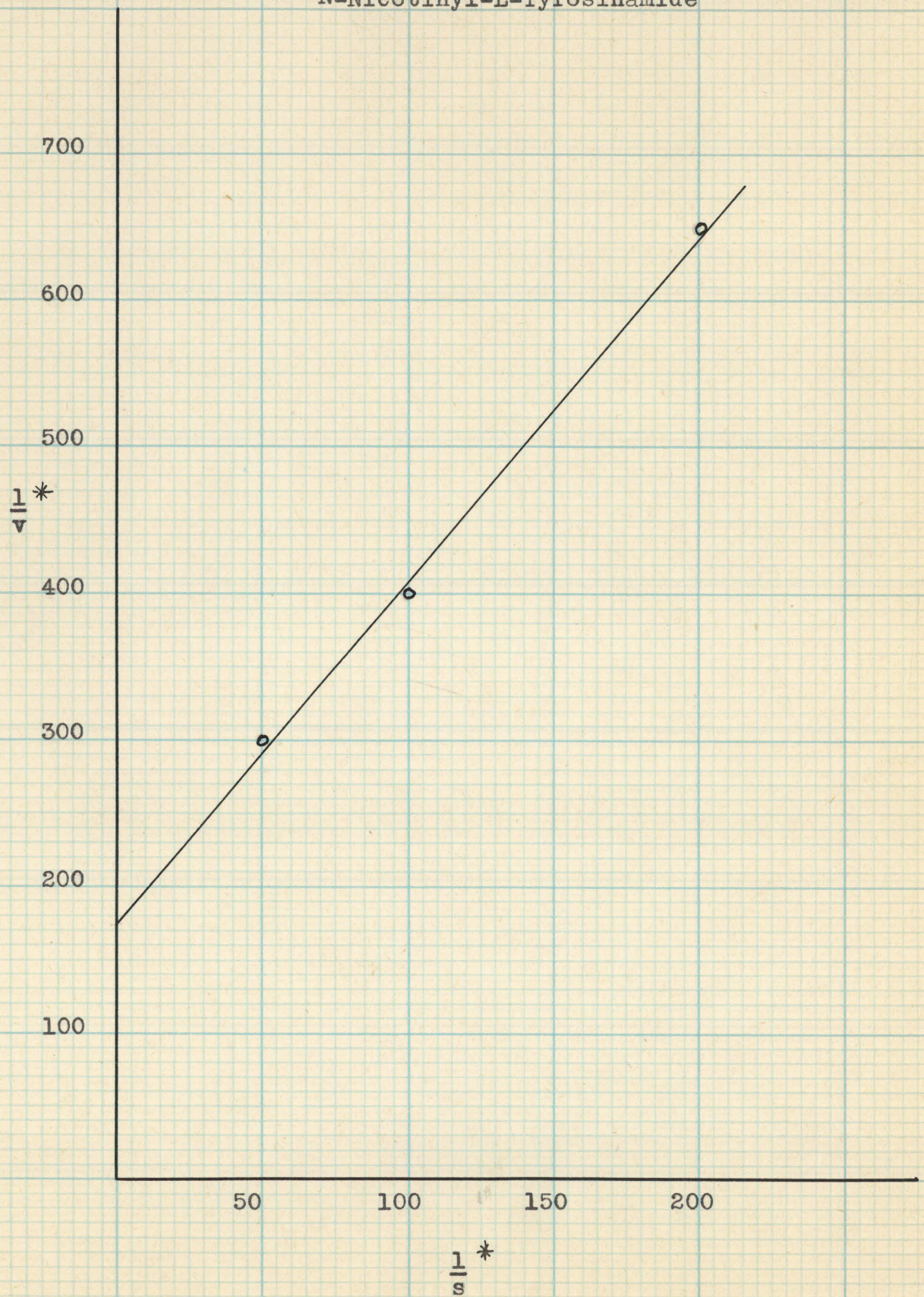


Fig.-17

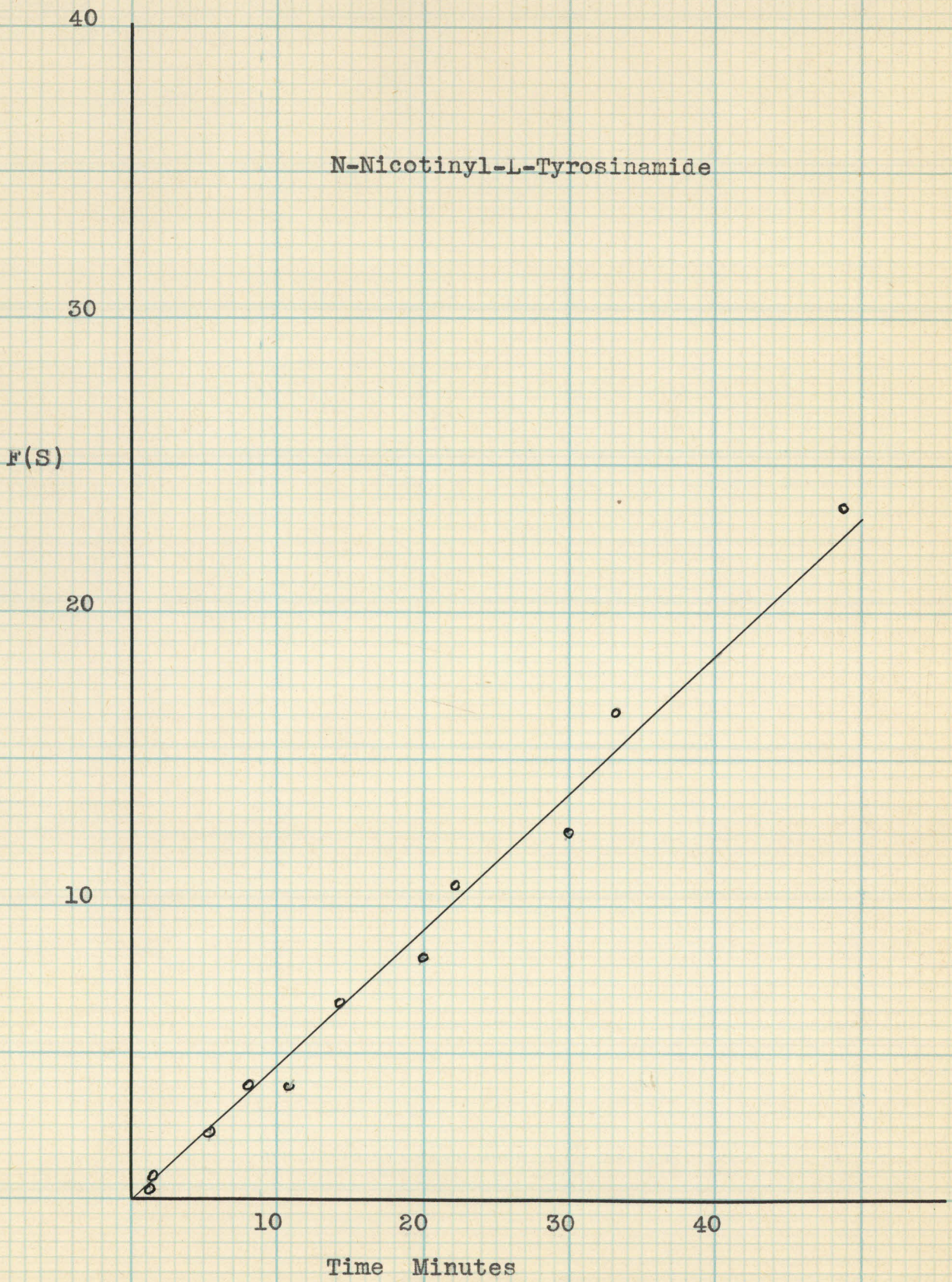

N-Nicotinyl-L-Tyrosinamide
40
30
F(S)
20
10
10
20
30
40
Time Minutes

Table VI

The Hydrolysis of N-Picolinyl-L-Tyrosinamide by Chymotrypsin

e	s_o	t	s	s_o-s	Log s
.075	2.50	1.2	2.45	.05	.389
		5.0	2.28	.22	.358
		10.0	2.11	.39	.325
		22.0	1.97	.63	.294
		33.5	1.63	.87	.212
		44.0	1.46	1.04	.164
		72.0	.96	1.54	-.017
.150	2.50	1.3	2.43	.07	.385
		9.5	1.86	.64	.270
		20.5	1.46	1.04	.165
		30.0	1.26	1.24	.101
		42.5	.92	1.58	-.036
		64.0	.59	1.91	-.229

Fig.-18

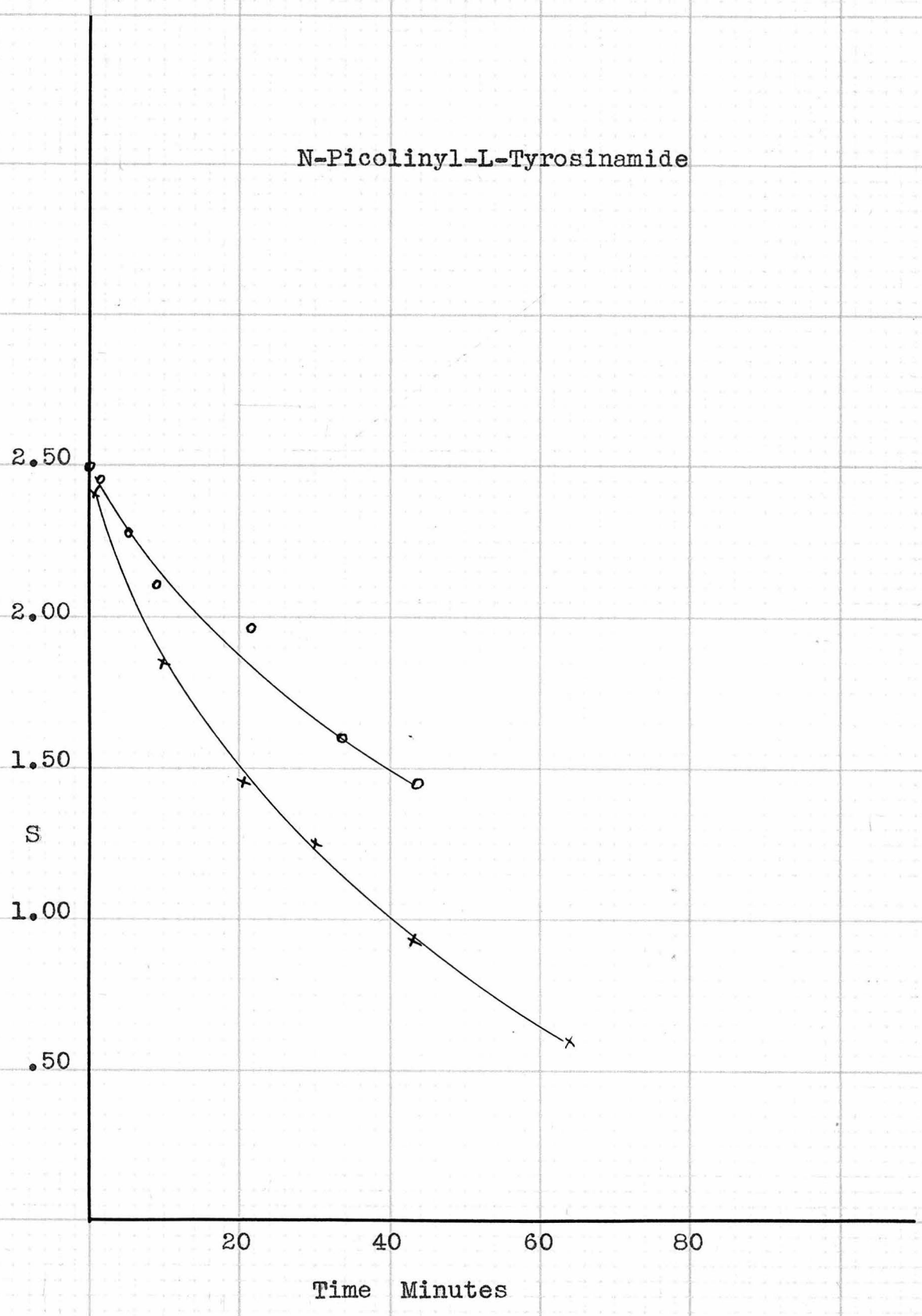

N-Picolinyl-L-Tyrosinamide
2.50
2.00
1.50
S
1.00
.50
20
40
60
80
Time Minutes

Fig.-19

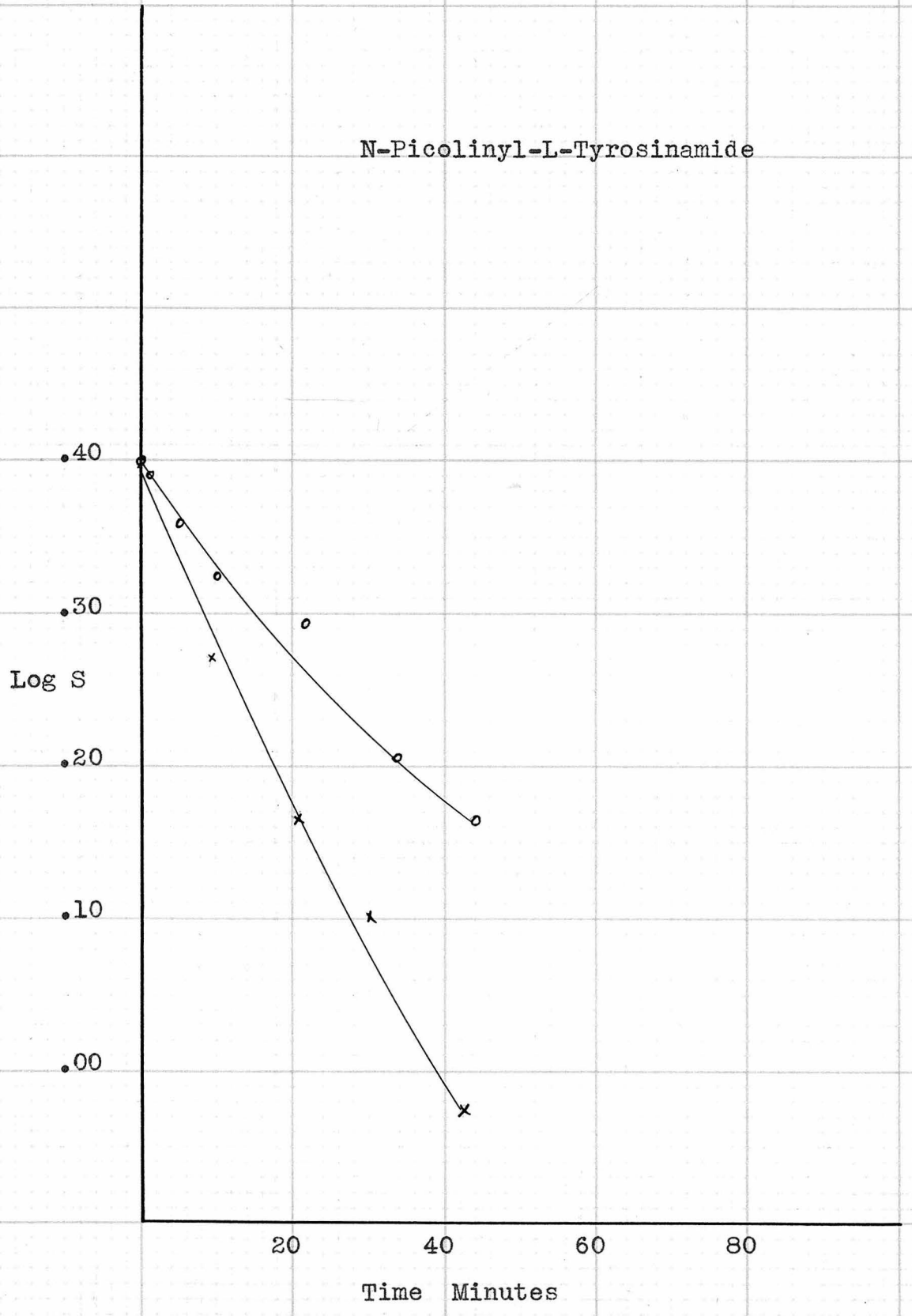

N-Picolinyl-L-Tyrosinamide
.40
.30
Log S
.20
.10
.00
20
40
60
80
Time Minutes

Table VII

The Hydrolysis of N-Nicotinyl-L-Tyrosinhydrazide by Chymotrypsin

$$e = .075 \frac{(\text{mg. enzyme N})}{(\text{ml.})}$$

s_o	t	s	Log s	s_o-s
5.00	1.2	4.95	.694	.05
	6.6	4.65	.668	.35
	10.6	4.50	.654	.50
	20.3	3.92	.593	1.08
	40.3	3.43	.536	1.57
	61.0	2.53	.404	2.47
	91.0	1.85	.268	3.15

Fig.-20

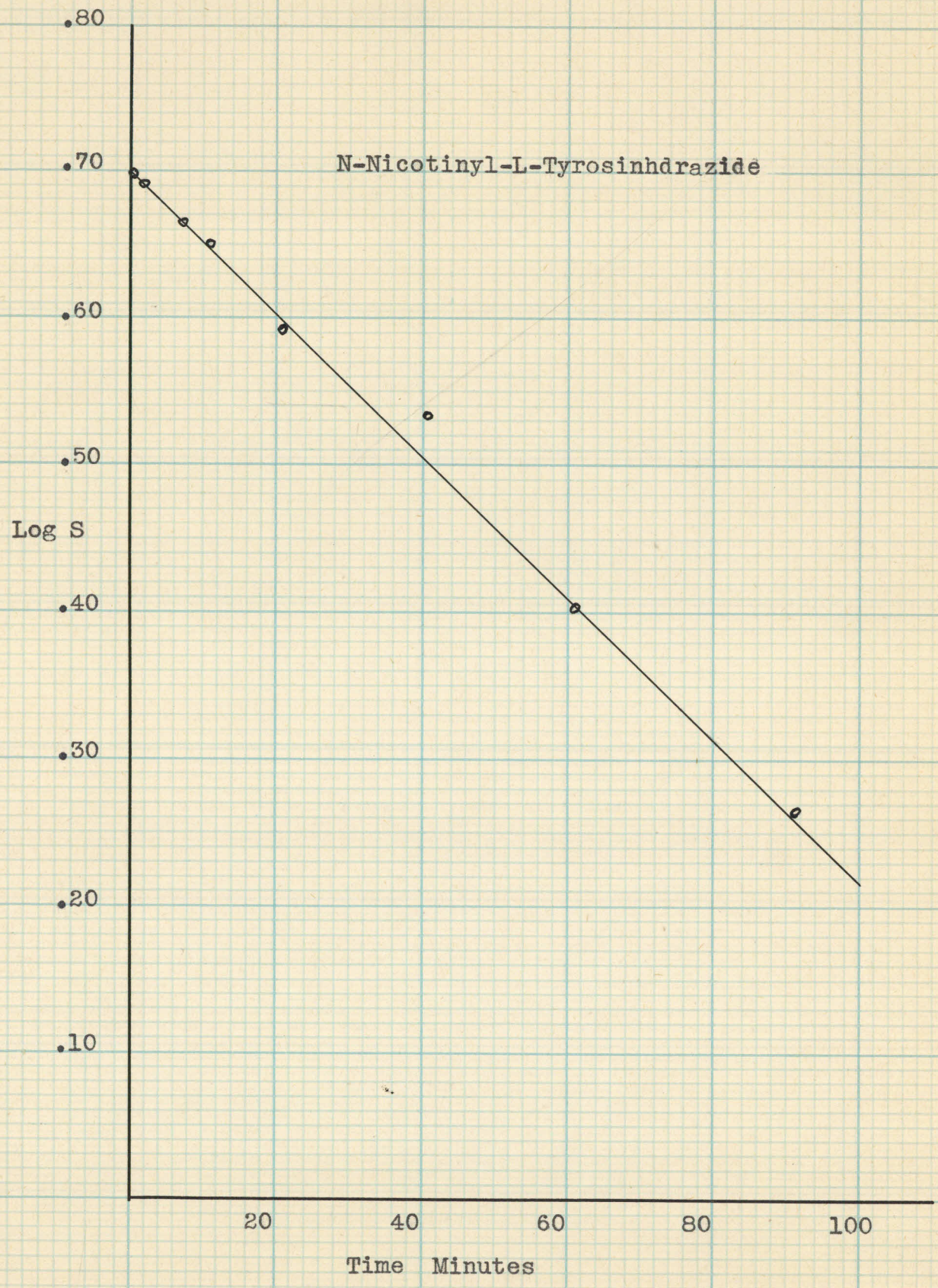

N-Nicotinyl-L-Tyrosinhdrazide
.80
.70
.60
.50
.40
.30
.20
.10
Log S
20
40
60
80
100
Time Minutes

Table VIII

Inhibition of the Chymotrypsin Catalyzed Hydrolysis of N-Acetyl-L-Tyrosinamide by the 'D' Isomer

$K_m = 28.2 \frac{(\text{micromoles})}{(\text{ml})}$

$K_I = 9.70 \frac{(\text{micromoles})}{(\text{ml})}$

$e = .150 \frac{(\text{mg. enzyme N})}{(\text{ml})}$

$i = 5.00 \frac{(\text{micromoles})}{(\text{ml})}$

s_o	t	s	Log s	s_o-s	$2.3(1+\frac{1}{K_I})K_m \text{Log} \frac{s_o}{s}$	F(s)*
10.0	1.3	9.89	.995	.11	.49	.6
	8.2	9.31	.970	.69	3.05	3.7
	14.7	8.94	.951	1.06	4.8	5.9
	22.5	8.52	.931	1.48	6.9	8.4
	36.3	7.61	.882	2.39	11.7	14.1
	54.3	6.56	.817	3.44	18.1	21.5
	85.4	5.26	.721	4.74	27.6	32.1
	111.7	4.56	.660	5.44	33.7	39.1
20.0	1.5	19.8	1.297	.2	.2	.4
	10.3	18.7	1.272	1.3	2.9	4.2
	22.7	17.3	1.238	2.7	6.1	8.9
	38.2	15.7	1.196	4.3	10.4	14.7
	54.5	14.4	1.158	5.6	14.1	19.7
40.0	6.5	39.1	1.592	.9	.9	1.8
	18.8	37.4	1.574	2.6	3.0	5.6
	34.3	34.7	1.541	5.3	6.1	11.4
	59.5	30.5	1.484	9.5	11.6	21.1
	91.0	26.3	1.420	13.7	17.9	31.6

Fig.-21

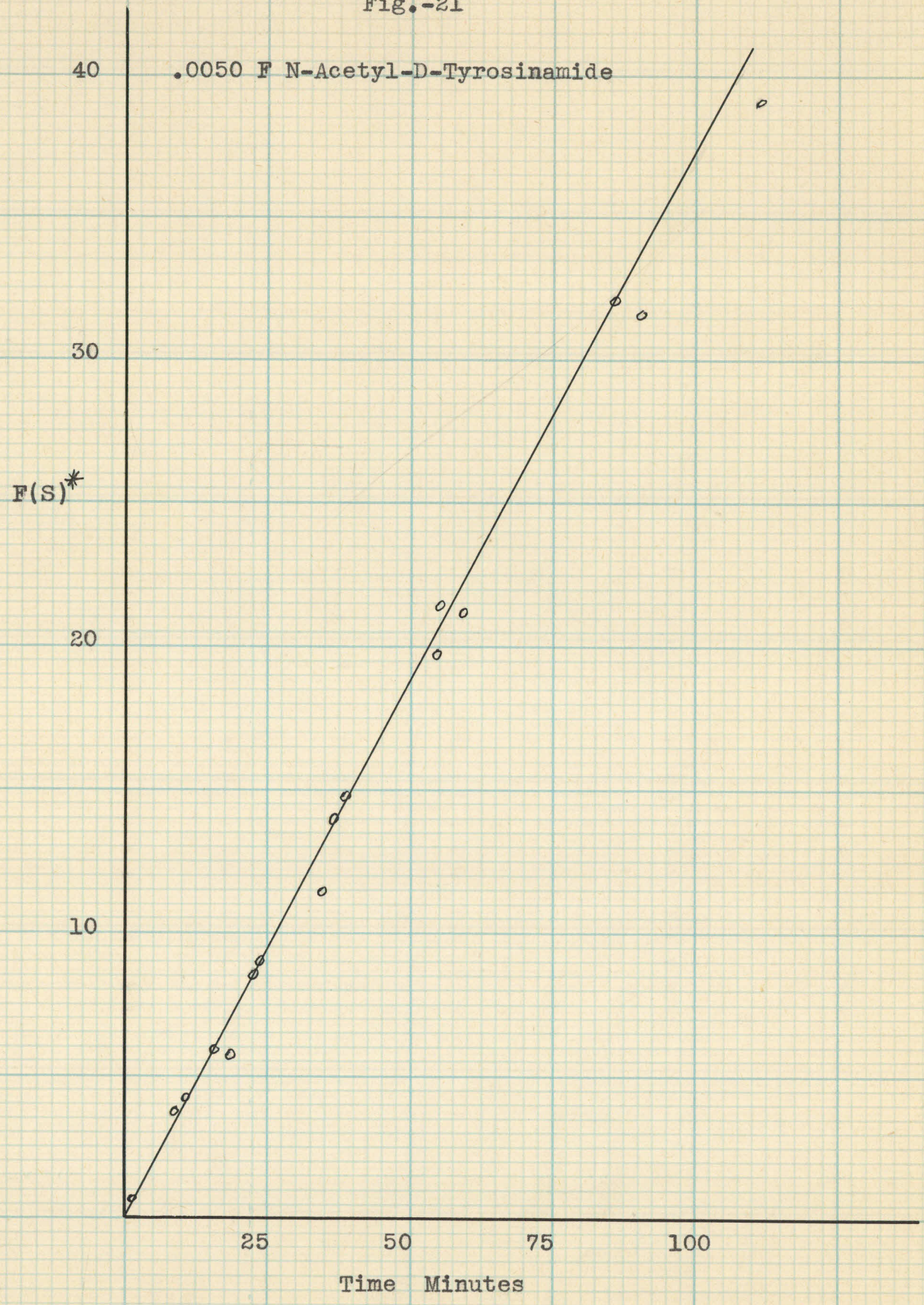

.0050 F N-Acetyl-D-Tyrosinamide
40
30
20
10
F(S)*
25
50
75
100
Time Minutes

Table IX

Inhibition of the Chymotrypsin Catalyzed Hydrolysis of N-Acetyl-L-Tyrosinamide by the 'D' Isomer (cont.)

$K_m = 28.2 \frac{(\text{micromoles})}{(\text{ml})}$

$K_I = 9.90 \frac{(\text{micromoles})}{(\text{ml})}$

$e = .150 \frac{(\text{mg. enzyme N})}{(\text{ml})}$

$i = 10.0 \frac{(\text{micromoles})}{(\text{ml})}$

s_o	t	s	Log s	s_o-s	$2.3(1+\frac{i}{K_I})K_m \text{Log} \frac{s_o}{s}$	F(s)*
10.0	1.5	9.90	.996	.10	.5	.6
	10.0	9.33	.970	.67	3.8	4.5
	21.5	8.72	.942	1.28	7.7	9.0
	39.5	8.00	.904	2.00	12.5	14.5
	77.0	6.14	.789	3.86	27.6	31.5
	140.0	5.49	.740	4.51	33.8	38.3
20.0	1.7	19.9	1.299	.1	.3	.4
	10.2	19.2	1.284	.8	2.3	3.1
	20.0	18.1	1.258	1.9	5.6	7.5
	30.7	17.1	1.233	2.9	8.8	11.7
	45.4	15.9	1.202	4.1	12.8	16.9
	68.8	14.2	1.153	5.8	19.3	25.1
	94.5	12.3	1.090	7.7	27.1	34.8
45.0	1.8	44.7	1.655	.3	.3	.6
	16.0	42.1	1.624	2.9	3.8	6.7
	25.2	40.5	1.608	4.5	6.0	10.5
	38.6	38.4	1.585	6.6	9.1	15.7
	55.4	36.2	1.559	8.8	12.4	21.2
	101.0	29.7	1.473	15.3	23.5	38.8

Fig.-22

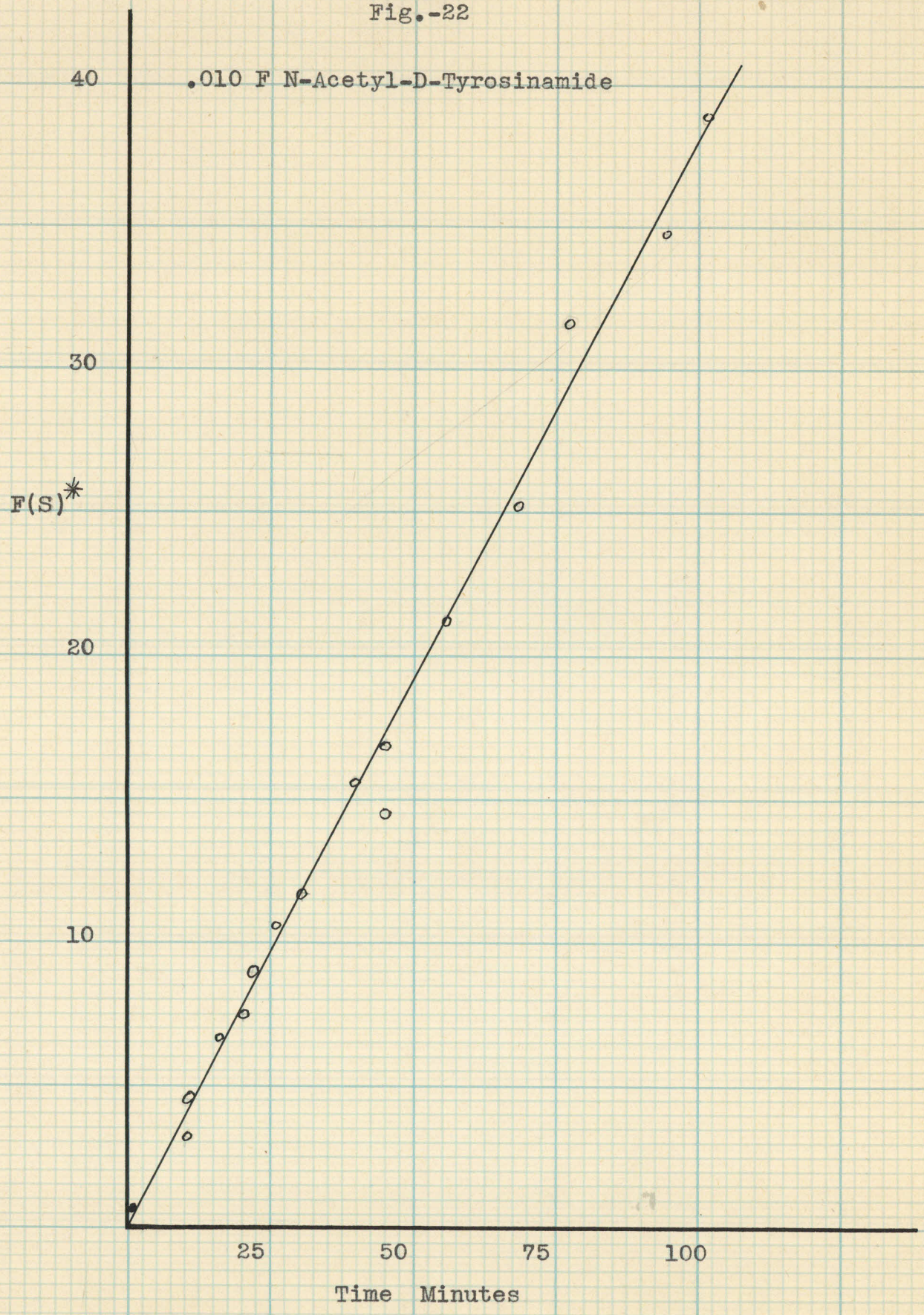

.010 F N-Acetyl-D-Tyrosinamide
40
30
20
10
F(S)*
25
50
75
100
Time Minutes

Table X

Inhibition of Chymotrypsin Catalyzed Hydrolysis of N-Acetyl-L-Tyrosinamide by 'D' Isomer (Cont.)

$K_m = 28.2 \frac{(\text{micromoles})}{(\text{ml})}$

$K_I = 10.4 \frac{(\text{micromoles})}{(\text{ml})}$

$e = .150 \frac{(\text{mg. enzyme N})}{(\text{ml})}$

$i = 20.0 \frac{(\text{micromoles})}{(\text{ml})}$

s_o	t	s	Log s	$s_o - s$	$2.3(1+\frac{i}{K_I})K_m \text{Log} \frac{s_o}{s}$	F(s)*
10.0	1.7	9.95	.998	.05	.4	.6
	14.7	9.50	.978	.50	4.2	4.7
	27.5	8.95	.952	1.05	9.1	10.2
	44.8	8.19	.914	1.81	16.3	18.1
	70.8	7.53	.877	2.47	23.4	25.9
	111.0	6.35	.803	3.65	37.5	41.2
20.0	2.0	19.9	1.299	.1	.4	.5
	11.0	19.3	1.286	.7	3.0	3.7
	22.2	18.7	1.272	1.3	5.5	6.8
	37.8	17.7	1.248	2.3	10.1	12.4
	63.3	16.2	1.210	3.5	17.5	21.0
	82.0	15.3	1.185	4.7	22.2	26.9
	113.0	13.6	1.134	6.4	31.9	38.3
40.0	1.7	39.8	1.600	.2	.4	.6
	14.4	37.7	1.577	2.3	4.9	7.2
	31.3	35.7	1.553	4.3	9.5	13.8
	53.5	33.3	1.523	6.7	15.2	21.9
	95.0	28.9	1.461	11.1	27.0	38.1

Fig.-23

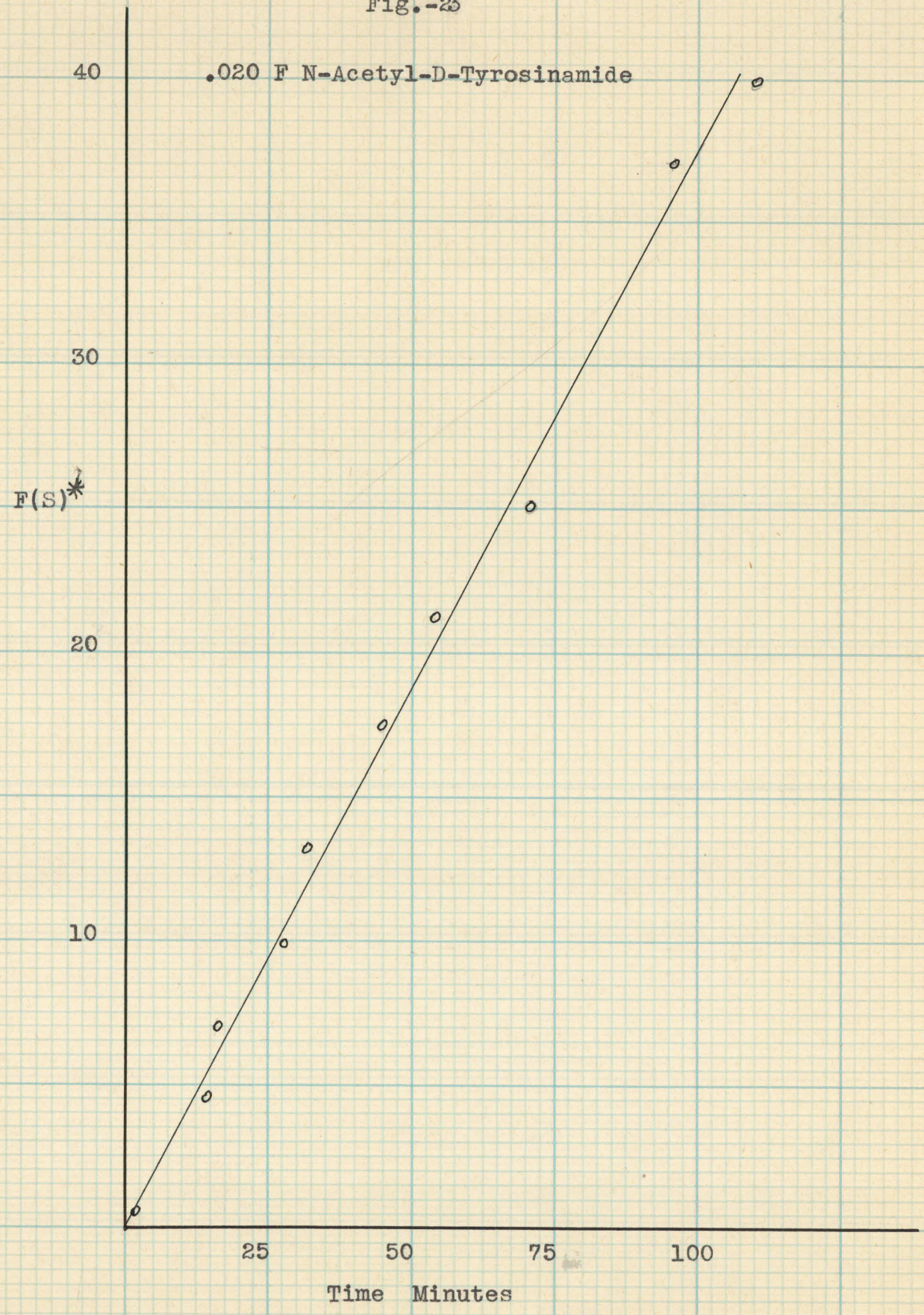

.020 F N-Acetyl-D-Tyrosinamide
40
30
20
10
F(S)*
25
50
75
100
Time Minutes

Table XI

Inhibition of Chymotrypsin Catalyzed Hydrolysis of N-Acetyl-L-Tyrosinamide by 'D' Isomer (Cont.)

$K_m = 28.2 \frac{(\text{micromoles})}{(\text{ml})}$

$K_I = 11.0 \frac{(\text{micromoles})}{(\text{ml})}$

$e = .150 \frac{(\text{mg. enzyme N})}{(\text{ml})}$

$i = 40.0 \frac{(\text{micromoles})}{(\text{ml})}$

s_o	t	s	Log s	s_o-s	$2.3(1+\frac{i}{K_I})K_m \text{Log} \frac{s_o}{s}$	F(s)*
10.0	1.8	9.95	.998	.05	.6	.65
	13.8	9.60	.982	.4	5.1	5.5
	25.5	9.30	.969	.7	9.3	10.0
	42.0	8.90	.950	1.1	15.3	16.4
	58.0	8.50	.930	1.5	21.0	22.5
	120.0	7.27	.862	2.7	41.4	44.1
20.0	1.5	19.9	1.299	.1	.6	.6
	12.8	19.4	1.288	.6	3.9	4.5
	22.1	18.9	1.276	1.1	7.5	8.6
	53.7	17.3	1.238	2.7	19.2	21.9
	170.0	13.2	1.121	6.8	54.3	61.1
40.0	1.8	40.0	1.602			
	12.8	39.2	1.593	.8	2.7	3.5
	23.3	38.2	1.582	1.8	6.0	7.8
	40.0	36.5	1.562	3.5	12.0	15.5
	61.0	34.2	1.534	5.8	20.7	26.5
	101.0	31.3	1.496	8.7	32.1	40.8
	157.0	27.4	1.438	12.6	49.5	76.9

Fig.-24

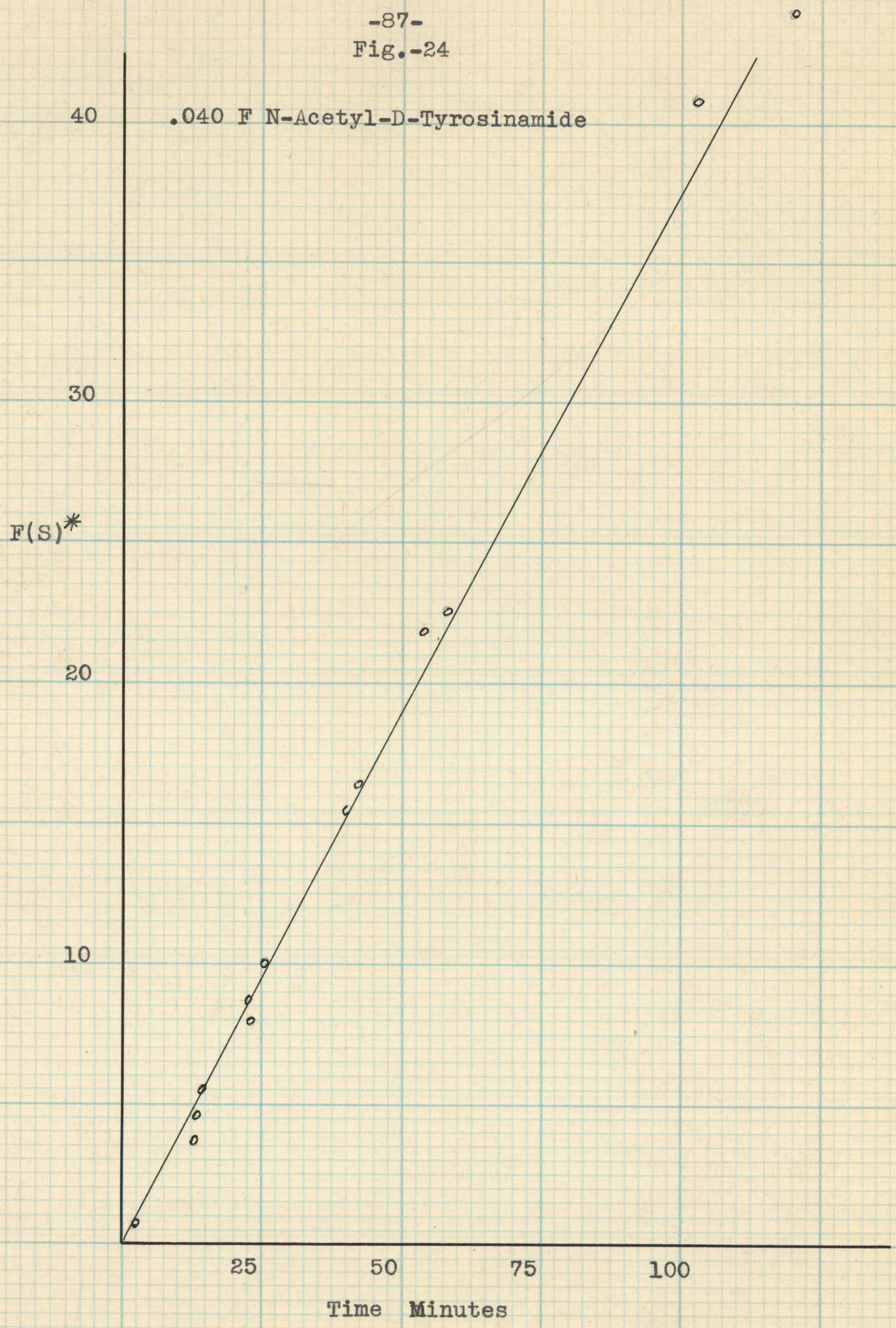

.040 F N-Acetyl-D-Tyrosinamide
40
30
20
10
F(S)*
25
50
75
100
Time Minutes

Table XII

Inhibition of Chymotrypsin Catalyzed Hydrolysis of N-Acetyl-L-Tyrosinamide by 'D' Isomer (Cont.)

$K_m = 28.2 \frac{(\text{micromoles})}{(\text{ml})}$

$K_I = 12.0 \frac{(\text{micromoles})}{(\text{ml})}$

$e = .150 \frac{(\text{mg. enzyme N})}{(\text{ml})}$

$i = 60.0 \frac{(\text{micromoles})}{(\text{ml})}$

K_I	s_o	t	s	Log s	s_o-s	$2.3(1+\frac{i}{K_I})K_m \log \frac{s_o}{s}$	F(s)*
12.0	10.0	1.5	9.98	.999	.02	.4	.4
		11.6	9.81	.992	.19	3.5	3.7
		30.3	9.40	.973	.60	10.5	11.1
		70.5	8.70	.940	1.30	23.4	24.7
		104.5	7.95	.901	2.05	38.6	40.7
		132.2	7.45	.873	2.54	49.5	52.0
*	*20.0	1.5	19.9	1.299	.1	.8	.9
		9.5	19.6	1.292	.4	3.5	3.9
		19.1	19.0	1.280	1.0	8.6	9.6
		30.2	18.6	1.270	1.4	12.5	13.9
		45.5	18.1	1.258	1.9	17.2	18.9
		59.1	17.4	1.240	2.6	24.2	26.8
		91.7	16.4	1.215	3.6	34.0	37.6
		154.0	14.3	1.156	5.7	57.0	62.7

* Some solid phase separated during this experiment.

Fig.-25

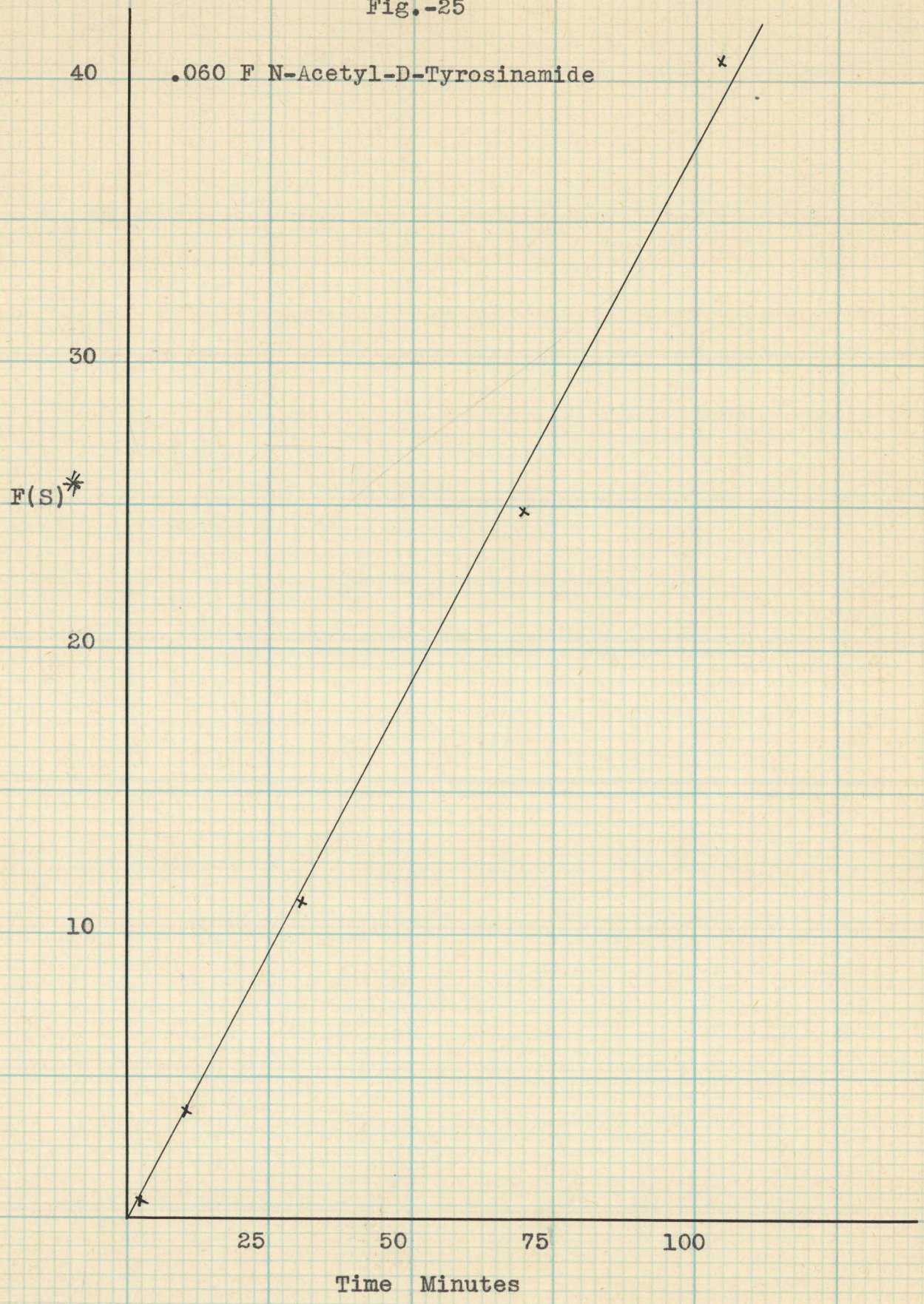

.060 F N-Acetyl-D-Tyrosinamide
40
30
20
10
F(S)*
25
50
75
100
Time Minutes

Fig.-26

N-Acetyl-L-Tyrosinamide Hydrolysis

Inhibition by N-Acetyl-D-Tyrosinamide

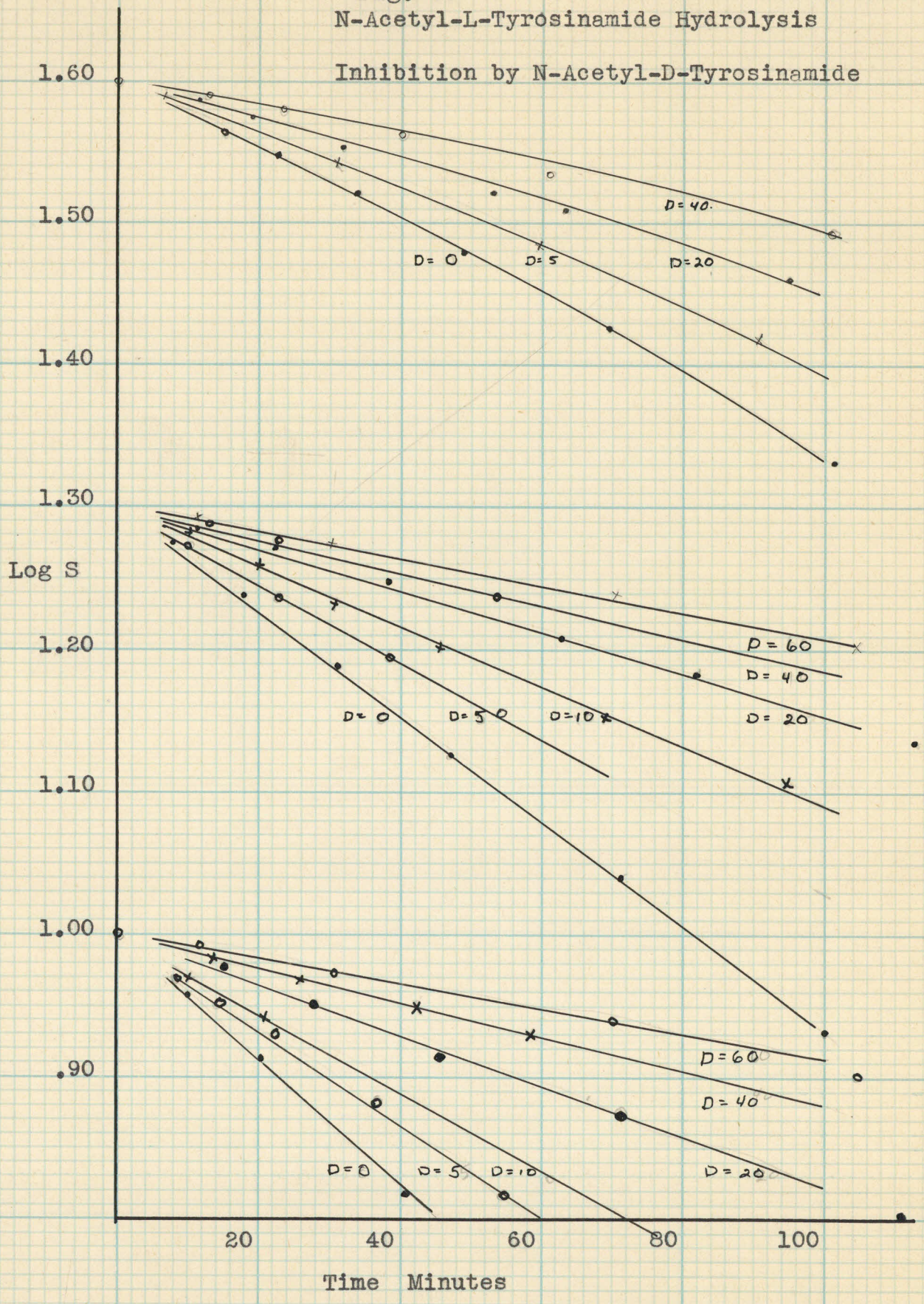


Fig.-27

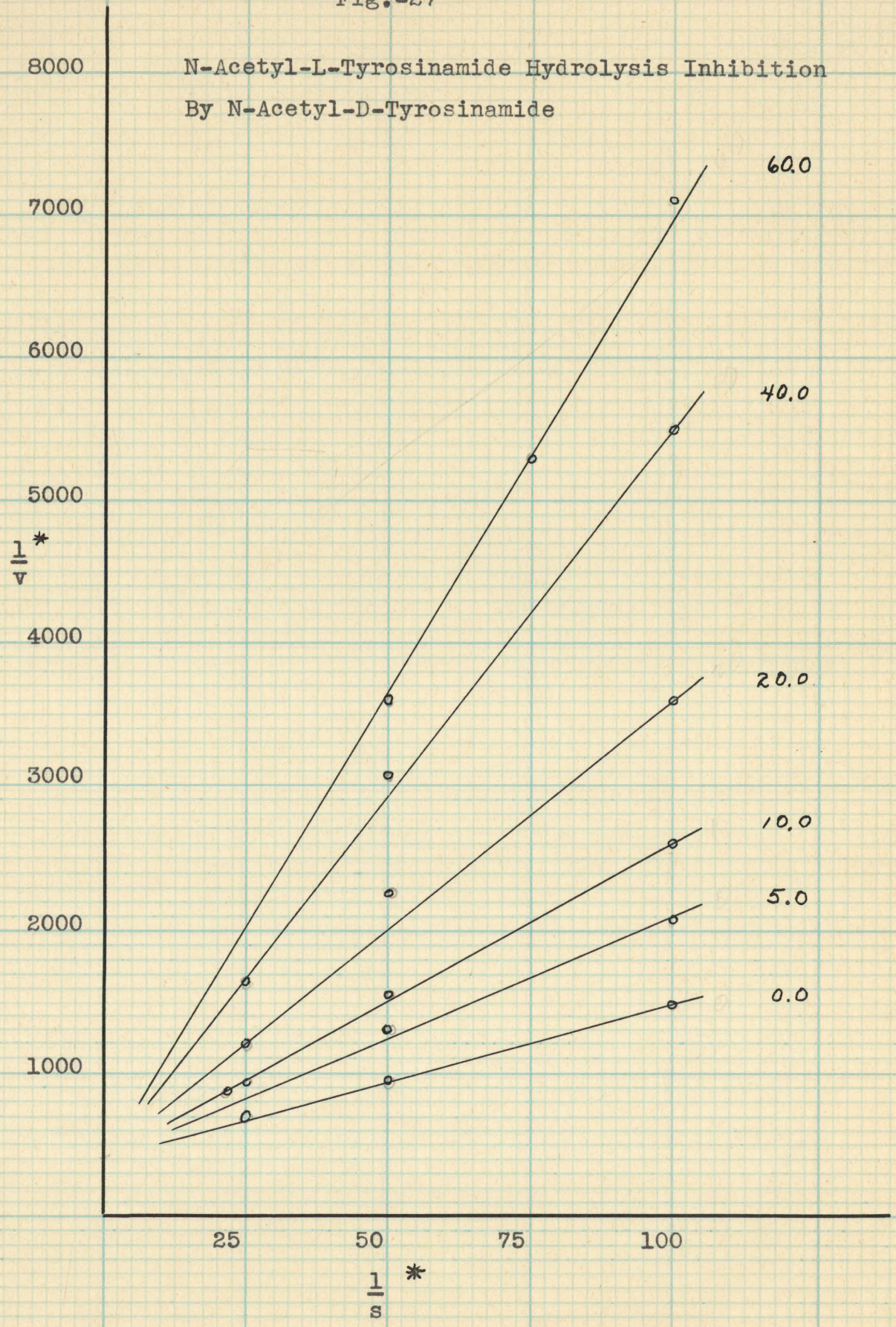

N-Acetyl-L-Tyrosinamide Hydrolysis Inhibition
By N-Acetyl-D-Tyrosinamide
8000
7000
6000
5000
4000
3000
2000
1000
1/v *
60.0
40.0
20.0
10.0
5.0
0.0
25
50
75
100
1/s *

Table XIII

The Hydrolysis of N-Acetyl-L-Tyrosineamide by Chymotrypsin in the Presence of N-Acetyl-D-Tyrosine Ethyl Ester

$K_m = 28.2 \frac{(\text{micromoles})}{(\text{ml})}$

$K_I = 5.2 \frac{(\text{micromoles})}{(\text{ml})}$

$i = 10.0 \frac{(\text{micromoles})}{(\text{ml})}$

$e = .150 \frac{(\text{mg. enzyme N})}{(\text{ml})}$

K_I	s_o	t	s	Log s	$s_o - s$	$2.3(1+\frac{i}{K_I})K_m \log \frac{s_o}{s}$	F(s)
5.20	10.0	1.5	9.94	.998	.06	.6	.7
		9.4	9.61	.983	.39	3.4	3.8
		18.2	9.33	.970	.67	5.9	6.6
		27.7	8.96	.953	1.04	9.1	10.1
		37.1	8.62	.936	1.38	12.4	13.8
		81.4	7.16	.856	2.84	27.6	30.4

Fig.-28

N-Acetyl-L-Tyrosinamide

N-Acetyl-D-Tyrosine Ethyl Ester

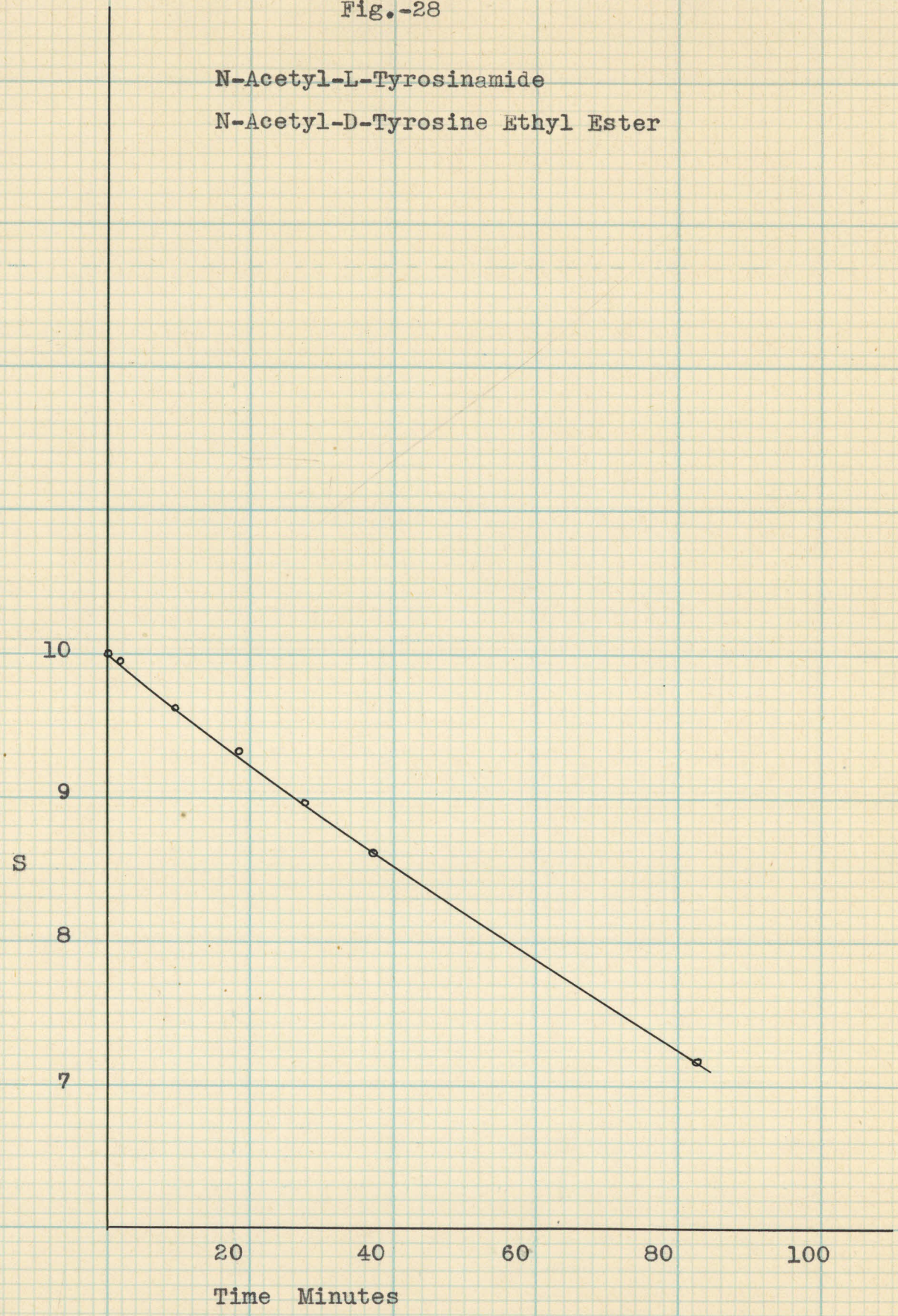


Fig.-29

N-Acetyl-L-Tyrosinamide

N-Acetyl-D-Tyrosine Ethyl Ester

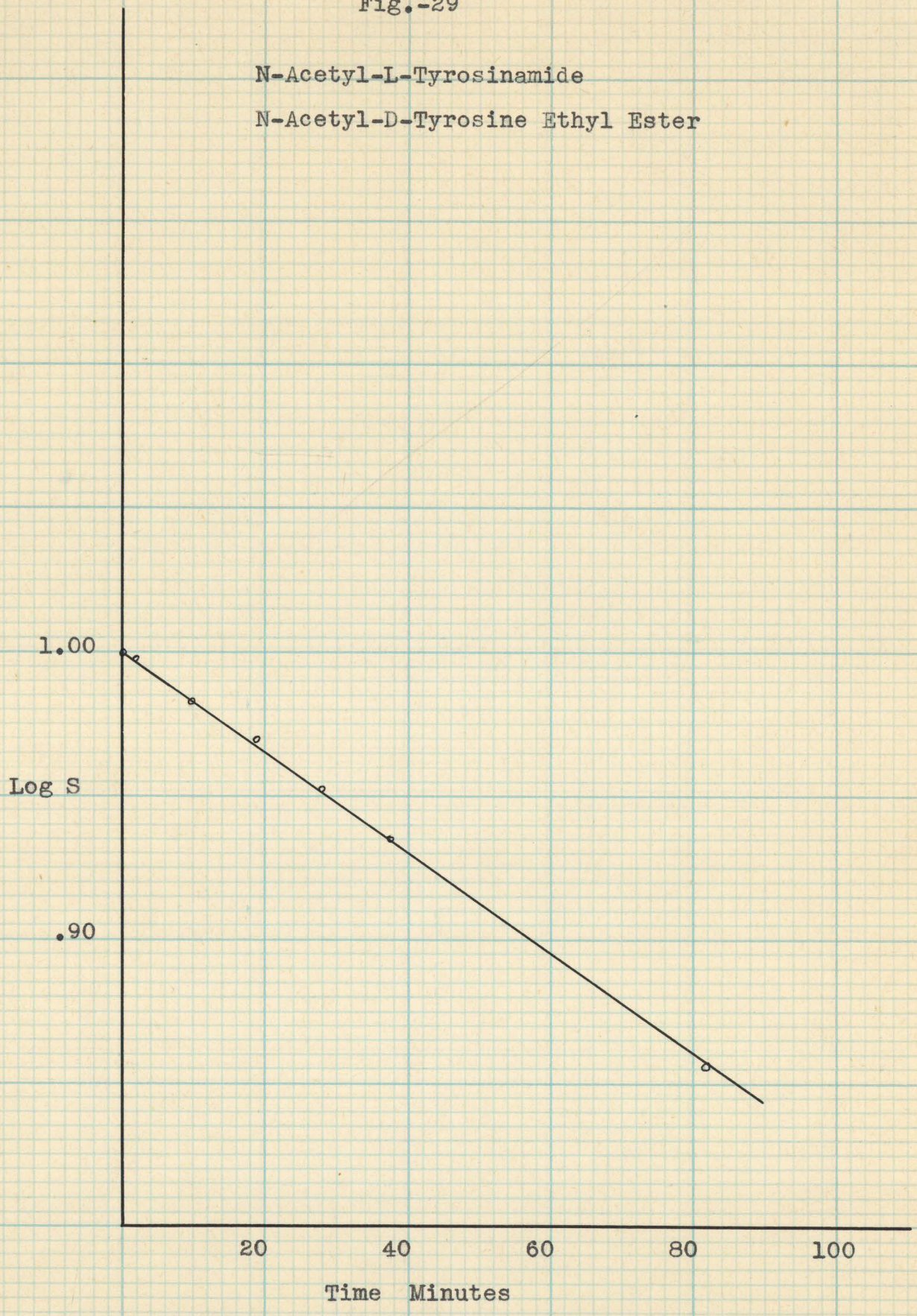
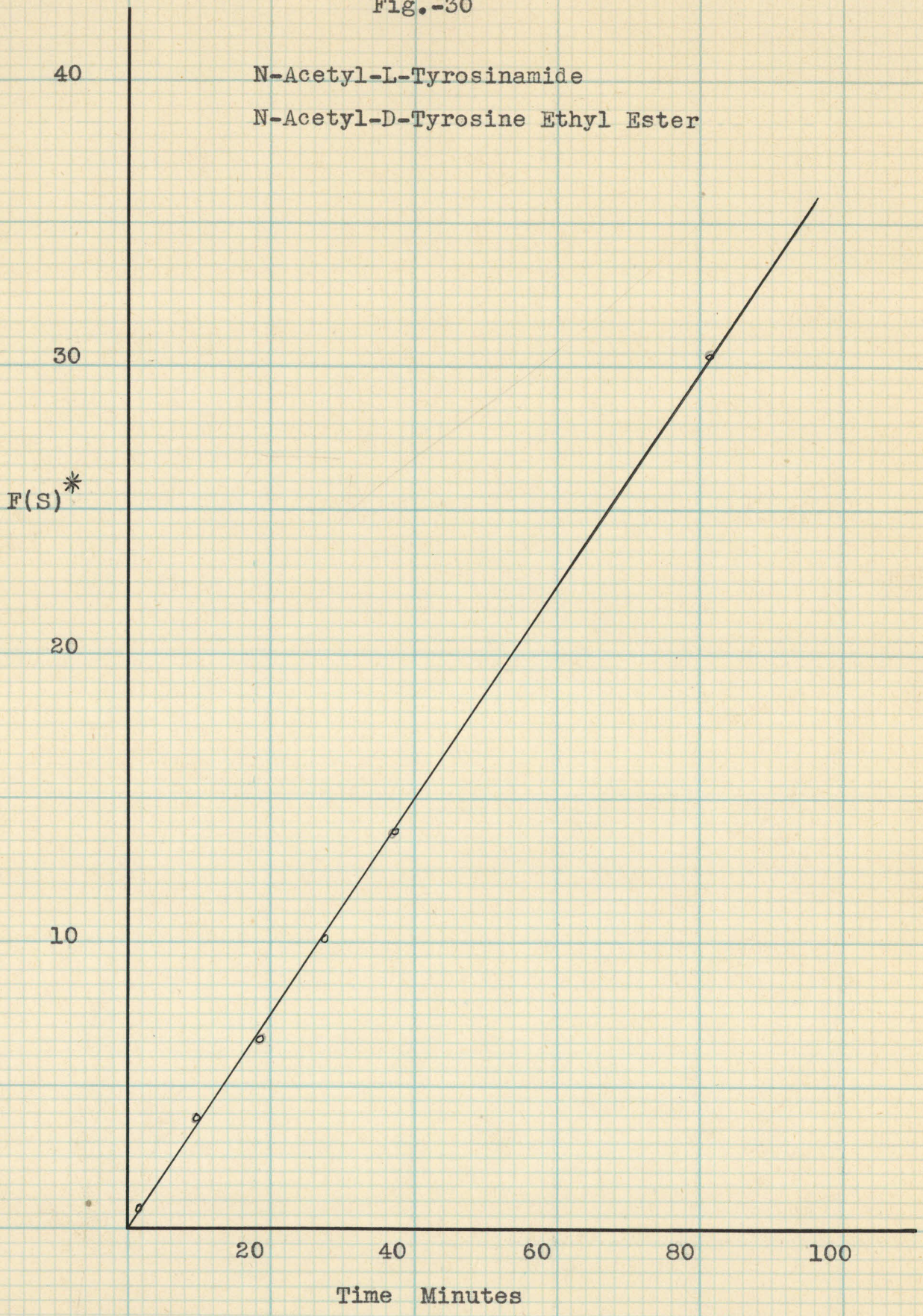


Fig.-30


N-Acetyl-L-Tyrosinamide
N-Acetyl-D-Tyrosine Ethyl Ester
40
30
20
10
F(S)*
20
40
60
80
100
Time Minutes

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Part IV

ADDENDUM

Addendum

In the course of the research program, a number of compounds and intermediates were prepared, purified, and analyzed, but were not mentioned in the main body of this thesis. This addendum is added with the thought that this information might prove useful to future workers in these laboratories:

N-Carbobenzoxy Glycyl-DL-Phenylalaninamide

7.50 gm. of N-carbobenzoxy glycinhydrazide was converted to the azide in the usual manner. The azide was added to an ethyl acetate solution containing 14. gm. of DL-phenylalanine ethyl ester. After standing at room temperature for 24 hours, the ethyl acetate solution was extracted with dilute HCl and washed with water. The ethyl acetate was evaporated under vacuum. The residue was recrystallized from methanol. The product was dissolved in 200 ml. of methanol and saturated with NH_3 at 0°. After 2 days at room temperature the solution was evaporated under vacuum to dryness. The residue was recrystallized from ethanol.

Melting Point: 164-165 (Corr.)

Analysis: ($C_{19}H_{21}O_4N_3$)

Calculated: C 64.3, H 5.98, N 11.8

Found: C 64.1, H 5.74, N 11.8

DL-Phenylalaninamide Acetate

1.00 gm. of N-carbobenzoxy-DL-phenylalaninamide was dissolved in 100 ml. methanol. .30 gm. of acetic was added, and the mixture reduced with H at 1 atmosphere in the presence of a palladium catalyst. After the theoretical amount of CO had evolved, the solution was filtered and evaporated to dryness under vacuum. The residue was recrystallized from a methanol ethyl acetate solvent.

Analysis: ($C_{11}H_{16}O_3N_2$)

Calculated: C 58.9, H 7.20, N 12.5

Found: C 58.9, H 7.54, N 12.6

N-Carbobenzoxy Glycinhydrazide

140 gm. N-Carbobenzoxy glycine ethyl ester was dissolved in 300 ml. methanol. 24 gm. of hydrazine was added, and the mixture held at room temperature for 24 hours. The hydrazide separated upon evaporating under vacuum. The product was recrystallized from water.

Melting Point: 114-115 (Corr.)

Analysis: ($C_{10}H_{13}O_3N_3$)

Calculated: C 53.8, H 5.87, N 18.8

Found: C 53.9, H 6.00, N 18.7

4-(4 Acetoxy Benzal) 2 Phenyloxazolone-5

This compound was prepared from p-hydroxybenzaldehyde and hippuric acid by the method of Erlenmeyer and Halsey (27).

Melting Point: 175-176 (Corr.)

Analysis: ($C_{18}H_{13}O_4N$)

Calculated: C 70.3, H 4.26, N 4.56

Found: C 70.5, H 4.41, N 4.74

N-Carbobenzoxy-DL-Phenylalaninamide

18.0 gm. of DL-phenylalanine ethyl ester was dissolved in 50 ml of ethyl acetate. 4.0 gm. of Mg O was suspended in the solution and 16.2 gm. of a 90 per cent solution of carbobenzoxy chloride in toluene was added slowly to the well agitated mixture. The solid phase was then filtered out, and the ethyl acetate solution washed with dilute HCl and water. The ethyl acetate was evaporated off under vacuum, and the residue was dissolved in methanol. The methanol solution was saturated with NH_3 and held for two days at room temperature. The methanol solution was evaporated under vacuum to dryness. The residue was recrystallized from aqueous ethanol.

Melting Point: 182-183 (Corr.)

Analysis: ($C_{17}H_{18}O_3N_2$)

Calculated: C 68.5, H 6.08, N 9.40

Found: C 68.5, H 6.38, N 9.76

Glycyl-DL-Phenylalaninamide Acetate

2.00 gm. of N-carbobenzoxy glycyl-DL-phenylalaninamide was dissolved in 100 ml. of methanol. 30 ml. of glacial acetic acid was added and the mixture treated with H_2 at 1 atmosphere pressure in the presence of a palladium catalyst for 12 hours. The solution was filtered and evaporated under vacuum to a syrup which crystallized upon the addition of a small amount of ethyl acetate. The product was recrystallized from a methanol-ethyl acetate mixture.

Analysis: ($C_{13}H_{19}O_4N_3$)

Calculated: C 55.5, H 6.80, N 14.9

Found: C 55.6, H 6.85, N 15.0