STUDIES ON THE MODE OF ENZYME CATALYSIS AND THE HYDROLYSIS OF BIS-P-DIMETHYLAMINOBENZALAZINE

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ABSTRACT

The alpha-chymotrypsin catalyzed hydrolysis of four L-tyrosinhydrazides at 25°C have been studied. The values of the kinetic constants K_s and k_3 for L-tyrosinhydrazide, acetyl L-tyrosinhydrazide, and nicotinyl L-tyrosinhydrazide have been evaluated by the usual procedure. The values of these constants for benzoyl L-tyrosinhydrazide have been obtained by the method of competitive hydrolysis.

The procedure for the quantitative determination of hydrazine by the reaction with p-dimethylaminobenzaldehyde to form the corresponding azine has been adapted to the study of the rate of the hydrolysis of alpha-amino acid hydrazides.

The rates of formation and of hydrolysis of bis p-dimethylaminobenzalazine have been studied under various conditions. The effects of dielectric constant, ionic strength, acid concentration, and temperature on the rate of hydrolysis have been investigated. The acid dissociation constants for p-dimethylaminobenzaldehyde in two distinct ethanol-water systems have been determined. The acid dissociation constants for the hydrazone and the azine of p-dimethylaminobenzaldehyde have been obtained. The value of the constant for the assumed aldehyde- hydrazine- hydrazone equilibrium has been calculated.

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Part I

STUDIES ON THE MODE OF ENZYME CATALYSIS

INTRODUCTION

The proteolytic enzymes are catalysts for the hydrolysis of many proteins. A study of the mode of action of such enzymes implies the determination of the structural characteristics of the enzyme which are responsible for the catalysis of the hydrolytic reaction. An approach to this problem of establishing the nature of the requisite unique configuration is an investigation of the requirements necessary for a reacting system. There are many parameters for such a system, and positive results are derivable only when a judicious selection of experimental conditions permits the observation of the system under controlled variations. One of these controlled variations has been successfully employed for the indirect examination of the structural characteristics of the enzyme alpha-chymotrypsin. This variation, the alteration of the substrate, has been used in this study.

The proteolytic enzyme alpha-chymotrypsin has the attributes necessary for an investigation of the mechanism of the enzymic catalysis. This enzyme possesses the desirable qualities of being available in highly purified crystalline form, of being stable in an aqueous solution for a period of time sufficient to secure reliable kinetic

data, and of being independent of the requirement of the presence of a coenzyme or activator for catalytic activity. The alpha-chymotrypsin used in this study was bovine alpha-chymotrypsin (Armour and Company).

A systematic investigation of the effects of the structural alterations of the substrates upon the reacting system involves the cataloging of the effects caused by substitutions of the functional derivative of the carboxyl group, the alpha-amino acid side chain, and the other substituent of the alpha-carbon atom. A generalized substrate may be represented by the formula R_2 $CHNH(R_1)COR_3$. It is the R_3 group, the group attached to the carboxyl moeity of the alpha-amino acid, which is hydrolysed by alpha-chymotrypsin. R_3 groups which have been found to be susceptible to hydrolysis include: amides (1-7), ethyl esters (1), hydrazides (1,8), methyl esters (1), and hydroxamide (1,9). Investigations have established that the R_2 group may be the residue corresponding to that found in L-tyrosine (1,2,6,7,9), L-phenylalanine (1,6), L-tryptophane (1,2,3,5), L-hexahydrophenylalanine (7), L-methionine (1), arginine (1), norleucine (1), or norvaline (1). Substrates with R_1 being benzoyl (1), acetyl (1,3,4,7,9), nicotinyl (1,3,4,5,8), carbobenzoxy (1), carbobenzoxyglycyl (1), carbobenzoxyglutamyl (1), chloroacetyl (6), and trifluoroacetyl (6) are known. Only a small number of the possible permutations for R_1, R_2, and

R_3 have been considered.

The effect of the stereoisomerism of alpha-amino acids upon enzymic hydrolysis has been extensively studied (10-13). To date the data obtained from investigations of a series of enantiomorphic pairs have shown that in every case the L-isomer is hydrolysed, and the D-isomer is not hydrolysed. This stereoisomeric specificity is not due to the inability of the enzyme and the D-isomer to interact, since the D-isomers act as competitive inhibitors for the L-isomers. However, no generalization stating that all D-isomers are not hydrolysed is justified. A broad statement of this type is simply not warranted on the basis of the small number of substrates studied and the fact that it is conceivable that the present experimental procedures are inadequate to detect the rate of hydrolysis of isomers of the unnatural configuration.

Inhibition of the hydrolysis reaction may be presumed to occur when the substrate is blocked from the formation of a reactive transition state with the active catalytic site of the enzyme. The hypothesis that the interaction of a trifunctional substrate and the enzyme takes place at three centers of the catalytically active site has received substantiation (3,5,6,12,14,15,16) and refines the definition of inhibition to mean inhibition at one or more of these centers. Thus, inhibition by the D-isomer of a specific

substrate is merely one case in which a molecule which is not measurably hydrolysed by the enzyme contains the structural requirements for interaction with one or more centers of the active site of the enzyme. It is clear that inhibition may be monofunctional, bifunctional, or trifunctional in so far as it is caused by a specie which is respectively capable of interacting with one, two, or all three of the centers. In this respect the hydrolysis products of non-acylated alpha-amino acids may act as monofunctional inhibitors. Similarly, the hydrolysis products of acylated alpha-amino acids may act as monofunctional and as bifunctional inhibitors. Inhibition by the hydrolysis products is presumed to be due to the alpha-amino acid residue and not to the other hydrolysis product, since no inhibition by these other products has been observed when they have been added independently to the reacting system.

The process of inhibition need not be the simple case in which the inhibitor occupies one or more of the centers of the active site of the enzyme and consequently prevents the formation of the enzyme-substrate complex. It is apparent that a substrate possessing similar functional groups may in effect act as its own inhibitor by interacting in a manner which prevents the formation of the reactive transition state of the enzyme-substrate complex. In addition, the formation of ternary complexes (5) involving the inhibitor, the substrate, and the enzyme can occur.

THE FORMULATION OF THE KINETIC EQUATIONS

The rate of reaction of the alpha-chymotrypsin catalysed hydrolysis of acylated alpha-amino acid hydrazides is a function of several variables. Since it was desired to limit the variation to that of the specific structure of the substrate, experimental conditions were selected and maintained so that, except for the nature of the substrate, the reaction system was maintained constant. The rate equations presented in this section were formulated with this basic assumption.

In the formulation of the rate equations used in this study the intermediate enzyme-substrate postulate has been assumed (17). The rate determining step for the overall hydrolysis reaction is presumed to be the macroscopically irreversible decomposition of the complex into free enzyme and products of hydrolysis. (It can be noted that the papain catalysed synthesis of acylated L- and D- alpha-amino acid hydrazides and phenylhydrazides (18-20) and the alpha-chymotrypsin catalysed synthesis of acylated L-tyrosyl-, L-tryptophanyl-, and L-phenylalanylphenylhydrazides (21) have been accomplished.) The enzyme liberated from this decomposition is held to be equivalent to the free enzyme before binding with the substrate into the intermediate

complex, and thus no loss of effective enzyme catalytic strength occurs as a result of the hydrolysis. In these studies the need for studying the effects of the inhibition due to any of the reaction products is obviated by the fact that in all cases any inhibition is precluded by the very small concentrations of the possible inhibitors.

The symbols used in the development of the rate equations (17, 22-27) are:

[E] = formal concentration of the free enzyme

[S] = formal concentration of the free substrate

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

P = products of the hydrolysis reaction

The rate equations are formulated as follows:

Let the reactions of the free enzyme, free substrate, and enzyme-substrate complex be represented thus:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad (1)$$

where k_1, k_2, and k_3 are the specific rate constants for the reactions expressed by the arrows.

If the decomposition of the complex is the rate determining step, then:

$$-\frac{d[S]}{dt} = k_3 [ES] \qquad (2)$$

The rate of formation of the complex is:

$$\frac{d[ES]}{dt} = k_1 [E] [S] - (k_2 + k_3) [ES] \qquad (3)$$

For the condition of a steady state, i.e.

where $- \frac{d[ES]}{dt} \ll -\frac{d[S]}{dt}$,

then, $\frac{d[ES]}{dt} \doteq 0 \doteq k_1[E][S] - (k_2 + k_3) [ES]$ (4)

Define K_s, the Michaelis-Menten constant, as:

$$K_s = \frac{[E][S]}{[ES]} \qquad (5)$$

For a steady state condition:

$$K_s = \frac{k_2 + k_3}{k_1} \qquad (6)$$

With the assumption that $E = E_o - ES$, the equation for the rate of disappearance of the substrate becomes

$$- \frac{d[S]}{dt} = k [ES] = \frac{k_3 [E_o] [S]}{K_s + [S]} = V \qquad (7)$$

A form of this equation (7) which relates the experimentally derived variables, S and V, in a manner more suitable for graphical presentation to obtain K_s and k_3 is (28-30)

$$\frac{1}{V} = \frac{K_s}{k_3[E_o]} \cdot \frac{1}{[S]} + \frac{1}{k_3[E_o]} \qquad (8)$$

The kinetics for the competitive hydrolysis of two substrates has been used herein for the determination of the constants for a substrate which were unobtainable by ordinary procedures. The method of competitive hydrolysis has been used previously to offer further evidence that certain substrates are hydrolysed at the same catalytically active site of the enzyme (2). The formulation of the rate equation for the case of competitive hydrolysis is as follows:

The Michaelis-Menten constants of the two substrates are defined:

$$K_{s1} = \frac{[E][S_1]}{[ES_1]} \quad \text{and} \quad K_{s2} = \frac{[E][S_2]}{[ES_2]} \tag{9}$$

The rate of disappearance of the total substrate is:

$$-\frac{d[S]}{dt} = -\frac{d[S_1]}{dt} - \frac{d[S_2]}{dt} = k_{31}[ES_1] + k_{32}[ES_2] \tag{10}$$

assuming that $-\frac{d[S_i]}{dt} \gg \frac{-d[ES_j]}{dt}$ $\quad i = 1,2$ $\quad j = 1,2$

The hydrolysis of the substrates is presumed to be competitive and thus:

$$K_{s1} = \frac{[E_o] - [ES_1] - [ES_2]\,[S_1]}{[ES_1]}$$

$$K_{s2} = \frac{[E_o] - [ES_1] - [ES_2]\,[S_2]}{[ES_2]} \tag{11}$$

Substitution yields:

$$[ES_2] = \frac{[E_o][S_2]K_{s1}}{K_{s1}K_{s2} + [S_2]K_{s1} + [S_1]K_{s2}} \quad (12)$$

and $$[ES_1] = \frac{[E_o][S_1]K_{s2}}{K_{s1}K_{s2} + [S_2]K_{s1} + [S_1]K_{s2}} \quad (13)$$

Thus, $$-\frac{d[S]}{dt} = \frac{k_{31}K_{s2}[E_o][S_1] + k_{32}K_{s1}[E_o][S_2]}{K_{s1}K_{s2} + [S_2]K_{s1} + [S_1]K_{s2}} = V \quad (14)$$

This may be rearranged to produce:

$$k_{31}[E_o] = \frac{VK_{s2}[S_1]}{K_{s2}[S_1] + \frac{k_{32}[E_o]K_{s1}[S_2]}{k_{31}[E_o]} - \frac{K_{s1}K_{s2}V}{k_{31}[E_o]} - \frac{K_{s1}[S_2]V}{k_{31}[E_o]}} \quad (15)$$

Equation (8) in one of its forms has been used to determine the values of K_s and k_3. It is apparent from this equation and from its integrated form, viz. $k_3E_ot = K_s\ln [S_o]/[S] + [S_o]-[S]$, that the order of the hydrolysis reaction is neither a pseudo first order reaction nor a pseudo zero order reaction; it is rather a combination of the two types of reactions in most instances. The determination of a velocity which can be associated with a substrate concentration necessitates the calculations, usually

by graphical means, of the tangents to the curves expressing the substrate concentration-time relationship. An alternate method is to calculate the velocities for several substrate initial concentrations at a point corresponding to zero time. These initial velocities are obtained by extrapolation to zero time from graphs of assumed zero order and first order reactions.

The determination of the values of K_s and k_3 from one of the forms of equation (8) is unambiguous if the reaction is one which actually proceeds via a composite of zero and first order kinetics. The accuracy of the values obtained in these cases is dependent only upon the accuracies of the experiments and subsequent calculations.

If, however, the reaction appears to proceed almost entirely via first order kinetics, one may obtain only a ratio of K_s to k_3 from this analysis procedure. (A distinction must be made between a reaction proceeding via pseudo first order kinetics and a reaction for which pseudo first order kinetics are assumed for the purpose of calculating velocities at zero time. In the latter case no assumption is made that the reaction itself proceeds via first order kinetics. The basic assumption is rather that at the early stages of the reaction a linear relationship between ln (S_o/S) and time exists for any particular substrate concentration. From this relationship an apparent first order

rate constant can be calculated. This apparent first order rate constant however varies with the initial substrate concentration; if it remained a constant independent of the initial substrate concentration, a true first order reaction would be occurring. One may use a linear relation between the time and the substrate concentration, viz. a zero order graph, in the same sense without assuming the reaction itself proceeded solely as a zero order reaction.) The conditions for this case are that $K_s \gg S_o$, since for this relationship equation (8) becomes an equation describing pure first order kinetics. It is obvious that only a ratio of K_s to k_3 obtains under such a circumstance.

Attempts have been made to interpret the activities of a series of substrates on the basis of the so-called first order proteolytic coefficients (31-35) and maximum first order proteolytic coefficients (36-37). It has been shown that neither of these methods is valid (36-38). It is therefore apparent that the ratio of K_s to k_3 cannot describe the enzyme-substrate system unambiguously.

For the case that $K_s \gg S_o$ and consequently for which the constants K_s and k_3 cannot be independently ascertained via a plot of equation (9), one may use a procedure based upon a study of the competitive hydrolysis of this substrate and a substrate for which the constants K_s and k_3 are known. It is evident from equation (15) that the k_3 value of one sub-

strate may be determined from the data of competitive hydrolysis, i.e. from rate of reaction data using mixtures of two substrates, when coupled with the known values of its ratio of K_s to k_3 and the K_s and k_3 values of the other substrate. The application of this competitive hydrolysis procedure cannot be made indiscriminately. The assumption of a condition of competitive hydrolysis demands that the condition be fulfilled whenever the equation describing it is applied. There are thus limits which must be placed upon the rate and affinity characteristics of the substrates as well as upon the relative amount of each which is used.

DISCUSSION OF THE EXPERIMENTAL RESULTS

In order that the kinetic constants determined from the rate of reaction data of this study have any meaning, i.e. in order that they may be compared with the kinetic constants for other substrates, all of the parameters of the system except the variation of the specific substrate were maintained constant. The parameters which were recognized and controlled so as to be constant were the pH, the temperature, the solvent, the ionic strength, and the enzyme concentration. In addition, the analytical procedure for the quantitative determination of the hydrazine was standardized.

For each of the substrates, the relative rates of hydrolysis at various pH values were determined first. All of the kinetic data for each substrate were then obtained at the pH for which the rate of reaction was a maximum. From graphs of assumed first and zero order reactions, i.e. from graphs of ln (S_o/S) vs. time and (S_o-S) vs. time, values for the initial velocity V_o were obtained. The initial velocities at various initial substrate concentrations were used to find the values of the kinetic constants K_s and k_3 from graphs of equation (8).

The values of K_s derived in this manner were used to secure the corrections to the time scale for the first and zero order reactions (7). The procedure for the calculation of the initial velocities at various initial substrate concentrations was repeated for both the first and zero order reactions using the corrected time scales. Finally, K_s and k_3 values were once again obtained from plots of equation (8).

For each of the four substrates studied the reaction system was controlled so that the solvent was water, the temperature was 25.0 ± 0.1°C, the solution was 0.02 F with respect to the amine component of the tris-(hydroxymethyl) aminomethane-tris-(hydroxymethyl)aminomethane · HCl buffer, the initial free enzyme concentration was 0.208 mg. protein nitrogen per ml., and the pH was maintained within 0.1 unit of the pH optimum for the particular system.

The kinetic constants of the four substrates, determined by a graphical treatment, are presented in table I. The kinetic data are given in tables II, III, IV, V, and VI. The graphical solutions using the equation

$$\frac{1}{V_o} = \frac{1}{[S_o]} \cdot \frac{K_s}{k_3[E_o]} + \frac{1}{k_3[E_o]}$$

are portrayed in figures 2, 4, 6, and 8. The determination of the pH optima are illustrated in figures 1, 3, 5, and 7.

When the hydrazides are compared with the amides and hydroxamides of similarly acylated L - tyrosine compounds, one important and distinct difference of the hydrazides is noted--all of the k_3 values for the hydrazides are appreciably lower. These differences must be due to the remarkable influence of the hydrazido group upon the mechanism of hydrolysis. A reasonable mechanism for the hydrolysis is an attack of a hydroxyl ion or a water molecule on the carbonyl carbon atom. Such a nucleophilic attack is facilitated by any resonance forms or inductive effects which increase the relative positive charge on this carbon atom. The hydrazido group acts as an electron donating center and in virtue of its adjacent position effectively diminishes the magnitude of a positive charge on the carbonyl carbon atom. The degree to which the hydrazido group functions in this manner is greater than that of the amido or hydroxamido groups.

The pH optimum for L - tyrosinhydrazide is ca 0.7 pH unit lower than the optima for the acylated hydrazides. Since it is reasonable, as has been noted, that the hydrolysis reaction is accelerated by an increasing positive charge on the carbonyl carbon atom, the low value of the pH optimum for this particular substrate may be interpreted as representing an acidity for which the charge is a maximum. However, the pH optimum does not occur at an acidity which would

represent a condition for maximum charge. This maximum results when the accumulative effects of groups acting as electron sinks for the carbonyl carbon atom is the greatest. The amino group acts in the capacity of an electron donor and opposes the buildup of a positive charge in the adjacent carbon position. Acylation of the amino group greatly decreases its basicity. Protonation of the group transforms it into an effective electron attracting site. If the rate of hydrolysis actually paralleled the concentration of the substrate having a protonated amino group, the pH optimum would be less than its value of 7.1. This conclusion is deducible from a comparison of the pKa value of the protonated amino group of an alpha-amino acid hydrazide, viz. pKa = 7.69 for glycylhydrazide (39). That this parallelism does not obtain is evidence that the pH optimum is the point of intersection of curves relating the separate dependence of the rate of hydrolysis of L - tyrosinhydrazide and of the activity of alpha-chymotrypsin on pH, the hydrolysis curve decreasing and the activity curve increasing at pH 7.1, the optimum value.

The value of k_3 for L - tyrosinhydrazide, 0.02×10^{-3} M per mg. protein nitrogen per ml., is remarkably low. A reasonable explanation is that since the more readily hydrolysable specie is not present in an overwhelming proportion, the observed rate is that for a mixture, one compon-

ent of which has a relatively low rate of hydrolysis. This hypothesis is subject to quantitative examination, if not in an enzymic system, at least in non-enzymic systems where the acidities may be varied. The results, as shown in figure 9, demonstrate that the log of the rate of hydrolysis is proportional to the pH of the solution and is not proportional hence to the hydrogen ion concentration. One may conclude, then, that in an acidic non-enzymic system it is essentially the protonated amino group specie which is being hydrolysed and that the rate determining step is an attack by a water molecule. This relationship can be contrasted with that of an acylated substrate, acetyl L-tyrosinhydrazide, which is hydrolysed under identical non-enzymic conditions. In this instance the rate of hydrolysis is directly proportional to the hydrogen ion activity. Acylation of the amino group reduces its basicity to such an extent that a reaction analogous to that of L-tyrosinhydrazide cannot occur. This result is illustrated in figure 10.

Although results derived from a non-enzymic system cannot justifiably be applied strictly to an enzymic system, one may at least consider their implications. The presence of substrate species having protonated and free amino groups can certainly be construed to mean that possibly there are two different enzyme-substrate complexes. Aside from any spatial or steric requirements, the center of the active

site which corresponds with the R_1 group appears to bind with more stability to groups of decreased electron density (40,41,15). Thus, the complex of the enzyme with the protonated amino group specie is the more stable of the two possible complexes. One may conclude, therefore, by making the assumption that the complex containing the protonated amino group specie is hydrolysed much more rapidly than is the other complex, i.e. by assuming an analogy between the non-enzymic and the enzymic systems, that the value of k_3 determined for L-tyrosinhydrazide by the customary use of equation (8) is not a k_3 for the substrate. The relationship between these two values is illustrated as follows:

Let $[S]_t$ = total formal free substrate concentration

$[SH^+]$ = protonated alpha-amino group formal concentration

$[S]$ = unprotonated alpha-amino group formal concentration

Define: $Ke = \dfrac{[SH^+]}{[S]}$ at the pH optimum

Then, $\dfrac{[S]_t}{[SH^+]} = \dfrac{1 + Ke}{Ke}$

Assume, $-\dfrac{d[SH^+]}{dt} = k_3 [ESH^+] = \dfrac{k_3^{SH^+}[E_o][SH^+]}{K_s^{SH^+} + [SH^+]} = V$

Now, V is the experimental velocity if $-\dfrac{d[SH^+]}{dt} \gg -\dfrac{d[S]}{dt}$

The constants derived by the conventional procedure are:

$$V = \frac{k_3^{exp}[E_o][S]_t}{K_s^{exp} + [S]_t}$$

However, the velocity connected with the total substrate disappearance is:

$$- \frac{d[S]_t}{dt} = V \frac{1 + Ke}{Ke} = VY$$

Substituting, the true equation for the total substrate becomes:

$$\frac{1}{VY} = \frac{1}{[S]_t} \frac{K_s^t}{k_3^t[E_o]} + \frac{1}{k_3^t[E_o]}$$

As a result of the corrected equation, the true k_3 for L-tyrosinhydrazide is $Y\, k_3^{exp.}$ and the true K_s^t is equal to $K_s^{exp.}$.

In so far as the K_s values may be interpreted as being measures of the relative stabilities of the enzyme-substrate complexes, one can specify that benzoyl L-tyrosinhydrazide forms a more stable complex than does nicotinyl L-tyrosinhydrazide. The presence of a negative charge at or near the catalytically active site (1) may seem to substantiate an argument on the basis of relative electrostatic repulsion. However, the K_s values for the substrates acetyl L-tyrosina-

mide (42), trifluoroacetyl L-tyrosinamide (6), and chloroacetyl L-tyrosinamide (6) indicate that the magnitude of such a repulsion effect is too small to affect the experimental K_s values. An explanation can be offered, however, on the basis of other energy considerations. The two states of the system to be examined with respect to free energy differences are the initial state of free enzyme and free substrate molecules and the final state of the activated transition complex. If the mating of the two entities is different, i.e. if the nicotinyl substituted substrate does not fit as well on the enzyme surface as does the benzoyl substituted substrate, the former will require an additional amount of energy for the formation of the transition state. This is simply a consequence of the fact that the creation of a hole in a liquid requires energy, and the amount of energy required is a direct measure of the size of the hole. The elimination of water molecules from both the enzyme and the substrates is necessary to assure a closeness of approach sufficient to permit interaction. The work required to remove the water molecules from the active site of the enzyme should be substantially identical for the two cases. However, the nicotinyl residue is more hydrophilic than is the benzoyl residue. Hence, more energy is required for this process in the case of the nicotinyl compound. In addition to these considerations of work energy requirements for

the creation of the transition state, one must examine the entropy variations for a suitable comparison. The chemisorption of the substrate on the enzyme (43) results in a decrease in entropy, a greater decrease accompanying a more rigid, or tighter fitting, complex. The release of the water molecules in this process causes an increase in the entropy. If a water molecule is involved in the transition state, a decrease in entropy occurs. For the sake of a comparison of entropy increments for the two cases only the increments due to chemisorption and water molecule release vary. Thus, the overall evaluation of the free energy changes obtaining when the transition states of the two systems are formed is certainly not a simple matter. Little success can be attributed to any such evaluation which ignores any of the factors without adequate reason. It is apparent, therefore, that a critical evaluation of the various contributions to the delta free energies of the formation of the two transition states is not possible at present.

As has been noted, the use of a competitive hydrolysis study in conjunction with the usual rate of hydrolysis study permits the evaluation of the kinetic constants K_s and k_3 in those cases in which $K_s \gg S_o$. This method is also applicable when the constants are theoretically operationally obtainable, i.e. when K_s is not $\gg S_o$, but when the calculation of unambiguous values is mechanically difficult. This

case is encountered for substrates for which the values of K_s and k_3 are such that a plot of S_o/V_o vs. S_o for equation (8) is nearly zero. The substrate benzoyl L-tyrosinhydrazide offered an example for both cases, being so insoluble that, even with a very small K_s value, not a sufficient amount could be used to determine K_s and k_3 independently from equation (8) and having values of K_s and k_3 such that a determination of independent values was impossible unless the range of concentrations could be greatly extended. For this substrate the general procedure was successfully employed, obtaining a K_s to k_3 ratio from a plot of equation (8) and a k_3 value from data of a study of competitive hydrolysis wherein the other substrate was acetyl L-tyrosinhydrazide.

EXPERIMENTS AND DATA

Buffer Solutions

The aqueous stock solutions of buffer were prepared from tris-(hydroxymethyl)-aminomethane which had been recrystallized twice from aqueous methanol (m.p. 169.0 - 169.5). These solutions were 0.20 F with respect to the amine component of the buffer. The varying pH values were obtained by the dropwise addition of concentrated hydrochloric acid. A dilution of 1 to 10 yielded substrate-enzyme systems which were 0.020 F with respect to the amine component of the buffer.

Aldehyde Reagent Solutions

The p-dimethylaminobenzaldehyde used for the aldehyde reagent solutions was, on different occasions, reagent grade (Matheson and Co.), reagent grade recrystallized from aqueous methanol, and practical grade purified by reprecipitation by sodium hydroxide from a hydrochloric acid solution and twice crystallized from aqueous methanol (m.p. 74.5-75). The solution was one containing 1 gm. of the aldehyde for 100 ml. of solution. The data from calibrations against known concentrations of hydrazine and from kinetic studies indicated that these solutions were sensibly constant even though they became colored upon

standing exposed to light for periods up to a week.

Enzyme Solutions

The enzyme solutions were prepared from alpha-chymotrypsin of bovine extract (Armour Co., Lot No. 10705). These solutions were kept at 25.0 ± 0.1°C for the time interval during which the aliquot portions were transferred to the reaction systems. No aliquots were taken from stock solutions which had been at 25°C more than one hour. The reaction systems contained 0.208 mg. protein nitrogen per ml., the protein nitrogen content having been determined by the Kjeldahl method after precipitation by trichloro-acetic acid.

Enzymic Reaction and Analysis Procedures

A 10 ml. volumetric flask containing 9 ml. of an aqueous solution of the substrate, of which 1 ml. was 0.20 F buffer, was placed in a Sargent Constant Temperature bath. The temperature was maintained at 25.0 ± 0.1°C. A period of 30 minutes was allowed for the temperature of these solutions to become equilibrated. The enzyme stock solutions were prepared in 5 ml. volumetric flasks, the dissolving of the enzyme being accomplished by gentle repeated inversions and swirlings to avoid excess foaming. At zero time a 1 ml. aliquot of the enzyme stock solution was transferred to the flask containing the substrate solu-

tion, and the mixture was gently inverted and swirled six to seven times. One ml. aliquots from this mixture were then transferred periodically into 10 ml. flasks containing 1 ml. of the aldehyde stock solution, 1 ml. of 1.87 N HCl, and ca 5 ml. of water. These azine reaction flasks were filled to the mark and inverted and swirled six to seven times. A minimum time of fifteen minutes was allowed for complete color development, i.e. complete azine formation. No appreciable changes in optical density were found if the development time was extended to 45 minutes. The optical densities of the bis-p-dimethylaminobenzalazine in aqueous acid solution at 455 mμ were determined using 1 cm. silica cells in a Beckman Model B spectrophotometer. The absorption at this wave length follows Beers' Law when the hydrazine to be determined varied from 1 x 10^{-5}F to 50 x 10^{-5}F.

Syntheses of the Substrates

Acetyl L-tyrosinhydrazide (I)

L-tyrosine was acetylated under Schotten-Bauman conditions and esterified by the usual procedure using HCl gas. (I) was obtained from an ethanol solution of the ethyl ester and 85% hydrazine hydrate refluxed for 2 hours, recrystallized twice from methanol, and dried in vacuo over phosphorous pentoxide, m.p. 227-228 (corr.)

Anal. Calcd. for $C_{11}H_{16}O_3N_3$: C,55.70; H,6.36; N,17.71

Found: C,55.69; H,6.33; N,17.79

Nicotinyl L-tyrosinhydrazide (II)

L-tyrosine ethyl ester was acylated using nicotinyl azide in an ethyl acetate solution. (II) was obtained by the method of (I), m.p. 242-243 (corr.).

Anal. Calcd. for $C_{15}H_{16}O_3N_4$: C,60.00; H,5.37; N,18.66

Found: C,60.14; H,5.35; N,18.76

L-tyrosinhydrazide (III)

(III) was obtained from the methyl ester by allowing a methanol solution with 85% hydrazine hydrate to stand at room temperature for 3 days, recrystallized twice from ethanol, and dried *in vacuo* over phosphorous pentoxide, m.p. 193-194 (corr.).

Anal. Calcd. for $C_9H_{13}O_2N_3$: C,55.40; H,6.71; N,21.52

Found: C,55.40; H,6.76; N,21.42

Benzoyl L-tyrosinhydrazide (IV)

(IV) was prepared from benzoyl L-tyrosine ethyl ester by the procedure of (I), m.p. 247-248 (corr.).

Anal. Calcd. for $C_{16}H_{19}O_3N_3$: C,64.20; H,5.72; N,14.04

Found:[1] C,64.21; H,5.63; N,14.07

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[1]Microanalyses by Dr. A. Elek, Elek Micro Analytical Laboratories, Los Angeles, California.

TABLE I

The Calculated Values of the K_s and k_3 Constants for the Alpha-Chymotrypsin Catalysed Hydrolysis of L-tyrosinhydrazide, Acetyl L-tyrosinhydrazide, Nicotinyl L-tyrosinhydrazide and Benzoyl L-tyrosinhydrazide at 25.0 ± 0.1°C.

Substrate	pH Optimum	K_s	k_3
Acetyl-L-tyrosinhydrazide	7.9	33.	0.90
Benzoyl-L-tyrosinhydrazide	7.9	2.0	0.48
Nicotinyl-L-tyrosinhydrazide	7.8	9.1	0.97
L-tyrosinhydrazide	7.1	5.7	0.02

K_s in units of 10^{-3}M

k_3 in units of 10^{-3}M/min. /mg. protein-nitrogen/ml.

The following symbols pertain to tables II-VI inclusive:

D_t = optical density at 455 mμ at time t

D_{oo} = optical density at 455 mμ corresponding to 100% hydrolysis

t' = time corrected for first order rate reaction

t'' = time corrected for zero order rate reaction

TABLE II

The Alpha-Chymotrypsin Catalysed Hydrolysis of L-tyrosinhydrazide at pH 7.1 and 25.0°C

$[S]_o \times 10^4$	t(min)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	$\log\frac{D_{oo}}{D_{oo}-D_t}$	t'	t''
41.2	15	.208	15.53	1.013	.00561	15	15
	45	.469	15.27	1.032	.0137	45	45
	75	.687	15.05	1.047	.0200	75	75
	105	.898	14.84	1.061	.0257	105	105
	135	1.08	14.66	1.074	.0310	130	135
	165	1.31	14.43	1.092	.0382	160	165
	195	1.48	14.26	1.104	.0430	190	195
				$V_o = 2.98 \times 10^{-8}$ M sec.$^{-1}$			
41.2	15	.201	15.54	1.013	.00561	15	15
	45	.456	15.28	1.032	.0137	45	45
	75	.663	15.08	1.045	.0191	75	75
	105	.867	14.87	1.059	.0249	105	105
	135	1.07	14.67	1.073	.0306	130	135
	165	1.28	14.46	1.090	.0374	160	165
	195	1.37	14.37	1.096	.0399	190	195
				$V_o = 2.98 \times 10^{-8}$ M sec.$^{-1}$			
30.6	15	.168	11.52	1.014	.00604	15	15
	45	.377	11.31	1.033	.0141	45	45
	75	.551	11.14	1.049	.0208	75	75
	105	.711	10.98	1.066	.0278	105	105
	135	.923	10.77	1.087	.0362	130	135
	165	1.05	10.64	1.098	.0406	160	165
	195	1.14	10.55	1.108	.0445	190	195
				$V_o = 2.50 \times 10^{-8}$ M sec.$^{-1}$			
30.6	15	.174	11.52	1.014	.00604	15	15
	45	.374	11.32	1.033	.0141	45	45
	75	.543	11.15	1.048	.0204	75	75
	105	.709	10.98	1.066	.0278	105	105
	135	.882	10.81	1.081	.0338	130	135
	165	1.05	10.64	1.098	.0406	160	165
	195	1.17	10.52	1.111	.0457	190	195
				$V_o = 2.50 \times 10^{-8}$ M sec.$^{-1}$			

TABLE II (cont.)

$[S]_o \times 10^{-4}$	t(min)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	$\log\frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
25.2	5	.057	9.57	1.006	.00260	5	5
	10	.095	9.53	1.010	.00432	10	10
	15	.120	9.51	1.013	.00561	15	15
	20	.150	9.48	1.016	.00689	20	20
	25	.175	9.45	1.019	.00817	25	25
	30	.200	9.43	1.021	.00903	30	30
	35	.223	9.41	1.023	.00988	35	35
	40	.245	9.38	1.027	.0116	40	40
	45	.273	9.36	1.029	.0124	45	45
				$V_o = 2.22 \times 10^{-8}$M sec.$^{-1}$			
24.7	15	.156	9.27	1.017	.00732	15	15
	45	.331	9.10	1.036	.0154	45	45
	75	.492	8.94	1.055	.0233	75	75
	105	.619	8.81	1.070	.0294	105	105
	135	.756	8.67	1.087	.0362	130	135
	165	.901	8.53	1.106	.0438	160	165
	195	1.02	8.41	1.121	.0496	185	195
				$V_o = 2.13 \times 10^{-8}$M sec.$^{-1}$			
24.7	45	.317	9.11	1.035	.0149	45	45
	75	.471	8.96	1.052	.0220	75	75
	105	.625	8.80	1.072	.0302	105	105
	135	.741	8.69	1.085	.0354	130	135
	165	.906	8.52	1.107	.0442	160	165
	195	.982	8.45	1.115	.0473	185	195
				$V_o = 2.13 \times 10^{-8}$M sec.$^{-1}$			
18.3	15	.127	6.66	1.018	.00775	15	15
	45	.254	6.54	1.037	.0158	45	45
	75	.374	6.42	1.057	.0241	75	75
	105	.509	6.28	1.082	.0342	100	105
	135	.597	6.19	1.097	.0402	130	135
	165	.698	6.09	1.115	.0473	160	165
	195	.795	5.99	1.133	.0542	185	195
				$V_o = 1.59 \times 10^{-8}$M sec.$^{-1}$			

TABLE II (cont.)

$[S]_o \times 10^{-4}$	t(min)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	$\log\frac{D_{oo}}{D_{oo}-D_t}$	t'	t''
18.3	15	.124	6.67	1.017	.00732	15	15
	45	.262	6.53	1.039	.0166	45	45
	105	.495	6.29	1.080	.0334	100	105
	135	.571	6.22	1.092	.0382	130	135
	165	.657	6.13	1.108	.0445	160	165
	195	.747	6.04	1.124	.0508	185	195
				$V_o = 1.59 \times 10^{-8}$M sec.$^{-1}$			
18.0	5	.046	6.83	1.008	.00346	5	5
	10	.066	6.81	1.010	.00432	10	10
	15	.090	6.79	1.014	.00604	15	15
	20	.114	6.77	1.016	.00689	20	20
	25	.130	6.75	1.019	.00817	25	25
	35	.170	6.71	1.024	.0107	35	35
	40	.187	6.69	1.029	.0124	40	40
	45	.208	6.67	1.030	.0128	45	45
				$V_o = 1.77 \times 10^{-8}$M sec.$^{-1}$			
16.5	15	.114	6.19	1.018	.00775	15	15
	45	.238	6.06	1.039	.0166	45	45
	75	.347	5.95	1.058	.0245	75	75
	105	.462	5.84	1.078	.0326	100	105
	135	.569	5.73	1.100	.0414	130	135
	165	.659	5.64	1.117	.0481	160	165
	195	.744	5.56	1.132	.0539	185	195
				$V_o = 1.53 \times 10^{-8}$M sec.$^{-1}$			
10.8	5	.035	4.09	1.010	.00432	5	5
	10	.045	4.08	1.012	.00518	10	10
	15	.061	4.07	1.015	.00647	15	15
	20	.074	4.06	1.017	.00732	20	20
	25	.087	4.04	1.022	.00945	25	25
	30	.100	4.03	1.025	.0107	30	30
	35	.114	4.02	1.027	.0116	35	35
	40	.122	4.01	1.030	.0128	40	40
	45	.136	3.99	1.035	.0149	45	45
				$V_o = 1.14 \times 10^{-8}$M sec.$^{-1}$			

TABLE II (cont.)

$[S]_o \times 10^{-4}$	t(min)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	$\log\frac{D_{oo}}{D_{oo}-D_t}$	t'	t''
12.2	15	.097	4.56	1.022	.00945	15	15
	45	.194	4.47	1.043	.0183	45	45
	75	.284	4.38	1.064	.0269	75	75
	105	.377	4.28	1.089	.0370	100	105
	135	.445	4.21	1.107	.0442	130	135
	165	.518	4.14	1.125	.0512	160	165
	195	.592	4.07	1.145	.0588	185	195
				$V_o = 1.25 \times 10^{-8}$M sec.$^{-1}$			
12.2	15	.092	4.57	1.020	.00860	15	15
	45	.191	4.47	1.043	.0183	45	45
	75	.278	4.38	1.064	.0209	75	75
	105	.357	4.30	1.084	.0350	100	105
	135	.438	4.22	1.104	.0430	130	135
	165	.510	4.15	1.123	.0504	160	165
	195	.561	4.10	1.137	.0561	185	195
				$V_o = 1.25 \times 10^{-8}$M sec.$^{-1}$			
7.2	5	.025	2.725	1.009	.00389	5	5
	10	.035	2.715	1.013	.00561	10	10
	15	.045	2.705	1.017	.00732	15	15
	20	.051	2.700	1.019	.00817	20	20
	25	.060	2.690	1.022	.00945	25	25
	30	.070	2.680	1.026	.0112	30	30
	35	.077	2.673	1.029	.0124	35	35
	40	.085	2.665	1.032	.0137	40	40
	45	.093	2.657	1.035	.0149	45	45
				$V_o = 8.72 \times 10^{-9}$M sec.$^{-1}$			

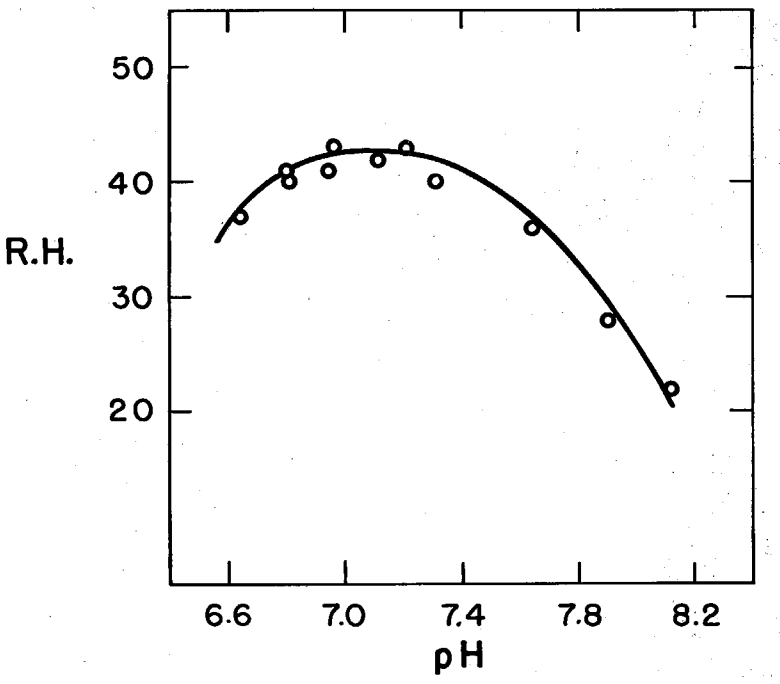

Fig. 1. pH-activity relationship of the system alpha-chymotrypsin-L-tyrosinhydrazide in aqueous solutions at 25°C and 0.02 M with respect to the amine component of a tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer. R.H.= relative activity.

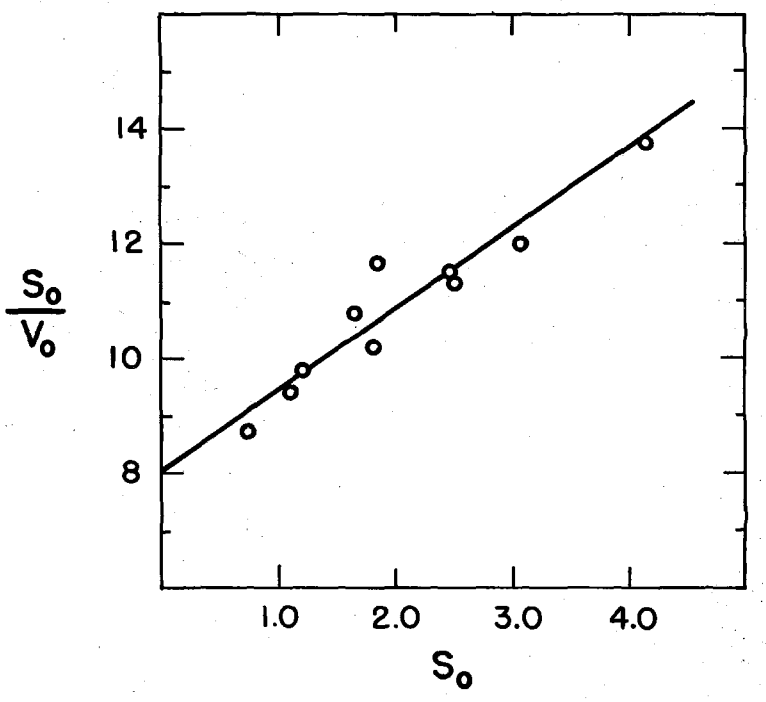

Fig. 2. S_o/V_o vs. S_o graph for the system of alpha-chymotrypsin-L-tyrosinhydrazide at pH 7.1 and 25.0°C. S_o is in units of 10^{-3}M. S_o/V_o is in units of 10^{+4} sec.

TABLE III

The Alpha-Chymotrypsin Catalysed Hydrolysis of Nicotinyl-L-tyrosinhydrazide at pH 7.8 and 25.0°C

$[S]_o \times 10^{-4}$	t(sec)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	$\log\frac{D_{oo}}{D_{oo}-D_t}$	t'	t''
43.6	60	.408	16.24	1.025	.0107	60	60
	120	.760	15.89	1.047	.0200	120	120
	180	1.08	15.57	1.070	.0294	175	180
	240	1.36	15.29	1.087	.0362	235	240
	300	1.66	14.99	1.110	.0453	290	305
	360	1.93	14.72	1.130	.0531	345	365
	420	2.18	14.47	1.151	.0611	405	425
				$V_o = 1.08 \times 10^{-6}$M sec.$^{-1}$			
38.2	60	.400	14.17	1.028	.0120	60	60
	120	.700	13.87	1.051	.0216	120	120
	180	1.00	13.57	1.073	.0306	175	180
	240	1.29	13.28	1.093	.0398	235	240
	300	1.54	13.03	1.117	.0481	290	305
	360	1.80	12.77	1.142	.0577	345	365
	420	2.05	12.52	1.162	.0652	400	425
	480	2.26	12.31	1.182	.0726	455	485
				$V_o = 1.01 \times 10^{-6}$M sec.$^{-1}$			
32.7	60	.370	12.12	1.028	.0120	60	60
	120	.655	11.83	1.054	.0228	120	120
	180	.920	11.57	1.080	.0334	175	180
	240	1.16	11.33	1.101	.0418	235	240
	360	1.66	10.83	1.152	.0615	345	365
	420	1.88	10.61	1.175	.0700	400	425
	480	2.00	10.49	1.190	.0756	455	485
				$V_o = 9.56 \times 10^{-7}$M sec.$^{-1}$			
27.3	60	.280	10.13	1.027	.0116	60	60
	120	.490	9.92	1.050	.0212	120	120
	180	.705	9.70	1.074	.0310	175	180
	240	.910	9.50	1.096	.0398	235	240
	300	1.11	9.30	1.119	.0488	290	300
	360	1.33	9.08	1.147	.0596	340	360
	420	1.50	8.91	1.168	.0674	405	420
	480	1.68	8.73	1.194	.0770	455	480
	540	1.87	8.54	1.222	.0871	495	545
				$V_o = 7.68 \times 10^{-7}$M sec.$^{-1}$			

TABLE III (cont.)

$[S]_o \times 10^{-4}$	t(sec)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	$\log\frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
16.4	60	.225	6.02	1.038	.0162	60	60
	120	.380	5.87	1.065	.0274	120	120
	180	.510	5.74	1.089	.0370	175	180
	240	.650	5.60	1.117	.0481	230	240
	300	.690	5.46	1.145	.0588	285	300
	360	.915	5.33	1.173	.0693	335	360
	420	1.05	5.20	1.202	.0799	385	420
	480	1.15	5.10	1.226	.0885	435	480
	540	1.31	4.94	1.266	.102	485	545
				$V_o = 5.33 \times 10^{-7}$M sec.$^{-1}$			
15.3	70	.146	5.69	1.026	.0112	65	70
	105	.214	5.63	1.037	.0158	105	105
	175	.334	5.51	1.058	.0245	170	175
	350	.611	5.23	1.117	.0481	330	350
	540	.887	4.95	1.179	.0751	500	540
	720	1.12	4.72	1.237	.0924	655	725
	900	1.42	4.42	1.321	.121	790	910
	1080	1.61	4.23	1.381	.140	940	1090
				$V_o = 4.44 \times 10^{-7}$M sec.$^{-1}$			
15.0	95	.243	5.49	1.044	.0187	95	95
	165	.389	5.34	1.074	.0310	160	165
	305	.672	5.06	1.132	.0539	290	305
	485	.995	4.73	1.213	.0839	445	485
	720	1.47	4.26	1.345	.129	635	725
	900	1.72	4.01	1.429	.155	765	910
	1080	1.88	3.85	1.488	.173	910	1090
	1260	2.04	3.69	1.554	.191	1030	1270
				$V_o = 5.08 \times 10^{-7}$M sec.$^{-1}$			
14.7	105	.267	5.34	1.053	.0224	105	105
	160	.369	5.24	1.073	.0306	155	160
	275	.585	5.02	1.118	.0484	270	275
	430	.867	4.74	1.185	.0737	395	430
	615	1.15	4.46	1.257	.0993	555	620
	785	1.42	4.19	1.342	.128	690	790
	970	1.62	3.99	1.407	.148	830	975
	1155	1.92	3.69	1.522	.182	960	1165
				$V_o = 5.07 \times 10^{-7}$M sec.$^{-1}$			

TABLE III (cont.)

$[S]_o \times 10^{-4}$	t(sec)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	$\log\frac{D_{oo}}{D_{oo}-D_t}$	t'	t''
10.9	60	.165	3.99	1.043	.0183	60	60
	120	.270	2.86	1.078	.0326	115	120
	180	.375	2.78	1.101	.0418	170	180
	240	.460	2.70	1.124	.0508	230	240
	300	.560	2.60	1.156	.0630	280	300
	360	.650	2.51	1.186	.0741	335	360
	420	.735	2.42	1.218	.0857	385	420
	480	.810	2.35	1.242	.0941	435	480
	540	.910	2.25	1.281	.108	485	540
				$V_o = 3.60 \times 10^{-7}$M sec.$^{-1}$			
10.0	75	.130	3.69	1.035	.0149	75	75
	125	.192	3.63	1.052	.0220	120	125
	240	.349	3.47	1.100	.0414	230	240
	350	.484	3.34	1.143	.0581	330	350
	535	.682	3.14	1.217	.0853	485	535
	720	.893	2.93	1.304	.115	640	720
	900	1.11	2.71	1.408	.149	775	900
	1080	1.23	2.59	1.475	.169	910	1090
				$V_o = 3.45 \times 10^{-7}$M sec.$^{-1}$			
10.0	75	.132	3.69	1.035	.0149	75	75
	115	.186	3.63	1.052	.0220	115	115
	240	.341	3.48	1.098	.0406	230	240
	355	.474	3.35	1.140	.0469	335	355
	540	.699	3.12	1.225	.0881	490	540
	720	.870	2.95	1.295	.112	640	720
	900	1.07	2.75	1.388	.142	775	900
	1080	1.22	2.60	1.470	.167	910	1090
				$V_o = 3.45 \times 10^{-7}$M sec.$^{-1}$			
9.67	90	.153	3.54	1.042	.0179	90	90
	160	.244	3.45	1.072	.0294	155	160
	285	.409	3.28	1.125	.0512	270	285
	500	.654	3.04	1.214	.0842	460	500
	735	.928	2.76	1.337	.126	645	740
	1030	1.19	2.50	1.475	.169	865	1040
	1450	1.57	2.12	1.740	.241	1150	1465
	1810	1.81	1.88	1.965	.293	1360	1830
				$V_o = 3.52 \times 10^{-7}$M sec.$^{-1}$			

TABLE III (cont.)

$[S]_o \times 10^{-4}$	t(sec)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	$\log\frac{D_{oo}}{D_{oo}-D_t}$	t'	t''
5.33	105	.103	1.94	1.052	.0220	105	105
	180	.162	1.88	1.085	.0354	175	180
	365	.280	1.76	1.160	.0645	340	365
	545	.378	1.66	1.230	.0899	495	545
	745	.492	1.55	1.315	.119	655	745
	1030	.633	1.41	1.446	.160	865	1030
	1455	.818	1.22	1.673	.224	1150	1455
	1810	.943	1.10	1.855	.268	1350	1830
				$V_o = 1.74 \times 10^{-7}$M sec.$^{-1}$			
5.00	75	.072	1.84	1.038	.0162	75	75
	115	.099	1.81	1.055	.0233	115	115
	180	.141	1.77	1.078	.0326	175	180
	360	.258	1.65	1.157	.0633	335	360
	725	.458	1.45	1.317	.120	640	725
	1090	.629	1.28	1.493	.174	905	1090
	1440	.775	1.13	1.691	.228	1140	1440
	1800	.896	1.01	1.893	.270	1350	1800
				$V_o = 1.73 \times 10^{-7}$M sec.$^{-1}$			
5.00	65	.075	1.83	1.043	.0183	65	65
	100	.101	1.81	1.055	.0233	100	100
	200	.163	1.75	1.092	.0382	190	200
	250	.267	1.64	1.164	.0660	235	250
	720	.456	1.45	1.317	.120	635	720
	1080	.650	1.26	1.515	.180	900	1080
	1450	.791	1.12	1.705	.232	1040	1450
	1800	.914	1.00	1.910	.281	1350	1820
				$V_o = 1.73 \times 10^{-7}$M sec.$^{-1}$			

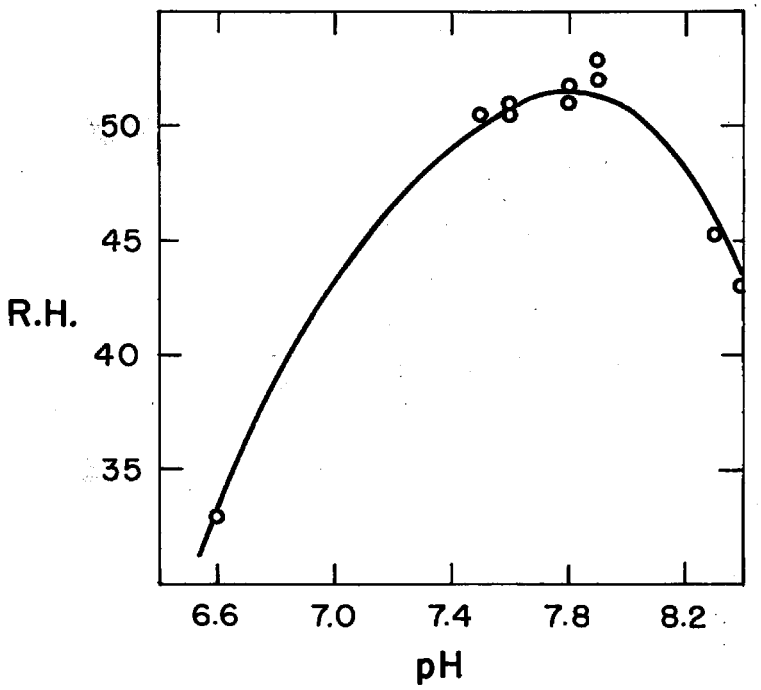

Fig. 3. pH-activity relationship of the system alpha-chymotrypsin-nicotinyl L-tyrosinhydrazide in aqueous solutions at 25°C and 0.02 $\overline{M}$ with respect to the amine component of a tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer. R.H.=relative activity.

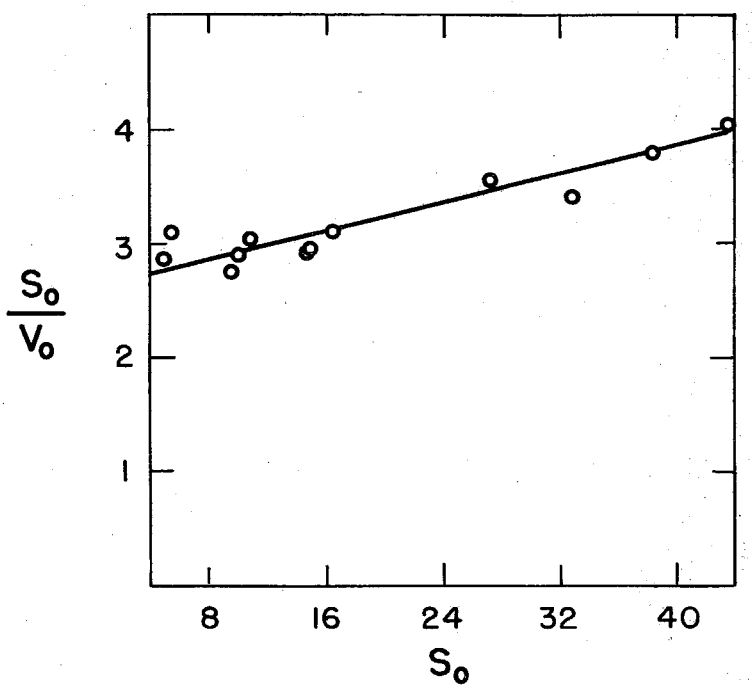

Fig. 4. S_o/V_o vs. S_o graph for the system of alpha-chymotrypsin-nicotinyl L-tyrosinhydrazide at pH 7.8 and 25.0°C. S_o is in units of 10^{-4}M. S_o/V_o is in units of 10^3 sec.

TABLE IV

The Alpha-Chymotrypsin Catalysed Hydrolysis of Acetyl-L-tyrosinhydrazide at pH 7.9 and 25.0°C

$[S]_o \times 10^{-4}$	t(sec)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	$\log\frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
66.7	60	.160	25.26	1.005	.00217	60	60
	150	.344	25.08	1.014	.00604	150	150
	240	.523	24.90	1.022	.00945	240	240
	360	.757	24.66	1.032	.0137	355	360
	480	.979	24.44	1.040	.0170	475	480
	690	1.35	24.07	1.056	.0237	675	695
	750	1.48	23.94	1.063	.0265	735	755
				$V_o = 5.33 \times 10^{-7}$M sec.$^{-1}$			
66.7	60	.161	25.26	1.005	.00217	60	60
	150	.334	25.09	1.014	.00604	150	150
	240	.509	24.91	1.022	.00945	240	240
	360	.738	24.68	1.032	.0137	355	360
	480	.980	24.44	1.040	.0170	475	480
	600	1.21	24.21	1.050	.0212	590	605
	690	1.37	24.05	1.056	.0237	675	695
	750	1.50	23.92	1.063	.0265	735	755
				$V_o = 5.33 \times 10^{-7}$M sec.$^{-1}$			
66.7	60	.160	25.25	1.005	.00217	60	60
	165	.362	25.06	1.015	.00604	150	150
	240	.520	24.90	1.022	.00945	240	240
	360	.752	24.67	1.032	.0137	355	360
	480	.982	24.44	1.040	.0170	475	480
	600	1.20	24.22	1.050	.0212	590	605
	690	1.37	24.05	1.056	.0237	675	695
	750	1.50	23.92	1.063	.0265	735	755
				$V_o = 5.33 \times 10^{-7}$M sec.$^{-1}$			
42.3	90	.176	15.98	1.011	.00475	90	90
	180	.275	15.88	1.018	.00775	180	180
	300	.486	15.67	1.031	.0133	295	300
	480	.716	15.44	1.047	.0200	470	480
	840	1.20	14.96	1.080	.0334	815	850
	960	1.35	14.81	1.090	.0374	930	970
	1080	1.48	14.68	1.101	.0418	1050	1090
				$V_o = 3.51 \times 10^{-7}$M sec.$^{-1}$			

TABLE IV (cont.)

$[S]_o \times 10^{-4}$	t(sec)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	$\log\frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
42.3	90	.147	16.01	1.009	.00389	90	90
	180	.276	15.88	1.018	.00775	180	180
	300	.456	15.70	1.029	.0124	295	300
	480	.674	15.49	1.043	.0183	470	480
	840	1.09	15.07	1.072	.0302	815	850
	960	1.34	14.82	1.090	.0374	930	970
	1080	1.42	14.74	1.097	.0402	1050	1090
				$V_o = 3.51 \times 10^{-7}$M sec.$^{-1}$			
42.3	90	.155	16.00	1.009	.00389	90	90
	180	.284	15.87	1.018	.00775	180	180
	300	.456	15.70	1.029	.0124	295	300
	480	.700	15.46	1.046	.0195	470	480
	840	1.16	15.00	1.077	.0322	815	850
	960	1.31	14.85	1.089	.0370	930	970
	1080	1.48	14.68	1.101	.0418	1050	1090
				$V_o = 3.51 \times 10^{-7}$M sec.$^{-1}$			
35.8	60	.120	13.53	1.009	.00389	60	60
	120	.212	13.44	1.016	.00689	120	120
	180	.304	13.35	1.023	.00988	180	180
	240	.390	13.26	1.029	.0124	240	240
	315	.500	13.16	1.038	.0162	310	315
	360	.565	13.08	1.044	.0187	355	360
	420	.642	13.01	1.049	.0208	410	420
	480	.720	12.93	1.054	.0228	470	480
	540	.800	12.85	1.062	.0261	530	545
				$V_o = 2.96 \times 10^{-7}$M sec.$^{-1}$			
25.3	90	.118	9.54	1.013	.00561	90	90
	210	.224	9.44	1.023	.00988	210	210
	330	.328	9.33	1.037	.0158	325	330
	570	.535	9.12	1.059	.0249	560	570
	930	.849	8.81	1.097	.0402	900	930
	1290	1.09	8.57	1.128	.0523	1230	1290
	1830	1.48	8.18	1.182	.0726	1700	1850
	2790	2.13	7.53	1.284	.109	2510	2815
				$V_o = 2.27 \times 10^{-7}$M sec.$^{-1}$			

TABLE IV (cont.)

$[S]_o x 10^{-4}$	t(sec)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	$\log\frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
25.3	90	.123	9.54	1.013	.00561	90	90
	210	.231	9.43	1.025	.0107	210	210
	340	.333	9.33	1.037	.0158	330	340
	570	.533	9.13	1.059	.0249	560	570
	975	.867	8.79	1.099	.0410	945	975
	1335	1.16	8.50	1.138	.0561	1270	1335
	1830	1.54	8.12	1.190	.0756	1700	1850
	2790	2.15	7.51	1.287	.110	2510	2815
				$V_o = 2.27 \times 10^{-7}$M sec.$^{-1}$			
25.3	90	.124	9.54	1.013	.00561	90	90
	210	.222	9.44	1.023	.00988	210	210
	330	.344	9.32	1.037	.0158	325	330
	570	.542	9.12	1.059	.0249	560	570
	930	.839	8.82	1.097	.0402	900	930
	1290	1.10	8.56	1.128	.0528	1230	1290
	1830	1.55	8.1	1.190	.0756	1700	1850
				$V_o = 2.27 \times 10^{-7}$M sec.$^{-1}$			
31.3	75	.130	11.82	1.011	.00475	75	75
	120	.198	11.75	1.018	.00775	120	120
	180	.280	11.67	1.024	.0103	180	180
	240	.358	11.59	1.031	.0128	240	240
	300	.440	11.51	1.039	.0166	295	300
	360	.510	11.44	1.045	.0191	355	360
	420	.575	11.37	1.051	.0216	410	420
	480	.670	11.28	1.059	.0249	470	485
	540	.735	11.21	1.066	.0278	530	545
				$V_o = 2.61 \times 10^{-7}$M sec.$^{-1}$			
22.4	75	.098	8.43	1.012	.00518	75	75
	120	.150	8.38	1.018	.00775	120	120
	180	.212	8.32	1.025	.0107	180	180
	240	.270	8.26	1.033	.0141	240	240
	300	.320	8.21	1.037	.0158	295	300
	360	.370	8.16	1.044	.0187	355	360
	420	.430	8.10	1.053	.0224	410	420
	480	.480	8.05	1.059	.0249	470	480
	540	.525	8.00	1.067	.0282	530	540
				$V_o = 1.95 \times 10^{-7}$M sec.$^{-1}$			

TABLE IV (cont.)

$[S]_o x10^{-4}$	t(sec)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	$\log\frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
16.9	60	.073	6.39	1.012	.00518	60	60
	180	.139	6.32	1.023	.00988	180	180
	300	.215	6.24	1.037	.0158	295	300
	540	.354	6.11	1.057	.0211	525	540
	1020	.613	5.85	1.104	.0430	970	1020
	1980	1.12	5.34	1.211	.0831	1820	1980
	2460	1.32	5.14	1.258	.0997	2220	2485
				$V_o = 1.57 \times 10^{-7}$M sec.$^{-1}$			
16.9	60	.073	6.39	1.012	.00518	60	60
	180	.136	6.32	1.023	.00988	180	180
	300	.204	6.26	1.032	.0137	295	300
	540	.334	6.13	1.055	.0233	525	540
	1020	.578	5.88	1.100	.0414	970	1020
	1980	1.06	5.40	1.197	.0781	1820	1980
	2460	1.28	5.18	1.250	.0969	2220	2485
				$V_o = 1.57 \times 10^{-7}$M sec.$^{-1}$			
16.9	60	.071	6.39	1.012	.00518	60	60
	180	.146	6.31	1.023	.00988	180	180
	300	.211	6.25	1.034	.0145	295	300
	540	.356	6.10	1.060	.0253	525	540
	1020	.633	5.83	1.109	.0449	970	1020
	1980	1.11	5.35	1.208	.0821	1820	1980
	2460	1.36	5.10	1.268	.103	2220	2485
				$V_o = 1.57 \times 10^{-7}$M sec.$^{-1}$			
13.4	60	.060	5.06	1.012	.00518	60	60
	120	.095	5.02	1.021	.00903	120	120
	180	.137	4.98	1.028	.0120	180	180
	240	.177	4.94	1.037	.0158	235	240
	300	.210	4.91	1.043	.0183	295	300
	360	.245	4.87	1.051	.0216	355	360
	420	.280	4.84	1.058	.0245	410	420
	480	.315	4.80	1.067	.0282	465	480
	540	.350	4.77	1.073	.0306	525	540
				$V_o = 1.18 \times 10^{-7}$M sec.$^{-1}$			

TABLE IV (cont.)

$[S]_o \times 10^{-4}$	t(sec)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	$\log\frac{D_{oo}}{D_{oo}-D_t}$	t'	t''
12.7	90	.067	4.78	1.015	.00647	90	90
	210	.132	4.72	1.028	.0120	210	210
	330	.188	4.66	1.041	.0175	325	330
	570	.296	4.55	1.067	.0282	550	570
	1050	.564	4.29	1.132	.0539	1000	1050
	1980	.907	3.94	1.232	.0906	1800	2000
	2760	1.19	3.66	1.327	.123	2430	2785
	3270	1.37	3.48	1.395	.145	2820	3300
				$V_o = 1.23 \times 10^{-7}$M sec.$^{-1}$			
12.7	90	.072	4.78	1.015	.00647	90	90
	210	.128	4.72	1.028	.0120	210	210
	330	.188	4.66	1.041	.0175	325	330
	570	.308	4.54	1.070	.0294	550	570
	1050	.552	4.30	1.129	.0527	1000	1050
	1985	.918	3.93	1.235	.0917	1800	2000
	2760	1.20	3.65	1.330	.124	2430	2785
	3270	1.38	3.47	1.400	.146	2820	3300
				$V_o = 1.23 \times 10^{-7}$M sec.$^{-1}$			
12.7	90	.071	4.78	1.015	.00647	90	90
	210	.131	4.72	1.028	.0120	210	210
	330	.186	4.66	1.041	.0175	325	330
	570	.305	4.54	1.070	.0294	550	570
	1050	.550	4.30	1.129	.0527	1000	1050
	2760	1.17	3.68	1.319	.120	2460	2785
	3270	1.36	3.49	1.390	.146	2820	3300
				$V_o = 1.23 \times 10^{-7}$M sec.$^{-1}$			

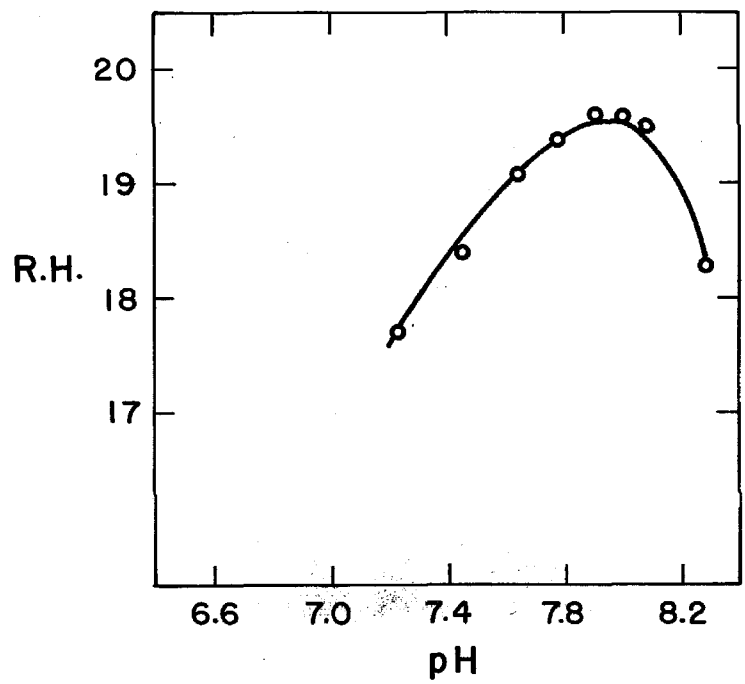

Fig. 5. pH-activity relationship of the system alpha-chymotrypsin-acetyl L-tyrosinhydrazide in aqueous solutions at 25°C and 0.02 M with respect to the amine component of a tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer. R.H.=relative activity.

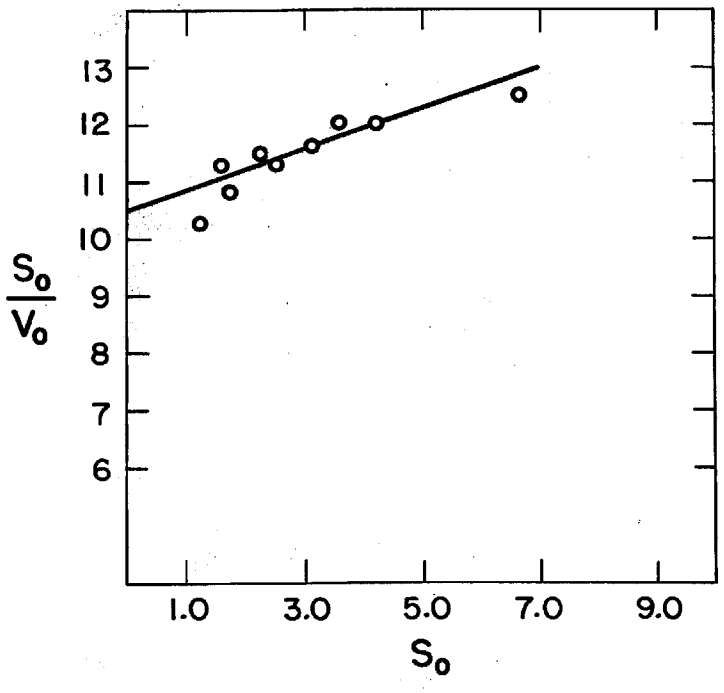

Fig. 6. S_o/V_o vs. S_o graph for the system of alpha-chymotrypsin-acetyl L-tyrosinhydrazide at pH 7.9 and 25.0°C S_o is in units of 10^{-3} M. S_o/V_o is in units of 10^3 sec.

TABLE V

The Alpha-Chymotrypsin Catalysed Hydrolysis of Benzoyl L-tyrosinhydrazide at pH 7.9 and 25.0°C

$[S]_o \times 10^{-4}$	t(sec)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	$\log\frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
2.00	60	.024	.205	1.116	.0477	57	60
	180	.043	.186	1.232	.0906	165	180
	300	.060	.169	1.355	.132	260	300
	420	.088	.141	1.625	.211	335	420
	540	.095	.134	1.710	.233	425	540
	780	.118	.111	2.083	.319	560	785
	900	.130	.099	2.335	.368	620	910
	1140	.150	.079	2.93	.467	720	1150
	1260	.157	.072	3.21	.507	755	1270
				$V_o = 5.23 \times 10^{-8}$M sec.$^{-1}$			
0.60	120	.030	.199	1.150	.0607	113	120
	240	.052	.177	1.293	.112	215	240
	360	.071	.158	1.448	.161	305	360
	480	.086	.143	1.600	.204	390	480
	600	.103	.126	1.816	.259	460	600
	720	.115	.114	2.010	.303	535	725
	840	.129	.100	2.29	.360	590	850
	960	.136	.093	2.49	.396	645	970
	1080	.147	.082	2.82	.450	690	1090
				$V_o = 5.23 \times 10^{-8}$M sec.$^{-1}$			
0.60	180	.041	.188	1.217	.0853	165	180
	360	.071	.158	1.448	.161	305	360
	540	.095	.134	1.710	.233	425	540
	720	.114	.115	1.992	.299	530	725
	900	.130	.099	2.335	.368	620	910
	1080	.146	.083	2.788	.445	690	1090
	1260	.155	.074	3.12	.494	770	1280
	1440	.165	.064	3.61	.558	835	1455
	1620	.175	.054	4.28	.631	875	1635
				$V_o = 5.23 \times 10^{-8}$M sec.$^{-1}$			

TABLE V (cont.)

$[S]_o \times 10^{-4}$	t(sec)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	$\log\frac{D_{oo}}{D_{oo}-D_t}$	t'	t''
1.00	120	.046	.336	1.137	.0558	108	120
	240	.080	.302	1.266	.102	215	240
	360	.115	.267	1.431	.156	305	360
	480	.138	.244	1.565	.195	395	480
	600	.163	.219	1.745	.242	470	605
	720	.183	.199	1.920	.283	540	725
	840	.200	.182	2.097	.322	605	850
	960	.220	.162	2.358	.373	620	970
				$V_o = 8.82 \times 10^{-8}$M sec.$^{-1}$			
1.00	60	.060	.322	1.186	.0741	56	60
	180	.066	.316	1.210	.0828	165	180
	300	.100	.282	1.355	.132	260	300
	420	.128	.254	1.504	.177	350	420
	540	.156	.226	1.690	.228	425	545
	660	.178	.204	1.874	.273	500	665
	780	.198	.184	2.075	.317	560	785
	900	.218	.164	2.330	.367	620	910
				$V_o = 8.82 \times 10^{-8}$M sec.$^{-1}$			
1.20	180	.079	.379	1.208	.0821	165	180
	360	.138	.320	1.432	.156	305	360
	720	.230	.228	2.07	.316	585	725
	900	.260	.198	2.31	.364	640	910
	1260	.307	.151	3.03	.481	780	1285
	1440	.328	.130	3.523	.547	850	1470
	1620	.345	.113	4.06	.609		1650
	1800	.365	.093	4.92	.692		1855
	1980	.367	.091	5.03	.702		2040
				$V_o = 9.95 \times 10^{-8}$M sec.$^{-1}$			
1.20	60	.035	.423	1.083	.0346	58	60
	180	.075	.383	1.195	.0774	170	180
	300	.110	.348	1.316	.119	265	300
	420	.144	.314	1.505	.178	350	420
	540	.179	.279	1.642	.215	440	545
	660	.200	.258	1.775	.249	515	665
	780	.223	.235	1.950	.290	590	785
	900	.242	.216	2.12	.326	640	910
	1020	.262	.196	2.335	.368	705	1030
				$V_o = 9.65 \times 10^{-8}$M sec.$^{-1}$			

TABLE V (cont.)

$[S]_o \times 10^{-4}$	t(sec)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	$\log\frac{D_{oo}}{D_{oo}-D_t}$	t'	t''
1.20	120	.056	.402	1.139	.0565	115	120
	240	.095	.363	1.260	.100	215	240
	360	.130	.328	1.395	.145	310	360
	480	.165	.293	1.563	.194	400	480
	600	.194	.264	1.735	.239	475	605
	720	.218	.237	1.934	.286	540	725
	840	.240	.218	2.10	.322	605	850
	960	.262	.196	2.335	.368	660	970
	1080	.280	.178	2.57	.410	710	1090
				$V_o = 9.96 \times 10^{-8}$M sec.$^{-1}$			
1.20	180	.080	.378	1.210	.083	165	180
	360	.130	.328	1.395	.145	310	360
	540	.175	.283	1.618	.209	440	545
	900	.252	.206	2.225	.347	640	910
	1080	.282	.176	2.60	.415	710	1090
	1260	.307	.151	3.13	.496	780	1270
	1440	.319	.139	3.29	.517	865	1455
	1620	.338	.120	3.81	.581	970	1635
				$V_o = 9.85 \times 10^{-8}$M sec.$^{-1}$			
1.60	60	.045	.566	1.080	.0334	58	60
	180	.096	.515	1.188	.0748	170	180
	300	.144	.467	1.310	.117	265	305
	420	.192	.419	1.460	.164	355	425
	540	.233	.378	1.617	.209	440	545
	660	.267	.344	1.778	.250	515	665
	780	.30	.311	1.965	.293	585	785
	900	.326	.285	2.145	.331	650	920
				$V_o = 13.4 \times 10^{-8}$M sec.$^{-1}$			
1.60	120	.074	.537	1.138	.0561	113	120
	240	.126	.485	1.262	.101	215	240
	360	.175	.436	1.403	.147	310	365
	480	.215	.396	1.545	.189	400	485
	600	.253	.358	1.709	.233	475	605
	720	.29	.321	1.905	.280	550	725
	900	.333	.278	2.200	.342	640	920
	1080	.37	.241	2.538	.404	725	1100
	1260	.405	.206	2.970	.473	795	1285
				$V_o = 13.3 \times 10^{-8}$M sec.$^{-1}$			

TABLE V (cont.)

$[S]_o x10^{-4}$	t(sec)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	$\log\frac{D_{oo}}{D_{oo}-D_t}$	t'	t''
1.60	300	.150	.461	1.326	.123	265	305
	540	.237	.374	1.636	.214	430	545
	720	.293	.318	1.924	.284	540	725
	900	.343	.268	2.281	.358	630	920
	1080	.372	.239	2.558	.408	725	1100
	1320	.417	.194	3.152	.499	820	1345
	1560	.446	.165	3.708	.569	905	1590
	1800	.465	.146	4.180	.621	990	1835
	2340	.505	.106	5.77	.761		2385
				$V_o = 13.6 \times 10^{-8}$M sec.$^{-1}$			
2.00	120	.094	.674	1.133	.0542	115	120
	240	.155	.609	1.255	.0986	215	240
	360	.216	.548	1.395	.145	310	365
	480	.270	.494	1.547	.189	395	485
	600	.320	.444	1.721	.236	475	605
	720	.367	.397	1.925	.284	540	725
	840	.400	.364	2.098	.322	605	855
	960	.432	.332	2.300	.362	670	980
	1080	.468	.296	2.580	.412	690	1100
				$V_o = 16.6 \times 10^{-8}$M sec.$^{-1}$			
2.00	180	.126	.638	1.197	.0781	165	180
	300	.188	.576	1.325	.122	255	300
	420	.244	.520	1.468	.167	350	425
	540	.295	.469	1.630	.212	440	545
	600	.340	.424	1.802	.256	450	605
	780	.382	.382	1.998	.301	575	785
	900	.423	.341	2.240	.350	640	920
	1020	.458	.316	2.415	.383	700	1040
				$V_o = 16.6 \times 10^{-8}$M sec.$^{-1}$			
2.00	180	.124	.640	1.193	.0766	165	180
	480	.216	.548	1.395	.145	410	485
	540	.300	.464	1.645	.216	430	545
	720	.365	.399	1.915	.282	540	725
	900	.424	.342	2.233	.349	640	920
	1080	.470	.294	2.595	.414	715	1100
	1260	.510	.254	3.005	.478	770	1285
	1440	.538	.226	3.375	.528	865	1470
				$V_o = 16.6 \times 10^{-8}$M sec.$^{-1}$			

TABLE V (cont.)

$[S]_o \times 10^{-4}$	t(sec)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	$\log\frac{D_{oo}}{D_{oo}-D_t}$	t'	t"
2.45	60	.077	.844	1.093	.0386	58	60
	180	.160	.761	1.210	.0828	165	180
	300	.240	.681	1.353	.131	265	305
	420	.310	.611	1.507	.178	350	425
	540	.368	.554	1.665	.221	430	545
	660	.425	.496	1.856	.269	510	665
	780	.485	.436	2.110	.324	570	795
	900	.532	.389	2.37	.375	630	920
	1020	.556	.365	2.525	.402	695	1040
				$V_o = 20.8 \times 10^{-8}$M sec.$^{-1}$			
2.45	120	.112	.809	1.141	.0573	115	120
	240	.200	.721	1.277	.106	215	240
	360	.278	.643	1.435	.157	310	365
	480	.340	.581	1.585	.200	395	485
	600	.408	.513	1.796	.254	470	605
	720	.455	.466	1.975	.296	540	725
	840	.500	.421	2.19	.340	605	855
	960	.531	.390	2.365	.374	670	980
	1080	.570	.351	2.63	.420	725	1100
				$V_o = 20.8 \times 10^{-8}$M sec.$^{-1}$			
2.45	180	.155	.766	1.202	.0799	165	180
	360	.275	.646	1.426	.154	310	365
	540	.366	.555	1.660	.220	430	545
	720	.450	.471	1.955	.291	540	725
	900	.515	.406	2.27	.356	640	920
	1080	.568	.353	2.61	.417	725	1100
	1260	.615	.305	3.025	.481	795	1285
	1440	.660	.261	3.53	.548	850	1480
	1620	.685	.236	3.91	.592	905	1670
				$V_o = 20.8 \times 10^{-8}$M sec.$^{-1}$			
4.00	60	.088	1.13	1.080	.0334	60	60
	180	.205	1.01	1.207	.0817	165	180
	300	.310	.91	1.340	.127	265	305
	420	.400	.82	1.487	.172	360	425
	540	.485	.73	1.673	.223	440	550
	660	.557	.66	1.850	.267	520	675
	780	.625	.59	2.068	.316	595	805
	900	.685	.53	2.300	.362	655	925
	1020	.743	.48	2.540	.405	715	1050
				$V_o = 27.3 \times 10^{-8}$M sec.$^{-1}$			

TABLE V (cont.)

$[S]_o \times 10^{-4}$	t(sec)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	$\text{Log}\frac{D_{oo}}{D_{oo}-D_t}$	t'	t''
3.20	120	.145	1.07	1.140	.0569	115	120
	240	.264	.96	1.271	.104	220	240
	360	.36	.86	1.418	.152	315	365
	480	.443	.78	1.565	.195	405	490
	600	.524	.70	1.745	.242	485	610
	720	.590	.63	1.936	.287	555	740
	840	.655	.56	2.179	.338	630	865
	960	.712	.51	2.392	.379	690	990
	1080	.770	.45	2.710	.433	735	1110
				$V_o = 27.3 \times 10^{-8}$M sec.$^{-1}$			
3.20	180	.206	1.01	1.207	.0817	165	180
	360	.355	.86	1.418	.152	315	365
	540	.485	.73	1.672	.223	440	550
	720	.585	.63	1.936	.287	555	740
	900	.67	.55	2.219	.346	665	930
	1080	.745	.47	2.595	.414	755	1110
	1260	.813	.41	2.975	.473	830	1310
	1440	.853	.37	3.295	.518	925	1495
	1620	.91	.31	3.932	.595	955	1685
				$V_o = 27.3 \times 10^{-8}$M sec.$^{-1}$			
4.02	60	.102	1.43	1.069	.0290	58	60
	180	.226	1.30	1.177	.0708	170	180
	300	.347	1.18	1.296	.113	270	305
	420	.448	1.08	1.415	.151	365	425
	540	.545	.98	1.562	.194	455	545
	660	.639	.89	1.720	.236	535	665
	780	.722	.81	1.888	.276	610	795
	900	.800	.73	2.095	.321	670	920
	1020	.888	.64	2.39	.378	735	1040
				$V_o = 32.6 \times 10^{-8}$M sec.$^{-1}$			
4.02	120	.168	1.36	1.125	.0512	115	120
	240	.297	1.23	1.244	.0948	220	240
	360	.408	1.12	1.365	.135	315	365
	480	.515	1.01	1.515	.180	410	490
	600	.608	.92	1.664	.221	490	610
	720	.694	.84	1.821	.260	560	735
	840	.770	.76	2.01	.303	640	855
	960	.845	.68	2.25	.352	700	990
				$V_o = 32.6 \times 10^{-8}$M sec.$^{-1}$			

TABLE V (cont.)

$[S]_o \times 10^{-4}$	t(sec)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	$\text{Log}\frac{D_{oo}}{D_{oo}-D_t}$	t'	t''
4.02	60	.093	1.44	1.063	.0265	58	60
	180	.226	1.30	1.177	.0708	170	180
	360	.408	1.12	1.365	.135	315	365
	540	.555	.97	1.577	.198	455	550
	720	.688	.84	1.821	.260	560	735
	900	.810	.72	2.125	.327	670	925
	1080	.902	.63	2.43	.386	770	1110
	1260	.974	.56	2.73	.436	860	1295
	1440	1.07	.46	3.33	.522	910	1495
				$V_o = 32.6 \times 10^{-8}$M sec.$^{-1}$			
6.53	60	.155	1.96	1.081	.0378	59	60
	180	.353	1.77	1.195	.0774	170	180
	300	.538	1.58	1.342	.128	270	305
	420	.703	1.42	1.493	.174	365	430
	540	.862	1.26	1.683	.226	450	555
	660	1.00	1.12	1.892	.277	530	680
	780	1.138	.98	2.163	.335	590	810
	900	1.276	.84	2.526	.402	650	945
				$V_o = 48.5 \times 10^{-8}$M sec.$^{-1}$			
6.53	120	.253	1.87	1.134	.0546	115	120
	240	.445	1.67	1.270	.104	220	240
	360	.620	1.50	1.413	.150	315	365
	480	.774	1.35	1.571	.196	410	495
	600	.930	1.19	1.782	.251	485	620
	720	1.08	1.04	2.036	.309	560	750
	840	1.21	.91	2.330	.367	620	875
	960	1.31	.81	2.517	.401	680	1005
	1080	1.41	.71	2.990	.476	720	1145
				$V_o = 48.2 \times 10^{-8}$M sec.$^{-1}$			
6.53	90	.208	1.91	1.110	.0414	85	90
	180	.360	1.76	1.203	.080	170	180
	360	.632	1.49	1.423	.153	315	365
	540	.895	1.22	1.737	.250	465	555
	720	1.095	1.02	2.078	.318	555	750
	900	1.27	.85	2.495	.397	650	945
	1080	1.43	.69	3.072	.487	720	1145
	1260	1.56	.56	3.688	.567	780	1335
	1440	1.65	.47	4.510	.654	835	1540
				$V_o = 48.8 \times 10^{-8}$M sec.$^{-1}$			

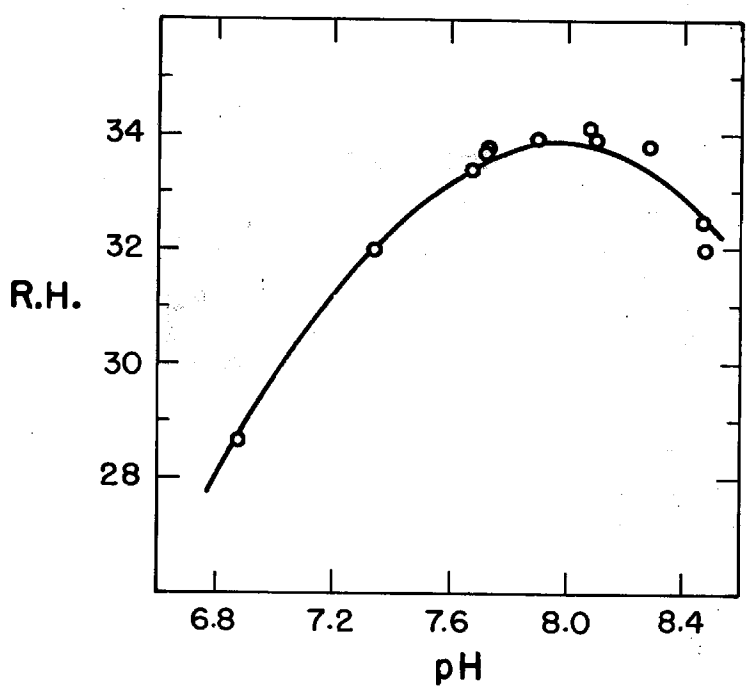

Fig. 7. pH-activity relationship of the system alpha-chymotrypsin-benzoyl L-tyrosinhydrazide in aqueous solutions at 25°C and 0.02 M with respect to the amine component of a tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer. R.H.=relative activity.

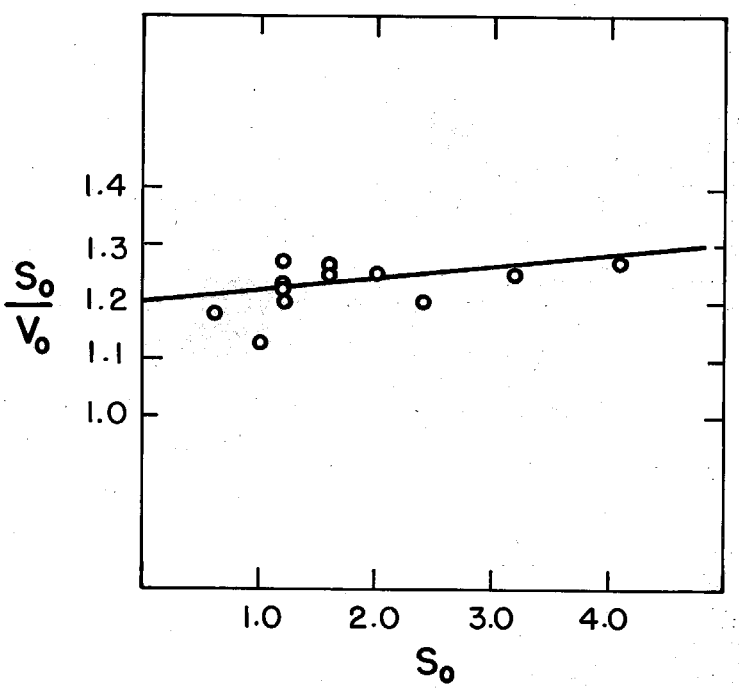

Fig. 8. S_o/V_o vs. S_o graph for the system of alpha-chymotrypsin-benzoyl L-tyrosinhydrazide at pH 7.9 and 25.0°C. S_o is in units of 10^{-4}M. S_o/V_o is in units of 10^3 sec.

TABLE VI

The Alpha-Chymotrypsin Catalysed Hydrolysis of Acetyl L-tyrosinhydrazide and Benzoyl L-tyrosinhydrazide at pH 7.9 and 25.0°C

$[S_1]_o \times 10^{-4}$	$[S_2]_o \times 10^{-4}$	t(min)	D_t	$D_{oo}-D_t$	$\frac{D_{oo}}{D_{oo}-D_t}$	log $\frac{D_{oo}}{D_{oo}-D_t}$	V_o	k_{31}
1.40	3.58	2	.140	1.76	1.080	.0334		
		4	.198	1.70	1.117	.0481		
		6	.250	1.65	1.152	.0615		
		8	.300	1.60	1.187	.0745		
		10	.340	1.56	1.218	.0857		
		12	.388	1.51	1.259	.100		
		14	.423	1.48	1.284	.109	1.27×10^{-7} M sec.$^{-1}$	1.40×10^{-6} M sec.$^{-1}$
1.40	2.68	2	.132	1.43	1.090	.0374		
		4	.185	1.37	1.137	.0558		
		6	.238	1.32	1.182	.0726		
		8	.280	1.28	1.218	.857		
		10	.318	1.24	1.256	.0990		
		12	.352	1.21	1.287	.100		
		14	.390	1.17	1.333	.125	1.16×10^{-7} M sec.$^{-1}$	1.50×10^{-6} M sec.$^{-1}$
1.40	4.47	1	.110	2.13	1.051	.0216		
		2	.145	2.09	1.072	.0312		
		3	.178	2.06	1.087	.0362		
		6	.273	1.97	1.137	.0558		
		7	.300	1.94	1.154	.0622		
		9	.350	1.89	1.185	.0737		
		10	.385	1.85	1.211	.0831		
		11	.405	1.83	1.223	.0874	1.53×10^{-7} M sec.$^{-1}$	1.85×10^{-6} M sec.$^{-1}$

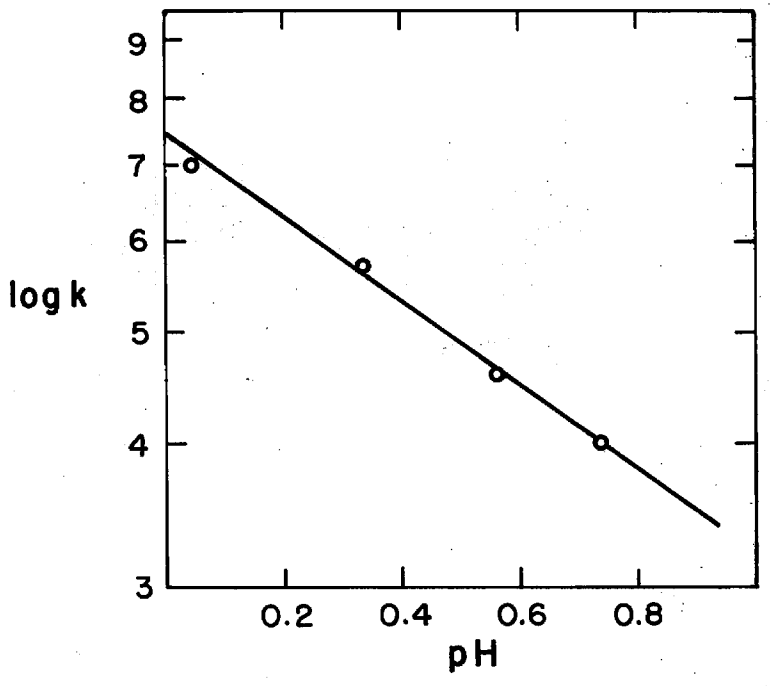

Fig. 9. Non-enzymatic hydrolysis of L-tyrosinhydrazide at 25.0°C in an aqueous solution. Ordinates are log relative rate constants. Abscissae are pH.

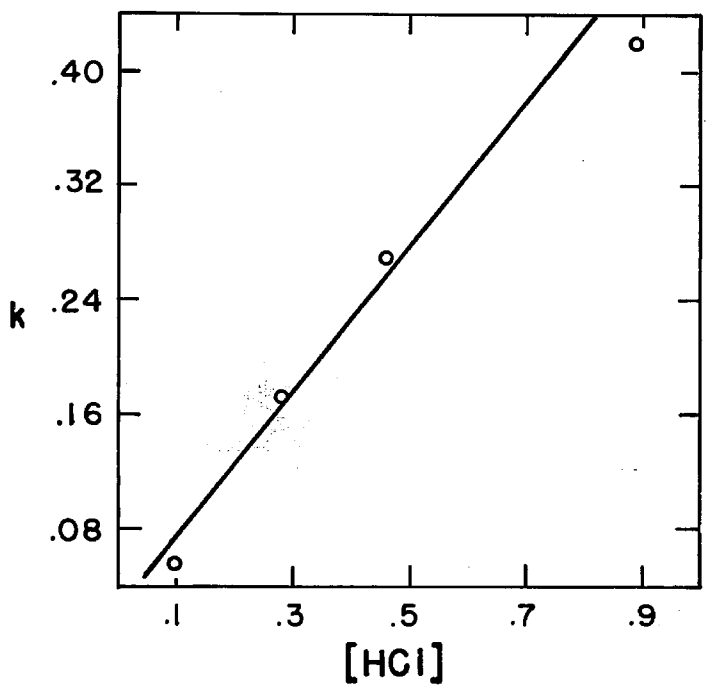

Fig. 10. Non-enzymatic hydrolysis of acetyl L-tyrosinhydrazide at 25.0°C in an aqueous solution. Ordinates are relative specific rate constants. Abscissae are in formal units.

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PART II

HYDROLYSIS OF

BIS-P-DIMETHYLAMINOBENZALAZINE

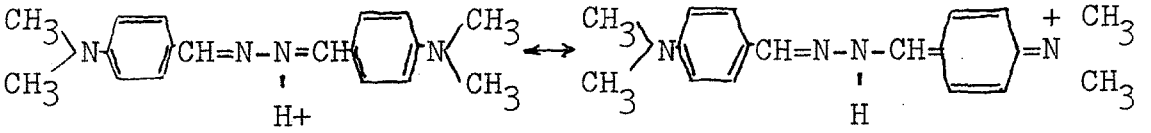
INTRODUCTION

The hydrazine liberated in the alpha-chymotrypsin catalysed hydrolysis of acylated L-tyrosinhydrazides, as described in Part I of this thesis, was quantitatively determined using the reaction of hydrazine with p-dimethylaminobenzaldehyde to form the corresponding azine. The method is extremely sensitive, the results are reproducible, and the procedure is simple (1,2,3).

Azine formation is a reversible reaction, the yield of azine depending upon the concentration of the p-dimethylaminobenzaldehyde and the acidity of the solution. Thus, the complete integration of the various aspects of this quantitative method required studies of the mechanism of the hydrolysis as well as of the parameters of the formation of the azine.

The bis-p-dimethylaminobenzalazine is a canary yellow solid. An ethanolic solution of it has a λ maximum at 400 mμ (12), whereas an aqueous acidic solution absorbs at a λ maximum of 455 mμ. It is the λ maximum at 455 mμ which is used for the spectrophotometric determination of hydrazine concentration. This species which absorbs at 455 mμ in the aqueous acidic solution is obviously a protonated form of the azine molecule (1). It is of interest, for the

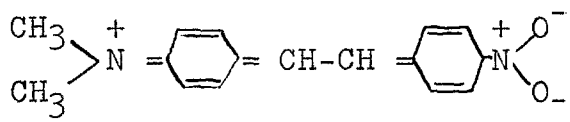
hydrolysis study particularly, that the position of attachment of the proton be known. If this problem is considered on the basis of a positive $\Delta\lambda$ maximum when the solvent is changed from ethanol to aqueous acid, then the protonation of an azo nitrogen atom is reasonable. The principal resonance forms for this case are:



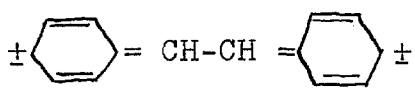
If the consideration is based on the known basicities of similar, isolated nitrogen groups, undoubtedly the protonation of the dimethylamino group would be presumed. That the former argument is the correct one and that the basicities of the individual groups are drastically altered in virtue of their positions in the azine molecule can be demonstrated by making use of the data of analogous compounds.

The wave length of the light absorbed by a molecule is inversely proportional to the difference in energies of the normal and excited states of the molecule, the excited state arising from a displacement of electric charge. A lower difference in energy, i.e. a lower frequency of absorbed light, corresponds to a greater charge displacement and a greater distance in space for the displacement. The introduction of suitable groups may lower the required energy for the transition of states. The absorptions of the para substituted derivatives of stilbene, wherein a shift in ab-

sorption to longer wave lengths is occasioned by the para substitution of the dimethylamino and nitro groups and the para-para' disubstitution of these groups, provide examples of this process (4). Each substitution causes a bathochromic shift, the effect of the disubstitution being greater than the sum of the individual effects of the groups. In this case the large positive $\Delta\lambda$ maximum results from the contribution of the structure:



Such a structure demonstrates that a much smaller increment of energy is required for the charge displacement than is necessary to produce a comparable structure from the parent molecule stilbene, viz.



The correlation of $\Delta\lambda$ maximum and the protonation reaction may be made in this same general manner. There is evidence which confirms the conclusion that any protonation which prevents the formation of quinoidlike structures of the type noted in the case of 4-nitro-4'-dimethylaminostilbene causes a decrease in the wave length of absorption. Examples of this type are: aniline (5), 4-nitro-4'-aminostilbene (6), 4-dimethylamino-2'-methylstilbene (7). One is able then to assign structures to the protonated and un-

protonated forms of a compound on this basis.

The basicity of the azo group nitrogen atom in compounds such as azo benzene (8) and p-nitroazobenzene (9) is very small, the latter compound possessing a pK_A of -3.06. However, the basicity of azo compounds having para substituted electron donor groups is enormously greater, e.g. the pK_A, or pK_{A1}, values of p-aminoazobenzene (10), p-dimethylaminoazobenzene (11), and 4-dimethylamino-4'-methylazobenzene (11) are 2.80, 2.2, and 2.3, respectively. One cannot conclude that the azo group has increased in basicity to these degrees without a consideration of the concomitant effects of the protonation on the spectra, since in these cases, as in the case of the bis-p-dimethylaminobenzalazine, the presence of the amino groups as the electron donating para substituents may simply mean that the pK_As of the amino groups of the molecules are being measured. The position of this proton can be established as being on an azo nitrogen atom on the basis of the criterion which has been suggested, viz. the criterion of wave length shift due to protonation (11,13). In all of these instances of the para amino group substituted azobenzenes a protonation results in a positive $\Delta\lambda$ maximum for the first protonation and in a negative $\Delta\lambda$ maximum for the second protonation. These results are in accordance with the initial protonation of an azo nitrogen atom and a subsequent protonation of the amino group. One can con-

clude that the position of the proton in the case of the azine is also on an azo nitrogen atom.

A study of the kinetics of the formation and of the hydrolysis of the azine requires a knowledge of the nature of the active species of the reactants. A reasonable interpretation of the kinetic data also necessitates the calculations of the concentrations of these active species, many equilibria between protonated and unprotonated forms being extant in the system. The influence of changes in the solvent may not be neglected in the considerations of these factors. The use of a water-ethanol mixture as the solvent thus directly affects the acidity function, the pK_As of the various reactants, and the rate constants in so far as the dielectric constant and solvolysis power effects are appreciable.

The modifications of reaction rates and mechanisms by solvent changes are primarily electrostatic phenomena. In general, the result of increasing the dielectric constant is to increase the reaction rate for reactant ions of like sign, to decrease the rate for reactant ions of unlike sign, to decrease the rate for the reaction of a positive ion with a dipolar molecule, and to increase the rate for a reaction of a negative ion with a dipolar molecule. Deviations from the predicted effects in low dielectric constant solvents are believed to be caused by the preferential orientation about the solute molecules of the molecules of

the higher dielectric constant component of a solvent mixture (14).

The comparative degrees of solvation of the reactants and of the transition state determine in a large measure the increments of heats and entropies of activation. The energy needed to separate ions is compensated for by the heat of solution of the ions. The orientation of the solvent molecules to a greater extent about the products of reaction rather than the reactants results in a decrease in entropy. The exact prediction of the effects of different solvents upon energy and entropy changes is not generally possible. However, generalizations derived from a treatment using simple electrostatic theory for ions reveal that the free energy decreases with increasing polarity of the solvent and that the entropy increment has a minimum value for solvents of moderate polarity but it is always a negative value (15). Values for the enthalpy increments are calculated from the equation $\Delta H = \Delta F + T\Delta S$. Such a treatment, based on Coulomb's Law, is not applicable in cases in which the solvent molecule forms essentially a covalent bond with a species, and the differences in bond energies must be included for the calculation of the total change of heat content when covalent bonds are broken and formed.

In the case of the reaction of an ion and a neutral molecule the solvation of the separate entities is not much

more than that of the transition state. The reaction rate is predicted to increase with decreasing dielectric constant from electrostatic considerations if the dielectric constant is varied by changing the proportions of a mixture (16).

The primary salt effect, the effect of ionic strength on the activity coefficients, is important when one or more species are ions. For dilute solutions where the total ionic concentration is less than 0.02 M the Debye-Huckel limiting law may be used to calculate activity coefficients (17). The use of this equation presumes that there are no complicating interactions of ions to form complexes. Although according to this theory the ionic strength should not affect a reaction in which one of the reactants is a neutral molecule, at higher ionic concentrations there may be deviations from predicted values due either to the non-applicability of the Debye-Huckel equation or to changes in the changes in the activity coefficients of neutral molecules. There are modified equations designed to permit the calculations of both of these effects (14,18).

The secondary salt effect is of primary importance in cases involving acid or base catalysis. This effect, which is that of ionic strength on the dissociation of weak acids and bases, thus can alter the rates of reactions dependent upon the concentration of a reactant or catalyst which is controlled by a dissociation equilibrium.

DISCUSSION OF THE EXPERIMENTAL RESULTS

Preliminary Studies

A knowledge of the consequences of varying acidities upon the components of the reaction system is required in order that the effect of this variable on the reaction may be correctly interpreted. The acid-base equilibria of each of the reactants, the azine, the aldehyde, the hydrazone, the hydrazine, and water, must be examined, and reasonable values for the activity of each reactant can be assigned only after a consideration of these equilibria.

In the case of the azine one is concerned with the identification of the species being measured as well as with its activity. For the system in which hydrazine is quantitatively determined using p-dimethylaminobenzaldehyde in an acidic mixture of water and ethanol it has been reported that the species predominantly present, the one absorbing at 455 mμ, is a mono-protonated azine, the proton being on one of the central nitrogen atoms (1). In the system in which the ethanol content of a 10 ml. mixture of ethanol and water is 6 ml. and the ionic strength is constant for varying acidities, the initial rate of hydroly-

sis, as measured spectrophotometrically at 455 mμ, is directly proportional to the concentration of the azine, and the logarithm of the apparent first order rate constant is a linear function of the pH of the solution. These data are presented in tables I and II and figures 1 and 2. This latter relationship is significant for the distinguishing of the form of the active species as well as being a criterion for the mechanism of the hydrolysis. Since the two conclusions are derived from the same pattern of analysis, they may be considered concomitantly.

The linear relationship of the pH and the log of the apparent first order rate constant establishes the rate determining step to be the hydrolytic decomposition of an azine species which has been protonated via a relatively rapid previous step (19). The mechanism by which a proton transfer is the rate determining step is thus proved to be incorrect. These conclusions can be demonstrated in this manner.

If the hydrolysis decomposition reaction is the rate determining step, then

$$\text{rate} = k[CHN_2CH^+] \quad \frac{f_{CHN_2CH^+}}{f_{TR}}$$

where k is the specific rate constant, $[CHN_2CH^+]$ denotes the concentration of the active protonated azine species,

and $f_{CHN_2CH^+}$ and f_{TR} are the activity coefficients of the protonated azine species and the transition state, respectively.

The rate of the reaction which was experimentally determined is

$$\text{rate} = k_a \, [CHN_2CH]_{455}$$

where k_a is the apparent first order rate constant and the subscript 455 denotes the spectrophotometrically followed azine concentration.

It follows that

$$k_a = k \quad \frac{(CHN_2CH^+) \quad f_{CHN_2CH^+}}{(CHN_2CH)_{455} \quad f_{TR}}$$

Assuming that $f_{CHN_2CH^+} = f_{TR}$ and using the acid-base equilibrium constant for these two azine forms the following equation results:

$$k_a \, (H^+) = K_A k$$

Thus, the log of the apparent first order rate constant is directly proportional to the pH.

A similar analysis, assuming the proton transfer to be the rate determining step, leads to the conclusion that the apparent first order rate constant is proportional to the acid concentration. Therefore, it follows that the azine species being hydrolysed is a form possessing one more

proton than the form whose absorption is spectrophotometrically measured at a wave length of 455 mμ.

In the case of the aldehyde the species having a protonated dimethylamino group may be presumed to be the active form on the basis of a consideration of the reactivity of the carbonyl carbon atom, which for the nucleophilic attack is measured by its relative positive charge. An electron donating dimethylamino group in the para position reduces this charge. A comparison of the dipole moments of benzaldehyde and p-dimethylaminobenzaldehyde reveals the magnitude of this effect, the respective magnetic dipole moments being 3.16 and 4.29 (20). When the dimethylamino group is protonated, not only is the electron donating effect removed but in addition the inductive effect of the positive charge tends to increase the positive character of the carbonyl carbon atom.

The Bronsted acid constant, defined by the equation

$$K_A = \frac{[B]\ (H^+)}{[BH^+]}$$

(21), has been determined so that the calculations of the concentration of the active species of the aldehyde under varying acid concentrations may be made. This constant has been obtained for the two water-ethanol systems, 10 ml. solutions containing 1 ml. and 6 ml. of ethanol, the latter being the system for the hydrolysis studies. The deriva-

tion of equilibrium constants for water-organic solvent systems must take cognizance of the possible aberrations of the hydrogen ion activity coefficient caused by varying compositions, different ionic strengths, and different acid concentrations. Studies have indicated that the activity coefficient is dependent upon the specific salt used to maintain a constant ionic strength (22). For systems at a constant ionic strength, as maintained by a particular salt, it has been noted that a linear relationship between the logarithm of the activity coefficient and the hydrochloric acid concentration is independent of the solvent composition (23). The procedure for the calculation of the value of K_A in this case has been to reproduce the experimental conditions for the published data for the water-ethanol system at a constant ionic strength of 1.0 M using sodium chloride so that the reported relations may be used (23), to extrapolate a value for log $\gamma(0)_1$, and to calculate the activity coefficients from the equation

$$\log \gamma_2 - \log \gamma(0)_1 = \alpha_{12} m_1$$

Here γ_2 is the activity coefficient to be calculated, $\gamma(0)_1$ is the activity coefficient of the hydrogen ion when the concentration of hydrochloric acid approaches zero for the ionic strength of 1.0 M in the same system, α_{12} is the slope of the straight line obtained from a plot of log $\gamma \pm$ vs. hydrochloric acid concentration at constant

ionic strength of 1.0 M, and m_1 is the molality of the hydrochloric acid (24). The activity coefficients of the hydrochloric acid obtained in this manner and the spectrophotometric data for the absorptions at three wave lengths were used to calculate K_A via the determinant method (25). The data and results are presented in table III.

The value of K_A of the aldehyde for the system containing 1 ml. of ethanol was calculated from spectrophotometric data at the wave length of 350 mu using the Debye-Huckel Law for activity coefficients,

$$\ln \gamma_{\pm} = - \frac{Z_H \, Z\text{-}A\mu^{\frac{1}{2}}}{1 + \beta \, a_i \mu^{\frac{1}{2}}}$$

For decadic logarithms and using the expressions of

$$A = \frac{0.509}{(dt)^{3/2}} \qquad \text{and} \qquad \beta = \frac{0.3286 \times 10^8}{(dt)^{\frac{1}{2}}} \qquad (26)$$

where $d = \frac{D}{78.54}$ and $t = \frac{T}{298.16}$,

this law becomes for a diunivalent electrolyte:

$$\log \gamma_{\pm} = \frac{0.56\mu^{\frac{1}{2}}}{1 + .339 \, a_i \mu^{\frac{1}{2}}}$$

The value of K_A was calculated by the following procedure:

$$\text{Let} \qquad K_A = \frac{K_A' \, \gamma_{H^+}}{\gamma_{CHO^+}} = \frac{[H^+][CHO]\,\gamma_{H^+}}{[CHO^+]\,\gamma_{CHO^+}}$$

where CHO and CHO^+ represent the unprotonated and the protonated forms of the aldehyde, respectively, and $\gamma_{CHO} = 1$.

By taking the logarithm and substituting the Debye-Huckel expression for log γ_{CHO^+} the equation becomes

$$\log K_A' \, \gamma_{H^+} = \log K_A + \frac{0.56\,\mu^{\frac{1}{2}}}{1 + .339\, a_i \mu^{\frac{1}{2}}}$$

The plot of log $K_A' \gamma_{H^+}$ vs. $- \dfrac{0.56\,\mu^{\frac{1}{2}}}{1 + .339\, a_i \mu^{\frac{1}{2}}}$ is a straight line of unit slope if the correct value of a_i is employed. This value of a_i required to meet this condition was obtained by a method of successive approximations and was found to be 7.2 Å. The value of K_A was derived from the intercept of the plot. The data for this determination are presented in table IV.

The active species of hydrazine was presumed to be the neutral molecule. Several similar reaction systems have been reported substantiating this assumption (27-29). The K_A for the first protonation was extrapolated from that reported for the ammonium ion in a similar system (30), the pK_A value of hydrazine being changed from 7.10 in water to 6.4 in the ethanol-water system used.

Hydrolysis Studies

The first section of the study of the hydrolysis of the azine was an investigation of the effect of acid concentration variation. Although the condition of constant ionic strength was maintained, there is no intended implication that the activity coefficients of the various reactants remain insensitive to differences in acid concentration. Changes in the activity coefficient of the hydrogen ion and of the salt used to maintain the condition of a constant ionic strength have been reported for many systems (31-33). Corrections have not been applied to these data, since such corrections would only be extrapolations from the data of analogous compounds and systems and would probably be subject to errors of the magnitude incurred by their neglect. It can be noted that where activity coefficient corrections have been applied in this study, viz. in the determination of the acid dissociation constant of p-dimethylaminobenzaldehyde, such uncertainties were removed by duplicating the system for which the data for the activity coefficients of the hydrogen ion were reported (23).

For each acid concentration the initial phase of each kinetic run was that of a first order rate reaction. However, the reaction deviated from that of the first order

type in increasing degree throughout the time interval of the run. This deviation is portrayed in table II and figure 10. It was, thus, only the data of the linear segment which were suitable for comparison to enable a deduction regarding the effect of acid concentration on the rate of hydrolysis. In this limited range the rate equation becomes

$$- \frac{d \ln(\text{azine})}{dt} = k_a$$

where the azine of the equation represents the particular species measured spectrophotometrically at a wave length of 455 mμ and k_a is the apparent first order rate constant. Since the hydrolysis rate is directly dependent upon the acid concentration, the constant k_a is a function of the acid concentration. There are in general two formulations for the dependence of the rate of an acid catalysed hydrolysis reaction upon the acidity of the system (19). The mechanism of the reaction can be represented either as a rate determining proton transfer followed by a rapid hydrolysis dissociation or as a fast proton transfer prior to a relatively slow, rate determining hydrolysis. These two reaction schemes are distinguishable by their intrinsically different dependencies upon the acid concentration. This subject has been treated in the section containing a discussion of the effects of the acid concentration on the

concentrations of the various species of the reactants. That the logarithm of k_a is a linear function of the pH of the system was the conclusion reached. This linear relationship is illustrated in figures 1 and 2. The Hammett acidity function expresses the same linear correspondence, and the value of the H_o function reduces to the pH value in solutions where the acid concentration is less than 1.0 F(34).

It can be noted that there were no measurable amounts of hydrolysis in the cases in which either no acid was added to the system or the acid was replaced by sodium hydroxide.

The gradually increasing deviations from the apparent first order rate relation suggested the operation of a reverse reaction. This was qualitatively established to be the case by adding increasing amounts of the p-dimethylaminobenzaldehyde to the system at the start of the kinetic runs and observing the increasing retardations of the rate of hydrolysis. These data are presented in figure 3.

A satisfactory quantitative treatment of the data required that a rate equation be derived which describes the variation of the azine concentration with time throughout the time interval of each kinetic run. In essence this means a rate equation which predicts the deviation from a first order rate reaction. The reaction was assumed to proceed in two discrete steps, a hydrolytic decomposition of the azine into the aldehyde and the corresponding hydrazone

followed by a hydrolytic decomposition of the hydrazone into hydrazine and the aldehyde. Of these compounds the azine was the only one which could be quantitatively measured by spectrophotometric means. The constants which were calculated from the data from the rate of hydrolysis and rate of formation kinetic studies were thus:

a k_a as defined by $- \frac{d(\text{azine})}{dt} = k_a\,(\text{azine})$

and a k_{-3} as defined by $\frac{d(\text{azine})}{dt} = k_{-3}(\text{aldehyde})^2(\text{hydrazine})$.

The first step was to relate the experimental k_{-3} to the k_{-1} of the hydrolysis reaction rate equation expressed as

$$- \frac{d(\text{azine})}{dt} = k_1(\text{azine}) - k_{-1}(\text{hydrazone})(\text{aldehyde})$$

The formulation of the rate equation in this manner states that the assumption has been made that for the general reaction described by

$$\text{azine} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{hydrazone} + \text{aldehyde}$$

and

$$\text{hydrazone} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{aldehyde} + \text{hydrazine}$$

the reactions governed by the specific rate constants k_1 and k_{-1} are slow when compared with the reactions governed

by the constants k_2 and k_{-2}, respectively. On the basis of these definitions, the nature of the dependence of the specific rate constants on the different species concentrations, and the assumption regarding the rate determining steps, one can proceed to relate the constants k_{-3} and k_{-1} as follows:

Let the rate of azine formation be expressed as

$$V_{-1} = k_{-1}(\text{aldehyde})(\text{hydrazone}) \qquad (1)$$

The experimentally determined dependence of this rate is described by the equation

$$V_{-1} = k_{-3}\,(\text{aldehyde})^2(\text{hydrazine}) \qquad (2)$$

Assume that k_2 and k_{-2} are of such a magnitude that there exists an equilibrium such that

$$K = \frac{(\text{aldehyde})(\text{hydrazine})}{(\text{hydrazone})} \qquad (3)$$

The equating of equations (1) and (2) and the substitution of equation (3) results in the relation

$$Kk_{-3} = k_{-1}$$

The substitution of Kk_{-3} for k_{-1} in the expression for the overall reaction yields the equation

$$-\frac{d(\text{azine})}{dt} = k_1\,(\text{azine}) - k_{-3}K(\text{aldehyde})(\text{hydrazone}). \quad (4)$$

The concentrations of the active species are represented in this equation, of course. Therefore, since the only quantity which was determined absolutely was the initial concentration of the total azine, equation (4) is applicable to experimental data only via the introduction of concentration correction factors. The assumption is made in all cases that the active specie of each compound is represented by only one form and that the various species of each compound are related via a base-conjugate acid equilibrium. If a_o is the initial azine concentration, X is the amount of azine hydrolysed, Y is the amount of subsequent hydrolysis of the hydrazone, and the Δ s are the concentration correction factors as defined by $\Delta_i = \frac{(H^+)}{K_A + (H^+)}$ for the case in which the conjugate acid is the active species and

$$\Delta_j = \frac{K_A}{K_A + (H^+)}$$

when the base is the active species, then equation (4) becomes

$$-\frac{d\ln(a_o - x)}{dt} = k_1 - \frac{k_{-3}K(X+Y)(X-Y)\,\Delta_{\text{aldehyde}}\,\Delta_{\text{hydrazone}}}{(a_o - x)\,\Delta_{\text{azine}}} \quad (5)$$

This equation may be rearranged to become

$$(x^2-y^2) = \frac{(k_1-k')(D_{455})(a_o)}{(D_{455})_o\ k_{-3}\Delta\text{aldehyde}} \cdot \frac{\Delta\text{ azine}}{K\ \Delta\text{ hydrazone}} \equiv ZA \quad (6)$$

where k' is $-\frac{d\ln(a_o-x)}{dt}$ and $(D_{455})_o$ is the optical density corresponding to the initial azine concentration. The value of

$$\frac{\Delta\text{ azine}}{K\ \Delta\text{hydrazone}}$$

is such that a graph of

$$(x^2-y^2) \text{ vs. } \frac{(k_1-k')(D_{455})(a_o)}{(D_{455})_o\ k_{-3}\ \Delta\text{ aldehyde}}$$

is linear and has a zero intercept. The quantity y^2 was not measured, but it can be calculated from the form of equation (6) which has a value of

$$\frac{\Delta\text{ azine}}{K\ \Delta\text{ hydrazone}}$$

such that the condition of a zero intercept is fulfilled.

The general procedure for the calculations of such values was as follows:

1. For each of the three kinetic runs for which the deviations from an apparent first order reaction were sufficiently large to allow significantly different values for k' to be determined, two of these values were used to calculate cor-

responding values for Z in equation (6).

2. An arbitrary line was drawn through the origin intersecting each of these two lines representing the two values of Z on graph of Z vs. (x^2-y^2).

3. The intersections yielded values of (x^2-y^2) corresponding to values of Z.

4. An equilibrium expression of

$$K = \frac{(\text{aldehyde})(\text{hydrazine})}{(\text{hydrazone})} =$$

$$\frac{(x+y)(y)}{(x-y)} \frac{\Delta \text{aldehyde}\ \Delta \text{hydrazine}}{\Delta \text{hydrazone}} = \frac{(x+y)(y)}{(x-y)} R$$

was assumed. The quantity R is a constant for a constant hydrogen ion activity.

5. The values of x and y derived from step (3) were used to calculate two values for K.

6. The procedure was repeated by successive approximations until a line was obtained whose intercepts yielded values of x and y such that the two calculated values of K were sensibly the same. The slope of this line was thus A, since it satisfied equation (6) and the values of x and y were limited by the equilibrium equation.

The values of $A \equiv \frac{\Delta \text{azine}}{K \Delta \text{hydrazone}}$ for the three acid concentrations permit the calculation of the values of K_A of the azine, of the K_A of the hydrazone, and of the equilibrium constant K. In the case of the azine, the K_A is that which represents the acid-base equilibrium between the acid form measured at a wave length of 455 mμ and its conjugate base. The protonated form of the hydrazone, the conjugate acid of the acid-base equilibrium, is presumed to be the active form. It can be noted that these K_A constants are Bronsted acidity constants as defined by

$$K = \frac{(H^+)[B]}{[BH^+]} \qquad (21).$$

The equilibrium constant is a concentration equilibrium constant. These three constants have been calculated by the method of determinants. The results are presented in table V.

The influence of the dielectric constant variations resulting from changing ethanol-water proportions on the initial first order rate constant ka is illustrated in table VI and figures 4 and 5. The general conclusions which may be drawn from these data are that the rate of reaction decreases approximately linearily up to between fifty and sixty volume percent ethanol and that the positive departure from linearity increases with increasing ethanol content thereafter. It has been established that the rate of

hydrolysis is directly proportional to the factor:

$$\frac{[CHN_2CH^+]}{[CHN_2CH]_{455}} \times \frac{f_{CHN_2CH^+}}{f_{TR}}$$

On this basis it was shown that the azine species which is hydrolysed is a form which possesses one more proton than the form measured spectrophotometrically. The dependence upon the concentration of water was not specifically designated in this equation, because the data were for conditions of a constant water concentration. The reaction is thus a bimolecular reaction between an azine ion and a neutral water molecule. As such the dependence of the rate upon the dielectric constant may be expressed as

$$\ln k = \ln k_o' + \frac{NZ^2e^2}{2\ DRT}\left(\frac{1}{r} - \frac{1}{r_{TR}}\right) \qquad (16)$$

The specific rate constant is expected to increase with decreasing dielectric constant, since the radius of the transition state is greater than that of the azine ion. The data presented in table VI and figures 4 and 5, however, show that the inverse of this statement holds. It is evident, therefore, that, assuming the equation for the dependence of the rate of hydrolysis on the dielectric constant is applicable to this case, the inverse result is caused by concomitant effects occasioned by dielectric constant variations produced by changing the ethanol-water proportion. Indeed,

the increases in the rate caused by the changes in the dielectric constant alone are not expected to be large, especially in this case wherein the azine ion and the transition state differ to a large extent only by a water molecule.

The rate of the hydrolysis reaction, being directly proportional to the concentration of a protonated azine species, is dependent upon the ability of the system to donate protons. It has been reported that the activity coefficients of hydrochloric acid for systems of ethanol and water decrease appreciably when the ethanol content is increased (35-37), that hydrochloric acid is completely ionized in ethanol-water systems containing up to twenty volume percent ethanol (35), and that its dissociation is very large in systems containing up to ninety volume percent of ethanol (38). The magnitude of the rate decreases indicates that explanations based upon diminutions of hydrogen ion formality and activity coefficients are quantitatively inadequate. The decreasing ability to donate protons for similar solvent systems has been interpreted as resulting from the breakdown of the quasi-crystalline tetrahedral structure of water by the introduction of the organic molecules and the consequent increase in proton affinity of the water molecules (39).

The examination of this problem in the light of the theory of absolute reaction rates, illustrating the depend-

ence of the specific rate upon the entropy and heat of activation, offers a reasonable explanation for this phenomenon. The theory states that the rate increases with decreasing energy of activation and decreases with decreasing entropy of activation. In essence the hydrolysis reaction may be considered as one in which an ion reacts with a molecule to form two ions. The activated complex represents a state of the incipient formation of the ions. Thus, this complex is solvated in a decreasing degree when the ethanol content of the system is increased and consequently the heat of activation increases concomitantly. The entropy of activation undergoes increasingly large decreases under these conditions in view of the fact that solvation by water molecules occasions small entropy decreases even though the solvation is greater in this case than for the ethanol molecules. It can be concluded, therefore, that both effects act to decrease the rate of reaction when the ethanol content of the system is increased.

The noted decreasing rate of decrease of the reaction rate can also be considered on this same basis and can be explained as the preferential orientation of water molecules about all species (14). Thus, the observed rate is not that which can be associated with the actual composition of the system, but rather it is one which can be described by a system with a higher water content, this effect being caused by the preferential orientation. A contributing factor to

this decreasing rate of the decrease of reaction rate is the gradual change of the acidic catalyst in the system from the hydronium ion to the protonated ethanol cation. The protonated ethanol cation is a stronger acid than is the hydronium ion, and hence the proton donating ability of a system increases with increasing proportions of the former. The concentration of the protonated ethanol cation becomes appreciable in systems containing more than eighty-five volume percent ethanol (40). This marked decrease in rate in this region of solvent composition is apparent from figures 4 and 5.

The dependence of the initial first order rate constant on the temperature is illustrated by the data presented in table VII and in figure 6. The values for the entropies and energies of activation have been calculated for seven systems differing only in hydrochloric acid formality. These values vary irregularly, since temperature changes generate alterations in the dielectric constant (41), in equilibria and in the activities of the various species. The prediction of the accumulative effect of these variations is not possible on the basis of the data obtained.

The influence of the ionic strength upon the initial first order rate constant has been measured, and the data have been presented in table VIII . One can discern from these data that there is sensibly no change in the

rate constant resulting from ionic strength variation. This conclusion is in accordance with the effect predicted from the equation relating the rate constant and the ionic strength, viz.

$$\ln k = \ln k_o + \frac{2 Z_A Z_B \alpha \mu^{\frac{1}{2}}}{1 + \beta a_i \mu^{\frac{1}{2}}}$$

EXPERIMENTS AND DATA

Synthesis of Bis-p-dimethylaminobenzalazine

This azine was prepared by the method described for the synthesis of benzalazine (42), recrystallized twice from boiling 95% ethanol, and dried in vacuo over phosphorous pentoxide, m.p. 258.0-259.0 (corr.).

Anal. Calcd. for	$C_{18}H_{22}N_4$:	C,73.44;	N,19.03;	H,7.53
Found[1]:		C,73.56;	N,18.85;	H,7.51

Aldehyde Solutions

The ethanol solutions were prepared from the p-dimethylaminobenzaldehyde described in Part I of this thesis.

Experimental Procedure

The solvent systems were acidic ethanol-water mixtures, the proportions being varied to secure the desired data. The 10 ml. volumetric flasks, containing all reactants except the azine, were equilibrated at 25.0°C in a Sargent Constant Temperature bath. At zero time a 1 ml. aliquot of the azine solution was added, and the mixture was inverted and swirled seven times. A 1 cm. silica cell was filled and placed in the constant temperature water-jacketed compartment of a Beckman Model DUR Spectrophotometer. The data were obtained using a Beckman Recording Quartz Spectrophotometer Amplifier and a Leeds and Northrup Company Type G Speedomax Recorder.

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[1]Microanalysis by Dr. A. Elek, Elek Micro Analytical Laboratories, Los Angeles, California.

TABLE I

Hydrolysis of the Azine. Effect of Acid Concentration Variations at 25.0°C in a System Containing 60% by Volume Ethanol

t(min)	D_1	D_2	D_3	D_4	D_5	D_6	D_7	D_8	D_9
1	0.64	0.70	0.73	0.75	0.75	0.74	0.74	0.70	0.635
2	.40	.51	.59	.63	.67	.68	.68	.665	.61
3	.26	.39	.485	.54	.60	.625	.64	.63	.585
4	.185	.31	.41	.47	.54	.58	.60	.60	.56
5	.14	.25	.35	.41	.49	.535	.56	.57	.54
6	.115	.205	.305	.365	.45	.50	.53	.54	.525
7		.175	.27	.33	.415	.47	.505	.515	.505
8		.15	.24	.30	.385	.44	.48	.49	.49
9		.13	.212	.27	.36	.415	.455	.47	.47
10		.115	.19	.25	.34	.395	.43	.45	.455
pH	0.88	1.18	1.40	1.58	1.88	2.18	2.40	2.58	2.88
k_a	0.22	0.15	0.10	0.08	0.05	0.037	0.028	0.022	0.018

D_i are optical densities at 455 mμ at time t

k_a in units of $min.^{-1}$

TABLE II

Hydrolysis of the Azine. Effect of Acid Concentration Variations at 25.0°C in a System Containing 60% by Volume Ethanol

t(min)	D_1	D_2	D_3	D_4	D_5	D_6	D_7
1	0.81		0.845	0.86	0.87	0.78	0.71
2	.755	0.785	.735	.70		.59	.43
3	.71	.725	.655	.585	.59	.465	.28
4	.675	.67	.59	.50	.505	.375	.195
5	.64	.625	.53	.435	.44	.305	.145
6	.61	.585	.48	.38	.39	.255	.115
7	.58	.55	.445	.34	.35	.215	.095
8	.55	.53	.41	.31	.31	.185	.082
9	.53		.385	.278	.285	.16	
10	.505	.46	.36	.253	.26	.145	
pH	2.59	2.28	1.88	1.58	1.58	1.28	0.88
k_a	.025	.039	.061	.084	.084	.12	.22

D_i are optical densities at 455 mμ at time t.

k_a in units of $min.^{-1}$

TABLE III

The Acid Dissociation Constant of p-dimethylaminobenzaldehyde at 25.0°C in the Water-Ethanol System Containing 6 ml. Ethanol

m_1	$\log \gamma_1$	γ_1	(H^+)	D_{350}	D_{340}	D_{330}
0.187	-0.0732	0.845	0.158	0.490	0.440	0.300
0.374	.0672	.856	.320	.365	.325	.220
0.561	.0613	.868	.487	.270	.240	.160
			K_A =	0.70	0.71	0.71

D_x are optical densities at noted wave lengths

TABLE IV

The Acid Dissociation Constant of p-dimethylaminobenzaldehyde at 25.0°C in the Water-Ethanol System Containing 1 ml. Ethanol

$[H^+]$	D_{350}	K_A'	μ	f_{H^+}	$-\log K_A' f_{H^+}$	$\log f_{CHO^+}$
0.024	1.33	0.033	0.024	0.86	1.548	0.0631
.036	1.10	.033	.036	.84	1.558	.0727
.048	0.94	.033	.048	.824	1.566	.0801
.060	.82	.033	.060	.816	1.571	.0858
.072	.72	.032	.072	.80	1.576	.0906
.084	.64	.032	.084	.80	1.579	.0949
.000	2.28					

$K_A = 0.033$

TABLE V

Determination of the Acid Dissociation Constants for the Azine and the Hydrazone and the Equilibrium Constant for the Hydrazine, the Aldehyde, and Hydrazone at 25°C.

D_{455}	X	k_1-k'	Z(F)	X	$[H^+]$	X_1^2	Y_1^2	K_1	X_2^2	Y_2^2	K_2
0.375	.485	0.0125	5.62×10^{-17}	0.141	0.0026	0.198	0.110	228	0.185	0.103	222
.415	.445	.0105	5.23	.198							
.430	.430	.0095	4.90	.185							
.525	.335	.0060	3.78	.112							
0.320	0.600	0.0215	4.20×10^{-17}	0.360	0.0052	0.360	0.070	684	0.336	0.067	677
.345	.575	.0185	3.90	.331							
.410	.510	.013	3.25	.260							
.545	.375	.008	2.66	.141							
0.355	0.595	0.028	2.26×10^{-18}	0.354	0.0133	0.354	0.096	995	0.225	0.083	1050
.380	.570	.026	2.17	.325							
.408	.542	.023	2.06	.294							
.445	.505	.021	1.76	.255							

k_1-k' in units of $min.^{-1}$

Calculated values of A:

$A(H^+ = 0.0026F) = 9.1 \times 10^5\ M^{-1}$
$A(H^+ = 0.0052F) = 3.26 \times 10^6\ M^{-1}$
$A(H^+ = 0.0133F) = 5.1 \times 10^7\ M^{-1}$

Calculated equilibrium constant values:

K_A (azine) $= 1.5 \times 10^{-2}$
K_A (hydrazone) $= 1.2 \times 10^{-3}$
$K = 1.3 \times 10^{-7} M$

TABLE VI

Hydrolysis of the Azine. Effect of Dielectric Constant Variations at 25.0°C

Vol.% EtOH	Mole Fraction H_2O	$\frac{10^2}{D}$	k_a''	k_a'	$\frac{k_a'}{[H_2O]}$	$\frac{k_a''}{[H_2O]}$
30	0.882	1.55	0.400	0.380	0.409	0.430
40	.83	1.69		.257	.310	
50	.764	1.87	.280	.190	.229	.337
60	.682	2.10	.210	.130	.171	.275
70	.582	2.40	.156	.100	.147	.229
80	.443	2.80	.112	.070	.120	.193
90	.265	3.33	.080	.045	.102	.179

k_a' = apparent first rate constant when $[H^+]$ = 0.133F

k_a'' = apparent first rate constant when $[H^+]$ = 0.0665F

TABLE VII

Hydrolysis of the Azine. Temperature Dependence

t(min)	D(2.49)	D(2.18)	D(1.78)	D(1.48)	D(1.18)	D(1.00)	D(0.78)
			T = 298.2°K				
1	0.80		0.845	0.87	0.78	0.74	0.70
2	.755	0.78	.735	.70	.59	.53	.42
3	.71	.72	.65	.59	.46	.385	.275
4	.675	.67	.585	.50	.37	.29	
5	.64		.53	.435	.305	.225	.143
6	.61	.58	.48	.385	.253	.18	.115
7	.575	.545	.445	.345	.215	.15	.095
8	.55		.408		.185	.128	.08
9	.525	.48	.38	.28	.16	.11	
10	.505	.455	.355	.255	.143	.095	.063
k_a	0.030	0.045	0.065	0.096	0.130	0.155	0.225
			T = 303.2°K				
1	0.79	0.84	0.82	0.77	0.68	0.63	0.56
2	.73	.74	.655	.565	.45	.39	.29
3	.67	.67	.54	.435	.32	.255	.168
4	.63	.605	.45	.35	.24	.18	.108
5				.285	.185		
6			.33	.243	.148		
7			.29	.21			
8			.26	.182			
9			.235	.16			
10			.215	.142			
k_a	0.037	0.058	0.102	0.138	0.190	0.210	0.292
ΔE (cal.)	7.5×10^3	9.1×10^3	$16.\times10^3$	$13.\times10^3$	$14.\times10^3$	$11.\times10^3$	9.5×10^3
log Z	3.94	5.21	10.5	9.5	8.9	7.0	6.2
ΔS (cal. deg^{-1})	-41.	-35.	-10.	-15.	-18.	-27.	-30.

TABLE VIII

Hydrolysis of the Azine. Effect of Ionic Strength Variations at 25.0°C in a System Containing 60 Vol. % Ethanol

time	D(0.133)	D(0.0665)	D(0.0399)	D(0.0266)	D(0.0133)	D(0.00665)	D(0.00399)	D(0.00266)	D(0.00133)
					1- μ = 0.133F				
1	0.64	0.70	0.73	0.75	0.75	0.74	0.74	0.70	0.635
2	0.40	.51	.59	.63	.67	.68	.68	.665	.61
3	0.26	.39	.485	.54	.60	.625	.64	.63	.585
4	.185	.31	.41	.47	.54	.58	.60	.60	.56
5	.14	.25	.35	.41	.49	.535	.56	.57	.54
6	.115	.205	.305	.365	.45	.50	.53	.54	.525
7		.175	.27	.33	.415	.47	.505	.515	.505
8		.15	.24	.30	.385	.44	.48	.49	.49
9		.13	.21	.27	.36	.415	.455	.47	.47
10		.115	.19	.25	.34	.395	.43	.45	.455
k_a	0.22	0.15	0.10	0.082	0.050	0.037	0.028	0.022	0.018
					2- μ = acid formality				
1	0.65	0.69	0.74	0.76	0.77	0.775	0.73	0.71	0.625
2	.40	.52	.60	.65	.70	.72	.69	.68	.605
3	.265	.405	.50	.56	.635	.665	.655	.64	.58
4	.185	.33	.43	.495	.575	.58	.62	.615	.565
5	.14		.375	.44	.525	.55	.585	.585	.545
6	.125	.225	.295	.40	.48	.515	.555	.56	.53
7		.19	.265	.36	.445	.48	.53	.535	.51
8		.165	.24	.33		.46	.505	.515	.495
9		.145	.22	.305		.43	.48	.49	.48
10			.20	.285		.41	.455	.475	.465
k_a	0.22	0.14	0.11	0.072	0.045	0.034	0.025	0.021	0.018

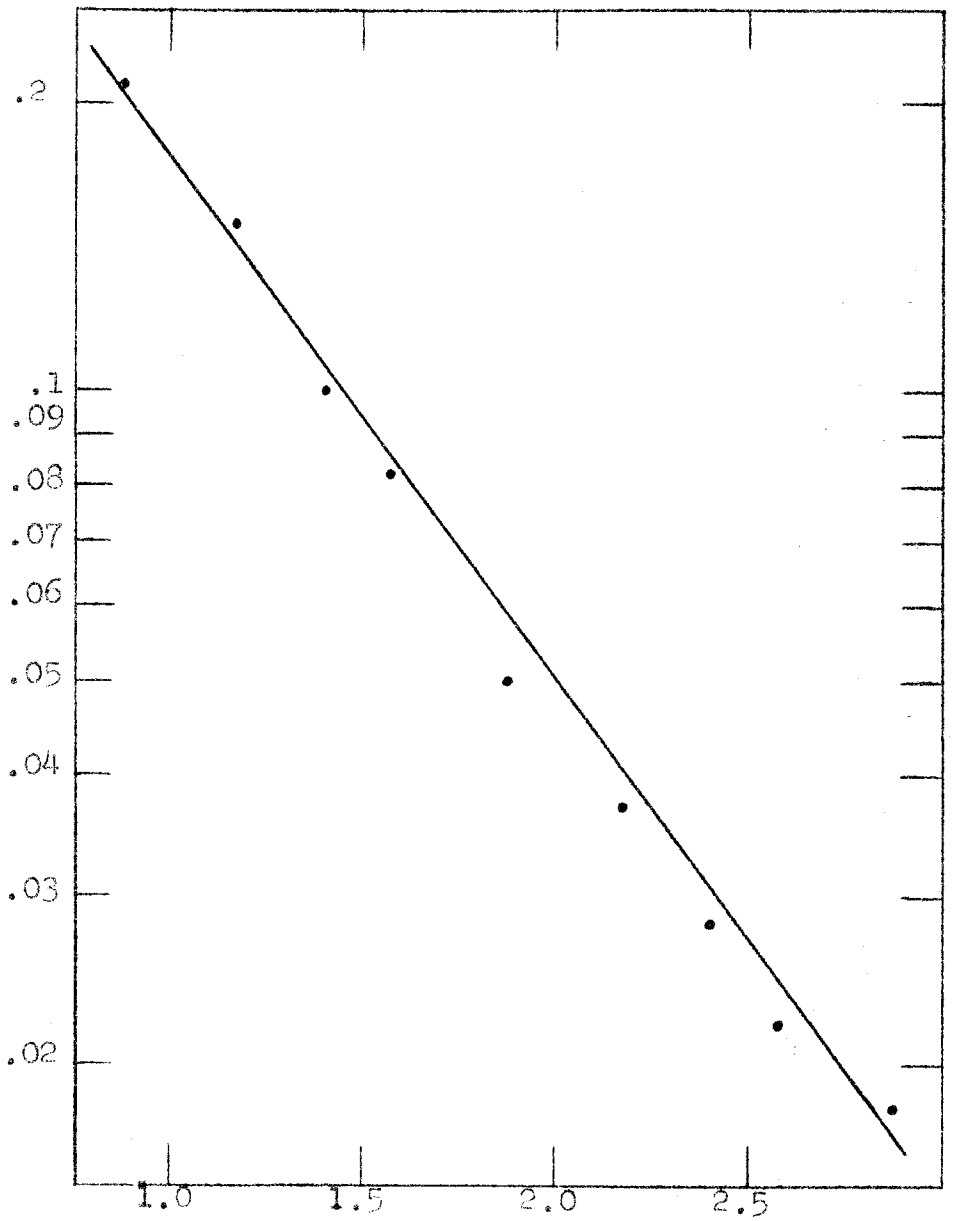

Fig. 1. Hydrolysis of the azine at 25.0°C. Dependence on acid concentration. Solvent: ethanol-water containing 60 vol. % ethanol. Initial azine concentration = 2.08×10^{-5}F. Ionic strength = 0.133F. Ordinates are log k_a. Abscissae are pH.

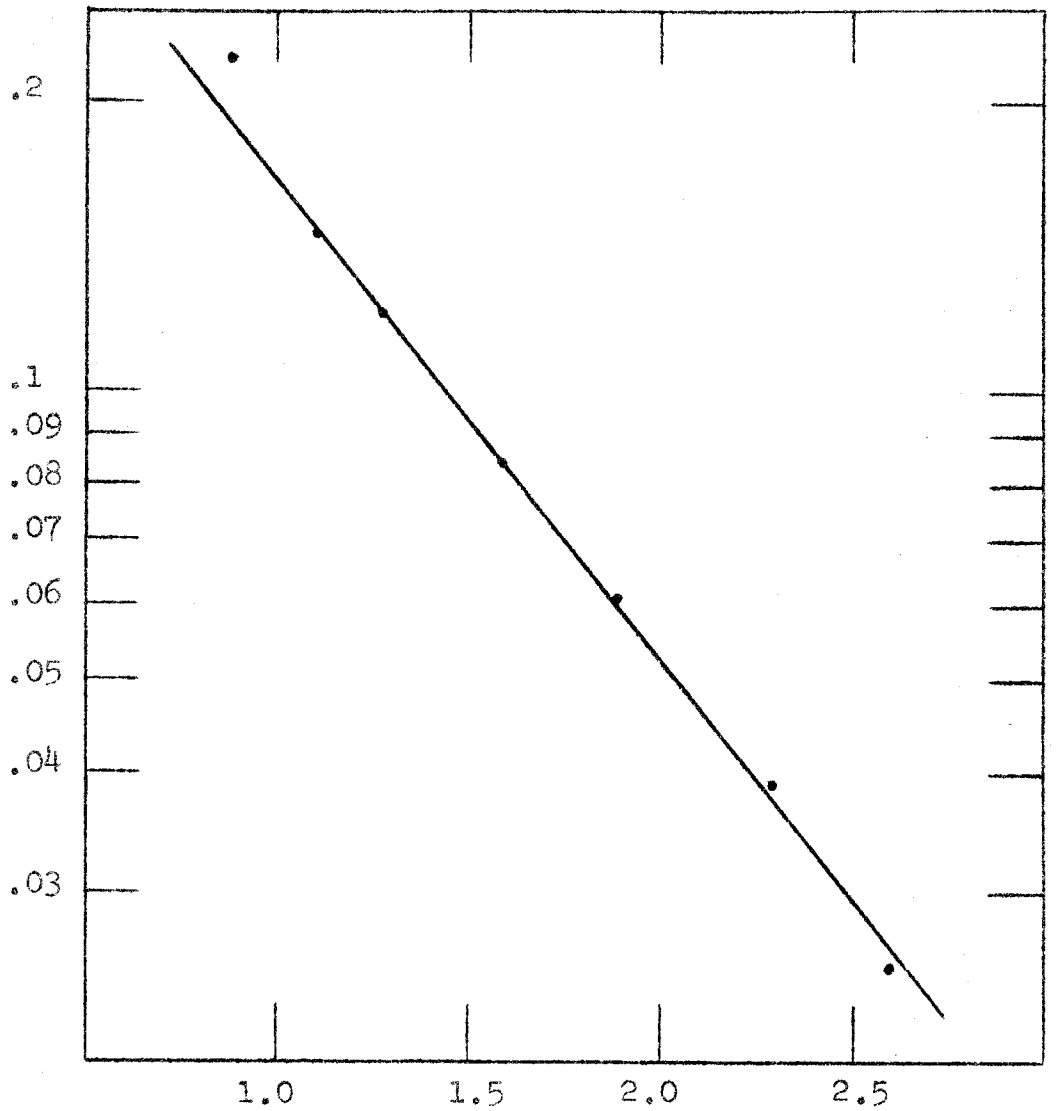

Fig. 2. Hydrolysis of the azine at 25.0°C. Dependence on acid concentration. Solvent: ethanol-water containing 60 vol. % ethanol. Initial azine concentration = 2.08×10^{-5}F. Ionic strength = 0.133F. Ordinates are log k_a. Abscissae are pH.

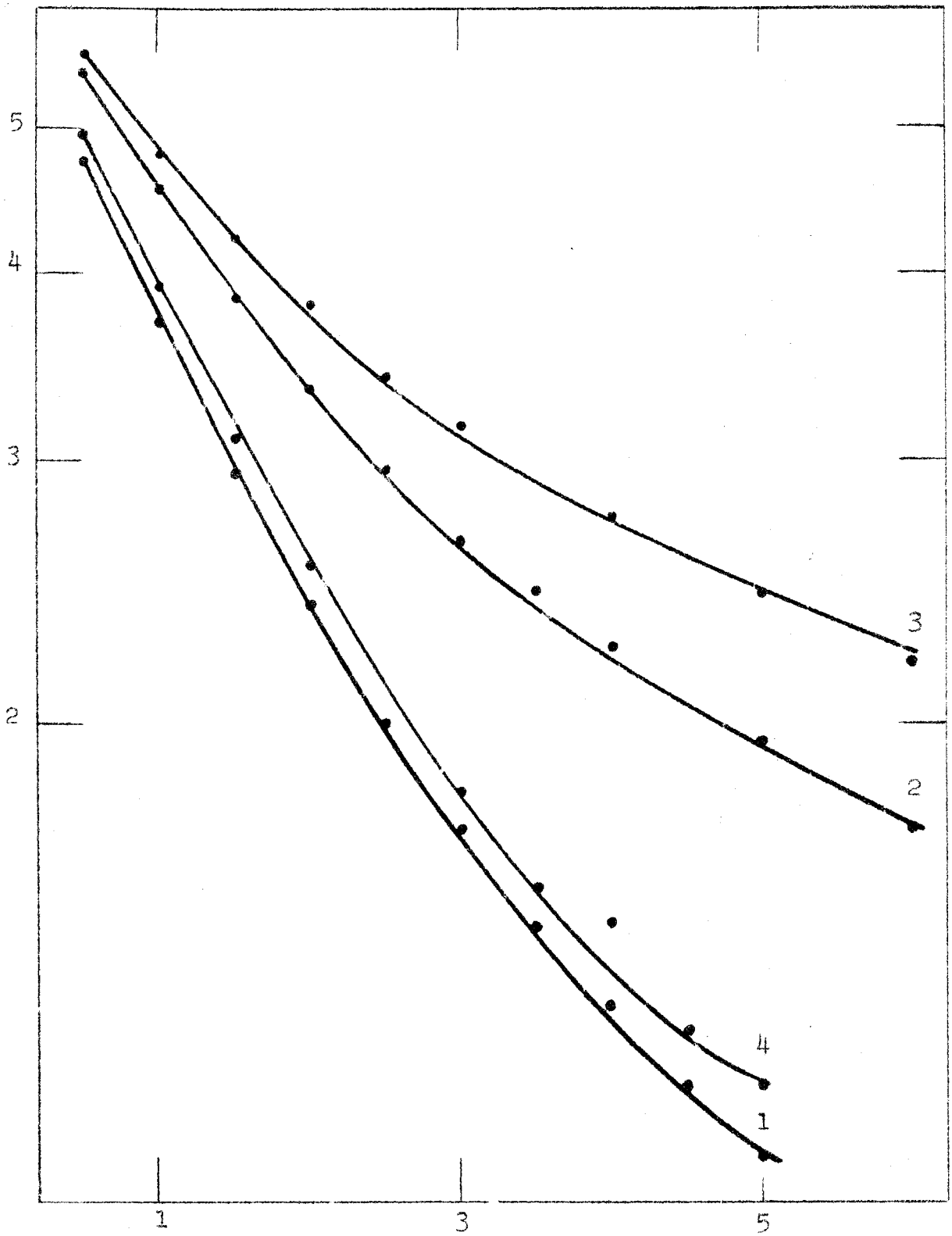

Fig. 3. Hydrolysis of the azine at 25.0°C. Retarding effect by initial addition of aldehyde and hydrazine to system. Solvent: ethanol-water containing 60 vol. % ethanol. Initial azine concentration = 2.08 x 10^{-5}F. Acid concentration = 0.133F. Curve 1: no additions; curve 2: 1 ml. 4.0 x 10^{-4}F aldehyde added; curve 3: 1 ml. 8 x 10^{-4}F aldehyde added; curve 4: 1 ml. 10^{-2} hydrazine added. Ordinates are log D_{455}. Abscissae are min.

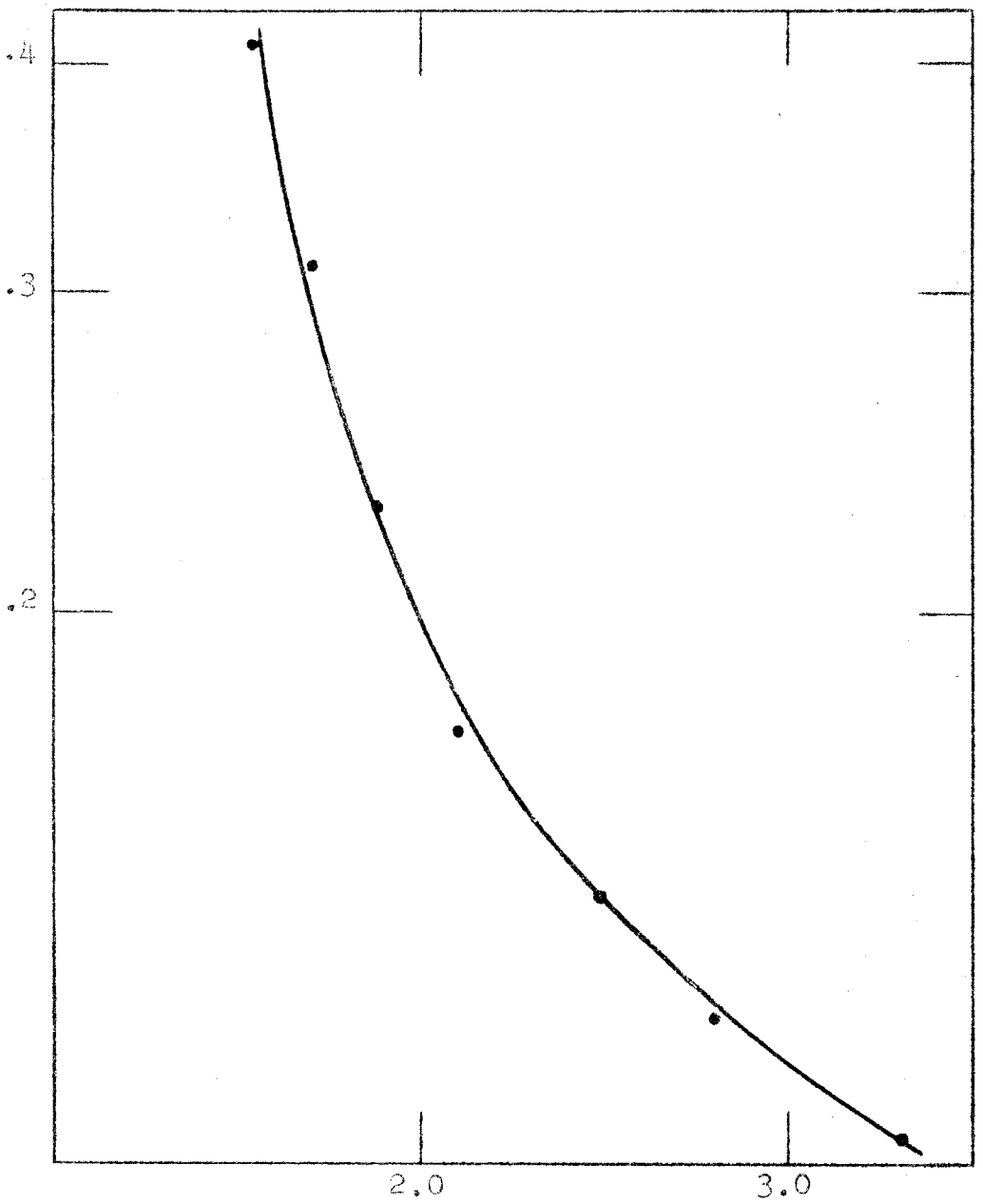

Fig. 4. Hydrolysis of the azine at 25.0°C. Dependence on dielectric constant. Solvent: ethanol-water. Acid concentration = 0.0665F. Ordinates are log $k_a/[H_2O]$. Abscissae are $10^2/D$.

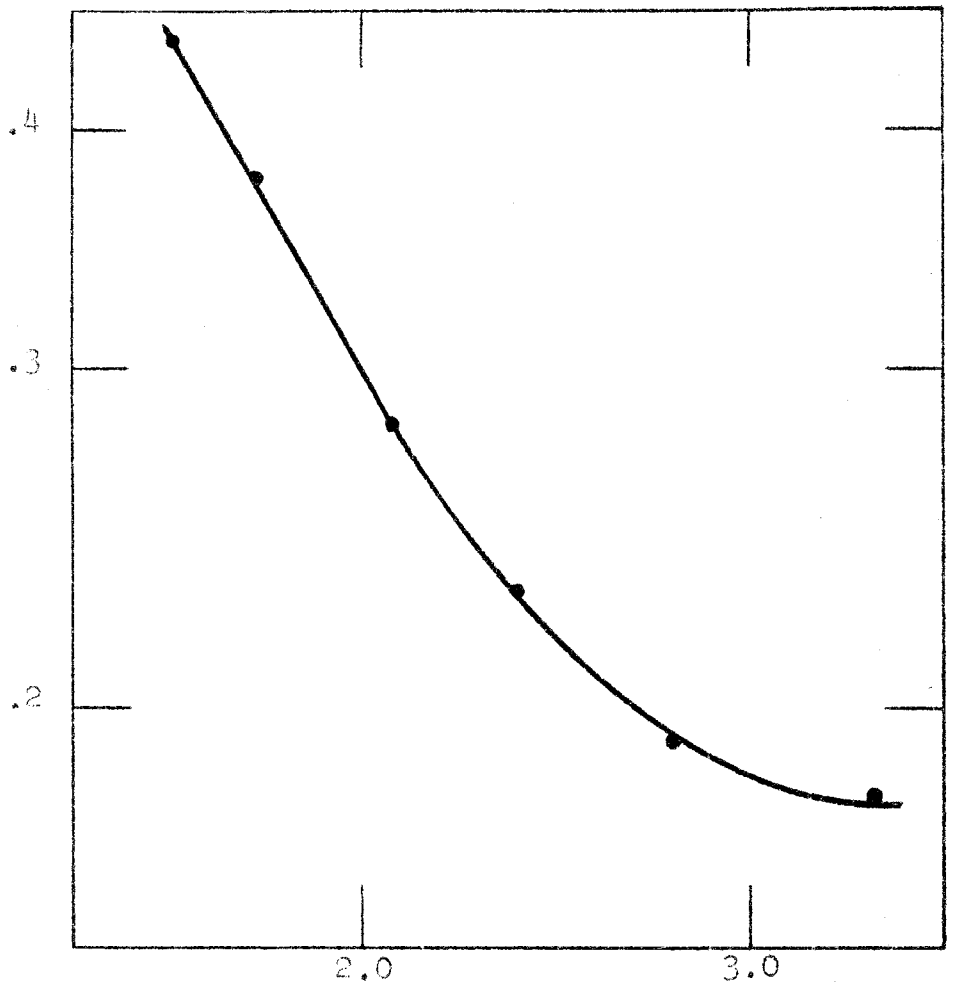

Fig. 5. Hydrolysis of the azine at 25.0°C. Dependence on dielectric constant. Solvent: ethanol-water. Acid concentration = 0.133F. Ordinates are log $k_a/[H_2O]$. Abscissae are $10^2/D$.

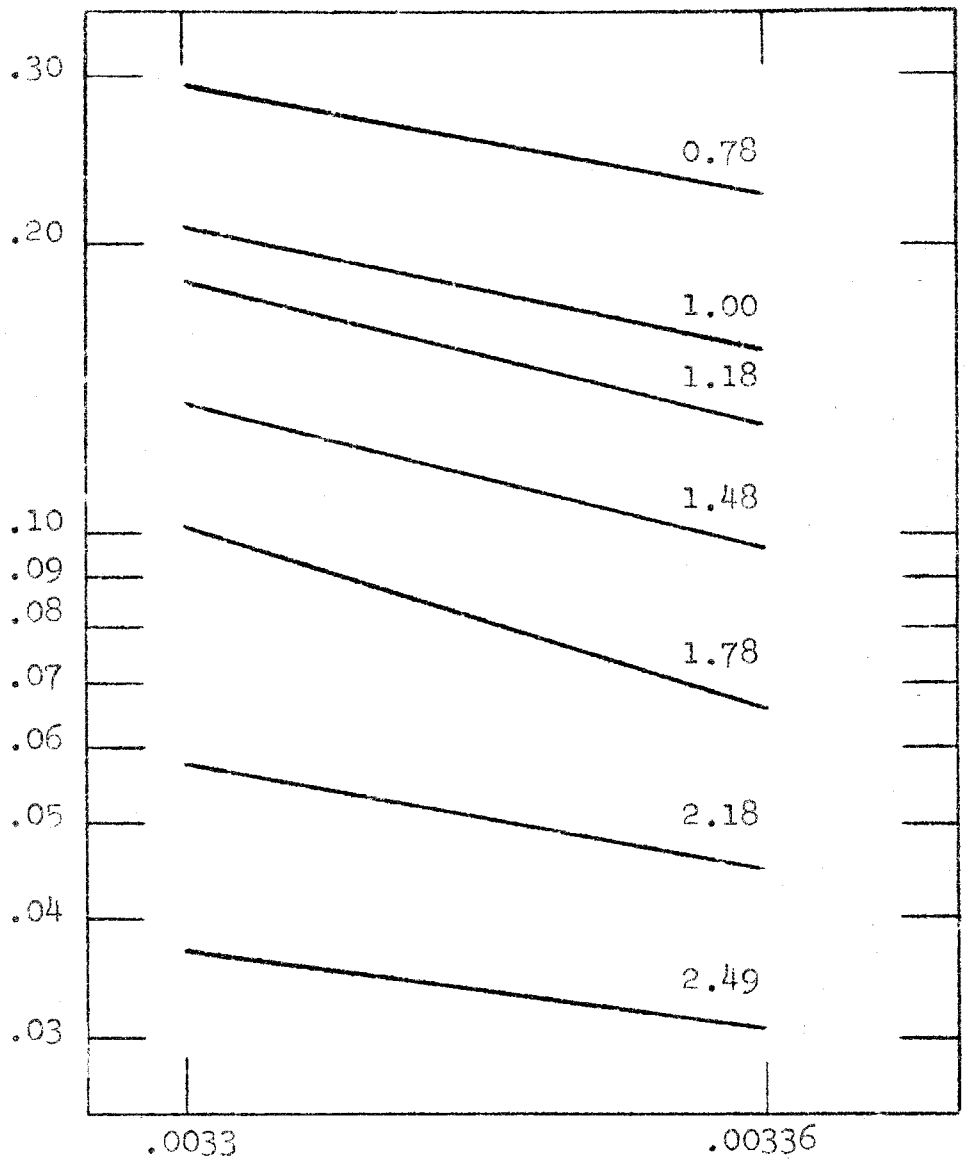

Fig. 6. Hydrolysis of the azine. Temperature dependence. Solvent: ethanol-water containing 60 vol. % ethanol. Ordinates are log k_a. Abscissae are 1/T. Ionic strength = 0.133F. Acidities of systems indicated by pH values at ends of curves.

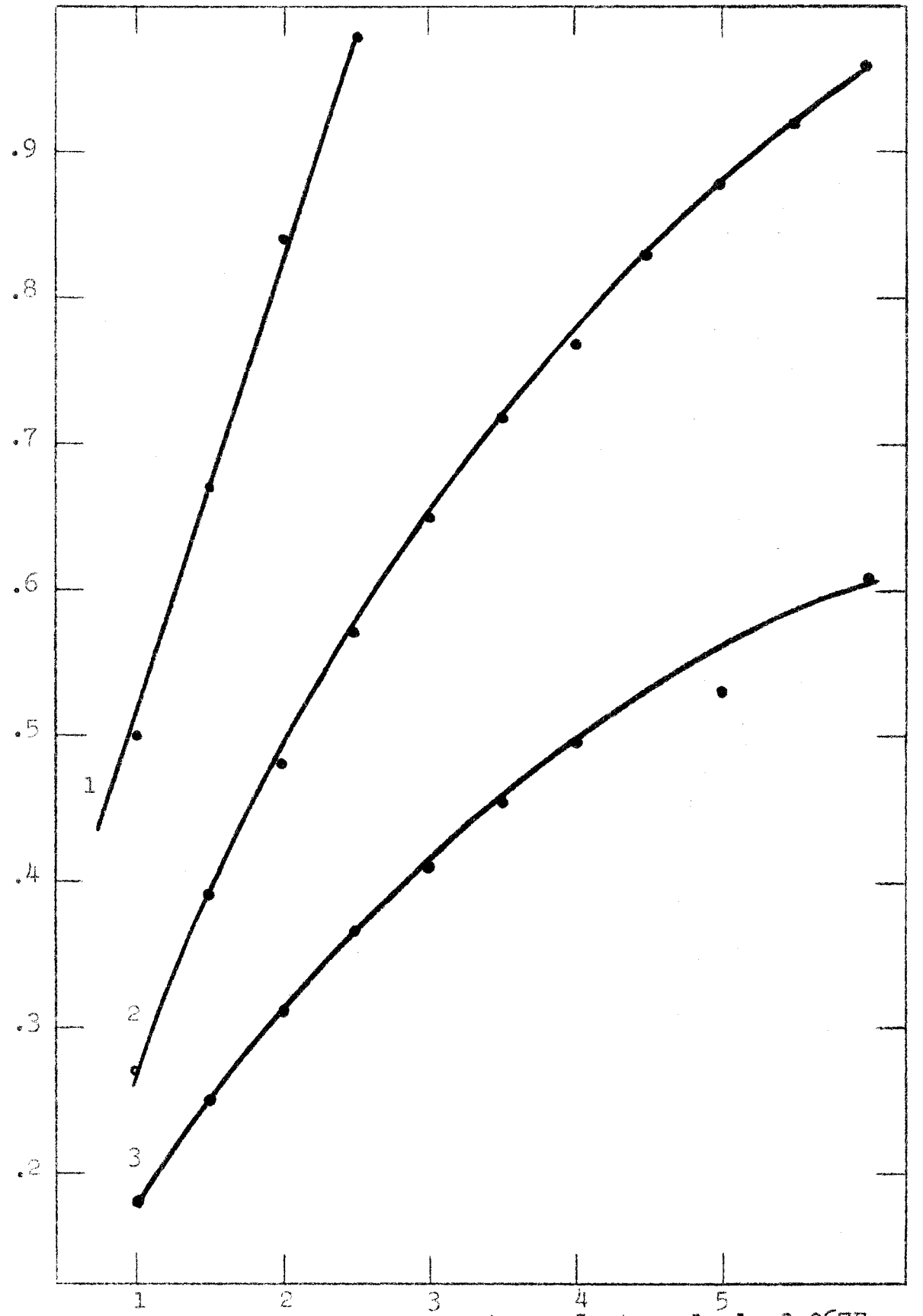

Fig. 7. Formation of the azine. System: 1 ml. 0.067F aldehyde, 8 ml. .216F HCl, and 1 ml. hydrazine. Curve 1: 50 x 10^{-5}F; curve 2: 30 x 10^{-5}F; curve 3: 20 x 10^{-5}F. Ordinates are D_{455}. Abscissae are min.

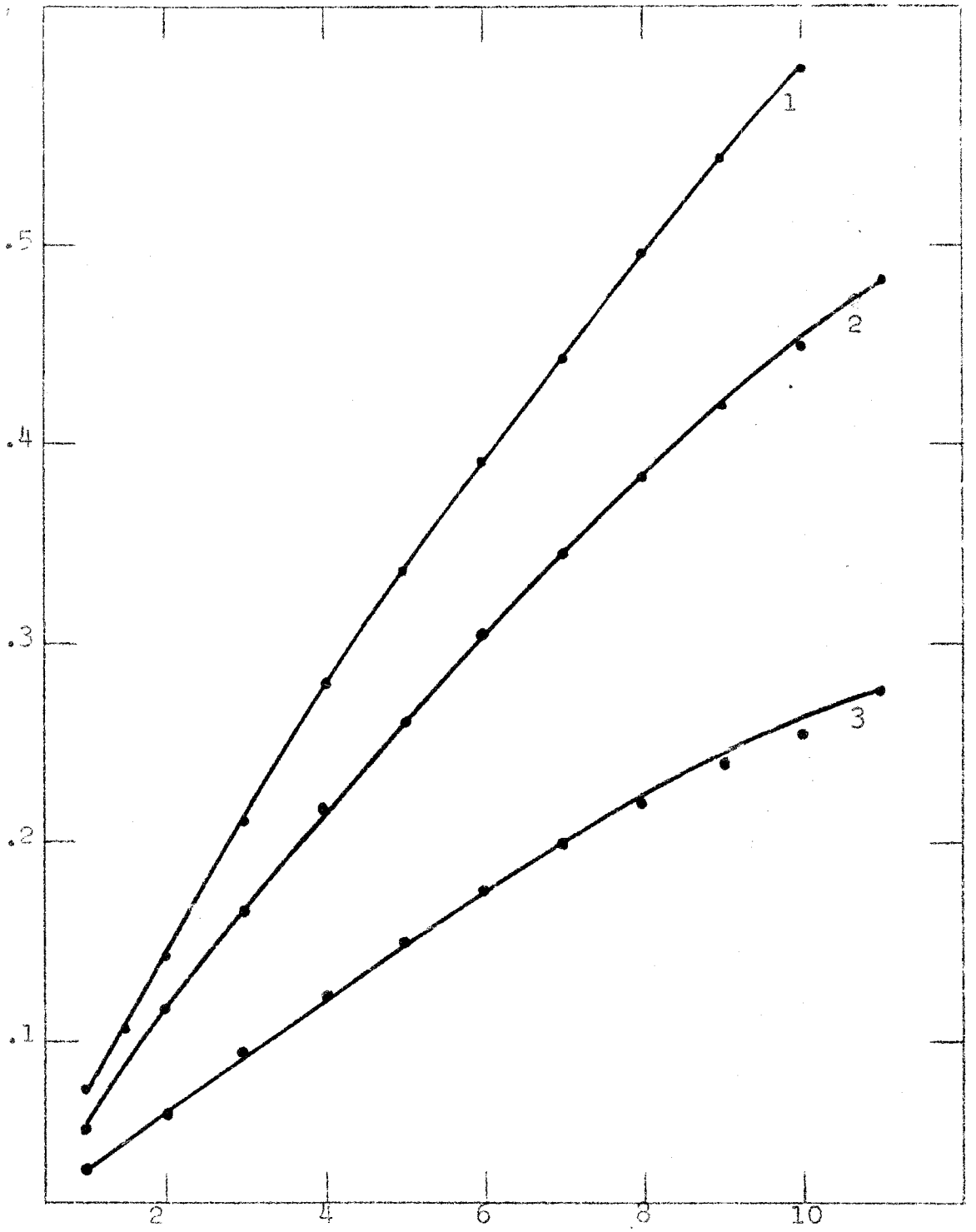

Fig. 8. Formation of the azine. System: 1 ml. 0.067F aldehyde, 1 ml. 1.33F HCl, 5 ml. EtOH, 2 ml. H_2O, and 1 ml. hydrazine. Curve 1: 25 x 10^{-5}F; curve 2: 20 x 10^{-5}F; curve 3: 12 x 10^{-5}F. Ordinates are D_{455}. Abscissae are min.

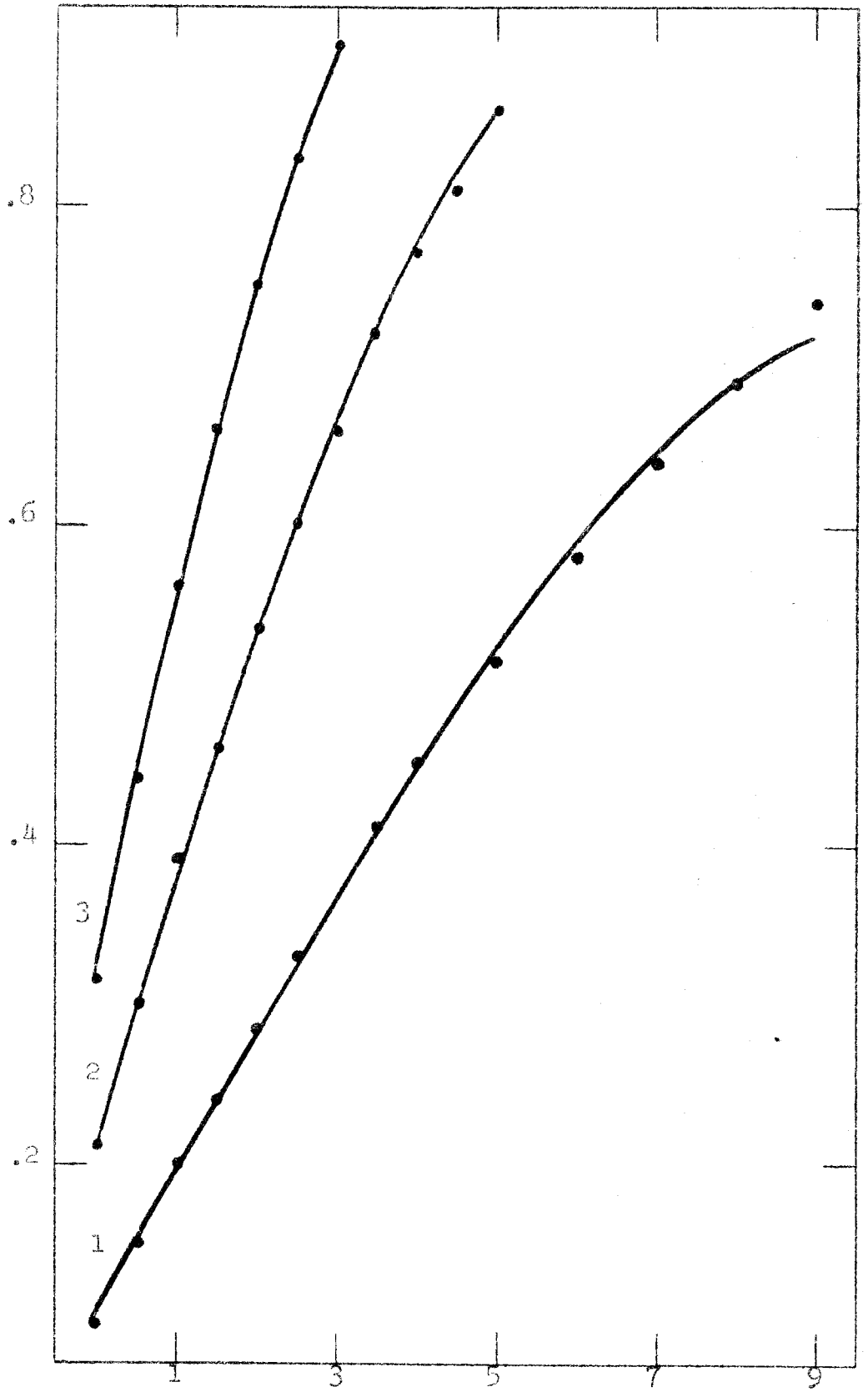

Fig. 9. Formation of the azine. System: 1 ml. 0.067F aldehyde, 1 ml. 25 x 10^{-5}F hydrazine, 5 ml. EtOH and 3 ml. aqueous HCl. Curve 1: 1 ml. 1.33F HCl; curve 2: 2 ml. 1.33F HCl; curve 3: 3 ml. 1.33F HCl. Ordinates are D_{455}. Abscissae are min.

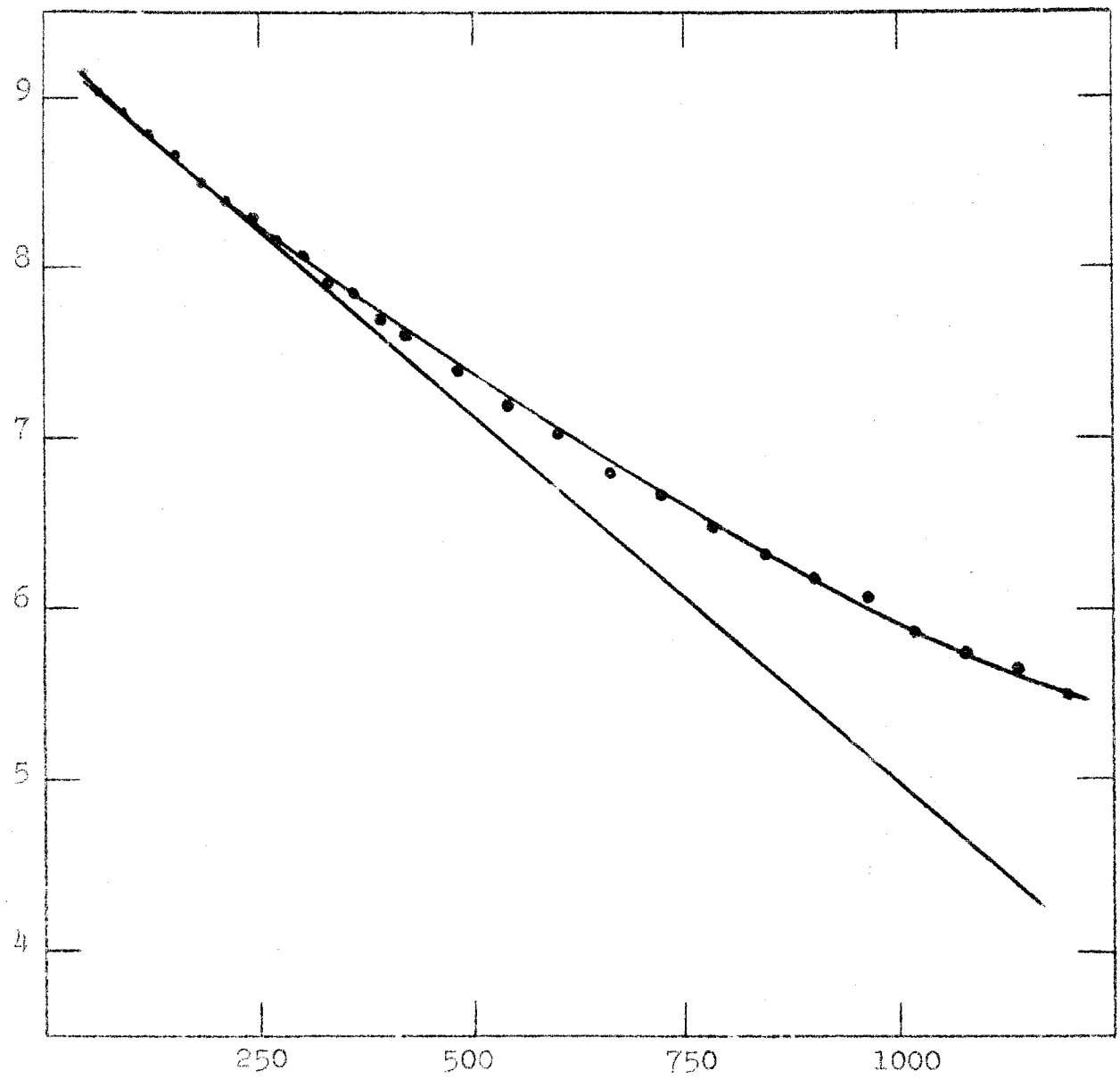

Fig. 10. Hydrolysis of the azine at 25.0°C. Solvent: ethanol-water containing 60 vol. % ethanol. Initial azine concentration = 2.08×10^{-5}F. HCl concentration = 2.6×10^{-3}F. Ionic strength 0.133F. Ordinates are log D x 10^2. Abscissae are seconds.

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PROPOSITIONS

1. It has been postulated that the difference in the K_s values of nicotinyl and benzoyl substituted substrates for the alpha-chymotrypsin catalysed hydrolysis is in part due to the difference in the hydrophilic character of these two substituents (1). I propose that this hypothesis may be tested by the use of mixed solvent systems.

(1) Huang, H. T., and Niemann, C., J. Am. Chem. Soc., 74, 101 (1952)

2. It has been postulated that the decrease in the value of the pH optima for non-acylated alpha-amino acid substrates for alpha-chymotrypsin catalysed hydrolysis as compared with the acylated substrates is due to the fact that the pH optima for these substrates represent the intersections of an enzyme activity curve and curves portraying the relative amounts of the active species of the substrates as a function of the pH (1). I propose that this postulate may be tested by the use of mixed solvent systems.

(1) Lutwack, R., Ph.D. Thesis, California Institute of Technology, 1954.

3. It has been postulated that for a series of substrates the relative closeness of fit of a particular substrate and

the enzyme in the substrate-enzyme complex is measured by the K_s value of the system. I propose that this postulate may be tested by the use of kinetic data from hydrolyses at different temperatures in various solvent systems.

4. It has been postulated that the solvation of the hydrogen ion changes in such a manner when the organic component of a binary mixture with water is increased that the concentration of the hydronium ion increases (1). I propose that this postulate may be tested by the method of proton magnetic resonance.

(1) Braude, E. A., J. Chem. Soc., 1944, 443.

5. The slow formation of bis-p-dimethylaminobenazlazine has been observed in an acidic solution of semicarbazide and p-dimethlyaminobenzaldehyde (1). I propose that a reasonable explanation of this phenomenon is the slow decomposition of semicarbazide to form hydrazine-dicarbonamide and hydrazine.

(1) Watt, G. W., and Chrisp, J. D., Anal. Chem., 24, 2006 (1952)

6. A value for the second acid dissociation constant for α - (β pyridyl) - piperidine has been reported (1). I propose that this value is incorrect.

(1) Linnell, R. H., J. Am. Chem. Soc., 76, 1391 (1954)

7. I propose that the hydration of alpha-chymotrypsin in aqueous solution may be studied by the method of proton magnetic resonance.

(1) Jacobson, B., Anderson, W. A., and Arnold, J. T., Nature, 173, 772 (1954)

8. The optical densities at 455 mu of bis-p-dimethylaminobenzalazine as functions of the concentrations of p-dimethylaminobenzaldehyde and acid have been reported (1). I propose that the conditions of the experiments were not satisfactory for the derivation of independent relationships.

(1) Wood, P. R., Anal. Chem., 25, 1879 (1953)

9. It has been reported that the values for K_s and k_3 for a substrate hydrolysed by alpha-chymotrypsin may be calculated from the kinetic data at only one substrate concentration (1). I propose that the proof intended to show that the kinetics follow the Michaelis-Menten equation is fallacious.

(1) Cunningham, L. W., J. Biol. Chem., 207, 443 (1954)

10. It has been reported that nicotinyl D-phenylalanyl-β naphthylamide is not an inhibitor for the L-isomer (1). I propose that the conditions of the experiment did not justify this conclusion.

(1) Ravin, H. A., Bernstein, P., and Seligman, A. M., J. Biol. Chem., 208, 1 (1954)