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Study of the Primary Salt Effect and of the Change in Dielectric Constant of the Medium on the Kinetics of the Urease-Urea Reaction

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A STUDY OF THE PRIMARY SALT EFFECT AND OF THE CHANGE IN DIELECTRIC CONSTANT OF THE MEDIUM ON THE KINETICS OF THE UREASE-UREA REACTION

by

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LIFE

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PREFACE

The author wishes to take this opportunity to express his thanks and appreciation to Dr. Hugh J. McDonald, under whose supervision this study was made.

The original conception of the problem, the encouragement throughout the progress of the work and the friendly interest shown by Dr. McDonald were factors which greatly assisted in the completion of this task.

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CHAPTER I

INTRODUCTION

For a number of years, the role played by enzymes, in the various fields of biochemistry, has provided a wealth of opportunities for original research. In recent years interest in enzyme systems has mounted steadily, and lately some significant progress has been made in the understanding of the mechanism of such systems. Although, the kinetics of many enzyme reactions has been studied, there is still no general agreement as to their mechanism because of their extreme complexity.

The purpose of this investigation is to study the kinetics of the enzymatic hydrolysis of urea by the enzyme, urease, in the light of two particularly important theoretical advances, namely, the Bronsted primary salt effect, and the effect of dielectric constant of the medium on this reaction. From these effects, it should be possible to determine whether the rate controlling step of this reaction involves ions as intermediates, or whether its course is influenced simply by molecular forms (55). Knowledge of this kind would provide a better understanding of the fundamental

mechanism of the urease-urea reaction.

The biochemical nature and importance of enzymes can not be overemphasized. Enzymes can be regarded as colloidal biological catalysts, which have been synthesized, so far, only in living organisms. They enable living cells to carry out the chemical processes necessary for their existence, at a sufficient velocity and at temperatures considerably lower than those which normally are required in the laboratory. Although, the enzymes usually act within the cell which formed them, they may, in many cases, carry out their particular functions outside the cell. Urease is such an enzyme.

Enzymes are indispensable for the chemical changes occuring in the living cell, and without them matter would be lifeless. No process associated with cellular activity, whether it be growth and proliferation of the lowliest of living organisms, or the functioning of the most highly organized of living structures, such as the central nervous system, can be imagined taking place without the participation of enzymatic reactions. In death, some enzymes are as active as in life, although their organization is completely altered. The autolytic changes taking place in the cell,

which through unfavorable environmental circumstances can no longer survive and proliferate, are controlled by enzymes which ultimately break down and lose their own activities. No aspect of the chemistry of living matter, whether it refers to such diverse phenomena as the bacterial fixation of nitrogen, the luminescence of bacteria, the synthesis of hormones and vitamins, or the digestion of cellulose in an animal's intestine, may be regarded as complete without due regard to the controlling influence of enzymes.

Enzymes often exhibit great specificity. A given enzyme will usually act only on a single substrate or a group of closely related substrates. In the case of urease, its specificity is absolute, since as far as is known will only catalytically hydrolyze urea. The activity of enzymes is influenced in a remarkable degree by factors, such as acidity, alkalinity, temperature, and the presence of certain inorganic salts. There is an optimum temperature for the action of an enzyme, above which the enzyme diminishes in activity, and eventually is destroyed. There is also an optimum pH at which the enzyme is most active. Owing to this sensitiveness to external factors, an enzyme very

frequently is able to change only a limited amount of substrate, for the products of its own activity accumulate, and by their accumulation, gradually inhibit the activity of the enzyme.

Not much of an enzyme is required to do its work, since they are active even in extremely minute quantities. Thus, the enzyme, urease, can hydrolyze a million times its own weight of urea, without an appreciable loss of activity. It might be pointed out that enzymes are easily poisoned by heavy metal cations, which react with the functional groups necessary for their activity, and urease is no exception.

The chemical structure of enzymes is not definitely known. Many regard them as proteins, but there is one school of thought that does not believe in this (97). They are of the opinion that enzymes exist in colloidal combination with cell proteins, and their sensitiveness to heat and other factors is interpreted as being due to this association. The molecule of an enzyme may consist of a colloidal carrier and a purely chemical active group, although complete separation of these has not been accomplished. There is no doubt that, owing to its close association with a colloid, the enzyme molecule has an extensive surface area,

and it appears reasonable that one of the first stages in the action of an enzyme is the concentration of the molecules of the substrate on this surface by adsorption.

It has been assumed that the first step in enzyme catalysis is the formation between enzyme and substrate of a compound which, after a definite time-interval, decomposes to form the reaction products and the original enzyme (99). The place of combination on the enzyme is left vacant until another substrate molecule enters the sphere of action, when the cycle of combination and decomposition takes place again. It is useful to accept this view of intermediate compound formation and breakdown, not only because the kinetics of enzymatic change can be interpreted on this basis, but because there is some evidence that the intermediate complex between substrate and enzyme does in reality exist. An example of such evidence comes from the facts concerning the protection by substrates of their enzymes from inactivation by heat and other agencies. Thus, urease is less sensitive to the inactivation by heat in the presence of its substrate, urea.

Urease, which can be considered a water soluble protein, catalytically hydrolyzes urea to ammonium carbonate,

which in turn decomposes to ammonia and carbon dioxide. Since this reaction proceeds well at a pH close to neutrality, the Bronsted primary salt effect (13, 14, 15, 16), and Scatchard's effect of changing environment (80, 81, 82) can be more correctly interpreted. The establishment of a case, either for, or against an ionic mechanism will represent a distinct advance in this important field, and would extend the understanding of enzyme reactions, in general.

CHAPTER II

A REVIEW OF THE LITERATURE

The fact that the hydrolytic activity of urease is affected by certain electrolytes and solvents is known, but there is no conclusive evidence in the published literature as to what kind of an effect it really is, and exactly how it operates.

As early as 1913, Armstrong and Horton (7) observed that sodium chloride and potassium chloride retarded the action of urease. They accounted for this effect of salts by assuming the enzyme, urease, to be a feebly acid substance, which in order to cause change combines with the feebly basic substance, urea, therefore, they thought that any basic cation would interfere with the enzyme. Armstrong and Armstrong (6) were probably the first workers to notice the effect of sodium and potassium phosphate buffers on the activity of urease. They found this effect to vary with the concentration of the phosphate buffer. Van Slyke and Zacharias (106) also found that the presence of salts, such as sodium chloride and phosphate would inhibit the action of urease. They concluded that the effect of sodium chloride

was to retard the combination of urease and urea. When they varied the concentration of the phosphate buffer, their results indicated that the combination of the enzyme and urea is retarded in a way which is directly proportional to the phosphate concentration.

Onodera (72) found that neutral salts inhibited the urease-urea reaction, and also proposed that the effect of these salts is due, at least to a certain degree, to the alkalinity of the cations. Groll (31) investigated the effect of a number of cations and anions on urease, and observed that the ammonium, potassium, sodium, strontium and barium cations appeared to retard its action, when in 0.05 molar concentration. With the same cation, the nature of the anion had little influence. It was his opinion that the effect of neutral salts on urease could be ascribed to the influence of their ions on the degree of dispersion of the enzyme, in the same manner as in colloids.

A few years later, Wester (113) also found that the inhibiting influence of anions such as chloride, bromide, iodide, nitrate and sulfate was slight on the ureolytic power of urease, but the effect did increase with increasing concentration of the anion. He found that the inhibiting ef-

fect of the potassium, sodium, magnesium and the barium ion was much greater, and it also increased with the higher concentration of the cation. No additive effect due to the ions was noted.

Up to 1920, although all of the investigators in this field found sodium chloride to inhibit the activity of urease, Rona and Gyorgy (77), who studied the action of poisons on urease, reported that they observed no inhibiting action of sodium chloride on the urease-urea hydrolysis. As a matter of fact, Kochmann (46) found that the calcium ion in a phosphate solution increased the activity of urease proportionately to its concentration.

Mystkowski (69), who studied the influence of sodium salts on the action of urease in buffered and unbuffered solutions, reported that certain sodium salts would accelerate the action of urease at low concentrations, but in every case retarded the activity at higher concentrations. Vande-Velde (107) observed that both potassium and sodium salts retarded the activity of urease when at 0.3 molar concentration, and that sodium salts were more inhibiting than the potassium salts, and that sulfates inhibited more than the chlorides. It was Ruchelmann (78), who first observed that

sodium chloride causes an activation of urease in small amounts, but did cause an inhibition when it was added in larger amounts. He also found that potassium chloride, rubidium chloride and lithium chloride inhibited the hydrolysis, and suggested that since urease is an ampholyte, it can react separately with many cations and anions.

Schmidt (83) observed that sodium and potassium fluoride caused an inhibition of the urease-urea system, which he thought was mainly due to the fluoride ion. A year later, Jacoby (38) noted that sodium iodide was effective in retarding urease activity when the iodide concentration was increased. Sizer and Tytell (84) have observed that urease activity increased and then decreased with increasing concentration of sodium sulfate.

In 1949, Harmon and Niemann (32), concluded from a study of the urease-catalyzed hydrolysis of urea in the presence of potassium phosphate buffers at pH 7, that the hydrolytic reaction was competitively inhibited by phosphate ion. Two years later, in a reinvestigation of the kinetics of the urease-urea system, Fasman and Niemann (24) admitted that this conclusion was incorrect and that an error was made in assuming that the potassium ion was incapa-

ble of interaction either with the enzyme or the enzyme-substrate complex. They also showed that at pH 7, the phosphate ion can apparently function as an activator, and the sodium or potassium ion as an inhibitor. Using a 0.05 molar concentration of phosphate buffer, they observed that on the addition of sodium or potassium chloride to the urease-urea system, a significant inhibition of the urease occurred, and that at equivalent buffer concentrations and with all other factors held constant, the activity of urease is greater in a potassium than in a sodium phosphate buffer.

According to Howell and Sumner (36), the composition of the buffer mixture in a reaction of urease and urea, may alter the optimum pH, and also the activity of urease. This variation may be due, in part, to a difference in the ionic strength of the medium, since this factor was not kept constant.

Ambrose, Kistiakowsky and Kridl (2), in their study on the inhibition of urease by sulfur compounds, have observed that sodium sulfate exerts a weak inhibitory action. Kistiakowsky and coworkers (42) have found that below pH 7, salts are without effect on the activity of urease, but from pH 7.0 to 7.5, the enzymatic activity decreases with increas-

ing concentration of the electrolyte. They suggest that the character of the electrolyte inhibition may be due to complexes formed by the alkali cations with the various buffer specie.

The rate of hydrolysis of urea by urease was studied by Kistiakowsky and Shaw (45), at pH 8.9 in the absence of buffers, by utilizing the buffering action of the products of the hydrolysis. They found the activity to be very sensitive to the ionic strength of the solution, and propose that the character of the retardation by salts is such as to suggest electrostatic interactions of the Debye-Huckel type.

The published literature reveals that almost all of the investigators, who studied the effect of neutral salts on the activity of urease, conducted their experiments at intermediate or high values of ionic strength, in a medium of only one value of dielectric constant, and very often at pH values other than seven. This severely limits the utilization of the data for the elucidation of the actual mechanism, and it may be the reason why the results in the literature concerning the salt effect on urease are frequently not in agreement, and even contradictory.

A further investigation of the literature revealed

that according to Ivanow (37), dilute aqueous solutions of alcohol and acetone would inhibit the activity of urease, but would not completely destroy its activity in solutions containing up to eighty per cent alcohol or acetone. The fact that ethyl alcohol inhibits the activity of urease was also obsrved by Marshal (63). Although the effect of twenty five volume per cent of dioxane in water will retard the activity of urease, Kistiakowsky and Rosenberg (43) report that there is no irreversible denaturation of the enzyme.

In this investigation, the study of the effect of neutral salts was made (74) on the ureolytic activity of urease at pH 7.00, at the lowest possible ionic strength, and in a media of varying dielectric constant (75) by using variously different mixtures of dioxane and water. It was felt that results obtained from such studies could be better interpreted according to the theories of Bronsted (15, 16) and Scatchard (80), and perhaps permit a better understanding of this reaction as viewed from a molecular-level standpoint.

CHAPTER III

THE EFFECT OF SALTS AND SOLVENT ON IONIC REACTIONS

The velocity of reactions in solution, whether catalytic or not, is very often affected by the addition of salts. It is now realized that only in a few cases are these effects due to any direct catalytic action by the added salt. Today the term, salt effect, is used to cover the various ways in which modern electrolyte theory bears on the interpretation of reaction kinetics in solution.

There are two main concepts which can be employed in the study of ionic reactions. The first is the general question of complete ionization of strong electrolytes and the electrostatic forces between ions. This fundamental idea underlying the modern theory of electrolytes has been incorporated into physical chemistry in the form given by Debye and Huckel (20). The second point concerns itself with the effect these electrostatic forces of ions play upon the equilibrium and velocity constants, involved in reactions between ions. In this direction Bronsted (15, 16)

and Scatchard (80, 81, 82) have arrived at satisfactory theoretical and quantitative conclusions.

It is beyond the scope of this thesis to examine the Debye-Huckel theory in detail, and reference will therefore be made only to those aspects of it which will be of direct service to us. The basic idea of their theory is that, because of electrostatic forces, every ion in solution is considered to be surrounded by an ionic atmosphere of oppositely charged ions. Assuming that the strong electrolyte is completely dissociated, Debye and Huckel (20) have shown that the value of the mean electrical potential, ϕ_A, at a distance r, from ion A, due to uneven distribution of the ions, is

$$(a) \qquad \phi = \frac{Z_A \epsilon e^{-Kr}}{Dr} \cdot \frac{e^{Ka}}{1 + Ka}$$

where Z_A is the ionic charge of the ion A, ϵ is the unit electronic charge, D is the dielectric constant of the medium, a is the distance of closest approach of other ions to the A ion, and K is given by the expression,

$$(b) \qquad K^2 = \frac{8N\epsilon\Pi u}{1000DkT}$$

where N is Avogadro's number, k is Boltzman's constant, T is the absolute temperature, and u is the ionic strength,

which is equal to one-half the sum of the concentration of all the ions in solution times the square of their valence charges.

Although it had been known that the activity coefficients of ions at normal concentrations were less than unity, it was Debye and Huckel (20), who satisfactorily explained this problem as being due to the forces of electrical attraction and repulsion exerted between ions. They derived what is now referred to as the Debye-Huckel expression for activity coefficients of ions which gives the relation between the activity coefficient and the ionic strength for dilute solutions,

$$(c) \qquad -\ln f_i = Z_i^2 A \sqrt{u}/(1 + \beta a_i u)$$

where f_i is the activity coefficient of the ion i, having a valence charge of Z, where u is the ionic strength, a_i the distance of closest approach, and where A and β are constants. For a very dilute solution, where u approaches zero, equation (c) may be written in the limiting form,

$$(d) \qquad -\ln f_i = Z_i^2 A \sqrt{u},$$

where A is a constant dependant on the dielectric constant of the medium, D, and the absolute temperature T, and related to the constants ϵ, π, N and k by the following:

$$(e) \qquad A = \frac{\epsilon^3}{DkT} \cdot \sqrt{\frac{2\pi N}{1000DkT}} .$$

Upon utilizing the known values of the above constants and including values for the dielectric constant and the temperature, and also introducing the factor for converting natural to ordinary logarithms, A is found to be 0.509 for aqueous solutions at 25.0°C. Therefore, equation (d) may be written in the form,

$$(f) \qquad -\log f_i = 0.509\, z_i^2 \sqrt{u}.$$

This simple relation represents the limiting form of the Debye-Huckel equation, which is based on the work required to separate ions in the process of dilution, and states that the activity coefficient of an ion is an exponential function of the square root of the ionic strength, its absolute value determined only by the sign and magnitude of the charge Z, and being independent of any specific property of the ion.

The application of these concepts to the kinetics of ionic reactions in solutions is due, to a large degree, to Bronsted (15, 16), whose theory of the influence of added salts on the rate of chemical change occuring between ions in solution will now be considered.

Bronsted (50, 51) formulated the important theory of the so-called, primary salt effect, from the influence of the ionic atmospheres on ionic reaction rates in solution by applying the concepts of Debye-Huckel regarding the activities of ions in liquid dielectrics. Chemical reactions between ions are rather sensitive to the addition of neutral salts, that is, electrolytes cause a deviation of the rate, where the velocity constant becomes dependent on the concentration of the electrolyte present. Bronsted has shown that activities should be substituted for concentrations in the ordinary kinetic rate equations.

The ionic reactions, which determine the velocity of the chemical change are assumed to form an intermediate activated complex, whose charge is the algebraic sum of all the charges of the reacting species. The deviations exhibited when ions interact are explained on the supposition that the velocities are inversely proportional to the activity coefficients of the intermediate complexes. These complexes, it is also assumed, are affected in the same manner by changes in the salt concentration as are ordinary ions. Although various methods have been used to develop Bronsted's important treatment of reactions in which ions are concerned, that

of Bjerrum (11) is probably the simplest.

The theory of the primary salt effect can be best understood, if we consider the ionic reaction,

$$A^{Z_A} + B^{Z_B} = X^{(Z_A+Z_B)} \longrightarrow \text{products},$$

where A and B are the reacting ions, X is the intermediate complex, and where Z_A, Z_B and (Z_A+Z_B) are the charges on A, B and X respectively. Then from the Debye-Huckel equation (f),

$$(g) \qquad -\log f_A = 0.509\, Z_A^2 \sqrt{u},$$

$$(h) \qquad -\log f_B = 0.509\, Z_B^2 \sqrt{u},$$

$$(i) \qquad -\log f_X = 0.509\, Z_X^2 \sqrt{u},$$

it follows then that,

$$(j) \qquad \log \frac{f_A f_B}{f_X} = -0.509 \sqrt{u}\,(Z_A^2+Z_B^2-Z_X^2).$$

Since the activated complex is made up of A and B, its charge will be the algebraic sum of A and B, that is Z_X is the sum of Z_A and Z_B, therefore, equation (j) becomes,

$$(k) \qquad \log \frac{f_A f_B}{f_X} = 0.509 \sqrt{u}(2Z_A Z_B), \text{ or}$$

$$(l) \qquad \log \frac{f_A f_B}{f_X} = 1.02\, Z_A Z_B \sqrt{u},$$

in the solvent, water, at 25.0°C.

Since ions affect the rate of an ionic reaction, the observed bimolecular velocity constant k_1, is given by the following expression (30),

$$(m) \qquad k_1 = k_o \cdot \frac{f_A f_B}{f_X},$$

where k_o is the rate constant at infinite solution in a given solvent at a constant temperature. In logarithmic form equation (m) is

$$(n) \qquad \log k_1 = \log k_o + \log \frac{f_A f_B}{f_X}, \text{ and}$$

on combining equation (l) and (n) we obtain

$$(o) \qquad \log k_1 = \log k_o + 1.02\, Z_A Z_B \sqrt{u}.$$

This last equation (o) puts Bronsted's concept of the primary salt effect on a quantitative basis. It follows that the plot of the logarithm of the observed velocity constant against the square root of the ionic strength, if in a dilute solution, at 25.0°C., should give a straight line of approximate slope, $Z_A Z_A$, and the intercept, on extrapolation to zero ionic strength, should be k_o. The magnitude and sign of the product $Z_A Z_B$, will depend on the nature of the reacting species. Therefore, the slope should vary according to the type of ionic reaction. This has been found

true, on a qualitative basis for a number of reactions (11, 15, 16, 19, 51). The primary salt effect in reactions between ions of the same sign is positive, i.e., the reaction is accelerated by an increase in the neutral salt concentration. On the other hand, this effect is negative if the reacting ions are of opposite sign, i.e., the reaction is retarded by an increase in the concentration of the salt.

This theoretical deduction of Bronsted has also been amply confirmed on a quantitative basis (50, 51, 52, 53), for it is possible to determine numerically the magnitude of the neutral salt effect. If the slope, $Z_A Z_B$, under these special conditions should be nearly +3, +2, +1, 0, -1, -2, or -3, the number and kind of charges on the reacting species can be predicted. For example, if the slope is zero, the reaction may either be between two molecules or a molecule and an ion of either charge. Or if the slope is plus one, the reacting ions have single charges of the same sign. If the slope is a minus one, the reacting ions possess single charges of opposite charge. For other slopes similar deductions can also be made.

It is important to remember, that although the velocities of various reactions involving ions provide a clear

confirmation of the Bronsted relationship, the equation (c) holds true only for very dilute solutions and where the charges on the ions of the added salt are low. At higher concentrations, the rate constant may change because of changes in the activity coefficients of the ions not given by the Debye-Huckel theory.

The problem of ionic bimolecular reactions has also been approached from the point of view of statistical mechanics by Christiansen (19), and he has shown that the collision theory is in full agreement with the theory of Bronsted.

Scatchard (80, 81, 82) has also made an outstanding contribution to the kinetics of ionic reactions in solution, when he developed the effect of changing solvent upon reaction velocities, in so far as the solvent can be considered homogeneous and of uniform dielectric constant. Basing his arguments on Christiansen's method of derivation, Scatchard (82) obtained a relation between the dielectric constant of the solvent and the reaction rate in solution.

If the activity coefficient of an ion, in a medium of known dielectric constant and ionic strength, depends primarily on its charge, then by making a reasonable assumption with regard to the dimensions of the intermediate complex

formed in a reaction between ions, it should be possible to determine its activity coefficient with a greater degree of accuracy. Scatchard (30, 79) assumed that the two reacting ions in a bimolecular reaction are spherical, and when they come in contact with each other, they form the intermediate activated complex, which retains the structure of a double sphere, each portion carrying its own individual charge. Thus for the reaction between ions A and B carrying the charges Z_A and Z_B, the activated complex, designated as X, will carry a charge (Z_A+Z_B), or

$$A^{Z_A} + B^{Z_B} = X^{(Z_A+Z_B)} \longrightarrow \text{products.}$$

By choosing a standard reference state of dielectric constant of the solvent as that of infinity, where the activity coefficients of the solutes, reactants and intermediate complex become unity, Scatchard (30) derived the following relation:

$$(p) \quad \ln \frac{f_A f_B}{f_X} = \frac{-Z_A Z_B \epsilon^2}{DkTr} + \frac{Z_A Z_B \epsilon^2}{DkT} \cdot \frac{K}{1+Ka},$$

where r is the radius of the complex and is written as $r = r_A + r_B$. The factor r, can be defined as the distance to which two ions must approach in order to react, and where K is a function of ionic strength as given by equation (b).

A word might be said about the assumption involved in Scatchard's derivation of equation (p). The activity coefficients of the ionic solutes are assumed to be functions of the dielectric constant of the medium, even at infinite dilution, and it is only at the standard reference state of the dielectric constant that the forces tending to decrease the activities of the ions vanish, and the activity coefficient for each ion becomes unity.

Now since the observed bimolecular rate constant, k_1, is given by,

$$(q) \qquad \ln k_1 = \ln k_o + \ln \frac{f_A f_B}{f_X},$$

then if we combine equation (p) and (q), we obtain

$$(r) \qquad \ln k_1 = \ln k_o - \frac{Z_A Z_B \epsilon^2}{DkTr} + \frac{Z_A Z_B \epsilon^2}{DkT} \cdot \frac{K}{1+Ka},$$

where k_1 is the observed rate constant, and where k_o is the specific rate constant in a medium of infinite dielectric constant, and where it is temperature dependent. Equation (r) is generally accepted as the Scatchard equation, and is used as the basis for theoretical interpretation of rate data between ionic reactants. Scatchard (79, 80, 81) points out that a, is the distance of closest approach of the ions and cannot differ largely from r, the distance to which two

ions must approach in order to react.

When the ionic strength of the medium is zero, then the terms involving K in equation (r) disappear, and we have

$$(s) \qquad \ln k_1 = \ln k_o - \frac{Z_A Z_B \epsilon^2}{DkTr} .$$

The observed rate constant k_1, when extrapolated to zero ionic strength, is then a function of the dielectric constant of the medium, at constant temperature. The plot of ln k_1, at u equal to zero, against 1/D should be linear with a slope of

$$(t) \qquad \frac{d \ln k_1}{d(1/D)} = \frac{-Z_A Z_B \epsilon^2}{kTr} .$$

It is at once apparent that if ions A and B are oppositely charged, the slope of the line is positive, whereas it should be negative if the reacting ions have like charges. In other words, equation (s) predicts an increase in the reaction velocity with increasing values of the dielectric constant of the medium for ions of similar charge, and a decrease of reaction rate with an increase of the dielectric constant for reacting ions of unlike sign.

Using a spherical model of the activated intermediate complex and choosing as the reference state of dielectric constant as unity, Laidler and Eyring (47) derived the following expression for the relation of the kinetic rate

constant with the dielectric constant and the ionic strength:

$$(u) \quad \ln k_1 = \ln k_o + \frac{\epsilon^2}{2kT(1/D-1)} \frac{Z_A^2}{r_A} + \frac{Z_B^2}{r_B} - \frac{(Z_A+Z_B)^2}{r_X} + \frac{2Z_AZ_B}{DkT} \cdot \frac{K}{1+Ka} + \frac{\xi\phi}{KT},$$

where the last term $\xi\phi/KT$, involves a non-electrostatic term. If this equation is compared with equation (r), based on Scatchard's double sphere model for the activated complex, it can be seen that, apart from the non-electrostatic term involving the ϕ's term, the only difference is in the second term on the right hand side of each expression. This difference is not serious, for if $r_A = r_B = r_X$, the two expressions are identical.

The experimental results of many workers, concerning the effect of dielectric constant on the rate of ionic reactions, are in close harmony with the theoretical deductions of Scatchard (4, 5, 95, 96). With the use of equation (t), one is able to calculate the radius of the intermediate complex, if the charges of the reacting ions and the slope of plot of ln k_1 against the reciprocal of the dielectric constant is known. Equally good agreement between experiment and theory has been reported in the published literature for

the radius of the intermediate activated complex for many ionic reactions (4, 5, 81). This can be considered a quantitative test for this theory of Scatchard.

It should be immediately obvious that according to the theories of Bronsted and Scatchard, which have been amply confirmed, the effect of ionic strength and the dielectric constant of the medium could be used to elucidate whether the rate-controlling step of the urease-catalyzed hydrolysis of urea at 25.0° C. is ionic. If it is ionic, the probable charges of the ions involved can then be determined.

CHAPTER IV

THE KINETICS OF ENZYMATIC REACTIONS

It would be beyond the scope of this thesis to consider in detail the complete picture of the kinetics of enzymatic reactions. This problem has been adequately treated in the literature (8, 12, 18, 22, 57, 58, 66, 70, 85, 94, 99, 103), however, a brief review will be presented as an aid in the interpretation of the experimental results.

The subject of the kinetics of enzyme reactions deals with the velocity at which such reactions occur. The velocity can usually be determined by the measurement of the rate of formation of the reaction products, or of the rate of disappearance of the substrate. Suppose that an enzyme E, is combining with a substrate S, to form the intermediate ES, thus

$$E + S \longrightarrow ES.$$

Then according to the mass-action law, the decrease in the concentration of the enzyme (E), and the concentration of the substrate (S), at any given instant is

$$(a) \qquad \frac{-d(E)}{dt} = \frac{-d(S)}{dt} = k(E)(S),$$

where k, is the rate constant, which is characteristic of the nature of the reaction and the conditions under which the reaction rate is measured.

If the two molecules, i.e., the enzyme and substrate are involved in determining the rate of the reaction, then the reaction is said to be of second order. If, however, the concentration of the enzyme is kept constant, then the rate of disappearance of the substrate at any moment is proportional to its concentration. Since in this manner, the rate is determined by the first power of the concentration of the substrate only, this reaction can be said to be of first order, that is,

$$(b) \qquad \frac{-d(S)}{dt} = k(E)(S) = k_1(S).$$

If the reaction between an enzyme, at constant concentration, and a substrate is such that the overall rate is constant even though the concentration of the substrate is varied over a period of time, where

$$(c) \qquad \frac{-d(S)}{dt} = k(E) = k_2,$$

then such a reaction is said to be zero order.

Most often an enzyme reaction will follow one of these kinetic equations of rate for a time, and then gradual-

ly drift from it. If the reaction has the characteristics of first order kinetics, then the first order equation (b), is used in its integrated form,

$$(d) \qquad k_1 = \frac{2.303}{t} \log \frac{a}{a - x}$$

where a, is the initial concentration of the substrate, and x, is the concentration of the substrate which has been converted into products during the interval of time, t.

The majority of enzymatic reactions are not simply zero or first order, kinetically, because the enzyme may be inactivated, or the rate may be inhibited by product, that is as the reaction proceeds more and more products are produced until an apparent appreciable reverse reaction occurs, changing the apparent rate of the forward reaction. This so-called inhibition by products, is usually due to an inactivation of the enzyme by a selective binding of the products to the active site rather than due to an approach to an equilibrium point. Since the nature of the reaction is very likely to change with time, it is advisable to measure the initial rate of an enzyme reaction.

The developement of the kinetic theory of enzyme action is rather an interesting one. Most workers in this

field assume the presence of an enzyme-substrate complex formed between the enzyme and substrate molecules. The concept of this intermediate complex has been confirmed and extensively developed by many investigators (12, 18, 65, 66, 70, 99, 103).

The rate of any enzyme-catalyzed process depends on the concentration of the enzyme and the substrate. In most cases, it is found that at a fixed concentration of the enzyme, the initial velocity increases with increasing substrate concentration until a limiting value is reached. The magnitude of the maximum rate finally obtained depends on the fixed enzyme concentration used. This is generally true except where the products of the reaction cause an inhibition of the rate. In order to explain this phenomena, Michaelis and Menten (66) proposed a kinetic scheme involving the formation of an intermediate complex between the enzyme and the substrate. This complex, after it is formed, decomposes to yield the reaction products and the free enzyme,

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_3} E + P.$$

Where E, is the enzyme, S is the substrate, ES the intermediate complex, P the products and k_1, k_2, and k_3 are the rate

constants. On the basis of this idea, Michaelis and Menten (66) obtained the overall reaction velocity as a function of enzyme and substrate concentration. Thus,

$$(e) \qquad v = \frac{-d(S)}{dt} = \frac{k_3(E)(S)}{K_m + (S)},$$

where K_m is called the Michaelis constant. Since according to the mass law, the concentration of ES increases as the substrate concentration is increased, the velocity v, increases hyperbolically with increasing substrate concentration to a maximum value, V_{max}, which is reached when all of the enzyme is bound as ES. Under this condition then (12, 18, 40),

$$(f) \qquad v = \frac{V_{max}(S)}{K_m + (S)},$$

where V_{max}, the maximum velocity is equal to $k_3(E)$, and K_m is equal to one-half the maximum velocity.

According to the original theory of Michaelis and Menten (66), K_m defines the equilibrium for the reversible formation of ES from E and S, regardless of the disturbance of the equilibrium by the subsequent decomposition of the intermediate complex, ES, into the enzyme and the products. While their expression for K_m bears some resemblance to an equilibrium expression,

$$(g) \qquad K_m = \frac{(E)(S)}{(ES)} = \frac{k_2 + k_3}{k_1},$$

it actually represents a condition of steady state in two consecutive reactions, the first one of which is reversible.

If the rate of formation of ES is considerably slower than the rate of decomposition, the enzyme-substrate complex formation becomes the rate determining step (70). This is true for low substrate concentration, when the overall velocity will be

$$(h) \qquad v = \frac{-d(S)}{dt} = k_1(E)(S),$$

which is the expression for a second order reaction, but since (E) is kept constant, it reduces itself to a first order expression. If the rate of formation of ES is considerably faster than the rate of disappearance of substrate, then the overall reaction velocity becomes

$$(i) \qquad v = \frac{-d(S)}{dt} = k_3(E),$$

and since (E) is kept constant, then $k_3(E)$ becomes a zero order kinetic constant. This is usually the case at high substrate concentrations.

Although the Michaelis constant, K_m, is a constant only for defined conditions, it has turned out to be very

useful. The theory with its subsequent extensions (12, 40, 58, 103), has laid a firm foundation in the interpretation of the kinetics of enzyme reactions. It is true that the most widely used methods for measurement of enzyme affinities depend on the measurements of the velocities of the reaction, and the kinetic element may in some cases give rise to the difficulties with regard to K_m, however, this does not affect its applicability.

The kinetics of the urease-urea reaction, as is the case for most enzymes, is also complex. It has been found by many investigators (48, 99) that the measured rate over a range of initial concentration of urea first increases linearly with concentration, reaches a maximum and then subsequently decreases. That an extension of the Michaelis and Menten treatment is necessary for the case of urea hydrolysis by urease, is indicated by the decreasing rate at high urea concentrations.

It is generally agreed that before urease hydrolyzes its substrate, urea, the latter is combined with the enzyme to form an intermediate complex. According to Van Slyke and Cullen (102, 103, 104), the action of urease can be expressed by a mass action formula, which they consider general

for enzymes. Their theory states that urease hydrolyzes urea by means of two successive reactions:

$$E + S \xrightarrow{k_1} ES \xrightarrow{k_3} E + \text{products.}$$

This process led them to formulate the following expression:

$$(j) \qquad t = (1/E)(1/k_1 \log a/(a-x) + x/k_3),$$

where a is the initial substrate concentration, x is the concentration of urea hydrolyzed in time t, E is the enzyme concentration, k_1 is the velocity constant of the combination of the enzyme and substrate, and k_3 is the rate constant of the decomposition of ES to enzyme and products. They found there was no effect of urea concentration on urease action as long as the urea concentration is varied between 0.015 and 1.5 molar, when buffered with phosphate. That is, the reaction is zero order where k_3 is equal to $x/(E)t$, and where $k_3(E)$ is the zero order kinetic constant. This is true because when the urea concentration is large and t is small, then x will be negligible so that log a/(a-x) approaches zero. When the concentration of urea is small, then x/k_3 becomes negligible and the overall rate expression becomes (99)

$$(k) \qquad tE = 1/k_1 \log a/(a-x),$$

which represents a first order equation when the enzyme concentration is kept constant.

Yamazaki (115) found that when the urea concentration was 0.05 molar or greater, the rate of hydrolysis by urease was independent of the urea left. However, when the concentration of urea was less than 0.05 molar, the rate was dependent on the substrate concentration down to at least 0.002 molar. Lovgren (59) considers a first order kinetic equation as probably the best for expressing the kinetics of the urease-urea hydrolysis. He does not claim, however, that it is valid for the whole course of the reaction, but only for initial rates at low urea concentrations. Sumner and Sumner (94) report that for a urea concentration below 0.33 molar, the activity of urease is a function of the substrate concentration or kinetically first order with respect to the urea, if the enzyme concentration is constant. Between 0.66 and 1.1 molar of urea, there is little or no difference of rate due to an increase of urea concentration, i.e., the reaction is zero order. The reason why these various investigators report slightly different values of urea concentration with respect to the kinetic order of the reaction is because they used different enzyme concentrations.

The rate of an enzyme reaction can be related to the concentration of the substrate, at constant enzyme con-

centration, by the general equation (48),

$$(1) \qquad v = \frac{-d(S)}{dt} = k(S)^n,$$

where v is the reaction velocity, k is the specific rate constant, (S) the concentration of the substrate, and n, the kinetic order of the reaction involved. If log v is graphed against log (S), the plot is a simple straight line, and the slope of the line will give the kinetic order of the reaction. Laidler and Hoare (48) using this method, found that the variations of the initial rate with concentration of urea corresponds to the results of early investigators. They found that below 0.12 molar concentrations of urea, the plot of log v vs. log (S) had a slope of unity, indicating that the rate varies linearly. At concentrations above 0.12 molar urea, the slope falls to zero, indicating an apparent zero order, and finally at higher concentrations of urea, the slope of the line becomes negative, indicating that the observed order is apparently negative. However this negative value of n does not necessarily mean that the actual reaction order is negative.

It is obvious that the kinetics of the urease-urea system falls into the same general pattern found for most en-

zymes. If the urease concentration is kept constant, then at low concentrations of urea, the measured initial rates of the reaction conform to first order kinetics. At high concentrations of urea, the observed kinetic order is zero. This, of course, is true only if the other experimental conditions, such as pH, temperature and ionic strength are kept constant.

CHAPTER V

EXPERIMENTAL PROCEDURE

The simplest way to measure the rate of the enzymatic hydrolysis of urea by urease is to permit the reaction to run long enough to obtain an exact measurement of the urea decomposed, but not to run it so long that the products begin to inhibit. A short reaction period from four to fifteen minutes is desirable, and has several advantages. First it limits the amount of urea decomposed, so that the concentration is great enough to support maximal activity of the urease. Secondly it prevents the accumulation of ammonia and carbon dioxide, which retard the reaction (7, 63). Finally it minimizes the opportunity of inactivation of urease during the reaction period. In other words, the activity of urease can be studied more accurately when initial rates up to two per cent of the total reaction are measured, rather than following the reaction curve all the way to the finish (48).

It was found that urease activity can be accurately determined if a urease preparation is allowed to react with a known amount of urea, in a phosphate buffer, at pH 7.0, at a

given temperature, for a short period of time. The action can easily be stopped by the rapid addition of strong acid or base, and the quantity of ammonia formed can be determined after aeration followed by titration or by Nesslerization (41, 48, 100). The course of the reaction can also be followed by determining the unreacted urea with the diacetyl method (73). Urease action has also been followed by the manometric determination of the carbon dioxide formed. Here a buffer at pH 6 must be employed, in order to release the carbon dioxide (98, 100).

In some of the preliminary kinetic runs, the following procedure was used. Exactly 5.00 ml of dilute urease solution was rapidly mixed with 5.00 ml of a standard urea solution, buffered by phosphate at pH 7.00 and containing a known amount of neutral salt. This was done in a constant temperature bath at 25.0°C. After a known time interval, usually four to six minutes, the reaction was killed by the rapid addition of 5.00 ml of a saturated potassium carbonate solution. With the use of a sintered glass bubbling tube, the alkaline reaction mixture was aerated with ammonia-free air, after one or two drops of capryl alcohol was added to prevent foaming. In this manner, the ammonia produced by the

reaction was swept out and trapped in a solution containing a known excess of standard acid. The amount of ammonia was then determined by the back titration of the excess acid with a standard solution of sodium hydroxide to the methyl red end point. After extensive experimentation with this method, it was discarded because it was too cumbersome and time consuming. However, it was later employed occasionally as a check.

Temperature control was provided by a water filled bath equipped with liquid cooling coils, electric heating elements, and an automatic temperature control. The temperature was controlled in such a manner that the bath could be maintained at 25.0°C to plus or minus 0.02°C, over at least a ten minute interval of time, which was necessary to perform one individual run. The temperature was read using a Bureau of Standards calibrated 0.1° mercury thermometer, which was equipped with a magnifying lens.

A Galco stopwatch with a Swiss movement, accurate to 0.2 of a second, was used to measure the time of the reaction runs. The same watch was used to measure the drainage time, when delivering solutions from pipets.

After a great number of preliminary experiments, the following procedure was found to yield reproducible re-

sults rather consistently. Exactly 5.00 ml of a solution containing a known amount of urea, neutral salt, dioxane and phosphate buffer at pH 7.0, was delivered into a clean and dry 100 ml volumetric flask. This flask along with another flask containing a dilute solution of urease was then clamped in the constant temperature bath at 25.0°C. After allowing at least twenty minutes for the solutions to reach thermal equilibrium, the reaction was started by rapidly adding 5.00 ml of the urease solution into the flask containing the urea. This was immediately stoppered and mixed for exactly twenty seconds, after which the reaction mixture was left in the bath for exactly four minutes. The reaction was stopped by the rapid addition, from a hypodermic syringe pipet, of 2.00 ml of 1.0 N hydrochloric acid. This was followed by rapid mixing for fifteen seconds, and then immediately diluted with ammonia-free water to exactly 100.0 ml.

The analytical procedure for most of the runs consisted of Nesslerization of the diluted solution of the reaction mixture with 2.00 ml of Nessler's reagent. After a ten minute waiting period, the optical density was then measured with a Beckman DU spectrophotometer at 450 mu and a 0.40 mm slit opening in 1.00 cm cuvettes. The absolute amount of

ammonium ion obtained either from a calibration curve or by comparison to two standards which were always determined along with the samples. Each set of measurements also included blanks, which were deducted from the ammonium ion concentration in the samples from actual experimental runs. Control experiments identical with other experiments, except that the acid was added immdiately, provided the necessary correction to the Nessler solution transmission measurements. It was found that the optical density of the same Nesslerized standards varied slightly depending on the amount of dioxane present, therefore, calibration curves were obtained for the various concentrations of dioxane used.

In order to verify the accuracy of the results obtained by the above analytical method, the unreacted urea was also quantitatively determined by the diacetyl method (73). This procedure consists of the addition of 2.00 ml of acid diacetyl reagent to the diluted reaction mixture and heating for ten minutes, in the absence of light, by means of a water bath. This was then cooled to room temperature, and the optical density of the yellow color which develops was measured at a wavelength of 480 mu with a Beckman DU spectrophotometer. Blanks and control experiments provided

the necessary corrections. The concentration of urea was determined both from a calibration curve and by comparison to standards, which were measured along with the experimental samples. This second analytical method provided an excellent check for many of the experimental runs. If care was exercised, both colorimetric methods gave a precision of less than one part per 100.

The urease solutions were prepared by diluting accurately aliquots of a 0.100% stock solution of enzyme. The stock enzyme solution was prepared from crystalline urease purchased from the Krishell Laboratories Inc., Portland, Oregon. One tenth of a gram of the urease was dissolved in all-glass distilled water to a total volume of 100 ml, after which it was stored at $0^{o}C$ until ready to use. During a two month period, it was observed that the loss of activity of the stock urease solution was only about five per cent. The diluted urease solution was usually prepared by taking a 5.00 or 10.00 ml aliquot of the stock solution and diluting it to exactly 100 ml. This was done one hour before using it, in order to stabilize it. Tests indicated that this was necessary for the diluted urease solution to attain its maximum activity, after that further aging caused only a very slow

loss of activity.

The activity of the diluted urease solutions used was determined by the method of Sumner and Hand (90), who have expressed urease activity in units. A unit is defined as the amount of urease which will produce one milligram of nitrogen from a urea-phosphate solution at pH 7, at 20°C, in five minutes. The activity of the urease solution in this work was determined in the following manner. Five milliliters of the urease solution was mixed with 5.00 ml of a 3.0% urea solution in 9.6% neutral phosphate. The amount of ammonia nitrogen from a 5.00 to 100 ml dilution of the stock enzyme produced was about one-tenth of a milligram. This corresponds to a weight of 7.5×10^{-4} milligrams of pure urease on the basis of Sumner's result where one gram of pure urease produces 133 mg of ammonia nitrogen. The molarity of the urease during a reaction run was about 1.5×10^{-10}, if the value of 483,000 is used as the molecular weight of the enzyme, urease (89).

The water used for the preparation of all solutions in these experiments was laboratory distilled water which had been redistilled through all-glass apparatus. Ordinary distilled water of most laboratories contains sufficient heavy

metal ions, such as copper, silver, mercury and cadmium, to cause it to be decidedly poisonous to dilute urease solutions (90). It was found that all-glass distilled water exerted no inactivation upon a 0.0050 per cent urease solution. The flasks, pipets and all the glassware employed for the reaction solutions had to be free of any toxic ions. The 100 ml volumetric flasks, in which the kinetic runs were made, were cleaned with warm dichromate-sulfuric acid solution, rinsed seven times with tap water, five times with ordinary distilled water and finally five times with ten milliliter portions of all-glass distilled water. The flasks were then dried at 110° C. for a period of three to five hours, after which they were stoppered and set aside until ready to use. Erratic and non-reproducible results would be obtained if this procedure was not followed.

Exacting reproducibility in the operation of each run with a number of various precautions were necessary in order to obtain standard deviations of individual runs at or below one per cent. Even though many changes in technique and added precautions were made, the deviations could not be completely eliminated. Each series of runs always included several made under standard conditions to avoid errors which

might occur from the dilution or inactivation of the stock enzyme solution. The rate constants were calculated from the relative rates which were observed under standard conditions, since the activity of the urease was found to be proportional to its concentration for the experimental conditions employed.

Hydrochloric acid was used to stop the reaction of urea hydrolysis by the enzyme, urease, because it is very toxic to urease and will destroy its activity completely even at concentrations of one-tenth normal (63, 115). Exactly fifteen seconds after the addition of the hydrochloric acid solution to the reaction mixture, the reaction mixture was immediately diluted to 100 ml with ammonia-free water. This was a precaution taken to prevent any possible hydrolysis of urea by the hydrochloric acid. Test runs showed that no acid hydrolysis of urea occurred, if this procedure was followed.

The phosphate buffers were prepared from sodium and potassium phosphate or from just sodium phosphates in such concentration that in the reaction mixture it would have an ionic strength of no less than 0.01000 molar. The capacity of such a buffer system, under the experimental conditions employed, was such that during a ten minute test run, the pH

change was never more than 0.1 unit from pH 7.0. Urease is greatly influenced by the type of buffer system used, however, it was found that the phosphate buffers gave the best capacity at low ionic strengths. Citrate and veronal buffers could not be used because of the higher ionic strengths required in order to keep the pH constant during the time required for an experimental run. Also, since the optimum pH of urease activity for the phosphate buffer is 7.0, and some other values for the citrate and veronal systems, it is better to use the phosphate system for the study of the effect of neutral salts on the rate of hydrolysis of urea catalyzed by the enzyme, urease. In all of the reaction runs, the pH was carefully controlled, since erroneous experimental results could result from uncontrolled changes of pH. After the urea-phosphate solutions were prepared, test runs were made where the pH was checked at the beginning and the end of the run. The pH's were measured with a Coleman pH Electrometer (Model 3), which had been previously calibrated against several standard buffer solutions at 25.0°C.

The quality of all of the chemicals used was reagent grade, 'Baker's Analyzed,' A.C.S. Standard, with no further purification.

The dioxane, used for varying the dielectric constant of the reaction media, was purified by refluxing commercial grade dioxane with aqueous hydrochloric acid for twelve hours, drying with potassium hydroxide pellets, then refluxing over sodium metal for another twelve hours (27). This was finally distilled by means of an all-glass still, during which time it was well protected from atmospheric moisture. Mixtures of the purified dioxane and all-glass distilled water were used to prepare the various media of different dielectric constants. Mixtures of 10.00, 20.00, 30.00, 40.00 and 50.00 per cent by weight were prepared. The dielectric constant values for these mixtures were obtained from Harned and Owen (33). The solubility of urease in various mixtures of dioxane and water was measured, and it was found that it was soluble to the extent of 0.04 per cent in an 86 per cent solution of dioxane. It was concluded that a 0.010 per cent solution of urease would be easily soluble in dioxane-water mixtures up to 50 per cent dioxane.

In most of the kinetic reaction runs, the initial concentration of urea was either 0.1428 or 0.01428 molar. These concentrations were chosen for two very good reasons. First, they represent a convenient initial concentration in

terms of ammonia nitrogen, since a 0.1428 and 0.01428 molar urea is equivalent to 0.4000 and 0.04000 milligrams per milliliter of ammonia nitrogen respectively, when 5.00 ml of the urea solution are diluted to exactly 100 ml. The second reason why these concentrations were used is because according to Laidler and Hoare (48), a 0.1428 molar urea solution should give excellent zero order kinetics, and 0.01428 molar urea solutions as initial concentration should produce good first order rate constants. This was actually verified experimentally with the use of the following less familiar method.

In general, the order of an enzyme reaction may be also determined by keeping the enzyme concentration constant and increasing the concentration of the substrate, and observing the change in reaction rate over a short period of time (3). Thus if the reaction rate is represented by

$$v = \frac{-d(S)}{dt} = k(E)(S)^n = k_1(S)^n,$$

where v is the velocity, (S) is the concentration of the substrate, (E) is the concentration of the enzyme, and k_1 is the rate constant. It should be apparent that n, the order of the reaction, can be evaluated by doubling the concentration of the substrate and determining the rate of the reaction in

in the two cases, if the order of the reaction does not change with a two fold increase in the substrate concentration. Then

$$\frac{V_{(2S)}}{V_{(1S)}} = \frac{k_1(2S)^n}{k_1(1S)} = 2^n.$$

With the use of this procedure, it was found that a 0.1428 and a 0.01428 molar urea initial concentration gave rather good zero and first order kinetics respectively, under the experimental conditions employed.

CHAPTER VI

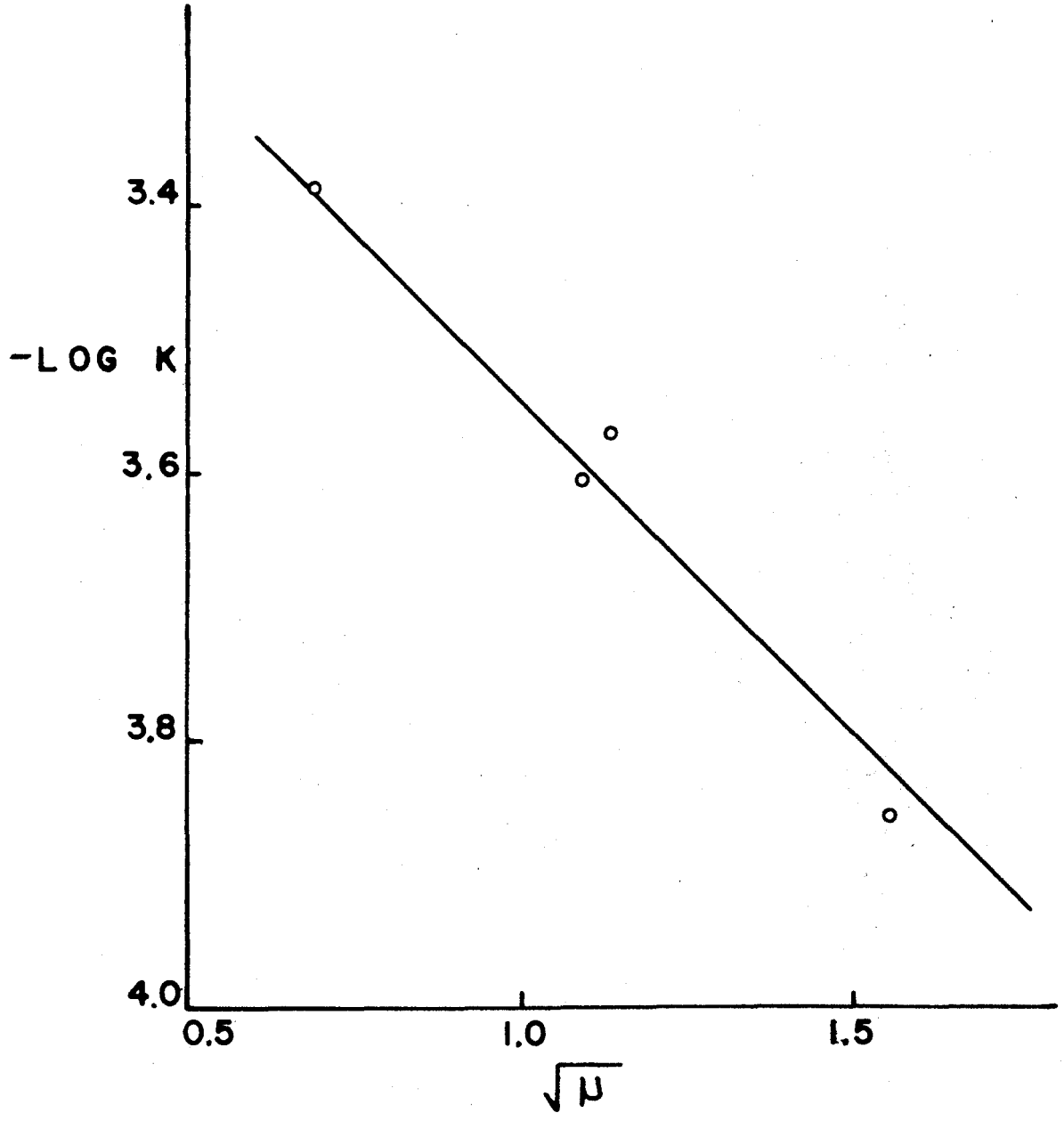
EXPERIMENTAL RESULTS AND DISCUSSION

It was as early as 1914 that Van Slyke and Zacharias (106) had studied the effect of phosphate concentration on the reaction between 0.10 per cent urease and 0.020 molar urea, and found the hydrolysis reaction rate to decrease when the phosphate concentration was increased. In an attempt to reinterpret their work in terms of the primary salt effect, first order constants were calculated from their experimental data. These along with the ionic strengths will be found in table I. When the logarithm of the rate constant is plotted against the square root of the ionic strength, the closest straight line through the points yields a slope of negative 0.5 (figure 1). Since the slope is neither zero nor minus one, and since the ionic strengths are rather high, this is in all probability not a Bronsted primary salt effect, but perhaps some sort of a secondary salt effect.

All of the kinetic runs in the experimental work in this dissertation were made at pH 7.00 and 25.0°C. For all the reactions, where 0.0143 molar urea concentration was used, first order constants were plotted. In reactions, where

FIGURE I

LOG K VERSUS THE SQUARE ROOT OF THE IONIC STRENGTH FROM THE DATA IN TABLE I



0.250, 0.122 and 0.143 molar urea were employed, zero order constants were calculated. The values of all the kinetic constants, expressed in moles per liter per second, have been calculated for only the initial four or five minutes of the reaction run. The following abbreviations will be used in tables of results and the figures:

k rate constant

u ionic strength

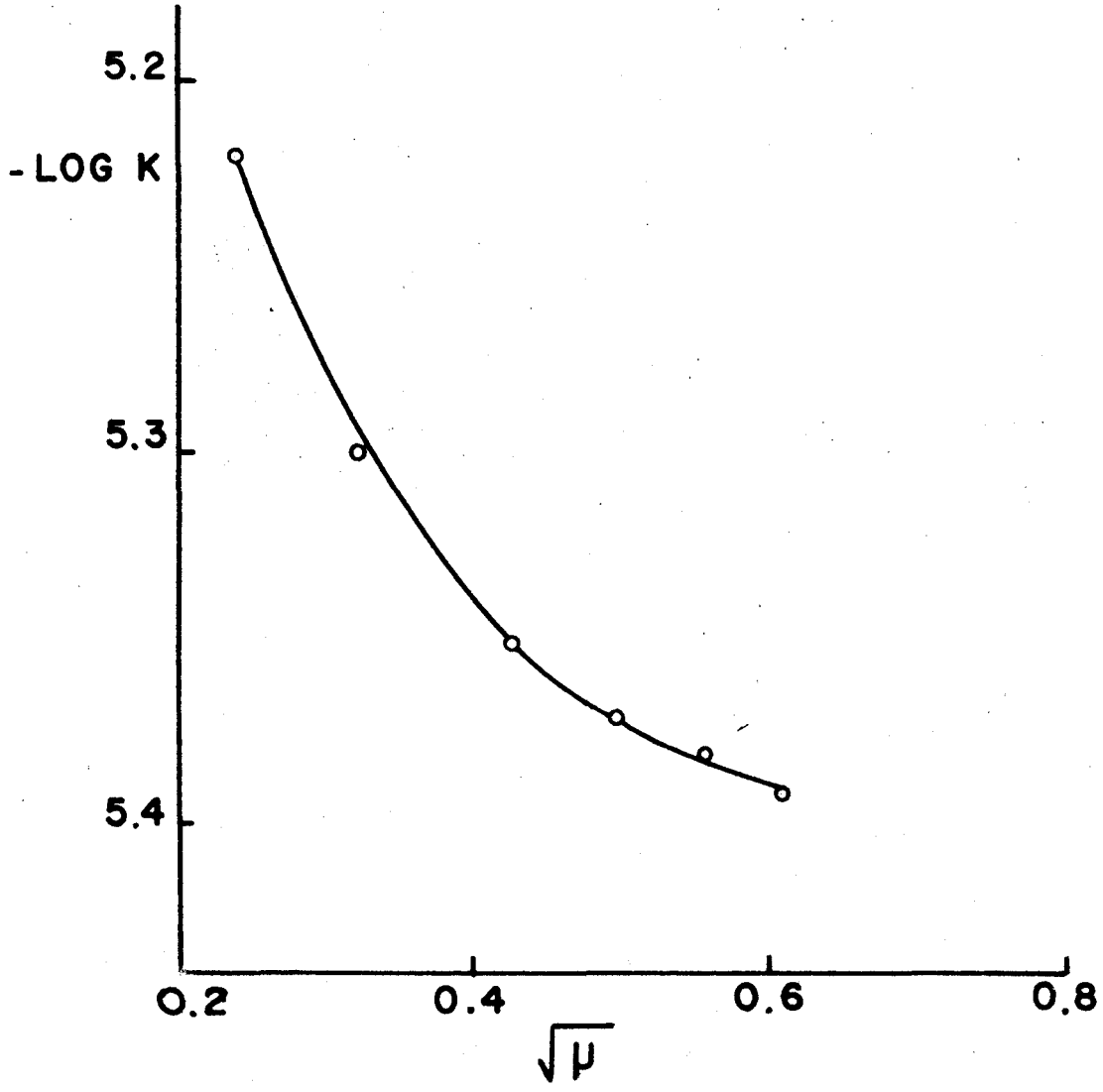
D dielectric constant.

All of the rate constants in the tables represent an average of three or four identical experimental runs. For the reactions involving 0.250, 0.122 and 0.143 molar urea, sodium, potassium phosphate buffers were employed, and for all the reactions where 0.0143 molar urea was used, only sodium phosphate buffers were utilized to obtain the initial value of ionic strength.

When sodium chloride was added from ionic strength 0.0545 to 0.367 molar, to the reaction mixture of 0.10 per cent urease and 0.250 molar urea (table II), it was found that the plot of the log of the zero order constant versus the square root of the ionic strength (figure 2) gave a curve with a negative slope. Although the rate is retarded by in-

FIGURE 2

LOG K VERSUS THE SQUARE ROOT OF THE IONIC STRENGTH FROM THE DATA IN TABLE II



creasing concentration of the salt, this does not appear to be a true primary salt effect, since this plot is not linear. When the same salt was used to vary the ionic strength from 0.0545 to 0.1045 molar for the reaction involving 0.10 per cent urease and 0.122 molar urea (table III), the plot of log k against the square root of the ionic strength gave a curve which was more linear with a slope of about a minus 0.8 (figure 3).

The effect of sodium chloride, potassium chloride and sodium sulfate, when varied from ionic strength 0.0545 to 0.1045 molar for the reaction between 0.020 per cent urease and 0.143 molar urea, was essentially the same (tables IV, V, VI). The plot of log k versus the square root of the ionic strength (figures 4, 5, 6) for all three salts was practically linear with a slope of approximately negative one. In tables II and III, different values of the zero rate constants were obtained at the same ionic strength because this is probably the region of urea concentration where zero kinetics has not been as yet attained, or because reference standards were not run with these as a check.

Since the effect of these salts (figures 4, 5, 6) might have been a primary salt effect, it was decided to stu-

FIGURE 3

LOG K VERSUS THE SQUARE ROOT OF THE IONIC STRENGTH FROM THE DATA IN TABLE III

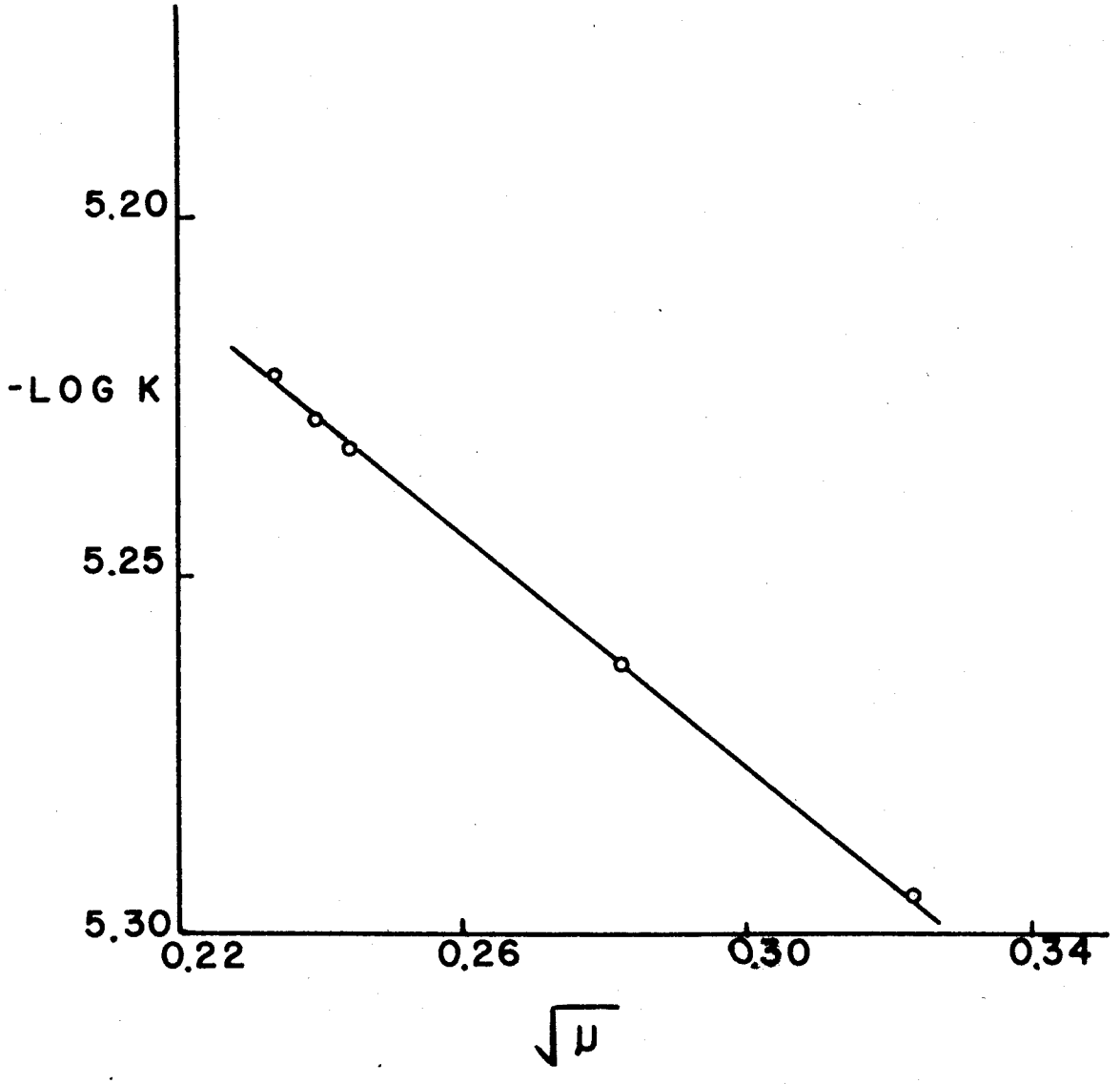


FIGURE 4

LOG K VERSUS THE SQUARE ROOT OF THE IONIC STRENGTH FROM THE DATA IN TABLE IV

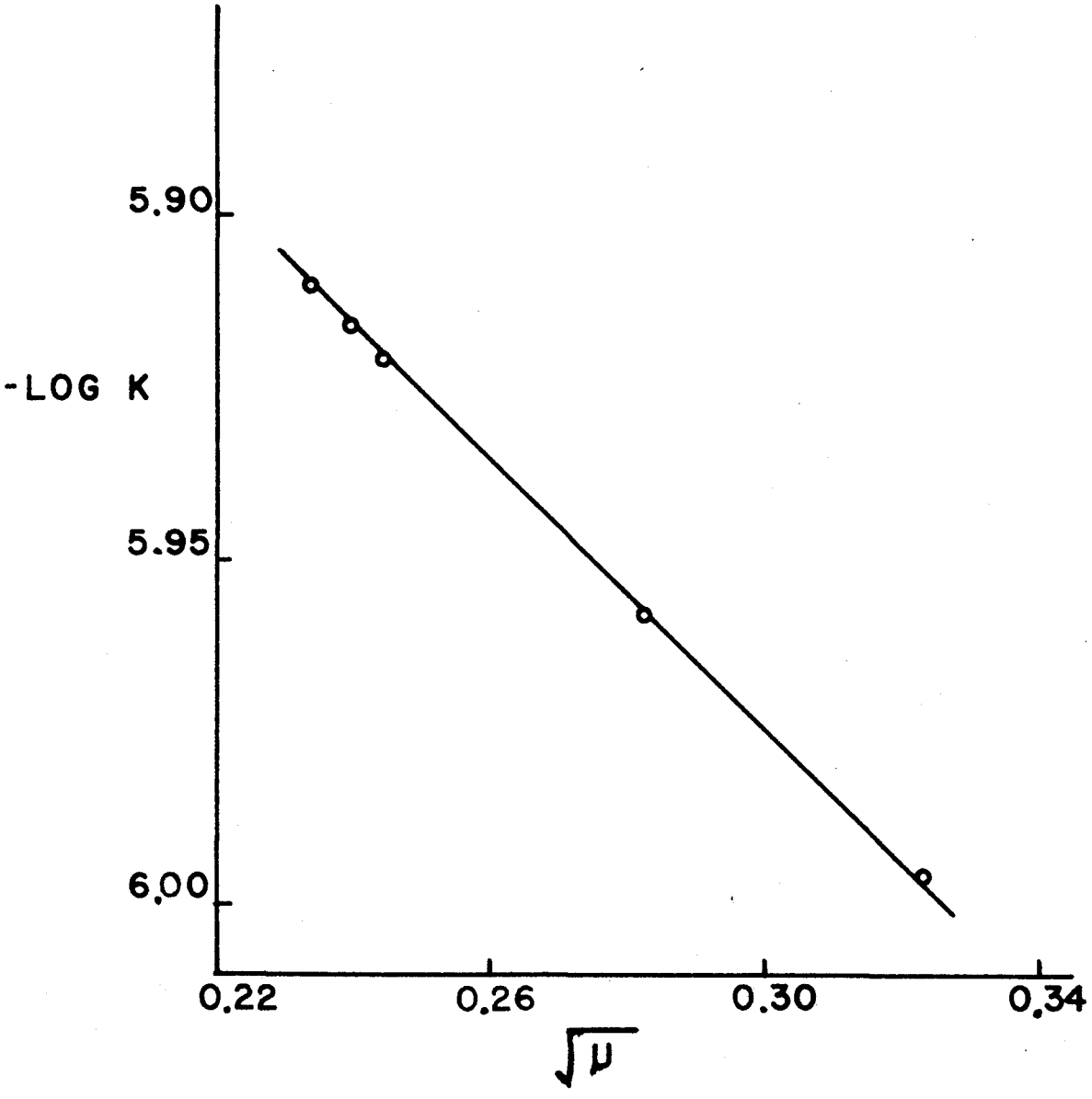


FIGURE 5

LOG K VERSUS THE SQUARE ROOT OF THE IONIC STRENGTH FROM THE DATA IN TABLE V

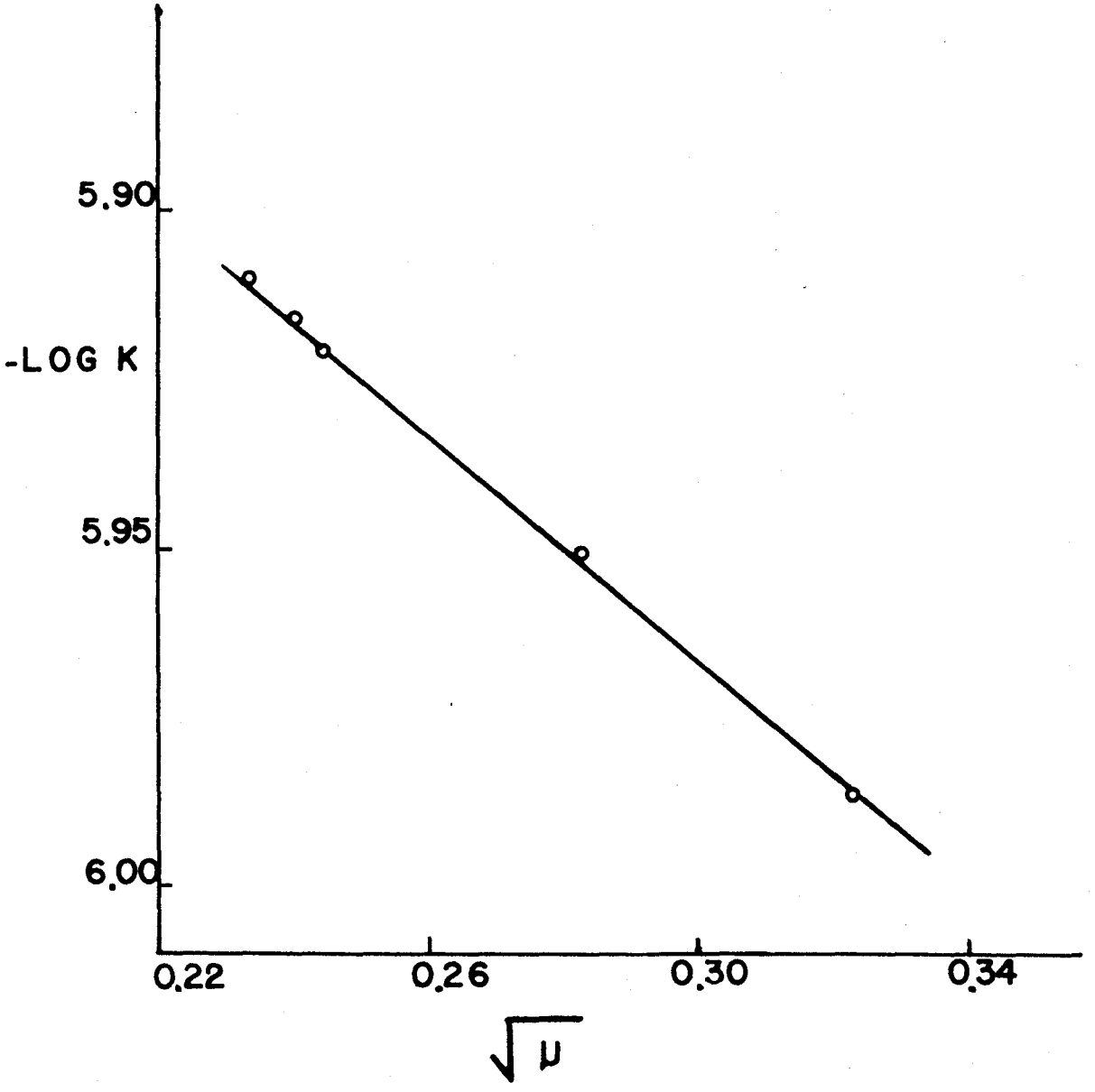
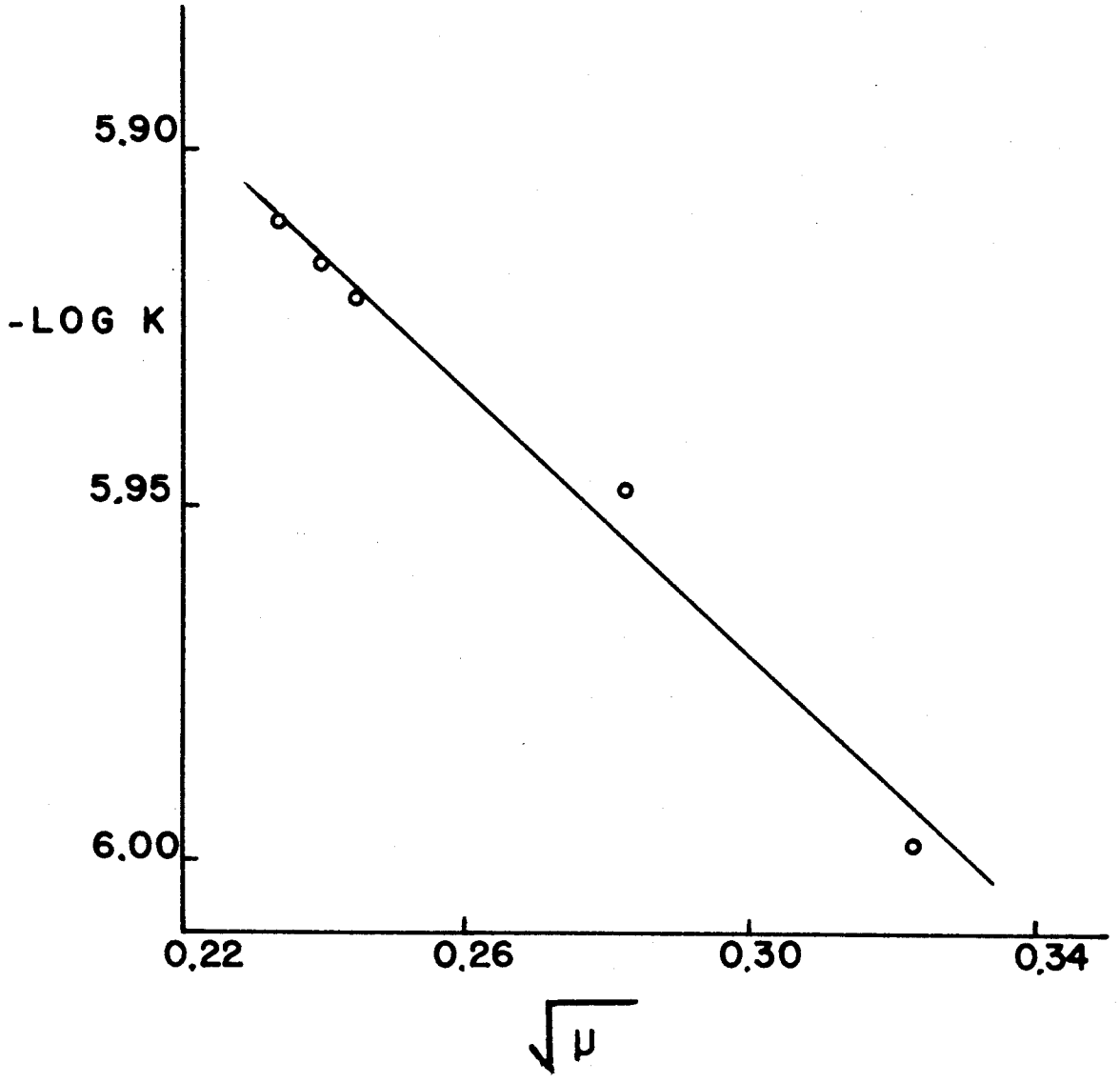


FIGURE 6

LOG K VERSUS THE SQUARE ROOT OF THE IONIC STRENGTH FROM THE DATA IN TABLE VI



dy this effect in a medium of lower ionic strength, where the Bronsted theory is more valid. Using sodium chloride to vary the ionic strength from 0.0273 to 0.0523 molar for the reaction between 0.050 per cent urease and 0.143 molar urea (table VII), it was found that the plot of log k against the square root of the ionic strength (figure 7) gave a curve which increased slightly and then began to decrease. The same results were obtained (figure 8) when the ionic strength was varied from 0.0109 to 0.0609 molar with sodium chloride, for the reaction between 0.010 per cent urease and 0.0143 molar urea (table VIII).

Because the effect of neutral salts on the urease-urea reaction appeared to accelerate the reaction at low ionic strength values and to slow it down at higher values of ionic strength, it was thought that the effect of the dielectric constant of the medium on this reaction would shed some light on this unusual turn of events. By varying the dielectric constant of the medium with the use of dioxane for the reaction between 0.025 per cent urease and 0.143 molar urea, at an ionic strength of 0.0273 molar (table IX), it was observed that the plot of the logarithm of the velocity constants at an ionic strength of 0.0273 molar, against the re-

FIGURE 7

LOG K VERSUS THE SQUARE ROOT OF THE IONIC STRENGTH FROM THE DATA IN TABLE VII

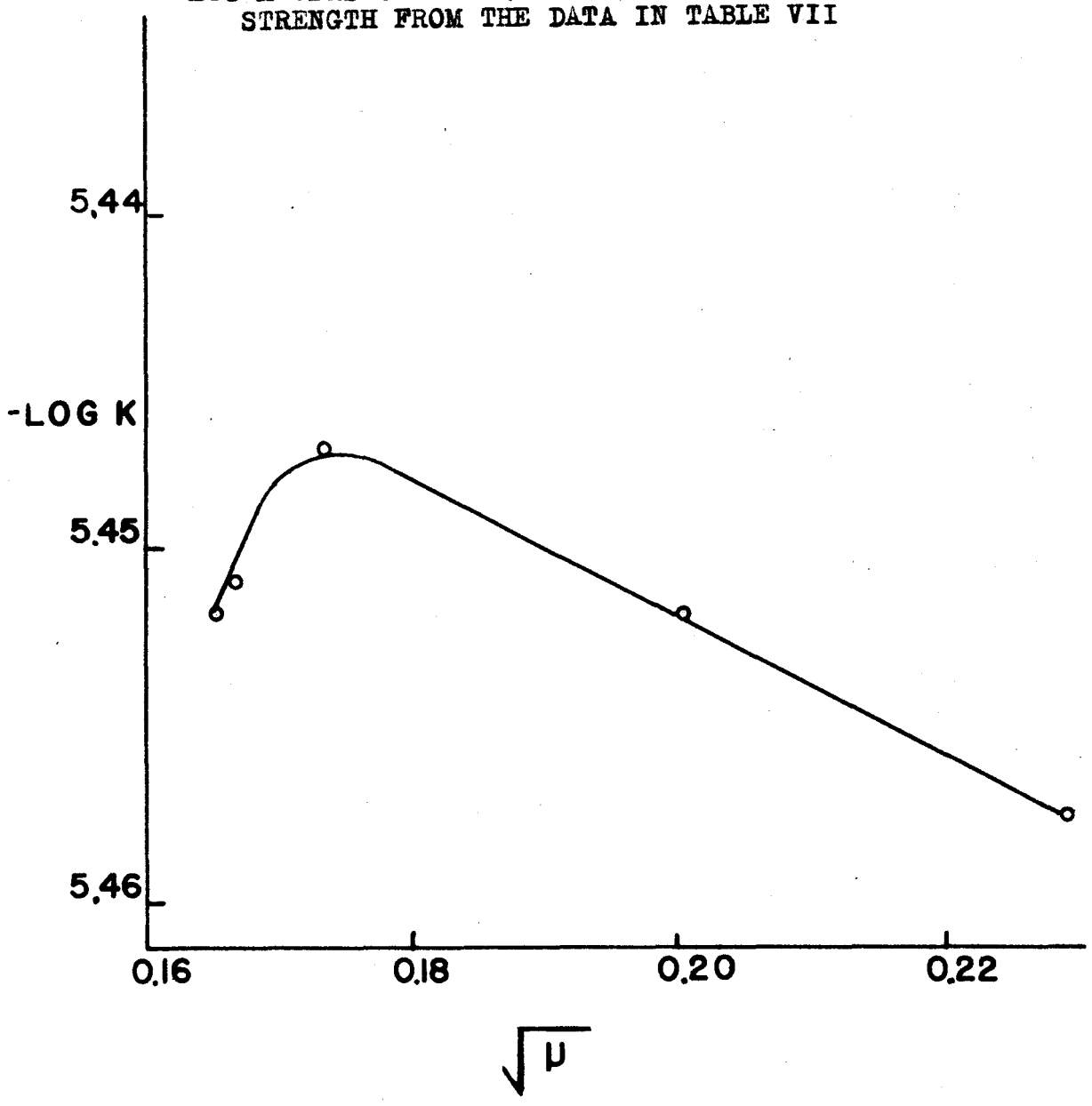
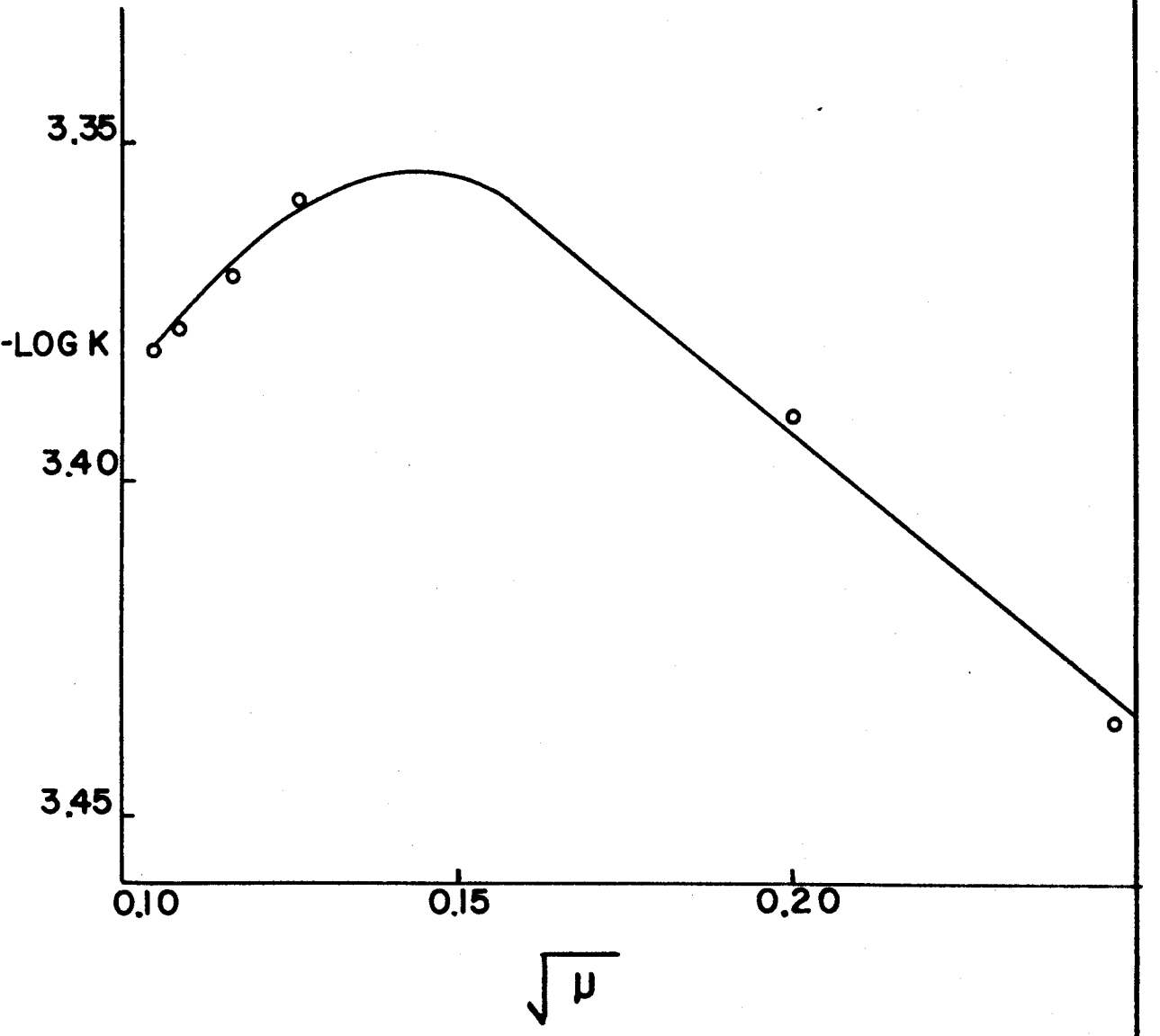


FIGURE 8

LOG K VERSUS THE SQUARE ROOT OF THE IONIC STRENGTH FROM THE DATA IN TABLE VIII

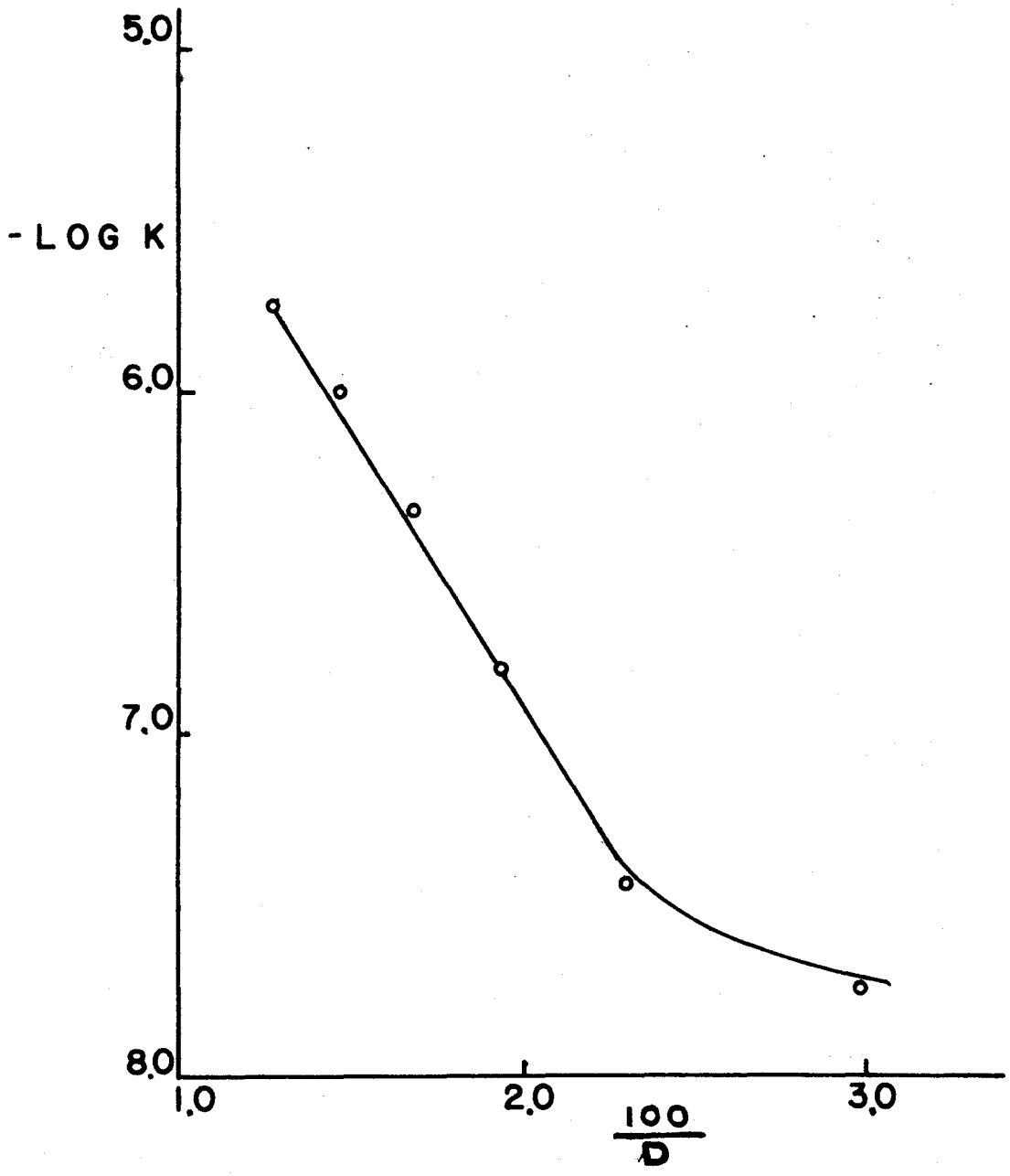


ciprocal of the dielectric constant (figure 9), gave a curve with a linear slope from 0.00 to 40.0 per cent dioxane, and which lost its linearity from 40.0 to 50.0 per cent dioxane.

This decrease of rate with decreasing values of the dielectric constant agrees with the theory that an increase in the ionic strength should increase an ionic reaction involving ions of the same sign. The fact that deviations at low dielectric constants from the straight line slope of log k versus the reciprocal of the dielectric constant occurred is not unusual. According to Glasstone, Laidler and Eyring (30), this is probably due to the treatment of the solvent as a homogeneous medium of uniform dielectric constant. It is probable that in a mixture of water with a solvent of low dielectric constant, the molecules of water will be preferentially oriented around the ions: the dielectric constant in the vicinity of the ion will thus be different from that of the bulk of the medium. As long as the solution contains a large proportion of water, the error will not be very significant. But when the dielectric constant is reduced by the addition of a relatively large amount of dioxane, the difference between the value in the bulk of the solution and that around the ion will be probably considerable. It was actually ob-

FIGURE 9

LOG K VERSUS THE RECIPROCAL OF THE DIELECTRIC CONSTANT FROM THE DATA IN TABLE IX



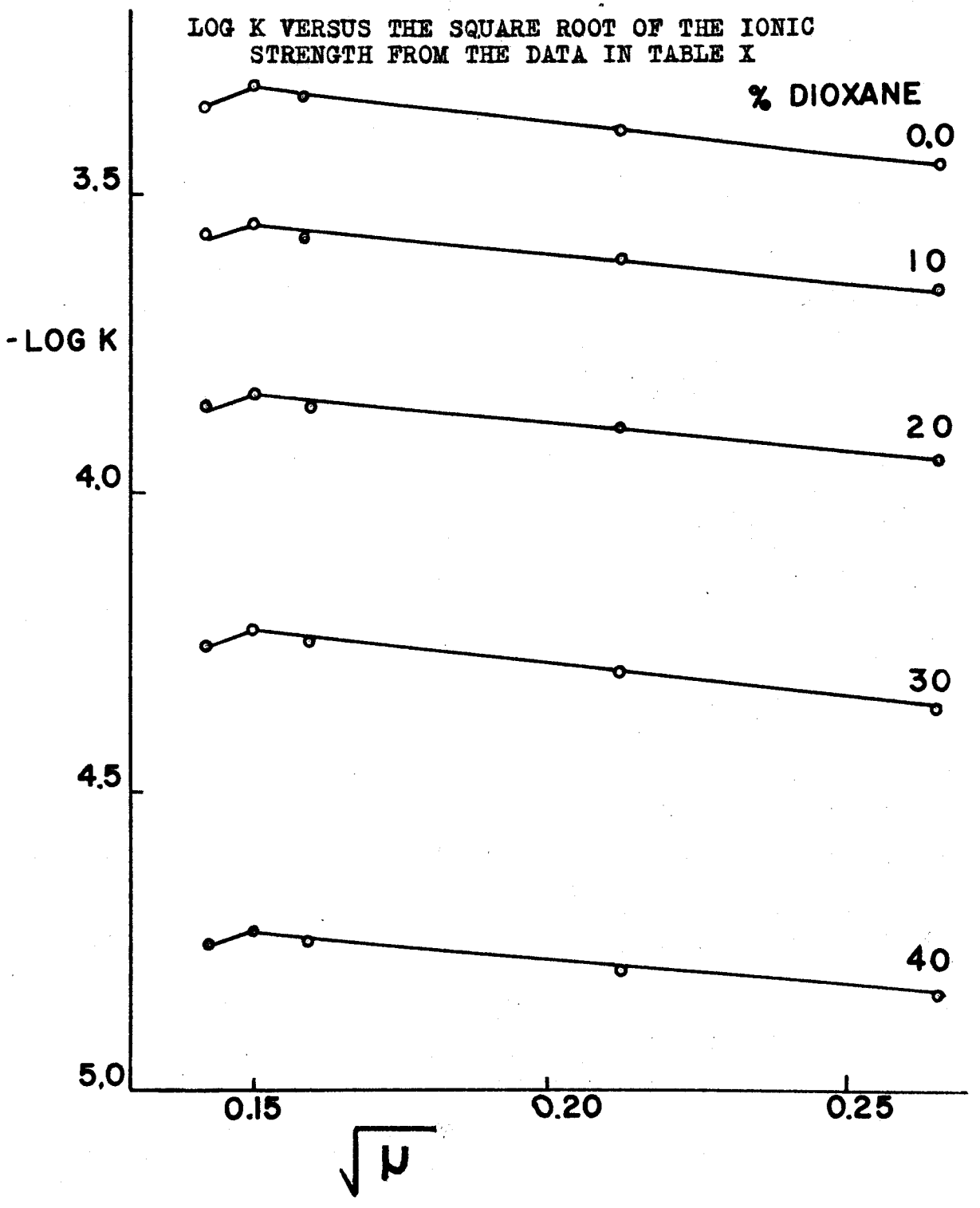
served that the reaction rate of the urease-urea hydrolysis at sixty per cent dioxane was almost the same as that found for forty per cent, and the rate in fifty per cent dioxane was actually below that of either forty or sixty per cent dioxane.

Dioxane (1,4-diethylene oxide) was chosen as the solvent for studying the effect of variation of the dielectric constant because of its low value of dielectric constant, its neutral character, its complete miscibility with water, and because the reactants and the neutral salts employed were soluble up to eighty per cent dioxane in water at the concentrations that were used in making the experimental kinetic rate measurements.

Varying the ionic strength from 0.0200 to 0.0700 molar with the salt, sodium chloride, the effect of dielectric constant was studied for the reaction between 0.0100 per cent urease and 0.0143 molar urea (table X). The results of these experimental kinetic runs gave a series of similar plots (figure 10), when log k was plotted against the square root of the ionic strength. It is quite obvious that for each different dioxane-water mixture, the slope of the curve is at first positive, then becomes zero for a small portion of the curve,

FIGURE 10

LOG K VERSUS THE SQUARE ROOT OF THE IONIC STRENGTH FROM THE DATA IN TABLE X



and finally becomes negative. This seems to indicate that the neutral sodium chloride accelerates the urease-urea reaction at low regions of ionic strength, but causes a retardation of the reaction at higher values of ionic strength.

Using exactly the same conditions as above, but varying the ionic strength from 0.0100 to 0.0200 molar, the effect of variation of the dielectric constant of the medium with dioxane was observed for the following neutral salts: sodium chloride (table XI), potassium chloride (table XII), sodium sulfate (table XIII), sodium bromide (table XIV), potassium bromide (table XV), potassium sulfate (table XVI), and tetramethyl ammonium bromide (table XVII). The log k's, from these particular set of kinetic runs, were plotted against the square root of the ionic strength for each of these neutral salts. These plots will be found in figures 11, 12, 13, 14, 15, 16 and 17. The initial linear portion of all of these curves, in the case of each salt and at different dielectric constant values, was found to possess a slope of plus one. This appears to be very definitely a primary salt effect because the slope of these curves of the logarithm of the rate constant versus the square root of the ionic strength is linear and approximately a whole number in

FIGURE II

LOG K VERSUS THE SQUARE ROOT OF THE IONIC STRENGTH FROM THE DATA IN TABLE XI

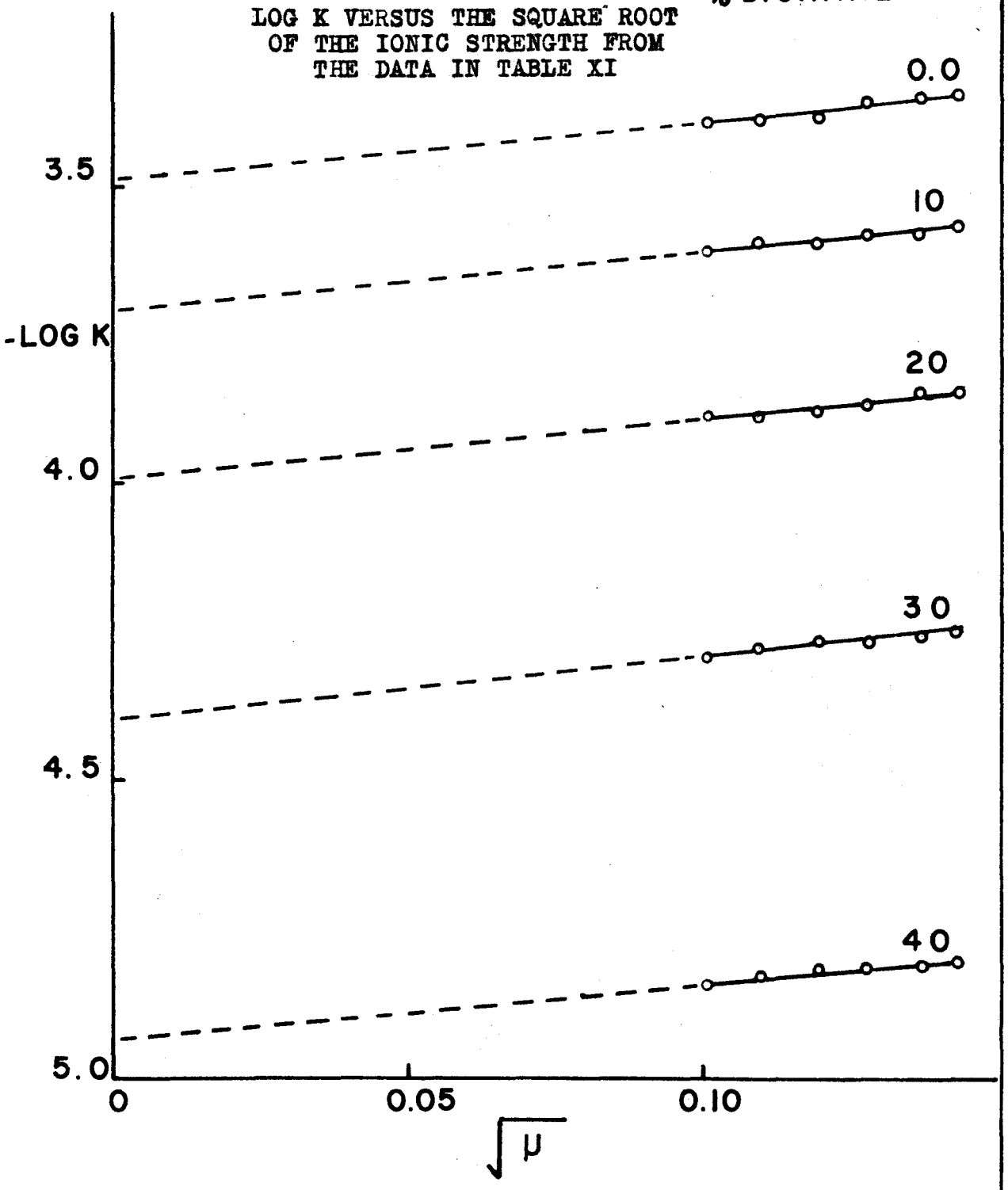


FIGURE 12

LOG K VERSUS THE SQUARE ROOT OF THE IONIC STRENGTH FROM THE DATA IN TABLE XII

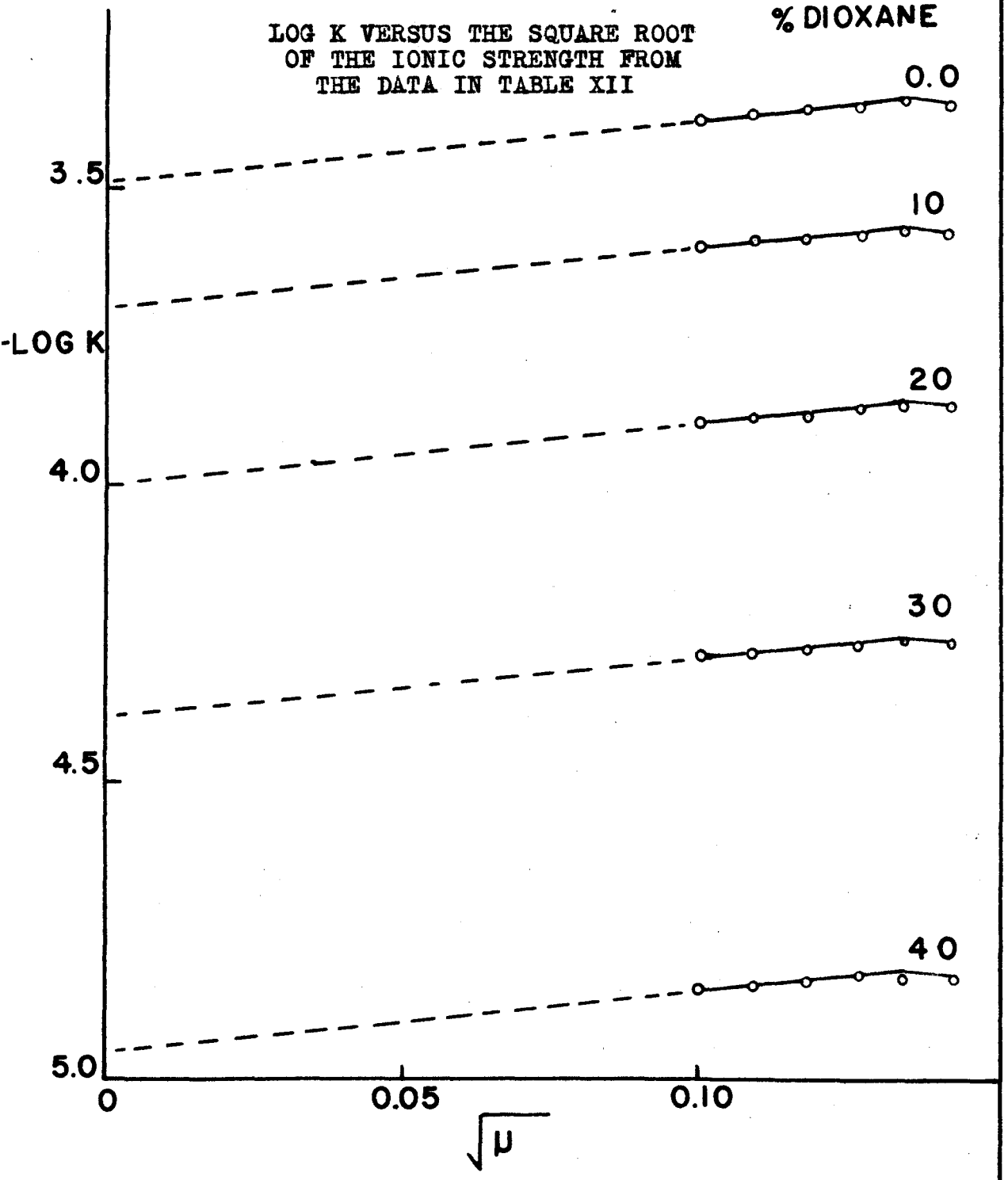


FIGURE 13

LOG K VERSUS THE SQUARE ROOT OF THE IONIC STRENGTH FROM THE DATA IN TABLE XIII

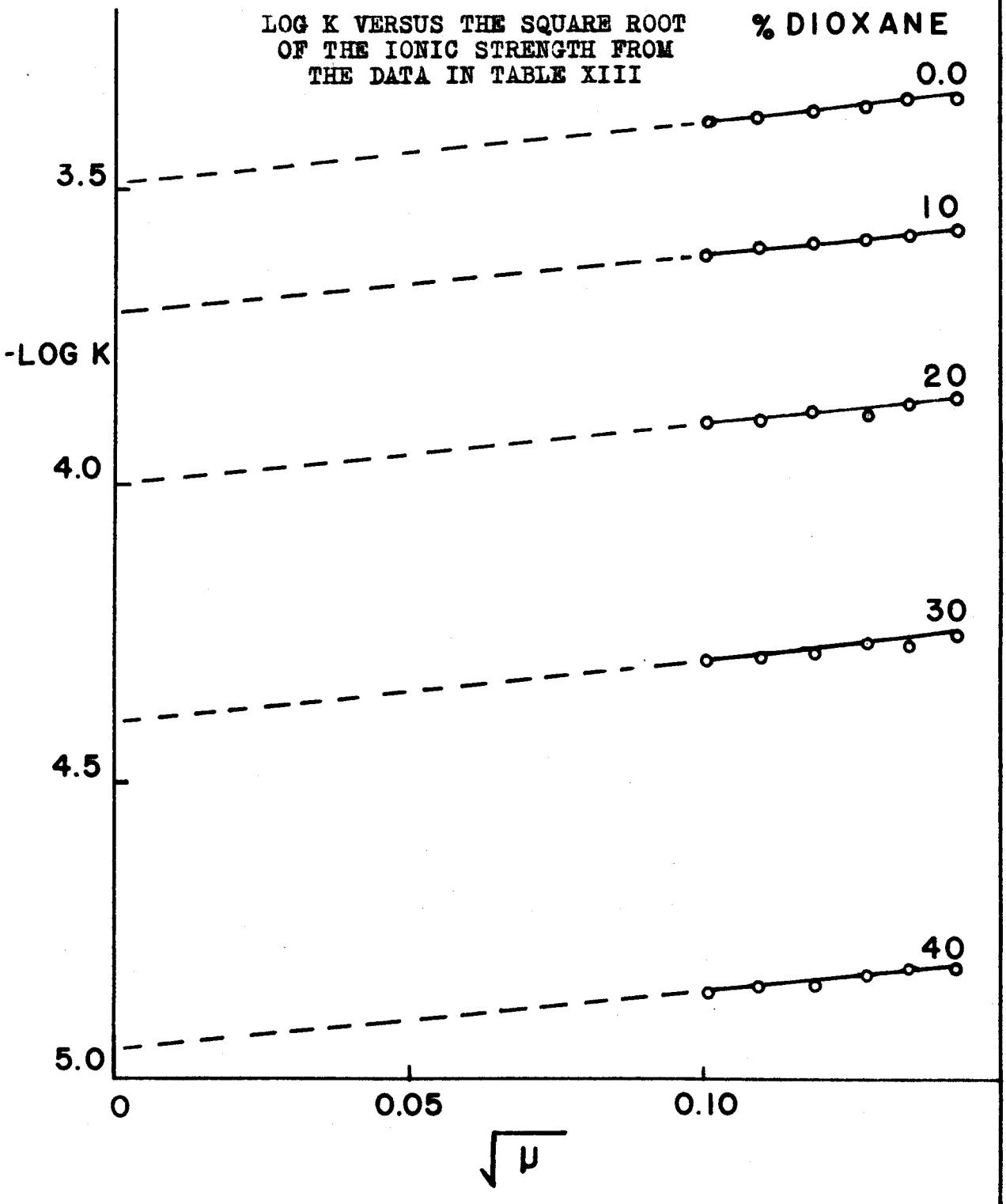


FIGURE 14

LOG K VERSUS THE SQUARE ROOT OF THE IONIC STRENGTH FROM THE DATA IN TABLE XIV

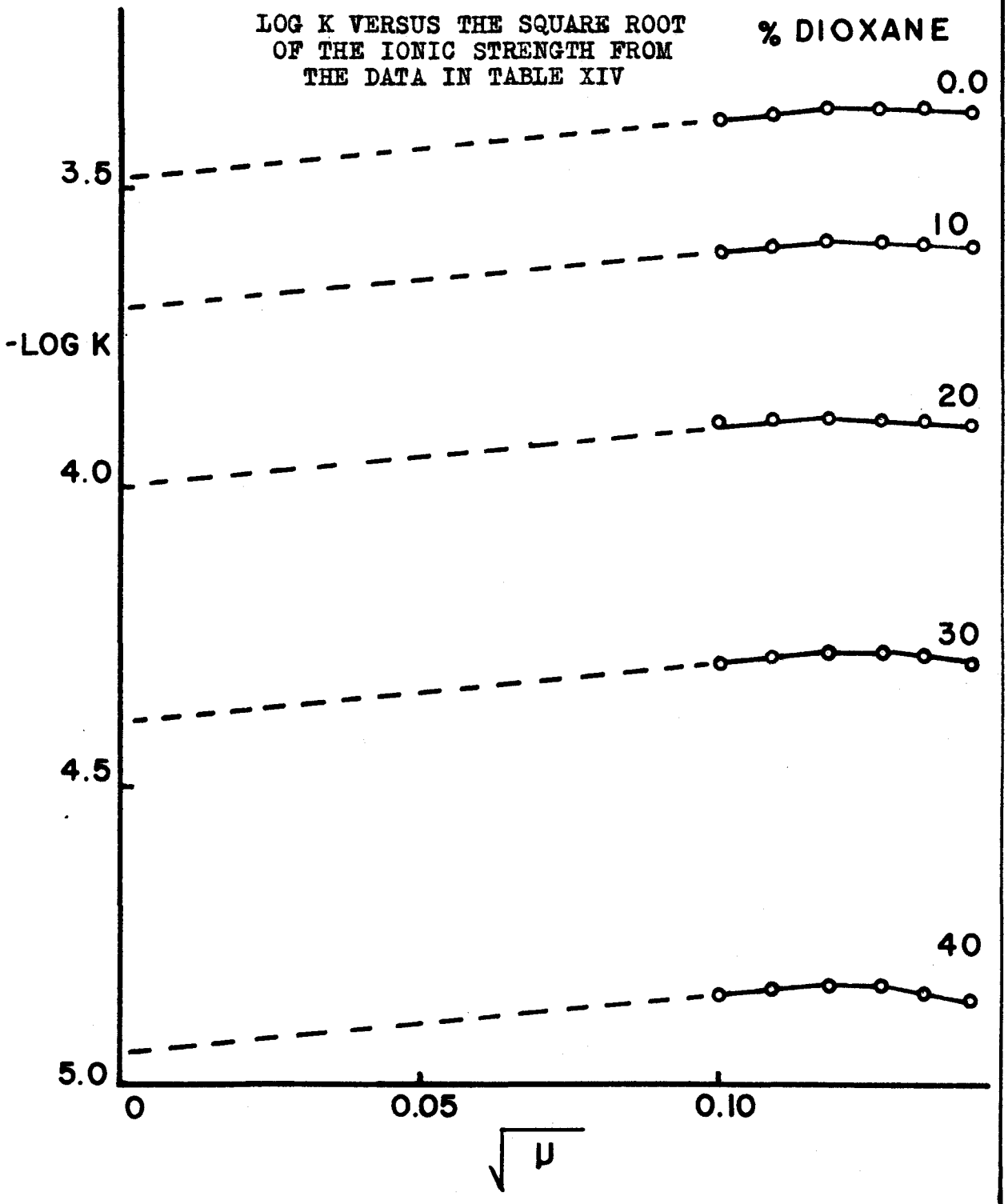


FIGURE 15

LOG K VERSUS THE SQUARE ROOT OF THE IONIC STRENGTH FROM THE DATA IN TABLE XV

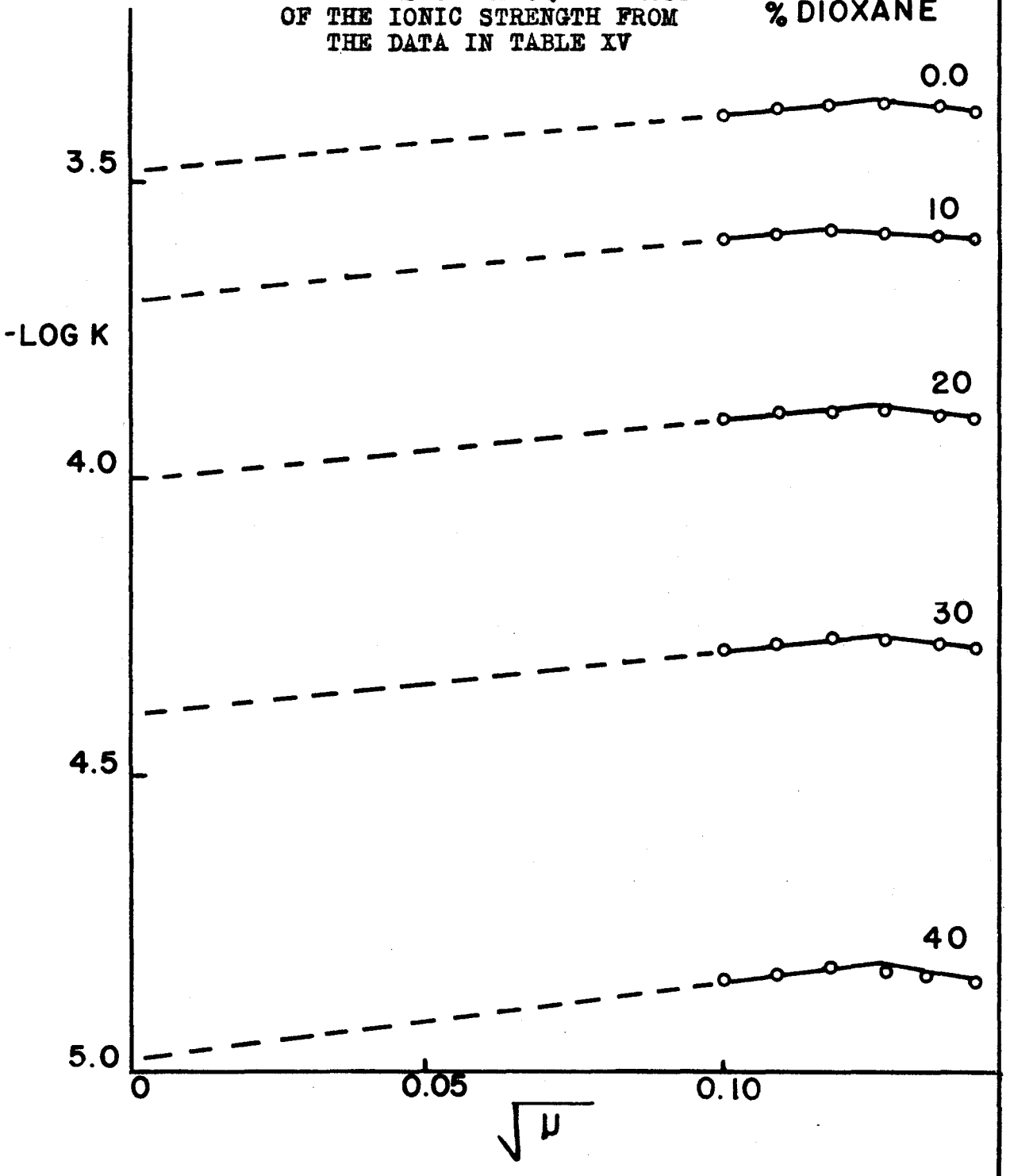


FIGURE 16

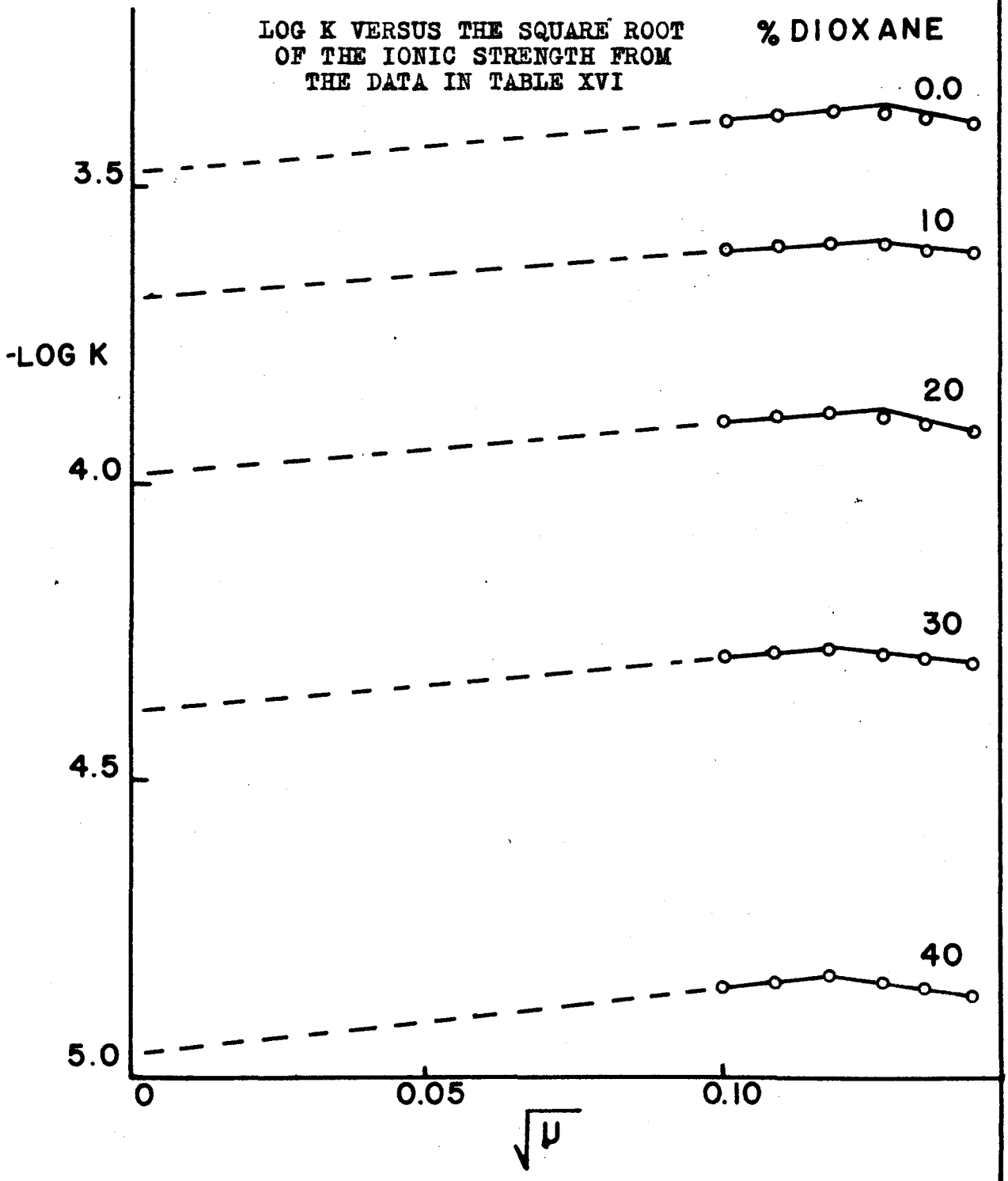
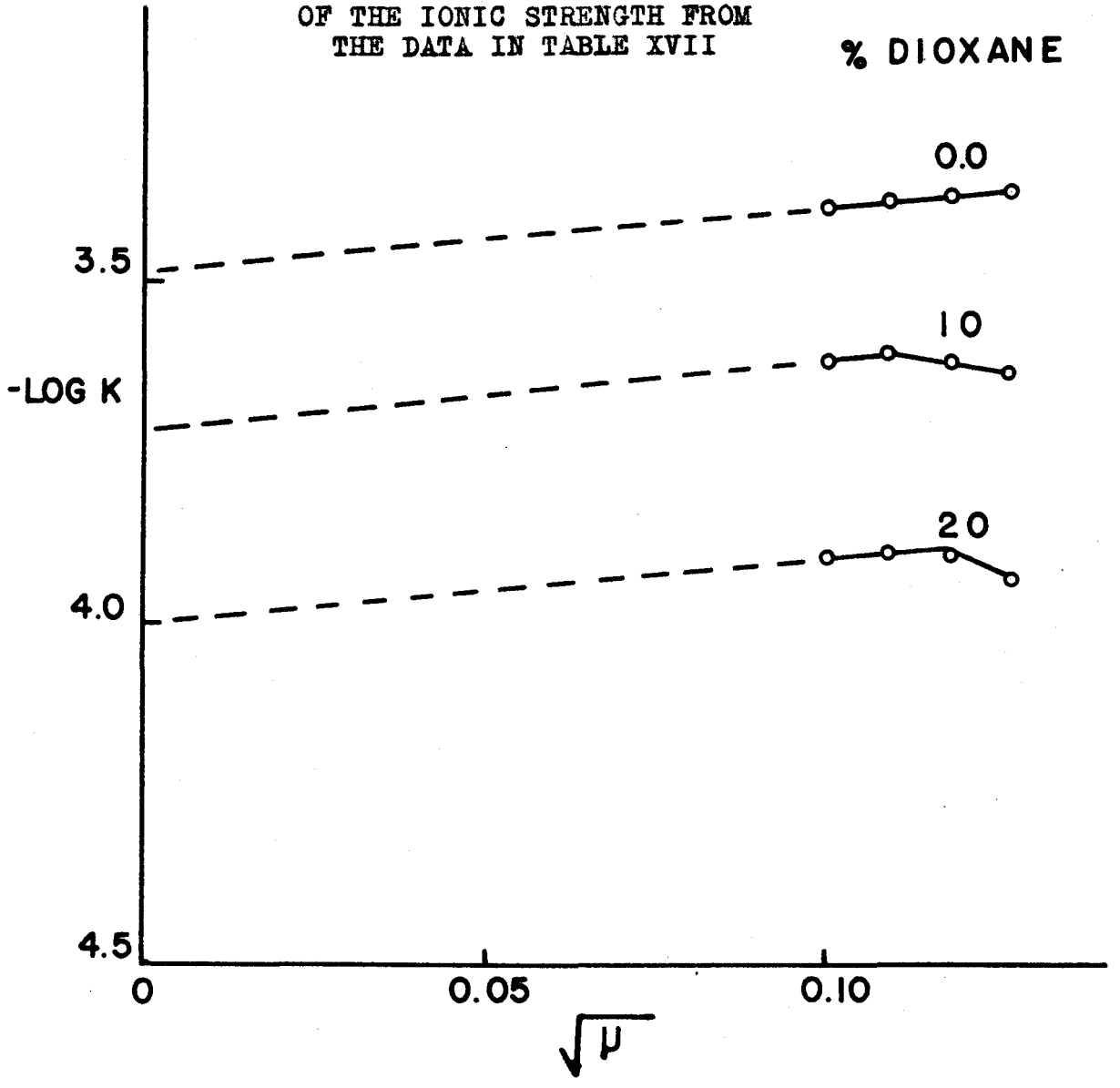

LOG K VERSUS THE SQUARE ROOT OF THE IONIC STRENGTH FROM THE DATA IN TABLE XVI
% DIOXANE
0.0
10
20
30
40
-LOG K
3.5
4.0
4.5
5.0
0
0.05
0.10
√μ

FIGURE 17

LOG K VERSUS THE SQUARE ROOT OF THE IONIC STRENGTH FROM THE DATA IN TABLE XVII



a region of ionic strength where the Bronsted theory is most valid.

In order to correlate the primary salt effect with that of the dielectric constant effect, the initial linear portions of all the curves of figures 11, 12, 13, 14, 15, 16 and 17, was extrapolated to zero ionic strength. In this manner, the log k's at zero ionic strength for all of these curves was determined. These can be found in table XVIII, along with the averaged values for each different value of dielectric constant for all of the neutral salts employed.

When the averaged values of the log k's at zero ionic strength for each dielectric constant value were plotted against the reciprocal of the dielectric constant (figure 18), a straight line was obtained with a negative slope of 137. This application of Scatchard's theory of effect of dielectric constant on an ionic reaction confirms what appears to be the primary salt effect in the case of the urease-urea reaction.

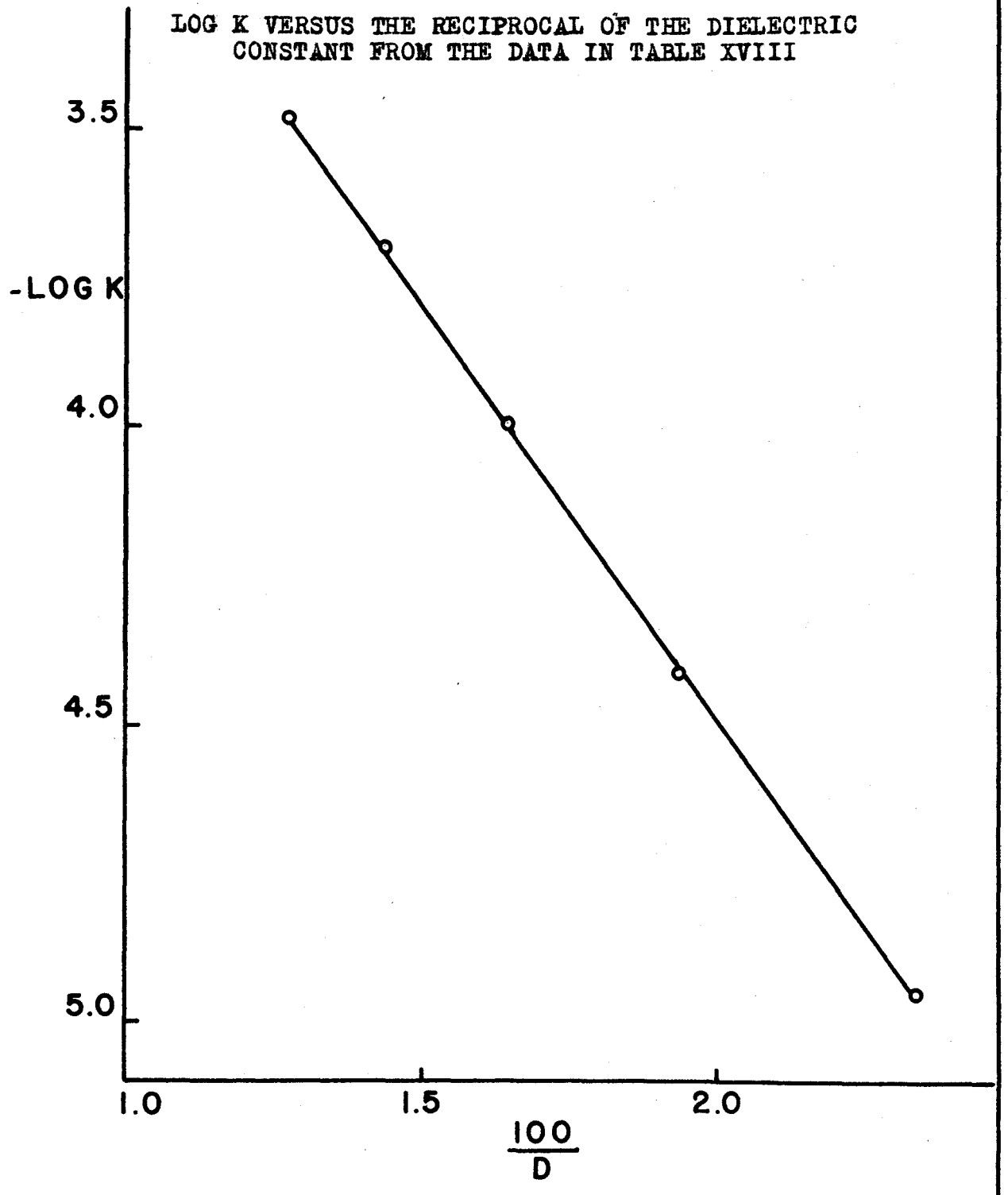
Since the radius of the activated intermediate comlex is given by the equation (30),

$$r = -Z_A Z_B e^2/2.303KT,$$

where Z_A and Z_B are the valence charges of the reacting ions

FIGURE 18

LOG K VERSUS THE RECIPROCAL OF THE DIELECTRIC CONSTANT FROM THE DATA IN TABLE XVIII



A and B respectively, where S is the slope, e is the electronic charge, K is Boltzman's constant and T is the absolute temperature. Then if the slope is known, it is possible to calculate the value of the radius, r. Using plus one for the value of $Z_A Z_B$ and substituting the other values required in the above equation, it was found that the radius of the intermediate complex was 35.7 A°. Although this value for r turns out to be slightly larger than that for ordinary ions or molecules, it is not at all unreasonable that the intermediate complex of urease-urea might be this large.

According to the theory of Bronsted's primary salt effect, a reaction between ions of the same sign, in a medium of low ionic strength, is accelerated by a small increase in ionic strength. The experimental results obtained for the catalytic hydrolysis of urea by the enzyme, urease, indicate rather strongly that the rate-controlling step of the overall reaction is an ionic one. Since the reaction rate is increased when the ionic strength is increased from 0.0100 to about 0.0180 molar, the ions reacting must have the same ionic charge. This is confirmed by the Scatchard theory of changing environment, that a reaction between ions of the same sign is retarded when the dielectric constant of the

medium is decreased. Because the slope of the plot of log k against the square root of the ionic strength is around plus one, which is equal to the product $Z_A Z_B$, then the charge on each of the reacting ions involved in the rate-determining step of the overall reaction must be either plus one or a negative one.

Let us suppose that for the experimental conditions employed that the rate-determining step of the overall urease-urea reaction is the formation of the intermediate complex, keeping in mind, however, that whatever the rate-determining step is, that is the step which the primary salt effect influences. Now since the salt effect was studied under conditions where good first order constants were obtained, there is a good possibility that the formation of the intermediate complex is slower than the decomposition of the intermediate complex to free enzyme and products for these particular experimental conditions.

It is realized that, in general, interpretation of kinetic data of enzymatic reactions by first order kinetics may be questioned. However, in a special case where the substrate concentration is decreased sufficiently to render the rate of combination of enzyme and substrate the rate-deter-

mining step, the interpretation of this step of the overall reaction by first order constants should be valid. The concentrations of urea and urease employed in the major portion of the experimental work in this dissertation yielded excellent first order constants both with respect to initial urea concentration and with respect to time (48).

It has already been pointed out that an enzyme combines with its substrate and while thus combined, the complex is decomposed to the free enzyme and products, thus

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} (ES) \xrightarrow{k_3} E + \text{products}.$$

Now according to Michaelis and Menten (66), the enzyme substrate combination is governed by K_m, the dissociation constant of the complex, according to

$$(a) \qquad K_m = \frac{(E)\ (S)}{(ES)} = \frac{(e - p)(a - x)}{p},$$

where e is the total concentration of the enzyme, p is that of the complex, and (a - x) is the substrate concentration, at equilibrium (70). If the rate of decomposition, v, of the intermediate complex is expressed by the velocity constant k_3,

$$(b) \qquad v = k_3(ES) = k_3 p,$$

where k_3 is only proportional to the concentration (ES) at time t. If at equilibrium, p, the concentration of the com-

plex is negligibly small in comparison to e and to (a - x), then K_m will be sufficiently high to force the equilibrium between free enzyme and combined enzyme far to the left. Combining equations (a) and (b) yields

$$(c) \qquad v = k_3 p = k_3 \frac{(e - p)(a - x)}{K_m},$$

and if e is much greater than p, then equation (c) reduces to

$$(d) \qquad v = \frac{-d(a - x)}{dt} = \frac{k_3 e(a - x)}{K_m},$$

which is the differential form of a kinetically first order rate equation. Integration of equation (d) therefore yields,

$$(e) \qquad k_3 et = 2.30\ K_m \log a/(a-x),$$

where a is the initial substrate concentration. It is evident that first order reaction constants should be observed in which the assumption of a high enzyme-substrate concentration and a low substrate concentration apply. Under these conditions, the rate of combination between enzyme and substrate will be the rate determining step (70). Since the dissociation constant, K_m, is given by the equation,

$$(f) \qquad K_m = \frac{k_2 + k_3}{k_1},$$

where k_1 is the rate constant for the combination of E and S to form ES, k_2 that for the reverse reaction and k_3, the rate

constant for the decomposition of ES to enzyme and products, then

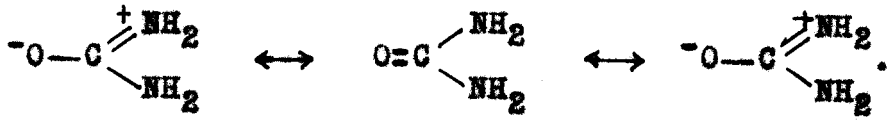
$$(g) \qquad \frac{k_3 e}{K_m} = \frac{k_3 k_1 e}{k_2 + k_3} = k.$$

For a special case, where k_2 is much smaller than k_3, equation (g) reduces to $k_1 e$, which is the same as 2.30 times the observed first order constant, k, which is the kinetic constant probably observed in the work of this dissertation on the primary salt effect of the urease-urea reaction.

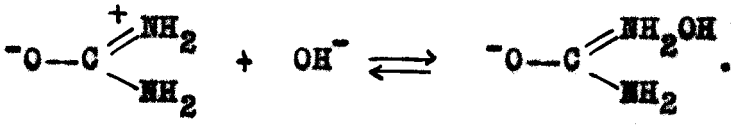
The enzyme, urease, it is generally agreed, is a protein. Therefore, it is an ampholyte with a definite isoelectric point, and can exist in a number of ionic forms. It so happens that the isoelectric point of urease is at pH 5.0 (91). Now all of the experimental kinetic runs which were made on the urease-urea reaction, were made at pH 7.0, which is on the alkaline side of the isoelectric point for urease. This means that the ionic charge of the enzyme molecule at this pH is predominantly negative. This does not necessarily mean that the urease molecule has only one negative charge, for it undoubtedly has a number of substrate binding groups, and therefore a number of such negatively charged sites. It may be that a negatively charged urea molecule reacts with one of these negatively charged sites on the

urease molecule to form the intermediate complex, which then rapidly breaks up to the free enzyme and the products of the hydrolysis.

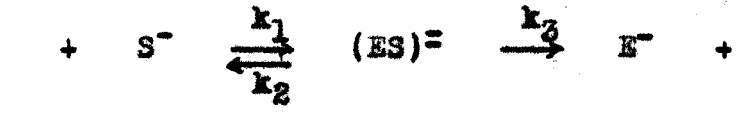
The question might arise as to how the urea molecule can attain one negative charge. According to Lenti (56), urea possesses the following resonance forms:



If this is the case, it may be that either of the charged resonant forms reacts with a hydroxide ion to produce a negatively charge urea-hydroxide ion, which might then react with the negatively charged site of the urease molecule. Thus



Then the reaction between the enzyme and the substrate may be written as an ionic reaction, thus



The author does not want to leave the impression that this is the whole picture of the urease catalyzed hydrolysis of urea. The very fact that high concentrations of buffer or neutral salts retard this reaction indicates that

this reaction is quite complex. This latter effect, however, may be due to secondary salt effects, where the equilibrium between one of the reactants and a third body is disturbed. The third body being some other ion as from a salt and the reactant being in all probability the enzyme. This is quite possible, since in the presence of a high concentration of foreign ions, the charged reacting site of the enzyme may interact with these ions and thereby hinder the combination of the substrate on this combining site.

Kistiakowsky and coworkers (42) have observed the effect of neutral salts and various buffers on the activity of urease, at ionic strengths as low as 0.024 molar. They observed that the reaction rate decreased rather uniformly with increasing ionic strength at pH 7.0 to 7.5, however, at pH 6.0 to 6.5, the decrease in reaction rate was hardly noticeable, if at all, when the ionic strength was increased by the addition of neutral salts to the reaction medium. This may mean that the secondary salt effect is smaller at pH 6, because this pH is closer to the isoelectric point of urease and the urease molecule does not exist in its particular ionic state of strongest catalytic properties.

Howell and Sumner (36) obtained a series of curves

showing the relation between urea concentration and rate at different pH values in 0.0125 molar phosphate buffer. When the Michaelis constants from these curves were plotted as pK_m against pH, a straight line with a slope of plus one was obtained. Dixon (21) suggests that this means that the enzyme either loses one positive charge or gains one negative charge, when it combines with urea. Or that when urea is activated by combination with the enzyme, urease, it acquires a negative charge. This is in full agreement with the idea that the negatively charged urease combines with a negatively charged urea molecule to form an intermediate complex.

The fact that the optimum pH of urease activity will vary for exactly the same concentration of different buffers may be due to ionic strength effects either of a primary or secondary nature. For example, the same concentrations of citrate and phosphate buffer will not give the same activity for urease. The reason for this is very likely a difference in ionic strength, since even at the same concentrations, these two buffers do not necessarily ionize to the same extent.

The results of many investigators on the salt effect on the urease-urea reaction, which have often been con-

tradictory, may be explained in terms of the primary and secondary salt effects. That is one worker may report that sodium chloride increases the activity of urease, and yet another investigator may observe that sodium chloride decreases the urease activity. The reason for this discrepancy between the observations of different investigators is self-evident when it is realized that they performed their experimental observations of urease activity under entirely different conditions of ionic strength.

In conclusion, although for the urease hydrolysis of urea, it appears as if the primary salt effect of all neutral salts is the same at very low values of ionic strength, the secondary salt effect seems to be characteristic of the particular salt used. In other words, there is an optimum value of ionic strength, for each different salt, where urease activity is at its highest level. This optimum ionic strength of the enzyme activity is due to a combination of both a primary and secondary salt effect.

CHAPTER VII

SUMMARY

This investigation was undertaken in an attempt to provide a better understanding of the fundamental kinetics involved in the mechanism of the urease-urea system.

In an investigation of the factors operative in urease-catalyzed hydrolysis of urea in dioxane-water mixtures of various dielectric constants, at 25.0°C. and buffered with sodium and potassium phosphate, it has been found that on the addition of neutral salts, the activity of urease is first increased and then decreased with increasing concentration of the neutral salt. Essentially the same effect was noted for the following salts: sodium chloride, potassium chloride, sodium sulfate, sodium bromide, potassium bromide, potassium sulfate, and tetramethyl ammonium bromide.

There is a definite effect of ionic strength on the reaction rate, when the ionic strength values of the medium are small. This primary salt effect was used to elucidate the possible ionic mechanism of the reaction. According to the Bronsted theory, the logarithm of the reac-

tion velocity constant, k, was plotted against the square root of the ionic strength, u. A linear relationship was obtained when the initial ionic strength was 0.0100 molar. From the slope of the straight line, which was found to be a positive one, it can be concluded that the probable charges of the ions in the kinetically-controlling step of the overall reaction are of the same sign, with each reactant ion having a single charge only.

It was also observed that there is a definite decrease in the reaction velocity, when the dielectric constant of the medium in which the reaction is taking place, is varied by the addition of dioxane. The reaction rate constants at zero ionic strength in mediums of different dielectric constants were obtained by plotting the logarithm of the rate constants against the square root of the ionic strength, and the curves thus obtained, were extrapolated to zero ionic strength. According to the theory of Scatchard, when the logarithm of the rate constants at zero ionic strength were plotted against the reciprocal of the dielectric constant of the medium, a linear relationship was obtained with a slope of a negative 137. Since the slope was negative, this verifies the fact that the

probable charges of the reactant ions in the rate-determining step of the reaction are of the same sign. From the slope it was possible to calculate the probable radius of the intermediate complex, which was found to be 35.6 A^{o}.

If it is assumed that the rate-controlling step of the overall reaction is the formation of the intermediate complex, when experimentally first order constants are observed, then this may possibly take place through the reaction of a negatively charged site on the urease molecule and a urea molecule, which has acquired a negative charge by way of an hydroxide ion. The decrease in the activity of urease at higher ionic strength values may very likely be due to secondary salt effects, which are produced by the interaction of the combining site of the urease molecule with foreign electrolyte ions. Also it is perhaps the operation of both the primary and secondary salt effects which cause a neutral salt to pass through an optimum ionic strength region of highest urease activity.

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APPENDIX

TABLES OF EXPERIMENTAL KINETIC DATA ON THE EFFECT OF NEUTRAL SALTS AND DIOXANE ON THE UREASE-UREA REACTION

TABLE I

THE EFFECT OF IONIC STRENGTH ON THE FIRST ORDER RATE CONSTANTS FOR THE REACTION BETWEEN 0.10% UREASE WITH 0.020 MOLAR UREA AT pH 7.0 AND 20.0° C.*

u	$\sqrt{u}$	k x 10^4/sec	-log k
0.48	0.693	4.08	3.389
1.20	1.095	2.51	3.603
1.28	1.132	2.69	3.570
2.40	1.549	1.38	3.860

*Data is that calculated from Van Slyke and Zacharias' experimental work (106).

TABLE II

THE EFFECT OF THE IONIC STRENGTH OF SODIUM CHLORIDE ON THE ZERO ORDER CONSTANTS FOR THE REACTION BETWEEN 0.10% UREASE WITH 0.250 MOLAR UREA AT pH 7.0 AND 25.0°C.

u	$\sqrt{u}$	k x 10^6/sec	-log k
0.0545	0.233	6.67	5.176
0.1045	0.323	6.55	5.184
0.1795	0.424	6.21	5.207
0.2420	0.492	5.97	5.224
0.3095	0.556	5.75	5.240
0.3670	0.606	5.59	5.252

TABLE III

THE EFFECT OF THE IONIC STRENGTH OF SODIUM CHLORIDE ON THE ZERO ORDER CONSTANTS FOR THE REACTION BETWEEN 0.10% UREASE WITH 0.122 MOLAR UREA AT pH 7.0 AND 25.0°C.

u	$\sqrt{u}$	k x 10^6/sec	-log k
0.0545	0.233	6.00	5.222
0.0572	0.239	5.92	5.228
0.0596	0.244	5.86	5.232
0.0796	0.282	5.47	5.262
0.1046	0.323	5.09	5.293

TABLE IV

THE EFFECT OF THE IONIC STRENGTH OF SODIUM CHLORIDE ON THE ZERO ORDER CONSTANT FOR THE REACTION BETWEEN 0.020% UREASE WITH 0.143 MOLAR UREA AT pH 7.0 AND 25.0°C.

u	$\sqrt{u}$	k x 10^6/sec	-log k
0.0545	0.233	1.23	5.910
0.0572	0.239	1.21	5.916
0.0596	0.244	1.20	5.921
0.0796	0.282	1.11	5.956
0.1046	0.323	1.01	5.996

TABLE V

THE EFFECT OF THE IONIC STRENGTH OF POTASSIUM CHLORIDE ON THE ZERO ORDER CONSTANTS FOR THE REACTION BETWEEN 0.020% UREASE WITH 0.143 MOLAR UREA AT pH 7.0 AND 25.0°C.

u	$\sqrt{u}$	k x 10^6/sec	-log k
0.0545	0.233	1.23	5.910
0.0572	0.239	1.21	5.916
0.0596	0.244	1.20	5.021
0.0796	0.282	1.12	5.951
0.1046	0.323	1.03	5.986

TABLE VI

THE EFFECT OF THE IONIC STRENGTH OF SODIUM SULFATE ON THE ZERO ORDER CONSTANTS FOR THE REACTION BETWEEN 0.020% UREASE WITH 0.143 MOLAR UREA AT pH 7.0 AND 25.0°C.

u	$\sqrt{u}$	k x 10^6/sec	-log k
0.0545	0.233	1.23	5.910
0.0572	0.239	1.21	5.916
0.0596	0.244	1.20	5.921
0.0796	0.282	1.13	5.048
0.1046	0.323	1.05	5.980

TABLE VII

THE EFFECT OF THE IONIC STRENGTH OF SODIUM CHLORIDE ON THE ZERO ORDER CONSTANTS FOR THE REACTION BETWEEN 0.050% UREASE WITH 0.143 MOLAR UREA AT pH 7.0 AND 25.0°C.

u	$\sqrt{u}$	k x 10^6/sec	-log k
0.0273	0.165	3.53	5.452
0.0276	0.166	3.54	5.451
0.0298	0.173	3.57	5.447
0.0398	0.200	3.53	5.452
0.0523	0.229	3.48	5.458

TABLE VIII

THE EFFECT OF THE IONIC STRENGTH OF SODIUM CHLORIDE ON THE FIRST ORDER CONSTANTS FOR THE REACTION BETWEEN 0.010% UREASE WITH 0.0143 MOLAR UREA AT pH 7.0 AND 25.0°C.

u	$\sqrt{u}$	k x 10^4/sec	-log k
0.0109	0.104	4.16	3.381
0.0114	0.107	4.19	3.378
0.0136	0.116	4.27	3.370
0.0159	0.126	4.37	3.359
0.0359	0.200	4.07	3.390
0.0609	0.247	3.65	3.437

TABLE IX

THE EFFECT OF DIOXANE ON THE ZERO ORDER CONSTANTS FOR THE REACTION BETWEEN 0.0250% UREASE WITH 0.143 MOLAR UREA AT IONIC STRENGTH OF 0.0273, pH 7.0 AND 25.0° C.

%Dioxane	100/D	D	k x 10^6/sec	-log k
0.0	1.27	78.5	1.77	5.752
10.0	1.43	69.7	0.990	6.004
20.0	1.64	60.8	0.462	6.335
30.0	1.93	51.9	0.157	6.804
40.0	2.33	43.0	0.037	7.44
50.0	2.91	34.3	0.017	7.78

TABLE X

THE EFFECT OF THE IONIC STRENGTH OF SODIUM CHLORIDE AND DIOXANE ON THE FIRST ORDER CONSTANTS FOR THE REACTION BETWEEN 0.010% UREASE WITH 0.0143 MOLAR UREA AT pH 7.0 AND 25.0°C.

u	k x 10^4/sec for the following % dioxane: 0.00	10.0	20.0	30.0	40.0
0.0200	4.54	2.72	1.40	0.560	0.177
0.0225	4.62	2.76	1.43	0.570	0.180
0.0250	4.57	2.73	1.41	0.564	0.177
0.0450	4.07	2.49	1.27	0.501	0.158
0.0700	3.67	2.24	1.15	0.447	0.144

$\sqrt{u}$	-log k for the following values of 100/D: 1.27	1.43	1.64	1.93	2.33
0.142	3.343	3.565	3.855	4.252	4.752
0.150	3.335	3.558	3.846	4.244	4.745
0.158	3.340	3.564	3.850	4.249	4.752
0.212	3.390	3.604	3.895	4.300	4.800
0.265	3.435	3.650	3.940	4.350	4.840

TABLE XI

THE EFFECT OF THE IONIC STRENGTH OF SODIUM CHLORIDE AND DIOXANE ON THE FIRST ORDER CONSTANTS FOR THE REACTION BETWEEN 0.010% UREASE WITH 0.0143 MOLAR UREA AT pH 7.0 AND 25.0°C.

u	k x 10^4/sec for the following % dioxane: 0.00	10.0	20.0	30.0	40.0
0.0100	4.12	2.48	1.27	0.507	0.141
0.0120	4.21	2.53	1.30	0.516	0.144
0.0140	4.29	2.57	1.32	0.528	0.147
0.0160	4.37	2.62	1.35	0.537	0.150
0.0180	4.46	2.67	1.37	0.547	0.153
0.0200	4.51	2.70	1.40	0.555	0.155

$\sqrt{u}$	-log k for the following values of 100/D: 1.27	1.43	1.64	1.93	2.33
0.100	3.385	3.605	3.895	4.295	4.850
0.109	3.376	3.597	3.885	4.287	4.841
0.118	3.368	3.590	3.878	4.277	4.832
0.127	3.358	3.581	3.870	4.270	4.824
0.134	3.351	3.574	3.862	4.262	4.815
0.142	3.346	3.569	3.854	4.256	4.810

TABLE XII

THE EFFECT OF THE IONIC STRENGTH OF POTASSIUM CHLORIDE AND DIOXANE ON THE FIRST ORDER CONSTANTS FOR THE REACTION BETWEEN 0.010% UREASE WITH 0.0143 MOLAR UREA AT pH 7.0 AND 25.0°C.

u	k x 10^4/sec for the following % dioxane: 0.00	10.0	20.0	30.0	40.0
0.0100	4.12	2.51	1.26	0.513	0.141
0.0120	4.21	2.57	1.28	0.522	0.143
0.0140	4.28	2.62	1.31	0.533	0.146
0.0160	4.36	2.67	1.33	0.542	0.148
0.0180	4.43	2.70	1.36	0.550	0.150
0.0200	4.38	2.70	1.36	0.540	0.145

$\sqrt{u}$	-log k for the following values of 100/D: 1.27	1.43	1.64	1.93	2.33
0.100	3.385	3.600	3.900	4.290	4.851
0.109	3.376	3.590	3.893	4.282	4.845
0.118	3.369	3.582	3.883	4.272	4.836
0.127	3.360	3.574	3.876	4.266	4.830
0.134	3.354	3.569	3.866	4.260	4.824
0.142	3.358	3.569	3.866	4.268	4.829

TABLE XIII

THE EFFECT OF THE IONIC STRENGTH OF SODIUM SULFATE AND DIOXANE ON THE FIRST ORDER CONSTANTS FOR THE REACTION BETWEEN 0.010% UREASE WITH 0.0143 MOLAR UREA AT pH 7.0 AND 25.0°C.

u	k x 10^4/sec for the following % dioxane: 0.00	10.0	20.0	30.0	40.0
0.0100	4.12	2.47	1.26	0.501	0.140
0.0120	4.20	2.51	1.29	0.513	0.142
0.0140	4.27	2.56	1.31	0.524	0.144
0.0160	4.36	2.61	1.33	0.533	0.147
0.0180	4.44	2.67	1.36	0.543	0.151
0.0200	4.52	2.72	1.39	0.552	0.153

$\sqrt{u}$	-log k for the following values of 100/D: 1.27	1.43	1.64	1.93	2.33
0.100	3.385	3.607	3.896	4.300	4.854
0.109	3.377	3.600	3.889	4.290	4.848
0.118	3.370	3.592	3.883	4.281	4.842
0.127	3.360	3.583	3.876	4.272	4.833
0.134	3.353	3.574	3.866	4.265	4.821
0.142	3.345	3.565	3.857	4.258	4.815

TABLE XIV

THE EFFECT OF THE IONIC STRENGTH OF SODIUM BROMIDE AND DIOXANE ON THE FIRST ORDER CONSTANTS FOR THE REACTION BETWEEN 0.010% UREASE WITH 0.0143 MOLAR UREA AT pH 7.0 AND 25.0°C.

u	k x 10^4/sec for the following % dioxane: 0.00	10.0	20.0	30.0	40.0
0.0100	4.12	2.49	1.27	0.507	0.141
0.0120	4.22	2.54	1.29	0.518	0.143
0.0140	4.30	2.59	1.32	0.527	0.146
0.0160	4.32	2.59	1.32	0.528	0.145
0.0180	4.28	2.56	1.30	0.524	0.143
0.0200	4.20	2.51	1.28	0.513	0.138

$\sqrt{u}$	-log k for the following values of 100/D: 1.27	1.43	1.64	1.93	2.33
0.100	3.385	3.604	3.896	4.295	4.851
0.109	3.375	3.595	3.889	4.286	4.845
0.118	3.366	3.587	3.879	4.278	4.836
0.127	3.364	3.587	3.879	4.277	4.839
0.134	3.369	3.592	3.886	4.281	4.845
0.142	3.377	3.600	3.893	4.290	4.860

TABLE XV

THE EFFECT OF THE IONIC STRENGTH OF POTASSIUM BROMIDE AND DIOXANE ON THE FIRST ORDER CONSTANTS FOR THE REACTION BETWEEN 0.010% UREASE WITH 0.0143 MOLAR UREA AT pH 7.0 AND 25.0°C.

u	k x 10^4/sec for the following % dioxane: 0.00	10.0	20.0	30.0	40.0
0.0100	4.12	2.52	1.25	0.514	0.142
0.0120	4.19	2.57	1.28	0.525	0.144
0.0140	4.27	2.62	1.30	0.535	0.147
0.0160	4.24	2.59	1.29	0.532	0.146
0.0180	4.19	2.56	1.28	0.525	0.144
0.0200	4.10	2.52	1.25	0.514	0.141

$\sqrt{u}$	-log k for the following values of 100/D: 1.27	1.43	1.64	1.93	2.33
0.100	3.385	3.599	3.903	4.289	4.848
0.109	3.378	3.590	3.893	4.280	4.842
0.118	3.370	3.582	3.886	4.272	4.833
0.127	3.373	3.587	3.889	4.274	4.836
0.134	3.378	3.592	3.893	4.280	4.842
0.142	3.387	3.599	3.903	4.289	4.851

TABLE XVI

THE EFFECT OF THE IONIC STRENGTH OF POTASSIUM SULFATE AND DIOXANE ON THE FIRST ORDER CONSTANTS FOR THE REACTION BETWEEN 0.010% UREASE WITH 0.0143 MOLAR UREA AT pH 7.0 AND 25.0°C.

u	k x 10^4/sec for the following % dioxane: 0.00	10.0	20.0	30.0	40.0
0.0100	4.12	2.50	1.26	0.508	0.141
0.0120	4.21	2.55	1.29	0.520	0.144
0.0140	4.28	2.59	1.32	0.527	0.146
0.0160	4.22	2.55	1.29	0.519	0.144
0.0180	4.14	2.50	1.27	0.509	0.141
0.0200	4.06	2.45	1.24	0.501	0.138

$\sqrt{u}$	-log k for the following values of 100/D: 1.27	1.43	1.64	1.93	2.33
0.100	3.385	3.602	3.896	4.294	4.851
0.109	3.376	3.594	3.889	4.284	4.842
0.118	3.369	3.587	3.880	4.278	4.836
0.127	3.375	3.594	3.889	4.285	4.842
0.134	3.383	3.602	3.896	4.293	4.851
0.142	3.392	3.611	3.907	4.300	4.860

TABLE XVII

THE EFFECT OF THE IONIC STRENGTH OF TETRAMETHYL AMMONIUM BROMIDE AND DIOXANE ON THE FIRST ORDER CONSTANTS FOR THE REACTION BETWEEN 0.010% UREASE WITH 0.0143 MOLAR UREA AT pH 7.0 AND 25.0°C.

u	$\sqrt{u}$	$kx10^4$/sec	-log k	%Dioxane	100/D
.0100	0.100	4.12	3.385	0.00	1.27
.0120	0.109	4.21	3.376	0.00	1.27
.0140	0.118	4.30	3.366	0.00	1.27
.0160	0.127	4.36	3.360	0.00	1.27
.0100	0.100	2.47	3.607	10.0	1.43
.0120	0.109	2.52	3.599	10.0	1.43
.0140	0.118	2.46	3.609	10.0	1.43
.0160	0.127	2.37	3.625	10.0	1.43
.0100	0.100	1.26	3.900	20.0	1.64
.0120	0.109	1.29	3.889	20.0	1.64
.0140	0.118	1.25	3.903	20.0	1.64
.0160	0.127	1.17	3.932	20.0	1.64

TABLE XVIII

THE EFFECT OF THE DIELECTRIC CONSTANT OF THE MEDIUM AT ZERO IONIC STRENGTH ON THE FIRST ORDER CONSTANTS FOR THE REACTION BETWEEN 0.010% UREASE WITH 0.0143 MOLAR UREA AT pH 7.0 AND 25.0°C.

	Extrapolated values of -log k, at zero u, for the following values of 100/D:				
Figure	1.27	1.43	1.64	1.93	2.33
11	3.482	3.704	3.996	4.398	4.938
12	3.490	3.700	4.000	4.390	4.958
13	3.486	3.710	4.000	4.498	4.950
14	3.480	3.700	3.998	4.392	4.945
15	3.482	3.700	4.002	4.398	4.975
16	3.477	3.685	3.980	4.380	4.960
17	3.480	3.707	4.000		
Average	3.482	3.701	3.997	4.409	4.954

APPROVAL SHEET

This doctoral dissertation submitted by William R. Pasterczyk has been read and approved by three members of the Department of Biochemistry, one member of the Department of Pharmacology and one member of the Department of Chemistry, of Loyola University.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that the necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

May 15, 1954
Date

Hugh J. McDonald
Signature of Adviser