

MONOACETYLATION OF 2,4-DIAMINO-6-NITROTOLUENE
KINETIC STUDIES WITH α -CHYMOTRYPSIN
EFFECT OF DILANTIN UPON ETHER NARCOSIS IN THE CAT

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ABSTRACT

The structure of the monoacetyl derivative of 2,4-diamino-6-nitrotoluene formed by reaction with an equimolar quantity of acetic anhydride in dry acetone at room temperature has been shown to be 2-amino-4-acetamido-6-nitrotoluene.

The kinetic constants for the enantiomorphous pair of acetyl-phenylalaninamides and α -chymotrypsin at 25° and pH 7.9 in aqueous solution have been determined. The relationships of these constants with others determined for similar enantiomorphous pairs is discussed. Equations for the competitive hydrolysis of two substrates at the same catalytically active site of an enzyme have been derived and have been tested experimentally.

The kinetic constants for L-tyrosine-hydroxamide and α -chymotrypsin have been determined at pH 6.9 and 25°. With this hydroxamide as substrate, the enzyme-inhibitor complex dissociation constants have been determined for a series of inhibitors at pH 6.9 and 25°. The differences between these constants and those at pH 7.9 is discussed and the charge state of the catalytically active site is inferred.

The effect of the anti-convulsant dilantin upon ether narcosis in the cat has been determined and is discussed in its relation to present theories of nervous propagation and narcosis.

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* This work done in collaboration with Dr. G. Fasman

PART I

THE MONOACETYLATION OF

2,4-DIAMINO-6-NITRO-

TOLUENE

Introduction

When equimolar quantities of acetic anhydride and 2,4-diamino-6-nitrotoluene are allowed to react in dry acetone at room temperature, a monoacetyl derivative is formed in good yield. Parkes and Farthing⁽¹⁾ state that the latter compound can be deaminated via diazotization to give after hydrolysis 2-nitro-4-aminotoluene, and infer that the monoacetyl derivative is 2-amino-4-acetamido-6-nitrotoluene. However, in the experimental section of their paper it is reported that, when 2,4-diamino-6-nitrotoluene is monoacetylated by three different procedures, including that described above, 2-acetamido-4-amino-6-nitrotoluene is the compound obtained, and that it can be converted into 2-amino-6-nitrotoluene in the usual manner.

The question as to which of the above conflicting statements is correct has been answered by the demonstration that the monoacetylation of 2,4-diamino-6-nitrotoluene proceeds under the conditions specified by Parkes and Farthing as methods a.) and c.) to form 2-amino-4-acetamido-6-nitrotoluene.

Discussion of Results

The monoacetyl derivative of 2,4-diamino-6-nitrotoluene was prepared by two converging routes from TNT. One route, that of Parkes and Farthing, is shown schematically in Table I,

I-III, while the other is I-IV-V-III. 4-Amino-2,6-dinitrotoluene (IV), which had been prepared by Rosicky⁽²⁾ from TNT, was acetylated to its known acetyl derivative⁽¹⁾(V). This compound, 4-acetamido-2,6-dinitrotoluene, was reduced to 2-amino-4-acetamido-6-nitrotoluene (III) which had the same melting point as the monoacetyl derivative prepared by the other route. No depression was observed when mixed melting points were taken.

Before it was realized that the product obtained by monoacetylation of 2,4-diamino-6-nitrotoluene was acetylated in the 4 position and that therefore deamination and hydrolysis would lead to the easily obtainable 4-amino-2-nitrotoluene, effort was expended in improving the yields of the deamination procedure. Since only tarry products could be obtained with the classical ethyl alcohol reduction of the diazonium chloride, hypophosphorous acid was tried. This mild and straightforward method was first described by Kornblum and Iffland⁽³⁾. The short life of the diazonium salt of hypophosphorous acid in the acid medium and the generally mild conditions are undoubtedly the reasons why very little coupling takes place. This procedure is recommended as a very clean method of deamination.

The 4-amino-2-nitrotoluene (VII) obtained by hydrolysis of 4-acetamido-2-nitrotoluene (VI) which was obtained by the hypophosphorous acid-sodium nitrite deamination of III, was identical

with that formed by reduction of 2,4-dinitrotoluene (VIII).

An authentic sample of the compound Parkes and Farthing reported to result from the deamination and hydrolysis of their monoacetyl derivative had been obtained by deamination of 4-amino-2,6-dinitrotoluene (IV) to the known 2,6-dinitrotoluene (IX) and subsequent reduction to 2-amino-6-nitrotoluene⁽²⁾. This compound was clearly different from the one prepared by deamination and hydrolysis of the monoacetyl derivative of 2,4-diamino-6-nitrotoluene since their mixed melting point was depressed 25° and spread over a 10° range.

Experimental*

2-Amino-4-acetamido-6-nitrotoluene (III) - First route - One hundred gm. of E. K. white label TNT was added slowly to 1250 ml. of 95% ethanol that had been saturated with ammonia and hydrogen sulfide at 5°. The suspension was stirred very vigorously during the addition and its temperature was kept below 5°. As the reaction proceeded the solution turned red, the color developing first at the surface of the finely divided TNT crystals. The reaction mixture was allowed to stand over night. Decantation through a filter was used to separate the

* All melting point values are corrected. All microanalyses were by Dr. A. Elek.

red solution from the yellow sulfur precipitate which was formed. One and one-half l. of water was added to the filtrate and the alcohol stripped off under reduced pressure. The sulfur precipitate was extracted with 1 l. of boiling water. After 1.5 l. of alcohol-water mixture had been stripped from the original filtrate, the extracts from the sulfur were added to it and the volume reduced further. This combined and concentrated filtrate was then heated to 95° , and filtered hot to remove remaining sulfur. Crude yield was 55.5 gm. (75%). After recrystallization from 2 l. water, the melting point of the shining red platelets was 132.5° - 134° with a yield of 45 gm. (61%). Parkes and Farthing reported a melting point of 135° .

Twenty and one-half gm. (0.123M) of the diamine prepared above was dissolved in 100 ml. of dry, redistilled acetone and 12.5 gm. (0.123M) of acetic anhydride was dissolved in 40 ml. of the acetone. The acetylating agent was added slowly to the well stirred amine solution at room temperature over a 40 minute period. After standing for three hours, the reaction mixture was evaporated to dryness on a water bath. The crude yellow-brown product was recrystallized from 720 ml. of 15% (volume) ethanol. Yield of purified product, clusters of fine yellow needles melting 155° to 158° , was 23.5 gm. (92%). Parkes and Farthing report a melting point of 155° . To obtain a product which analyzed correctly as the anhydrous amine, drying in vacuo at 110° was needed. This product melted at 160° - 161° .

Analysis calculated for $C_9H_{11}O_3N_3$: C, 51.7; H, 5.3; N, 20.1. Found: C, 51.6; H, 5.4; N, 20.1.

Alternate route: 4-amino-2,6-dinitrotoluene (IV) prepared by Fred Rosicky⁽²⁾, orange needles melting at 171° - 172° (Holleman and Boeseken⁽⁵⁾ reported 171°), was acetylated by the same procedure used above to monoacetylate 2,4-diamino-6-nitrotoluene except a slight excess of acetic anhydride was employed. The yield of V, 4-acetamido-2,6-dinitrotoluene, was 66% of white needles melting from 223° - 225° . An analytical sample, melting point 224.5° - 225° , was obtained by recrystallizing from toluene. Parkes and Farthing reported a melting point of 221.5° .

Analysis calculated for $C_9H_9O_5N_3$: C, 45.2; H, 3.8; N, 17.6. Found: C, 45.3; H, 3.9; N, 17.5.

2-Amino-4-acetamido-6-nitrotoluene (III), melting point 160° - 161° , was produced in 60% yield when V was reduced using the same conditions used to prepare II, 2,4-diamino-6-nitrotoluene, from TNT. The melting point of a mixture of III prepared by these two routes was 160° - 161° . An analytical sample was prepared as with the first route by drying in vacuo at 110° .

Analysis calculated for $C_9H_{11}O_3N_3$: C, 51.7; H, 5.3; N, 20.1. Found: C, 51.6; H, 5.4; N, 20.0.

4-Amino-2-nitrotoluene (VII) -- 520 ml. of 50% hypophosphorous acid was chilled to ice temperature and 52.3 gm. (0.25M) of III added slowly and dissolved. 19 gm. (0.27M) of sodium nitrite was dissolved in 60 ml. of ice water and this solution was slowly added to the acid solution of the amine salt. The time of addition was governed by the rate of dissipation of the nitrogen bubbles. The solution turned red but remained clear and no solid phase formed. Water was added to bring the volume to 1250 ml. and the hydrogen ion concentration to four formal. The amide bond was hydrolyzed at 95°. In one experiment the time of hydrolysis was three hours and the product isolated could be separated into a neutral and a basic component. The neutral component was shown to be the acetyl derivative of the basic one by further hydrolysis and by acetylation of the basic component. When the time of hydrolysis was eight hours, only the basic component could be isolated and that in 40% yield. The melting point of yellow orange platelets after recrystallization from water was 78°-79°. Beilstein and Kuhlberg⁽⁶⁾ reported a melting point of 77.5° for their product from the reduction of 2,4-dinitrotoluene.

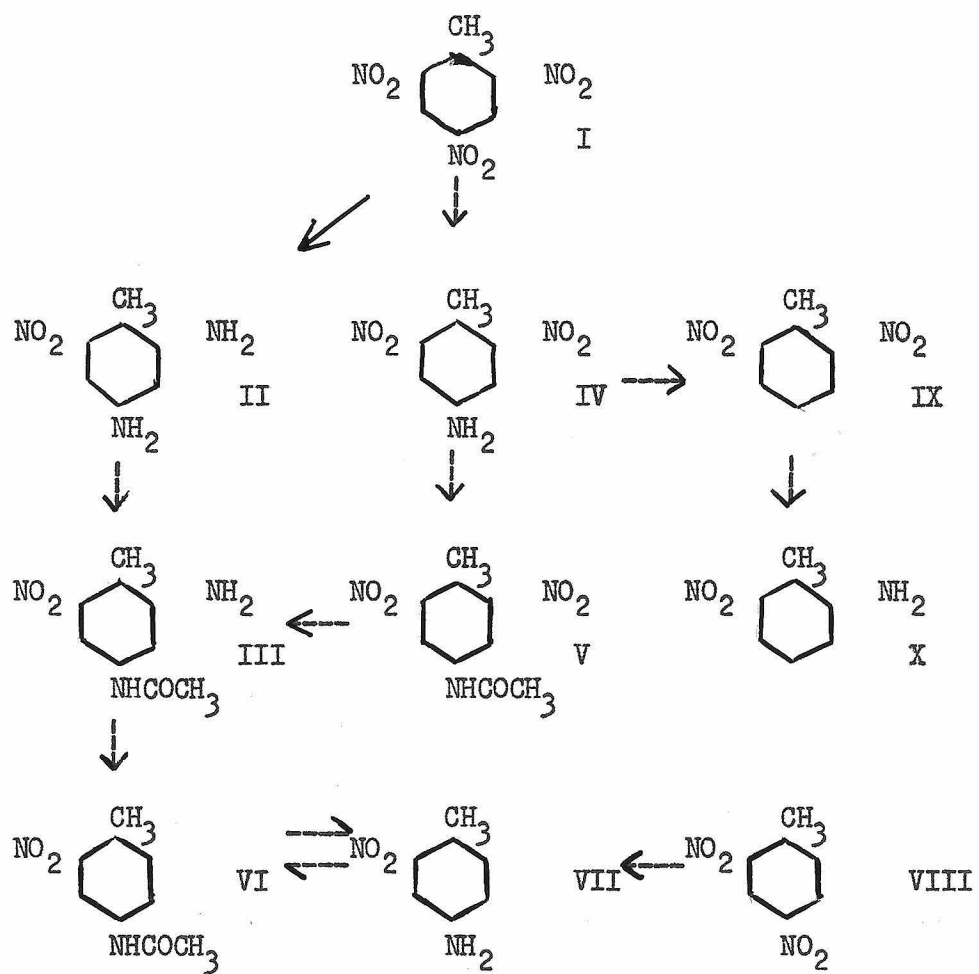
Analysis calculated for $C_7H_8O_2N_2$: C, 55.3; H, 5.3; N, 18.4. Found: C, 55.2; H, 5.4; N, 18.5.

After recrystallization from 50% aqueous ethanol the orange needles of the acetyl derivative melted from 145° - 146° . Bogert and Kropff⁽⁷⁾ reported a melting point of 77.5° for 4-acetamido-2-nitrotoluene.

Analysis calculated for $C_9H_{10}O_3N_2$: C, 55.7; H, 5.2; N, 14.4. Found: C, 55.8; H, 5.1; N, 14.4.

Another route for preparing 4-amino-2-nitrotoluene was that of Bogert and Kropff⁽⁷⁾. Twenty-five ml. of ethanol was saturated with ammonia and hydrogen sulfide at 5° . Two gm. of 2,4-dinitrotoluene, melting point 70° - 71° as prepared by Fred Rosicky, was added with swirling. After half an hour the solution was poured into 50 ml. of water. Alcohol was stripped off and the orange precipitate was dissolved in dilute hydrochloric acid, treated with norite and filtered hot. The solution was then neutralized with sodium hydroxide pellets. Yellowish plates started forming and became orange as they increased in size. The compound was purified by recrystallization from water and the crystals were dried in vacuo. Melting point of the orange platelets was 78° - 79.5° and when mixed with the basic component from deamination and hydrolysis of the monoacetyl derivative of 2,4-diamino-6-nitrotoluene, the melting point was still 78° - 79.5° .

Table I



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PART II

KINETIC STUDIES WITH

α -CHYMOTRYPSIN AT pH 7.9

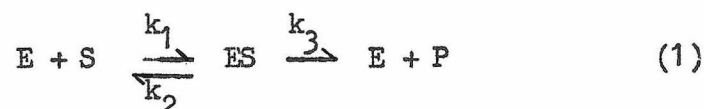
Introduction

The discovery of Warburg⁽¹⁾ in 1905 that pancreatic extracts capable of catalyzing the hydrolysis of proteins were also able to facilitate the hydrolysis of amino acid esters foreshadowed the observation by Neurath⁽²⁾ and others that the proteolytic enzyme chymotrypsin, first crystallized by Kunitz and Northrop⁽³⁾ in 1933, had specific esterase activity. As a result of these discoveries the structural requirements of specific substrates and inhibitors of this enzyme have been extensively investigated. The relative stability of this enzyme has also allowed investigations of the effect of temperature and pressure on its activity⁽⁴⁾.

The effects of any changes in an enzyme catalyzed system, whether in the structure of the substrate, inhibitor or enzyme or in the environment of the reaction -- i.e., temperature, pressure, pH, ionic strength, etc., are best expressed by changes in the kinetic constants of the enzyme catalyzed reaction. In work with chymotrypsin, the constants usually evaluated are: the Michaelis-Menten constant,⁽⁵⁾ K_S , the rate of break-down of the enzyme-substrate complex to yield products, k_3 , and the enzyme-inhibitor dissociation constant, K_I . Assumptions made in the following derivations will be indicated where they are significant.*

* These derivations are based on those of Haldane.⁽⁶⁾

The reaction system is as follows: The enzyme and substrate unite to form a complex which breaks down in some manner (probably attack by OH^- or H_2O) to yield the products and the enzyme. In this treatment the affinity of the products for the enzyme will be ignored since for the extent of hydrolysis used in the experiments recorded and for the products produced the effect is negligible.* For competitive inhibition there is another equilibrium to consider: enzyme and inhibitor in equilibrium with an inhibitor-enzyme complex.



A further assumption is that the concentrations of "S" and "I" are much greater than the concentration of "E" so that:

$[\text{S}]$ = total concentration of "S" $\stackrel{\circ}{=}$ free concentration of "S",
 $[\text{I}]$ = " " " " "I" $\stackrel{\circ}{=}$ " " " "I",
 $[\text{E}]$ = " " " "E",
 $[\text{ES}]$ = " " " enzyme-substrate complex,
 $[\text{EI}]$ = " " " enzyme-inhibitor complex,
 $([\text{E}] - [\text{ES}] - [\text{EI}])$ = concentration of free enzyme.

The next assumption is that a steady state is reached in which

* See Huang and Niemann⁽⁷⁾ for a treatment of a case where affinity of products for enzyme is not negligible.

$$\frac{d}{dt} [ES] \text{ and } \frac{d}{dt} [EI] < \frac{-d[S]}{dt}.$$

Then;

$$(k_2 + k_3) [ES] = ([E] - [ES] - [EI]) [S] k_1, \quad (3)$$

or

$$K_S [ES] = ([E] - [ES] - [EI]) [S], \quad (3a)$$

$$\text{where } K_S = \frac{k_2 + k_3}{k_1}$$

$$\text{and } K_I [EI] = ([E] - [ES] - [EI]) [I], \quad (4)$$

$$\text{where } K_I = \frac{k_5}{k_4}.$$

Dividing equation (4) by equation (3) and solving for $[EI]$,

$$[EI] = \frac{[I]}{K_I} \frac{K_S}{[S]} [ES]. \quad (5)$$

Substituting for $[EI]$ in equation (3a),

$$K_S [ES] = [E] - [ES] \left(1 + \frac{K_S [I]}{[S] K_I}\right), \quad (6)$$

which may be solved for $[ES]$:

$$[ES] = \frac{[E] [S]}{K_S + [S] \left(1 + \frac{K_S [I]}{[S] K_I}\right)}. \quad (7)$$

From the assumption of a steady state it is seen from equation

(1) that $\frac{-d[S]}{dt} = k_3 [ES]$. Then,

$$-\frac{d[S]}{dt} = \frac{k_3 [E] [S]}{[S] + K_S \left(1 + \frac{[I]}{K_I}\right)}. \quad (8)$$

Since in this system $[ES]$ has a maximum value of $[E]$, $-\frac{d[S]}{dt}$ has a maximum value $k_3 [E] \equiv V$.

Thus,

$$-\frac{d[S]}{dt} = \frac{V [S]}{[S] + K_S (1 + \frac{[I]}{K_I})} \quad (9)$$

This may be inverted to give

$$\frac{1}{V} = \frac{1}{V} + \frac{1}{V} \frac{K_S}{[S]} (1 + \frac{[I]}{K_I}), \quad (10)$$

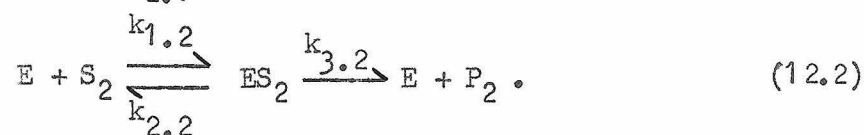
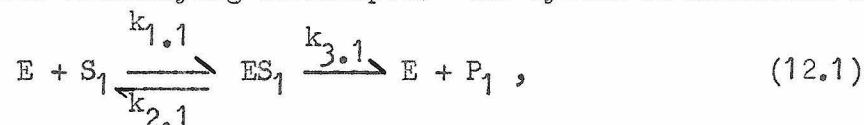
which may be integrated to give

$$k_3 [E] t = [S]_0 - [S] + 2.3 \log_{10} K_S (1 + \frac{I}{K_I}) \frac{[S]_0}{[S]}. \quad (11)$$

If $\frac{1}{V_0}$ (initial velocity reciprocal) is plotted against $\frac{1}{[S]_0}$, a straight line is obtained if the system can be formulated as above⁽⁸⁾. The slope of the line is $\frac{K_S}{V} (1 + \frac{[I]}{K_I})$ and the intercept at $\frac{1}{[S]} = 0$ is $\frac{1}{V}$. In practice the K_S and V (for a given enzyme concentration) are determined in this manner with $[I] = 0$. A competitive inhibitor when tested in this manner will give a family of straight lines having the same intercept as given by the substrate alone but with a slope of $(1 + \frac{[I]}{K_I})$ times the original slope.

Several series of cross-checks -- that is the determination of the K_I of an inhibitor using two or more substrates have all yielded the information that there is only one kind of hydrolytic site on the chymotrypsin molecule⁽⁹⁾. Recent

studies of inhibition by di-isopropyl-fluoro-phosphate and related phosphate esters of the esteratic and proteolytic function also lend credence to this belief⁽¹⁰⁾. Therefore it seemed appropriate to develop the kinetic expressions for simultaneous competitive hydrolysis of two substrates at the same site of a hydrolytic enzyme. The symbols used below are defined as above, the only changes being the addition of obvious identifying subscripts. The system is formulated as:



With the assumption of a steady state, i.e.,

$$\frac{d[ES_1]}{dt} \ll \frac{d[S_1]}{dt} \quad \text{and} \quad \frac{d[ES_2]}{dt} \ll \frac{d[S_2]}{dt} ,$$

$$([E] - [ES_1] - [ES_2]) [S_1] k_{1.1} = [ES_1] (k_{2.1} + k_{3.1}) \quad (13.1)$$

$$([E] - [ES_2] - [ES_1]) [S_2] k_{1.2} = [ES_2] (k_{2.2} + k_{3.2}) . \quad (13.2)$$

By dividing equation (13.1) by (13.2), substituting K_{S_1} and

K_{S_2} for their corresponding expressions, and solving for $[ES_2]$,

$$[ES_2] = [ES_1] \frac{K_{S_1} [S_2]}{[S_1] K_{S_2}} . \quad (14)$$

Equation (13.1) then becomes,

$$\left[[E] - [ES_1] \left(1 + \frac{K_{S_1} [S_2]}{[S_1] K_{S_2}} \right) \right] [S_1] k_{1.1} = [ES_1] (k_{2.1} + k_{3.1}). \quad (15.1)$$

Then,

$$[ES_1] = \frac{[E][S_1]}{K_{S_1} + [S_1] \left(1 + \frac{K_{S_1} [S_2]}{[S_1] K_{S_2}} \right)}. \quad (16.1)$$

Since $-\frac{d[S_1]}{dt} = k_{3.1} [ES_1]$ and

$$\lim_{S_1 \rightarrow \infty} -\frac{d[S_1]}{dt} = k_{3.1} [E] = v_1, \quad (17.1)$$

$$-\frac{d[S_1]}{dt} = \frac{v_1}{1 + \frac{K_{S_1}}{[S_1]} + \frac{K_{S_1} [S_2]}{[S_1] K_{S_2}}}. \quad (18.1)$$

In a completely analogous manner it can be shown that

$$-\frac{d[S_2]}{dt} = \frac{v_2}{1 + \frac{K_{S_2}}{[S_2]} + \frac{K_{S_2} [S_1]}{[S_2] K_{S_1}}}. \quad (18.2)$$

Therefore the total velocity v_T is:

$$v_T = -\frac{d[S_T]}{dt} = \frac{v_1 \frac{[S_1]}{K_{S_1}} + v_2 \frac{[S_2]}{K_{S_2}}}{\frac{[S_1]}{K_{S_1}} + 1 + \frac{[S_2]}{K_{S_2}}}. \quad (19)$$

This may be solved for $\frac{1}{v_T}$,

$$\frac{1}{v_T} = \frac{\frac{[S_1]}{K_{S_1}} + \frac{[S_2]}{K_{S_2}}}{V_1 \frac{[S_1]}{K_{S_1}} + V_2 \frac{[S_2]}{K_{S_2}}} + \frac{1}{[S_T]} \frac{\frac{[S_1]}{K_{S_1}} + \frac{[S_2]}{K_{S_2}}}{V_1 \frac{[S_1]}{K_{S_1}} + V_2 \frac{[S_2]}{K_{S_2}}} , \quad (20)$$

which is in the form of equation (10) with an apparent maximum rate v_T ,

$$v_T = \frac{V_1 \frac{[S_1]}{K_{S_1}} + V_2 \frac{[S_2]}{K_{S_2}}}{\frac{[S_1]}{K_{S_1}} + \frac{[S_2]}{K_{S_2}}} , \quad (21)$$

with an apparent K_{S_T} of

$$K_{S_T} = \frac{\frac{[S_1]}{K_{S_1}} + \frac{[S_2]}{K_{S_2}}}{\frac{[S_1]}{K_{S_1}} + \frac{[S_2]}{K_{S_2}}} . \quad (22)$$

Equation (22) simply means that when initial conditions are investigated for competitive hydrolysis, the apparent K_{S_T} is such that its reciprocal is the weighted average of the reciprocals of the individual K_S values. The apparent k_{3_T} for the competitive reaction is the average of the individual k_3 values weighted by the function $\frac{[S]}{K_S}$ as an investigation of equation (21), will reveal. Some interesting consequences of

these expressions will be discussed later.

An integral expression similar to equation (11) may be derived as follows for either one of the substrates. Equation (18.1) may be solved for $-V_1 dt$,

$$-V_1 dt = d[S_1] + \frac{K_{S_1}}{[S_1]} d[S_1] + \frac{K_{S_1} [S_2]}{[S_1] K_{S_2}} d[S_1] \quad (23)$$

When equations (18.1) and (18.2) are put in the form of their lowest common denominator (as was done to obtain (19)), their quotient may be expressed as:

$$\frac{\frac{d[S_2]}{dt}}{\frac{d[S_1]}{dt}} = \frac{k_{3.2}}{k_{3.1}} \frac{K_{S_1} [S_2]}{K_{S_2} [S_1]} \quad (24)$$

which may be written in the form

$$\frac{d[S_2]}{[S_2]} = \frac{d[S_1]}{[S_1]} \mathcal{H} \quad (25)$$

where

$$\frac{k_{3.2}}{k_{3.1}} \frac{K_{S_1}}{K_{S_2}} = \mathcal{H} \quad .$$

Then,

$$\log [S_2] = \mathcal{H} \log [S_1] + \log C, \quad (26)$$

or

$$[S_2] = C [S_1]^{\mathcal{H}}, \quad (26a)$$

where $C = \frac{[S_2]_0}{[S_1]_0}$. Substituting for $[S_2]$ in equation (23)

and integrating from $[S_1]_0$ to $[S_1]$,

$$k_{3.1} [E] t = ([S_1]_0 - [S_1]) + K_{S_1} \log \frac{[S_1]_0}{[S_1]} + \frac{K_{S_1}}{K_{S_2}} \frac{c}{\kappa} \left[[S_1]_0^\kappa - [S_1]^\kappa \right]. \quad (27)$$

This equation differs from that for a single substrate only in the added term in the exponential.

The enzyme-inhibitor dissociation constant K_I is a true equilibrium constant and the free energy change for the reaction $E + I \longrightarrow EI$ may be calculated as $-\Delta F = RT \ln \frac{1}{K_I}$. Since in the case of the proteolytic enzymes a way has not been found yet to measure either k_1 , k_2 , or their quotient directly for the reaction $E + S \longrightarrow ES$, one is left with the choice of k_3 greater than, equal to, or less than k_2 for the complete reaction of equation (1). However, certain systematic structural changes in inhibitors and substrates alter the constants K_S , K_I , and k_3 in such a manner that the most logical explanation is that k_2 is larger than k_3 and that therefore K_S is indeed a quasi equilibrium constant for α -chymotrypsin and the more active of the substrates discussed below.

α -Chymotrypsin catalyses the cleavage of the bond indicated⁽¹¹⁾ in $R(R')CHCO \longrightarrow R''$ where R may be hydrogen, chlorine, hydroxyl, amino or acylamido, R' may be such that $R'CHNH_2CO_2H$ is L-norvaline, norleucine, methionine, tryptophan, phenylalanine or many substitution products thereof as well as

hexahydrophenylalanine, while $R''H$ may be an alcohol, ammonia, hydroxylamine or hydrazine. The different combinations that have been studied so far vary widely in affinity for the enzyme and also in susceptibility to hydrolysis. Derivatives of L-tryptophan and L-phenylalanine and its substitution products may be called the "typical" substrates for α -chymotrypsin because of their relatively greater affinities and susceptibilities to hydrolysis. The pH at which the reaction system is buffered affects the relative affinities of compounds of varying R with the other variants held constant. For example, compounds with free amino groups have pH optima more acid than those with acylamido groups for R. This phase of the specificity is discussed more fully in Part III of this thesis. The relative susceptibility of the bond indicated above to hydrolysis in the presence of the enzyme decreases in the order ester > hydroxylamide > amide > methylamide.

Present data indicate that for derivatives of amino acids there is complete stereochemical specificity; i.e., only those amino acid derivatives belonging to the L series are subject to chymotryptic hydrolysis. However in all cases but one reported so far, the K_I for the D antipode is less than the K_S for the L substrate. It is interesting to note in this instance - the carboethoxy-tyrosinamides⁽¹²⁾ - that

this acyl group and the carbobenzoxy group are the two which disturb the antipodal specificity of the papain catalyzed synthesis of acyl phenylalanine phenylhydrazides⁽¹³⁾.

There are of course inhibitors in the L family too. Some of these have no hydrolyzable bond - viz., acetyl-L-tryptophan, while others have such a bond but hydrolysis proceeds so slowly that they can be treated as inhibitors in a system with a more susceptible substrate. A case in point is acetyl-L-tryptophan methylamide in the presence of acetyl-L-tryptophanamide. A more detailed discussion of the structural requirements of inhibitors is reserved for Part III of this thesis.

To further the understanding of the mode of action of chymotrypsin, the kinetic constants for the enantiomorphic pair of acetyl phenylalaninamides have been determined. The equations for competitive hydrolysis developed in the introduction have been tested for two pairs of substrates: acetyl-L-tyrosinamide, acetyl-L-phenylalaninamide; and acetyl-L-tyrosinamide and acetyl-L-tryptophanamide.

Discussion of Results

H. T. Huang and C. Niemann detected certain characteristic differences in K_S and K_I values when the structures of substrates and inhibitors were changed in a systematic manner^(7,9,14). For acyl-L-tyrosinamides and acyl-L-tryptophanamides, a change

of acyl groups from acetyl to nicotinyl resulted in a two-fold decrease of K_S while for the corresponding D enantiomorphs the same change brought about a two-fold decrease of K_I . The change in $-\Delta F$ for the formation of the enzyme-inhibitor constants was 400 calories per mole for the acyl shifts noted above. Comparison of the "K" values showed that substitution of β -indolyl-methyl for para-hydroxybenzyl decreased them five-fold. This is an increase of $-\Delta F$ of 1000 calories for the inhibitors. For the enantiomorphic pairs above, the constant for the D compound was nearly one-half of that for the L substrate.

It was desired to extend these studies to include the corresponding phenylalaninamides and while H. T. Huang was investigating the nicotinyl phenylalanine amides the present author determined the constants for the acetyl compounds ⁽¹⁵⁾. The results are tabulated below.

	$\frac{K_{S,I}}{}$	$\frac{k_3}{}$	$\frac{-\Delta F}{}$
Acetyl-L-phenylalaninamide	34	0.8	2000
Acetyl-D-phenylalaninamide	14		2530
Nicotinyl-L-phenylalaninamide	18	2.1	2380
Nicotinyl-D-phenylalaninamide	7		2930

The $-\Delta F$ values for the substrates are valid of course only with the assumption that $K_S \stackrel{0}{=} \frac{k_2}{K_1}$. The substitutions of

nicotinyI for acetyl and D for L in the acyl-phenylalanin-amides have the same effect on the ratios of the $K_{S,I}$ values as they had in the tyrosine and tryptophan series. The loss of the para-hydroxyl group increased the $K_{S,I}$ values only by 10% whereas it decreased k_3 by almost 70%.

In view of the fact that the constants for acetyl-L-tyrosinamide and acetyl-L-tryptophanamide had been determined with a reasonable degree of precision^(7,16), it was decided to check the hypothesis that there was only one kind of enzymatic site on the α -chymotrypsin molecule by employing the equations for competitive hydrolysis derived in the introduction. Two series of experiments each with two different ratios of substrates are summarized in Table I. The hydrolyses for these experiments are presented in Figures 7 to 16. The K_{S_T} values found experimentally agree very well with the calculated ones, whereas the k_{3_T} values were about 10% high. This is still within the limits of experimental error but part of this discrepancy may be explained.

The high values of k_{3_T} found experimentally can be explained partially by studying the consequences of the equations for competitive hydrolysis and the method used for evaluating initial velocities. If the experimental values of k_3 and K_S for acetyl-L-tyrosinamide and acetyl-L-tryptophanamide are substituted in equation (24), it is seen that the

ratio of hydrolysis of the tryptophan derivative to that of the tyrosine derivative is 1.2 times the ratio of their concentration. However, from equation (19) it is seen that a higher percentage of the tryptophan derivative decreases the total rate. Thus, as the hydrolysis proceeds, the ratio of tyrosine substrate to tryptophan substrate increases and so does the apparent rate constant k_{3T} . Since the initial velocity is estimated graphically or analytically from a series of eight determinations of total product concentration, each initial velocity computed is higher than it would have been for a single substrate with the same kinetic constants. This point is illustrated in Table 3 which compares percentage of hydrolysis rates and relative rates for two different ratios of $\frac{\text{acetyl-L-tryptophanamide}}{\text{acetyl-L-tyrosinamide}}$. As a further check on the validity of the expressions derived for competitive hydrolysis, the pair acetyl-L-tyrosinamide and acetyl-L-phenylalaninamide was studied. The constants found for these experiments are given in Table 2. The individual hydrolyses are presented graphically in Figures 17 to 19.

Experimental*

Acetyl-L-phenylalaninamide - Two grams of acetyl-L-phenylalanine methyl ester⁽¹⁵⁾ was added to 50 ml. of methanol saturated with ammonia at 0°. After standing for two days at

* All melting point values are corrected. All microanalyses by Dr. A. Elek.

room temperature, the solvent was evaporated to dryness and the solid recrystallized twice from water. The melting point of the fine white needles was 176° - 177° , $[\alpha]_D^{25} = +27^{\circ}$ (C, 1% in methanol).

Analysis calculated for $C_{11}H_{14}O_2N_2$:

C, 64.1; H, 6.9; N, 13.6

Found: C, 64.2; H, 6.8; N, 13.6

Acetyl-D-phenylalaninamide - One gram of acetyl-D-phenylalanine methyl ester⁽¹⁵⁾ was ammonolyzed as above. The melting point of the fine white needles after two recrystallizations from water was 176° - 177° , $[\alpha]_D^{25} = -27^{\circ}$ (C, 1% in methanol).

Analysis calculated for $C_{11}H_{14}O_2N_2$:

C, 64.1; H, 6.9; N, 13.6

Found: C, 64.2; H, 6.8; N, 13.6

Acetyl-L-tyrosinamide - Ammonolysis of 10 grams of acetyl-L-tyrosine ethyl ester⁽¹⁶⁾ gave 4.1 grams of acetyl-L-tyrosinamide. The small rhombs melted at 225.5° - 226° after recrystallization from methanol and drying in vacuo over P_2O_5 . The melting point of the product was not depressed when mixed with an authentic specimen⁽¹⁶⁾. $[\alpha]_D^{25} = +50.7$ (C, 0.8% in water).

Acetyl-L-tryptophanamide - Ammonolysis of 10 grams of acetyl-L-tryptophan methyl ester⁽⁷⁾ gave 8.0 grams of acetyl-

L-tryptophanamide which melted at 189° - 191° after recrystallization from water and drying in vacuo over P_2O_5 . A mixed melting point with an authentic specimen⁽⁷⁾ showed no depression. $[\alpha]_D^{25} = + 20.5^{\circ}$ (C, 2% in methanol).

Enzyme - The enzyme used in all of these experiments was an Armour product, lot 90402. Several analyses yielded a value of 10.4% for the protein nitrogen content, which was determined as follows: An accurately weighed sample of the chymotrypsin preparation, dissolved in water, was precipitated with an equal volume of 5% trichloroacetic acid. The precipitate was collected on a previously weighed sintered glass crucible and dried. The crucible was reweighed and the nitrogen content of the precipitate determined.

$$\% \text{ prot. N} = \frac{\text{wt. of ppt.}}{\text{wt. of samples}} \quad \% \text{ N in ppt.}$$

All of the experiments in this part of the thesis were conducted with an enzyme concentration of 0.208 mg. protein nitrogen per ml. of reaction solution. The dry enzyme preparation was put into solution shortly before use and placed in a thermostat at 25.0° .

Buffer - A tris-(hydroxymethyl)-amino-methane-hydrochloric acid buffer was employed to keep the pH between 7.8-7.95 since it had been shown that the maximum activity of chymotrypsin for these substrates included this range^(7,15,16). The technical amine from Commercial Solvents Corporation was

recrystallized twice from ethanol to a melting point of 168° - 168.5° . A stock solution 0.2 F in the amine component was prepared and 1.00 ml. was used for the 10.0 ml. reaction solutions.

Temperature - The temperature was maintained at $25.0^{\circ} \pm 0.1$ by a "Sargent" water bath thermostat for all hydrolyses.

Analysis - The method used for analysis was potentiometric formol titration of 1.00 ml. aliquots taken at selected time intervals⁽¹⁷⁾. A Beckman pH meter model G, with special narrow bore electrodes, models 290 - 11x (glass) and 270 - 6 (calomel), was employed to measure the pH. Titration curves were plotted of pH versus ml. of 0.0100 N sodium hydroxide standard solution (prepared carbonate free from 50% sodium hydroxide⁽¹⁸⁾). The point of inflection was chosen as the endpoint of each titration. A sample set of titration curves for one hydrolysis is shown in Figure 1.

Calculations - The initial value for each substrate concentration was determined by extrapolation of the hydrolysis curves to zero time. This value is the formol titration of the buffer, enzyme, inhibitor if present, and the substrate. The main component is of course that due to the buffer and for this reason the buffer concentration was maintained as low as was consistent with a satisfactory buffering. For the first few points of each determination this buffer blank amounted to

as much as 90% of the total titration and decreased to less than 50% at the end for higher substrate concentrations.

The most critical part of the determinations was the evaluation of the initial velocity from the primary data. The reason for this is that the order of the reaction is neither zero, first, nor even constant. As may be seen from an examination of the equations for the enzymatic hydrolysis, first order kinetics are approximated if the initial concentration is much less than the K_S . However, the value of K_S for substrates used in these studies is such that concentrations small enough to give a good first order approximation would create other experimental limitations. With substrate concentrations considerably greater than the K_S value, zero order kinetics are approximated but unfortunately, the low solubility of the substrates precludes such concentrations. For the above reasons the initial velocity of most α -chymotryptic hydrolyses must be evaluated graphically.

The values of the kinetic constants k_3 , K_S , and K_I for single substrate hydrolyses and k_{3T} and K_{ST} for competitive hydrolyses were obtained from appropriate plots of equations (10) and (20).

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Table 1

α -Chymotrypsin Catalyzed Competitive Hydrolysis of
Acetyl-L-Tryptophanamide and Acetyl-L-Tyrosinamide^a

Mole % S ₁ ^b	Mole % S ₂ ^c	K _S ^d		k ₃ ^e	
		Exptl.	Calcd.	Exptl.	Calcd.
100	0	5.3 ^f		0.50 ^f	
50	50	9.0	9.1	0.86	0.78
25	75	13.8	13.9	1.3	1.2
0	100	30.5 ^g		2.4 ^g	

a., in aqueous solutions at 25° and pH 7.9; b., acetyl-L-tryptophanamide; c., acetyl-L-tyrosinamide; d., in units of 10⁻³ molar; e., in units of 10⁻³ mole. l.⁻¹ min.⁻¹ (mg. prot. N. ml.⁻¹)⁻¹; f., cf. ref. 7; g., cf. ref. 16.

Table 2

α -Chymotrypsin Catalyzed Competitive Hydrolysis of Acetyl-L-Phenylalaninamide and Acetyl-L-Tyrosinamide^a

Mole % S ₁ ^b	Mole % S ₂ ^c	K _S ^d		k ₃ ^e	
		Exptl.	Calcd.	Exptl.	Calcd.
100	0	34 ^f		0.7 ^f	
50	50	33	32	1.7	1.6
0	100	30.5 ^g		2.4 ^g	

b., acetyl-L-phenylalaninamide; f., this thesis; all other footnotes see Table 1 above.

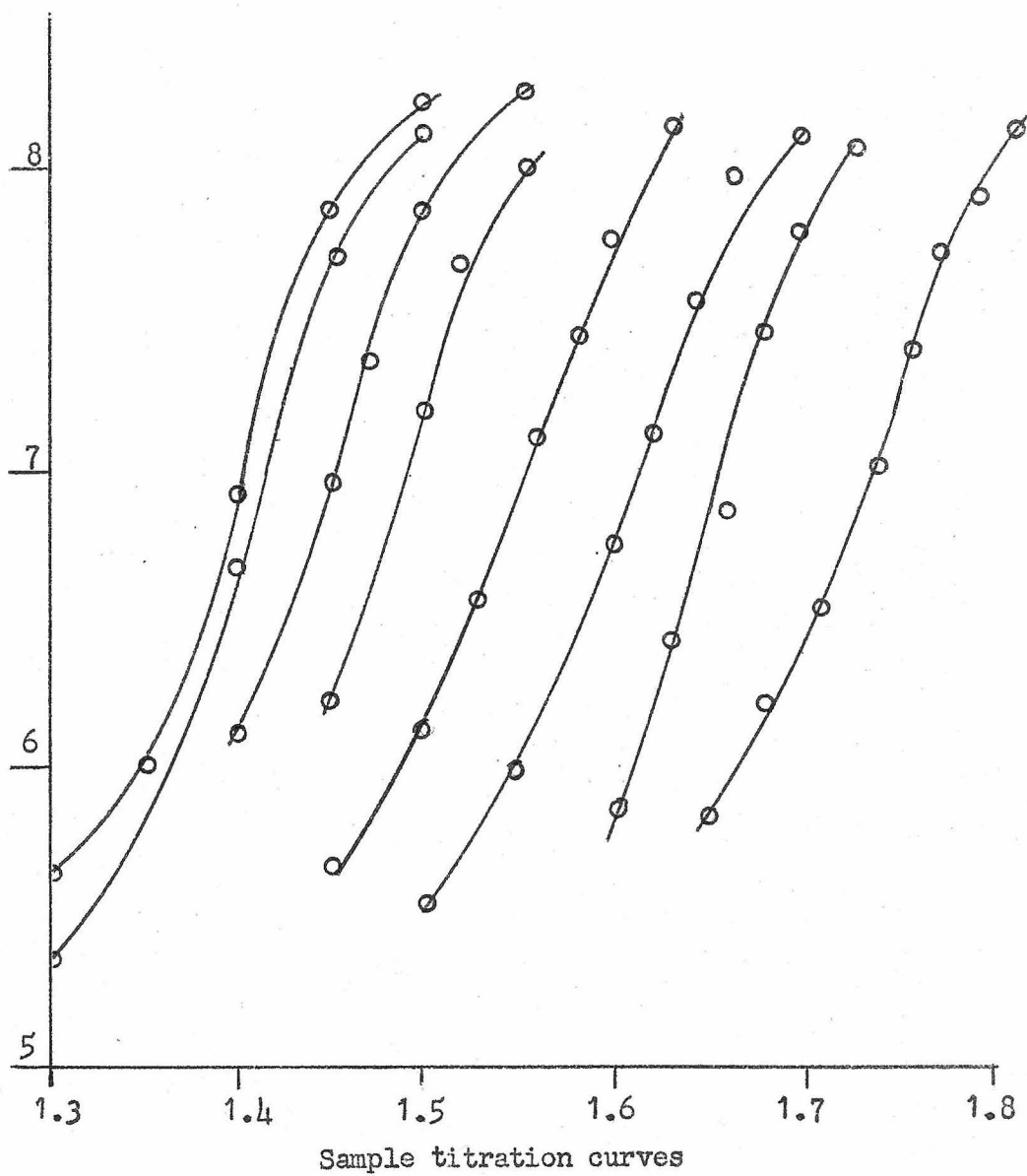
Table 3

The Relative Velocities of Hydrolysis in the
Competitive Hydrolysis of Acetyl-L-Tryptophan-
amide and Acetyl-L-Tyrosinamide^a

S_T ^b	S_1 ^c		S_2 ^d		Total Velocity ^e
	% of S_T	% of v_T	% of S_T	% of v_T	
20	25	29	75	71	1.26
60	25	29	75	71	1.71
20	50	55	50	45	1.00
60	50	55	50	45	1.25

a., calculated from equations (19) and (24); b., in units
of 10^{-3} molar; c., Acetyl-L-tryptophanamide; d., Acetyl-
L-Tyrosine amide; e., relative to initial velocity of
competitive hydrolysis of 10^{-2} molar S_1 and 10^{-2} molar S_2 .

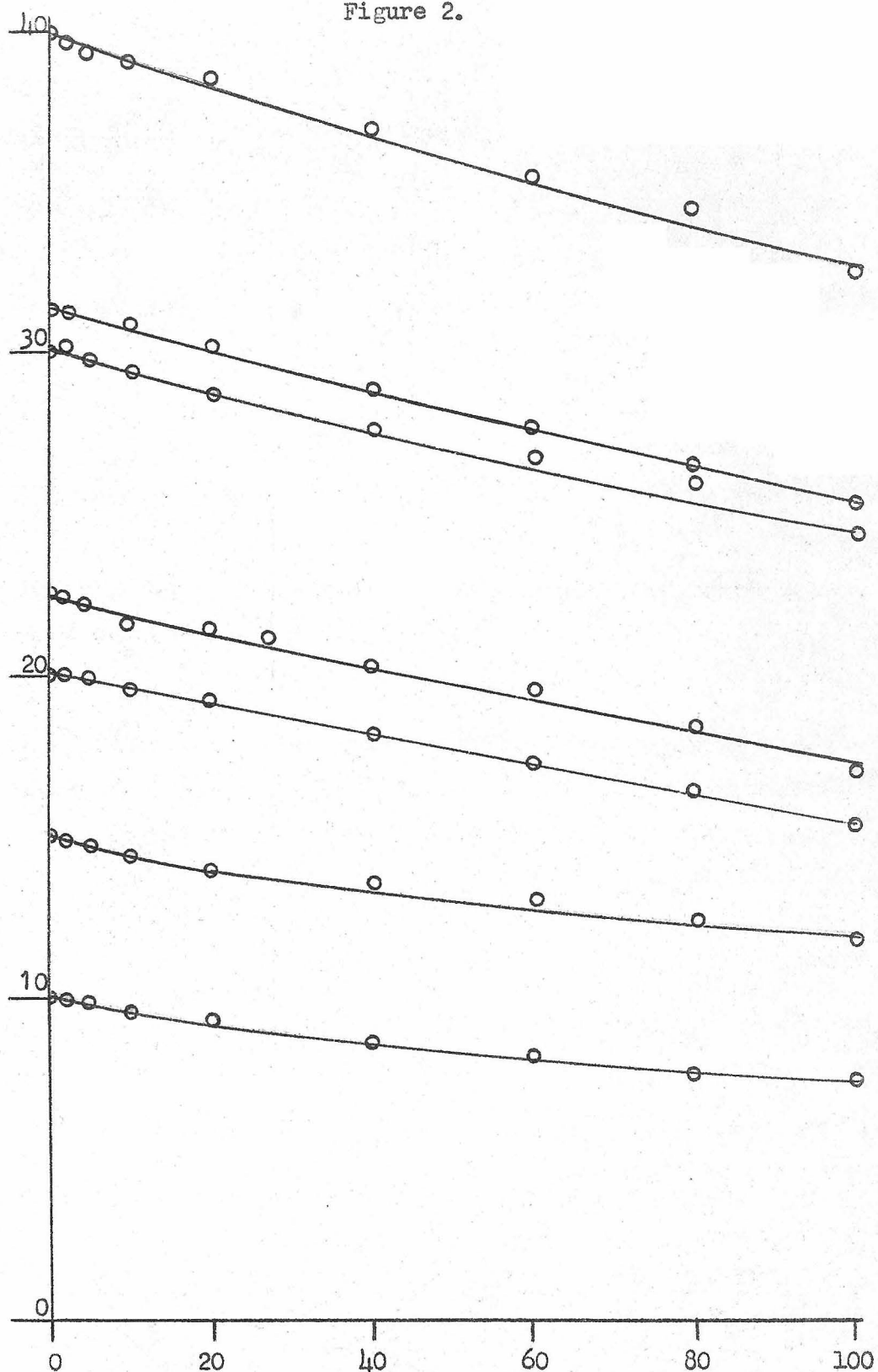
Figure 1



Ordinate: pH of formaldehyde solution of one ml. aliquots plus added base.

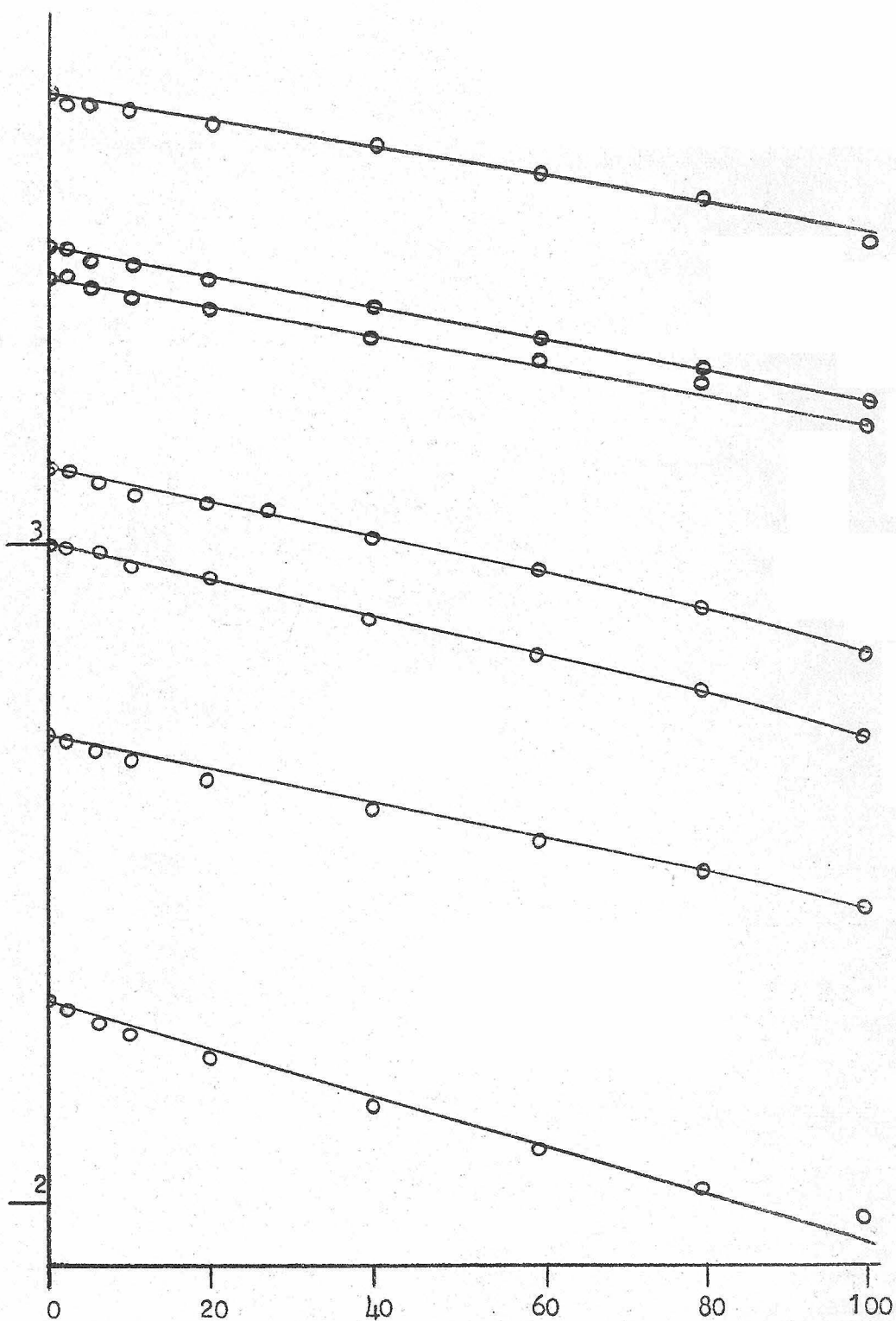
Abcissa: Ml. of 0.01 N NaOH added.

Figure 2.



Chymotryptic hydrolysis of acetyl-L-phenylalaninamide.
Ordinate: Substrate concentration in units of 10^{-3} M.
Abcissa: Time in minutes.

Figure 3.

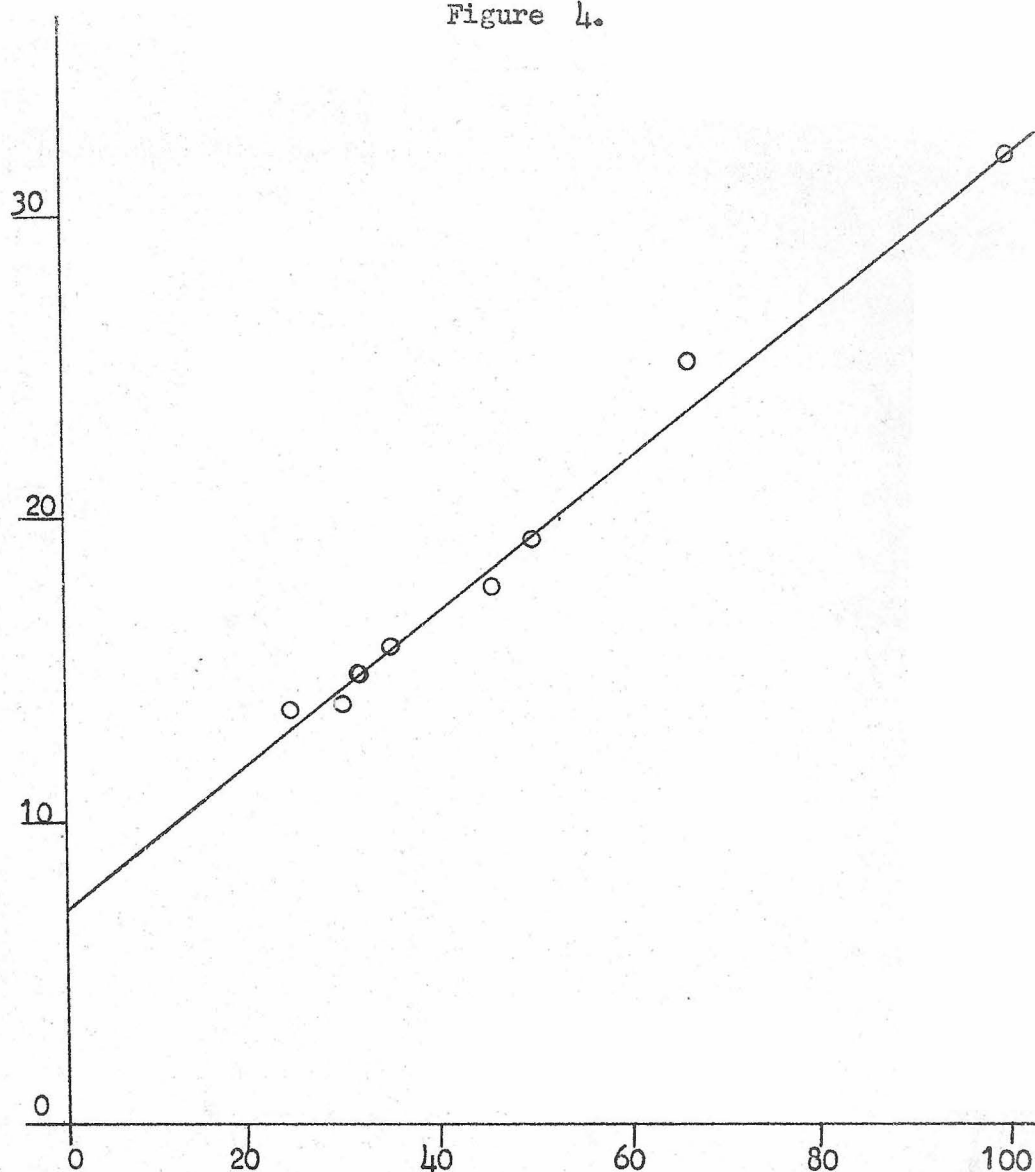


Chymotryptic hydrolysis of acetyl-L-phenylalaninamide

Ordinate: Natural log of substrate concentration in units of 10^{-3} M.

Abcissa: Time in minutes.

Figure 4.



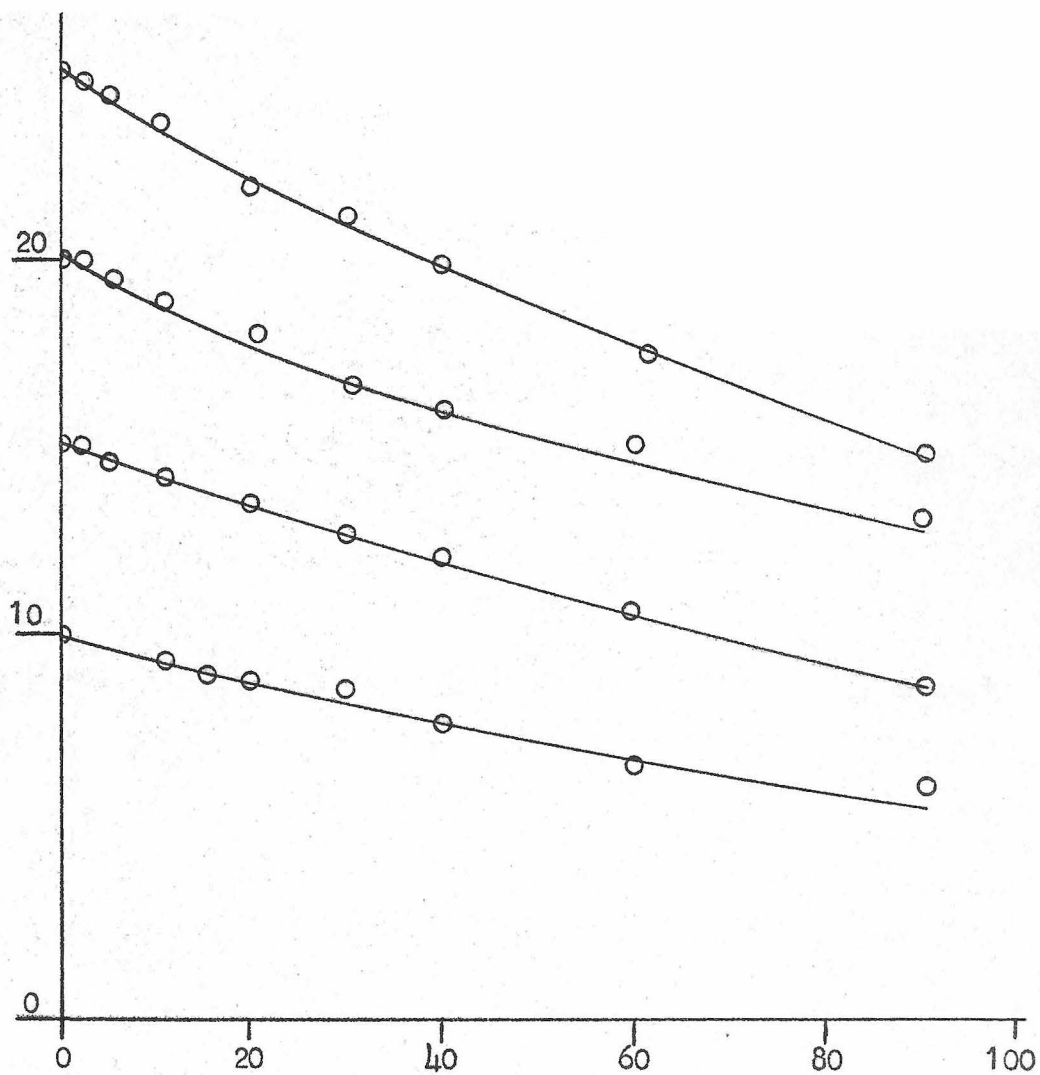
Determination of kinetic constants for the
chymotryptic hydrolysis of acetyl-L-phenylalaninamide

Ordinate: Initial velocity reciprocal in units of 10^{-3} min.
l./mole.

Abscissa: Reciprocal of substrate concentration in units of
l./mole.

$K_S = 35 \times 10^{-3}$ M; $k_3 = 0.7 \times 10^{-3}$ mole l^{-1} min $^{-1}$ (mg. prot. N.ml $^{-1}$) $^{-1}$

Figure 5.

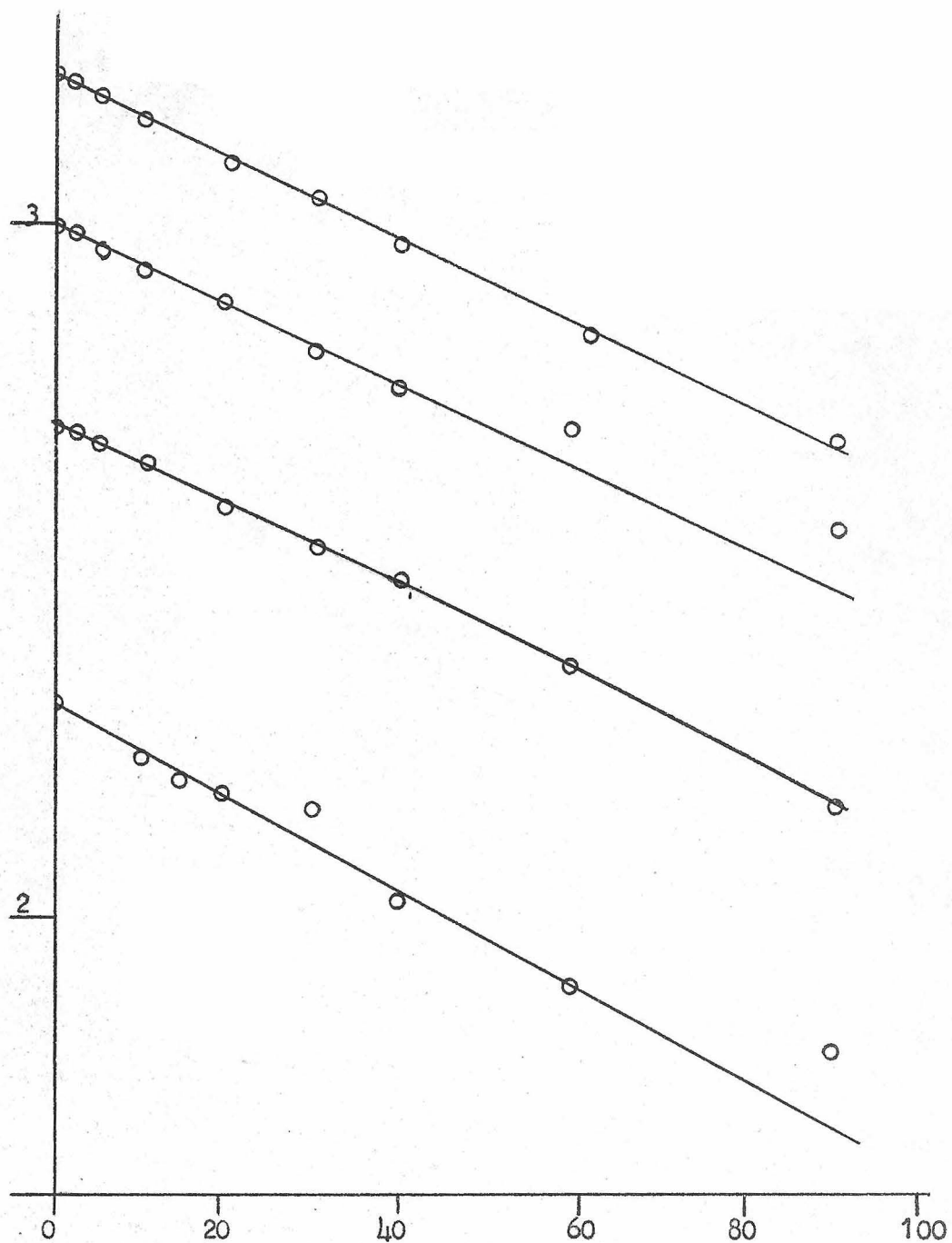


Inhibition by acetyl-D-phenylalaninamide of the
chymotryptic hydrolysis of acetyl-L-tyrosinamide

Ordinate and abscissa as in Figure 2.

Inhibitor concentration 10^{-2} M.

Figure 6.

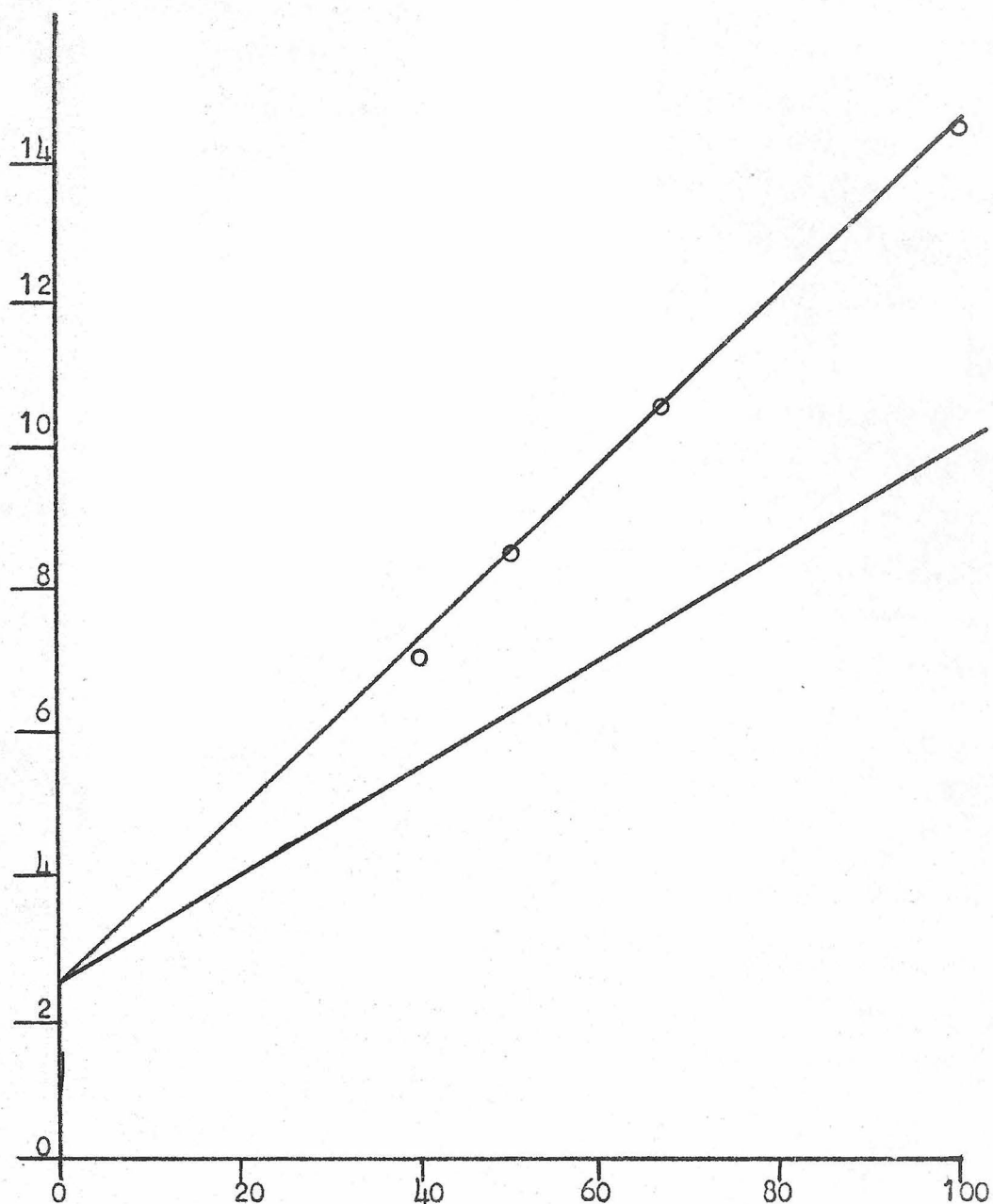


Inhibition by acetyl-D-phenylalaninamide of the
Chymotryptic hydrolysis of acetyl-L-tyrosinamide

Ordinate and abscissa as in Figure 3.

Inhibitor concentration $10^{-2} M$.

Figure 7.



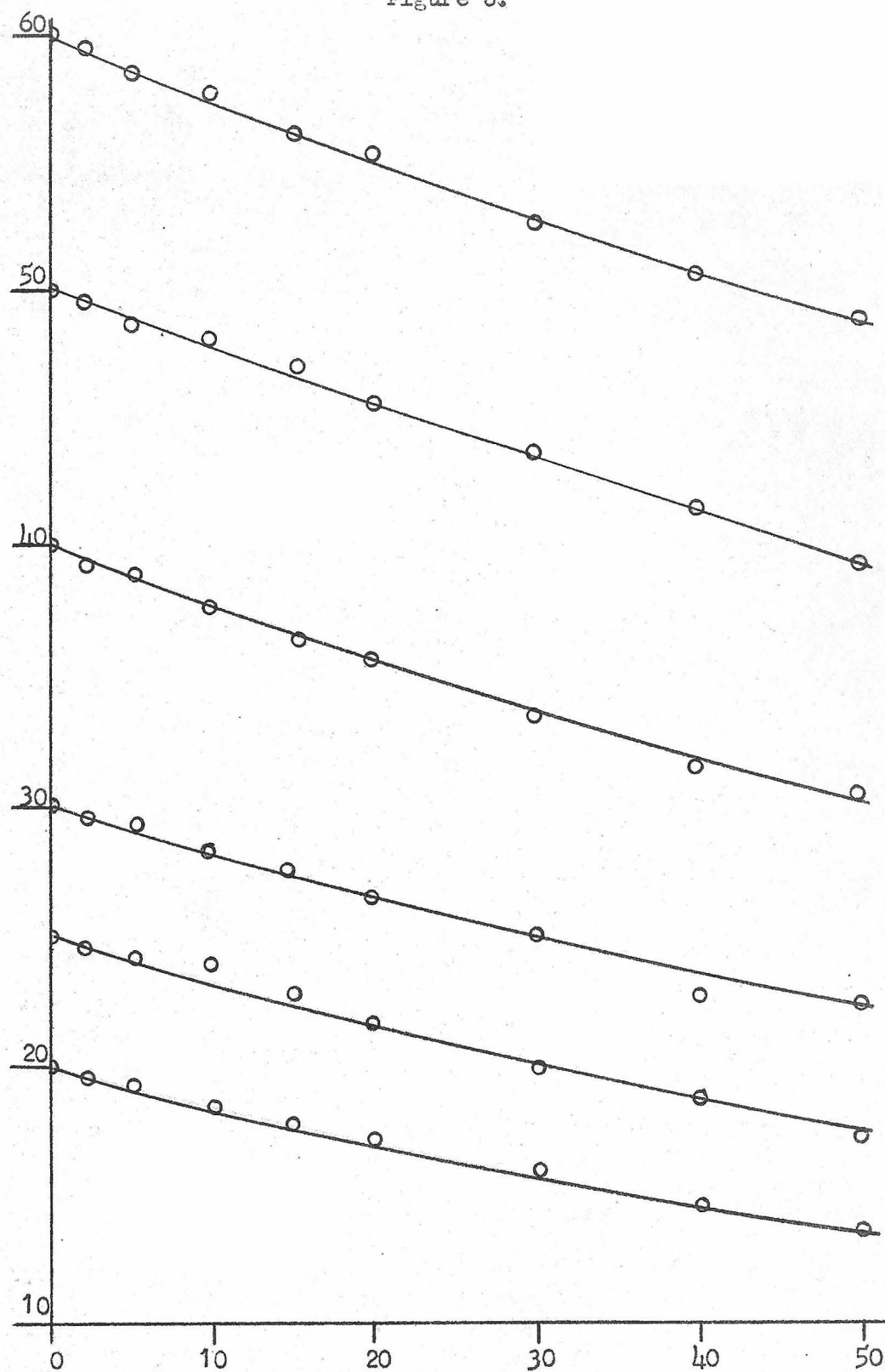
Determination of K_I for acetyl-D-phenylalaninamide

Ordinate and abscissa as in Figure 4.

Base line represents $I = 0$, upper line is for $I = 10^{-2} M$

$$K_I = 12 \times 10^{-3} M.$$

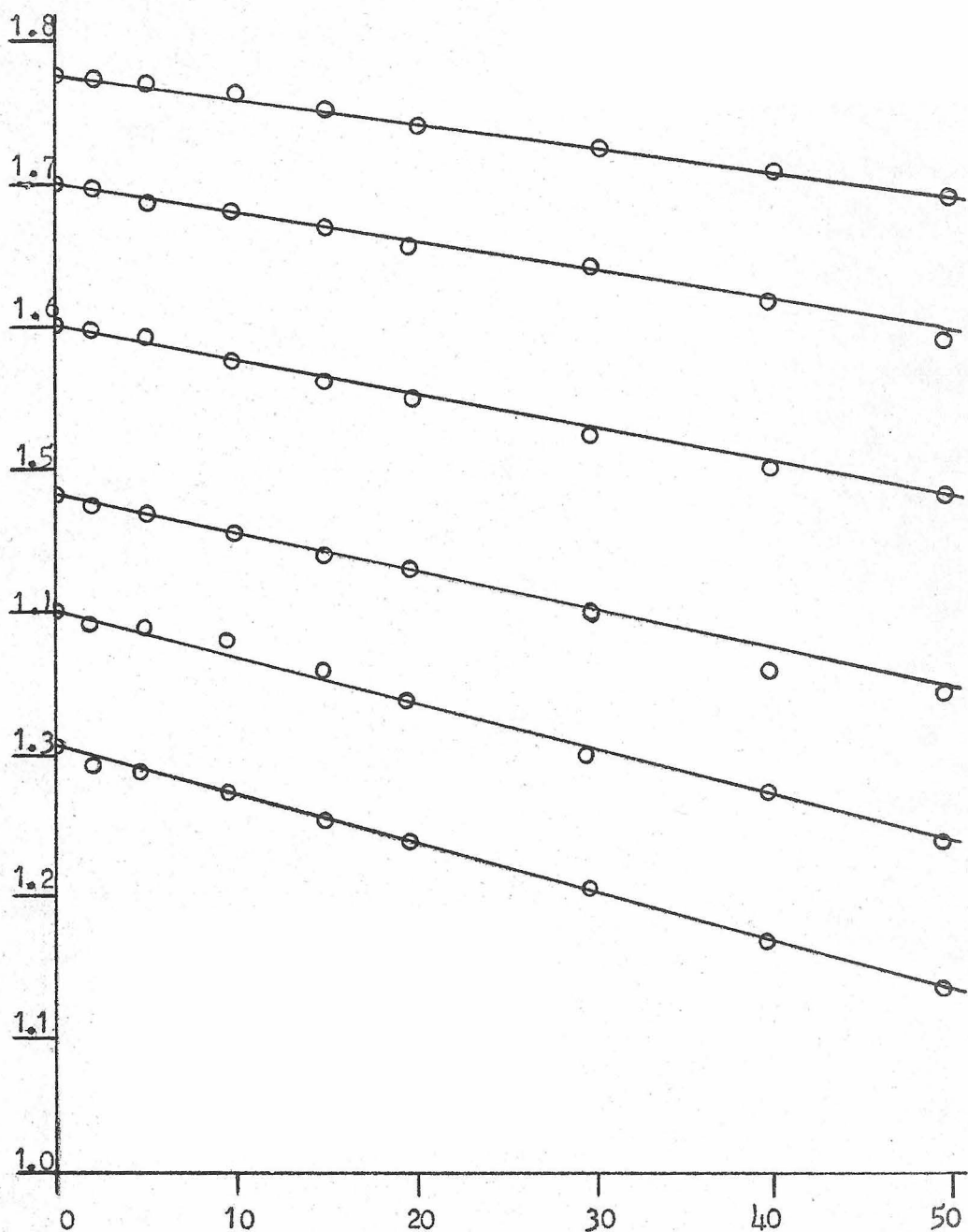
Figure 8.



Competitive chymotryptic hydrolysis of acetyl-L-tryptophanamide (25%) and acetyl-L-tyrosinamide (75%).

Ordinate and abscissa as in Figure 2.

Figure 9.

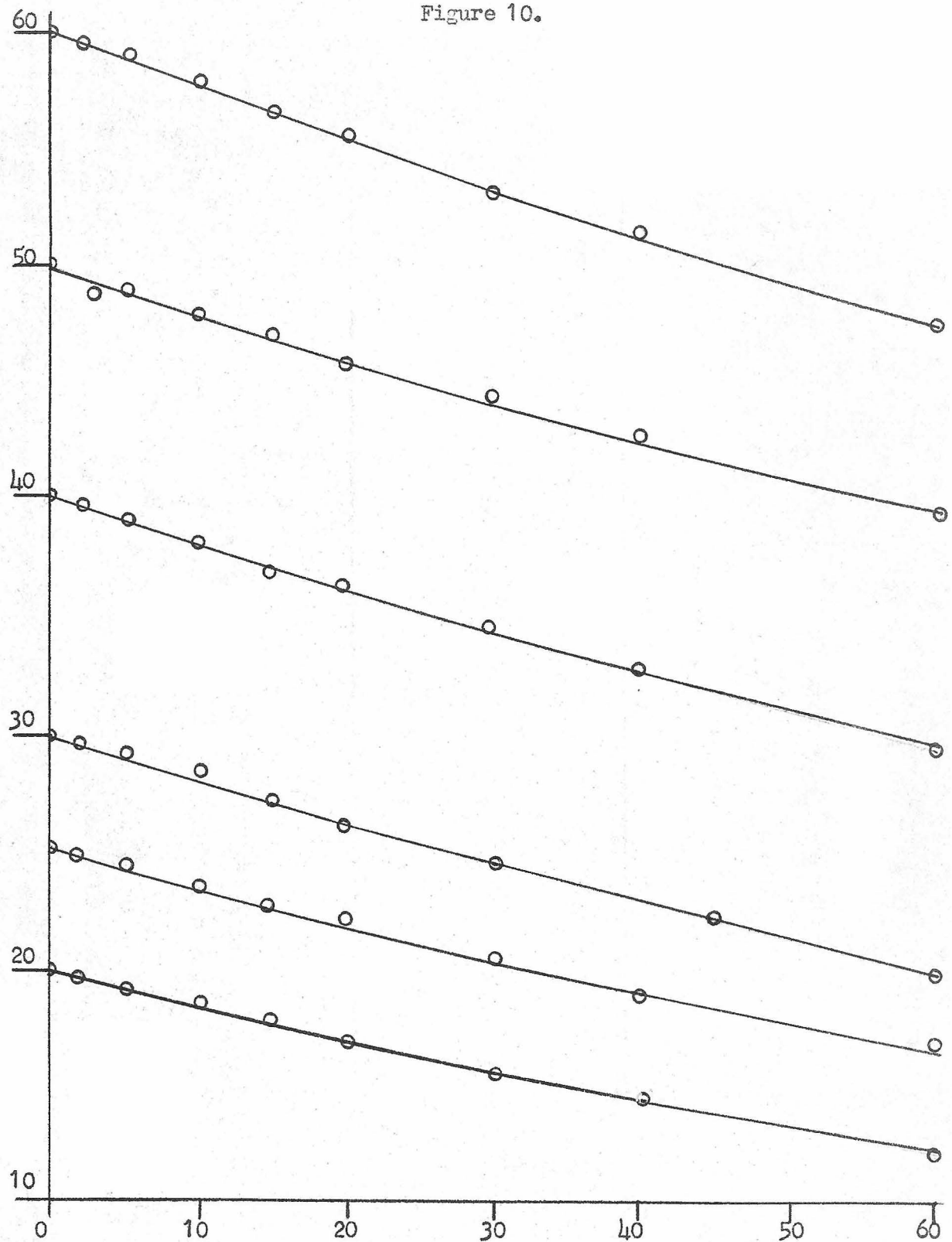


Competitive chymotryptic hydrolysis of acetyl-L- tryptophan-
amide (25%) and acetyl-L-tyrosinamide (75%)

Ordinate: Log base 10 of substrate concentration in units of
 10^{-3} M.

Abcissa: Time in minutes.

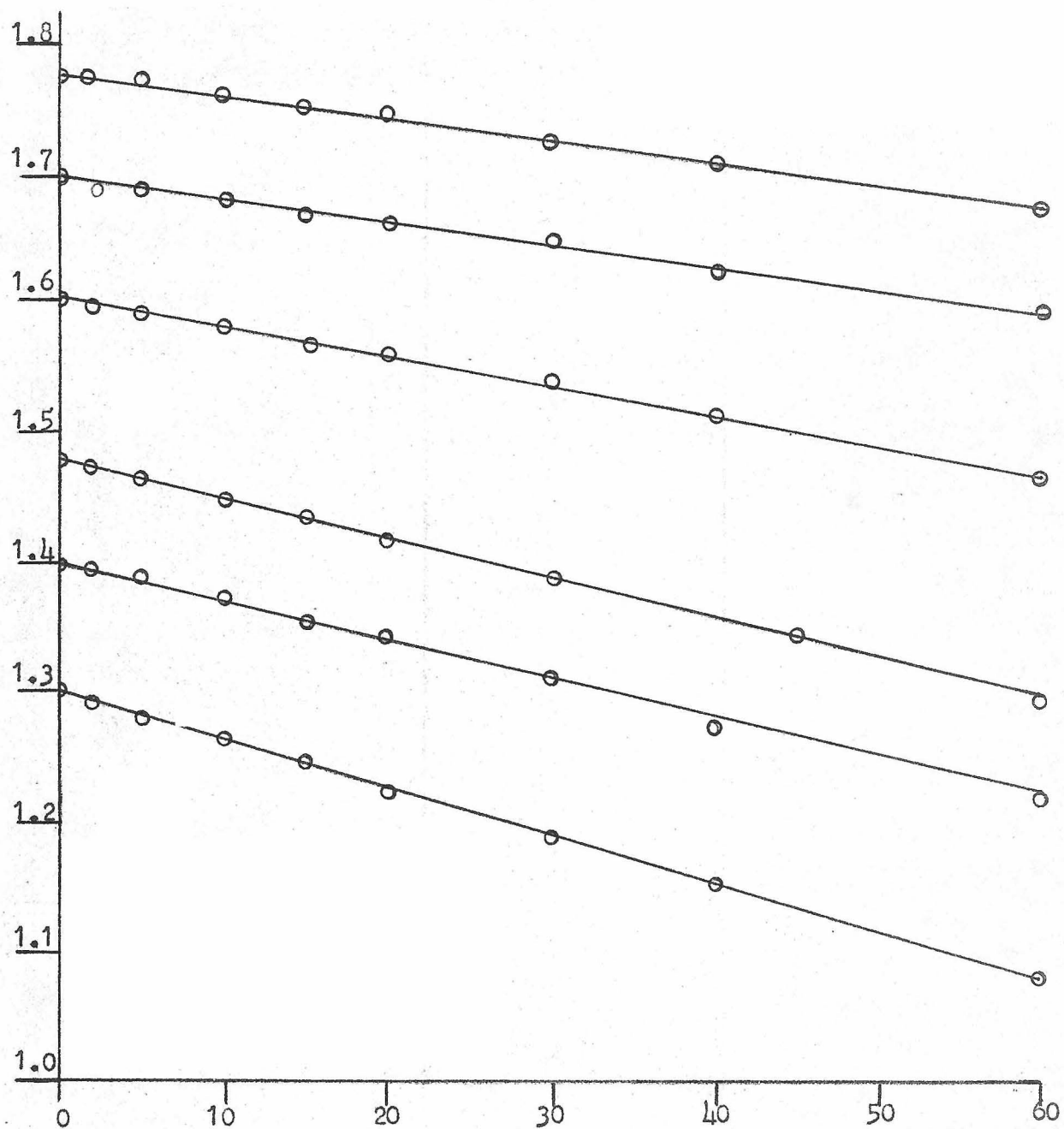
Figure 10.



Competitive chymotryptic Hydrolysis of acetyl-L-tryptophanamide (25%) and acetyl-L-tyrosinamide (75%)

Ordinate and abscissa as in Figure 2.

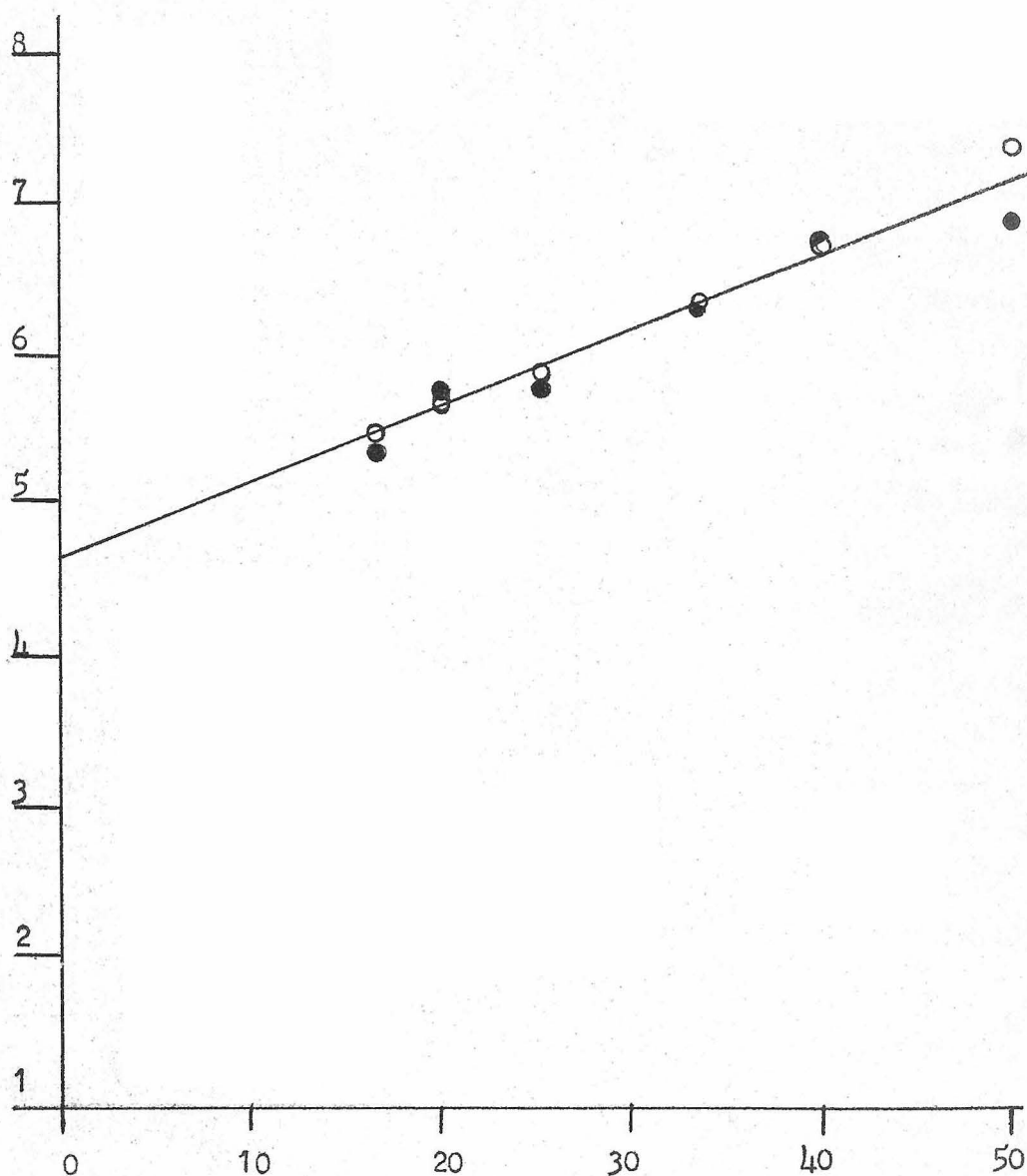
-41-
Figure 11



Competitive chymotryptic hydrolysis of acetyl-L-tryptophan-
amide (25%) and acetyl-L-tyrosinamide (75%)

Ordinate and abscissa as in Figure 9.

Figure 12.



Determination of apparent kinetic constants for the competitive chymotryptic hydrolysis of acetyl-L-tryptophanamide (25%) and acetyl-L-tyrosinamide (75%)

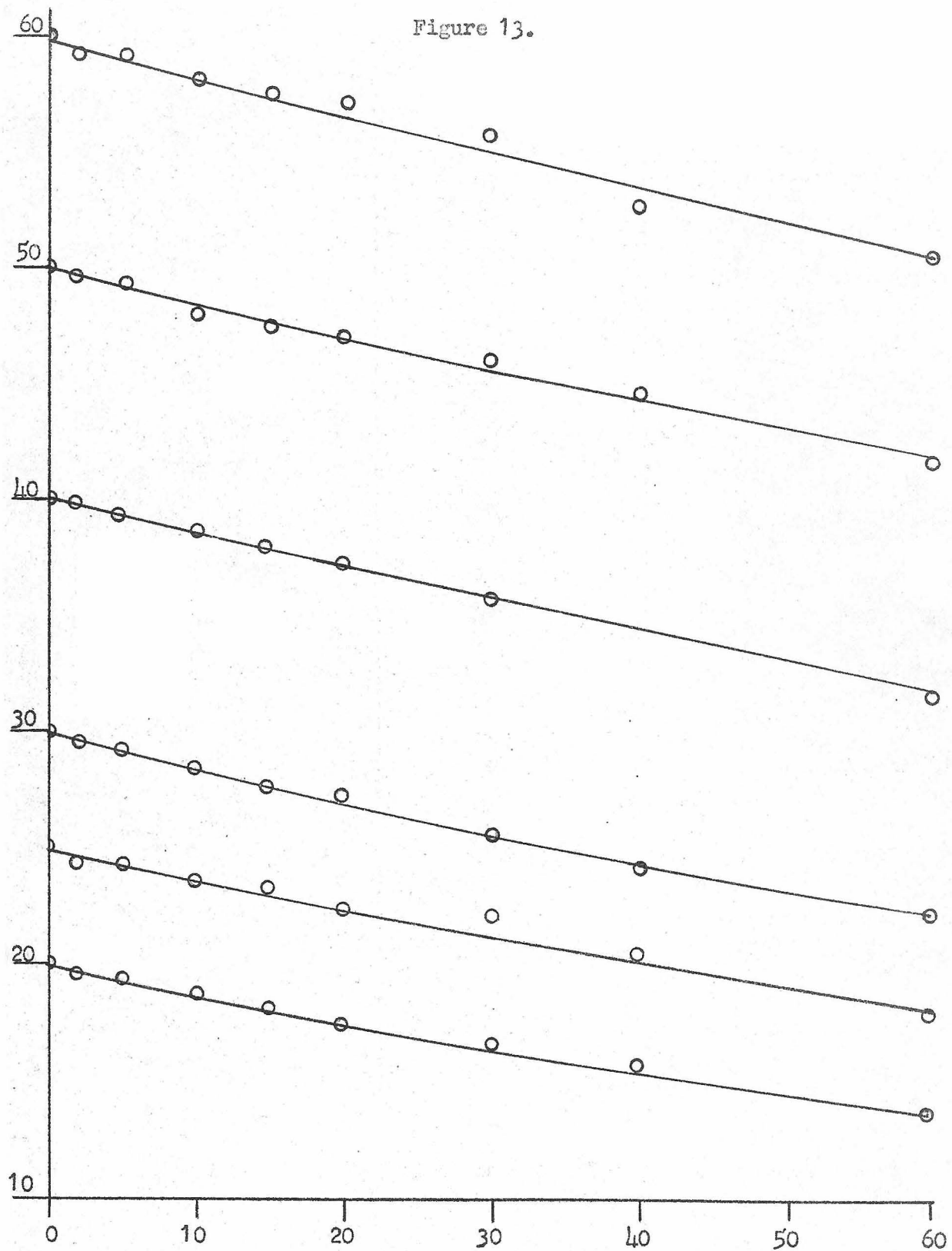
Ordinate and abscissa as in Figure 4.

Open and closed circles for two series of experiments.

$$K_{S_T} = 13.8 \times 10^{-3} \text{ M}$$

$$k_{3_T} = 1.3 \times 10^{-3} \text{ mole, l}^{-1} \text{ min}^{-1} (\text{mg. prot. N ml}^{-1})^{-1}$$

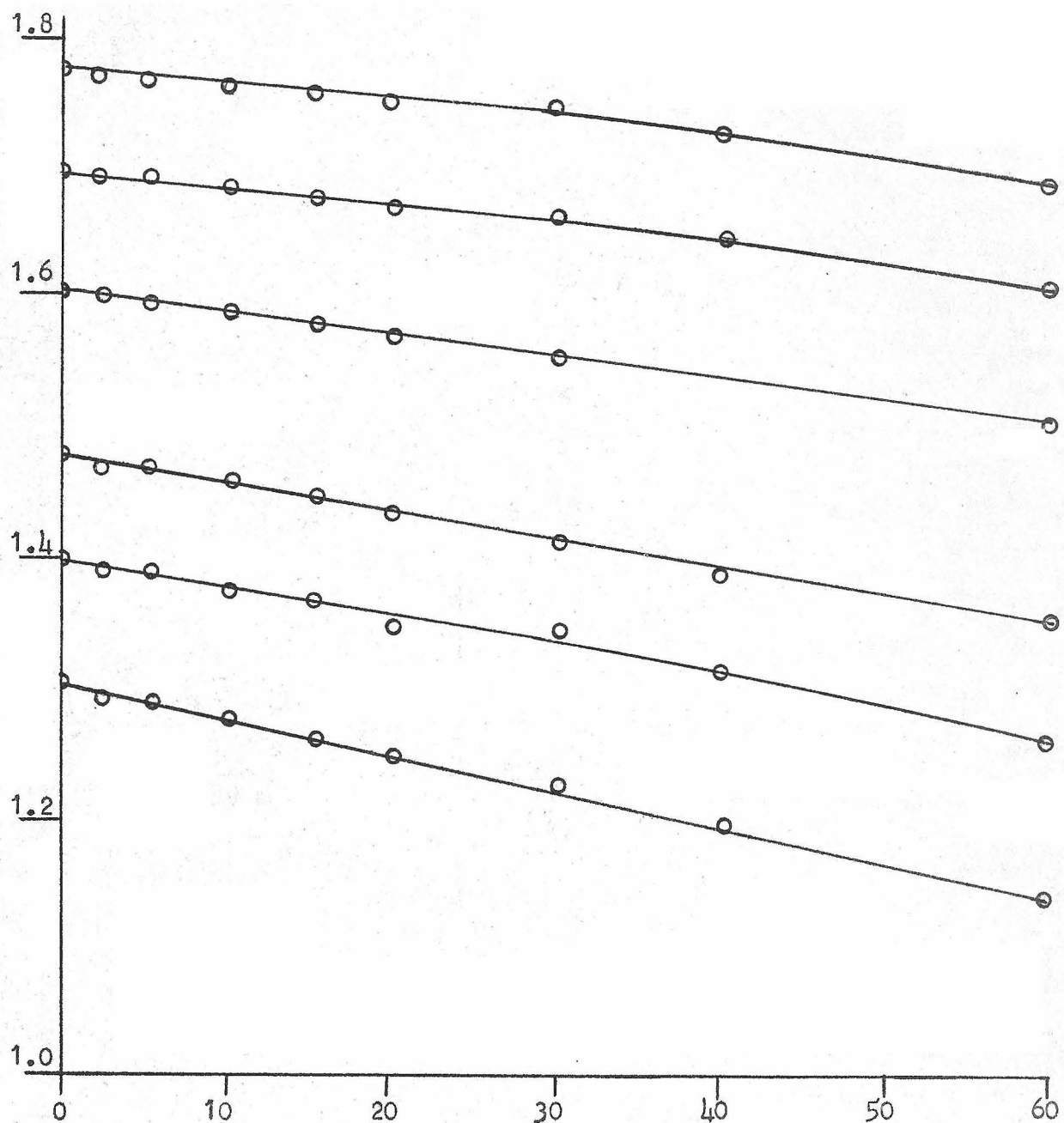
Figure 13.



Competitive chymotryptic hydrolysis of acetyl-L-tryptophanamide (50%) and acetyl-L-tyrosinamide (50%)

Ordinate and abscissa as in Figure 2.

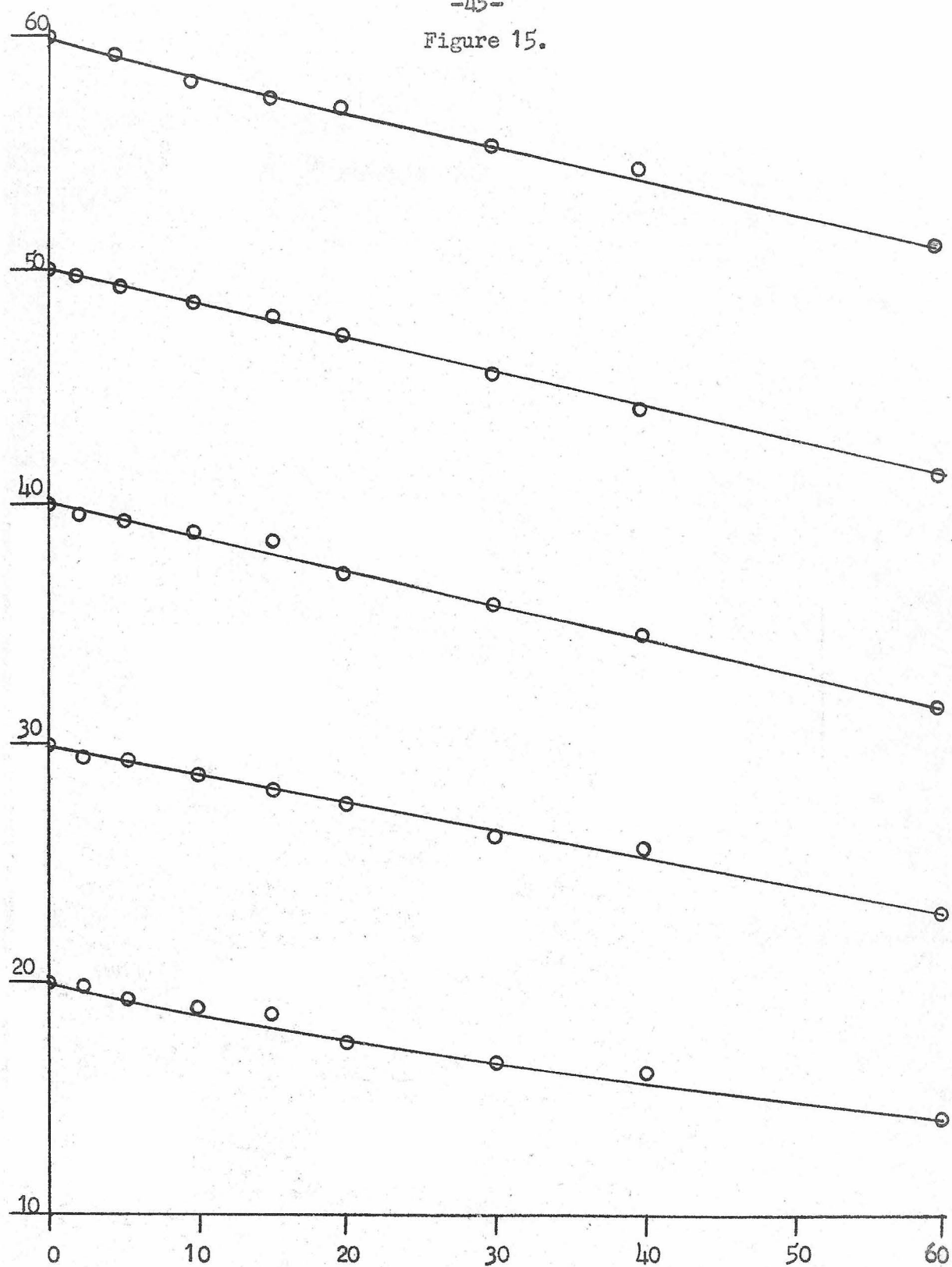
Figure 14.



Competitive chymotryptic hydrolysis of acetyl-L-tryptophanamide (50%) and acetyl-L-tyrosinamide (50%)

Ordinate and abscissa as in Figure 9.

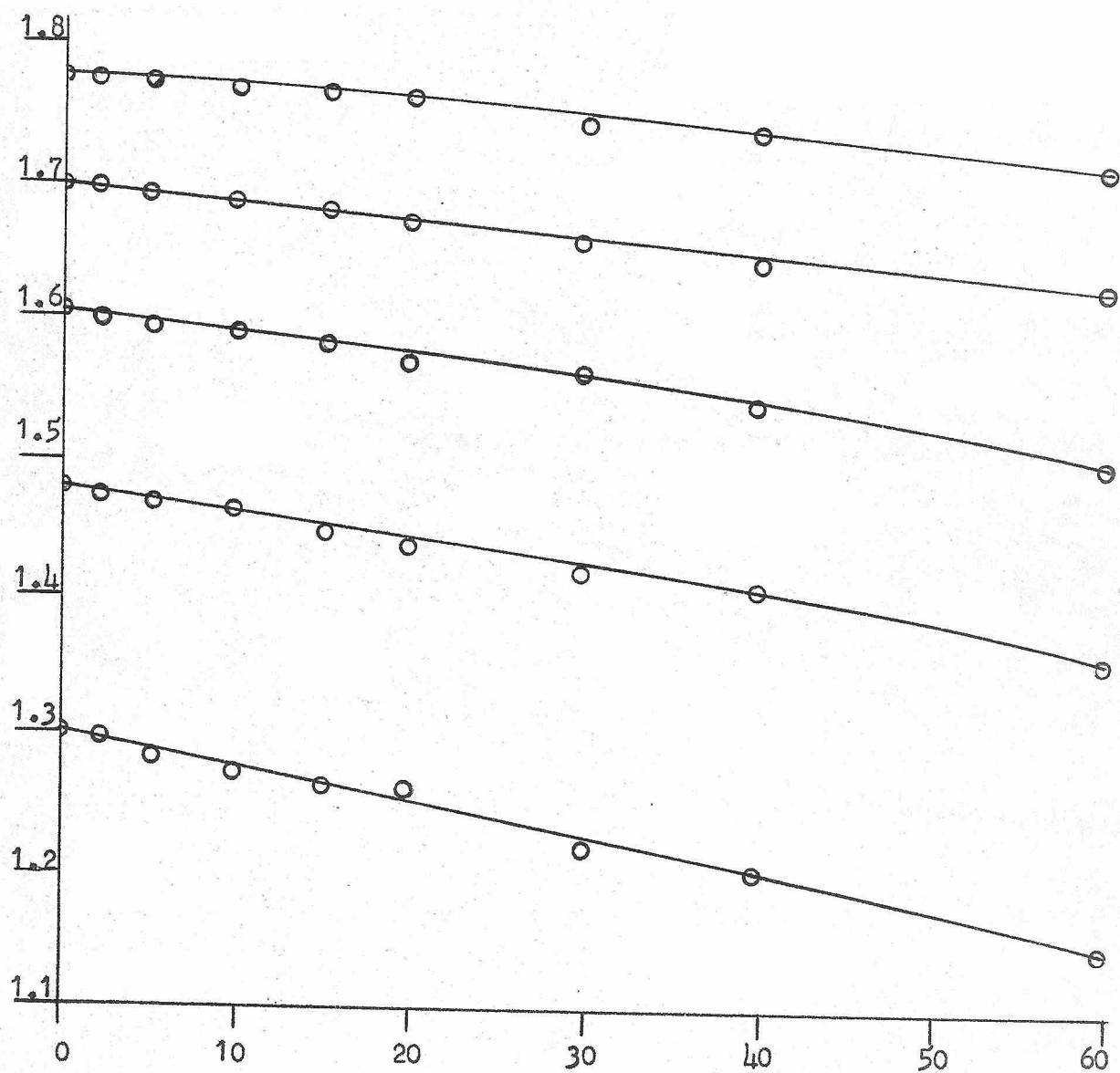
Figure 15.



Competitive chymotryptic hydrolysis of acetyl-L-tryptophanamide (50%) and acetyl-L-tyrosinamide (50%)

Ordinate and abscissa as in Figure 2.

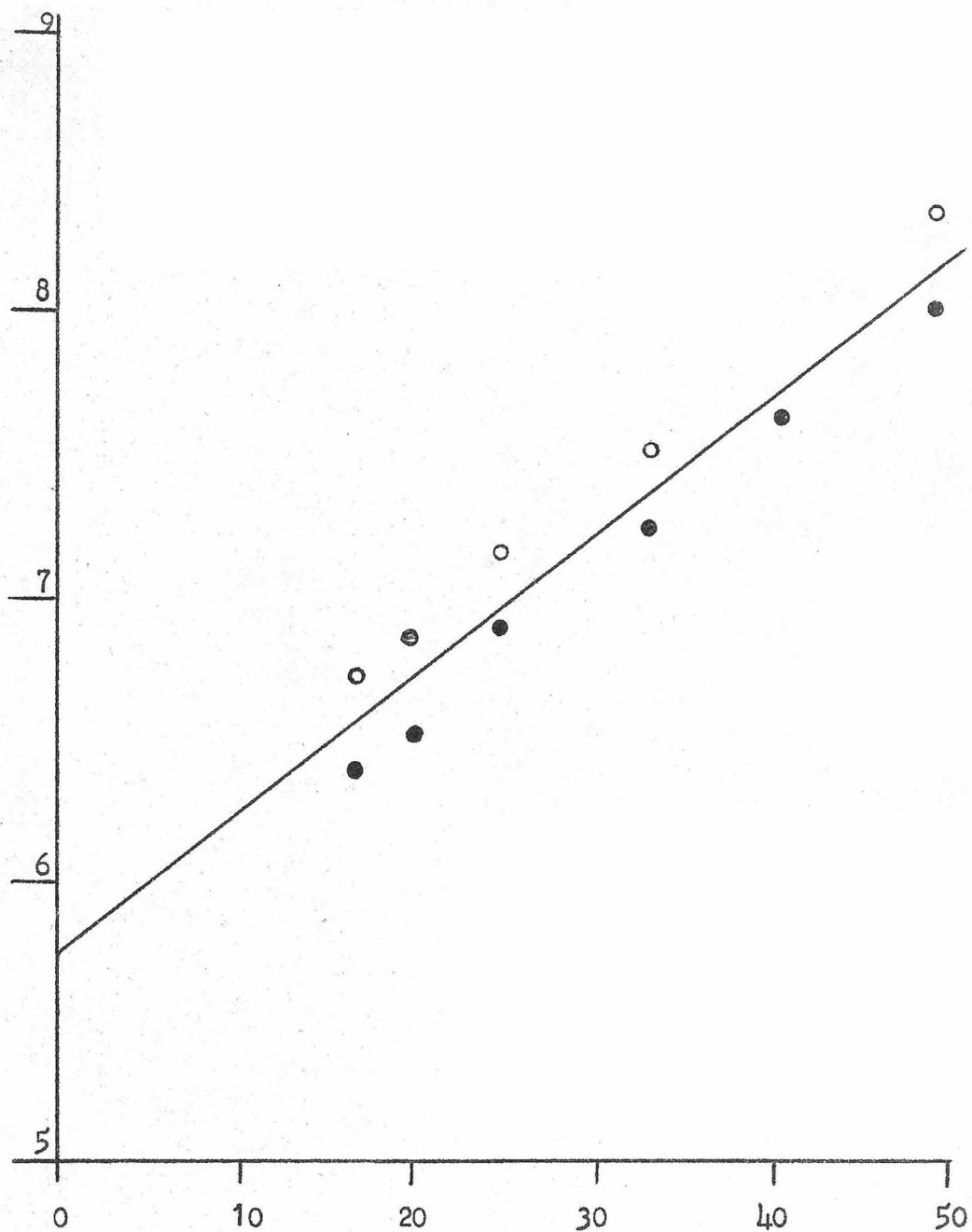
Figure 16.



Competitive chymotryptic hydrolysis of acetyl-L-tryptophanamide (50%) and acetyl-L-tyrosinamide (50%)

Ordinate and abscissa as in Figure 9.

Figure 17.



Determination of apparent kinetic constants for the competitive Chymotryptic Hydrolysis of acetyl-L-tryptophanamide (50%) and acetyl-L-tyrosinamide (50%)

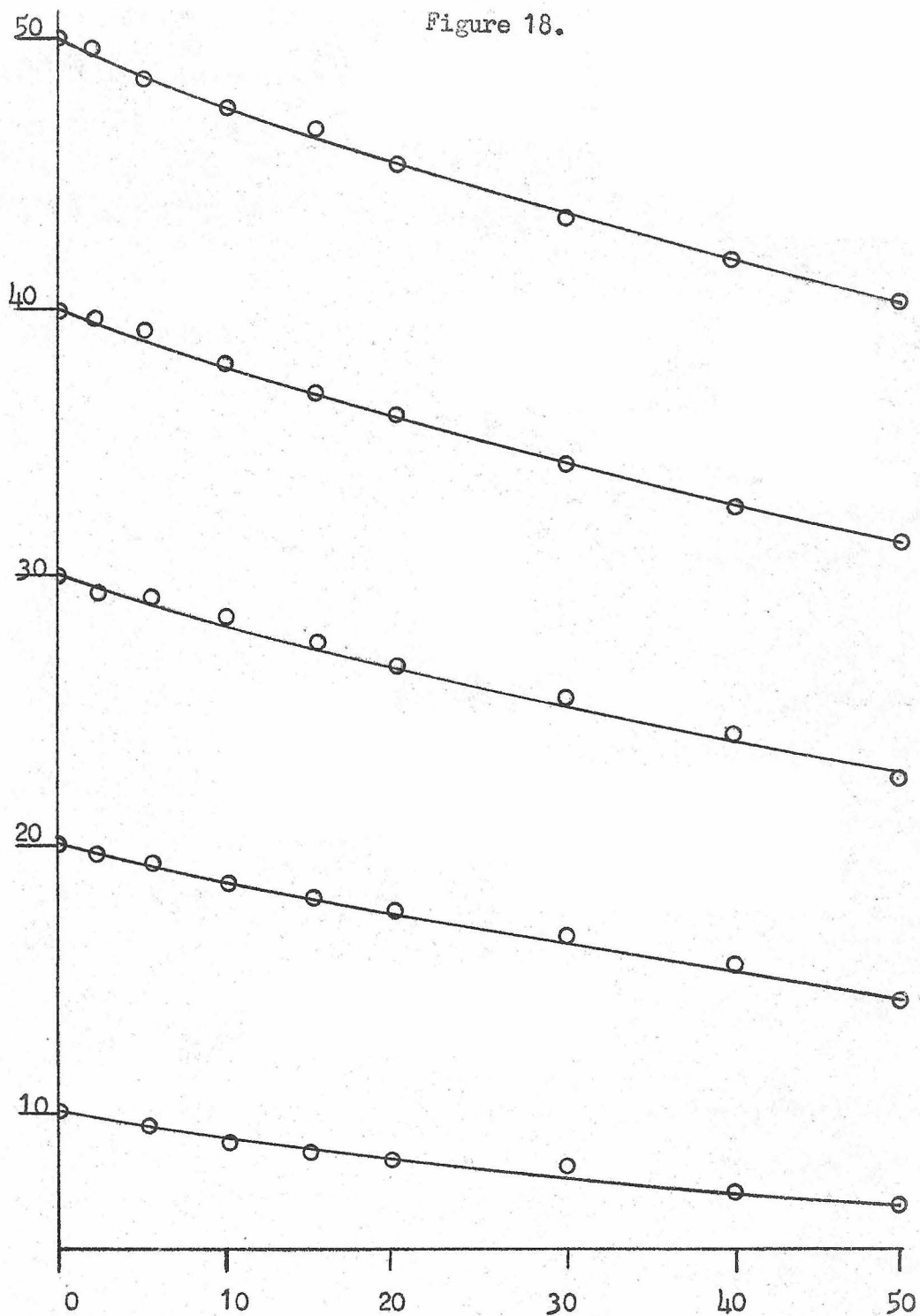
Ordinate and abscissa as in Figure 4.

Open and closed circles for two series of experiments.

$$K_{ST} = 9.1 \times 10^{-3} \text{ M}$$

$$k_3 = 0.86 \times 10^{-3} \text{ mole l}^{-1} \text{ min}^{-1} (\text{mg. prot. N ml}^{-1})^{-1}$$

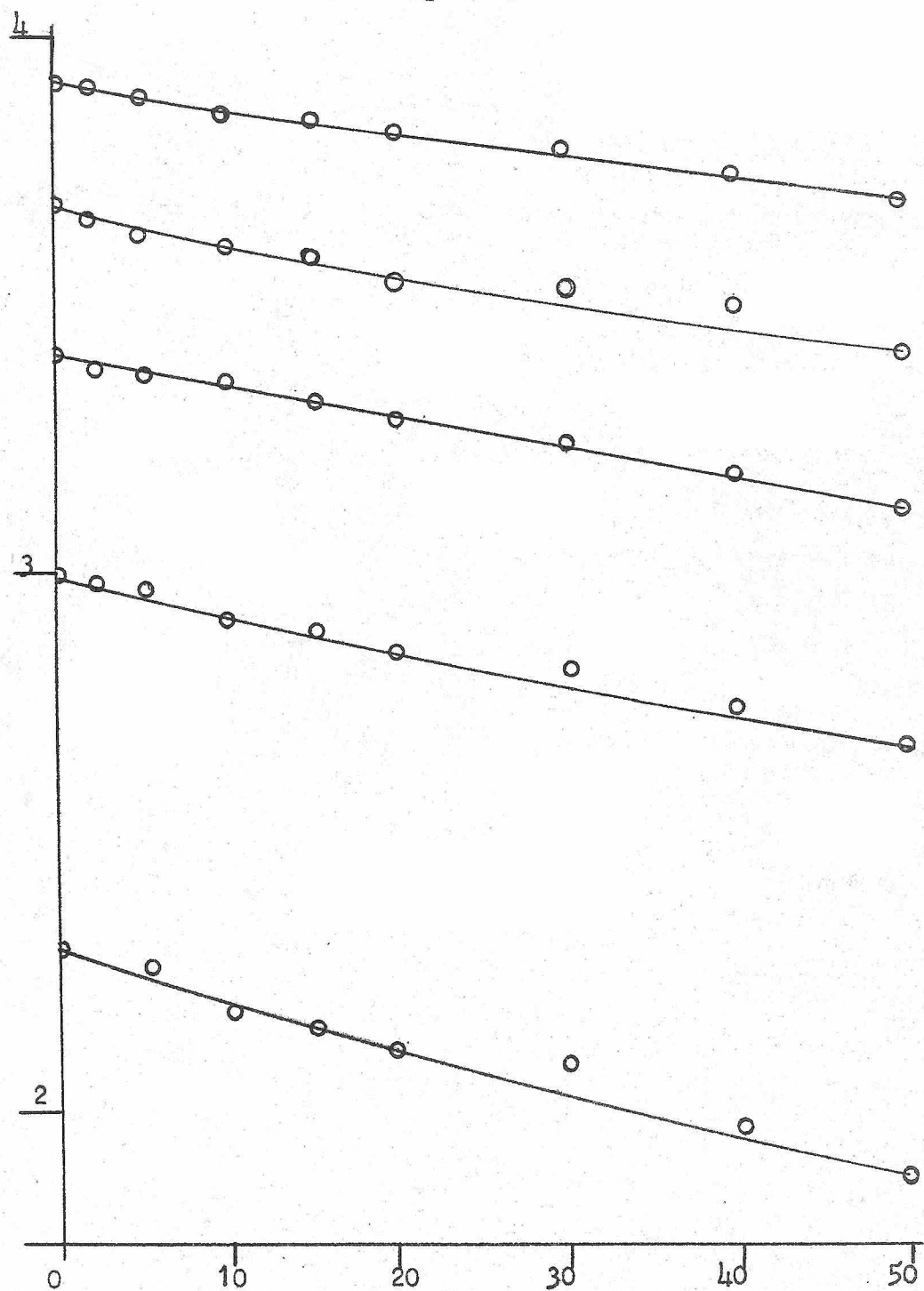
Figure 18.



Competitive chymotryptic hydrolysis of acetyl-L-phenylalaninamide (50%) and acetyl-L-tyrosinamide (50%)

Ordinate and abscissa as in Figure 2.

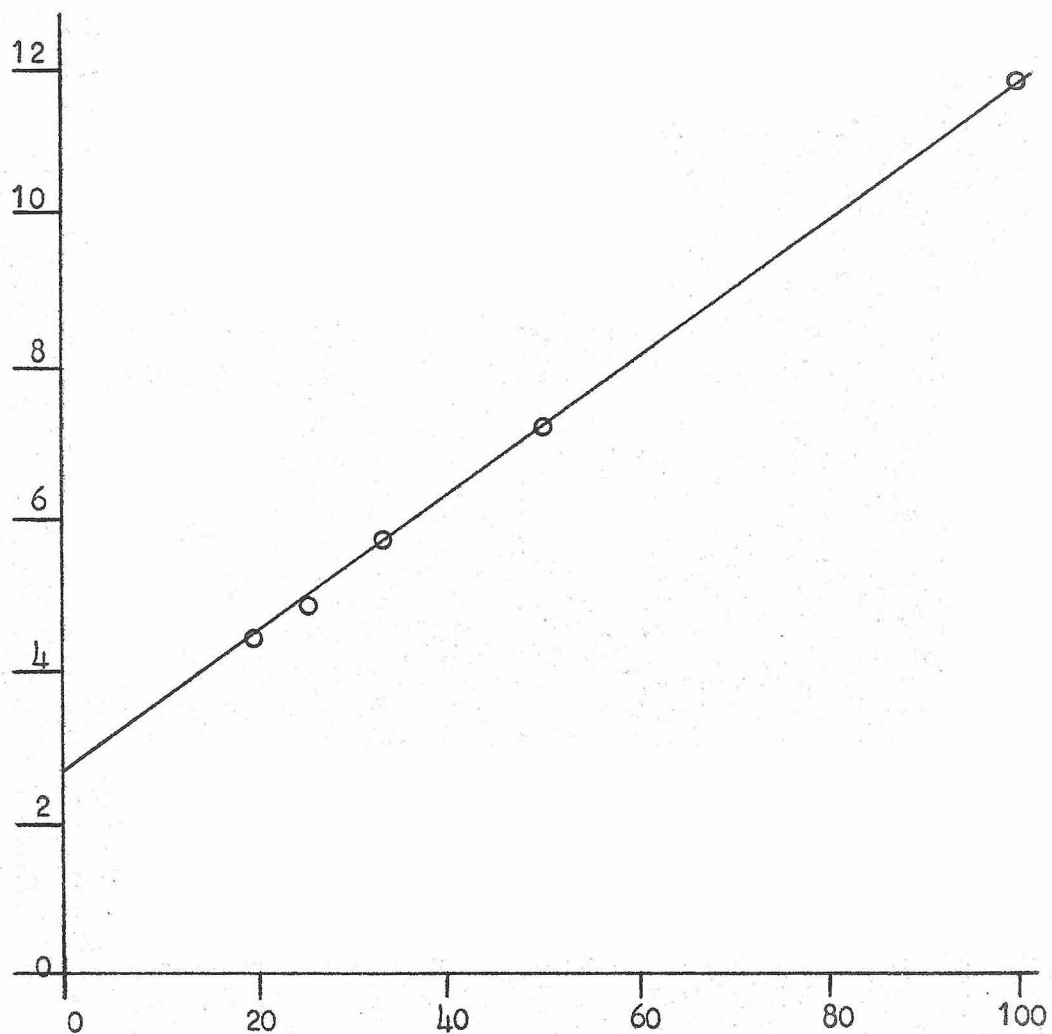
Figure 19.



Competitive chymotryptic hydrolysis of acetyl-L-phenylalaninamide (50%) and acetyl-L-tyrosinamide (50%)

Ordinate and abscissa as in Figure 3.

Figure 20.



Determination of apparent kinetic constants for the competitive chymotryptic hydrolysis of acetyl-L-phenylalaninamide (50%) and acetyl-L-tyrosinamide (50%)

Ordinate and abscissa as in Figure 4.

$$K_{S_T} = 33 \times 10^{-3} \text{ M}$$

$$k_{3_T} = 1.7 \times 10^{-3} \text{ mole l}^{-1} \text{ min}^{-1} (\text{mg. prot. N ml}^{-1})^{-1}$$

PART III
KINETIC STUDIES WITH
 α -CHYMOTRYPSIN AT pH 6.9

Introduction

The phenomenon of an optimum pH is a characteristic feature of enzyme catalyzed reactions. This is not surprising since the enzyme is a protein which contains groups whose electric charges are dependent upon the concentration of hydrogen ions. Hydrolytic reactions present still other reasons for a pH dependency since the products of the hydrolysis are usually substances whose structures are profoundly affected by the pH.

The pH optima have been used widely as a means of classifying enzymes. It must be realized however, that the optimum pH for an enzymatic reaction depends upon both the substrate and the enzyme. Thus the pH optimum for the chymotryptic digestion of insulin is 8.6⁽¹⁾, while with L-tyrosine-ethyl-ester it is 6.2⁽²⁾. It may be noted also that for the chymotryptic synthesis of the phenylhydrazide from acetyl-L-phenylalanine and phenylhydrazine the optimum pH is 5.5⁽³⁾. Most pH optima that have been obtained refer only to the overall rate of reaction. Therefore their interpretation is difficult since there is no reason to believe that the formation of the enzyme-inhibitor complex and its subsequent breakdown to free enzyme and products are both affected by pH changes to the same degree or even in the same direction.

Enzyme-inhibitor dissociation constants also are affected

by a change in the pH. This is of course to be expected since specific inhibitors of α -chymotrypsin are closely related in structure to substrates. The following classes of substances have been found to be competitive inhibitors of α -chymotrypsin:

1. Antipodes of specific substrates,
2. Other degradation and substitution products of specific substrates (including the hydrolysis products) which are not hydrolyzed by α -chymotrypsin. For example, indole, tryptamine, acetyl-tryptamine, β -indolyl-3-propionic acid and its amide, D and L-tryptophan, D and L-tryptophanamide and D and L-tryptophan-methylamide are a few of the inhibitors related to the specific substrate acetyl-L-tryptophanamide.

Certain esters of phosphoric acid inhibit α -chymotrypsin⁽⁴⁾ but this inhibition seems to be irreversible and not competitive.

Since a competitive inhibitor of α -chymotrypsin may be neutral, acidic, or basic, a study of the effect of pH on the enzyme-inhibitor complex dissociation constant for several of each charge type might reveal information as to the possibility of charges near the catalytic site of the enzyme. L-Tyrosine-hydroxamide was investigated as a specific substrate with which to investigate enzyme-inhibitor dissociation constants at a pH other than 7.9 since pH optima for α -chymotrypsin substrates had been demonstrated to depend upon the state of the α -amino-nitrogen.

Discussion of Results

It was found that the concentration of L-tyrosine-hydroxamide was proportional to the absorption of light by its complex with ferric iron. The molar extinction at the maximum, 505 m μ , is 1.05×10^3 for a solution 0.02 F in FeCl_3 , 0.028 F in HCl, 0.01 F in tris-(hydroxymethyl)-aminomethane and from 10^{-4} to 10^{-3} F in L-tyrosine-hydroxamide. The solvent was 85% methanol, 15% water. Figure 1 contains the spectrum for a solution of the above composition and Figure 2 demonstrates the linear relationship between concentration of the substrate and extinction.

As shown by Figure 3, the pH optimum for L-tyrosine-hydroxamide was found to be 6.9. At this pH the kinetic constants for the substrate are:

$$K_S = 32 \pm 2 \times 10^{-3} \text{ M},$$

$$k_3 = 3.0 \pm 0.3 \times 10^{-3} \text{ ml}^{-1} \text{ min}^{-1} (\text{mg. N})^{-1} \text{ ml}.$$

The individual hydrolyses for the determination of the kinetic constants are shown in Figures 4 and 5 and Figure 6 is the plot used to evaluate K_S and k_3 . Figure 7 shows the experimental points for many hydrolyses superimposed, with suitable translations along the time axis, on a plot of the integrated rate equation.

The dissociation constant, K_I , of the enzyme-inhibitor complex was determined for a series of compounds related to acetyl-L-tryptophanamide. Table III compares the values of K_I

and $-\Delta F$ for the enzyme-inhibitor complexes at pH 6.9 with those at pH 7.9. It is seen that the inhibitors for which there is a marked change (an increase) in affinity for the enzyme in going from pH 7.9 to pH 6.9 are the free carboxylic acids. This suggests strongly that there is a negatively charged center at or near the catalytically active site of the enzyme and that a change of one pH unit diminishes the negative charge considerably.

Experimental

L-Tyrosine-hydroxamide⁽⁵⁾ - 19.5 gm. (0.10 mole) of L-tyrosine methyl ester⁽⁶⁾ was suspended in 200 ml. of methanol and chilled to ice temperature. 7.65 gm. (0.11 mole) of hydroxylamine-hydrochloride was also placed in 200 ml. of methanol and chilled. 110 ml. of 1.0 N sodium methoxide in methanol was added to the hydroxylamine-hydrochloride solution. The free hydroxylamine solution was separated from a sodium chloride precipitate by centrifugation and added slowly to the solution of the ester. The resultant solution was allowed to stand in the cold room for three days and then concentrated by evaporation in a dry air stream. The white crystals which formed were collected, dried and recrystallized several times from water. The yield of purified product, melting at 161-162° with decomposition, was 12.5 gm. (64%). $[\alpha]_D^{25} = +63^\circ$ (C, 0.3% in water).

Analysis calculated for $C_9H_{12}O_3N_2$:

C, 55.1; H, 6.1; N, 14.3.

Found*: C, 55.1; H, 6.0; N, 14.4.

Inhibitors - With the exception of the D-tryptophan, which was prepared by Mr. Richard Bernhard, the inhibitors used in this study were prepared by Dr. H. T. Huang who also determined their kinetic constants at pH 7.9^(7,8). For the D-tryptophan, $[\alpha]_D^{25} = +32.5^\circ$ (C, 0.5 in water). Berg⁽⁹⁾ reported 32.45° .

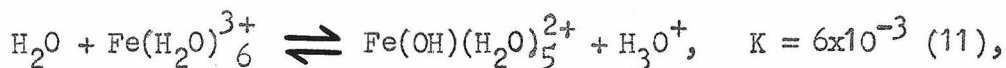
Enzyme - The enzyme preparations were exactly as described in Part II of this thesis except that the final concentration of enzyme in all experiments in this part of the thesis was 0.104 mg. prot. N/ml.

Buffers - For the determination of the various kinetic constants at pH 6.9, the final buffer concentration was 0.10 F in tris-(hydroxymethyl)-aminomethane with sufficient hydrochloric acid to give the required pH. Cacodylic acid-sodium cacodylate buffers were used for the lower pH region of the pH-activity curve and suitable tris-(hydroxymethyl)-aminomethane buffers for the higher region. The two buffers were used in an overlapping manner (hydrolyses were performed with tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffers down to pH 6.6 and with cacodylate up to pH 7.4) and, as shown by Figure 3, a smooth curve can be drawn through all of the points.

* Microanalysis by Dr. A. Elek.

Temperature - The temperature was maintained at 25.0° ± 0.1 by a "Sargent" water bath thermostat for all hydrolyses.

Analysis - The absorption of light by the complex of the hydroxamide with ferric iron was used to determine the substrate concentration at selected time intervals.⁽¹⁰⁾ Examination of the spectrum of this ferric complex in different environments reveals that it is not due simply to the hydrated $\text{Fe}(\text{RCONHOH})^{3+}$ ion. When the acid strength is high enough to suppress the hydrolysis of ferric ion, which may be expressed as:



and the anionic species is perchlorate, the absorption in the visible almost vanishes. However, if a small amount of dilute hydrochloric acid is added to a perchloric acid solution of the complex, the extinction coefficient increases markedly. This point is illustrated by Table I.

The use of alcohols for dilution to volume greatly enhances the light absorption in the visible, even in the presence of chloride ion. This is well shown in the spectra of Figure 1 and the extinction values of Table II. A series of solvents miscible with water was used to dilute solutions containing ferric ion, hydrogen ion, chloride ion and L-tyrosine-hydroxamide (duplicate solutions containing no hydroxamide were prepared, of course, for the spectrophotometer zeroing) in 50%

(by volume) aqueous methanol. There was no correlation between extinction of light at 505 m μ and dielectric constant. The presence of a free hydroxyl group however, results in maximum absorption. Table II is a tabulation of the composition of these various solutions along with the dielectric constant of the solvent and the resulting extinction values.

For the kinetic experiments, a stock solution was prepared of the following composition: 0.2 F in ferric chloride, 0.2 F in hydrochloric acid in a solution of 50% (by volume) aqueous methanol. Prior to an enzymatic hydrolysis one ml. of this solution was pipetted into each of a sufficient number of ten ml. volumetric flasks and about seven and one half ml. of methanol added. A one ml. aliquot of the hydrolytic reaction mixture was pipetted into a prepared flask and the dilution to final volume was with methanol. A standard blank was prepared with concentrations of buffer and enzyme equivalent to those in the hydrolytic system and one ml. of this was pipetted into one of the volumetric flasks containing ferric chloride in aqueous methanolic solution. When diluted to volume, this latter solution was used to zero the spectrophotometer.

In order to be sure that the acid liberated in the course of the hydrolysis did not change the extinction coefficient, sufficient acetic acid was added to a buffered solution of the substrate to simulate the hydrolysis. No change could be found

in the extinction.

All of the spectra and individual extinction values measured in this study were determined with a Beckmann Model B spectrophotometer using one centimeter matched cuvettes (both "Corex" and fused silica pairs were used).

Calculations - The initial velocities were estimated as in Part II from the zero order and first order plots of substrate concentration vs. time. The kinetic constants were also determined as in Part II from appropriate plots of initial velocity and initial substrate reciprocals using equation (10).

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Table I

Light Extinction of Ferric-Hydroxamide Complex
in Various Solutions

Solution ^a	Optical Density for one cm. path at 505 m μ
8 ml. of water	0.176
8 ml. of methanol	0.790
7 ml. of water, 1 ml. 6F perchloric acid	0.050
7 ml. of methanol, 1 ml. 6 F perchloric acid	0.470
7 ml. of water, 1 ml. 0.6 F hydrochloric acid	0.508
7 ml. of methanol, 1 ml. 1 N ammonia	0.800

a. Total solution 10 ml. consisting of 1 ml. of Fe^3 , 0.2 F, perchloric acid, 2 F, and 1 ml. of L-tyrosine-hydroxamide solution, 10^{-2} F. Blank for spectrophotometer same as above except concentration of hydroxamide is zero. Remaining 8 ml. of solution as in table.

Table II

Effect of Solvent upon Light

Extinction of Ferric-Hydroxamide Complex

Solvent ^a	Dielectric Constant ^b	Optical Density for one cm. path at 505 m μ ^c
Formamide	84	0.770
Water	80	0.656
Ethylene Glycol	41	0.818
Methanol	34	0.970
Ethanol	26	0.990
Isopropanol	26	0.980
Propanol	22	0.985
Acetone	21	0.353
Tertiary Butanol	11	0.988
Ethylene Glycol-Mono- Benzyl-Ether		0.818
1, 4, Dioxane		0.485

a. Arranged in approximate order of decreasing dielectric constant. b. I.C.T. values. c. One ml. of a solution 0.2 F in FeCl_3 , 0.2 F in HCl in 50% aqueous methanol, one ml. of an L-tyrosine-hydroxamide solution 10^{-2} F (concentration zero for blank), plus solvent as indicated to ten ml.

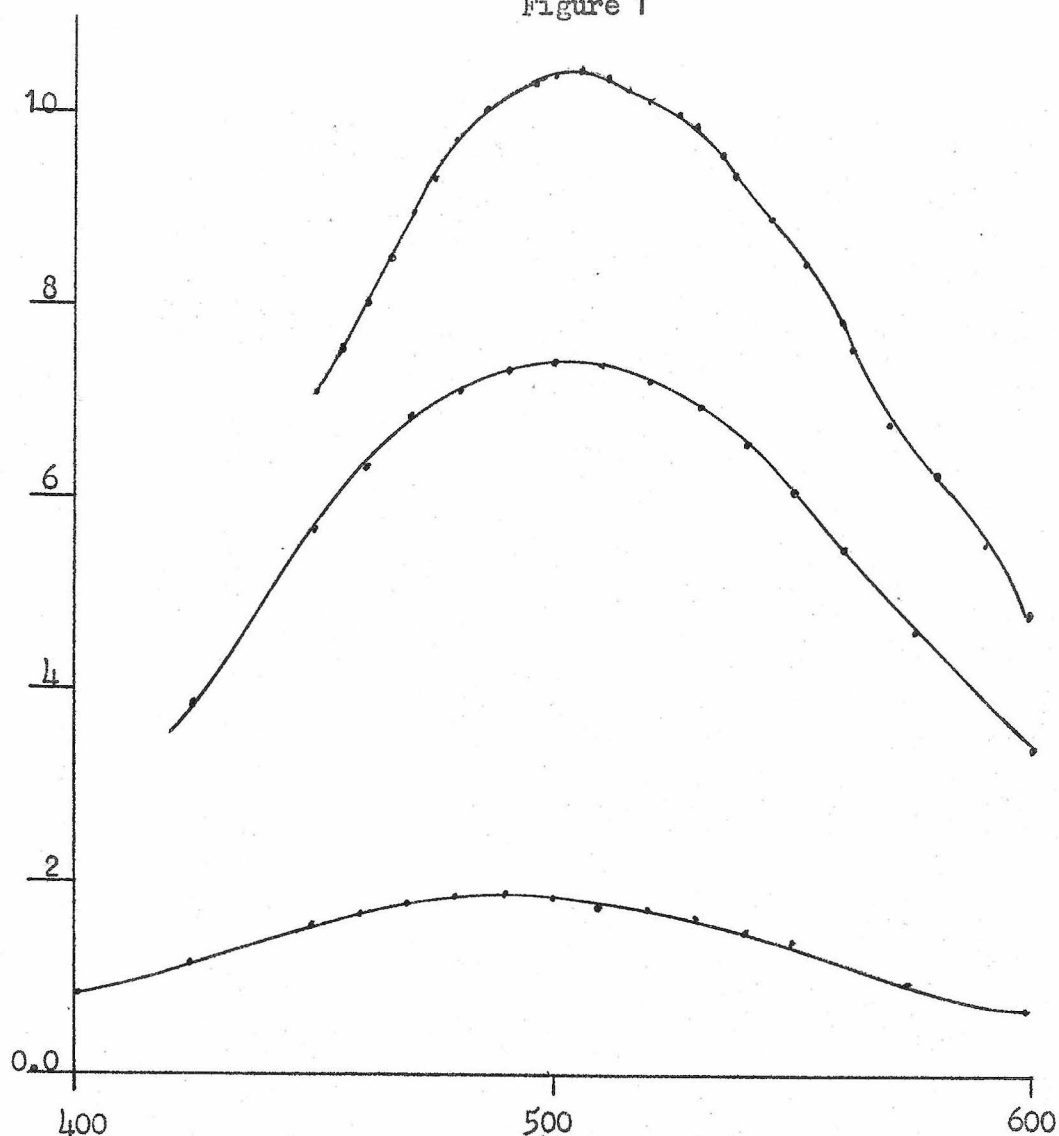
Table III

Change of Enzyme-Inhibitor Constants
With pH Change

Inhibitor	K_I		$-\Delta F^a$		d ΔF^b
	pH7.9	pH6.9	pH7.9	pH6.9	
Acetyl-D-Tryptophanamide	2.7	2.0	3.50	3.67	170
Acetyl-D-Phenylalaninamide	14.0	11.	2.53	2.66	130
D-Tryptophanamide	3.2	5.0	3.39	3.14	-250
Tryptamine	2.5	2.2	3.54	3.62	80
Acetyl-D-Tryptophan	4.8	1.1	3.15	4.02	870
Acetyl-L-Tryptophan	17.5	2.0	2.39	3.67	1280
Indolyl-3-propionic acid	13.	1.2	2.56	3.98	1420
D-Tryptophan		6.0		3.02	

a. In kilocalories per mole. b. In calories per mole with a positive sign indicating greater affinity at pH 6.9.

Figure 1



Spectra of ferric-hydroxamide complex
in several environments

Ordinate: Extinction values for a 10 mm. light path and a concentration of L-tyrosine-hydroxamide of 10^{-3} F.

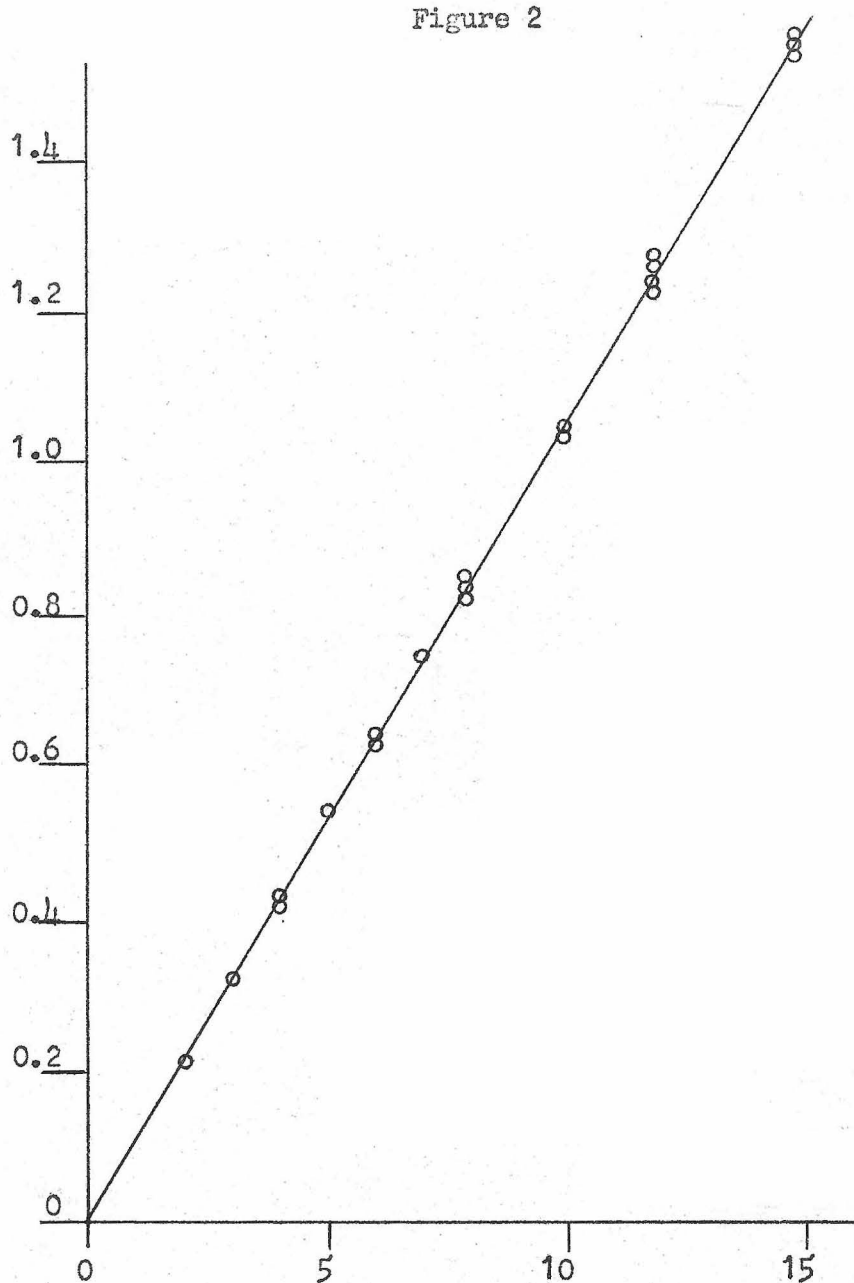
Abscissa: Wave length in $m\mu$.

Upper spectrum: $FeCl_3$, 2×10^{-2} F; HCl , 2×10^{-2} F; tris-(hydroxymethyl)-aminomethane, 10^{-2} F; in 50% aqueous methanol.

Middle spectrum: Fe^{III} , 2×10^{-2} F; $HClO_4$, 2×10^{-1} F; in 85% methanol.

Lower spectrum: Concentration of all species the same in a completely aqueous system.

Figure 2

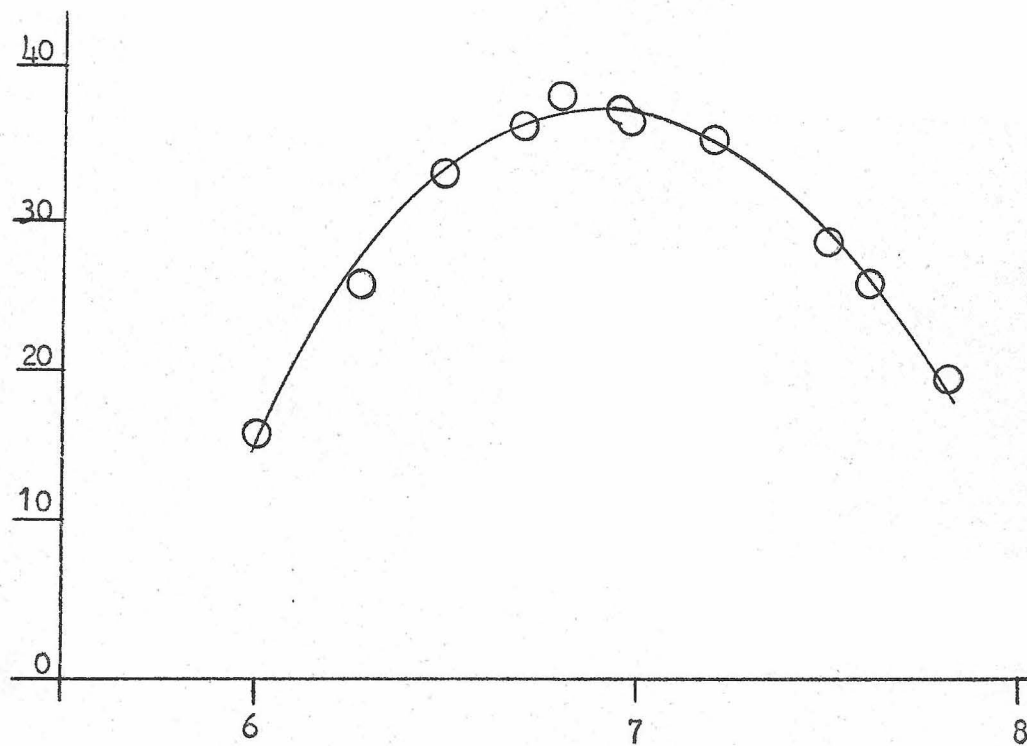


Linear dependence of extinction upon concentration

Ordinate: Extinction in a 10 mm. light path of light of wave length $505\text{ m}\mu$.

Ordinate: Concentration of L-tyrosine-hydroxamide, in reaction mixtures before 10 fold dilution and addition of ferric chloride solution, in units of 10^{-3} M. Concentrations of other constituents as in upper spectrum of figure 1.

Figure 3

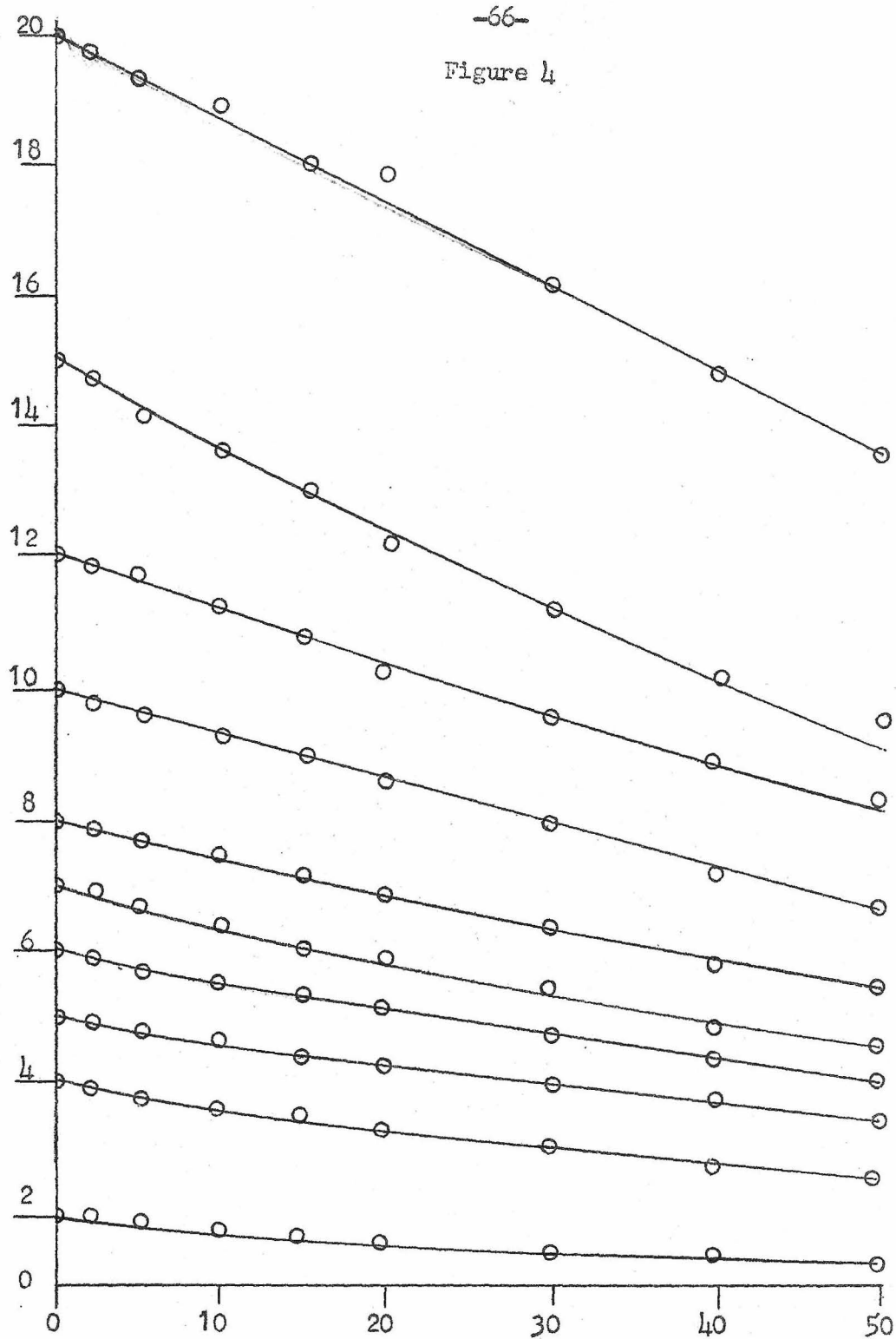


Dependence of activity upon pH

Ordinate: % hydrolysis in one hour of a solution 5×10^{-3} F in L-tyrosine-hydroxamide and with an enconcentration of 0.104 mg. prot. N/ml.

Abscissa: pH of reaction mixture.

Figure 4



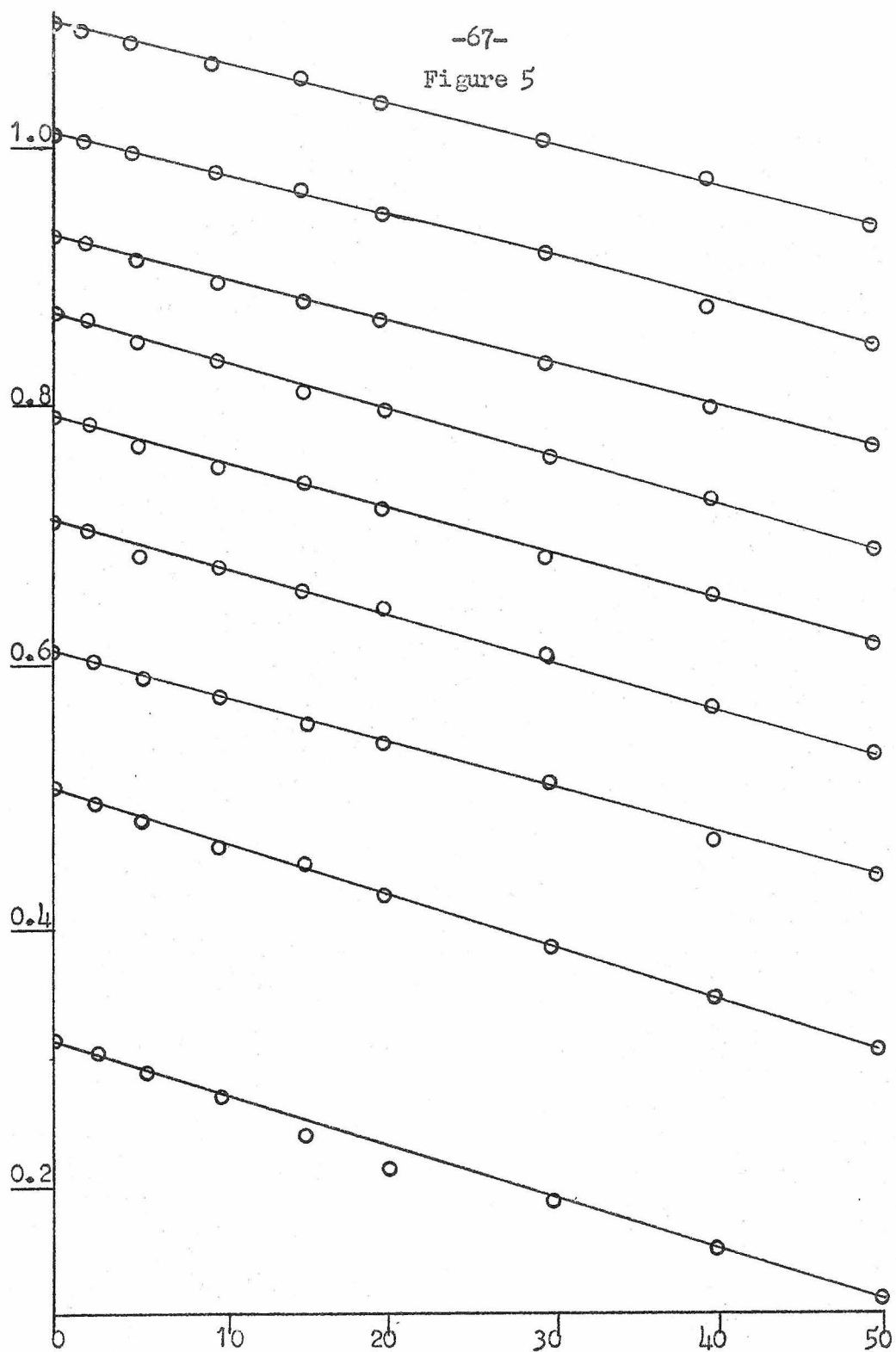
Chymotryptic hydrolysis of L-tyrosine-hydroxamide

Ordinate: Substrate concentration in units of 10^{-3} M.

Abscissa: Time in minutes.

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Figure 5

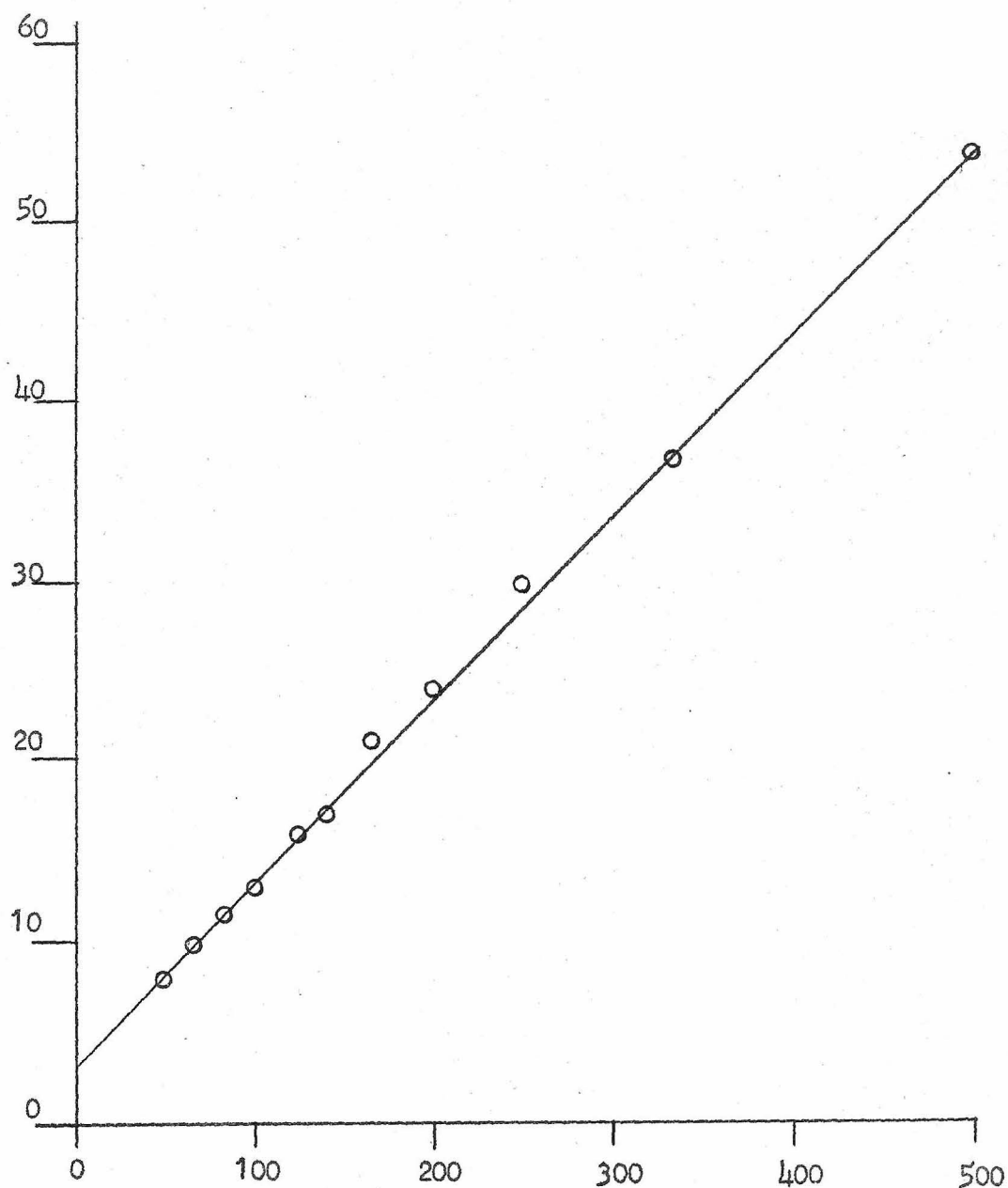


Chymotryptic hydrolysis of L-tyrosine-hydroxamide

Ordinate: Log base 10 of substrate concentration in units of 10^{-3} M.

Abscissa: Time in minutes.

Figure 6



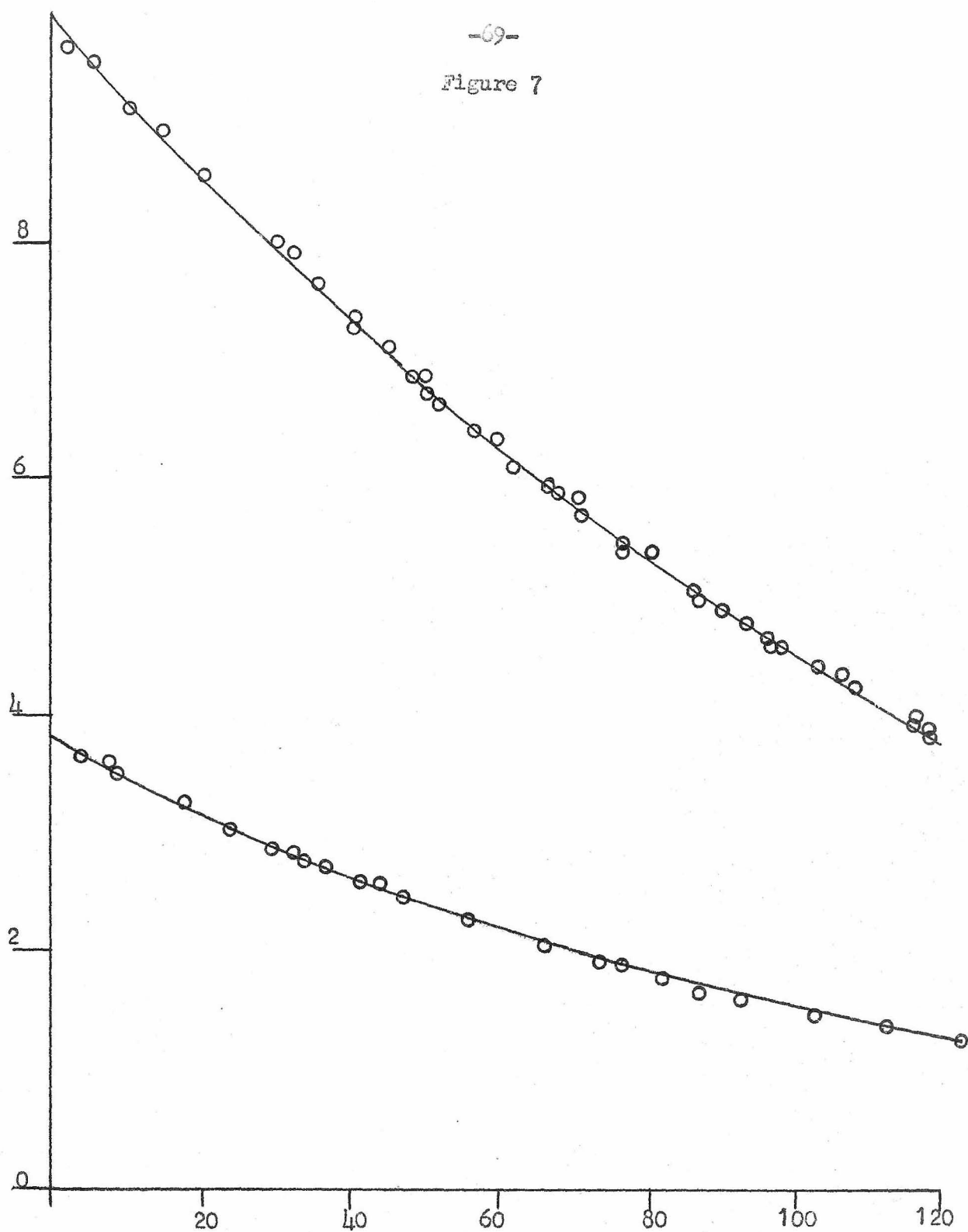
Determination of the kinetic constants for the chymotryptic hydrolysis of L-tyrosine-hydroxamide

Ordinate: Initial velocity reciprocal in units of 10^{-3} min. l./mole.

Abscissa: Reciprocal of initial substrate concentration in units of l./mole.

$$K_S = 32 \times 10^{-3} \text{ M}, k_3 = 3.0 \times 10^{-3} \text{ mole l}^{-1} \text{ min}^{-1} (\text{mg. prot. N ml}^{-1})^{-1}$$

Figure 7

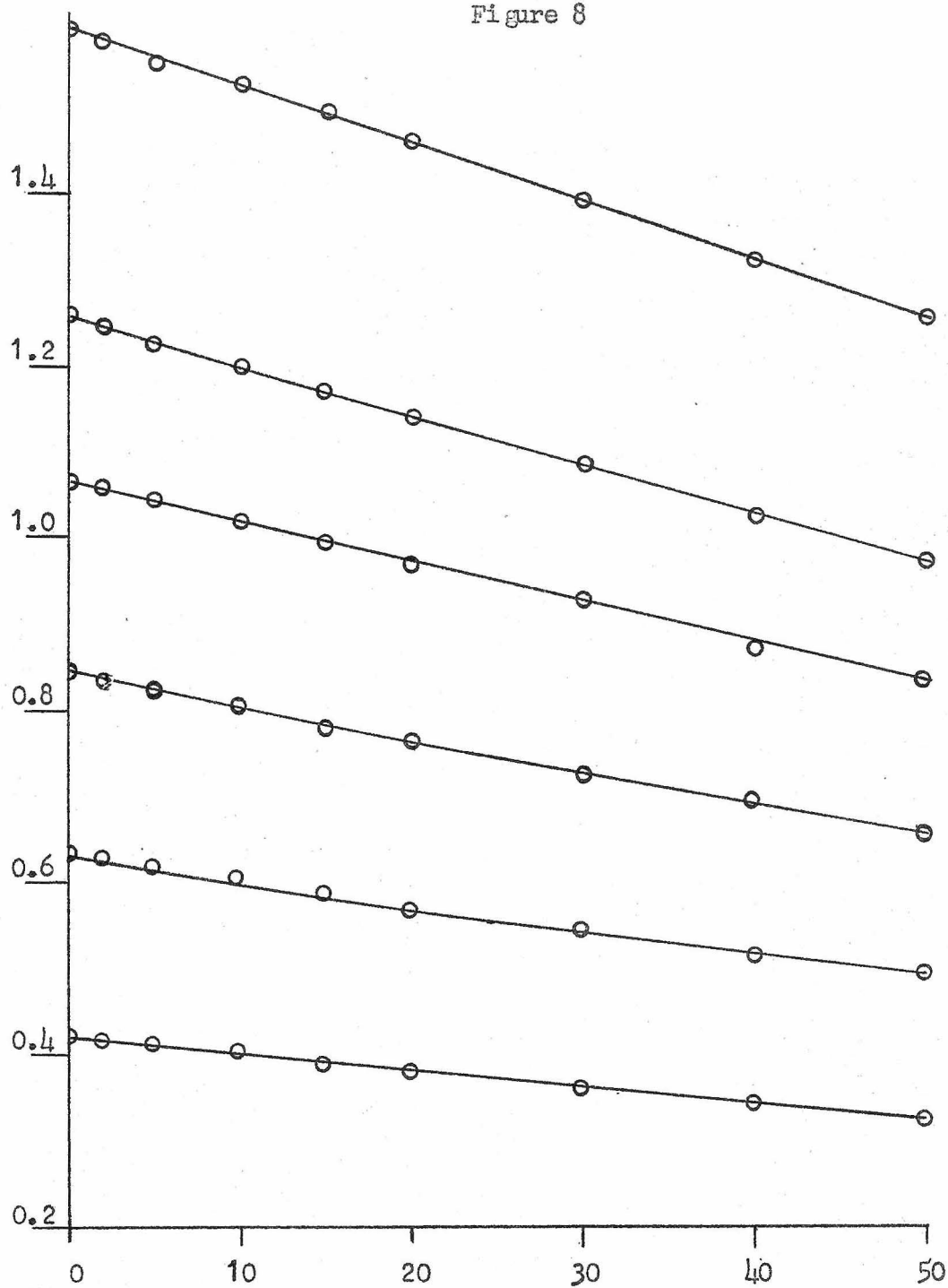


Experimental points and theoretical curve.

Curve: A plot of equation (11) part II with constants as in figure 6, Part III.

Points: From figure 4. Ordinate and abscissa as in figure 4.

Figure 8



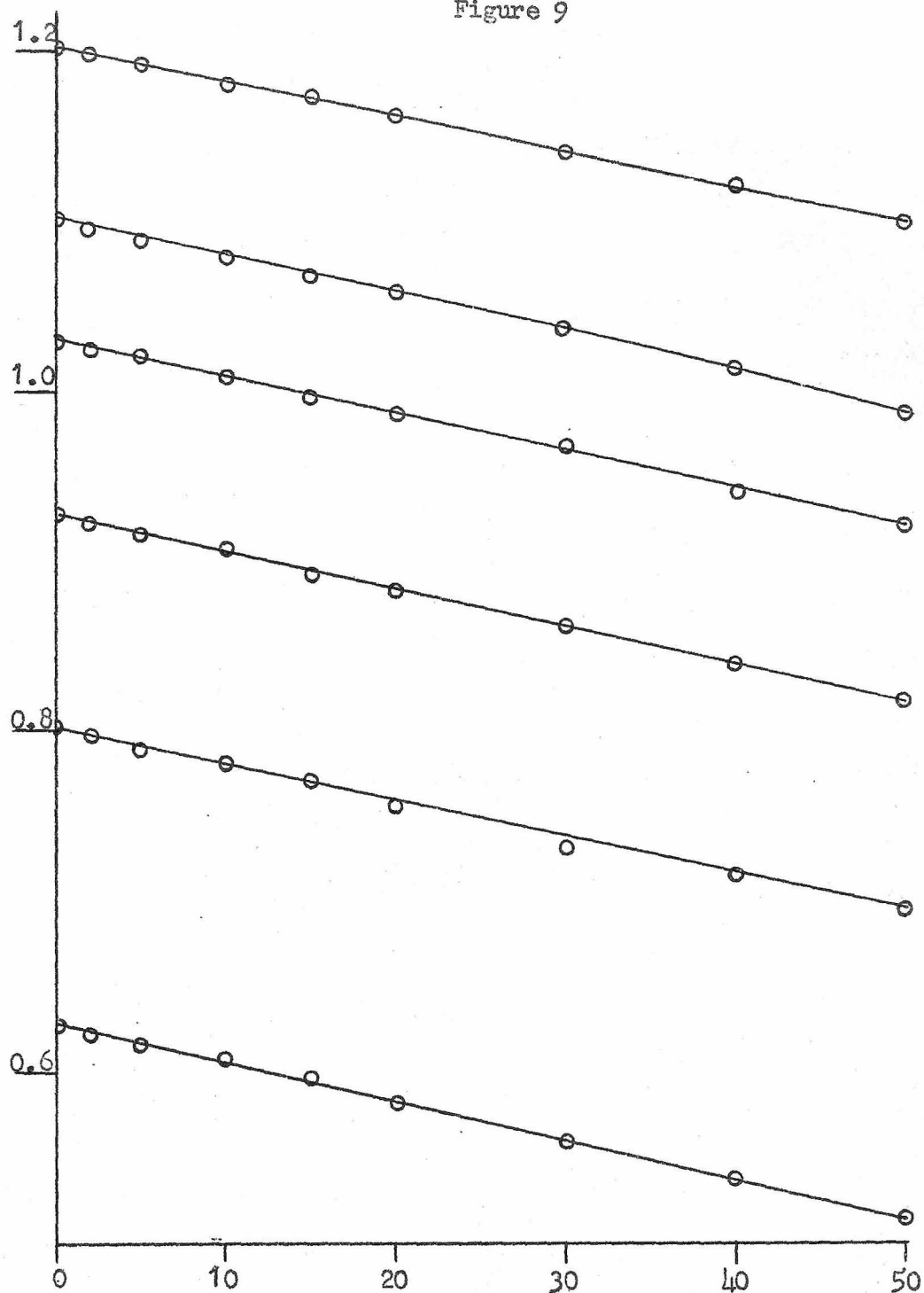
Inhibition by acetyl-D-tryptophanamide of the chymotryptic hydrolysis of L-tyrosine-hydroxamide

Ordinate: Extinction values.

Abscissa: Time in minutes.

Inhibitor concentration: 1.5×10^{-3} M.

Figure 9



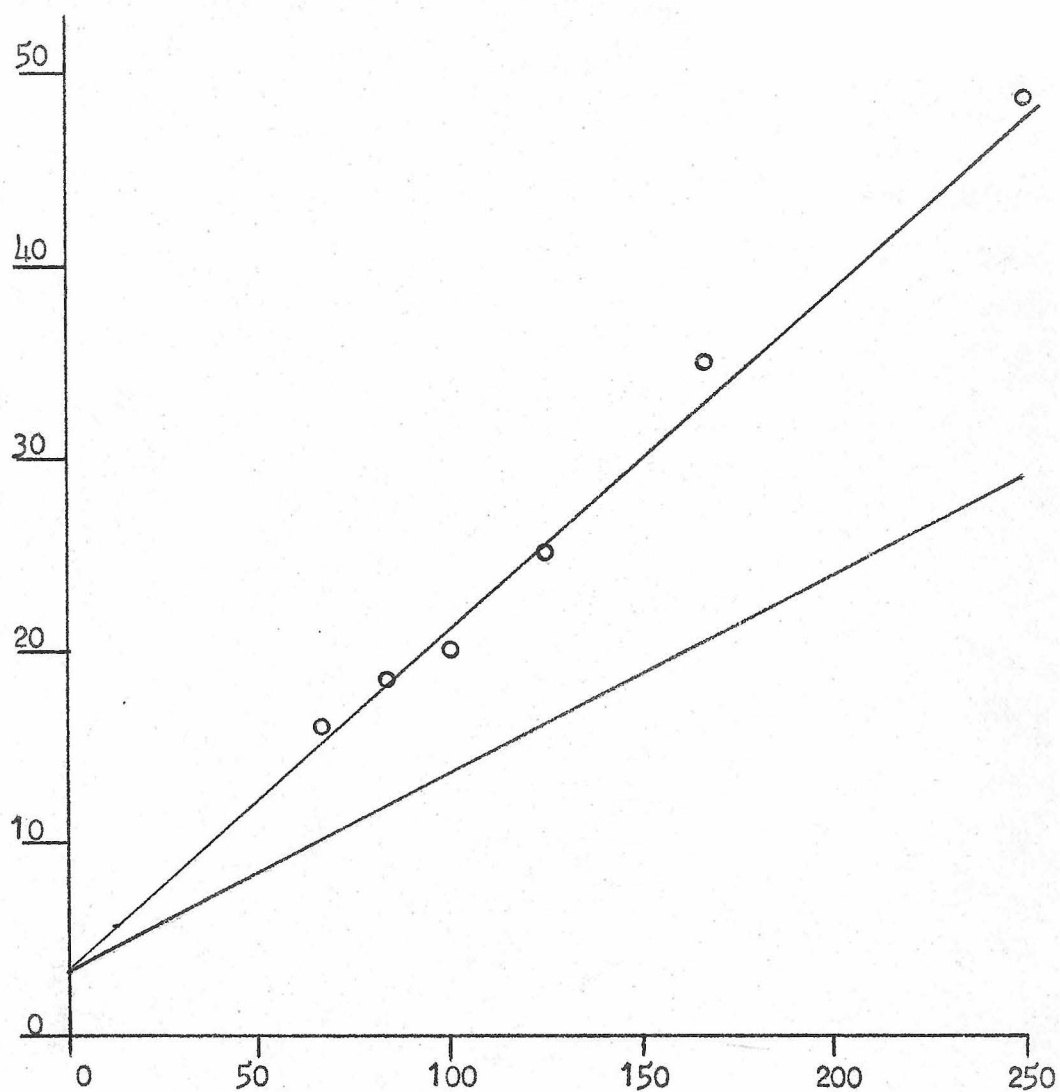
Inhibition by acetyl-D-tryptophanamide of the chymotryptic hydrolysis of L-tyrosine-hydroxamide

Ordinate: Log base 10 of extinction values x10.

Abscissa: Time in minutes.

Inhibitor concentration: 1.5×10^{-3} M.

Figure 10

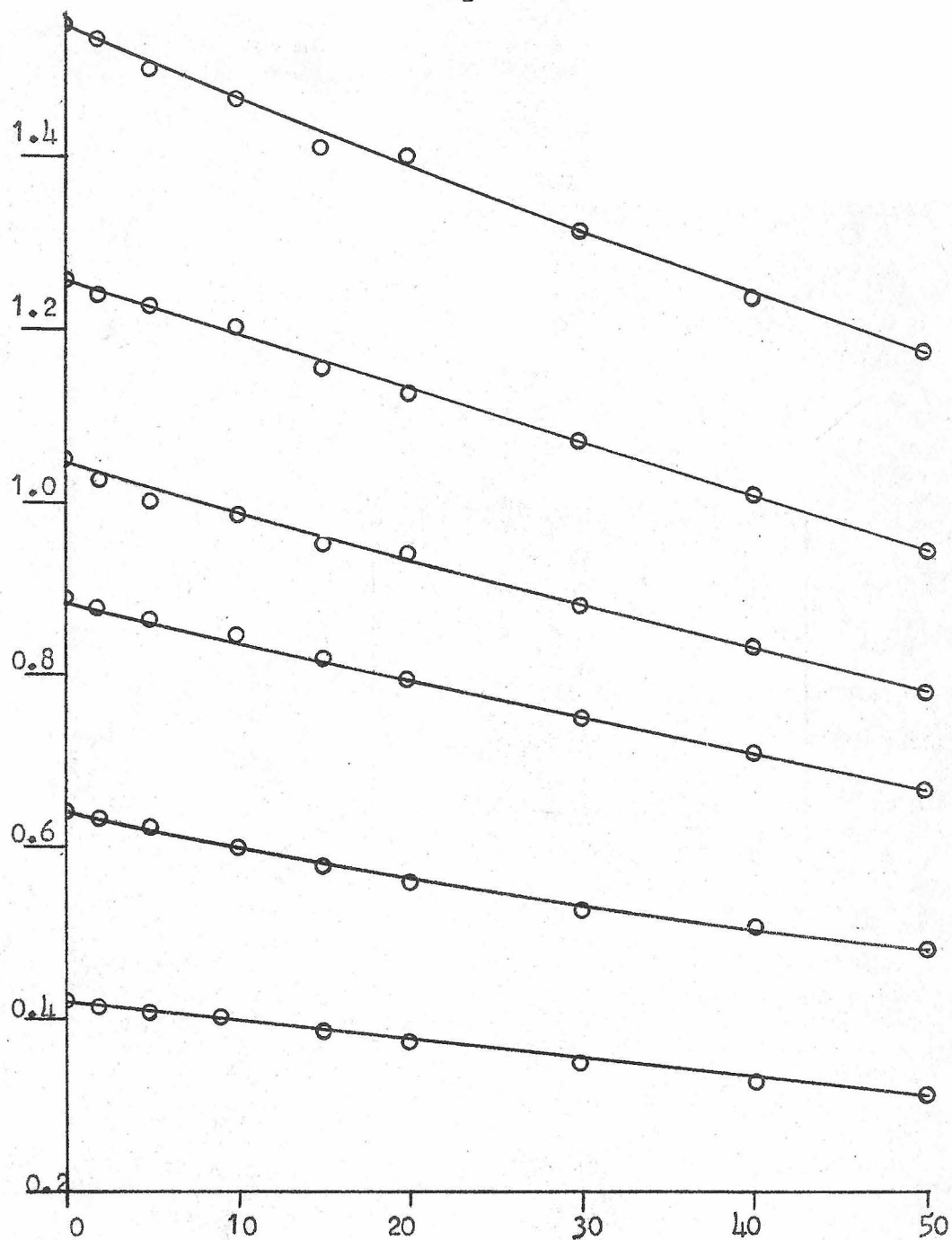


Determination of K_I for acetyl-D-tryptophanamide

Ordinate and abscissa as in figure 6.

$$K_I = 2.0 \times 10^{-3} \text{ M.}$$

Figure 11

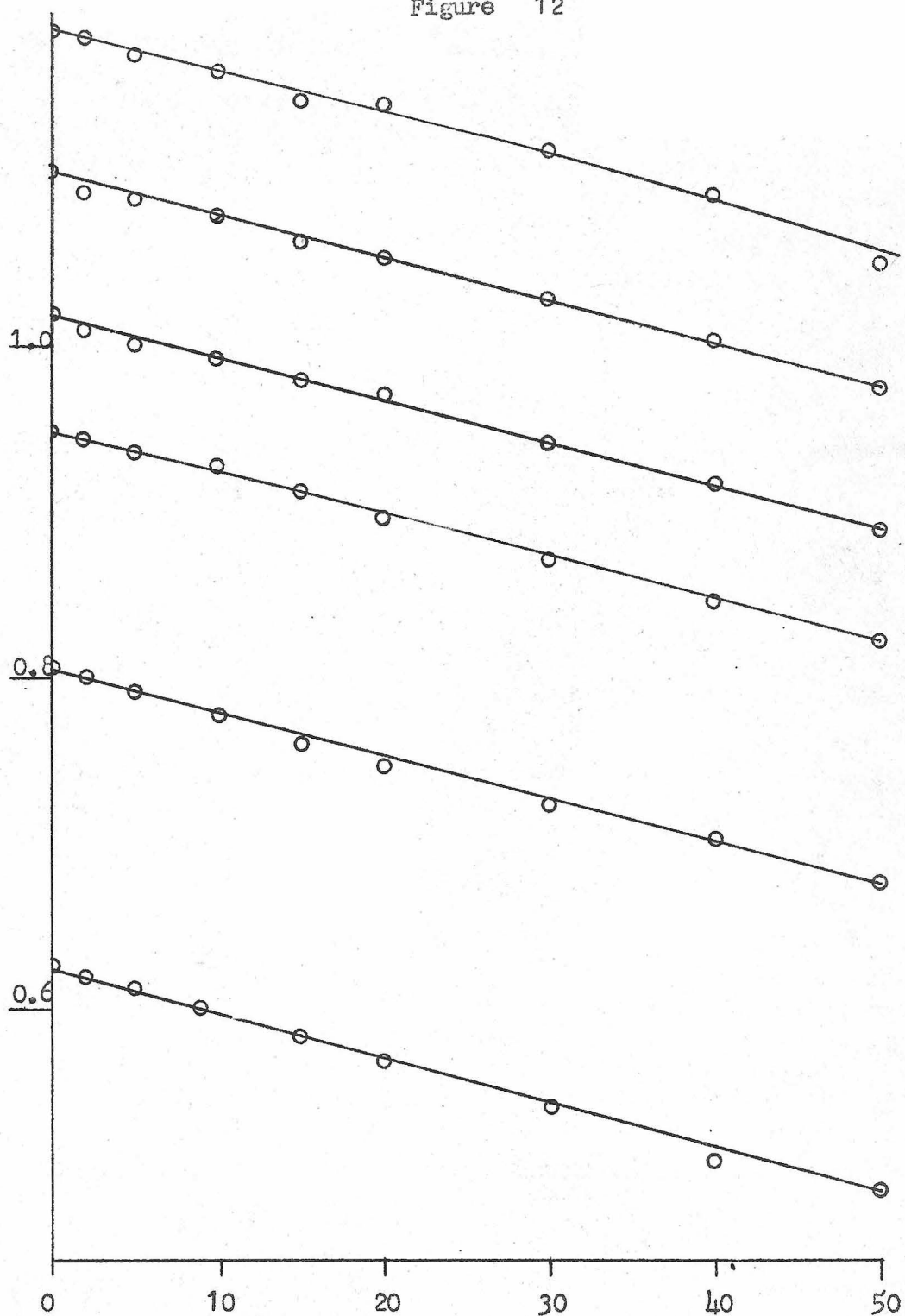


Inhibition by acetyl-D-phenylalaninamide of the chymotryptic hydrolysis of L-tyrosine-hydroxamide

Ordinate and abscissa as in figure 8.

Inhibitor concentration: 5.0×10^{-3} M.

Figure 12

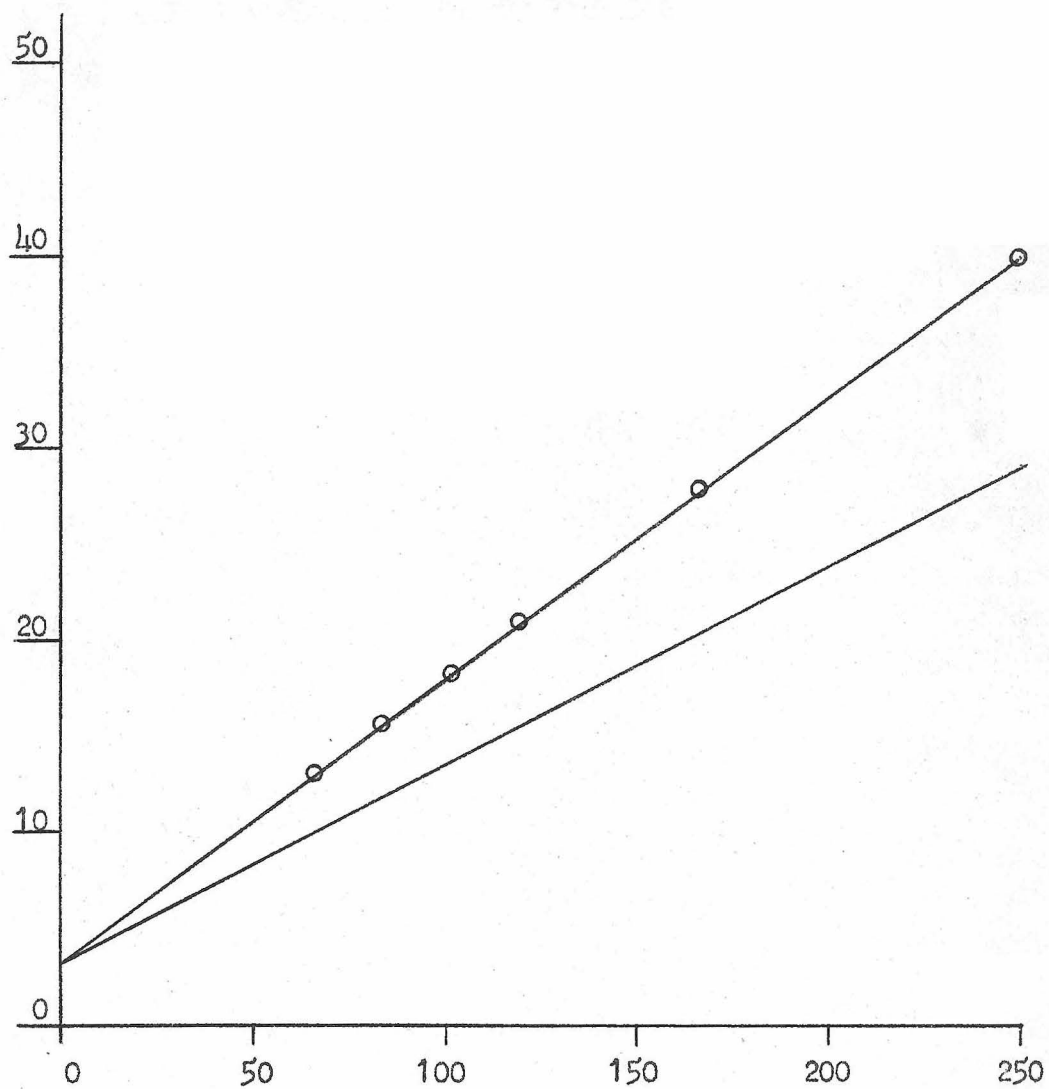


Inhibition by acetyl-D-phenylalaninamide of the chymotryptic hydrolysis of L-tyrosine-hydroxamide

Ordinate and abscissa as in figure 9.

Inhibitor concentration: 5.0×10^{-3} M.

Figure 13

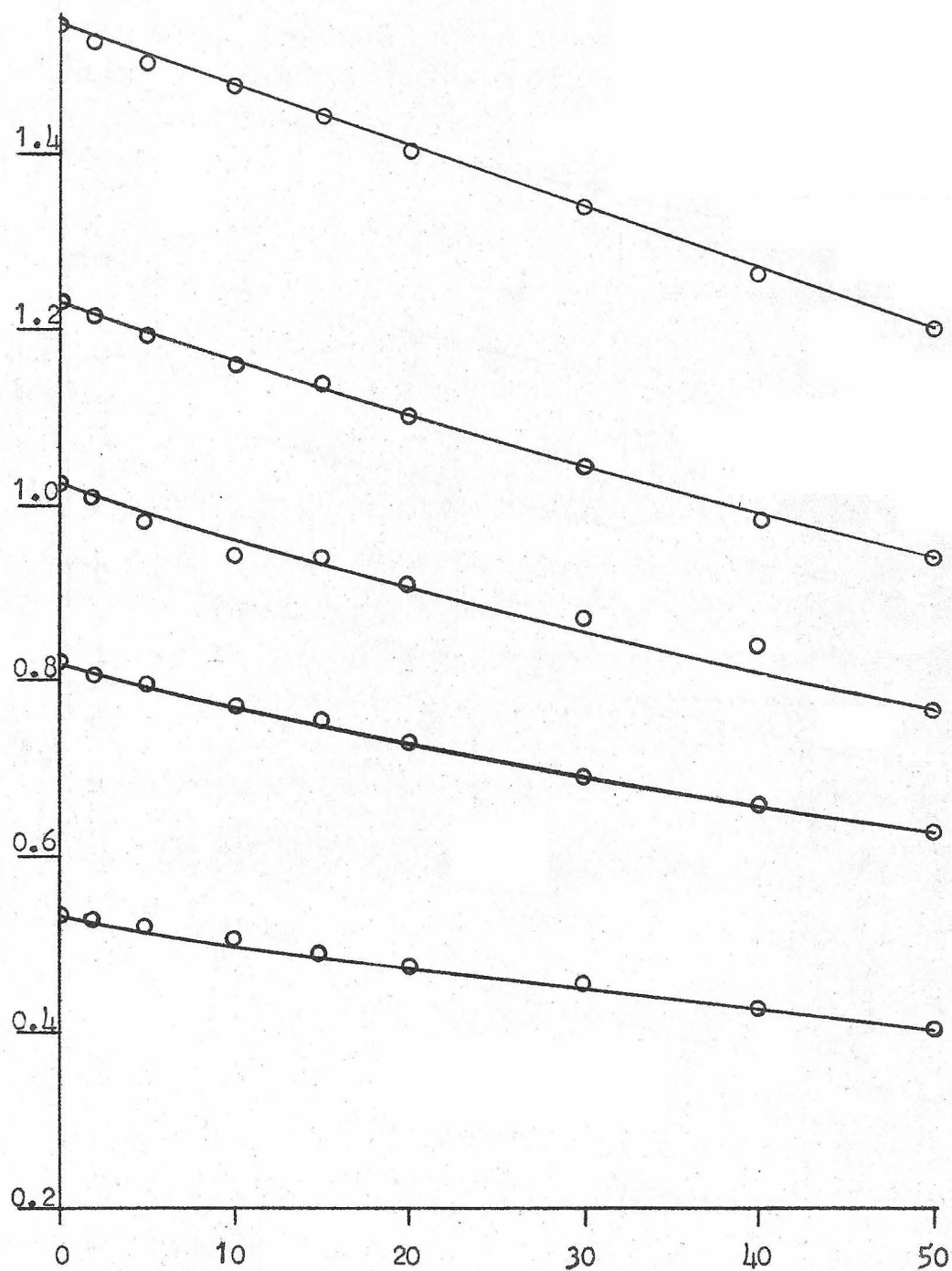


Determination of K_I for acetyl-D-phenylalaninamide

Ordinate and abscissa as in figure 6.

$$K_I = 11 \cdot 10^{-3} \text{ M.}$$

Figure 14

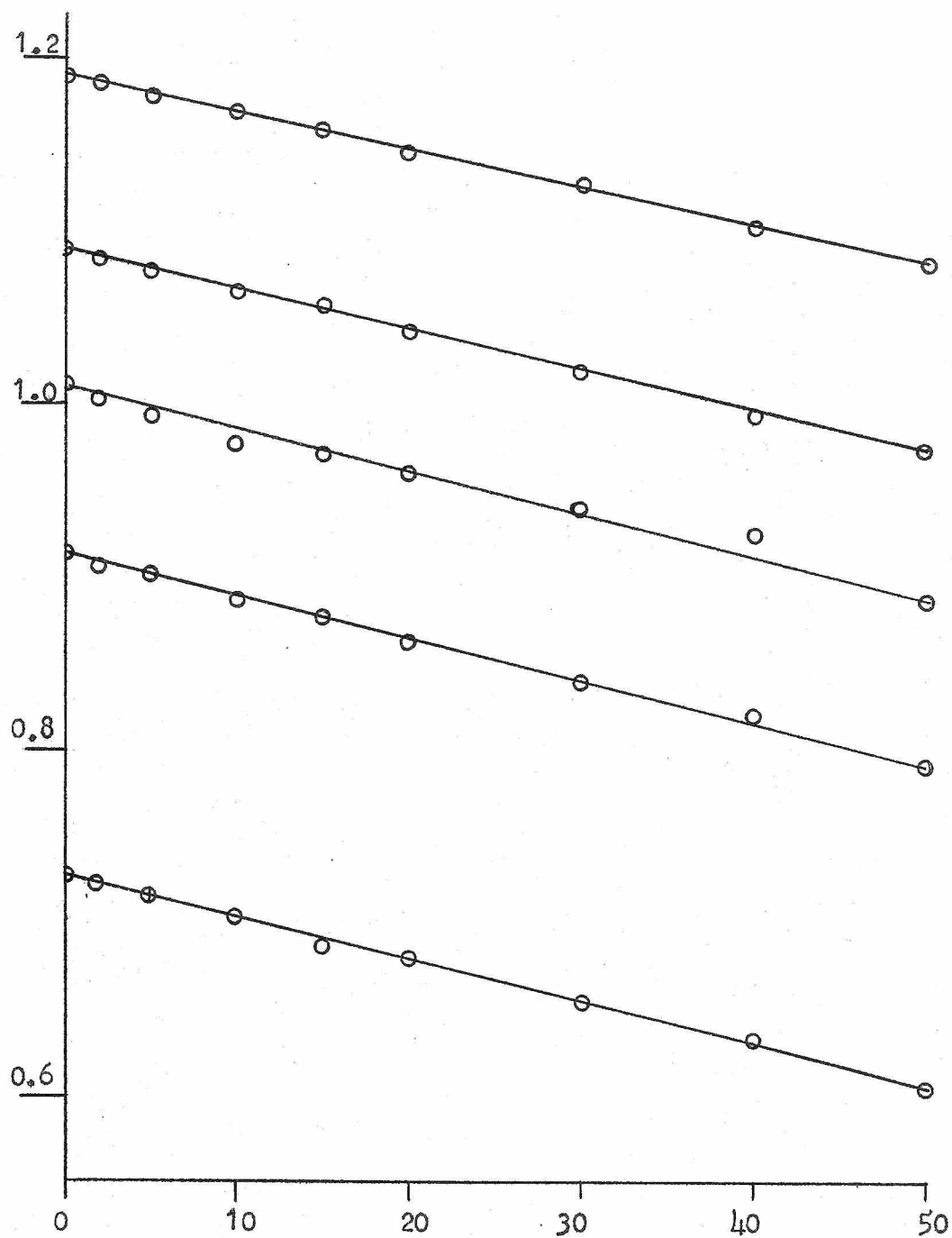


Inhibition by D-tryptophanamide of the chymotryptic hydrolysis of L-tyrosine-hydroxamide

Ordinate and abscissa as in figure 8.

Inhibitor concentration; 3.0×10^{-3} M.

Figure 15

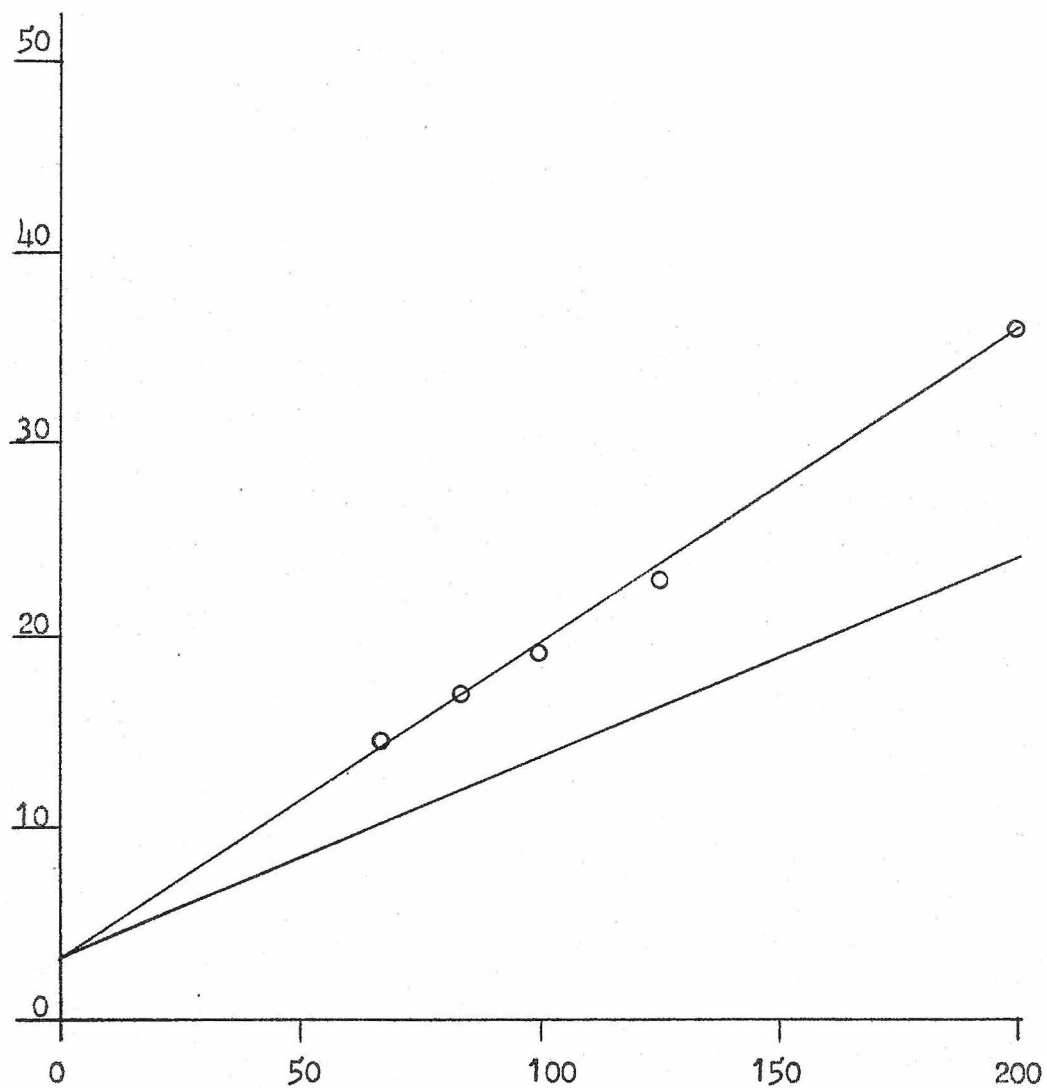


Inhibition by D-tryptophanamide of the chymotryptic hydrolysis of L-tyrosine-hydroxamide

Ordinate and abscissa as in figure 9.

Inhibitor concentration: 3.0×10^{-3} M

Figure 16

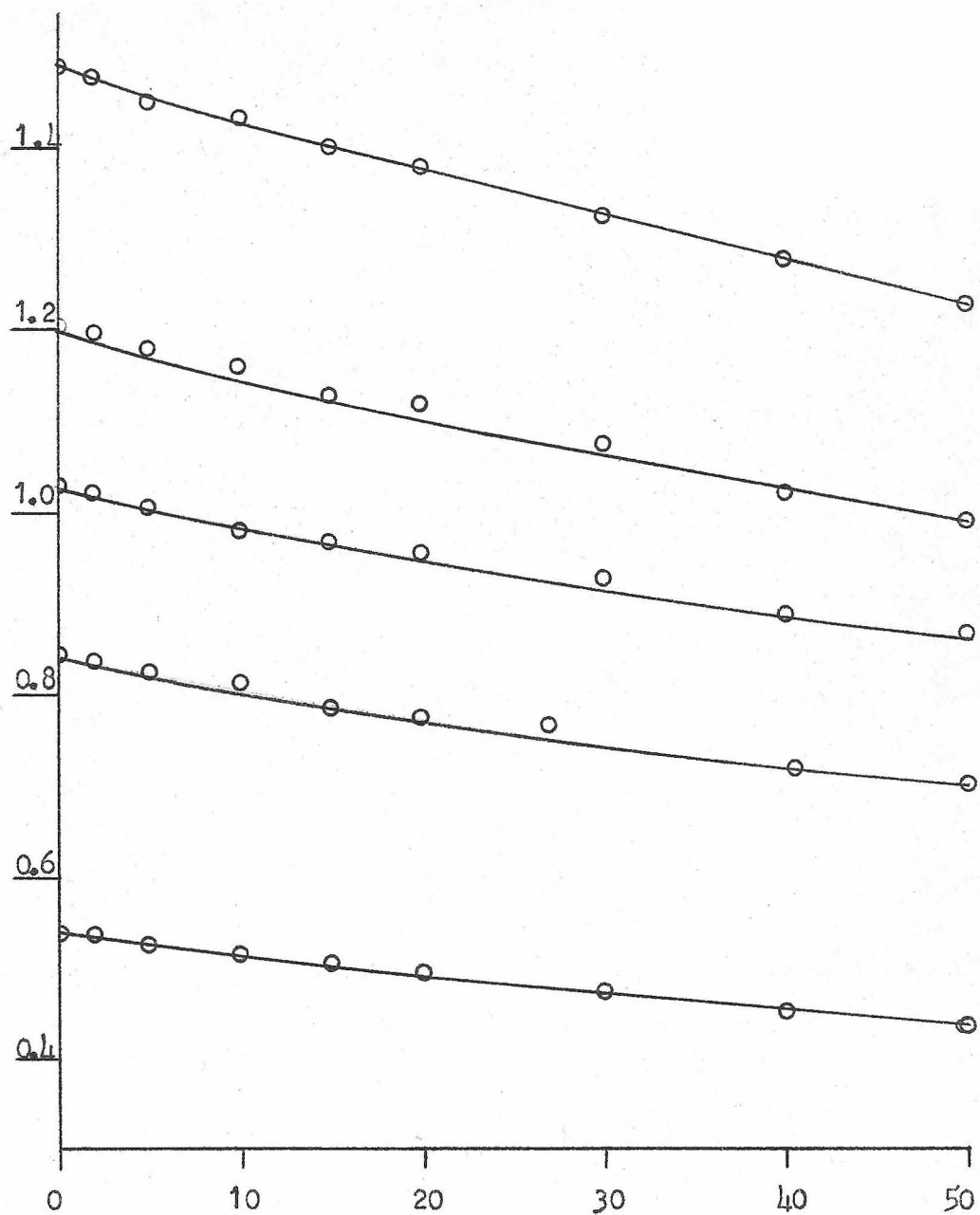


Determination of K_I for D-tryptophanamide

Ordinate and abscissa as in figure 6.

K_I 5.0×10^{-3} M.

Figure 17

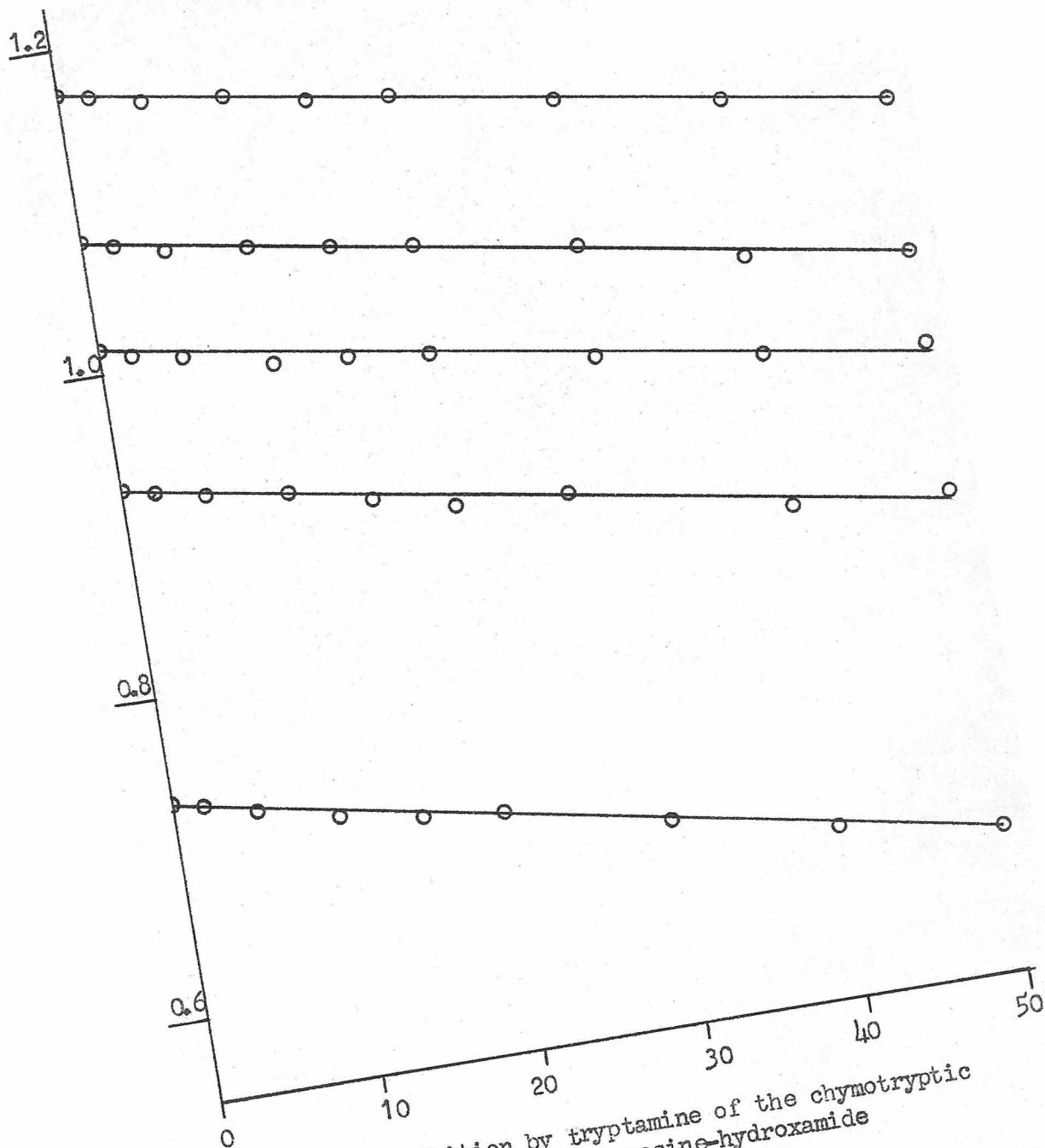


Inhibition by tryptamine of the chymotryptic hydrolysis of L-tyrosine-hydroxamide

Ordinate and abscissa as in figure 8.

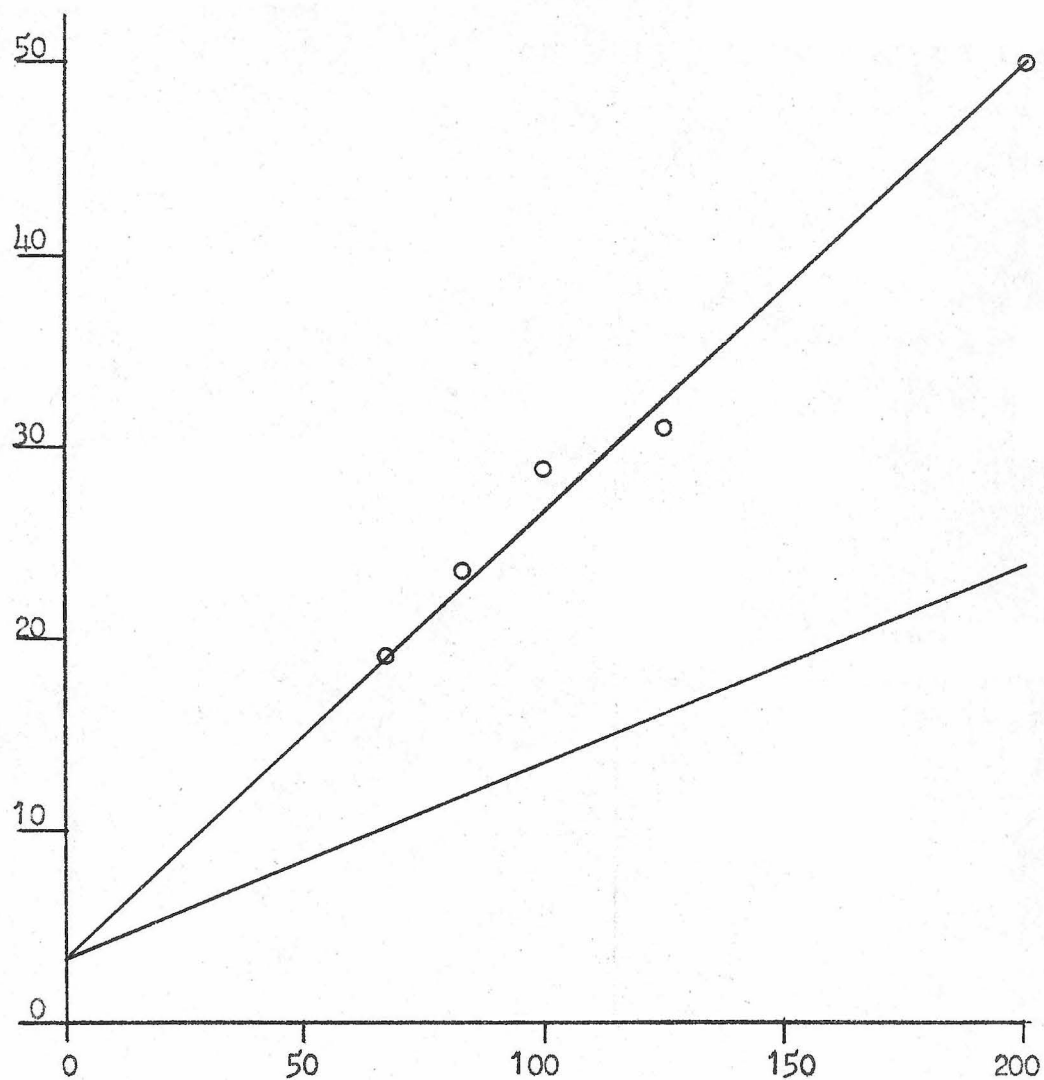
Inhibitor concentration: 3.0×10^{-3} M.

Figure 18



Inhibition by tryptamine of the chymotryptic
hydrolysis of L-tyrosine-hydroxamide
Ordinate and abscissa as in figure 9.
Inhibitor concentration: 3.0×10^{-3} M.

Figure 19

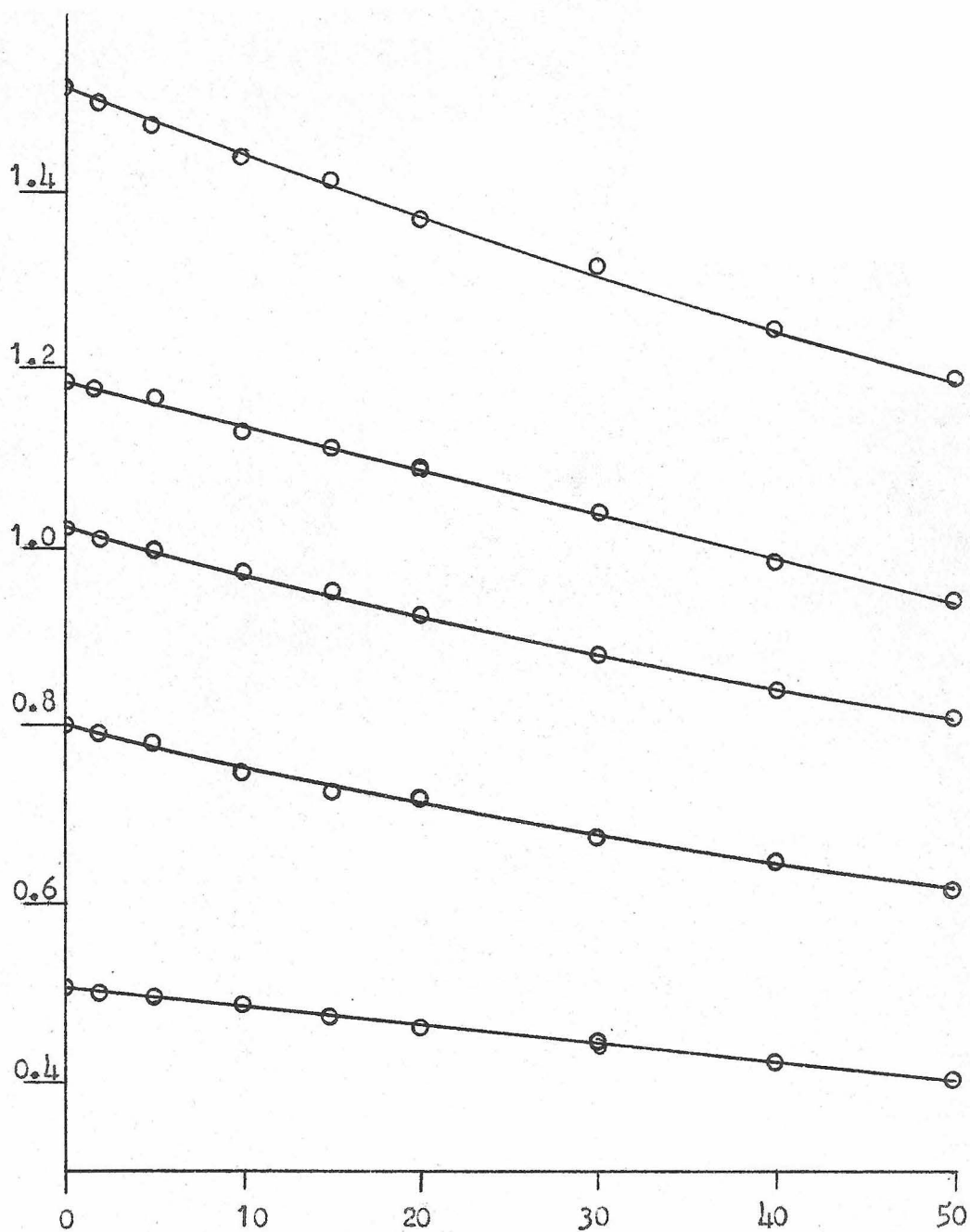


Determination of K_I for tryptamine

Ordinate and abscissa as in figure 6.

$$K_I = 2.2 \times 10^{-3} M.$$

Figure 20

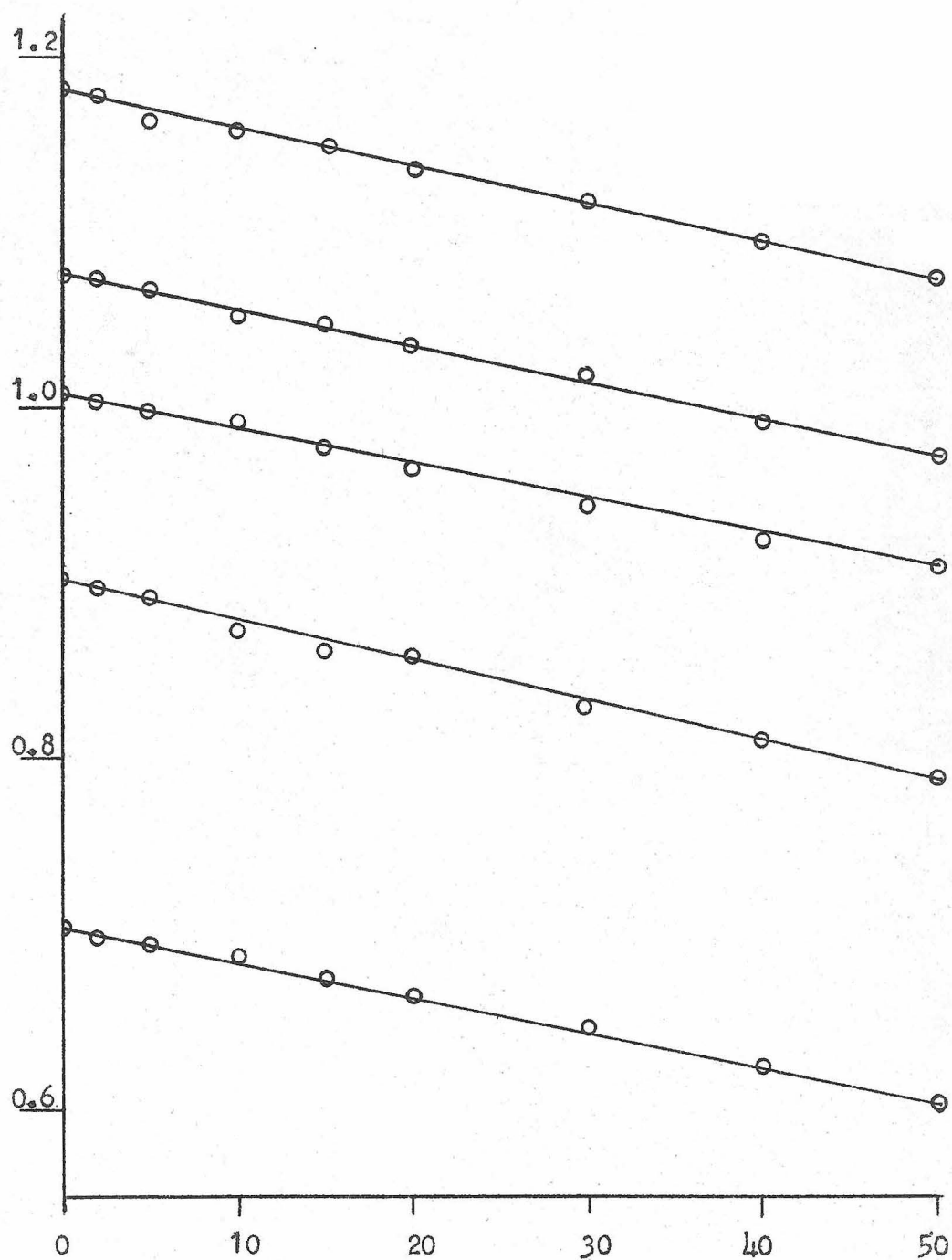


Inhibition by acetyl-D-tryptophan of the chymotryptic hydrolysis of L-tyrosine-hydroxamide

Ordinate and abscissa as in figure 8.

Inhibitor concentration: 1.0×10^{-3} M.

Figure 21

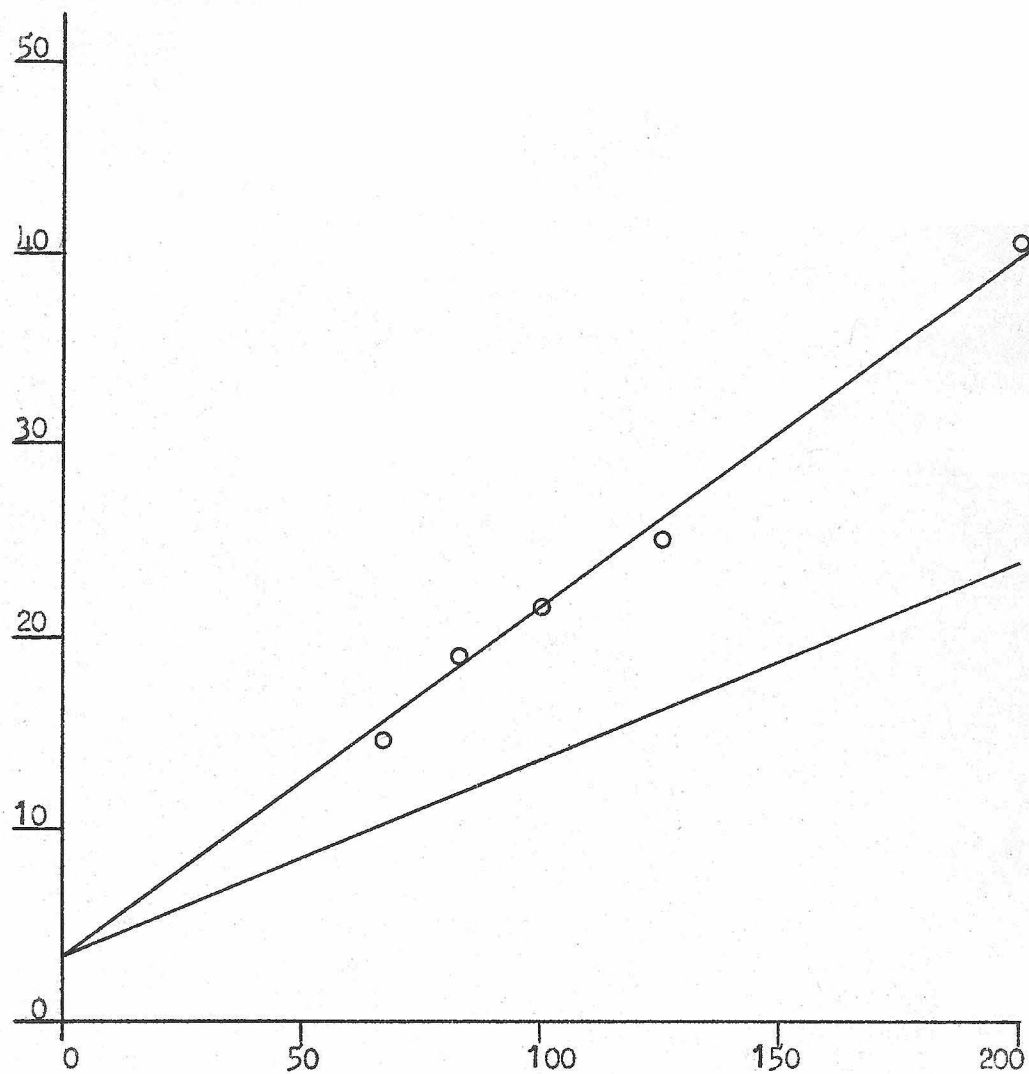


Inhibition by acetyl-D-tryptophan of the chymotryptic hydrolysis of L-tyrosine-hydroxamide

Ordinate and abscissa as in figure 9.

Inhibitor concentration: 1.0×10^{-3} M.

Figure 22

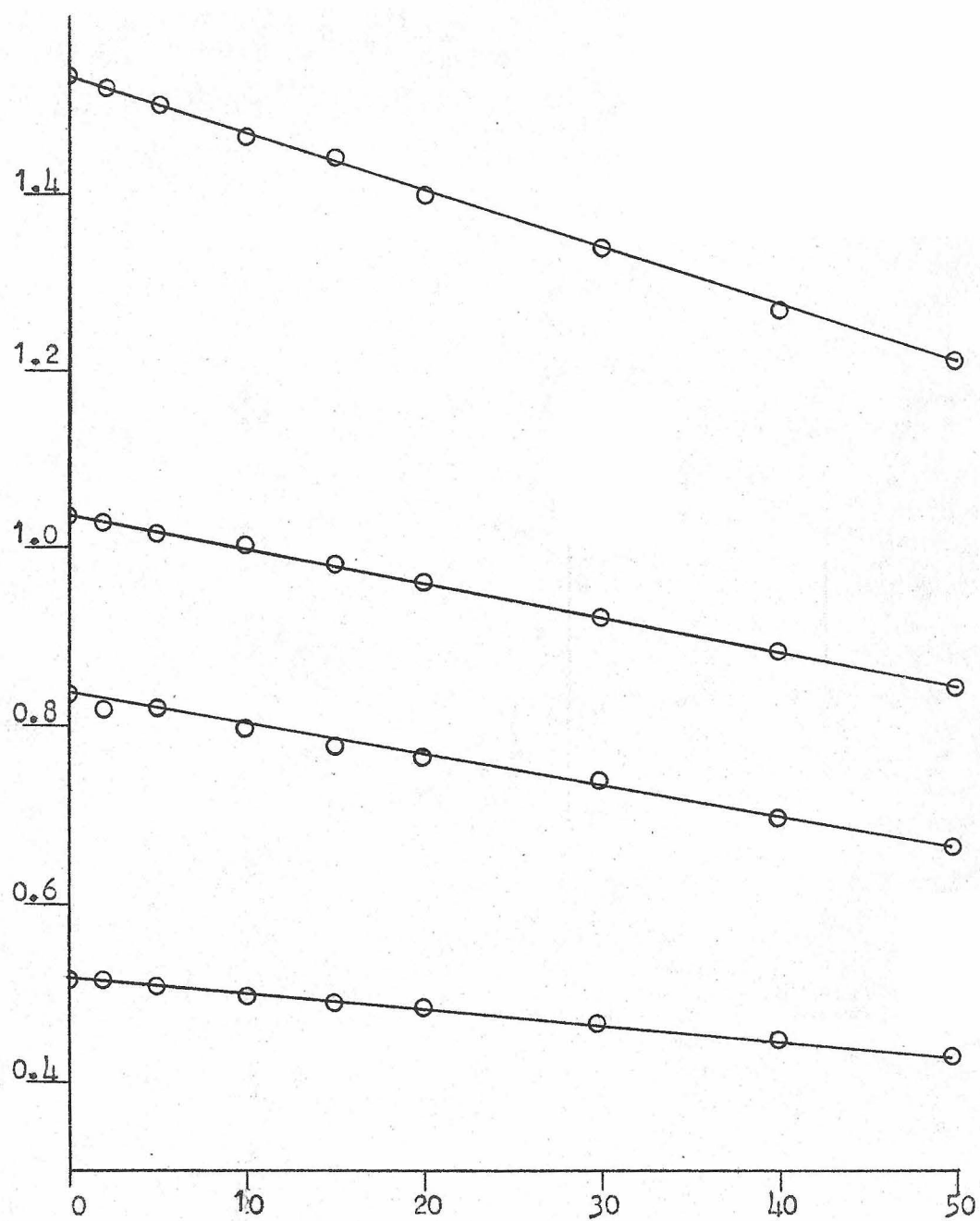


Determination of K_I for acetyl-D-tryptophan

Ordinate and abscissa as in figure 6.

$$K_I = 1.1 \times 10^{-3} \text{ M.}$$

Figure 23

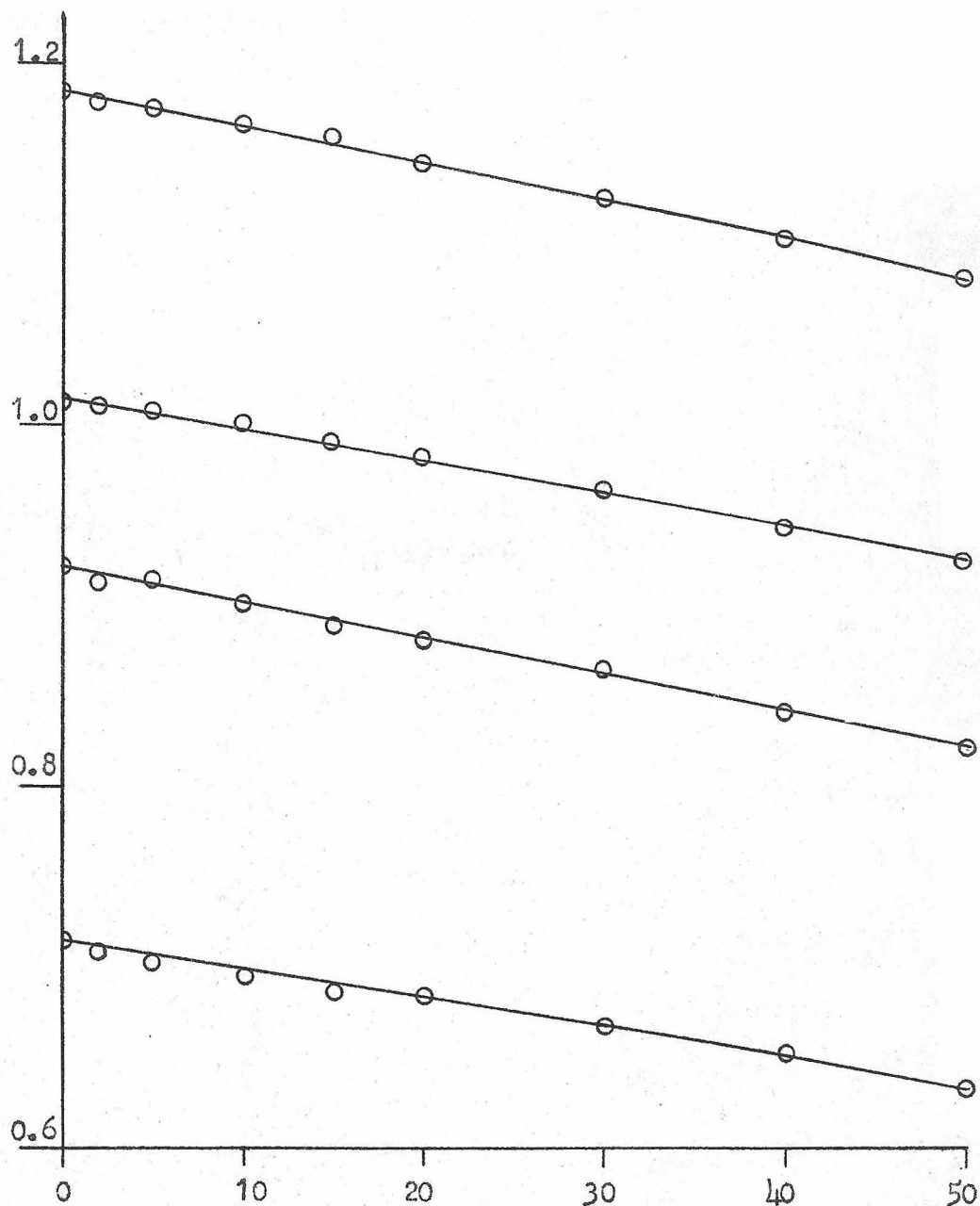


Inhibition by acetyl-L-tryptophan of the chymotryptic hydrolysis of L-tyrosine-hydroxamide

Ordinate and abscissa as in figure 8.

Inhibitor concentration: 2.0×10^{-3} M.

Figure 24

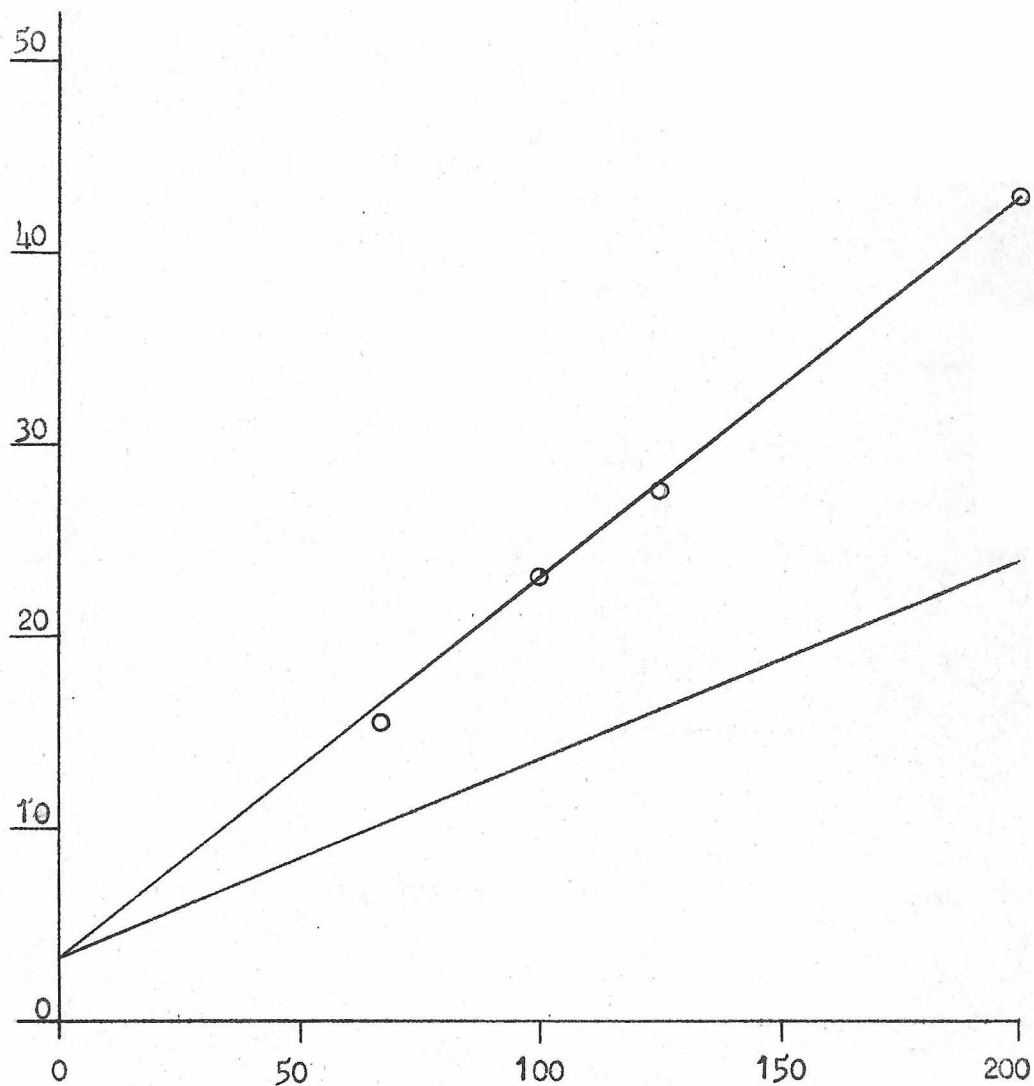


Inhibition by acetyl-L-tryptophan of the chymotryptic hydrolysis of L-tyrosine-hydroxamide

Ordinate and abscissa as in figure 9.

Inhibitor concentration: 2.0×10^{-3}

Figure 25

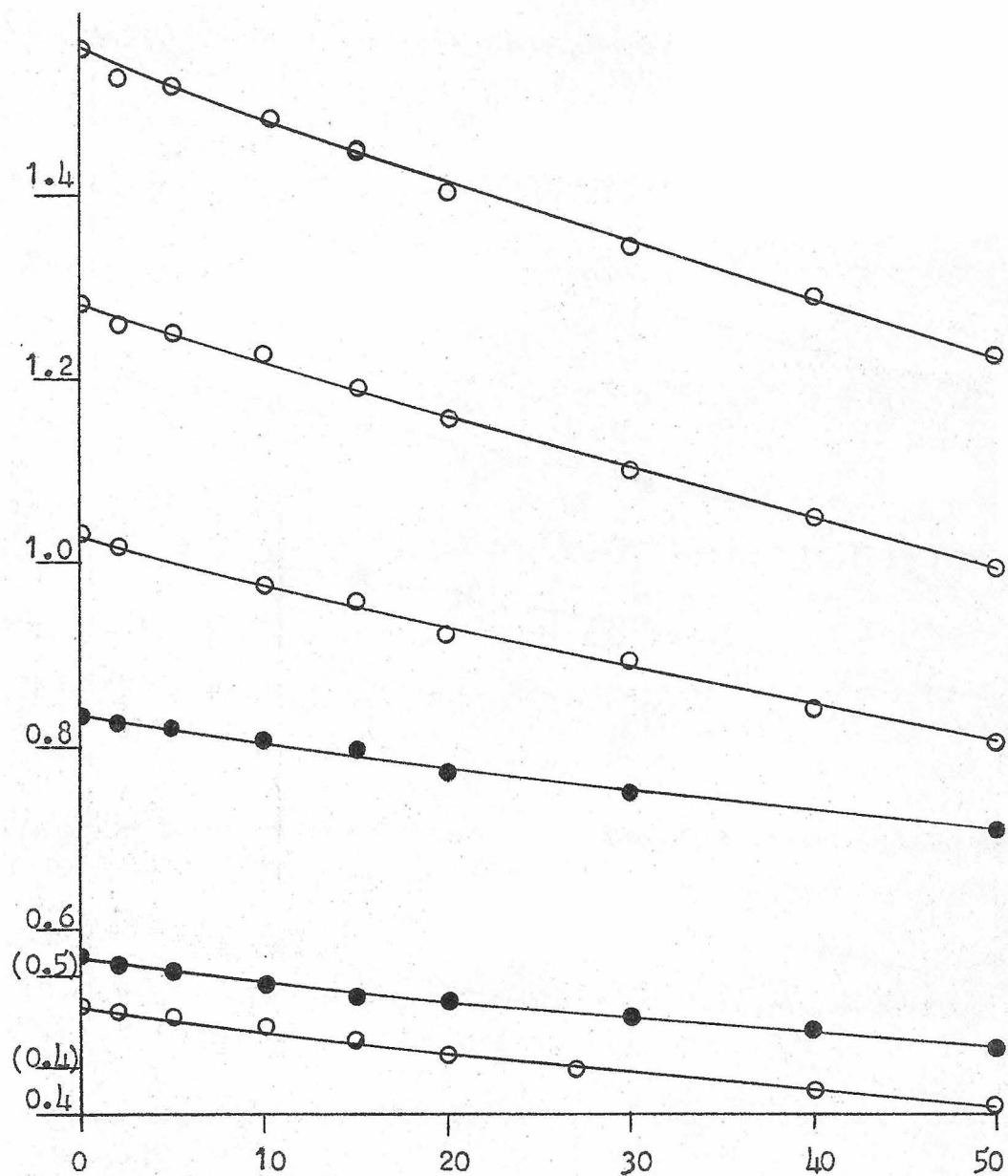


Determination of K_I for acetyl-L-tryptophan

Ordinate and abscissa as in figure 6.

$$K_I = 2.0 \times 10^{-3} \text{ M.}$$

Figure 26

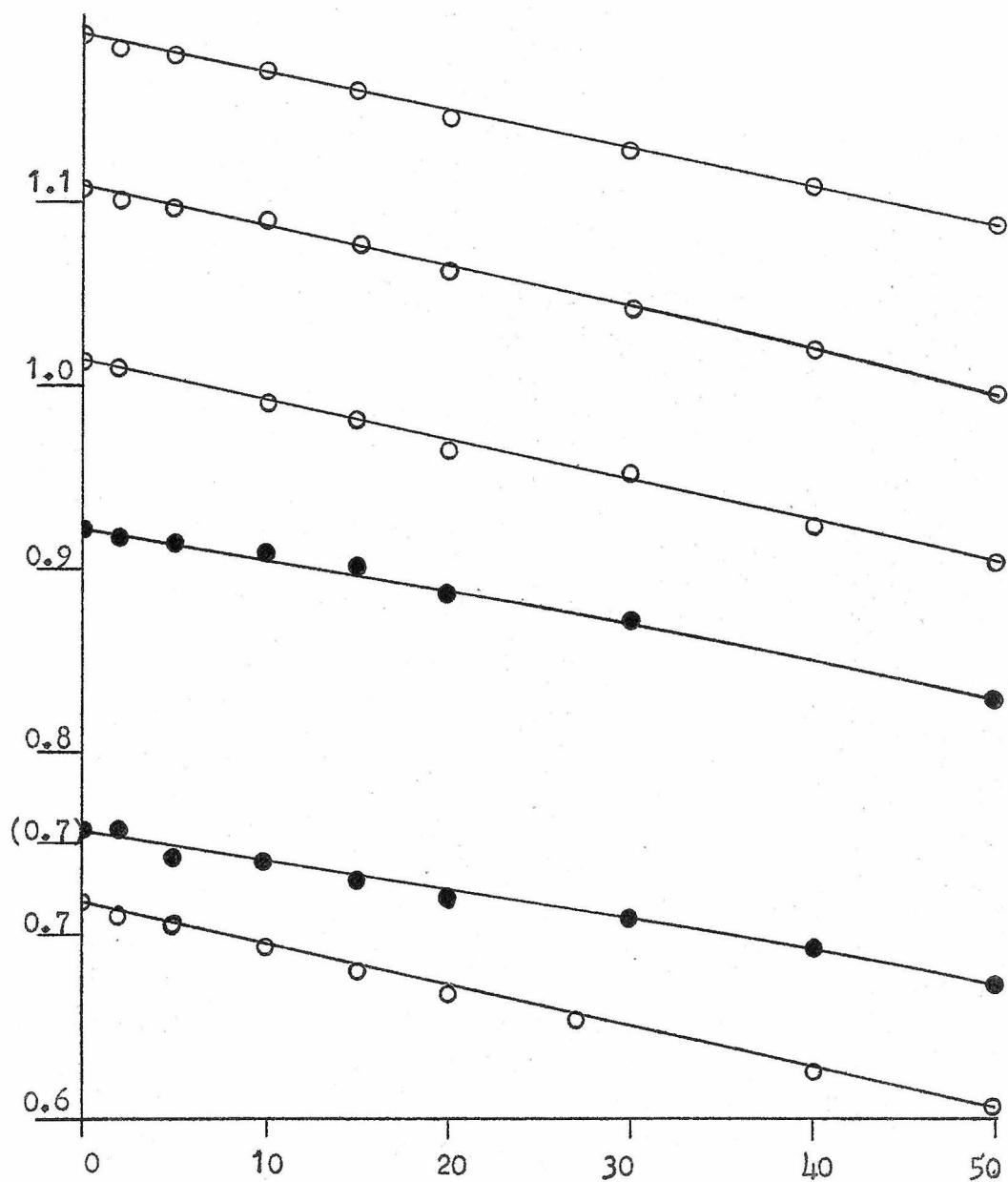


Inhibition by β -indolyl-3-propionic acid of the chymotryptic hydrolysis of L-tyrosine-hydroxamide

Ordinate and abscissa as in figure 8. Numbers in () refer to closed circles.

Inhibitor concentration: 1.0×10^{-3} M for open circles and 1.26×10^{-3} for closed circles.

Figure 27

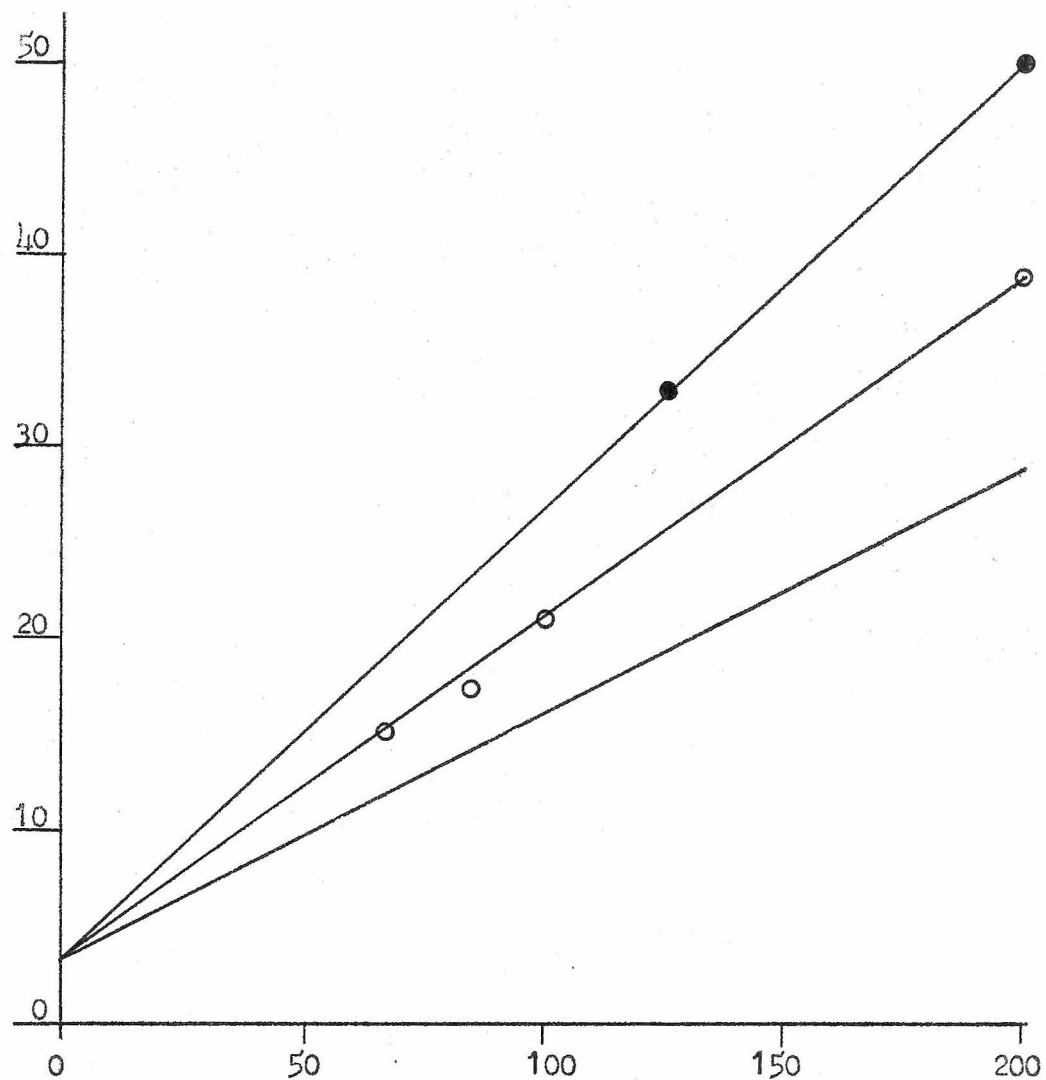


Inhibition by β -indolyl-3-propionic acid of the chymotryptic hydrolysis of L-tyrosine-hydroxamide

Ordinate and abscissa as in figure 9. () for closed circles.

Inhibitor concentration as in figure 26.

Figure 28

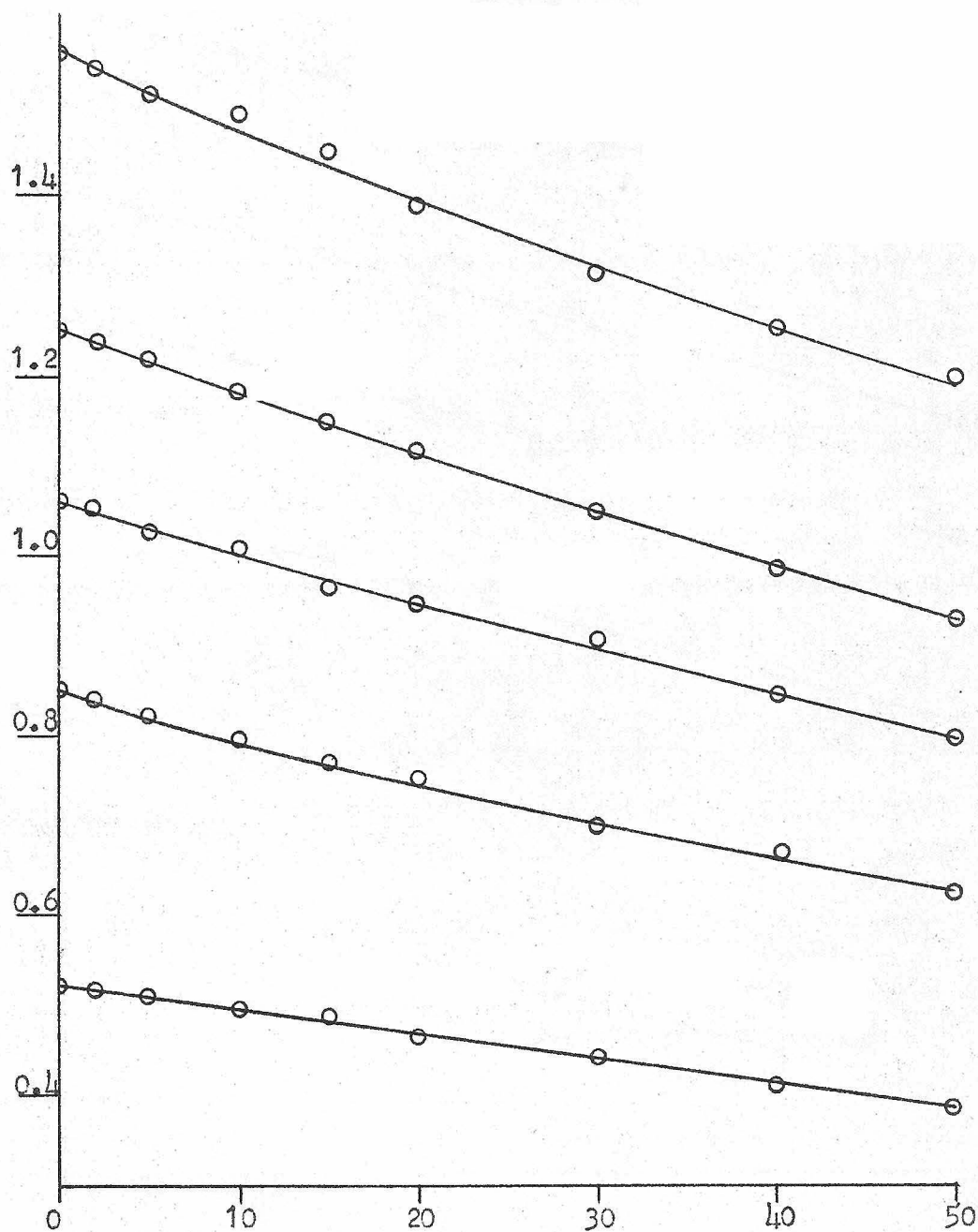


Determination of K_I for β -indolyl-3-propionic acid

Ordinate and abscissa as in figure 6.

$$K_I = 1.2 \times 10^{-3} \text{ M.}$$

Figure 29

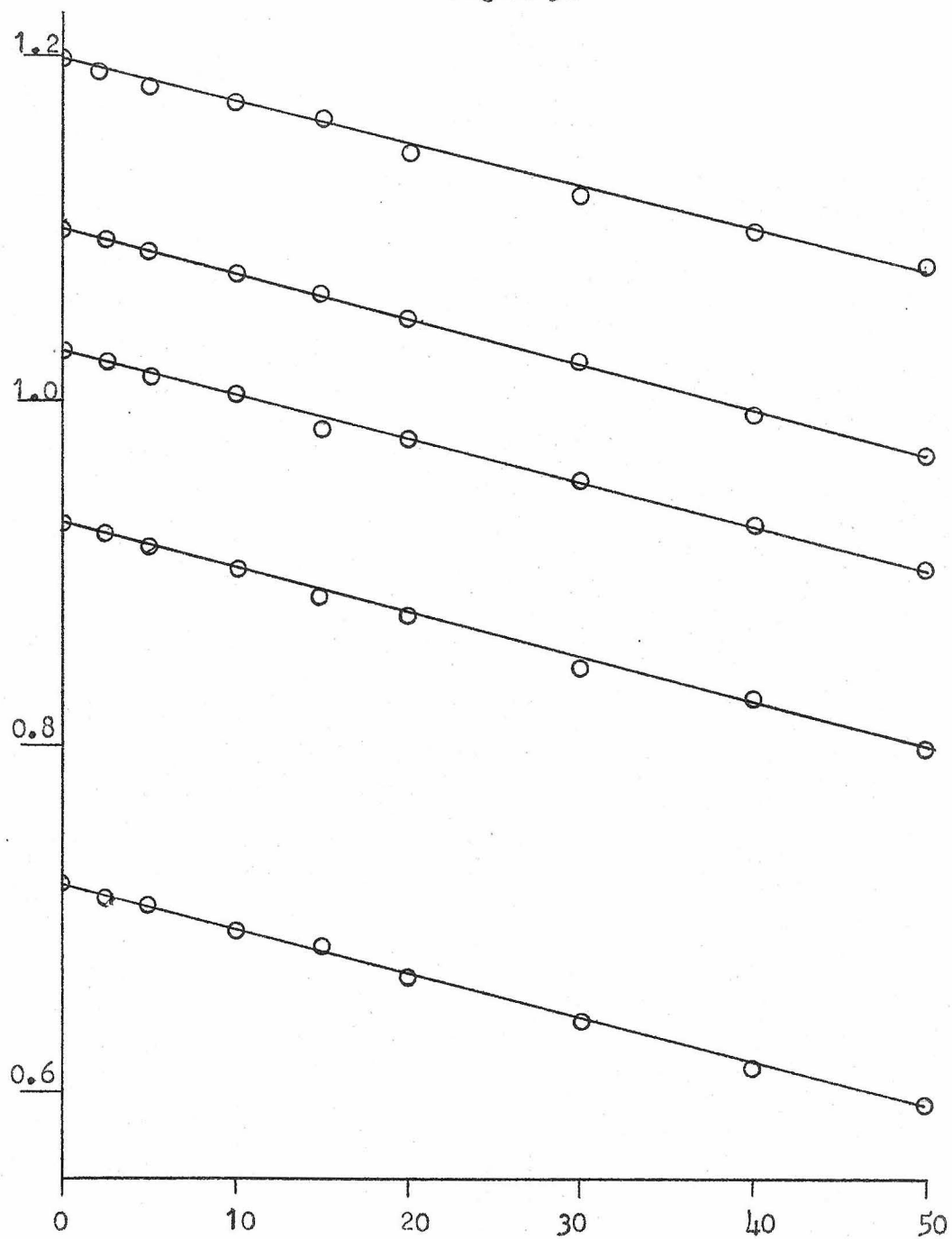


Inhibition by D-tryptophan of the chymotryptic hydrolysis of L-tyrosine-hydroxamide

Ordinate and abscissa as in figure 8.

Inhibitor concentration: 2.4×10^{-3} M.

-92-
Figure 30

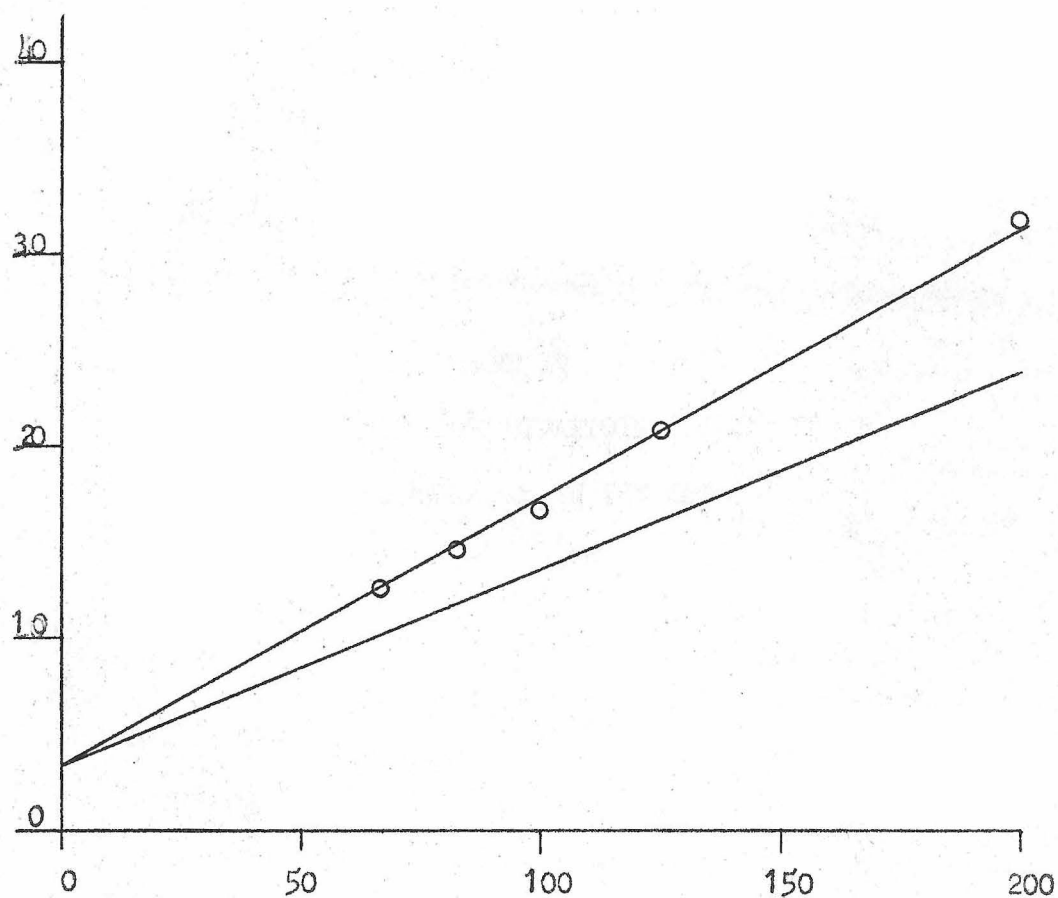


Inhibition by D-tryptophan of the chymotryptic hydrolysis of
L-tyrosine-hydroxamide

Ordinate and abscissa as in figure 9.

Inhibitor concentration: 2.4×10^{-3} M.

Figure 31



Determination of K_I for D-tryptophan

Ordinate and abscissa as in figure 6.

$$K_I = 6.0 \times 10^{-3} \text{ M}$$

PART IV

THE EFFECT OF DIPHENYL HYDANTOIN (DILANTIN) ON
ETHER NARCOSIS IN THE CAT

THE EFFECT OF DIPHENYL HYDANTOIN (DILANTIN) ON
ETHER NARCOSIS IN THE CAT

Introduction

The thesis that various parts of the neuron differ in sensitivity to oxygen lack led van Harreveld⁽¹⁾ to postulate and confirm that a "depolarization potential" could be led off during cord asphyxiation. This depolarization potential is due to the fact that certain grey matter elements in the spinal cord, i.e., nerve cells, are depolarized more rapidly by oxygen lack than the axons and other white matter, which have much lower oxygen requirements^(2,3). It was found that the grey matter developed a negativity with respect to the anterior root or posterior or lateral column.

Several investigations⁽⁴⁾ showed that narcotics effect the polarization state of the peripheral nerve; using the depolarization potential as an indicator, van Harreveld⁽⁴⁾ observed the effect of narcotics on the central nervous elements. Depolarization can only be detected if part of the neuron depolarizes. For uniform depolarization of the complete neuron no effect is observed. Therefore to measure the polarization state, the asphyxial depolarization potential was employed.

From the observation that diphenyl hydantoin (dilantin) in a dose of 50 mg. per kg. bodyweight depresses the asphyxial

depolarization potential, the conclusion was drawn that this compound has a depolarizing action on the nervous elements in the spinal cord of cats. Since the administration of dilantin does not produce a depolarization potential, it was further suggested that the spinal neuron is depolarized uniformly⁽⁵⁾. Asphyxial depolarization differs from the dilantin induced depolarization in that it affects first the neuron parts which are more sensitive to oxygen lack⁽¹⁾. The administration of dilantin has no marked effect on the kneejerk or the flexion reflex of the lightly narcotized preparation.

The narcotic doses producing comparable states of nervous depression caused varying degrees of depression of the asphyxial depolarization potential for the narcotics diallyl barbituric acid, chloral, etc. Cocaine, which causes increased spinal reflex activity, was also found to cause depression of the depolarization potential. These experiments⁽⁵⁾ showed that synaptic conduction is not incompatible with a considerable uniform depolarization of the spinal neuron. Earlier work had shown that the depolarization and membrane stabilizing effects of narcotics are not related⁽⁶⁾.

Ether has a pronounced depolarizing action on peripheral nerve^(7,8,9), as well as on spinal cord elements⁽⁴⁾. Ether also depolarizes the spinal neuron uniformly and its effect is therefore similar to the depolarization caused by dilantin.

Lorente de No⁽⁹⁾ succeeded in restoring conduction in peripheral nerve narcotized with ether, by repolarizing the nerve membrane with an externally applied anodal current. This indicates that depolarization is an important factor in the narcotic effect of ether on peripheral nerve. Assuming the same to be true for the narcotic effect of ether on central synaptic conduction, it can be expected that diphenyl hydantoin, which itself does not depress spinal reflex action, but which depolarizes cord elements, will potentiate the narcotic effect of ether on reflex activity.

The central narcotic effect of pentobarbital does not seem to depend on depolarization. Pentobarbital in narcotic doses (50 mg. per kg. bodyweight) causes only a moderate depolarization of the spinal cord elements⁽⁴⁾. Eccles⁽¹⁰⁾ and Brooks and Eccles⁽¹¹⁾ found that pentobarbital hampers the elaboration of a conducted impulse from the synaptic potential. Increasing the amount of pentobarbital administered necessitates increasingly higher synaptic potential for the initiation of a conducted impulse in the motoneurone. Finally even the fully developed synaptic potential becomes incapable of eliciting a conducted impulse. Since the narcotic effect of pentobarbital thus seems to depend not on depolarization but on a "membrane stabilizing effect", there is less reason to expect a potentiating effect of dilantin on pentobarbital narcosis.

Van Harreveld⁽¹²⁾ found that there was no evidence of a potentiation of pentobarbital narcosis in the cat by diphenyl hydantoin. Not only was in no instance a potentiation of the narcotic effect of pentobarbital by dilantin observed, but in most experiments there were indications of an antagonism between these drugs, which resulted in an increase of the knee-jerk contractions after diphenyl hydantoin administration. This seems to indicate that depolarization is of little importance for the narcotic effect of pentobarbital, since even the combined depolarizing action of this compound and of dilantin are insufficient to depress the kneejerk.

Discussion of Results

The work reported in this thesis is an investigation of the effect of dilantin on ether narcosis in the cat.

The effect of the injection of diphenyl hydantoin in a dose of 50 mg. per kg. bodyweight during a series of determinations is shown in Fig. I (Table 7). The four control determinations before the injection showed only minor variations. The first two determinations after the injection were about 40% lower than the control values. From then on the minimum ether concentration necessary to suppress the kneejerk increased again to reach the original level after about an hour. It was

found constantly that the greatest depression of the minimum ether concentration was not reached immediately after the injection of dilantin, but 20-40 minutes later. It was surprising that the effect of dilantin on the narcotizing ether concentration is of relatively short duration (not more than 60 to 90 minutes). Table 1 shows the results obtained in 5 experiments of this kind. The mean decrease of the minimum ether concentration to narcotize the kneejerk due to the administration of dilantin was $44 \pm 2\%$.

Preliminary experiments summarized in Tables 2-4 had shown that there was an induction period for the ether potentiating effect of dilantin. It was also noted that a concentration of greater than 25 mg. per kg. bodyweight was necessary for the ether potentiating effect of dilantin. Complete recovery from the effect of dilantin upon ether narcosis in a relatively short time was observed. An early observation indicated that for intravenous injection of dilantin, water was a better diluent than Ringer's solution in that circulatory collapse was less frequent.

Tables 5-10 contain data for experiments wherein dilantin was administered both intra-arterially and intra-venously in a concentration of 50 mg. per kg. bodyweight.

In two control experiments (Tables 11 and 12) the amount of alkali which would be necessary to dissolve diphenyl hydantoin in a dose of 50 mg. per kg. bodyweight was administered.

In these experiments the minimum ether concentration required to narcotize the kneejerk had a tendency to slowly and gradually increase in time. These experiments demonstrate the expected potentiating effect of diphenyl hydantoin in a dose of 50 mg. per kg. bodyweight on the narcotic effect of ether on the cat although given alone dilantin does not depress spinal reflex activity.

There is another important effect of the injection of dilantin. This is an immediate increase in the size of the kneejerk. This phenomenon is more clearly demonstrated in those experiments in which the drug was injected swiftly into the peripheral circulation. Figures II and III demonstrate that the increase in strength of the kneejerk is not an artifact. From the first triangle to the second on Figure II dilantin was injected into the manometer circuit. At the third this solution was expelled into the peripheral circulation and at the fourth the ether apparatus was turned on. The time interval between the third and fourth was approximately one minute. Figure III, upper trace depicts the effect of merely shutting off the supply of ether while the lower trace is for a control experiment where 10 ml. of 0.2% sodium hydroxide was injected intra-arterially. The triangles mark the time the ether was turned off and sodium hydroxide injected respectively.

From their effect on the asphyxial depolarization potential it was concluded that both diphenyl hydantoin and ether have a depolarizing action on the nervous elements in the spinal cord^(4,5). Although diphenyl hydantoin given alone has little effect on spinal reflex activity it does potentiate the narcotic effect of ether as has been shown above. This supports the thesis that the depolarizing effects of ether and dilantin are additive and that depolarization is an important factor in the narcotic effect of ether on central conduction. Although depolarization seems to play an important part in the effect of ether on central as well as on peripheral⁽⁹⁾ nervous structures, it is not the only effect of this compound. The threshold of excitation of peripheral nerve increases very considerably when subjected to ether vapors⁽⁷⁾. This is not due to the resulting depolarization since asphyxial depolarization of peripheral nerve was found to produce either a decrease of the threshold^(13,14) or to leave the excitability unchanged⁽⁷⁾. It seems therefore that ether has a "membrane stabilizing" effect in addition to its depolarizing effect. Both effects may cooperate in producing the narcotic effect of ether.

The effect of diphenyl hydantoin on ether narcosis has been shown to be quite different from its effect on pentobarbital narcosis.

Section 2

Experimental and Data

The animals were prepared under light ether narcosis. The neck was opened and the trachea immediately canulated so that artificial respiration could be administered when necessary. The left external jugular was canulated to facilitate the administration of the drugs and Ringer's solution required to maintain the blood pressure. The left carotid was cleaned so that samples of blood could be removed for ether determination. The right carotid was then canulated in order that the blood pressure could be read continuously. The animal was made spinal by ligating the dura at Th10-Th12. The condyle of the right femur was drilled to take a 3/16" screw. The femur was then fastened to a wooden brace by means of a screw, taking care not to impair the movements of the shank. The kneejerk was elicited by an electro-magnetic hammer⁽¹⁵⁾ which tapped the quadriceps tendon at regular intervals (ca. 3 sec.). The blood pressure manometer was connected and the movements of the shank were recorded on a kymograph.

Ether was administered by passing the air for artificial respiration over a bottle of ether resting in a warm water bath. The bottle had a controllable by-pass for fine regulation of ether concentration. The minimum ether concentration in the blood necessary to suppress the kneejerk was determined before and after the administration of dilantin. An ether concentration

was offered in the respired air sufficient to decrease slowly and finally to suppress the kneejerk (Figure IV - 1A, 1B, 1C). At the moment that the tap on the tendon failed to elicit a reflex contraction a 2 cc. sample of blood was taken from the carotid artery. A 2 cc. pipette was connected by a small length of rubber hose with a No. 19 hypodermic needle. The needle was forced through the arterial wall and the arterial pressure filled the pipette quickly and with a minimum loss of ether from the blood. The ether bottle was removed from the respiratory path by a clamp at the beginning of the sampling. The animal was allowed to recover from the deep narcosis until the kneejerk returned to a moderate size. After this the ether bottle was again placed in the path and another sample taken at the proper time. In this way a determination of the minimum ether concentration in the blood necessary to narcotize the kneejerk was made at 10 to 15 minute intervals. The animal was then allowed to come under a fairly light narcosis and dilantin was added through either the jugular vein or the right carotid. The usual dose was 50 mg. per kg. bodyweight dissolved in a 0.2% sodium hydroxide solution. When added intra-venously a time interval of 10-15 minutes was necessary as the blood pressure dropped too fast if there was a shorter interval. When added arterially, the

solution was added to the clamped manometer tube and then forced into the peripheral system suddenly. It was necessary to add epinephrine (10^{-5}) through the jugular to combat a pressure decrease. Intra-arterial injection in order to dilute the drugs in the blood of circulation before reaching the heart is to be preferred. After injection of the dilantin the ether was administered until the kneejerk failed again. A blood sample was withdrawn as before for analysis. This was repeated in fifteen minute intervals as many as eight times. In order that the rate of narcosis would be approximately the same each time, the control valve was not touched when the animal was recovering. Rather, a clamp was applied to the tube going to the ether bottle. Cats were used exclusively in these experiments.

Ether determinations were made essentially in the manner of Friedman⁽¹⁶⁾. By this method the ether in the samples was determined to within ± 1%.

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14. Lehman, J. E., Am. J. Physiol., 119, 111 (1937).
15. Johnson, C. A., Am. J. Physiol., 82, 75 (1927).
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Table I

Minimum Concentration of Ether (gm. Ether/gm. Blood
x 100) in the Circulating Blood, Necessary to Suppress the
Kneejerk

No.	Mean of the Control Values	Lowest Value after Diphenyl Hydantoin Administration	% Decrease of Ether Concentration	Time of Minimum (minutes)
1	0.088	0.052	-41%	30
2	0.132	0.065	-51%	50
3	0.107	0.065	-39%	25
4	0.052	0.028	-46%	30
5	0.122	0.070	-43%	20
Mean			-44%	

1. 50 mg./kg. bodyweight

Note: Time taken for the minimum is taken from the time at the middle of the injection to the lowest value obtained for the ether required for the abolishment of the knee reflex.

Tables 2 - 10

Ether Concentration vs. Time for Dilantin Injection

Unless otherwise stated the Dilantin concentration was such that 10 ml. contained 50 mg./kg. bodyweight in 0.2% NaOH and was added intravenously.

Table 2

Sample No. ¹		% Ether
1		0.160
2		0.152
	Dilantin added ²	
3		0.129
	Dilantin added ²	
4		0.092
5		0.086

Table 3

Sample No. ¹		% Ether
1		0.129
2		0.140
3		0.127
	Dilantin added ³	
4		0.139
5		0.129
	Dilantin added ³	
6		0.141
	Dilantin added ³	
7		0.114
	Dilantin added ³	
8		0.121
	Dilantin added ³	
9		0.127
	Dilantin added ³	
10		0.115

1. Fifteen minutes between consecutive ether samples. Dilantin given over fifteen minute intervals. Ether samples taken approximately five minutes after dilantin injection.
2. 35 mg./kg. bodyweight - dissolved in Ringer's solution plus sufficient NaOH to dissolve the sample.
3. 12.5 mg./kg. bodyweight - dissolved in H₂O plus sufficient NaOH to dissolve the sample.

Table 4

Sample No. ¹	% Ether
1	0.100
2	0.098
3	0.113
4	0.098
Dilantin added ²	
5	0.085
6	0.093
7	0.100
8	0.100
Dilantin added ³	
9	0.058
10	0.082
11	0.096

Table 5

Time Sample Taken (minutes)	% Ether
0	0.091
15	0.089
31	0.086
48	0.086
63-78	Dilantin added
84	0.058
100	0.052
111	0.070
127	0.075
143	0.084
161	0.084

1. See footnote 1 on previous page.
2. 50 mg./kg. bodyweight - dissolved in 0.1% NaOH.
3. 25 mg./kg. bodyweight - dissolved in 0.1% NaOH.

Table 6

Time Sample Taken (minutes)		% Ether
0		0.133
18		0.133
37		0.124
55		0.137
106-115	Dilantin added	
129		0.091
144		0.098
160		0.065
177		0.070
198		0.079
212		0.091

Table 7

Time Sample Taken (minutes)		% Ether
0		0.110
16		0.107
38		0.104
50		0.107
58-72	Dilantin added	
78		0.070
91		0.065
102		0.089
118		0.103
133		0.103
187		0.112
212		0.107

Table 8

Time Sample Taken (minutes)		% Ether
0		0.079
30		0.052
41		0.084
57		0.093
65-80	Dilantin added	
86		0.084
98		0.084
112		0.075
125		0.070
138		0.058
151		0.051
166		0.068

Table 9

Time Sample Taken (minutes)		% Ether
0		0.061
14		0.047
26		0.049
36		0.051
44-45	Dilantin added ¹	
50		0.047
62		0.037
73		0.028
84		0.033
95		0.033
107		0.037
122		0.028
133		0.040

1. Intra-arterially.

Table 10

Time Sample Taken (minutes)		% Ether
0		0.149
12		0.110
26		0.121
38		0.107
56-57	Dilantin added ¹	
62		0.086
73		0.070
85		0.098
98		0.107
110		0.098
125		0.112

Table 11

Ether Concentration vs. Time for NaOH
Blank (0.2%) 10 ml.

Time Sample Taken (minutes)		% Ether
0		0.135
24		0.126
41		0.154
53		0.158
64		0.145
84		0.171
90-99	NaOH added	
108		0.175
124		0.161
137		0.182
150		0.191

1. Intra-arterially

Table 12

Ether Concentration vs. Time for NaOH
Blank (0.2%)-10 ml.

Time Sample Taken (minutes)		% Ether
0		0.140
15		0.117
22		0.112
32		0.168
45-55	Ether Recovery	
60-70	NaOH added	
79		0.117
90		0.135
98		0.149
111		0.135

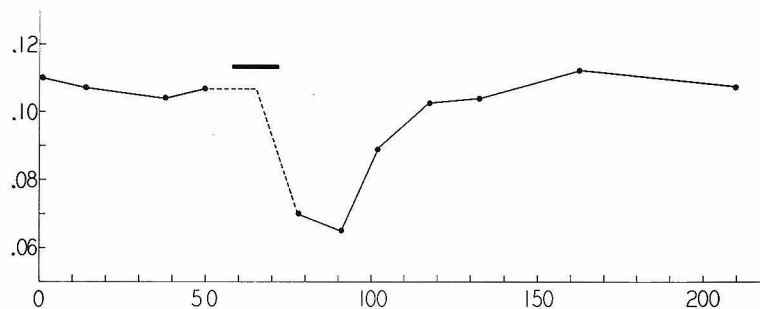


Figure I

The Effect of Dilantin on Ether Narcosis
of the Kneejerk of the Cat

Ordinate - percentage ether in blood necessary
to suppress the kneejerk.

Abscissa - time in minutes.

Bar, 58-72, represents the time of injection
of dilantin.

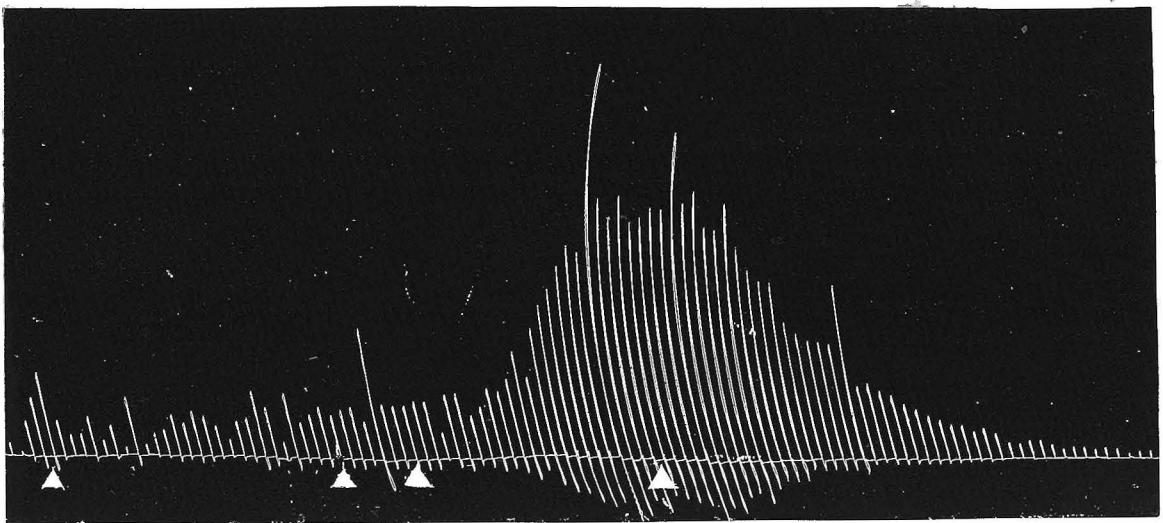


Figure II

To Illustrate the Initial Increase of the Kneejerk
Upon Dilantin Injection.

First to second triangle - dilantin injection into the
manometer: third triangle - solution expelled into the
peripheral circulation: fourth triangle - ether administered.

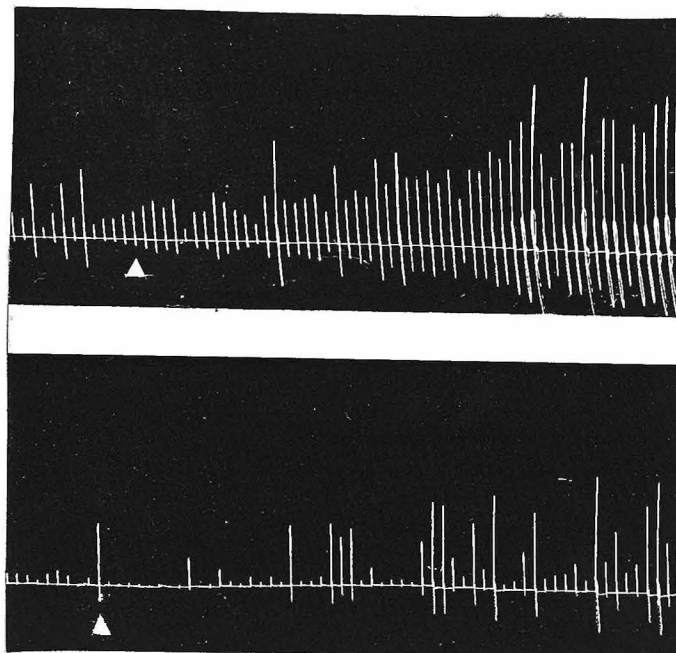


Figure III

Control Experiments with Ether and Sodium Hydroxide

Upper trace: Ether control. At triangle ether was turned off.

Lower trace: Sodium hydroxide control. At triangle the ether was turned off and sodium hydroxide added.

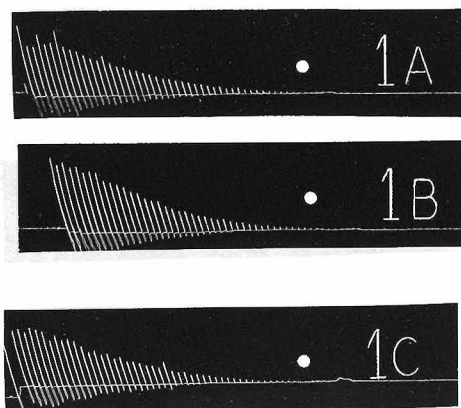


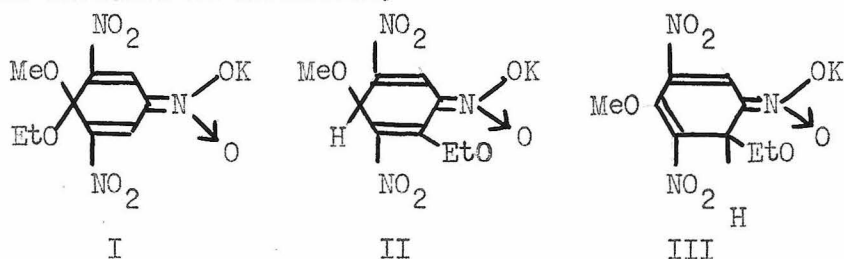
Figure IV

Effect of Ether on the Kneejerk

At the circles, where the kneejerk failed, blood samples were taken.

PROPOSITIONS

1. The structure of the salt formed by reacting tri-nitro-anisole and potassium ethoxide or tri-nitrophenetole and potassium methoxide has been stated to be I below⁽¹⁾. The proof was ambiguous however, because the structure could be the tautomeric mixture II and III below. This ambiguity could be resolved by decomposing the salts in aqueous media that have been enriched in deuterium.



1. Meisenheimer, J., Ann., 323, 205 (1902).

2. Since glyoxalic acid forms a very stable hydrate, the crystal structure of this hydrate should be determined in order to ascertain whether or not the oxygen of the water is bonded directly to the aldehydic carbon atom.

3. It has been reported⁽²⁾ that L-phenylalanine does not exchange carbonyl oxygen with water in the presence of α -chymotrypsin whereas carbobenzoxy-L-phenylalanine does. In light of the fact that such compounds as L-tyrosine-ethyl-ester⁽³⁾ and L-tyrosine-hydroxamide⁽⁴⁾ are hydrolyzed by this enzyme these experiments such be repeated with a longer time allowed for exchange.

2. Sprinson, D. B. and Rittenberg, D., Nature, 167, 484 (1951).

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

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4. The argument of Nachmansohn and Wilson⁽⁵⁾ that the reason a thousand fold increase in concentration of di-isopropyl-fluorophosphate over that needed to inhibit completely acetylcholine-esterase is needed to block nerve conduction is lack of penetration of the drug into the nerve might be checked by experiments with desheathed nerve in the manner of Crescitelli⁽⁶⁾, since, in general, substances are retarded by the sheath.

5. Nachmansohn, P. and Wilson, I.B., Adv. in Enz., 12, 259 (1951).

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5. Since it has been found⁽⁷⁾ that the hydrogenolysis of gem dialkylcyclopropanes opens the ring almost exclusively at the bond opposite the di-substituted carbon atom it is proposed that the structure of 1, 1-dimethylcyclopropane be investigated by the method of electron diffraction to see if the bond is longer than normal.

7. Shortridge, R.W., Craig, R.A., Greenlee, K.W., Derfer, J.M., and Boord, C.E., Jour. Am. Chem. Soc., 70, 946 (1948).

6. The enzyme-inhibitor constants for α -chymotrypsin should be measured by an equilibrium method. Determinations of the distribution constants between an aqueous phase and a suitable organic phase for an inhibitor both with and without enzyme present should be possible. If an inhibitor is chosen that has sufficiently high affinity for the enzyme, the equivalent weight of the enzyme can be determined by this method.

7. It is proposed that faulty metabolism of a group of brain cells resulting in abnormally high membrane potentials may be the trigger mechanism for epilepsy. In support of this as a working hypothesis are the following facts:⁽⁸⁾

a. It has been known for a long time that externally produced potentials will cause a pseudo epileptic fit.

b. Drugs such as dilantin and the barbiturates which are useful in decreasing attacks in epileptics have been demonstrated to decrease membrane potential.

c. The feeding of glutamic acid has been shown to decrease frequency of attacks and this substance is known to be involved in the metabolism maintaining normal membrane potentials.

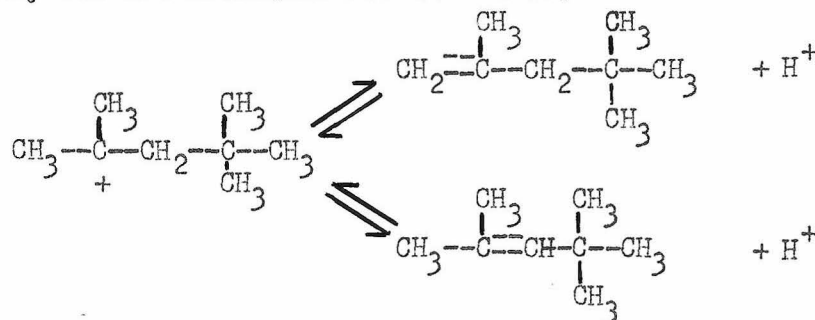
8. Toman, J.E.P., and Goodman, L.S., Phys. Rev., 28, 409 (1948).

8. The ferric-hydroxamide complex should be studied carefully to find out what particular species is responsible for the observed red color in certain solutions.⁽⁴⁾

9. The chymotryptic hydrolysis of acylated and nonacylated substrates should be studied carefully as a function of pH, with special attention paid to the constant k_3 , to see if there appears to be a change in mechanism from hydroxyl to hydrogen ion type.

10. For the formation of "diisobutylene" from isobutylene with sulfuric acid catalyst, Alexander⁽¹⁰⁾ proposes a mechanism and states that the experimental results⁽¹¹⁾ are opposed to those

predicted on the basis of hyperconjugation. However, using the theory of Hughes and Ingold⁽¹²⁾ that hyperconjugation in the transition state is important, neither isomer is favored over the other and therefore the explanation for the experimental facts is most probably that for the production of one isomer six electron pairs are available to form the double bond while only two are available for the other.



9. Alexander, E. R., Ionic Organic Reactions, John Wiley and Sons, New York.

10. Whitmore, F.C., and Church, J.M., Jour. Am. Chem. Soc. 54, 3710 (1932).

11. Dhar, M.L., Hughes, E.D., Ingold, C.K., Mandour, A.M.M., Maw, G.A., and Woolf, L.I., Jour. Chem. Soc., 2093 (1948).

11. Since the telephone has proved itself to be an excellent means of communication, it would seem advantageous to install more of these instruments in the Gates and Crellin Laboratories and dispense with the tintinabulation of the bells, bells, bells, bells ad nauseum.