THE DISSOCIATION OF UREASE BY 1,2-ETHYLENE GLYCOL

By

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PREFACE

Urease undergoes reversible dissociation into enzymatically active subunits in the presence of ethylene glycol. This paper is a rate investigation using acrylamide gel electrophoresis and ultracentrifuge techniques.

I am indebted to Dr. George Gorin for his supervision and guidance in the project. I am also thankful to Dr. D. P. Blattler and Dr. S. F. Wang for suggestions and help.

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CHAPTER I

INTRODUCTION

Urease dissociates into subunits in a variety of conditions. The present study deals with the dissociation and reassociation of urease in the presence of 1,2-ethylene glycol. The rate of the dissociation may be related to the percentage of glycol and the pH of the medium. Enzymatic activity is retained.

Chapter I contains a partial survey of the literature concerning urease and a brief review of protein dissociation. A paper reporting the principal results has been written in a form suitable for publication, possibly in Biochemical and Biophysical Research Communications. This constitutes Chapter II of the thesis. The paper omits descriptions of many experimental details, which are covered in Chapter III along with some new experiments. Chapter IV contains results.

Literature Survey

This chapter contains a survey of the literature concerning urease for the period August 1966 through 1968 and a brief discussion of enzyme dissociation. The survey of the urease literature brings up to date that compiled by Chin (2). It is based on the subject index of Chemical Abstracts. It excludes references, unless they contain other relevant information, regarding the occurrence of urease in biological systems and urease extracted from sources other than jack beans. Special

attention has been accorded to the dissociation of urease because of its pertinence to the subject of the thesis. The discussion of enzyme dissociation is not comprehensive. It describes the behavior of select enzymes in general terms, for comparison to that of urease. The bibliography of the urease survey is prepared separately from the rest of the references (p. 11).

Isolation

Many procedures have been employed to extract urease from jack beans and to purify it. Many factors influence the yield.

Babson (1) extracted urease from 600 g of jack bean flour with 2.2 l of water. The slurry is mixed for 15 minutes and its pH maintained at 7.0 by the addition of 2.5 N NaOH. After centrifugation the urease is separated from the supernatant by dialysis against 0.05 M phosphate buffer, at pH 7.5 for two days. The solution can be frozen and lyophilized or spray dried forming a dry stable form of urease.

Lynn (16) purified urease using ion-exchange chromatography and molecular sieves of polyacrylamide gel and Sephadex G-200. Urease recovered from a XE-64 ion-exchange column contained the total enzymatic activity that had been put on the column but with a 75% reduction in weight. This product had a specific activity of 581 Sumner units per mg. Chromatography on Sephadex G-200 also was effective. All of these experiments were done with the enzyme from one sample of jack bean meal. When other samples were used a substantially lower specific activity was obtained, ranging from 105 to 291 units per mg. A previously described isolation procedure by Gorin et al. gave material having a specific activity of 175 Sumner units per mg.

Assays

Urease catalyzes the decomposition of urea to NH_3 and CO_2. Several methods have been devised to measure the catalytic activity of the enzyme.

Gorin and Chin (8) developed an assay based on the hydrolysis of urea in pH 9.0 TRIS buffer. The urea solution containing the urease to be assayed is allowed to react for 2 minutes. The reaction is stopped by adding a measured excess of HCl. The quantity of product, NH_3, is then determined by back titration with NaOH to a methyl-orange end point. The unit of activity was defined, in accordance with the recommendations of the I.U.B., as "the quantity of enzyme that catalyzes the decomposition of one micro equivalent of the bond involved in the reaction in one minute." The specific activity of several samples of urease was found to be 1920 international units per mg. This value was based on a nitrogen determination and a value of 15.8 for the percentage of nitrogen in urease. The specific absorbancy at 278 mμ was found to be 0.754.

The addition of 2-mercaptoethanol to the solvent used to extract urease from jack beans increased the yield. The increase varied with the meal used. The specific absorbancy of urease changed when extracted with 2-mercaptoethanol present. The change in specific activity, when calculated from the proper absorbancy, was small.

In a companion paper Chin and Gorin (4) examined Sumner's assay procedure which used as a medium pH 7.0 phosphate buffer. This procedure is more limited and less accurate than Gorin and Chin's method. The phosphate salts used for Sumner's method must be very pure to pre-

vent inhibition of the urease by heavy metal ions. The product, NH_3, causes a gradual increase in pH of phosphate buffer which changes the rate of the reaction when urease of too high activity is used.

Kaltwasser and Schlegel (2) developed a spectrophotometric activity assay that utilizes the enzyme catalyzed reaction for the conversion of α-ketoglutarate to glutamate. Equimolar quantities of NH_3 and NADH are needed for the reaction. The catalyst is glutamic acid dehydrogenase. The consumption of NADH is followed spectrophotometrically. The NH_3 is produced by urease, and the activity of the urease is determined.

Du Pont (6) patented a similar spectrophotometric assay. NH_3 is produced by urease and that reacts with the coenzyme of another enzyme.

Kanter (13) developed a urease test outfit based on the Berthlot reaction.

Inhibition

The enzymatic activity of urease can be reduced or eliminated by a large variety of chemicals and conditions such as heat and extreme acidic or basic conditions.

Mueller and Pflanzen (17) found chloramphenicol inhibited urease and that the inhibition was partially reversible. Cysteine protected urease from this inhibition. Splitting chloramphenicol into dichloroacetate and nitrophenylaminopropanediol destroyed its inhibitory powers.

Kleczkowski and Dabrowska (15) found D-phenylalanine or phenylurea inhibited urease, but L-phenylalanine did not. Inhibition was greater at pH 6.8 than at pH 8.0.

Reifer (20) extracted and partially purified an apoinhibitor and a

coinhibitor of urease from poplar leaves. The inhibition was irreversible but could be prevented by L-cysteine, reduced glutathione, and 2-mercaptoethanol.

Hase and Kobashi (11) studied the inhibition of hydroxamic acids on urease from Proteus vulgaris. Caprylohydroxamic acid was the most potent inhibitor. The optimal range of inhibition was from pH 8.0 to 9.0 which corresponds to the enzymatic activity range. Variation in the number of carbon atoms in n-aliphatic hydroxamic acid caused changes in its inhibitory powers with a maximum at 8 carbons. Substitutions of $-CH_3$, $-Cl$, $-NO_2$, $-OH$, $-OCH_3$, and $-OC_4H_9$ at the "para" position in benzohydroxamic acid had the same inhibitory powers as benzohydroxamic acid. Less inhibition was found for ortho derivities.

Pianotti (19) evaluated the inhibitory effects of thirty-five urea analogues for Proteus vulgaris urease. Thiourea, N-methylthiourea and N-acetylthiourea were good inhibitors which could not be reversed by 10^{-3} M cysteine. Aryl substituted analogues were not nearly as good inhibitors as thiourea.

Reaction

Kinetic and related studies have been made which elucidate the nature of the urease catalyzed hydrolysis of urea.

Blokhra (3) showed Glansdorff and Prigogine's theorem on minimum entropy production for the decomposition of urea by urease was valid.

Skujins and McLaren (22) showed the rate of the hydrolysis of urea in the presence of water vapor followed the water absorption isotherm of urease, not of urea. Urease, urea, and urea containing C^{14} were mixed, frozen, lyophilized, and introduced into a chamber having a

measured water vapor pressure. The rate of the reaction was followed by the amount of $C^{14}O_2$ produced which did not begin until 60% relative humidity had been attained.

Lynn (16) found that the kinetics of ureolysis at various pH values followed Michaelis-Menton kinetics, but each preparation of urease had different constants. The temperature dependence of the constants was also dependent on the enzyme preparation. It was suggested that the pH dependence of the reaction was related to the ionization of histidine and the interaction of sulphydryl groups.

Pancholy and Saksena (18) found that the ultrasonic absorbtion coefficients of urea and urease alone were small but in the same solution they have a large value with a strong frequency dependence that dropped off rapidly as the frequency was increased from 4 to 40 mc.

Fishbein (7) found dihydroxyurea was hydrolyzed by urease about 100 times more slowly than urea.

Chernitskii, Konev, and Volotovskii (5) found the life time of phosphoresence of tryptophan in urease was 5.8 to 6.4 sec.

Khramov (14) found the kinetics of urease in Proteus vulgaris were similar to pure bacterial urease. Michaelis constants were calculated. Ionic strength had the same effect on bacterial and soybean urease.

Urease Dissociation

Urease dissociates into subunits. The process is effected by various conditions. In some conditions enzymatic activity is retained. This phenomenon is common to many enzymes as will be seen in the section on enzyme dissociation.

Gorin, Mamiya, and Chin (10) dissociated urease into subunits of molecular weight 50,000 to 60,000 by treatment with sodium dodecyl sulfate (SDS). The enzymatic activity was destroyed. The rate and the extent of the reaction is critically dependent on the ratio of SDS to urease. At low concentrations, 0.5 mg SDS per 1 mg urease, conversion of native urease, which has a sedimentation coefficient s of 18, to a subunit of s value 3.3 was not complete after 36 hours. With higher ratios the reaction was rapid and complete. The product had an s value of 2.0. The percent inactivation, which was not reversible, during the reaction approximately paralleled the dissappearance of the 18 s unit. Along with the dissociation, there took place an increase in viscosity, indicating a change to more asymmetric molecular shape.

Reithel and Robbins (21) produced urease subunits with a s value of 3.0 by titrating urease in phosphate buffer with HCl to pH 2.0 and dialyzing against the same. These samples varied in molecular weight depending upon the concentration of the urease present. With 0.043 percent protein the molecular weight was 322,000 while with more concentrated solutions the molecular weight decreased. If the urease was titrated with 0.1M KCl present a s value of 5.6 to 6.0 was obtained. After dialysis s values of 6 and 12 were obtained. S values of 6 and 12 corresponded to molecular weights of 157,000 and 185,000 respectively. Variations of the calculated molecular weight with the centrifuge speed indicated polydispersity.

Gorin, Chin, and Wang (9) dissociated urease with pH 3.5 acetate buffer to a subunit of one half the original molecule. The reaction was complete in one hour. Enymatic activity decreased with time to nearly zero in 24 hours. The inactivation did not correspond to the

dissociation. When assayed at pH 7.0 after 1 hour at pH 3.5 70 percent of the activity was retained. The viscosity change in one hour was low hence the molecular weight could be calculated from s values.

Tanis and Naylor (23) prepared an enzymatically active subunit of urease, s 12, by dialysis against .02 M phosphate buffer for four days or by reducing the pH to below 4.9. In the first method total conversion is not attained by extending the time period, but by the latter method the reaction is complete. The range of conversion was 0.5 pH units wide. Lowering the pH caused a decrease in the s value by an amount not explainable by ionic strength. The 12 s form, assayed at pH 4.5 - 4.8, has 5 percent of the original activity. However, the activity is totally recovered when the pH is raised from 4.0 to 7.0 in phosphate buffer. All of the activity from twice recrystallized urease at pH 7.0 is in the heavier unit when analyzed on a sucrose density gradient. If the pH is lowered to pH 4.8 the activity is also in the lighter unit. The 12 s and 18 s forms are converted to each other without loss of activity.

Blattler, Contaxis, and Reithel (2) dissociated urease using 1,2 propanediol while retaining enzymatic activity. At 90% propanediol in pH 9.2 TRIS buffer urease dissociates in a few minutes into subunits of molecular weight 236,000.

Enzyme Dissociation

Enzymes dissociate under a variety of conditions with or without loss of activity. In some cases the activity loss is irreversible.

Some useful general references are the following. Klotz (5) compiled a list of 54 papers dealing with protein subunits. Reithel (8)

wrote a comprehensive review on "The Dissociation and Association of Protein Structures." Sund (10) wrote a review on "The Quarternary Structure of Proteins." The parts of Sund's article, which are relevant to the subject of this thesis, are summarized below. The Annual Review of Biochemistry was searched from 1966 through 1968 to bring Sund's review up to date but no reviews on the subject were found.

Four major structural classifications have been proposed for proteins, primary, secondary, tertiary, and quaternary. The primary structure is the sequence of amino acids in the polypeptide chain. The secondary structure is the result of hydrogen bonding between CO and NH groups of the polypeptide chain. The tertiary structure is the spatial arrangement of the polypeptide chain due to interactions from side chains. The quaternary structure is concerned with the number and arrangement of polypeptide chains in the molecule.

Dissociation can take place into subunits consisting of several polypeptide chains or into single chains which may or may not be identical.

Denaturation, a change in the native structure of the molecule, is accompanied by a loss of biological activity. Globular enzymes can be changed to a structure which may approximate a random coil model. Denaturation can be caused by many agents which include organic solvents, salts, urea, guanidine hydrochloride, detergents, heavy metal ions, and changes in temperature or pH. The process in most cases cannot be reversed. Denaturation is often accompanied by dissociation into subunits. The number of different subunits can usually be determined by ultracentrifugation, electrophoresis, or chromatography.

Dissociation of enzymes is not always a denaturation process.

Aldolase dissociates, below pH 3, into three subunits with considerable unfolding. One hundred percent of its activity returns on dialysis or rapid dilution. The three subunits associate rapidly and activity is recovered as the molecule assumes its original compact state. Lactic dehydrogenase, in 5M guanidine hydrochloride, dissociates into four subunits. The process can be reversed with reactivation.

Glutamic dehydrogenase, molecular weight 2×10^6, dissociates spontaneously upon dilution. An equilibrium is present between the molecule and subunits of molecular weights 10^6, 5×10^5, and 2.5×10^5. All subunits are enzymatically active. A variety of substances can displace the equilibrium in either direction. Information can be obtained from viscosity measurements about the geometry of the subunit if the dissociation is accompanied by little conformational change. The intrinsic viscosity decreases with the geometrical anisotropy. The glutamic dehydrogenase subunit is about half as long as the origional molecule and has a lower intrinsic viscosity. The dissociation was probably a transverse cleavage.

Denaturing agents cause the dissociation of some enzymes directly into single polypeptide chain while others dissociate via multiple polypeptide chain subunits. In most cases polypeptide chains in enzymes are held together by non-conalent bonds. Disulfide bonds are not common. In some enzymes the number of active enters and the number of chains are the same while in others the number of chains is greater.

Enzymes dissociate into subunits with retention of activity, with reversible loss of activity, or with irreversible loss of activity.

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CHAPTER II

REVERSIBLE DISSOCIATION OF UREASE BY AQUEOUS 1,2-ETHANEDIOL

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Blattler (1) dissociated urease, M. W. 480,000, into a subunit of one half molecular weight, 240,000, in 1,2 propanediol. Several diols and triols were tested and some produce the same effect. 1,2-ethylene glycol was used for dissociation and reassociation experiments. At 33 percent and 50 percent glycol concentrations dissociation of one half of the urease will take place in 13 hours and 13 minutes, respectively with little enzymatic deactivation. These periods are not altered by changing the pH from 7 to 9. Reassociation will take place in 10 percent glycol at pH 7.

Experimental

Urease was extracted from jack beans grown by Mr. Ernest Nelson of Route 1, Waldron, Arkansas, using Sumner's procedure modified by Gorin (3).

TRIS buffer contained 10g of Tris (Hydroxymethyl)Aminomethane and 1g EDTA per liter. pH 7 buffer contained 3.130g $Na_2HPO_4 \cdot 7H_2O$, 1.150g $Na_2HPO_4 \cdot H_2O$ and 0.3723g Na_2 EDTA per liter. All chemicals were reagent

grade. All water was distilled and deionized.

Ethylene glycol was added to pH 7 and pH 9 buffers containing 25 mg of urease per ml. Reactions were run at 33 percent, 45 percent, and 50 percent glycol, by volume.

At appropriate times during the reaction, at 20°C, aliquots were diluted with pH 9 buffer to 10 percent glycol where no dissociation takes place. Aliquots were analyzed at the same time on an acrylamide gel electrophoresis apparatus described by Blattler (2), at about 16°C. The gel which contained 5g acrylamide and .250g N,N'-Methylenebisacrylamide, Eastman Organic Chemicals, per 100 ml of tris buffer, 6.0g tris and 0.6g H_4EDTA per liter, was catalyzed by 0.1g $K_2S_2O_8$ (reagent grade) and 0.050 mls of N, N, N',N'-tetramethylethylenediamine, E-C apparatus Corp. Phila., Pa. The voltage gradient on the gels was 14.5 volts/cm^2. Urease in the gel was dyed with amido black.

Ultracentrifuge analysis was done at 20°C at 420,040 r.p.m. with a 2° sector cell on a Beckman Model E analytical ultracentrifuge. Viscosity correction for $\underline{s}$ values to $S_{20,\omega}$ were done as H. K. Schachman (4). Viscosity and density values were taken from Timmermans (5).

To see it dissociation was related to enzymatic deactivation, urease in 50 percent glycol was assayed over a period of 200 minutes.

Results

Figure 1a shows an acrylamide gel pattern of a reaction done in 50 percent glycol at pH 9. Results were the same at pH 7. The half life period was about 12 minutes. A half life period of 1.75 hours was obtained for 45 percent glycol.

Figure 1b an acrylamide gel pattern of a reaction done in 33 per-

cent glycol at pH 7 and 9. The sample corresponding to 13 hours was judged as having equal amounts of urease and dissociation product. There was no difference in pH 7 and pH 9 reaction rates. Photography distorts the intensity and quantity of dye in the spots so it is not obvious from the figure that the 13 hour samples have equal amounts of 480,000 M.W. and 240,000 M.W. species.

Figure 3 shows a graph of enzymatic activity of urease vs time in a 50% glycol solution. Since it takes 12 min for one half of the urease present to dissociate in 50% glycol, at 200 min almost all should be dissociated. Very little deactivation has taken place.

The dissociation rate is greatly effected by the ethylene glycol concentration but not by pH changes of 7 to 9.

Figure 4 shows an ultracentrifuge pattern of two urease samples. One sample was dissociated in 33 percent glycol and diluted to 10 percent glycol with pH 7 buffer. The other was dissociated in 33 percent glycol and diluted to 10 percent glycol with pH 9 buffer. The one diluted with pH 7 buffer shows reassociation by having only one peak with an $\underline{s}$ value corresponding to that of urease. The sample diluted with pH 9 buffer shows two peaks with $\underline{s}$ values corresponding to that of urease and its dissociation product. The $\underline{s}$ value of the product corresponds to a one half of molecular weight of urease, assuming no change in molecular form has taken place.

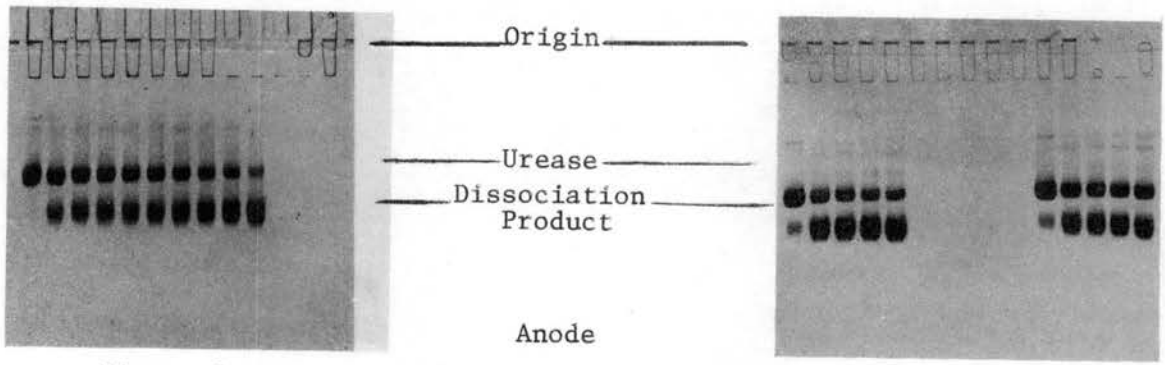

Figure 1a

Figure 1b

Figure 1c

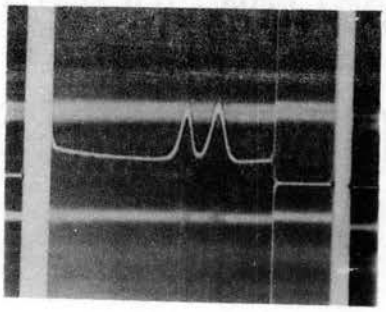

Figure 2

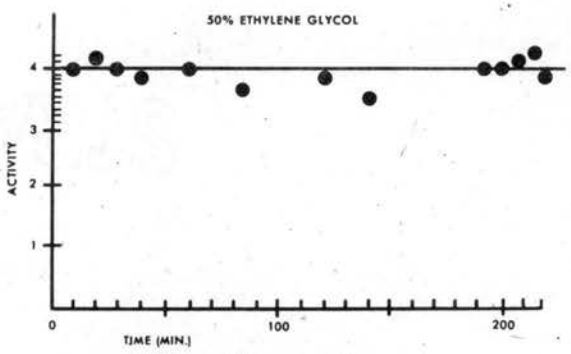

Figure 3

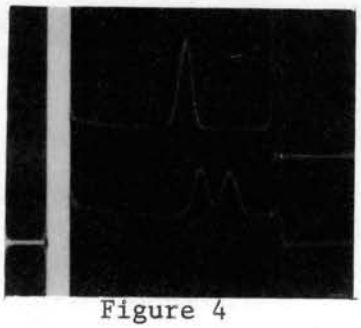

Figure 4

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CHAPTER III

EXPERIMENTAL

Buffers

TRIS buffer contained 10 g tris (hydroxymethyl)aminomethane and 1 g ethylenediaminetetraacetic acid per liter. The TRIS buffer used for electrophoresis contained 6 g tris (hydroxymethyl)aminomethane and 0.6 g ethylenediaminetetraacetic acid per liter. The pH of both TRIS buffers was about 9.0. Phosphate buffer, pH 7.0, contained 3.130 g $Na_2HPO_4 \cdot 7H_2O$, 1.150 g $NaH_2PO_4 \cdot H_2O$, and 0.3723 g disodium (ethylenedinitrilo)tetracetate dihydrate per liter. All chemicals were reagent grade. All buffers were made with deionized distilled water.

Urease Purification

Urease was extracted from jack beans by the method of Mamiya and Gorin (7) except that the extracting medium contained 10^{-3}M EDTA (0.2126 g Na_2 EDTA$\cdot 2H_2O$ and 0.163 g Na_4 EDTA) and 10^{-2}M 2-mercaptoethanol, and the solution from which the urease was recrystallized contained 10^{-3} M Na_2 EDTA and 10^{-2}M 2-mercaptoethanol. Urease dissolved in the extracting solution was centrifuged, and acetone was added to the supernatent causing the urease to crystallize. The urease was purified by recrystallization.

Urease was assayed for enzymatic activity by the method of Gorin

and Chin (4). The protein concentration was estimated spectrophotometrically from the absorbance at 278 mμ, using a specific absorbancy of 0.64. Some time after preparation I had been completed it was realized that the enzyme crystals separated by centrifugation would contain some residual acetone, which contributed appreciably to the absorbance of urease at 278 mμ. This caused the calculated specific activity to be low. In preparation II acetone was replaced with ethanol in the last recrystallization to eliminate this problem.

Preparation I

Preparation I was extracted from 1967 jack beans. Its activity was 11,908 $U_a^{25^\circ}$ per ml and contained a protein concentration of 8.72 mg per ml. The calculated specific activity 1365 $U_a^{25^\circ}$ per mg, was low because of small amounts of acetone present which absorb at the same wave length as urease.

Preparation II

Preparation II was extracted from 1968 jack beans. Its activity was 6,250 $U_a^{25^\circ}$ per ml, and contained a protein concentration of 3.4 mg per ml. Its specific activity was 1845 $U_a^{25^\circ}$ per mg.

Density Measurements

Since the dissociation of urease depends very critically on the glycol concentration, in most experiments solutions were made up by weight, even though the specifications of composition is by volume. The densities of buffer solutions, of urease solutions, and of glycol solutions were determined by weighing appropriate amounts in volu-

metric flasks. The densities of water and glycol (12) at 20°C were 0.9971 g/cm^3 and 1.1088 g/cm^3. The densities of the two buffers and urease solution are as follows:

Phosphate buffer	1.0001 g/cm^3
TRIS buffer	1.0006 g/cm^3
Urease (25 mg/ml)	1.0026 g/cm^3

From this data the densities of urease solution and buffers were taken to be 1.000 g/cm^3. Solutions at various percentages of glycol were made up by volume and their densities were as follows:

50 percent glycol	1.062 g/cm^3
33 percent glycol	1.0419 g/cm^3

These densities were used to calculate the weight of various volumes of the solutions needed when decreasing the percent glycol reaction mixtures to 10 percent for stopping the reaction.

Preparation of Urease Solutions in Aqueous Glycol

The urease suspension was centrifuged at 16,000 r.p.m. for 10 minutes in polyethylene test tubes, and the precipitate was then dissolved in an appropriate volume of buffer, usually 0.5 ml, to get a concentration of about 25 mg/ml. The solutions were allowed to stand for at least 30 minutes to insure complete dissolution of urease. The appropriate amount of urease solution was weighed into a one-dram glass shell vial. Glycol was then added, taking care to avoid prolonged localized high concentrations.

By using 100λ and 10λ disposable micropipettes small quantities of

solution can be delivered or extracted as needed. With practice, using 100λ pipettes, solution deliveries can be made within an accuracy of 1 mg. With 10λ pipettes greater accuracy can be attained. One-dram glass shell vials reduce the evaporation to less than one percent in 10 minutes. The vials are capped after weighing.

Dissociation Reaction

Appropriate quantities of ethylene glycol were mixed with urease solution prepared as described earlier. Glycol, 0.277 g for 33 percent and 0.554 g for 50 percent, was mixed with 0.500 g of urease solution. The vial was capped and kept in a 20°C water bath until an aliquot was to be taken out.

At convenient time intervals 0.0522 g of the reaction was taken out and diluted to 10 percent glycol, 0.115 g for 33 percent solution and 0.200 g for 50 percent solutions, with TRIS buffer. It was found that at 10 percent glycol and pH 9 the reaction stopped. At the end of the reaction all of the samples were analyzed by acrylamide gel electrophoresis.

For ultracentrifuge analysis the entire reaction solution was diluted to 10 percent glycol at an appropriate time.

Reassociation

Urease is dissociated in either 50 or 33 percent glycol in phosphate buffer as described previously and then reassociated by reducing the glycol concentration to 10 percent while maintaining the pH at 7.0.

Urease was allowed to dissociate in 33 percent glycol, pH 7.0, for 12 hours at room temperature. The solution was then divided into three

parts. In the first 0.2084 g of solution was mixed with 0.46 g of a 33 percent glycol solution, pH 7.0, to give a urease dilution equal to a dilution to 10 percent glycol. In the second 0.2084 g of the solution was diluted to 10 percent glycol with 0.46 g of phosphate buffer. In the third 0.2084 g of the solution was diluted to 10 percent glycol with TRIS buffer. The pH of the mixture was about 9 since the concentration of the TRIS buffer is much higher than the phosphate buffer. All three samples were analyzed by ultracentrifugation.

In a similar experiment the rate of the reassociation was found with acrylamide gel techniques. A solution of urease in 50 percent glycol phosphate buffer was made as described earlier and dissociation was allowed to proceed for three hours at which time it should be complete. Aliquots of 0.0522 g were diluted with 0.200 g of phosphate buffer to reduce the glycol concentration to 10 percent. The dilution procedure was repeated at intervals over a period at 8.5 hours keeping the aliquots in a 20°C constant temperature bath. At the end of the 8.5 hour period 0.020 ml samples of the aliquots were run on acrylamide gel. Since the electrophoresis apparatus contains TRIS buffer the samples were changed from pH 7.0 to pH 9.0 when they were put in the gel cavities.

Electrophoresis

Electrophoresis was done in an apparatus described by Blattler (1). Acrylamide gel was supported vertically by rectangular glass plates which were immersed in a temperature controlled buffer. The gel was cast so that 15 sample cavities were formed in the top edge of the gel. The current flows through the buffer on one side of the glass support

plates into the top of the gel. Then it flows through the length of the gel and out of the bottom into the buffer on the opposite side of the glass plates. Buffer was pumped from one compartment to the other to maintain a constant pH.

A 5 percent acrylamide gel contains 5 g of acrylamide and 0.25 g of bisacrylamide dissolved in 100 ml of TRIS buffer. The catalysts for the gelling were 0.05 ml of TMED and .67 ml of 10% $(NH_4)_2\ S_2O_8$ solution. The two catalysts were mixed separately with portions of the acrylamide solution and combined in a nitrogen atmosphere.

Operation

The apparatus was in operation for a 2 hour period at 250 volts with the temperature maintained at about 16°C. The amount of urease used for electrophoresis analysis was 0.01 ml of sample which was deposited in the gel cavities with 10λ disposable micropipettes. For dilute samples 20λ pipettes were used. About 0.002 g of sucrose was dissolved in the samples, ranging from 0.165 ml to 0.250 ml, to increase the density. When deposited in cavities the solution could be seen to flow to the bottom.

Staining of Gels

Since urease in the gel was clear it was stained with amido black. The gel was immersed in a solution containing 500 ml of methanol, 500 ml of water, 100 ml of glacial acetic acid, and 0.025 g amido black. The gel was left in the stain for at least twelve hours. The background dye was washed out using the same solution without amido black. The wash solution was changed every twenty-four hours until the portions

of the gel, not containing urease, were clear.

Amido Black Assay

In previous experiments the reaction half period for urease was taken to be the time corresponding to the sample which had equal intensities for urease and its dissociation product. For more accurate results a spectrophotometric method for determining the relative percent of urease to its dissociation product in acrylamide gel stained with amido black was developed from a method described by Kuno and Kihara (6).

Technique

Gels containing urease stained with amido black as previously described were cut up and each piece put in 2.5 ml of 1.0 N NaOH. The sections of the gel, about 7 x 5 mm rectangles containing the urease spots, were slowly mixed with the NaOH for 12 hours in sealed vials on an immersion rotor. The dye was then equally distributed through both gel and solution.

The supernatent was spectrophotometrically assayed at 620 mμ in a 1 ml quartz cell. The blank was a similarly made solution containing no stained urease. The optimum urease in each gel square for assay was about 6×10^{-2} mg.

Ultracentrifuge Sedimentation Measurements

Ultracentrifugations were at 20°C at 42040 revolutions per minute on a Beckman Model E analytical ultracentrifuge. S values were calculated from measurements with a Nikon profile projector. The expression

for the sedimentation coefficient, $\underline{s}$, taken from Schachman (9), is defined as follows:

$$\underline{s} = \frac{1}{\omega^2} \times \frac{dx}{dT}$$

x is the distance from the center of rotation, T is the time, and ω is the angular velocity. After integrating the above equation and converting 42040 r.p.m. to radians per second the following was obtained:

$$\log \frac{x_2}{x_1} = (T_2 - T_1) \frac{\underline{s}}{1.98 \times 10^{-9}}$$

By plotting log x against time the slope is $\underline{s}/1.98 \times 10^{-9}$.

Schachman (9) also gives the following formula for converting $\underline{s}$ values obtained at different temperatures and in solvents of different viscosities to the value at $20^{\circ}C$ that would be obtained in water, $S_{20,\omega}$:

$$S_{20,\omega} = \underline{s} \left(\frac{\eta_T}{\eta_{20}}\right)\left(\frac{\eta}{\eta_o}\right)\left(\frac{1 - \bar{v}\rho_{20,\omega}}{1 - \bar{v}\rho_T}\right)$$

$\frac{\eta}{\eta_{20}}$ is the ratio of the viscosity of water at temperature T to that at $20^{\circ}C$. η/η_o is the ratio of the viscosity of the solvent to that of water. $\bar{v}$ is the partial specific volume. $\rho_{20,\omega}$ is the density of water at $20^{\circ}C$, and ρ_T is the density of the solvent. All $\underline{s}$ values used in this thesis are corrected to $20^{\circ}C$ in H_2O.

Density and viscosity values for $20^{\circ}C$ from Timmermans (11) were plotted and interpolated for 10 and 33 percent glycol. The following results were obtained:

	Viscosity	Density	Correction Factor
10 percent	1.35	1.017	1.40
33 percent	2.55	1.049	2.96

By multiplying the s by the correction factor the $S_{20,\omega}$ value is obtained.

Molecular Weight From S Values

Schachman (9) gives an equation for calculating molecular weights when the molecule sedimenting is a rigid sphere.

$$M = \frac{4690\ (S_{20,\omega})^{3/2}\ [\eta]^{1/2}}{(1 - \bar{v}\rho)^{3/2}}$$

M is the molecular weight, η and ρ are the viscosity and density of the solvent. Since η, ρ, and $\bar{v}$ are the same for identical solvents, the equation indicates the s value of the dissociation product of urease with a m.w. of ½ urease, if it retains the shape of native urease would be equal to the s value of the urease times $(\frac{1}{2})^{2/3}$.

CHAPTER IV

Results

Behavior of Urease in 10 Percent Glycol-TRIS Buffer

The following experiment was done to show that diluting the medium to 10 percent glycol with TRIS buffer stopped the dissociation process.

Urease in 33 percent glycol and TRIS buffer was allowed to react for 33 hours. At appropriate intervals aliquots were withdrawn and diluted to 10 percent glycol with TRIS buffer. At the end of the 33-hour period the aliquots were analyzed on acrylamide gel. The aliquots were kept for 51 hours and analyzed again. The two gels were compared after staining with amido black. There was no change in the relative percent of urease to dissociation products between corresponding aliquots on the two gels.

Dissociation in 33 and 50 Percent Glycol

About 12 hours are required for one half of the urease to dissociate in 33 percent glycol in solutions containing 17 mg of urease per ml of reaction solution. In 50 percent glycol and 13 mg of urease per ml the half period was about the same.

In 33 percent glycol and TRIS buffer one experiment was done with preparation I urease. The half period was 13 hours. With preparation II urease three identical experiments were done. The half periods obtained were 13 hours, 11 hours and 19 hours.

In 33 percent glycol and phosphate buffer one experiment was done with preparation I urease and one with preparation II urease. The half periods were 13 hours and 12 hours respectively.

In 50 percent glycol and TRIS buffer one experiment with preparation I urease gave a half period of 13 minutes.

In 50 percent glycol and phosphate buffer one experiment with preparation I urease gave a half period of 10 minutes.

In 33 percent glycol in TRIS buffer two experiments were done at a reduced urease concentration of 4.2 mg per ml. The one with preparation II urease showed a half period of 16 hours. The other with a different urease sample gave a half period of 19 hours.

In one experiment at 45 percent glycol and TRIS buffer with preparation I urease the half period was 1.75 hours.

Determinations of the half period was done by a visual comparison of the relative intensities of the amido black stained urease spots in acrylamide gel. These results were verified by amido black spectrophotometric assays.

Amido Black Assay

Amido black assays were made of stained urease in gels prepared with known ratios of urease concentrations. The results give data on the accuracy of the method.

Urease solutions of volumetrically determined ratios were run on an acrylamide gel and assayed spectrophotometrically. The results are given in Table I. A similar experiment was done with a ratio of urease of 1:4:6. The absorbance divided by the ratio was 0.091,0.089, and 0.096, respectively.

TABLE I

ASSAY OF UREASE OF KNOWN CONCENTRATION

Ratio of Urease Conc.	Absorbance	Absorbance Ratio
10	0.496	0.050
10	0.537	0.054
8	0.389	0.049
8	0.434	0.054
6	0.291	0.049
6	0.314	0.052
4	0.186	0.047
2	0.091	0.045

Amido black assays were made of urease dissociated in 33 percent glycol-TRIS buffer, Figure 5, Table II; 33 percent glycol-TRIS buffer at reduced urease concentration, Figure 5, Table III; and 50 percent glycol-phosphate buffer, Figure 6, Table IV. The half periods were as predicted by the visual method.

The results of Figure 5 are different from what might be expected from having one reactant. If the reaction was of first order, the two lines would be superimposed, and the half period would be the same at both urease concentrations.

It was not known if urease absorbed as much dye per mg as its dissociation product hence when assays of reactions were done the absorbance of the urease and dissociation product from each sample were summed. The sum was about the same over a wide range of percentages of product

TABLE II

DISSOCIATION IN 33 PERCENT GLYCOL-TRIS BUFFER, AT 17 MG OF UREASE PER ML

Time in Hours	Absorption Urease	Absorption Product	Absorption Sum	Percent Urease
0.33	0.320	0.029	0.349	91.7
10	0.170	0.174	0.344	49.4
13	0.148	0.193	0.341	43.4
16	0.129	0.217	0.346	37.3
19	0.112	0.230	0.342	32.7
23	0.099	0.256	0.355	27.9
33	0.057	0.293	0.350	16.3

TABLE III

DISSOCIATION IN 33 PERCENT GLYCOL-TRIS BUFFER, AT 4.2 MG OF UREASE PER ML

Time in Min.	Absorption Urease	Absorption Product	Total Absorption	Percent Urease
0.33	0.157	0.007	0.164	95.8
10	0.108	0.059	0.167	64.7
13	0.092	0.067	0.159	57.8
16	0.083	0.076	0.159	52.2
19	0.071	0.083	0.154	46.1
23	0.063	0.098	0.161	39.1
33	0.38	0.113	0.151	25.2

TABLE IV

DISSOCIATION IN 50 PERCENT GLYCOL-PHOSPHATE BUFFER

Time in Min.	Absorption Urease	Absorption Product	Absorption Sum	Percent Urease
1	0.064	0.009	0.073	87.5
7	0.048	0.031	0.079	60.8
10	0.038	0.039	0.077	49.4
15	0.036			45.0
32	0.019	0.068	0.087	21.8
46	0.011	0.069	0.080	13.8
60	0.007	0.072	0.079	8.4

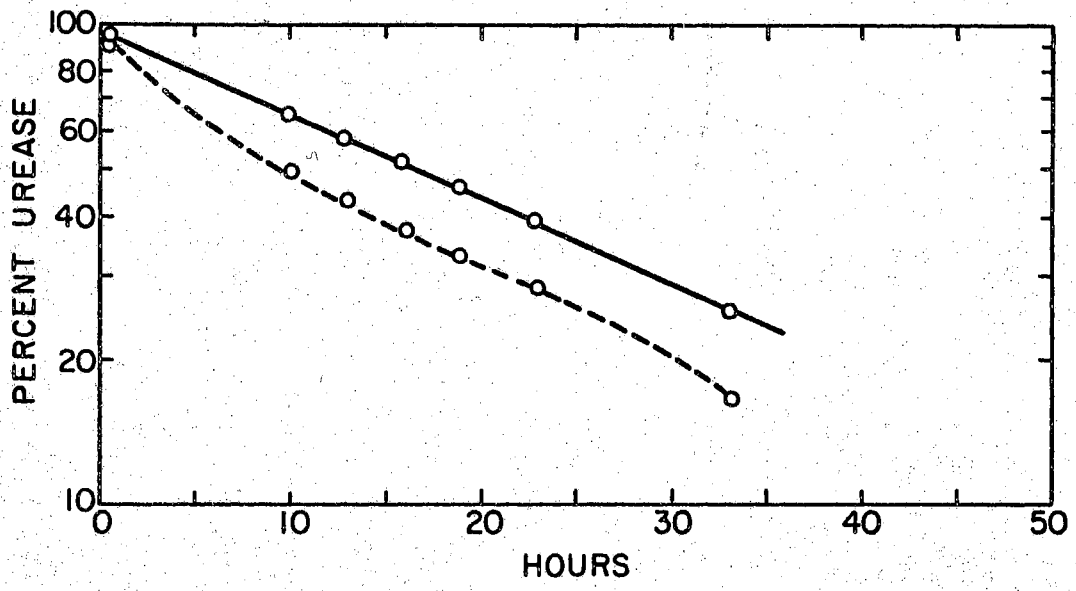

Figure 5. Dissociation in 33 Percent Glycol-TRIS Buffer; Solid Line 4.2 Mg Urease Per Ml Dashed Line 17 Mg Urease Per Ml.

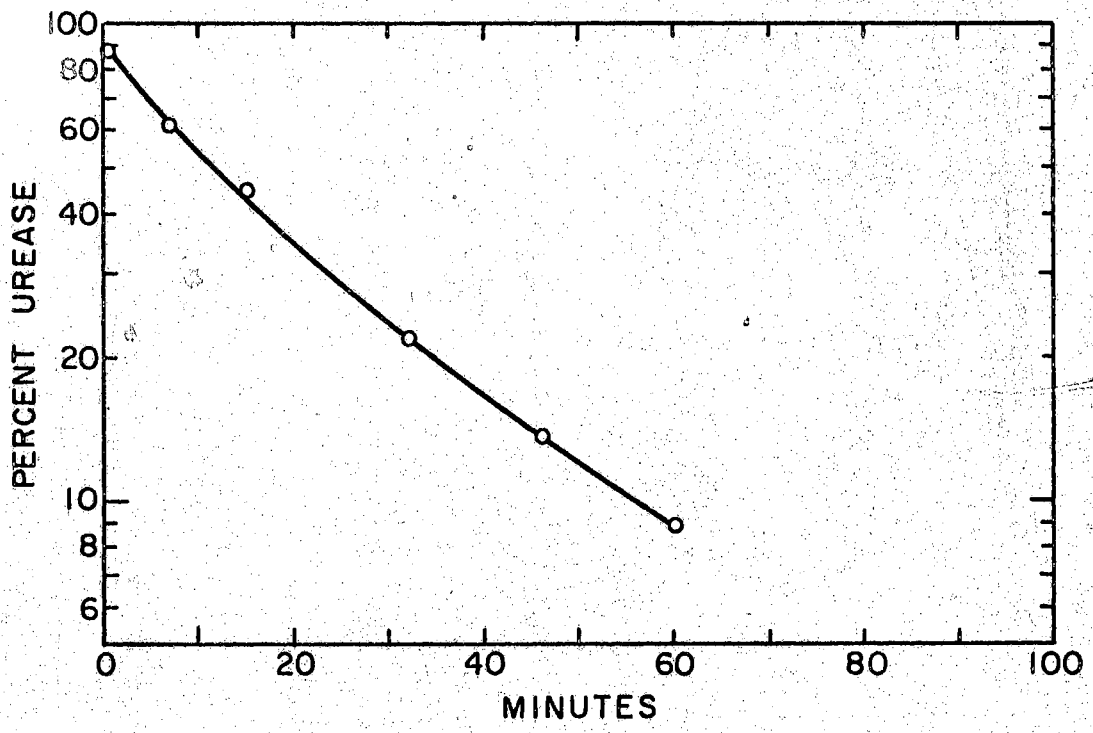

Figure 6. Dissociation in 50 Percent Glycol-Phosphate Buffer.

and reactant indicating dissociation did not change the ability of urease to attach to amido black.

Activity During Dissociation

The dissociation of urease does not alter its activity as seen in Chapter II in 50 percent glycol. The activity during dissociation in 33 percent glycol was followed and found not to decrease during the reaction.

A reaction mixture was made as described earlier in 33 percent glycol. The urease was assayed for enzymatic activity before and after the glycol was added. The method was that of Gorin and Chin (4) as described in the literature search.

The assay showed no decrease in activity during or after the glycol was added. The ratio of the activities before the reaction, 5 minutes after, 1 hour, 5 hours, 19 hours, and 31 hours were 1.00, 1.04, 1.01, 1.00, 1.01, and .95 respectively. The listed values are an average of three assays at each time interval when 0.050 ml of the reaction was diluted into 50.0 ml of phosphate buffer.

The activity of the urease before the glycol was added was multiplied by two thirds to adjust for dilution when the glycol was added. A 33 percent glycol solution has 99 percent of the volume of the separate glycol and buffer components, hence no correction factor was needed.

Reassociation Analyzed by Ultracentrifugation

Urease in 33 percent glycol phosphate buffer was divided into three parts after 12 hours. The first and second aliquots were diluted

to 10 percent glycol with TRIS and phosphate buffers. They were centrifuged 30 hours later. The one diluted with TRIS showed two peaks which had s values of 18.0 and 11.8 corresponding to urease and its dissociation product. The one diluted with phosphate buffer had only one peak with a s value of 19.2 corresponding to urease. The third sample was diluted by the same volume using a pH 9 33 percent glycol-TRIS solution. This sample showed two peaks. Reassociation had taken place in phosphate buffer. The result is shown in Figure 4.

Reassociation Analyzed by Electrophoresis

Urease was dissociated in 50 percent glycol-phosphate buffer for three hours at which time it should be complete. Aliquots during a period of 8.5 hours were diluted to 10 percent glycol with phosphate buffer. All aliquots were analyzed at the same time on an acrylamide gel. The half period for the reassociation was about 2.25 hours. Table V contains data from an amido black assay of this experiment. Figure 7 is a graph of the inverse of the percent dissociation products vs time which should be linear for a second order reaction.

Dimerization of Urease

The conditions which produced the urease dimer were investigated. It was observed that urease prepared by the method of Mamiya and Gorin (7) contained a band which moved more slowly than urease under electrophoresis. This band was thought to be the urease dimer observed by Creeth and Nichol (3) which showed an s value corresponding to a molecular weight twice that of native urease. It was found that urease in 0.01M pH 7.0 phosphate buffer at room temperature, when exposed to the

TABLE V

REASOOCIATION IN 10 PERCENT GLYCOL AND PHOSPHATE BUFFER

Time in Hours	Absorption Urease	Absorption Product	Absorption Sum	Percent Product	10^{-2} / Percent Product
0	0.023	0.191	0.214	89.3	1.12
0.25	0.042	0.173	0.215	80.5	1.24
0.5	0.069	0.155	0.224	70	1.43
1.0	0.078	0.169	0.247	68.5	1.46
1.5	0.094	0.140	0.234	59.8	1.67
2.5	0.115	0.092	0.207	45	2.22
3.5	0.152	0.072	0.231	34.3	2.92
4.5	0.155	0.075	0.227	31.7	3.15
6.5	0.215	0.062	0.277	22.3	4.48
8.5	0.204	0.046	0.250	18.3	5.47

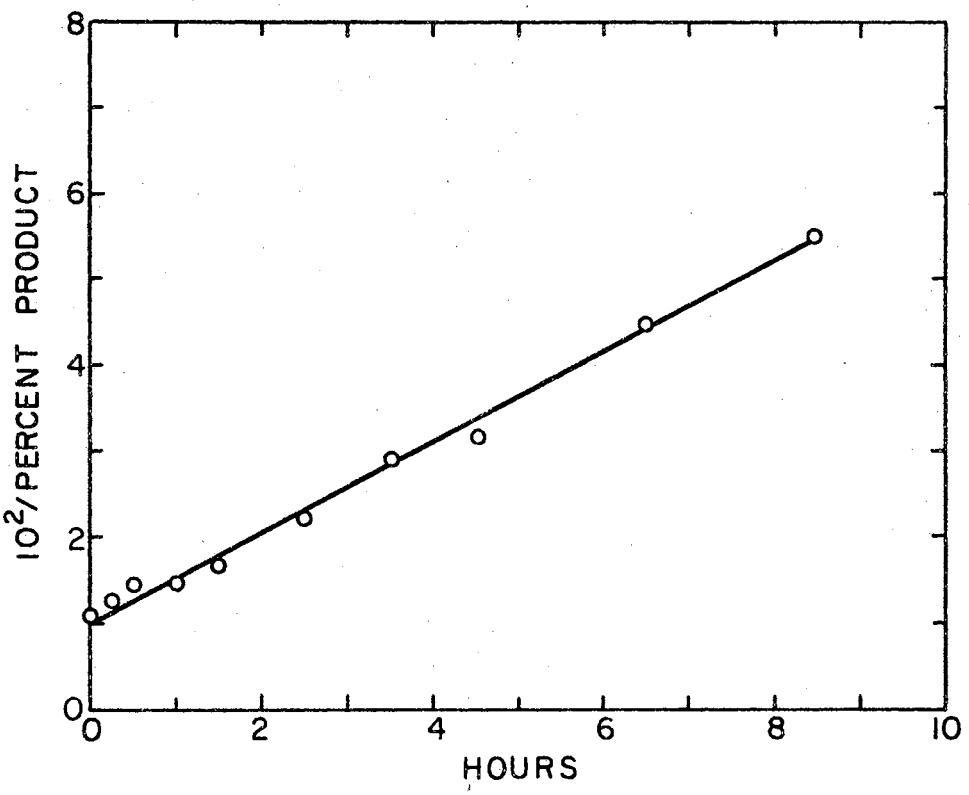

Figure 7. Reassociation in 10 Percent Glycol-Phosphate Buffer.

air for 48 hours in a thin layer of 3 to 4 mm, would produce thirty to forty percent dimer, as shown on acrylamide gel electrophoresis, with no loss in enzymatic activity. An appreciable increase in the dimer concentration could not be produced by exposing it longer.

Exposure of urease to several oxidizing agents did not increase the dimer. It was thought that the dimerization process involved oxidation or reduction which could be accelerated by adding an oxidizing or reducing agent. To a solution of 0.02M phosphate at pH 7.0 containing 8 mg per ml of urease was added 10^{-4}M and 10^{-3}M 2-mercaptoethanol, 10^{-3}M and 10^{-4}M dithiothreitol, 10^{-3}M H_2O_2, 10^{-4}M $K_3Fe(CN)6$, and 10^{-4}M KIO_3. None gave a method for producing the dimer. It was thought that dimerization was caused by trace heavy metals in the chemicals used. Urease was exposed to 10^{-1}M Fe^{+3}, Fe^{+2}, Mn^{+2}, and Cu^{+2} ions with no increase in dimer.

An attempt was made to separate the dimer from other components. Aged samples of urease in pH 7.0, 0.01M phosphate buffer were separated by acrylamide gel electrophoresis. The dimer band was cut out and pulverized by forcing the gel through a syringe without the needle. This was soaked for 24 hours in 0.01M phosphate buffer containing EDTA then analyzed by gel electrophoresis. This showed small amounts of the dimer had been extracted. The monomer and dimer failed to separate using the preparative ultracentrifuge with a sucrose density gradient. The fractions were analyzed by electrophoresis and showed the monomer and dimer had migrated at the same speed.

Conclusion

Urease dissociates into subunits of one half the original molecu-

lar weight in the presence of 1,2-ethylene glycol. The dissociation rate is not affected by a change of pH from 7 to 9. The rate is critically dependent on the percent glycol with a half period of 12 hours for 33 percent glycol and 12 minutes for 50 percent glycol. Enzymatic activity is retained during the dissociation process.

Reassociation takes place in 10 percent glycol in pH 7.0 phosphate buffer but not in pH 9.0, TRIS buffer. The half period for the reassociation in 10 percent glycol-phosphate buffer is 2.25 hours.

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