STUDIES ON UREASE

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INTRODUCTION

Urease, the enzyme that catalyzes the hydrolysis of urea, is one of the most specific and active enzymes. It is rather easily prepared and may be obtained in a crystalline form of high purity. It is not very stable, but when handled in the proper way it is no worse than many other enzymes. Urease is an SH enzyme; mercapto groups may be a part of the active sties, be involved in the process of association and dissociation, and take part in several other reactions.

The work described in this thesis is part of a comprehensive study of the enzyme, which was begun several years ago. Five papers on this general subject have been contributed by investigators in the Chemistry Department of the Oklahoma State University. This thesis comprises five additional papers on the subject. One of these has been published and two are in press. The other two have been prepared in a form suitable for publication.

Chapter II of this thesis presents the Literature Survey, which covers almost all the articles about urease published since the 19th century up to August, 1966, except those dealing with biological action. Chapter III concerns the mercapto groups that are responsible for the enzymatic activity of urease; it reproduces the article that has been published (Biochimica et Biophysica Acta 99, 418-426 (1965)). Chapter IV and Chapter V present two related papers, that describe a novel

method of assaying urease, compare it with other methods, and report on the specific enzymatic activity; these papers are in course of publication in Analytical Biochemistry. Chapter VI and Chapter VII discuss the dissociation of urease by sodium dodecyl sulfate, and in acetate buffer of pH 3.5. Finally, Chapter VIII reports some fragmentary results and some additional details that were not included in the papers.

The bibliography for each chapter is given at the end of the chapter.

CHAPTER II

LITERATURE SURVEY

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Introduction

A search has been made of the literature dealing with urease up to August, 1966; more than 500 papers have been found. Those dealing with the enzyme action *in vivo* and the occurrence of urease in various species of microorganism will not be considered in detail; this leaves 265 papers that are cited in this survey. Two review articles written by Sumner (211,228) and one by Varner (248) give good coverage of most aspects of the subject up to 1959; a review by Laidler (114) in 1954 reviews the chemical kinetics of urease action. The subjects covered in these reviews are treated briefly, but a complete bibliography is given.

1. Important events in the history of urease

In 1860 Pasteur (161) recognized that some yeasts were responsible for the ammoniacal fermentation of the urea in urine. In 1876 Musculus (142) began to do experiments with urease solutions he had prepared from bacteria. In 1890 Miquel (141) reported the occurrence of urease in many organisms and studied some properties of this enzyme. In 1909, Takeuchi (231) found that the soybean was a source of urease; a more systematic study of soybean urease was then conducted by Van Slyke and his collaborators (246). In 1916 Mateer and Marshall (138) discovered that jack beans contained sixteen times more urease than the soybean.

Sumner started to study urease in 1917 (209,215) and isolated the first crystals of urease in May, 1926 (205). Many biochemists doubted that he had truly isolated an enzyme in the crystalline state, and in the succeeding four years Sumner spent considerable time demonstrating

his experiments before several research groups, both domestic and foreign. Other enzymes were then obtained in crystalline form (209), and Sumner's ideas gradually gained general acceptance. Sumner, Northrop and Stanley shared the 1946 Nobel prize in chemistry for the isolation of crystalline enzymes and of tobacco mosaic virus. Sumner and his coworkers have published more than 27 articles on urease; most of their results have been confirmed by subsequent work.

Sumner and Poland's demonstration that urease contained mercapto groups (227) was one of the first pieces of information concerning the chemistry of this enzyme. Hellerman and his associates (65) showed in 1943 that the mercapto groups in urease might be divided into three categories of different reactivity. In the period 1949-1956 Kistiakowsky, Laidler and their coworkers conducted a comprehensive investigation of the chemical kinetics of urease action and of its inactivation.

At present, there are about ten research groups engaged in studying this very specific and active enzyme. As yet, not so much is known about urease, and some exciting developments may be expected in the near future.

2. Classification of urease

Urease is a trivial name. Since the enzyme catalyzes the hydrolysis of urea, the systematic name recommended by the International Union of Biochemistry is urea amidohydrolase (75). It has been given the Commission Number EC 3.5.1.5. The first figure of the Commission Number, 3, shows to which of the six main divisions the enzyme belong, i.e. hydrolases; the second figure, 5, indicates the sub-class, the type of bond hydrolyzed, C-N bonds other than peptide bonds; the third

figure, 1, indicates the sub-sub-class, the type of substrate hydrolyzed, linear amides; and the fourth figure, 5, is the serial number of the enzyme in its sub-sub-class.

3. The unit of enzyme activity

Sumner and Graham (214) defined the urease unit as that amount of enzyme which would produce 1 mg of ammonia nitrogen from urea (at pH 7.0) in 5 min at 20°. This unit is still commonly used today. In 1961 the International Union of Biochemistry recommended that the enzyme activity unit be defined as the quantity of enzyme which catalyzes the decomposition of one microequivalent of the bond involved in the reaction in 1 min. Chapter IV of this thesis describes a new method for assaying urease and defines the activity unit in accordance with this recommendation.

Since the enzyme unit can be defined arbitrarily, some authors have used other definitions of the activity unit (149,244,245).

4. Urease in the metabolism cycle

Although the role of urease in metabolism is still quite unclear, it probably is an important link in the nitrogen cycle of nature (228). It is assumed that urease adsorbed on soil colloids is responsible for the hydrolysis of urea in soils (19). In the soybean, urease and arginine concentrations vary in almost parallel fashion during germination, suggesting that urease is involved in arginine metabolism (229). Mycobacterium smegmatis and M. phlei, which contain an enzyme capable of hydrolyzing guanidine derivatives to urea, also have a high urease activity; it can reasonably be presumed that the urease in bacteria

allows them to utilize the urea in animal wastes as a source of ammonia (265). A soil microorganism degrades alloxan or alloxanic acid to glyoxalic acid and urea; the action of urease then supplies nitrogen for growth (55).

Occurrence and Isolation of Urease

1. Occurrence of urease in sources other than jack bean

Jack bean is the source of most urease used for chemical studies, but urease also occurs in microorganisms, plants and animals.

Miquel (141) in 1890 demonstrated the occurrence of urease in many species of microorganisms. Bacillus pasteurii was reported to contain as much as 1% of its dry weight of urease (121). Sakaguchi and Shizume (177) found urease in several species of yeast. Abadie (1) has made a detailed study of the urease in yeast and yeast-like organism. A review that emphasizes the urease activity of pathogenic bacteria and protozoa has been given by Seneca et al. in 1962 (183). Jefferies (86) investigated 26 species of bacteria and showed that bacterial urease does not lose activity upon rupture of the cell.

The first practical source of urease was found by Takeuchi (231) to be the soybean, which contains about 0.012% urease (138). The dehusked seeds of Cajanus cajan were used by Nath and Pradhan (145,146) of India to prepare their crystalline urease. Damodaran and Sivaramakrishnan (21) have listed the contents of urease in jack bean, squash, watermelon seed, snake gourd, soybean, colocynth, horsegram, bitter gourd and white gourd. The distribution of urease in plant seeds has also been reported by Tai (230) and by Manzanille (136).

Reports of the occurrence of urease in lower animals have been made since 1930 (129,165,176). In 1944 Weil (255) declared that the erythrocytes of rat, rabbit, and man, and also the spleen and liver of rat are able to decompose urea. The existence of urease in the tubular gastric glands and in the subcellular fractions of stomach were reported later (72,126,127). The occurrence of urease in the gastric mucosa has been questioned by many investigators (28,107,108,122), who suppose the activity to be of bacterial origin.

The urease content of microorganisms and plants may change during growth. The urease elaborated by Micrococcus ureae and Proteus vulgaris is mainly formed during aging and disintegration of the cells (131). In the cotyledons of Citrullus, an initial rise of urease during growth is followed by an abrupt drop (258).

The urease derived from different sources may have different properties. Kornberg and Davies (107) showed that bacterial urease has electrophoretic and immunologic properties different from plant urease; Nikoloff (153-156) confirmed this conclusion. Arora and Guha (6) claimed that the urease from Mycobacterium tuberculosis was more resistant to high temperature and stable at -10°.

2. Preparation of non-crystalline urease

In 1914 Van Slyke and Cullen (246) obtained a solid urease preparation by extracting soybean meal with water and then pouring it into 10 volumes of acetone. The precipitate was then dried in vacuum, pulverized and kept in the dry powder state. Jacoby and Sugga (83) prepared a dry urease powder from soybean by a similar procedure in 1915. Later, Reveltella (173) modified this procedure. Kay and Reid

(91) in 1934 described a method of preparing dry urease powder from jack beans. Archibald and Hamilton (4) in 1943 used the Van Slyke - Cullen method to prepare urease but purified the product by dialysis to remove canavanine.

Ramirez and Monge (170) used glycerol and phosphate buffer to extract urease from watermelon seeds, evaporated the extract and kept it as a syrup.

3. Isolation of crystalline urease by Sumner's method

Sumner worked for 9 years to develop a method of isolating urease (209). Many solvents were used for extracting and every mean of concentrating enzymes known at that time was tried. The main problem was to extract more urease from the jack bean meal and less of other proteins. At last, Sumner succeeded by using 32% acetone.

The isolation process of Sumner (205) involves stirring 100 g of jack bean meal with 500 ml of 31.6% acetone and filtering by gravity in an ice chest at 3-6°. After standing overnight urease crystals separate from the filtrate and can be removed by centrifuging. The process is really simple, but obtaining a good yield of crystalline urease will depend, firstly, upon having a meal rich in extractable urease, and secondly, upon careful avoidance of contamination by heavy metals.

For keeping the extractable urease in the active state, heating and the introduction of heavy metals should be avoided in the isolation process. Kirk and Sumner (93) recommended grinding the beans first in a coffee mill, and then in a pulverizer made of stainless steel at least in the grinding parts. In 1927 Sumner (207) observed that urease

was inactivated by the heavy metals in common distilled water, therefore the water should be carefully distilled in an all-glass still.

Even when these precautions are observed, some jack bean meals fail to yield crystalline urease. Sumner and Hand (218) reported in 1928 that jack bean meal containing 175 S.U./g gave a good yield of urease, but meal containing less than 91 S.U. gave no crystals. In the same year, Sumner and Holloway (222) encountered two samples of jack beans which contained the requisite amount of urease but which produced no crystals. The urease in these two samples could be extracted with water but difficulty with 32% acetone or 30% alcohol. The failure of some famous biochemists to obtain crystalline urease by Sumner's procedure, which led them to deny his great success (36,81,82,209), might be due to their use of such unsatisfactory beans.

In 1937 Kitagawa and Fujii (100) reported that no crystalline urease could be obtained by Sumner's method from Japanese jack bean. Later Kitagawa and Hunatzu (101) claimed that jack bean contained two kinds of urease, one of which is non-crystallizable and only slightly soluble in dilute acetone.

Sumner's method has been modified by later investigators in order to increase the yield and purity. A typical example is the method devised by Gorin's group in 1960 and 1962 (49,50), and then modified further by Mamiya and Gorin in 1965 (135). In these papers every step is described in detail and the conditions are specified clearly. Mamiya and Gorin (135) also found that addition of 2-mercaptoethanol to the 32% acetone could increase the yield especially from "bad" or old meal. At a much earlier time, Sumner and Holloway (222) have suggested the use of acetic acid in treating low-yield meals, but the improvement

was limited.

Another modification was used by Uehara and Kobashi (242) in 1959; this consisted of adding 17% more acetone to the extract of 32% acetone; cysteine or glutathione were used in the process. The yield was reported to be 51.8%

4. Purification of crystalline urease

Sumner developed a method of recrystallization (206), but Dounce's procedure (30) is more satisfactory. It consists of dissolving the crystals from each 100 g of meal in 3 ml of distilled water, centrifuging or filtering, adding 5% by volume of 0.5M citrate buffer at pH 6.0 and 20% volume of ice-cold acetone with rapid stirring. The crystallization is complete after 30 min and can be repeated.

In 1959 Sophianopoulos (201) claimed that the ease with which urease was denatured made repeated crystallization unprofitable as a method of purification. Knappen and Krampitz (102) purified urease by chromatography on a 1 x 50 cm column of CAM powder. Shadaksharaswamy and Hill (185) reported that fractionation of urease on carboxymethylcellulose was preferable to repeated crystallization. The total recovery of urease was said to be around 90%.

5. Other methods of isolating crystalline urease

For the meal of jack beans grown in Japan, an alternative isolating method was described by Hanabusa (57,58,59). The principal steps included water extraction, fractional $(NH_4)_2SO_4$ precipitation, adsorption on calcium phosphate gel and elution with phosphate buffer; a second fractional precipitation with $(NH_4)_2SO_4$ and dialysis; acetone

precipitation, solubilization of the insoluble precipitate by 2-mercapto-ethanol-containing phosphate buffer; finally, crystallization with acetone and recrystallization according to Dounce. This method is much more laborious than Sumner's method, but it was reported to be suitable for jack bean meals of low activity and to give a yield of 25%.

With a procedure similar to that of Sumner and Dounce, Nath and Pradhan (145,146,150) isolated urease from the dehusked seeds of Cajanus cajan; 40% ethanol was used for the extraction and after 24 hr, ethanol or acetone was added to the supernatant to cause crystallization.

In 1966 Stewart and Craig (202) obtained a low molecular weight urease of 8.5s in high yield by adding NaCl and polyethyleneglycol-400 to a water extract of jack-bean meal, dialyzing the resultant precipitate in Tris-sulfate buffer, and chromatographing the supernatant on DEAE-cellulose. 0.1% 2-mercaptoethanol was used in this process.

6. Storage of urease

Urease preparations may lose their activity rather rapidly and the factors that influence this process are as yet not completely understood. For this reason, it may be best to prepare the enzyme from jack bean meal shortly before use.

Various means of improving the preservation of enzyme activity have been described. In 1944, Kingsley (92) prepared the urease solution in saturated salt and stated that this could be kept for several weeks. Kistiakowsky et al. (95) stored the urease in 50% glycerol at 2^{o} and claimed that only 10% of the specific activity was lost in 4 months. In the present work, the most satisfactory method of storage was to keep the crystals in the citrate-acetone mother liquor (see

Chapter IV). Raices (169) reported that urease extracted with permutite in slight acidic solution and then shaked with glycerol could be stored for 2 years and 8 months. Riesel and Katchalski (174) first prepared a water insoluble urease by reacting it with p-chloromercuribenzoate; it was reactivated by cysteine before use. They reported that this preparation retained most of its activity even after 5 months. Durand (31) fixed urease on betonite in acid medium and released it into the solution by increasing the pH.

Physico-chemical Properties of Urease

1. Molecular weight, size and shape

The molecular weight of crystalline native urease was found by Sumner, Gralén and Eriksson-Quensel to be 473,000 (216) or 483,000 (217). This value was calculated from the sedimentation coefficient at 20°, $\underline{s}_{20,\underline{w}}$, 18.6 x 10^{-13} cm sec^{-1} $dyne^{-1}$; the diffusion coefficient at 20°, $\underline{D}_{20}$, 3.46 x 10^{-7} cm^2 sec^{-1}; and the partial specific volume, $\underline{\bar{V}}$, 0.73 ml g^{-1}. By the sedimentation equilibrium method, Reithel and Robbins (171) found the value to be 500,000. An early determination by the osmotic pressure method indicated an average molecular weight of 700,000 (61).

From electron microscopy studies (243), it has been estimated that urease has an average volume of 0.59 x 10^6 $Å^3$ with an axial ratio, 3.2 and the longer diameter, 104 Å.

From the x-ray pattern of crystalline urease (44), it was concluded that the molecule consists of folded chains of amino acid residue.

Chapter VI and VII of this thesis describe molecular weight

determinations based on the sedimentation coefficient and intrinsic viscosity.

2. Isoelectric point

Urease is moderately soluble in water and precipitates at its isoelectric point. The isoelectric point of urease was determined by Sumner and Hand (219) to be 5.0-5.1 in acetate buffer using the minimal solubility method. A value of 5.0-5.1 was also obtained by Gorter and Maaskant (52) using the technique of surface films, by Wills and Wormall (261) from measurement of the inhibition with suramin, and by Creeth and Nichol (20) from electrophoretic-mobility data at pH 3.5-9.0.

3. Absorption spectrum

Kubowitz and Haas (110) found that solutions of crystalline urease have the same absorption spectrum as simple proteins and observed that the absorption spectrum in the ultraviolet coincided with the destruction spectrum. Ito (78) in 1936 also observed this phenomenon. According to the studies of Landen (117), the molar absorbancy coefficient is 798,000 at about 270 mμ. A quantitative reinvestigation of this property is described in Chapter IV.

4. Radiation sensitivity

Tauber (236) found that urease was inactivated by ultraviolet light. The quantum yield as determined by Landen (117) is 0.008 molecules/quantum from 313 to 254 mμ, increasing at shorter wavelength. The inactivation is irreversible (162). By means of ultracentrifuge studies, McLaren et al. (139) showed that the decrease in activity of the

irradiated solutions was about equal to the decrease in concentration of the main component. Successive applications of heat and U.V. and vice versa to dry urease produced no partially damaged molecules (118). According to de Bornier et al. (25), exposure of aqueous solutions of urease to U.V. radiation causes a transitory increase of activity and a decrease of the Michaelis constant; it was suggested that the effect was due to unmasking of the active sites in the molecules.

Urease is inactivated by irradiation with x-rays in dilute aqueous solutions. Its sensitivity depends upon the purity and concentration of enzyme (240). Lewis et al. (123,124) found an inactivation yield of 0.042 molecules/100 ev. It was concluded that the inactivation is mainly the result of oxidation of the SH groups by free radicals. Added amino acids protected the enzyme; cysteine and methionine were the most, alanine and tryptophan the least, effective. Addition of EDTA and blocking the SH groups afforded protection. Other proteins also were effective. The inactivation-dose relationship was found to be exponential by Gorin et al. (51); their value of the inactivation yield is 0.016 molecules/100 ev.

The inactivation of urease by γ-rays has also been investigated. The degree of inactivation increases both with increasing purity of enzyme and with water content, and it is decreased by SH compounds (235). In aqueous suspension, the inactivation is by indirect action to the radiation products and thus occurs exponentially to the dose of γ-rays (63,233); a linear relation obtains, however, between the γ-ray dose and the decrease in SH groups (234). The viscosity changes in irradiated urease solution are interpreted by Dickens and Shapiro (27) as indicating intramolecular rearrangement.

Urease is about five times more sensitive to neutron irradiation than to x-rays (33). From the inactivation by deuterons, the molecular weight per active site was determined by Setlow (184) to be about 100,000.

Urease is slowly inactivated by tritiated water; 34% inactivation was attained after 6 hr (7). The activity could be restored by SH compounds.

5. Chemical composition

The fact that trypsin and pepsin digest crude as well as crystalline urease (213,224,225,236,237,264) was used by Sumner and by other investigators as evidence that urease was a protein. Some contrary results were also reported (249).

Urease is a protein of the globular type. Sumner reports, for twice crystallized urease, C, 51.6%, H, 7.1%, N, 16.0%, S, 1.2% (208). The nitrogen content for four-time-crystallized urease was taken to be $15.8 \pm 0.2\%$ (50). A sulfur content of 1.2% was also reported by Gorin et al. (50).

The amino acid composition of urease has been determined qualitatively by paper chromatography (60,160,254). Quantitative analysis by column chromatography in an automatic amino acid analyzer (171) has given these results: Lys_{218}, His_{107}, Arg_{166}, Asp_{451}, Thr_{284}, Ser_{222}, Glu_{381}, Pro_{188}, Gly_{371}, Ala_{369}, $(CySH/2)_{85}$, Val_{267}, Met_{114}, $Ileu_{338}$, Leu_{315}, Tyr_{99}, Phe_{105}, $(NH_3)_{519}$, Try_{46}.

Oki (158) has found the N-terminal residue of urease to be phenylalanine and the C-terminal residue serine. He claimed that there are six such end groups per molecule of urease.

6. Mercapto groups in urease

The presence of mercapto groups in the urease molecule was first demonstrated by Sumner and Poland (227) in 1933 by the nitroprusside test. Smythe (200) in 1936 observed that the inactivation of urease with iodoacetamide was greater than the iodoacetate, and concluded that reaction with SH groups was involved. Three years later, Hellerman (64) found that the SH groups of crystalline urease could be oxidized with prophyrindin, giving a product which no longer gave nitroprusside test, but still possessed its original urease activity. Addition of yet more porphyrindin, however, caused inactivation. In 1943 Hellerman and his co-workers (65) made the results more quantitative. They concluded that urease contained three categories of mercapto groups: (a) 22-23 groups that react readily with p-chloromercuribenzoate, porphyrindin or o-iodosobenzoate without affecting the enzyme activity; (b) 22-23 groups that react with additional p-chloromercuribenzoate with the concomitant loss of enzyme activity; and (c) some 60 more groups that react with o-iodosobenzoate in concentrated guanidine hydrochloride.

Desnuelle and Rovery (26) showed that phenylisocyanate also reacted readily with 22-23 groups per mole of urease without loss of enzyme activity. However, when about half of the next 22-23 less reactive SH groups had reacted, the enzyme activity was lost completely.

Gorin et al. (50) found that 47 moles of N-ethylmaleimide (NEM) or p-chloromercuribenzoate reacted with urease in 4M guanidine hydrochloride, and this value is in good agreement with the number of the first two categories of SH groups; however, these reagents gave no evidence for the presence of the c-category groups. With ferricyanide

a somewhat higher titer was obtained, but this may be due to occurrence of oxidation beyond the disulfide state. Hill and Elliott (68) reported that their most active urease fraction separated on CM-cellulose contained 41 SH groups per mole, but this determination was made in sulfite-containing buffer.

Further studies of the reactivity of mercapto groups in urease have been conducted as part of this thesis. They are described in Chapter III.

7. Polymer, subunits and the role of mercapto groups

From ultrafiltration studies, Garbar and Riegert (46) showed, in 1935, that the particles of urease were of different dimensions or in different states of aggregation. From ultracentrifuge studies, Kuff et al. (111) showed that only about half of crude jack bean urease sedimented with the same velocity as crystalline urease; the remaining activity was divided among three faster discrete components. Creeth and Nichol (20) have obtained, in addition to the 19s component corresponding to the native monomer form, two other components of 28s and 36s, which they supposed to be the dimer and trimer of the 19s component. Similar results were obtained by means of dextran gels column (194), sucrose density gradient (181) and CM-cellulose chromatography (68). A 12s component also has been found (20,181), which is catalytically active. Sehgal et al. (181) believe this to be the "monomeric" form of the enzyme.

When treated with 6M guanidine hydrochloride urease of 483,000 M.W. dissociates into 6 subunits of 83,000 M.W., but the activity is lost (172). On the basis of some diffusion studies, Hand (61) claimed

in 1939 that a still smaller active subunit existed, with weight of 17,000, but this result has not been confirmed by any later investigator.

Creeth and Nichol (20) concluded that the polymerization to 28s and faster-sedimenting forms probably resulted from intermolecular disulfide interchange. Sulfite reversed the polymerization (20); in the presence of sulfite, only a single symmetrical peak of 19s appeared in the ultracentrifuge pattern (152). The same result was obtained with 2-mercaptoethanol (135).

An insoluble form of urease has been reported, which is probably a polymer. The existence of an insoluble form which possesses urease activity was first observed by Jacoby (80). Sumner and Graham (214) in 1925 obtained insoluble but active urease from the crude solution by adding 30% ethanol and NaCl. After his success in crystallizing urease, Sumner (210) found that the crystals could be converted to an insoluble form, which gave a strong nitroprusside test and was rather resistant to digestion by trypsin at pH 7.0 and 30°. The insoluble form of urease also exists in natural sources. The preparation of urease from such sources requires some special technique (242).

Inactivation and Reactivation

A large number of investigations have been made in which urease preparations of varying degrees of purity were subjected to various treatments and the effect on the enzyme activity was determined. Many of these studies were conducted before very much was known about enzymes and the nature of enzyme action and may not be very useful in the light of present knowledge. Nevertheless, they will be reviewed briefly in

this section.

1. Enzymatic activity and oxidation-reduction

Urease is most active in a mildly reducing medium. It is inactivated by oxidation and also strong reduction. Sizer and Tytell (198) investigated the activity of crystalline urease as a function of the oxidation-reduction potential. They found that the maximum activity was obtained when E_η = -150 mV.

The oxidation of mercapto groups to disulfide bond may cause urease to become polymerized and inactivated. Hellerman et al. (66) thought that the reaction is reversible, but from the preceding section it is known that the oxidation is reversed only by some reducing agents. The oxidation can be effected by oxygen and other oxidizing agents, such as naphthoquinone, I_2, Cu_2O (5) H_2O_2, O_3 (238), NO_3^- (89), and some oxidized products of diamines (35,164).

2. Inactivation by metal ions

Soon after Sumner succeeded in isolating crystalline urease, he demonstrated that urease was inactivated by certain heavy metals in the distilled water (207). In 1930, Sumner and Myrbäck (226) reported that about 5 Ag^+ ions would cause 50% inhibition and that reactivation could be effected by hydrogen sulfide. Then, in 1951 Ambrose et al. (3) came to the conclusion that only 3-4 Ag^+ ions would suffice for complete inhibition. These values are in marked contrast to the comparatively large number of mercapto groups present in the urease molecule. In the same year, Fasman and Niemann (38) claimed that Na^+ and K^+ ions in the phosphate buffer also inhibit urease, while phosphate

ions activate it. Kistiakowsky et al. (95) suggested that the inhibition might be exerted by complexes of alkali metal and phosphate ions.

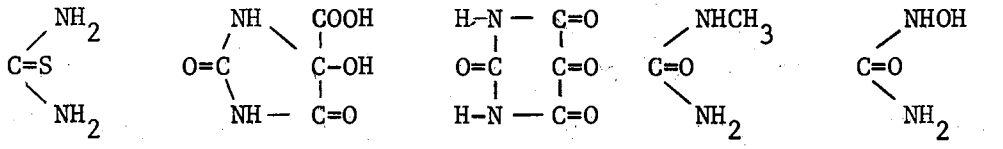
The activity of metal ions in the inactivation of urease has been shown by Shaw (187) and by Shaw and Raval (191) to decrease in the following order: $Ag^{+} > Hg^{++} > Cu^{++} > Cd^{++}$; $Zn^{++} > Ni^{++} > Co^{++} > Fe^{++} > Mn^{++}$; Pb^{++} was not located but was less active than Cu^{++}. This order closely parallels the coordination-stability sequence and is related to the metal sulfides insolubility. The inactivation by metal ions is non-competitive, but can be reversed by some SH compounds (174), by bromide ions (3), by H_2S (226), and by urea (37).

In 1946 Henry and Smith (67) observed that glassware which had been in contact with cleaning solution was poisonous to urease even after repeated rinsing. In the paper electrophoresis of urease Methfessel (140) observed that loss of urease activity was caused by heavy metals in the paper, electrodes, and buffer.

3. Inactivation by substituted ureas

According to Desay (24) the inactivation of urease at higher urea concentrations is due to the presence of $NH_3CO(NH_2)_2$. Suramin, hexa-sodium sym-bis (m-aminobenzoyl-m-amino-p-methylbenzoyl-1-naphthylamino-4,6,8-trisulfonate) carbamide, is a potent inhibitor; its sulfonic acid groups are necessary for the inactivation, and SH compounds or basic amino acids do not protect the urease (260). Thiourea inhibits competitively at pH 6.0 and both competitively and noncompetitively at pH 2.6 (97). It appears that two moles of thiourea are required for both kinds of inhibition. At pH 5.0 in acetate buffer urease is strongly inhibited by alloxanic acid, but alloxan (mesoxalyl urea) show

little inhibitory effect (54). Shaw and Raval (190) showed that the inhibition of urease by methylurea is dependent on pH, substrate concentration, and inhibitor concentration. At pH 8.9 in unbuffered solutions and at pH 7.0 in maleate buffer, the inhibition is noncompetitive; in maleate buffer at low pH, it is competitive. The inhibitory order in Tris-H_2SO_4 buffer at pH 7.4 is: urea>methylurea>sym. and asym. N-dimethylurea (201). Hydroxyurea is a potent noncompetitive inhibitor of urease; 50% inhibition occurs at a concentration of 5×10^{-5} M (45). This inhibition has been shown by Fishbein and Carbone (41) to be irreversible from dilution studies.



Thiourea Alloxanic acid Alloxan Methylurea Hydroxyurea

4. Inactivation by other chemical agents

Urease is inactivated by fluorides, halogens, borates (211), phosphate, maleate (95), bisulfites (2), quinones, polyhydric phenols (166), iodosobenzoate, porphyrindin, p-mercuribenzoate (65), N-(3-chloromercury-2-methoxypropyl)-5-methyl-3-isoxazolecarboxamide (182), hydroxamic acid (105), certain war gases and blistering agents (40), phenothiazine (18) and some basic triphenylmethane dyes (43), and ascorbic acid (34,47,137,167,211). Most of these reagents affect the SH groups of urease and the enzyme can be protected or reactivated by SH compounds in most cases.

Urease is also inactivated by the alkylation of SH groups by some alkylating agents (53). O,S-Diacetylthiamine was reported to inactivate

urease by transferring the acetyl group to SH (241). A similar result was obtained by thiamine propyl disulfide and its homologs with alkyl-mercapto radicals (90,103). All these inactivations are reversed and prevented by cysteine.

Urease is also inhibited by penicillin (239) and unsaturated fatty acids or their peroxides (259). It completely loses its activity after 20 min in 98.5% acetic acid (56).

5. End-product inhibition

Robinson (175) first showed that the automatic control of urease activity was through the inhibiting effect of its end product, NH_3. Hoare and Laidler (70) later demonstrated that the inhibition is non-competitive and of first order. Kistiakowsky and Thompson (99) pointed out, however, that it was not NH_3 but the ammonium phosphate ions that caused the inhibition. The inhibitory effect of NH_3 has also been studied in this work (see Chapter IV).

6. Effects of pH and temperature on inactivation

At pH 4.3 urease is inactivated slowly, but as pH is made more acidic the rate of inactivation increases rapidly (228). Langmuir and Schaefer (119,120) found that unfolding to form a monolayer caused complete inactivation.

Hofstee (71) reported that the activity of twice recrystalized urease was reversibly increased by moderate heating and decreased by storage in the cold, but an earlier report (74) showed that urease suffers only a slight loss of its enzyme activity after prolonged exposure to the temperature of liquid air. The activity was not

destroyed by heating at 80° for 5 min, but it vanished completely at 90° for 5 min (125). Dry heat-inactivation studies were interpreted as indicating that the molecule is not unfolded in the active state (184).

7. Activators, coenzymes, and reactivation

Reports of the existence of activators (or auxo substances or promoters) (81,82,84,85) and of coenzymes (87,159) for urease were made before 1930. Sumner and his coworkers (223,227,228), however, argued that there could be no activation of urease unless there had first been inactivation. The so-called activators or coenzymes probably protect by removing some inactivating substances, especially the heavy metals; some proteins, amino acids and gum arabic function by binding the heavy metals and thus release the catalytic ability (163). Many other investigators agree with this viewpoint (22,130,157,192,193).

Sakai (178,179) declared that a low concentration of $NaNO_2$ (below 8×10^{-3} M) promotes urease action, while higher concentration retards it. Bohadur and Saxena (8) reported that $Pb(OAc)_2$ in small concentration, was a definite activator for 40 min of reaction; after this period, it acted as an inhibitor.

Flame spectrophotometric studies of the crystalline urease from Cajanus indicus revealed traces of Mg (151).

Kinetics and Mechanism of Urease Action

A pioneer study of the kinetics of urease action was conducted in 1914 by Van Slyke and Cullen (245,246). Since that time, many other investigations have been made. An especially thorough study was

conducted in 1949-1956 by Kistiakowsky and Laidler and their coworkers. However, the nature and mechanism of enzyme action are so complex that they can not be elucidated by a study of the kinetics alone; conversely, the kinetics can not be properly understood until more is known about the chemical structure of this and other enzyme. For this reason, the review that follows will be a compilation of the pertinent references, and will not attempt to evaluate them critically; for convenience, the subject will be divided quite arbitrarily into several sub-headings.

1. Substrate specificity.

Sumner (211) stated that urease interacts only with urea and is absolutely specific. Werner (257) claimed that the decomposition of monobutylurea was catalyzed by urease, but Sumner said this was untrue. It was also reported that urease acted on biuret (188,231), but it was later proven that urea was present in the biuret preparations (189). The decaboxylation of mesoxalic acid is catalyzed by urease at pH 2.2, but this seems to be a general effect by SH compounds, not dependent on the utilization of the enzymatically active sites (15,16).

In 1965 Fishbein et al. (42) announced the discovery of a second substrate, hydroxyurea. The initial velocity of hydroxyurea hydrolysis is 120 times slower than that of urea. Other hydrolytic enzymes do not catalyze the hydrolysis. The methyl-substituted hydroxyurea analog, acetohydroxamic acid, is a powerful inhibitor of urease, indicating the existence of a special affinity of this type of structure.

2. Order of the reaction and the Michaelis constant

As has been stated, the kinetics of enzyme reactions are in general

complicated. However, in special conditions a comparatively simple apparent reaction order may be shown. Thus, Sizer (196) investigated the action of urease upon urea at 0.2-50° and stated that the rate of reaction was a linear function of time, i.e. the kinetics were of zero order. Sumner expressed the rate in phosphate buffer (211) by the relation:

$$\underline{X}=\underline{K}\cdot\underline{t},$$

where $\underline{X}$ is the quantity of urea decomposed, or ammonium or carbon dioxide produced, in time $\underline{t}$ and $\underline{K}$ is a constant. He determined the turnover number of pure urease to be 460,000 at 20° and pH 7 (211).

On the other hand, from the studies of urease action in Tris-H_2SO_4 buffer at pH 7 Wall and Laidler (251) found that at low concentrations the activity increases more rapidly than the concentration, while at higher concentrations the opposite was true. They believed that the low-concentration effect was due to the rapid inactivation of the enzyme and the high-concentration effect was due to the inhibition of the reaction by the ammonium ions produced.

If the hydrolysis is conducted at pH 8.9 by utilizing the buffering action of the products of the hydrolysis, the rate of reaction was reported by Kistiakowsky and Shaw (98) to be a linear function of the time up to 10 min. From thermal analysis of the urease reaction, Tamura (232) confirmed that the rate was linear in distilled water, but not in buffer, for a few min and to pH 8.7.

The effect of urea concentration upon the rate of hydrolysis has been studied by Kistiakowsky and Rosenberg (96) over a 5000-fold concentration range at several pH values in phosphate buffer. They observed that the rate first increased, reached a maximum and then fell

off slowly with rising urea concentration. The high concentration effect may result from an inhibition of the reaction by ammonium ions, or from an inhibition by urea (96,250). In Tris-sulfate buffer it was found by Wall and Laidler (250) that, at pH 7.1 and 8.0 the Michaelis-Menten law is obeyed up to a substrate concentration of about 0.30M; at higher substrate concentrations there is a falling-off of the rate as the concentration is increased. At pH 4.3 in acetate buffer the rate shows a dependence on substrate concentration which does not follow the Michaelis-Menton equation (99). Kistiakowsky and his co-workers (96,99) thus suggested two types of active sites effective on each side of pH 7.0 with Michaelis constants 0.42 mM and 8.4 mM respectively.

Using the crystalline urease isolated from Cajanus indicus Nath and Pradhan reported a first order reaction for all substrate concentrations (147) and also obtained results similar to those of Kistiakowsky et al. (148,149).

3. Effects of pH and ionic strength.

The kinetic studies of Van Slyke and Zacharias (247) indicated that the decomposition of urea by urease was most rapid in neutral solution. Howell and Sumner (73) measured the pH optimum in various buffers and found that it varied from 6.4 to 7.6 depending on the buffer ions and substrate concentration. In Tris-sulfate buffer, Wall and Laidler (250) found a sharp optimum at pH 8.0. According to the authors, this buffer had neither an inhibiting nor activating effect on the enzyme (250,252). A series of papers by Kistiakowsky and coworkers reports investigation of the rate from pH 4.0 to 8.9. The dependence of activity over the range from pH 4.3 to 8.9 was interpreted as indicating that the

neutral form of urease is the active one and that it is not in equilibrium with the inactive acidic or basic forms (99). At pH 4.0, the enzyme is denatured quite rapidly (99). At pH 8.9, urease shows lower activity but is not irreversibly denatured; indeed the pH can be raised to 10 without altering the activity of the enzyme upon return to lower pH (98).

The rate of hydrolysis is effected by neutral salts and is accelerated by the ionic strength of the solution (99). This influence is much greater at pH 8.9 than at pH 7.0 (98).

From the pH-activity curves and the effect of pH on K_m and V it is possible to calculate the pK values of the ionizable groups in the enzyme which combine with the substrate and of the groups which influence the reactivity of the enzyme-substrate complex without affecting the formation of the complex. This type of analysis indicates that groups having pK values of 6.1 and 9.2 undergo a change of ionization in the urease-urea-water complex (113), and that a group having a pK value of 9.0 influences V without affecting K_m (29,143).

4. Effect of temperature and energy of activation.

The dependence of rate constant on temperature can in many cases be described by the Arrhenius equation:

$$k = A\, e^{-E/RT},$$

where E is the energy of activation. It is uncertain to what extent this rate of reaction can be applied to enzyme action; in most cases, the equation was assumed to hold and E was calculated on this basis.

According to Sizer (197), the energy of activation in the range

of 0.2-50° was 11,700 cal/mole in the presence of mild oxidants and 8,700 cal/mole in the presence of reductants. Kistiakowsky and Lumry (94) reported later that the activation energy was 8,830 cal/mole at all temperatures. However, sulfite ions inhibit urease reversibly in such a way that the apparent activation energy rises to 15,000 cal/mole at 5°; the Arrhenius plot in these conditions is not linear.

The activation energy of urease in phosphate buffer decreases with increasing urea concentration. Laidler and Hoare (116) postulated the formation of urea-urease-water complex and that the urea would displace the water reversibly at high concentrations; also, it was suggested that the urease molecule "opened out" during the complex formation.

The activation energy of urease in Tris-sulfate buffer has been determined by Wall and Laidler (250) to be 6,800 cal/mole at pH 7.13 and 8,500 at pH 8.00 with 0.005M substrate; 9,700 at pH 7.13 and 11,100 at pH 8.00 with 0.25M substrate.

5. Studies of intermediates and products.

The reaction mechanism of urease action proposed early by Bersin and Koster (11) and by Brandt (14) was:

$$\text{Urease} + \text{urea} \xrightarrow{NH_3} \text{Urease-urea complex} \xrightarrow[\text{Urease}]{H_2O} H_2NCOOH \rightarrow HH_3 + CO_2$$

NH_3 is liberated and carbamic acid is formed in the first step of the mechanism; carbamic acid then decomposes to NH_3 and CO_2.

The existence of carbamate as an intermediate has been the subject of controversy among investigators. Its presence was first demonstrated by Yamasaki (263) in 1920. After extensive studies, Sumner et al. (221) showed that when urea was decomposed by crystalline urease in the

absence of buffer, i.e., > pH 7.0, ammonium carbamate was formed and then decomposed to NH_3 and CO_2. If neutral phosphate was present no carbamate could be detected. Sumner (211) therefore suggested that NH_3 and CO_2 were the products first produced and that NH_3 and CO_2 united in the absence of buffer to form ammonium carbamate, while in the presence of buffer no carbamate results. In 1959 Slocum et al. (199) showed that O^{18} was transferred from labeled phosphate and arsenate to the CO_2 produced during the hydrolysis of urea. It was concluded that urease may bring about the formation of carbamyl phosphate and carbamyl arsenate, but not as intermediates. According to these investigators, urease also catalyzes a urea-dependent exchange of O^{18} between water and phosphate. However, no P^{32}-labeled carbamyl phosphate is formed during the hydrolysis of urea in the presence of p^{32}-labeled phosphate. In 1966, Kull and Jones (112) reinvestigate the oxygen-exchange between water and phosphate during the hydrolysis of urea by urease and found that under the condition reported there was no incorporation of O^{18} from H_2O^{18} into orthophosphate buffer, in contradiction to the aforementioned results.

On the other hand, Wang and Tarr (253) showed that the O^{18} content of the CO_2^{18} produced was only half of that amount in H_2O^{18} used. It was concluded from this that carbamic acid and NH_3 are the first products of the hydrolysis and that carbamic acid then decomposes to NH_3 to CO_2. But Varner (248) pointed out that the same result would be obtained from any mechanism that did not involve the formation of carbonic acid or the replacement of the urea oxygen. Gorin (48) confirmed the formation of carbamate under conditions where recombination of NH_3 and CO_2 did not occur.

From studies of the isotopic exchange between urea and $N^{15}H_3$ in the hydrolysis reaction, Kistiakowsky and Thompson (99) claimed that NH_3 was not hydrolyzed from urea molecules in the first reaction step, hence, that urea combined with the enzyme without loss of ammonia. They suggested that NH_3 was split off only in the last and slower reaction step and some urea was resynthesized from carbamic acid and NH_3.

The existence of a urease-urea complex is not detectable directly. Isotopic exchange studies led Singleton et al. (195) to believe that urea or its fragment was incorporated into urease at the active site, but Robinowitz et al. (168) excluded this possibility by the experiments with C^{14}-labeled urea. Lynn and Yankwich (132,133) studied the kinetic isotope effects in the urease-catalyzed hydrolysis of C^{13}-urea. They found that changes in reaction conditions that were without effect on the gross rate did, nevertheless, bring about changes in the kinetic isotope effect. They concluded that the mechanism probably involved a temperature-dependent interconversion of two or more types of active sites.

Cyanic acid was also mentioned as a possible intermediate (39,134). Sumner and Hand (220) as well as some other investigators (79,204) demonstrated that this was not true, however.

6. The nature of active site

The mercapto groups are essential for the enzyme activity of urease, but they are possibly also involved in maintaining the active conformation (262). Laidler suggested (113) that a basic group of urease is involved in the formation of the enzyme – substrate complex, and an acidic group participates in the subsequent break-down of the complex.

Since diisopropyl phosphofluoridate does not inactive urease, serine does not participate in the catalytic reaction (10). Singleton et al. (195) postulated the participation of an aldehyde group in the catalyic reaction by the reversible formation of an imino group, but their observations, based of $N^{15}H_3$ exchange, is rather indirect evidence.

From their kinetic studies, Laidler and Hoare (115) deduced that urea and water were adsorbed upon separate sites on the enzyme before reacting. Kistiakowsky and his co-workers have suggested two type of active sites, one effective in acid and other in base (96,99). Nakamura (144) assumed an active center and a binding center located on the different sites of the enzyme molecule but bound together by an activity-conducting system.

Ambrose et al. (3) concluded that there were 3-4 active sites per molecule of urease from the Ag^+ ions inhibition studies. Kobashi and Hase (104) suggested the existence of 2 active sites from experiment on the inhibition by hydroxamic acid. The conclusion reached in the present work are given in Chapter III.

Assay

Many methods of assaying urease have been proposed. There are, in addition, several studies that are pertinent to the problem. This section of the literature review is limited rather strictly to studies intended especially for assay purposes.

Methods in which the rate can be measured continuously are advantageous in that they permit many measurements to be taken on one sample, which increases the precision. Also, they make it easier to establish the kinetics; if these are of zero order, the amount of

product is directly proportional to the enzyme concentration and inversely proportional to the time, which simplifies the calculation.

Unfortunately, the determination of ammonia does not lend itself to continuous determination. For this reason, most assays are of the fixed interval type; they are discussed first. In the second subsection, "continuous" assay methods are reviewed.

The qualitative assay of urease activity in microorganism has been made by special methods (9,12,69,180,255).

1. Fixed-interval assays

In assays of the fixed-interval type the sample of urease is allowed to act upon an excess of urea solution at a given temperature for a definite time interval, at the end of which the amount of product is determined.

Van Slyke and Cullen (245) stopped the reaction by adding acid; the ammonia produced was aerated off and estimated either by titration or by Nesslerization. Krebs and Henseleit (109) estimated the enzyme activity by measuring the CO_2 produced manometrically. A modification of this method was described by Weil and Russel (256). A more systematic and detailed description of manometric and titrimetric methods, with improvements, has been given by Van Slyke and Archibald (244). In this article, a colorimetric method also is described, which is based on the determination of the time required to obtain a change in indicator color.

The assay of Sumner and Graham (214) is essentially the same as that proposed by Van Slyke and his collaborators. They used 3% urea in 0.68M phosphate buffer as substrate, mixed 1 ml of it with 1 ml of

urease solution, and then allowed the reaction to proceed at 20° for 5 min; 1 ml of 1M HCl was then blown into the digest to stop the reaction. With samples of crystalline urease, Sumner diluted the solution to be assayed to about 1 S.U./ml and determined the ammonia by direct Nesslerization; with crude urease, the ammonia was first aerated into acid (212).

Gorin et al. (49,50) modified the above method by titrating the digest mixture with 0.1M HCl to the end point of Alka-ver indicator. Further discussion and improvements of this method are given in Chapter IV.

Other assay methods of the fixed-interval type include the microdiffusion method used by Sehgal et al. (181), in which the ammonia was evolved in Conway dishes and titrated with acid. Cederangolo et al. (17) employed the same method and trapped the diffused ammonia on an acid film, which was then analyzed with Nessler's reagent. A radiometric method was developed by Shatalova and Meerov (186) based on the determination of $C^{14}O_2$ from C^{14}-urea.

2. Continuous assays.

The method of Kono (106) employed a high-frequency oscillator to measure the amount of NH_4NCO_3 produced with time. A similar method employs continuous recording of the differential electric resistance of urea solutions during enzymic hydrolysis (13). The authors claimed that this method was superior in precision to the classical colorimetric method.

A calorimetric method, which measures the temperature change with time has been proposed (77); the Michaelis-Menten law was obeyed up to

a certain concentration, but at high concentrations the rate decreases. Durand (32) made use of the microcalorimeter of Calvet and Prat for the calorimetric assay.

A continual spectrophotometric determination for NH_3-producing systems has been developed by Stutts and Fridovich (203). It is based on the stimulation by ammonia of the peroxidation of o-dianisidine, catalyzed by horseradish peroxidase. The rate of peroxidation was determined by measuring the absorbancy at 460 mμ, 26°, and pH 9.3.

A potentiometric method has been described by Katz (88), which employes a sensitive glass electrode to determine the NH_4^+ liberated. Katz has utilized his assay method to study the metal ions inhibition of urease (89).

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CHAPTER III

THE REACTION OF UREASE WITH N-ETHYLMALEIMIDE AND WITH SILVER ION

(A paper entitled "Urease. IV. Its Reaction with N-Ethylmaleimide and with Silver Ion" has been published in Biochim. Biophys. Acta 99, 418 (1964) and is reproduced below.)

Summary

Native urease (urea amidohydrolase EC 3.5.1.5; mol. wt. 480,000) reacts rapidly with 21 moles of N-ethylmalemide (NEM) with no loss of activity. Further reaction takes place slowly, and a 90% decrease in activity occurs at 7-8 additional moles of NEM combine with the enzyme. Native urease binds Ag^+ ion strongly; with 10^{-9} M enzyme in citrate buffer of pH 6 about 8 ions per molecule cause 50% inhibition. If the 21 reactive mercapto groups are previously combined with N-ethylmaleimide only 4 Ag^+ ions are required for 50% inhibition. It is concluded that there are about 8 "active sites" per 480,000 - mol. wt. unit.

Introduction

This paper reports some experiments on the reaction of native urease (urea amidohydralase, EC 3.5.1.5)[1,2] with N-ethylmaleimide (NEM), with $AgNO_3$ and with both these substances. These experiments were undertaken to study the reactivity of the mercapto groups in the enzyme and to test certain conclusions reached in earlier investigations.

The presence of mercapto groups in urease was first demonstrated

by SUMNER AND POLAND[3]. HELLERMAN et al.[4] did an interesting quantitative study and concluded that the enzyme contains three categories of mercapto groups: (a) 22-3 groups that react readily with p-chloromercuribenzoate, porphyrindin or o-iodosobenzoate without affecting the enzyme activity; (b) 22-3 groups that react with additional p-chloromercuribenzoate and with the concomitant loss of enzyme acitivity, and (c) some 60 more groups that react with o-iodosobenzoate in concentrated guanidine hydrochloride (the nomenclature "a, b and c groups" will be utilized in the rest of this paper).

According to DESNUELLE AND ROVERY[5], phenyl isocyanate, like the reagents mentioned above, reacts first with the a groups of urease, and thus treatment of the enzyme with 22-3 moles of reagent causes little change in activity; however, addition of about half again as many moles of reagent inhibits the enzyme completely. It was concluded that not all the b groups are essential to activity, and that isocyanate reacts more specifically than p-chloromercuribenzoate with the essential groups.

GORIN et al.[6] found that 48 moles of NEM or p-chloromercuribenzoate reacted with urease in 4M guanidine hydrochloride, and this value is in good agreement with the number of (a + b) groups; however, these reagents gave no evidence for the presence of the c groups. With ferricyanide a somewhat higher titer was obtained but it increased with the concentration of reagent (55 to 64 equivalents/mole) and this may be due to occurrence of oxidation beyond the disulfide state.

Urease is very strongly inhibited by Ag^+ ion. In an early study, SUMNER AND MYRBÄCK[7] reported that about 6 Ag^+ ions per molecule would cause 50% inhibition. However, AMBROSE et al.[8] later came to the conclusion that only 3-4 ions would suffice for complete inhibition. These

values are in marked contrast to the comparatively large number of mercapto groups present in the molecule, and raise interesting but puzzling questions about the nature of the active sites.

The present investigation provides some clarification of these questions; in some respect the results confirm the conclusions of previous investigations, but they indicate the probable presence of some eight active sites in the enzyme molecule.

Materials and Methods

Urease preparation

Several samples of crystalline urease were prepared by the procedure previously described.[6,9] After the final crystallization, the enzyme was usually dissolved in 0.02M phosphate buffer, and the solution was stored at 5-10°. For the inhibition experiments with silver, the crysatlline enzyme was dissolved in water and diluted with citrate buffer. Activity measurements were made by the procedure of GORIN et al.[6]; the samples assayed had concentrations of about 4 units/ml. The first assay was made within 72 hours from the time of preparation, and the determination was then repeated occasionally. All experiments were done with preparations less than 30 days old, which had lost no more than 20% of the original activity. Urease concentrations were determined spectrophotometrically, using the value 3.70×10^5 (cm^2 $mole^{-1}$) for the molar absorbancy at 272 mμ (adjusted to mol. wt. 480,000)[6].

Other chemicals

NEM was obtained from the California Corporation for Biochemical Research, Los Angeles, California. Guanidine hydrochloride, originally

of "Aero" technical grade from the American Cyanamid Company, Bound Brook, N. J., was purified as described elsewhere[10]; the absorbance of a 1M solution was less than 0.1 at 250 mμ and negligible at 300 mμ. Nessler's reagent was prepared as described by FISTER[11]. The water used in all operations done with urease (except the initial extraction of jack-bean meal) and in the preparation of all solutions was purified by taking ordinary distilled water that had been obtained by condensing steam and passing through an ion-exchange resin, distilling it from 0.2% $KMnO_4$-0.2% K_2CO_3, and then redistilling it a third time. Deaerated water was made by boiling triple-distilled water and then allowing it to cool with a stream of nitrogen passing through. Buffer solutions were made up as follows, per liter of solution: 0.68M (9.6%) phosphate, pH 7.0--68.00 g of Na_2HPO_4 and 28.00 g of KH_2PO_4; 0.1M phosphate, pH 7.0 -- 2.458 g of Na_2HPO_4 and 4.620 g of $NaH_2PO_4 \cdot H_2O$; 0.02M phosphate, pH 7.0-- 1.657 g of Na_2HPO_4 and 1.150 g of $NaH_2PO_4 \cdot H_2O$; 1M citrate, pH 6.0-- 279.4 g of $Na_3C_6H_5O_7.2H_2O$ and 10.51 g of $H_3(C_6H_5O_7).H_2O$; 0.1M citrate, pH 6.0-- 27.94 g of $Na_3C_6H_5O_7.2H_2O$ and 1.051 g of $H_3(C_6H_5O_7).H_2O$.

Reaction of urease with NEM

For the determination of mercapto groups in 4M guanidine hydrochloride, the following directions are representative. Dissolve 15.7 mg of NEM in 0.1M deaerated phosphate buffer to make 50 ml (2.5×10^{-3}M). Dissolve 17.5 g of guanidine hydrochloride in about 20 ml of 0.1M phosphate buffer, adjust the pH to 7.0 with NaOH, and dilute to 25.0 ml (7.3M in guanidine). Mix 1 ml of about 10^{-5}M urease in 0.02M phosphate with 2 ml of guanidine solution. Mix 1 ml of urease-guanidine solution

with 0.25 ml of NEM solution and measure the absorbance (A) against 1 ml of urease-guanidine + 0.25 ml of buffer. Also mix 0.25 ml of NEM solution with 1 ml of guanidine solution and measure the absorbance (A') against 0.25 ml of buffer solution + 1 ml of guanidine solution. The number of SH groups per mole is calculated from the expression: $(A'-A)/620\underline{M}$, where 620 is the molar absorbance of NEM[12] at 300 mμ and $\underline{M}$ the molarity of urease.

Experiments with native urease were done similarly, except that the guanidine hydrochloride was omitted. Activity changes in the course of the reaction were followed by taking two aliquot portions, one of which was measured in the spectrophotometer, while the other was tested for activity.

Reaction with Ag^+ ion

Sufficient urease solution to give a concentration approximately $2.5 \times 10^{-9}\underline{M}$ was added to 2 liters of 0.1$\underline{M}$ citrate buffer of pH 6.0 in a polyethylene bottle. The bottle was put in a thermostat at $20\pm0.1^{\circ}$ and its contents were stirred mechanically with a slow-speed motor. After two hours a 20-ml aliquot was withdrawn and its activity tested in the following way. 1 ml of 31.5% urea was added quickly with vigorous stirring and exactly five minutes later the reaction was stopped with 1 ml of 7$\underline{M}$ sulfuric acid; a 100-μl aliquot was withdrawn with a micropipette and added to about 10 ml of ice-cold water in a 25-ml volumetric flask; then 10 ml of Nessler's reagent was added, the volume made up to the mark with ice-cold water, and the color was allowed to develop for 10 minutes at room temperature. The transmission was then determined with a Coleman colorimeter at 415 mμ. The amount of ammonia was

calculated by comparison with appropriate standards, which were treated similarly to the samples.

Standard $AgNO_3$ solution, about 10^{-5}M, was added to the urease solution in 1-2 ml portions; after 5-10 minutes (no longer, to minimize the "recovery reaction", see the Results and Discussion sections), a 20-ml aliquot was withdrawn for assay. This was repeated until 5-10% of the original activity remained. Then 0.05M KBr was added until the activity was restored to above 90% of the original value.

Reaction with NEM followed by Ag^+ ion

1 ml of approximately 10^{-5}M urease in 0.02M phosphate buffer was mixed with NEM in 1:22 molar ratio. After 2.0 hr the reaction mixture was diluted 2,000 times and $AgNO_3$ solution added as described in the section above.

Results

Table I summarizes some characteristics of the enzyme preparations used in the present work.

NEM reacted quite rapidly with urease in 4M guanidine hydrochloride at pH 7.0. After about 2 min the absorbance of the reaction mixture reached a steady value, which corresponds to the reaction of 46.5 ± 2.5 moles per mole of enzyme. Fig. 1 shows the data from a representative experiment.

NEM reacted also with native urease, but the course of the reaction was rather different: the rate was quite rapid at first, then decreased to a low but measurable value, and finally became so slow it could not be clearly distinguished from the spontaneous hydrolysis of the reagent.

TABLE I

PROPERTIES OF THE UREASE PREPARATIONS

Preparation No.	Times recrystallized	Specific activity (units/mg)	Mercapto groups per mole in 4M guanidine-HCl
I	4	151	46
II	4	157	44
III	5	154	51
IV	5	158	--
V	5	152	45
VI	4	153	--
			Mean 46.5 $\pm$ 2.5

Fig. 1 shows some representative results, obtained with an initial NEM: urease (molar) ratio of 90. A clear inflection is seen at 20-23 moles, following which about 7 moles were consumed in 2 hours; extrapolation of the intermediate-rate portion of the curve gave, in the present case, 22.5 moles. The average of several determinations done with different preparations was 21.0 ± 0.3 moles for the amount consumed rapidly, and 8 moles for the additional amount consumed in 2 hours. At an initial NEM: urease ratio of 25, it was again found that 21 moles were consumed rapidly, after which not enough reagent remained to give appreciable further reaction.

Fig. 2 shows the results of a representative experiment, in which both NEM consumption and activity were measured. The initial NEM:urease ratio was about 90. It is seen that 20-21 groups reacted in the first 1-2 min and that the activity was then essentially undiminished (control experiments showed that when the reaction mixture was diluted to the level suitable for assay, i.e., about 200 times, the rate of reaction with NEM was reduced to a negligible value).

In the subsequent two hr, an additional 7-8 moles of NEM reacted, and 90% of the activity was lost. The activity decreased to naught in about two additional hr, during which time the change in absorbancy corresponded to 1-2 moles of NEM. This change, though measurable, is within the experimental uncertainty and must be regarded as insignificant.

The results obtained by reacting Ag^+ ion with native urease were somewhat variable, but those shown in Fig. 3 are quite representative. The concentration required to cause 50% inhibition in this case was 7.5 gram-ions per mole of urease; the average of 7 experiments done with

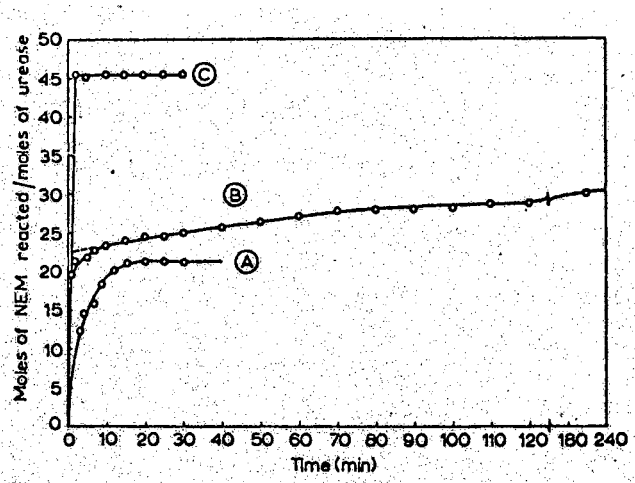

Fig. 1. Reaction of urease with NEM. Curve A: urease, $1 \cdot 10^{-5}$ M; NEM, $2.5 \cdot 10^{-4}$ M; 21.3 mercapto groups. Curve B: urease, $1 \cdot 10^{-5}$ M; NEM, $8.8 \cdot 10^{-4}$ M; extrapolation corresponds to 22.5 mercapto groups. Curve C: urease, $3.3 \cdot 10^{-6}$ M; NEM, $3.1 \cdot 10^{-4}$ M; guanidine-HCl, 4 M; 46 mercapto groups.

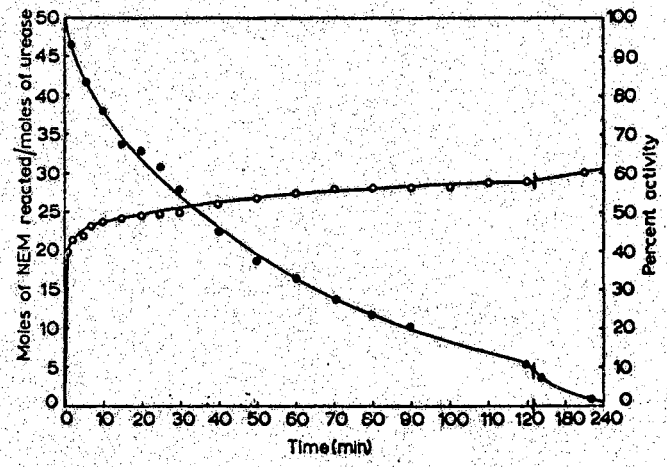

Fig. 2. Reaction of $1.24 \cdot 10^{-5}$ M urease with $1.08 \cdot 10^{-3}$ M NEM. Empty circles, left ordinate; full circles, right ordinate.

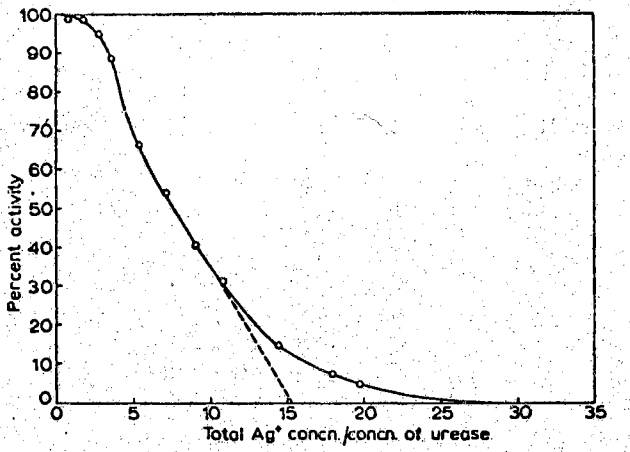

Fig. 3. Inhibition of urease with Ag^+ ions.

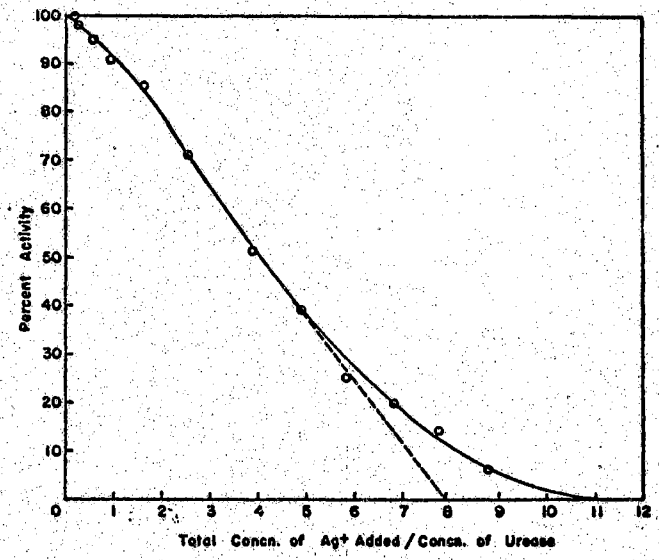

Fig. 4. Inhibition of NEM-treated urease with Ag^+ ions.

six preparations was 7.7±0.3. Complete (>98%) inhibition required much higher amounts of Ag^+ ion, about 25 gram-ions; this amount cannot be estimated accurately because the activity tends toward zero asymptotically. If KBr was added to a urease-Ag^+ mixture with 5-10% of the original activity, the inhibition could be completely reversed.

It was also observed that, in partially inhibited samples, some restoration of the activity occurred spontaneously with the passing of time. For example, the activity of a urease solution which has been reduced to 20% of its original value by treatment with Ag^+ increased twofold, i.e., to 40% of the original value, after standing two days.

Urease which had been treated with 22 moles of NEM reacted with Ag^+ ion in a quite different way from that of the native enzyme. A representative experiment is shown in Fig. 4. A plot of percent activity against Ag^+ ion added was more nearly linear, from the origin to about 25% activity, and extrapolation of this line to zero gives a value of 8 Ag^+ ions per mole of modified urease. The average of four experiments was 8.0±0.3. The activity of partially inhibited, NEM-modified urease showed no tendency to increase on standing, in contrast to the behavior of the native enzyme.

Discussion

The consistency of the specific activity values for the urease preparations used in this work and the good agreement with the values reported earlier[6] give some assurance that one is dealing with a well-defined, reproducible enzyme. The highest specific activity reported by SUMNER[2] was 133 units/mg, and this value in turn is higher than what has been reported by many other investigators[13] (for other values see Ref. 9).

Because of the uncertainties which still remain regarding what variables affect urease activity, it may not be possible to make a precise comparison between the values obtained in different investigations, but it can be asserted that the present enzyme preparations compare favorably with those that have been previously described. An investigation currently in progress indicates that urease activity is proportional to concentration only within a narrow range and attention is therefore called to the fact that the assays were done at a rather high level of activity, about 4 units/ml.

The reaction of NEM with urease in 4M guanidine hydrochloride was used as a control on the reproducibility of the enzyme preparations, which is seen to be fair. The average result, 46.5 ± 2.5 (moles consumed per mole of urease) is in good agreement with the value found previosuly with other preparations[6]. Also it corresponds well to the sum of (a + b) groups found by HELLERMAN *et al.*[4]

On the basis of their reactivity toward NEM, three categories of mercapto groups can be recognized in the native enzyme: (a) 21 groups that react rapidly, with no loss of enzyme activity; (b_1) 7-8 groups that react more slowly with the concomitant loss of 90% of the activity; and, by difference, (b_2) 16-20 groups that do not react at an appreciable rate. The distinction between *a* and *b* groups is very clear because the rates of reaction differ by a factor of about 60, as indicated by the midpoints of the respective sections in the NEM-consumption curves -- 1 minute or less and about 60 minutes, respectively. The separation of b_1 and b_2 groups is less well defined because of interference from the spontaneous hydrolysis of NEM, but is nevertheless qualitatively clear. So far as the reactive *a* groups are concerned there can be

little doubt that they correspond to those found by HILLERMAN *et al.*[4] However, NEM makes possible the further distinction into b_1 and b_2, that was not evidenced with *p*-chloromercuribenzoate.

The results obtained with Ag^+ ion can most easily be interpreted by referring first to the reaction with NEM-treated urease. As can be seen in Fig. 4, the activity in this case decreases in nearly linear fashion with the amount of Ag^+ ion added, to about 70% inhibition. This indicates that the Ag^+ ion must be nearly completely bound. Since the absolute concentration of enzyme is only 2.5×10^{-9} M, the binding constant must be high, approx. 10^{10} or greater. The number of strongly binding sites is indicated by extrapolating the linear portion of the line to 100% inhibition and the result, as has already been stated, is 8 per molecule.

The inhibition of native urease in similar conditions, exemplified in Fig. 3, does not follow a linear course. However, it is clear from the comparison with NEM-treated urease that the native enzyme contains a greater number of strongly-binding groups. The first inflection in the curve indicating that the native enzyme contains some sites; not essential for activity, that bind Ag^+ even more strongly than the active sites; these are apparently removed, at least for the most part, by reaction with NEM. The active sites, in turn, must bind Ag^+ ions more strongly than many of the other mercapto groups; since extrapolation of the linear portion of the curve to zero activity gives a value of 15 per molecule (this extrapolation is of course only approximate); this value is less than one third the sum of the (*a* + *b*) groups.

These results are not in agreement with the conclusion reached by AMBROSE *et al.*[8] that urease would be completely inhibited by reaction

with 4 gram-ions of Ag^+ (per 480,000 g) and it is appropriate that some comment be made on this point. The enzyme samples employed by AMBROSE *et al.* contained 60-85% of inactive material and the assumptions were made that this material was denatured urease and that it had the same affinity for Ag^+ as the native enzyme; furthermore, the data were treated on the basis that a single type of binding site was involved. This might have seemed a reasonable basis for calculations at the time, but it is now clear that the assumptions are inadequate and that the results based on them cannot be regarded as quantitatively significant.

The correspondence between the number of $\underline{b}_1$ groups and that of strongly-binding sites in NEM-treated urease is suggestive and provides a reasonable basis for the hypothesis that urease contains 8 active sites, each involving 1 mercapto group. These sites bind Ag^+ ion more strongly than the $\underline{b}_2$ and some of the *a* mercapto groups and it may be surmised that some other functional group, such as amino or carboxylate, at or near the active sites also coordinates with the Ag^+ ion, giving a chelate bond.

The very high reactivity of the *a* groups toward several reagents' strongly suggests that they are on the "surface" of the molecule, i.e., on portions of the polypeptide chain that can come into intimate contact with the solvent. As has been seen, some but not all these groups bind Ag^+ ions more strongly than the active sites. On the other hand, the $\underline{b}_2$ mercapto groups which react very slowly or not at all with NEM and do not compete with the active sites for Ag^+ ion may well be located in the interior of the molecule.

So far as the location of the active sites themselves is concerned, the exceptionally high catalytic activity of urease suggests that they

would be at the surface, and their ready reaction with Ag^+ ion is consonant with this view. The slow reaction of the sites with NEM argues in the opposite sense, but not with equal force--there can be several reasons why the reaction of NEM at the active sites might be hindered, for example, unfavorable steric interactions.

A recently completed study[15] has shown that urease in 6M guanidine hydrochloride dissociates into particles of weight about 80,000, i.e., that the 480,000-mol. wt. unit obtained by the usual procedure for isolating urease is an aggregate comprising 6 subunits. The conclusions reached in the present work are not in complete agreement with this but, on the other hand, the discrepancy is not large. There is reasonable hope that further experimentation will soon remove the remaining margin of uncertainty.

Acknowledgment

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CHAPTER IV

A NEW METHOD OF ASSAY AND THE SPECIFIC ENZYMATIC ACTIVITY OF UREASE

(A paper entitled "Urease. VI. A New Method of Assay and the Specific Enzymatic Activity" will be published in Anal. Biochem. and is reproduced below.)

This paper describes a novel method of assaying urease (urea amidohydrolase, EC 3.5.1.5) which affords certain important advantages over other methods. This method utilizes titration with alkali and will therefore be called the "alkalimetric method." Also presented is a definition of the unit of activity in accord with the recommendations recently made by the International Union of Biochemistry (IUB) (1,2). In the companion paper which follows (3) alternative assay methods are considered, in particular the method of Sumner (4), which has been widely used in the past. The present paper also reports values of the specific activity found recently for highly purified urease preparations and summarizes pertinent earlier results.

Materials

The studies reported in this paper extended over a considerable period of time, in the course of which some changes were introduced in the procedure for isolating urease. The most recent results were obtained with the materials and by the procedure described by Mamiya and Gorin (5). After three or four crystallizations, the urease crystals were stored as a suspension in the acetone-citrate mother liquor at 4°.

Freshly prepared crystals were completely soluble in 100-200 parts of 0.02M phosphate buffer, aged crystals usually gave a small amount of insoluble residue; to separate any such residue, the solutions were routinely centrifuged at 27,000g for 30 min and decanted before use in other experiments. Typically, urease crystal stored in mother liquor at 4° lost less than 5% of the activity in one month; urease solutions (ca. 1%) in EDTA-containing buffer stored at 4° lost less than 5% of the activity in one week; the solutions were stored no longer than 2 weeks. Determination of the specific activity was made on crystals less than 2 days old with solutions prepared in the same day.

TRIS buffer, 0.1M, pH 9.0±0.1, contained 12.114 g of tris(hydroxymethyl)-aminomethane, 57.0 ml of 0.2M HCl and 0.3723 g of disodium ethylenedinitrilotetraacetate (EDTA) (Eastman Chemicals) per liter; phosphate buffer, 0.02M, pH 7.0, 1.657 g of Na_2HPO_4, 1.150 g of $NaH_2PO_4 \cdot H_2O$ and 0.3723 g of EDTA per liter. The water was obtained by redistilling deionized steam distillate in an all-Pyrex still. All chemicals except EDTA were of A.C.S.-reagent grade.

Methods

Alkametric assay method

The substrate was prepared by dissolving 3.00 g of urea in sufficient TRIS buffer to give 100 ml. Urease solutions were diluted to the proper concentration range for assay with 0.02M phosphate buffer and allowed to stand at least 2 hr. Then, 1 ml of urease solution and 1 ml of substrate solution were mixed; after exactly 2 min the reaction was stopped by adding 2 ml of 0.1M HCl with a Folin pipette. The excess HCl was then backtitrated with 0.05M NaOH to the methyl orange end-point

(volume of NaOH required in ml=$\underline{V}_s$); a 10-ml burette was used and readings were estimated to 0.002 ml with the aid of a magnifier. A blank made up from 1 ml of substrate and 1 ml of phosphate buffer was treated in the same way (volume of NaOH required in ml=$\underline{V}_b$). The activity of the sample in IUB units, $\underline{U}_a$ (see next section) was calculated by the equation:

$$\text{Activity} = 500\ (\underline{V}_b - \underline{V}_s)\underline{M}_{OH}; \qquad (1)$$

$\underline{M}_{OH}$ is the molarity of the NaOH solution.

Determination of nitrogen by Kjeldahl and azotometric methods

The Kjeldahl determination was conducted in a Parnas-Wagner apparatus (6). Additional details were as follows: For the digestion, 1 ml of 0.5-1.0% urease solution was mixed with 1.9 g of K_2SO_4 - HgO mixture, 1 ml of water and 2 ml of concentrated H_2SO_4; the solution became clear in 1-2 hr and the heating was continued 0.5 hr longer. The 0.01$\underline{M}$ HCl used for the titration was standardized by subjecting an acetanilide standard to the procedure (National Bureau of Standards sample 141A, 10.36% N).

The azotometric determination was conducted with the apparatus and by the procedure described by Matsuda and Sekita (7); since this reference may not be readily available, some details will be given. Digestion was effected in a 50-ml L-necked flask. The azotometer consists of the following parts, fused to one another: a stopcock (A), a 15-ml bulb (B), a stopcock (C), a 50-µl capillary and 15-ml elongated bulb (D), and a stopcock (E). The sample, about 25 µl of 0.5-1.0% urease, was digested with 2 ml of H_2SO_4 - K_2SO_4 - glucose mixture (60 ml of concd H_2SO_4 + 360 ml of 10% K_2SO_4 + 1.2 g of glucose). To the digestion

mixture was then added 4 ml of NaOH-NaCl solution (10 ml of 10M NaOH + 90 ml of saturated NaCl). Bulb (B) of the azotometer was evacuated and then filled 2/3 full of saturated NaCl solution. Then the digest was sucked through stopcock (E) into bulb (D); the digestion flask was rinsed with 4 ml of $NaHCO_3$-NaCl solution (50 ml of 6% $NaHCO_3$ + 50 ml of saturated NaCl) and this also was sucked into bulb (D). Carbon dioxide gas from a cylinder was bubbled through the apparatus for at least 5 min; 1 ml of freshly prepared ice-cold NaOBr solution (20 ml of 10M NaOH + 0.5 ml of Br_2) was then sucked into bulb (D). Stopcocks (C) and (E) were then closed and the contents of bulb (D) shaken for 1 min; residual CO_2 was absorbed by the alkali, leaving a bubble of N_2. The end of the apparatus closest to stopcock (E) was then immersed in saturated NaCl solution and the stopcock opened; this admitted some solution and thus relieved the partial vacuum created by absorption of the CO_2. Finally, stopcocks (C) and (A) were opened, and by gentle blowing and then suction, the N_2 bubble was forced into the capillary. The stopcocks were then closed and the apparatus allowed to come to equilibrium in a constant-temperature bath; after 20 min the volume of N_2 was measured and corrected to STP from the table given in the reference cited (7). The aforementioned acetanilide standard was used to calibrate the capillary.

Some General Principles and Definition of the Activity Unit

The alkalimetric assay method is of the "fixed-interval" type, i.e., the enzyme and substrate are allowed to interact for a predetermined period (2 min), at the end of which the amount of product is determined. It will be demonstrated that the amount of product is

proportional to the enzyme concentration within a specified range. This implies that the rate of reaction is constant within that range, i.e., that the kinetics are of zero-order. The units of activity are then measured by the amount of product formed, which is stoichiometrically related to the amount of substrate consumed, divided by the time.

The International Union of Biochemistry has recently recommended that the enzyme activity unit be defined as the quantity of enzyme that catalyzes the decomposition of one microequivalent of the bond involved in the reaction in 1 min (1). The reaction here under consideration is the hydrolysis of urea, which may be represented by the equation:

$$NH_2CONH_2 + H_2O \rightarrow 2NH_3 + CO_2. \qquad (2)$$

Since two bonds are hydrolyzed, one unit has been defined as the amount which catalyzes the hydrolysis of 0.5 μmoles of urea and liberates 1 μmole of ammonia.

Most of the measurements have been conducted at 25°, which was the temperature recommended in 1961 (1); this was changed to 30° in 1964 (2). The temperature is of course one of the factors determining the size of the unit--in general, the higher the temperature the smaller the unit. Some measurements have been made both at 30° and at 20° and factors will be given in the next section for interconverting these units.

The size of the unit also depends on such factors as the pH, the ionic strength, and the specific nature of the buffer salts. In order to facilitate differentiation of the units determined by the alkalimetric method in the conditions specified below from the units determined by other methods, symbols such as $\underline{U}_a^{25}$ will be used to denote the former.

Results

Fig. 1 presents representative results of experiments done to ascertain the kinetics of the reaction. The amounts of ammonia liberated by varying concentrations of urease are plotted as a function of time, and it can be seen that the plots are nearly linear to about 100 μmoles of ammonia liberated, after which there is a downward trend. This effect might be due to inhibition by product, and to test this possibility the activity of urease was determined in the presence of varying amounts of added ammonia. Table I gives the results, which show that ammonia does indeed inhibit the reaction, and very strongly. For this reason, the rate of reaction can be expected to remain constant only at low conversions.

Fig. 2 shows that amounts of ammonia liberated by increasing concentrations of urease in a 2 min reaction period. As would be expected, the results fall on a straight line up to about 50 μmoles of ammonia liberated per min and show a downward curvature beyond that point. An upper limit of 45 $\underline{U}_a^{25}$ was accordingly set for the assay system; although this limit might be raised by shortening the reaction period, this would lower the precision owing to the increased relative uncertainty in the timing of the assay operations. A lower limit of 15 $\underline{U}_a^{25}$ was set for precise measurements since the determination of lower activities would also be subject to greater relative uncertainties. Lower activities could of course be determined with no loss of precision by lengthening the assay period, but there is little reason to do so, because the enzyme concentration is already very low; also, lowering the concentration further would increase the danger of inactivation by impurities.

Twenty determinations made at the extremes of the recommended range,

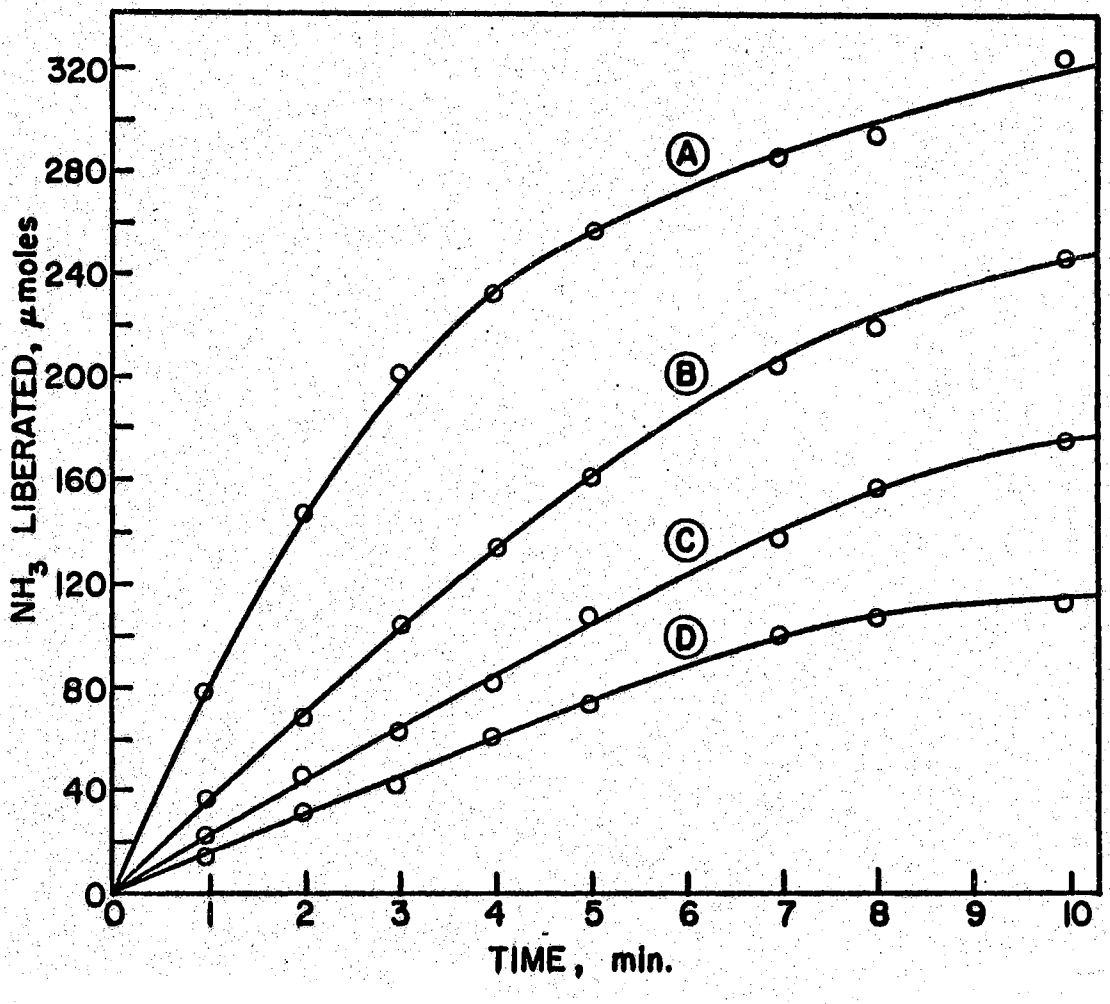

Fig. 1. Ammonia liberated in the alkalimetric assay at 25°. Concentrations of urease x 10^8 M: curve A, 10.5; curve B, 4.2; curve C, 2.8; curve D, 2.2.

TABLE I

UREASE ACTIVITY IN THE PRESENCE OF ADDED AMMONIA

36 U added to alkalimetric substrate at 25°

NH_3 added (μ moles)	Additional NH_3 in 2 min (μmoles)
0	72
59	27
118	23
236	13
472	7

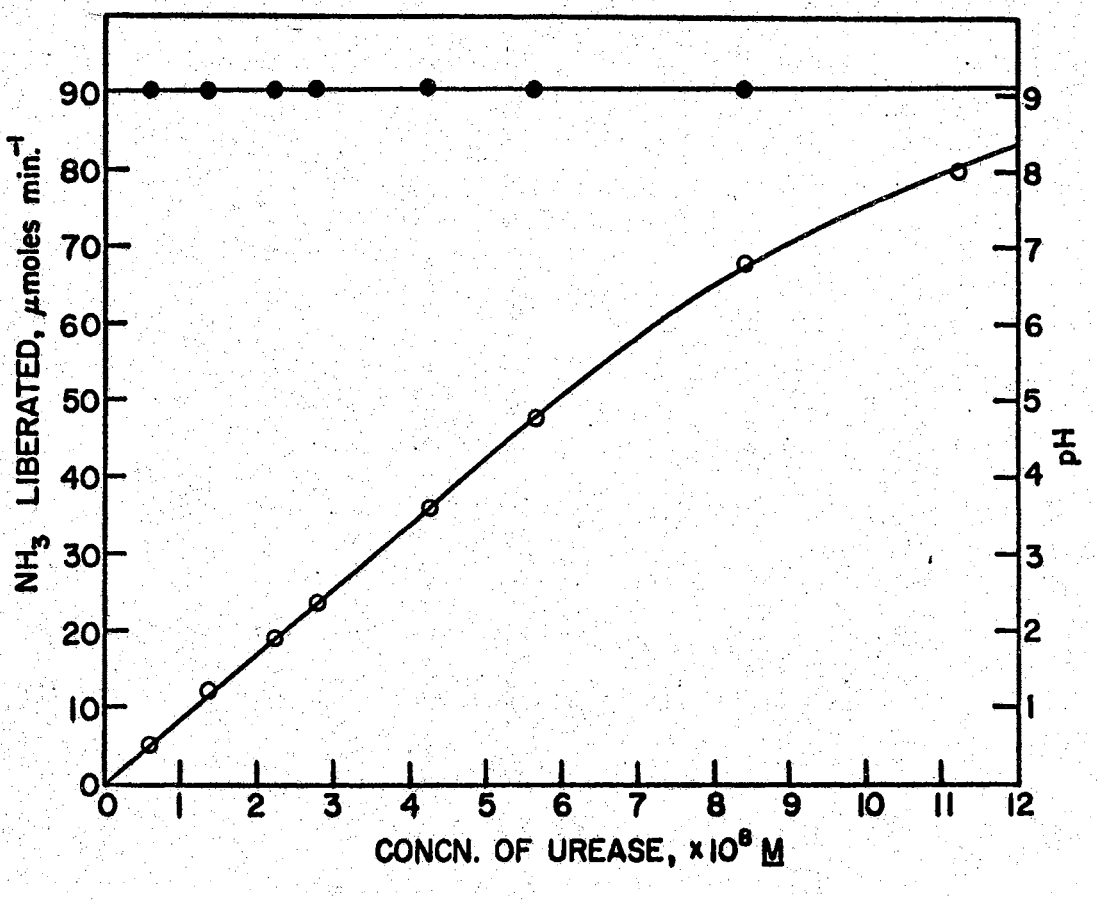

Fig. 2. Alkalimetric assay of urease at 25°. Left ordinate, empty circles: ammonia liberated, per min; right ordinate, full circles: pH of reaction mixture at end of 2-min reaction period.

15-45 $\underline{U}_a^{25}$, had an average precision of ±1% and an accuracy of ±2%.

Parallel determinations made at 20°, 25°, and 30° gave the following results: $\underline{U}_a^{25} = 1.22\ \underline{U}_a^{30}$; $\underline{U}_a^{25} = 0.79_4\ \underline{U}_a^{20}$.

Table II reports some results that make it clear why 10^{-3} M EDTA was added to the substrate; it was found to have a marked "stabilizing" effect on the activity. A sample diluted with EDTA-containing buffer showed an appreciable increase in activity over the first 2 hr and the activity then remained constant for at least 24 hr. On the other hand, an aliquot of the same sample diluted with buffer containing no EDTA showed no initial increase in activity and a 35% decrease over a 24-hr period. The data in the table also show that EDTA "protects" the urease from inhibition by 10^{-6} M Cu^{++} and Pb^{++} ions, but not from Hg^{++} and Ag^{+}.

Table III reports carefully determined values of the specific absorbancy index for five samples of urease prepared according to the directions of Mamiya and Gorin (5) without adding 2-mercaptoethanol to the extracting solvent. The determination of protein nitrogen was done both by the Kjeldahl method (6) and by the azotometric method of Matsuda and Sekita (7), which gave as good results with smaller samples. In these samples, the absorbancy maximum was at 278 mμ and the same result was also found in many other samples, obtained since 1962; the average value of the specific absorbancy (1 mg of enzyme/ml, 1 cm thickness) was 0.754±0.002. The ratio of the absorbances at 278 and 272 mμ was 1.07±0.01. The samples had an absorption minimum at 251 mμ; the ratio of the absorbances at 278 and 251 mμ was 1.99±0.06 (see Discussion).

The specific acitivity of samples 1964I-1966I was 1,920±20 $\underline{U}_a^{25}$. Most previous determinations of the activity have been made by Sumner's method and it was thought desirable to assay the present samples by

TABLE II

"ACTIVATING" AND STABILIZING EFFECTS OF EDTA ON UREASE

Urease concentration 3.5×10^{-8} M

Time after dilution, hr.	Inhibitor added	Activity: No EDTA 10^{-2} U_a^{25}/mg	Activity: 10^{-3} M EDTA 10^{-2} U_a^{25}/mg
0.25		14.3	15.4
0.75		13.8	16.3
2		13.8	17.9
4		12.4	18.4
6		12.0	18.2
24		8.96	18.0
1	10^{-6} M Pb^{++}	8.6	15.5
2		14.8	17.0
1	10^{-6} M Cu^{++}	1.8	17.3
2		2.8	17.8
1	10^{-6} M Hg^{++}	0.5	4.9
2		1.1	4.8
1	10^{-6} M Ag^{+}	0	0
2		0	0

TABLE III

SPECIFIC ACTIVITY OF UREASE PREPARATIONS (NO MERCAPTOETHANOL)

Preparation No.	Specific absorbancy[a] at 278 mμ	Specific Activity	
		Alkalimetric U_a^{25}/mg	Acidimetric S.U./mg
	(Assayed with 10^{-3}M EDTA)		
1966 I	0.759	1,860	170
1965 V	0.754	1,940	172
1964 III	0.755	1,960	173
1964 II	0.750	1,920	181
1964 I	---	1,920	172
	Ave.	1,920±20	174±3
	(Assayed without 10^{-3}M EDTA)		
1963 VI	0.752	---	153
1963 I+VI[9]	---	---	154±2[b]
1962 I-X	---	---	160±5[b]
1961 I-VIII[8]	---	---	149±8[b]
Ave.	0.754±0.002		154

[a]Based on nitrogen determination and 15.8% N content.

[b]Band on specific absorbancy at 272 mμ, 0.771.

this method (3) in order that a comparison to earlier work might be made. The average value of the specific activity was 174 Sumner units/mg. For purposes of comparison, Table III summarizes the results obtained with twenty-five preparations of urease prepared in 1961-62 (8,9); the specific activity found for these preparations was, as can be seen, 154 Sumner units. These results were obtained by a procedure which differed from that used in the present work in two respects; 0.05% serum albumin was used as protector instead of EDTA and the assay was conducted without waiting for 2 hr after dilution. When this change of procedure was made, comparison experiments showed that the present procedure would give results about 15% higher, and application of this correction brings the two sets of results into excellent agreement.

Table IV reports values of the specific activity for samples of urease that were prepared like those reported in Table III, except that 2-mercaptoethanol was added to the extraction medium. This change in the procedure may substantially increase the yields of urease, especially from certain samples of meal (5); for the meal used in the present work, the increase was by a factor of 2. It was reported that these preparations had a higher specific activity than those prepared without 2-mercaptoethanol; however, this result was calculated using a specific absorbancy of 0.771. Determination of the nitrogen content showed that the specific absorbancy of mercaptoethanol-treated urease was significantly different; when calculated on the proper basis, it is seen that the specific activity is somewhat lower than, but very close to, that of urease prepared without mercaptoethanol. Preliminary measurements have shown that mercaptoethanol-treated urease has a lower mercapto-group content.

TABLE IV

SPECIFIC ACTIVITY OF 2-MERCAPTOETHANOL-TREATED UREASE

Preparation No.	Absorbancy[a] at 278 mμ	Specific Activity	
		Alkalimetric $\underline{U}_a^{25}$/mg	Acidimetric S.U./mg
1965 IV	---	1,640[b]	151[b]
1965 III	0.639	1,590	150
1965 II	0.640	1,670	149
1965 I	0.640	1,700	158
1964 III	0.641	1,630	150
Ave.	0.640±0.01	1,650±30	152±3

[a]Based on nitrogen determination and 15.8% N content.

[b]Calculated from the specific absorbancy, 0.640.

Discussion

As has been pointed out, the results of a fixed-interval assay will be proportional to enzyme concentration only if the kinetics of reaction are of zero-order, i.e., the reaction rate is constant with time. In order to achieve this, the pH should be held constant, since the activity is in general quite dependent on the pH. In the case of urease, keeping the pH constant is very difficult, except in the special circumstance described below, because the enzyme action converts a neutral substrate to a basic product. In Sumner's assay method (4) the pH change is minimized by using very concentrated phosphate buffer, but this is not wholly satisfactory (for further discussion, see Ref. 3).

The alkalimetric assay method utilizes the observation, first made by Kistiakowsky and Shaw (10) that the pH of initially neutral, unbuffered urea-urease solution quickly rises to pH 9 and then remains nearly constant. The product at this pH is mostly ammonium carbamate, mixed with some ammonium carbonate and bicarbonate:

$$H_2NCONH_2 + 2H_2O \rightarrow (NH_4)^+ + (NH_2CO_2)^- + H_2O \rightleftharpoons 2(NH_4)^+ + (CO_3)^= \rightleftharpoons (NH_4)^+ + NH_3 + (HCO_3)^-. \qquad (3)$$

The products themselves constitute a buffer system which keeps the pH nearly constant as the reaction proceeds. By lightly buffering the substrate at pH 9 initially, one avoids a subsequent change in pH; this is shown by the pH data reported in Fig. 2. Addition of excess hydrochloric acid at the end of the assay interval stops the reaction and converts the carbamate and ammonia to ammonium ion; then, backtitration with sodium hydroxide measures the unreacted acid. Equation (1) can be

simply derived from the definition of the IUB unit and the foregoing stoichiometric relationships.

The buffering action is conveniently provided by TRIS, which has two advantages; it has no specific inhibitory effect on urease (11), in contrast to phosphate (12), and it is easily obtained in highly purified form. The pH chosen for the assay is, unfortunately, higher than the pH optimum for the enzyme, which is about 8.3 in TRIS buffer (11); but it will be clear from the foregoing discussion that to maintain the latter value without change during the assay would require a concentrated buffer, and this would introduce other problems.

The effect of EDTA can reasonably be ascribed to its ability to complex metal ions, which may be present in the buffer solution as well as in the enzyme preparation itself. The great susceptibility of urease to metal-ion inhibition has been well documented (13). It will be seen from the data in Table II that the effects are in part time-dependent; this phenomenon will be investigated further. For the present, it suffices to have shown that the addition of EDTA to the diluting buffer and substrate prevented these effects and afforded protection from reasonable amounts of some possible inhibitors.

The excellent agreement obtained for the value of the specific activity in a large number of urease preparations, obtained over a period of some years by different workers from different samples of meal in somewhat varying conditions affords considerable assurance that very nearly homogeneous preparations of enzyme have been obtained. The values given above for the specific absorbancy and activity of urease are, in our opinion, more accurate than any values reported previously. In the 1962 paper from this Laboratory (8), the absorbancy of urease

was reported at 272 mμ and it is our qualitative recollection that those samples (obtained in 1960-1961) had a somewhat broader maximum, so that the absorption at 272 and 278 was nearly the same. It may be surmised that those samples contained some impurity that slightly increased the absorbance at 272 mμ (it should be noted that the discrepancy is within the estimated uncertainty of the 1962 value). Since adventitious impurities are likely to absorb at shorter wavelengths, the maximum/minimum ratio might be a useful additional index of purity; the preparations listed in Table III show good reproductibility with respect to this criterion.

In the report on the effect of 2-mercaptoethanol on the isolation of urease (5), it was assumed that the products obtained with and without mercaptoethanol were the same. The results reported in the present paper show that this is not the case; the products are quite similar, but their differences are quite outside the limits of experimental error. Work on the further characterization of mercaptoethanol-treated urease is in progress. The preliminary results lead us to believe that these preparations contain a substantial amount of mixed-disulfide bonds, formed in the course of the isolation procedure either by oxidation, or by mercaptan-disulfide interchange with teleomeric forms of urease.

The ratio of the activities found by the alkalimetric method and by Sumner's method provides an empirical factor that can be used to compare the results: 1 Sumner unit $\simeq$ 11.0 $\underline{U}_a^{25}$.

Summary

(1) The unit of activity for urease has been defined in conformance with the recommendations of the International Union of Biochemistry

(=$\underline{U}_a$).

(2) A new assay method has been devised and tested. The substrate is 3% urea in TRIS-hydrochloride buffer of pH 9.0. In the range 15–45 $\underline{U}_a^{25}$ the results are proportional to enzyme concentration; the accuracy is ±2%.

(3) Activity measurements have been made at 20° and 30° as well as 25°.

(4) The specific abosrbancy of purest urease is 0.754 at 278 mμ (0.02$\underline{M}$ phosphate, pH 7), its specific activity is 1920 $\underline{U}_a^{25}$ (174 Sumner units)/mg.

(5) Urease isolated with the aid of 2-mercaptoethanol has a specific absorbancy of 0.640 and a specific activity of 1650 $\underline{U}_a^{25}$/mg.

Acknowledgment

This work was supported by Grants AM 06941 and GM 11,573 as well as Career Development Award 5K3-GM 13,489 (to G.G.) from the National Institutes of Health, Department of Health, Education, and Welfare. We are indebted to Dr. G. Mamiya and Professor K. Sekita for information and advice.

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CHAPTER V

SOME OBSERVATIONS ON THE ASSAY METHOD OF SUMNER

(A paper entitled "Urease. VII. Some Observation on the Assay Method of Sumner" will be published in Anal. Biochem. and is reproduced below.)

Sumner, who first isolated the enzyme urease (urea amidohydrolase, EC 3.5.1.5) in crystalline form, proposed an assay method and a definition of the activity unit that are still widely used (1). A modification of Sumner's method that utilizes titration with acid was proposed some years ago by one of the present authors (2,3). Sumner's method is of the "fixed interval" type, and the range of validity of such methods is, usually, limited. The principal purpose of this paper is to ascertain the conditions in which the assay gives results that are proportional to the enzyme concentration.

The International Union of Biochemistry (IUB) has recently made suggestions concerning the definition of enzyme units (4,5). The relation between Sumner units and IUB units will be established and the latter units then used. The relative merits of Sumner's method, the alkalimetric method described in the preceding paper (6), and some other assay methods that have been proposed will also be discussed.

Materials and Methods

Chemicals

The urease preparations, other chemicals and the 0.02M phosphate

buffer used in this work were the same as described in the preceding paper (6). Phosphate buffer, 0.68M, pH 7.0, contained 67.99 g of Na_2HPO_4, 28.00 g of KH_2PO_4 (A.C.S.-reagents) and 0.3723 g of disodium ethylenedinitrilotetraacetate (EDTA) per liter.

Sumner's assay method

The water used in these experiments was obtained from deionized steam distillate by distilling it from 0.2% alkaline permanganate and then distilling it a third time in an all-Pyrex still. The substrate contained 3.00 g of urea in sufficient 0.68M phosphate to make 100 ml. The urease solution was diluted to the proper range for assay with 0.02M phosphate, allowed to stand at least 2 hr. The urease solution, 1 ml, and the substrate, 1 ml, were mixed; after exactly 5 min, 1 ml of 1M H_2SO_4 was added quickly. A 10-25 λ aliquot (= V, in λ) was then analyzed with Nessler's reagent.

Nessler's determination was conducted according to the directions of Fister (7). The calibration curve was constructed from standard $(NH_4)_2SO_4$ solution containing 10 mg of ammonia-N/ml; aliquots were added to 10 ml of ice-cold water in 25-ml volumetric flasks, then 10 ml of Nessler's reagent was added and additional ice-cold water to the mark. The flasks were transferred to a thermostat at 21° and the transmittance at 415 mμ was measured after 10 min with a Coleman Model 6A Spectrophotometer. The amount of ammonia-N liberated was determined from the calibration curve and the dilution factor (= 3000/V).

Acidimetric assay method

The reaction of enzyme and substrate was conducted in exactly the

same way as described for Sumner's assay method, except that singly redistilled deionized steam distillate could be used for making up the solutions. After the 5-min reaction period the assay mixture was titrated as quickly as possible with 0.1M HCl and bromcresol green indicator to a greenish-yellow end point.

Definition of Activity Unit and Calculation of Activities

The products of enzyme-catalyzed hydrolysis ~~are mainly ammonium~~ and bicarbonate ions:

$$H_2NCONH_2 + 2H_2O + (H_2PO_4)^- \longrightarrow 2(NH_4)^+ + (HCO_3)^- + (HPO_4)^=.$$

By Nessler's determination, the ammonia is determined directly. In the acidimetric method, two moles of acid are consumed per mole of urea hydrolysed.

Sumner defined the unit of activity as the amount that liberates 1 mg of ammonia-nitrogen at 20° in 5 min (1). The IUB recommendation is that the unit be the amount that catalyzes the decomposition of 1 μequivalent of the bond involved in 1 min (2). The symbol $\underline{U}_s^{20}$ will denote the unit so defined, determined at 20° (although a temperature of 30° is recommended (3), the definition can apply to any temperature); the subscript is intended to help differentiate this unit from those determined in other substrates (6). The stoichiometric relation between the two units is:

$$1 \text{ Sumner unit} = 1000/14.01 \ (\mu\text{moles } NH_3/\text{mg N}) \times 1/5 \ (\text{min}^{-1}) \ \underline{U}_s^{20}$$
$$= 14.28 \ \underline{U}_s^{20}$$

The activity measured by Nessler's determination is calculated by the expression:

$$\text{Activity (in } \underline{U}_s) = 1000\ \underline{W}/(14.01 \times 5) = 14.28\ \underline{W};$$

where $\underline{W}$ is the weight of ammonia-nitrogen, in mg, liberated in the assay. The activity measured by the acidimetric assay is given by:

$$\text{Activity (in } \underline{U}_s) = 200\ (\underline{V}_s - \underline{V}_b)\ \underline{M}_{HCl};$$

where $\underline{V}_s$ and $\underline{V}_b$ are the volumes of acid of molarity $\underline{M}_{HCl}$ consumed by the sample and blank, respectively.

Results

Fig. 1 shows the results of a representative experiment that was done to determine in what range the amount of ammonia liberated in the assay would be directly proportional to the enzyme concentration. It is seen that the line is sensibly constant up to about 40 µmoles/min, i.e., 40 $\underline{U}_s$ (a total of 200 µmoles in the 5-min period). The pH of the reaction mixture at the end of that period was measured, and the results are also plotted in this graph; it is seen that the pH changes appreciably, even in the range in which the product is proportional to enzyme concentration.

The precision attained in the present work by Nessler's determination was not very satisfactory; it averaged ±3%, in the calibration determinations as well as in the measurement of enzymatic activity. The precision attained in the acidimetric method averaged ±2% in a large number of trials.

Discussion

Urease converts neutral urea to basic ammonia, and the activity of the enzyme is very high. This makes it difficult to keep the pH

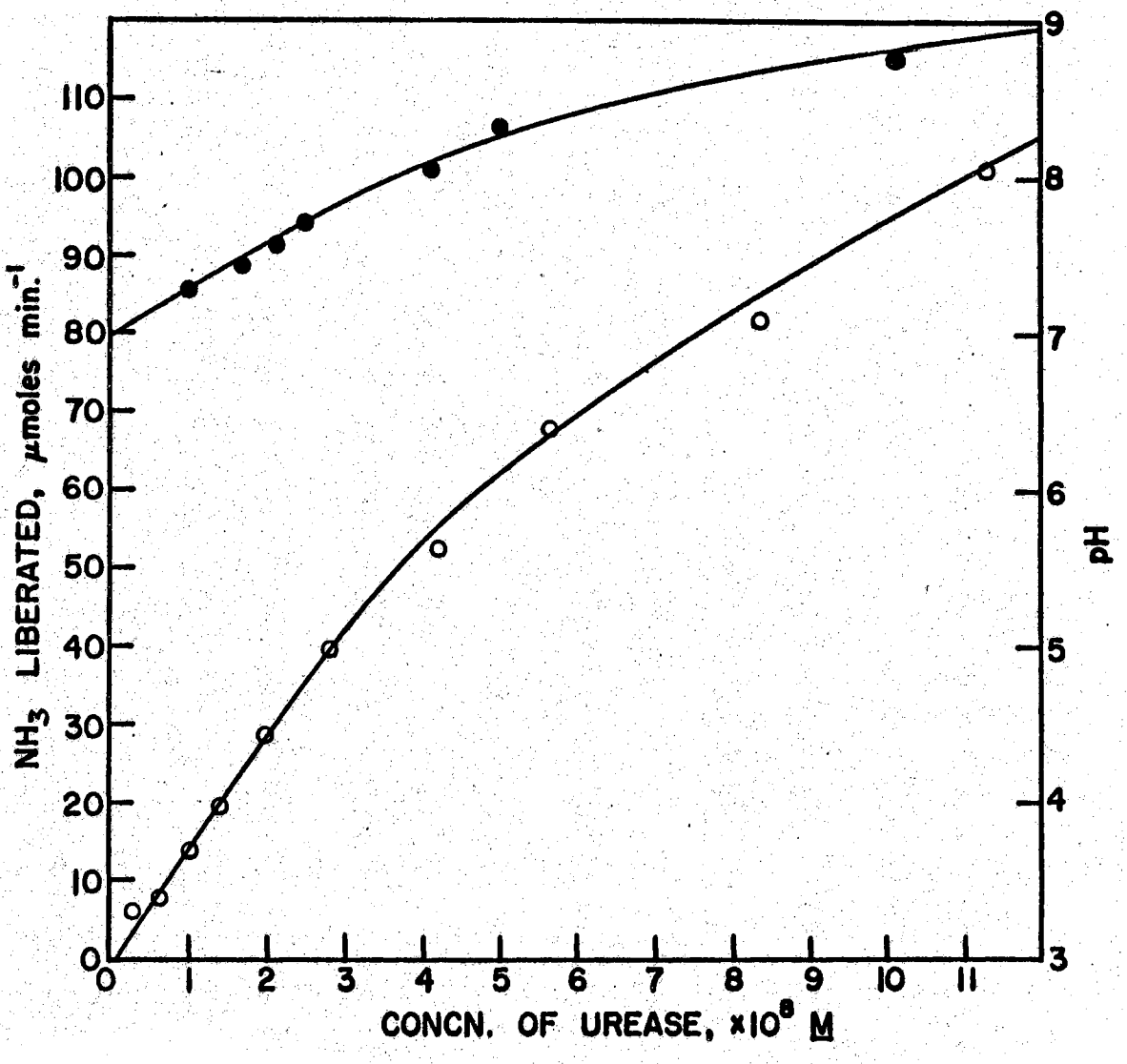

Fig. 1. Sumner's assay of urease at 25°. Left ordinate, empty circles: ammonia liberated, per min, in Sumner's substrate; right ordinate, full circles: pH of reaction mixture at end of 5-min reaction period.

constant for purposes of quantitative assay. If the urease solution is made relatively concentrated, the pH change will be excessive; if, on the other hand, the urease solution is made dilute, the danger of accidental inhibition or denaturation becomes very great. Thus the assay, which is simple in principle, has given considerable trouble in practice.

In Sumner's assay, the pH change is minimized by using very concentrated buffer. The data in Fig. 1 show, however, that substantial changes in pH occur nevertheless. At the same time, other complications are introduced, for the components of the buffer exert specific effects on the enzyme. According to Kistiakowsky *et al.*, $(H_2PO_4)^-$ is a strong inhibitor (8), while the other components, $(HPO_4)^=$, Na^+, K^+, and NH_4^+ possibly have some effect, albeit less marked (9). Apparently, these effects balance to some extent, for it may be seen that the results of the assay are proportional to the enzyme concentration over a range greater than that in which the pH remains constant; it is a tribute to Sumner's insight that he chose conditions in which this degree of balance would be realized. However, the complexity of the kinetics must be considered a disadvantage. Other practical disadvantages of the method were the cooperative inconvenience and limited reproducibility of Nessler's determination.

The acidimetric assay method was developed to avoid the defects of Nessler's determination. Unfortunately, its range is limited. The original papers describing this method (2,3) stated that it could be applied to as much as 4 Sumner units (56 U_s), but the present data show that this is not correct; for accurate determinations the limit should be about 35 U_s. At the other extreme, the difference in titer between sample and blank should be no less than about 0.06 ml of 0.1M acid,

corresponding to 12 $\underline{U}_s$.

A disadvantage of Sumner's substrate is that the necessity for high concentrations of phosphate and the susceptibility of urease to inhibition places a very stringent requirement upon the purity of the phosphate salts. Sumner, in at least some of his work (10), found it advisable to use 2% gum arabic as a "protector" against inhibition, and it seems likely that inhibition may have been a cause of the low results obtained in much other work (see Reference 2 for bibliography). It should be noted that $10^{-3}\underline{M}$ EDTA was used in all of the work reported in this paper; reference should be made to the preceding paper (6) for a demonstration of the effect of this substance.

In view of the foregoing considerations, the writers feel justified in concluding that Sumner's assay method, although valid in the proper range, compares unfavorably with the alkalimetric method described in the preceding paper (6) and in recommending the latter method when the highest precision is required. Since the acidimetric modification of Sumner's method involves fewer operations, however, it may continue to find useful application when the saving of time and labor is an important consideration; care should be exercised not to exceed its range. The factor given in the preceding paper (6) makes it possible to compare and interconvert the results of the two methods.

Summary

(1) The following relationship has been established: 1 Sumner unit = 14.28 $\underline{U}_s^{20}$ when $\underline{U}_s^{20}$ is the unit defined in accordance with the recommendations of the International Union of Biochemistry.

(2) Sumner's method of assay gives results proportional to the enzyme

concentration to about 35 U_s.

(3) The useful range of the acidimetric modification of Sumner's method is 12-35 U_s.

(4) The relative merits of the aforementioned methods have been discussed.

Acknowledgment

This work was supported by Grants AM 06941 and GM 11,573 as well as Career Development Award 5K3-GM 13,489 (to G.G.) from the National Institutes of Health, Department of Health, Education, and Welfare.

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CHAPTER VI

THE INTERACTION OF UREASE WITH SODIUM DODECYL SULFATE

(This paper has been written in the form suitable for publication as a short communication in Biochim. Biophys. Acta.)

A molecular weight of 483,000 has been reported for urease[1] (urea amidohydrolase, EC 3.5.1.5.), but there are reasons for believing that this value refers in fact to comparatively stable aggregates of smaller subunits[2,3]. This communication reports some experiments in which urease was treated with SDS; it augments a previous preliminary report[4]. The results indicate that SDS causes dissociation of the 500,000-weight particles, thus supporting the view that they are aggregates.

Urease was prepared as previously described (2-mercaptoethanol was added to the extracting solvent)[5], crystallized three or four times. It was dissolved in 0.02M phosphate (pH 7)-10^{-3}M EDTA[6]. Activity was determined by the acidimetric modification of SUMNER's method; the specific activity was 2,060 IUB units/mg at 20° (equivalent to 150 SUMNER units)[6]. SDS was a specially prepared sample, containing >98% C_{12} (kindly supplied by Dr. M. Konort, Lever Brothers Laboratories, Edgewater, N. J.).

The results depended on the concentration of the reagents, their ratio, and the length of interaction. Fig. 1a shows the ultracentrifugal

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Abbreviations: SDS, sodium dodecyl sulfate; EDTA, disodium ethylenedinitrilotetraacetate; IUB, International Union of Biochemistry.

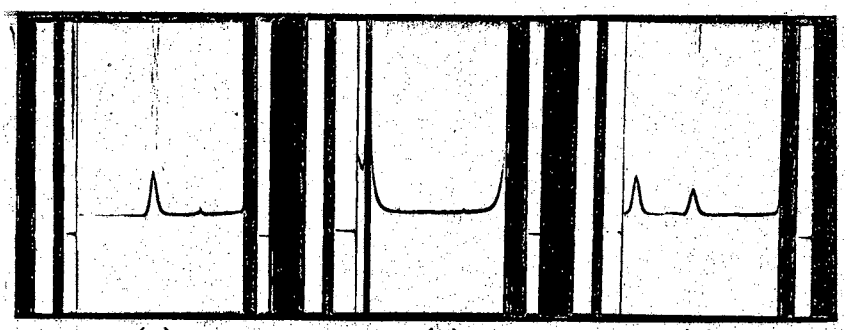

Fig. 1. Ultracentrifugal patterns of native urease and SDS-urease: (a) native urease; (b) SDS/urease ratio 4.5, 6 hr after mixing; (c) ratio 0.5, after 6 hr. Speed 59,780, 75° schlieren angle, (a) and (c) taken 20 min after attaining speed, (b) after 40 min.

pattern given by native urease, 6 mg/ml; the sedimentation coefficient $s_{20,w}$ was 18. Fig. 1b shows the pattern obtained in the presence of SDS in the proportion 4.5:1 by weight, 6 hr after mixing; the urease was completely converted to a product with sedimentation constant $s_{20,w}$~2. This reaction mixture had essentially no enzymatic activity both when this was determined after dilution with SDS-containing buffer and after dilution with buffer alone (SDS *per se* did not have much effect on the activity, since urease diluted with SDS-containing buffer and assayed *immediately thereafter* exhibited 85-90% as much activity as a control with no SDS).

When the proportion of SDS was reduced to 0.5:1, the results were quite different. After 6 hr, ultracentrifugal analysis (Fig. 1c) gave two peaks, of $s_{20,w}$ 3.3 and 17, respectively, and not much further change occurred in, say, 36 hr. About 70% of the activity remained 6 hr after mixing, 50% after 36 hr. These results indicate that SDS in the aforementioned ratio converts some 30% of the native urease into an *s*-3 product (the areas of the peaks in Fig. 1a and 1c indicate ~40% conversion; the peaks in Fig. 1c should not be directly compared to one another, for the area of the slower peak reflects in part the mass of bound SDS).

Fig. 2 shows results at some intermediate ratios. Figs. 2a,b were obtained at SDS/urease ratio 1.6, respectively 1 and 6 hr after mixing; Fig. 2c,d at ratio 1.2, respectively 6 and 36 hr after mixing. The latter ratio is seen to be just short of that needed to affect complete conversion to the low-*s* (2.1-2.3) product. Fig. 3 represents the changes in viscosity and activity for the 1.2 ratio as a function of time. The change is gradual and there is at least a qualitative

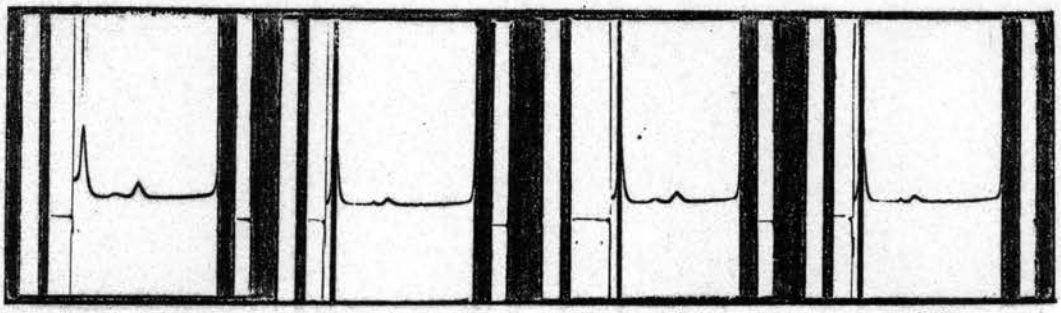

Fig. 2. Ultracentrifugal patterns of SDS-urease. 59,780 rpm, 75^{0} angle. SDS/urease ratio 1.6: (a) 1 hr after mixing; (b) after 6 hr. Ratio 1.2: (c) after 6 hr; (d) after 36 hr. All pictures taken 20 min after attaining speed.

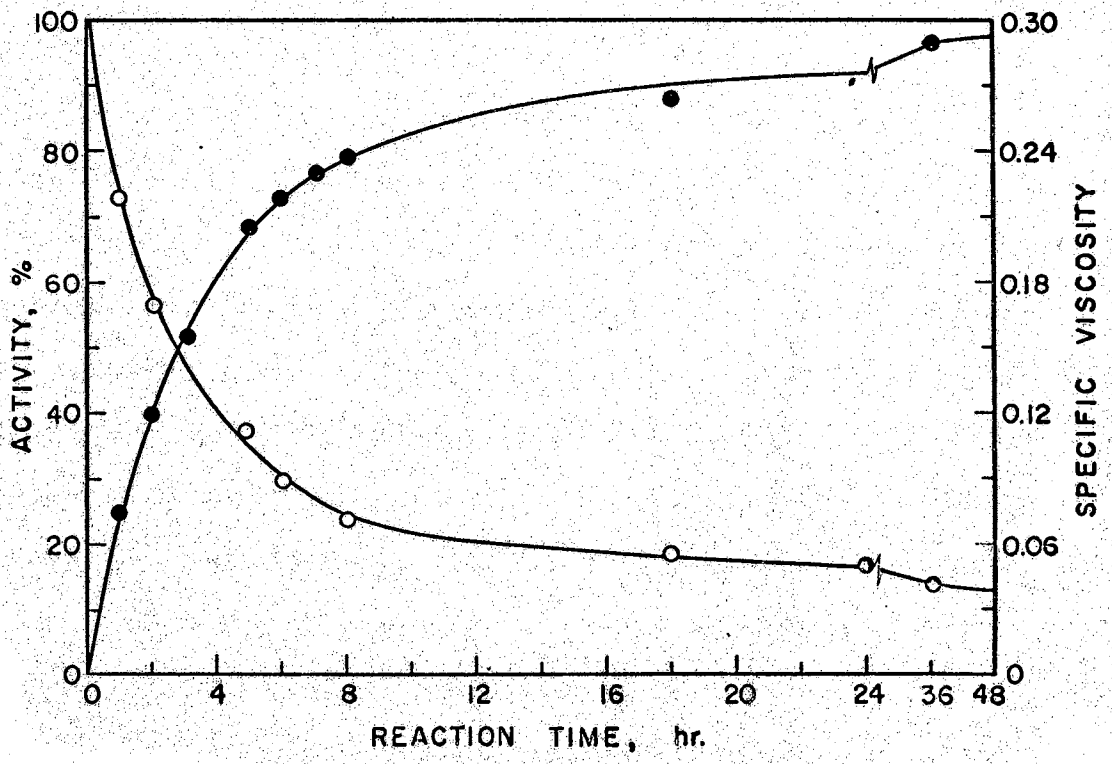

Fig. 3. Changes in viscosity and activity of SDS-urease in ratio 1.2. Left ordinate, empty circles: activity, % of control containing no SDS; right ordinate, full circles: specific viscosity.

correspondence between the decrease in activity, the increase in viscosity, and the conversion from *s*-17 to *s*-2. At ratio 1.6, the course of the reaction was qualitatively the same, but the rate of reaction naturally was faster and the activity after 36 hr very nearly 0.

To estimate the weight of the sedimenting species, use was made of the expression derived by SCHERAGA AND MANDELKERN[7]:

$$M = [s_o N \eta_o / \beta \ (1 - \bar{V})]^{3/2} \ [\eta]^{1/2}$$

This was first applied to native urease, for which $\bar{V}$=0.73[1]. The following values were obtained: s_o 18.6, $[\eta]$=0.0405 dl/g. The axial ratio calculated by Polson's equation[8] for prolate ellipsoids is 3.98 and the corresponding value of β[7], 2.2×10^6. M is then 520,000, which is in fair agreement with the accepted value[1] (REITHEL AND ROBBINS[9] have found a value close to 500,000 by sedimentation equilibrium measurements).

Measurements were then made on the 1:1.2 SDS/urease mixture, 48 hr after mixing, by which time the viscosity had become constant; dilutions were made as needed for extrapolating to infinite dilution. $[\eta]$ was provisionally estimated on the basis that the complex contained all the SDS and found to be 0.10; s_o = 3.8; $\bar{V}$ was taken as 0.776 (based on a value of 0.83 for SDS[10], assuming additivity). This leads to an axial ratio of 9.5, $\beta = 2.4 \times 10^6$, M=89,000; the weight of the urease subunit would then be 40,000. The assumption that the complex contained all the SDS is very much open to question, but the result does not depend critically on this assumption, because there are compensatory changes in $[\eta]$ and $\bar{V}$; if, for purposes of illustration, one assumes an SDS/urease ratio of only 0.6, the weight of the urease subunit would be 50,000. There are other uncertainties as well, but the results leave

little doubt that SDS causes dissociation to a subunit of much smaller weight. REITHEL et al.[2] found a value of 83,000 for the weight of the subunits obtained by treating urease with 6M guanidine hydrochloride; SDS causes dissociation to subunits that may be yet smaller and certainly are no larger.

Unfortunately, the action of SDS on urease is slow and, at least to a large extent, irreversible; for these reasons and the fact that there remain theoretical uncertainties in the interpretation of buffer-detergent-protein systems, a more quantitative study of the phenomenon is not being attempted at this time.

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CHAPTER VII

THE DISSOCIATION OF UREASE IN ACETATE BUFFER OF pH 3.5

(This paper is written in the form suitable for publication in a journal.)

Urease (urea amidohydrolae, EC 3.5.1.5), with a molecular weight of 483,000, has been dissociated into 6 subunits by treatment with 6M guanidine-hydrochloride (1), but this treatment caused complete loss of the enzymatic activity. Creeth and Nichol (2,3) reported that their urease preparations contained, in addition to the 19s component corresponding to the 483,000 form, two higher components, 28s and 36s, and two lower components, 12s and 4-6s. The existence of an active 12s component was demonstrated by Sehgal et al. (4) with the sucrose density gradient method and by Hill and Elliott (5) with CM-cellulose chromatography; in these investigations, the lower component existed together with the native and still higher forms and comprised only a few per cent of the total. Stewart and Craig (6) have recently announced obtaining a low molecular-weight urease of 8.5s in high yield by an isolation procedure which precipitated the enzyme from an aqueous extract with 0.1% 2-mercaptoethanol, NaCl and polyethyleneglycol-400 and then subjected the product to DEAE-cellulose chromatography; however, no detailed description of the work has yet appeared.

In the present investigation urease was dissociated in acetate buffer of pH 3.5. The resultant subunits still had enzymatic activity,

which was lost rather slowly. On raising the pH to 7, the original activity could not be entirely restored. The loss of activity at pH 3.5 and the loss of reversibility did not develop at the same rate. Sedimentation velocity and viscosity determinations indicate that the inactivation is accompanied by unfolding of the peptide chains in the dissociated state. The subunits obtained at pH 3.5 are estimated to have a weight of 240,000.

Materials and Methods

Urease was prepared from jack bean meal by the procedure of Mamiya and Gorin (7) without adding 2-mercaptoethanol to 32% acetone. After four crystallizations, the urease crystals were stored as a suspension in the acetone-citrate mother liquor at 4°. The specific activity of a typical preparation was 1,890 $\underline{U}_a^{25}$ or 172 S.U. (8).

Disodium ethylenedinitrilotetraacetate (EDTA) was obtained from Eastman Chemicals; all other chemicals were of A.C.S.-reagent grade. Acetate buffer solutions were prepared according to Walpole (9); all other buffer solutions were described in earlier papers (8,10,11).

Solutions of urease were prepared by centrifuging, separating the acetone-citrate mother liquor and dissolving the urease crystals in a suitable amount of triply distilled water (7). Aliquots of this solution were immediately mixed with 0.2M acetate buffer to obtain the desired concentrations of urease as well as of buffer. After standing for the specified time at room temperature, 20-4°, an aliquot of the reaction mixtures were analyzed in a Spinco Model E Ultracentrifuge at 20° and 59,780 rpm using schlieren optics; another aliquot was brought to pH 7 with 1M NaOH and ultracentrifuged also.

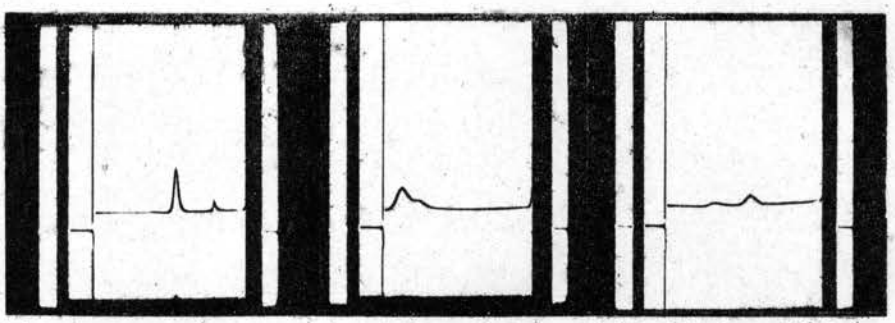
The enzymatic activity was determined both by Sumner's method (12) in pH 7.0 phosphate buffer and by the alkalimetric method of Gorin and Chin in pH 9.0 TRIS buffer (8). Measurements were also made in pH 3.5 acetate buffer; in this case, 1 ml of diluted urease was mixed with 1 ml of 3% urea in buffer, after 5 min, 1 ml of 1M H_2SO_4 was added to stop the reaction, and the amount of liberated ammonia was determined by Nesslerization.

The measurement of viscosity was made in a Cannon-Fenske 50 semi-micro viscometer at 20°. The specific gravities of solvents and urease solutions were determined with a Nicol pycnometer.

Results

Urease at 0.5% concentration in phosphate buffer at pH 7 gave the ultracentrifugal pattern shwon in Fig. 1a; the principal peak has a sedimentation coefficient s of 18.4 (this and all subsequent values are in Svedbergs), in accordance with the findings in several previous investigations (2,4,5,7,13). A minor component of s-27 also is present; this disappears on treatment with 2-mercaptoethanol (7) or sulfite (2,3) and is probably a disulfide dimer. The activity of the urease determined by the alkalimetric method of Gorin and Chin was 1,890 International Units at 25° ($=U_a^{25}$) (8). The activity was also measured by the method of Sumner at 25° and found to be 2,460 International Units ($=U_s^{25}$), equivalent to 172 Sumner units at 20° (12).

In preliminary experiments, urease crystals were dissolved in water and immediately mixed with acetate buffers of various concentrations and pH -- more than 20 combinations were tested, varying from pH 3.1 to 5.7 and from 0.02 to 0.2M. After specified intervals, the



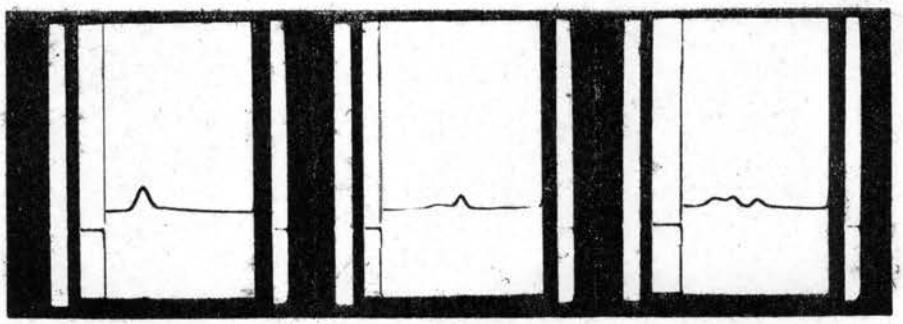
(a) 18.4s 27.5s (b) 4.5s 8.5s (c) 11.2s 19.2s

Fig. 1. Ultracentrifugal patterns of native and dissociated urease: (a) native urease at pH 7.0 in 0.02M phosphate buffer; (b) urease at pH 3.1, (c) at pH 4.2 in 0.1M acetate buffer for 1 hr. Speed 59,780 rpm, 75° schelieren angle. All pictures taken 20 min after attaining speed.

solutions were subjected to ultracentrifugal analysis. At the same time, the enzymatic activities were determined; this was done by first diluting an aliquot of the acetate buffer with 0.02M phosphate buffer of pH 7, and then assaying the latter by the aforementioned methods. Different results were obtained in each medium and the relationship between the variables is not as yet clear. For this reason, the results will not be reported in detail. In general, lowering the pH and increasing the buffer concentration favored dissociation, as indicated by the development of lower-s peaks, and the activity also decreased, but not in a parallel manner. Since the effect of pH is very marked, it is necessary that this be very carefully established.

Some representative results are shown in Fig. 1b,c. At pH 3.1 and 0.1M acetate, a peak of s = 4.5 was obtained in 1 hr's time, but it can be seen that there are other components; nearly all the activity was lost in this time. Over a longer period of time, a single peak of s = 4.3 was obtained. At pH 4.2, partial conversion to an 11.2s product occurred in 1 hr's time, with little loss of activity.

The results that seem most promising were obtained at an intermediate pH, pH 3.5, and 0.1M acetate. In this medium, rapid conversion to a product of 9.8s occurred in 1 hr's time (Fig. 2a) and there was little loss of the enzymatic acitivity assayed at pH 7 (10% or less). If an aliquot portion of the pH 3.5 solution, after 1 hr, was adjusted to pH 7 with 1M sodium hydroxide, the pattern shown in Fig. 2b was obtained, i.e., the 18s peak was restored to about 3/4 its original area (after correction for dilutions), but a smaller peak of 11s also was found; in addition, traces of more slowly sedimenting material may be seen. Fig. 2c shows the pattern obtained when the urease solution of



(a) 9.8s (b) 11.3s 18.2s (c) 10.5s 17.8s

Fig. 2. Ultracentrifugal patterns of dissociated and reassociated urease: (a) urease at pH 3.5 in 0.1M acetate buffer for 1 hr; reassociated urease by bringing back to pH 7 after (b) 1 hr, (c) 6 hr in 0.1M acetate buffer of pH 3.5. Speed 59,780 rpm, 75° schlieren angle. All pictures taken 20 min after attaining speed.

pH 3.5 was kept for 6 hr, then adjusted to pH 7 and analyzed; the area of the 18*s* peak was further reduced, smaller-*s* peaks become more pronounced.

Urease exhibits enzymatic activity even at 3.5. When this was measured as soon as possible after preparing the solution, the activity was some 40% of that exhibited at pH 7. The activity was determined again at intervals with the results represented in Fig. 3, curve A; as can be seen, a *gradual* decrease occurred, about 30% in 6 hr and 50% in 24 hr.

The activity was also determined after restoration of the enzyme to pH 7, and the results of these experiments were most surprising. As can be seen in Fig. 3, curve B, the loss of the activity measured in these conditions was much faster, more than 50% in 6 hr and 95% in 24 hr. It should be stressed that the assays at pH 3.5 and 7 were made with aliquots of the *same* solution, and that appropriate control experiments were made to exclude the possibility that the activity measured at pH 7 was lowered by accidental contamination.

The viscosity of 0.5% urease in 0.1*M* acetate of pH 3.5 increased gradually with time. The reduced viscosity was 0.049 dl/g at the beginning and it reached a limiting value of 0.164 dl/g in 30 hr. The sedimentation coefficient decreased gradually at the same time.

When the viscosity and concentration had reached sensibly constant values, the solution of urease was diluted to ascertain the effect of concentration. The results are shown in Fig. 4; the extrapolated value, i.e., the intrinsic viscosity $[\eta]$ was 0.066 dl/g, and $s^{o}_{20,w}$, 9.8. The corresponding values for native urease at pH 7.0 were 0.045 and 18.6.

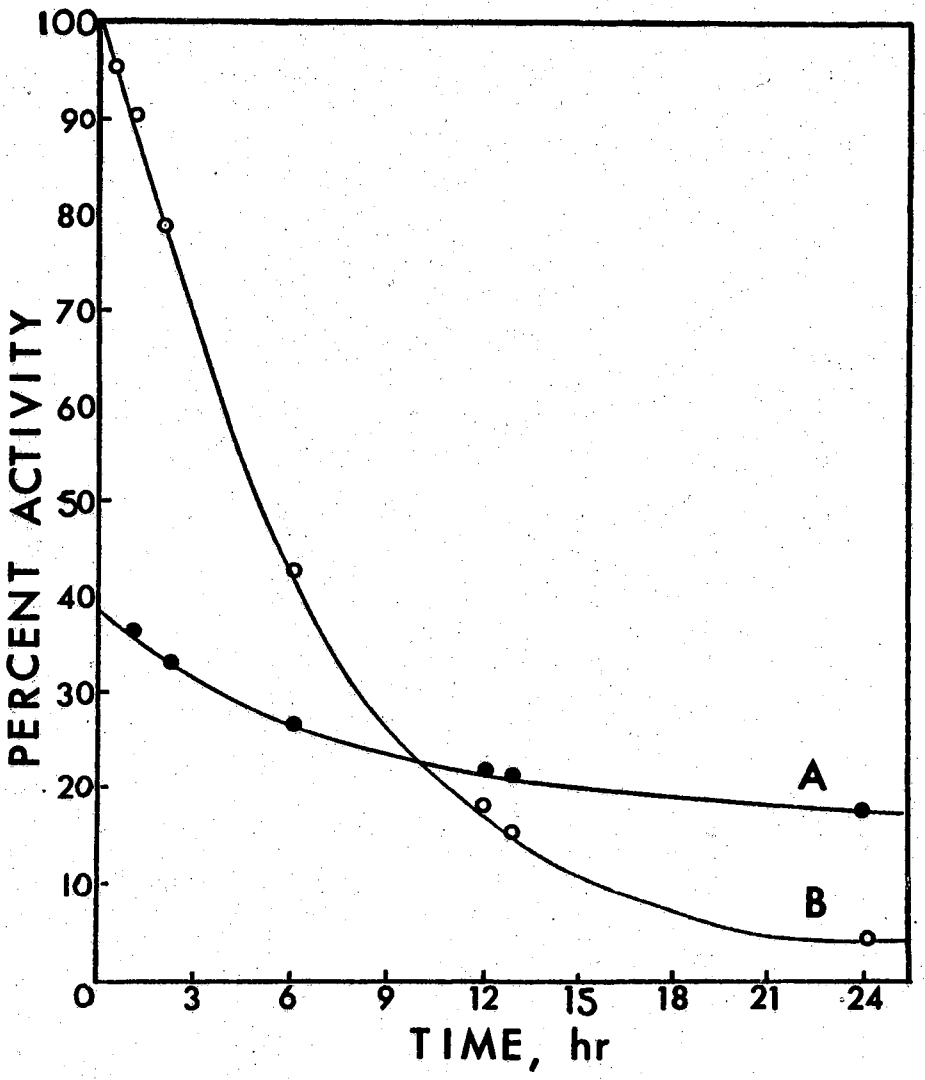


Fig. 3. Changes of enzymatic activity with time for dissociated and reassociated urease: •, curve A, in 0.1M acetate buffer of pH 3.5 and assayed at pH 3.5 in 0.1M acetate buffer; o, curve B, diluted with 0.02M phosphate buffer and assayed at pH 7 in phosphate buffer or at pH 9 in Tris buffer.

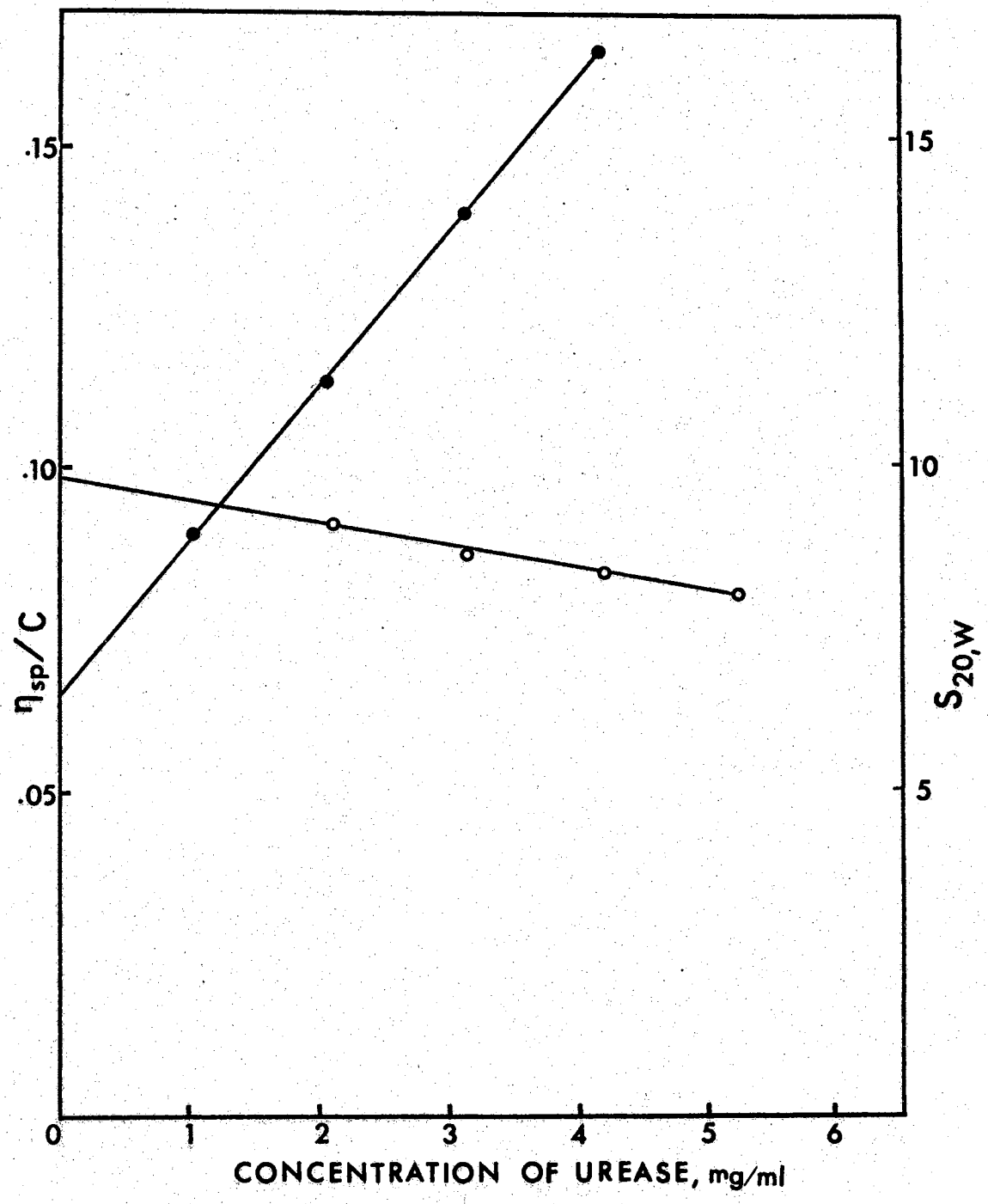


Fig. 4. The reduced viscosity and $s_{20,w}$ of urease in 0.1M acetate buffer of pH 3.5. ●, left ordinate, reduced viscosity; o, right ordinate, $s_{20,w}$

The weight of the subunit of urease produced in acetate buffer of pH 3.5 was then calculated according to the equation of Scheraga and Mendelkern (14);

$$\underline{M} = \left(\frac{\underline{s}_o \underline{N} \eta_o}{\beta(1-\underline{\bar{V}})} \right)^{3/2} [\eta]^{\frac{1}{2}}$$

The result was 240,000. Where η_o in the equation was determined to be 1.009×10^{-2}, $\underline{\bar{V}}$ is 0.73 as determined by Sumner (13), ρ is 1.000 and the axial ratio is 7.1 by Polson's equation (15). The parameter β thus is found to be 2.30 from Perrin's table (14). $\underline{N}$ is Avagadro number.

Discussion

The present data indicate that urease of mol.-wt. 483,000 can be cleaved into 2 subunits by treatment with acetate buffer of pH 3.5, and that these subunits have enzymatic activity. The first observation is not surprising; several proteins and enzymes have been dissociated into subunits by the combined effect of pH and of interaction with the buffer components. The isoelectric point of urease is 5.0-5.1 in acetate buffer (16); this means that at pH 3.5 the charge on the molecule is opposite that at pH 7 or 9, and this might well cause a drastic change.

For this very reason, it is on the other hand quite surprising that the molecule still has enzymatic activity; while the catalytic activity is less than at pH 7, it is, on the absolute basis, still very high. Even more surprising is the observation that the rates of loss of the activity measured at pH 3.5 is considerably less than the loss of activity measured at pH 7.

This leads to the conclusion that the mechanisms of the enzymatic

action at the two pH values must be different. Since the mechanism of action of urease is not understood, one can only speculate on the nature of this difference by analogy with what is known about other enzymes. It is now generally accepted that the superior catalytic activity of enzymes is due to the concerted action of two or more groups, which are held in an appropriate relative position by the so-called secondary and tertiary structure. In the case of a hydrolytic reaction, such as that catalyzed by urease, it is reasonable to suppose that the groups in question might be basic and acidic respectively. Now, it is possible that in the more acid medium the acidic group of the active site would no longer be necessary, since the acidic buffer component can substitute for it. The catalytic action would be less efficient, but on the other hand it would not be critically affected by alterations in the tertiary structure. This is in accordance with the experimental facts.

Summary

Native urease is dissociated into 2 subunits of 240,000 mol.-wt. in 0.1M acetate buffer of pH 3.5. The resultant subunits still have enzymatic activity, which is lost rather slowly. On raising the pH to 7, the original activity cannot be entirely restored. The loss of activity at pH 3.5 and the loss of reversibility do not develop at the same rate. Sedimentation velocity and viscosity determinations indicate that the inactivation is accompanied by unfolding of the peptide chain in the dissociated state.

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CHAPTER VIII

SUPPLEMENTARY MATERIAL

This chapter presents some fragmentary information that was obtained in the course of the work previously described but that was not included in the papers for various reasons.

Characteristics of the Jack Bean Meals

The paper of Mamiya and Gorin (1) mentions the fact that jack bean meals from various sources gave different yields of urease. One of the important factors is the means of grinding, another the age of the meal. The following observations summarize briefly the behavior of the various meals; all the jack beans referred to below were grown by Mr. Ernest Nelson, Route 1, Waldron, Arkansas.

(1) Beans purchased in 1959, ground in 1960. The beans were first chopped to pea-sized pieces in a motor-driven stainless-steel food chopper (Model 4222, Hobart Mfg. Co., Troy, Ohio) and then ground to a fine powder of about 16 mesh in a stainless-steel hammer mill (Micro-pulverizer Type CF, Metal Disintegrating Co., Pulverizing Machinery Division, Summit, N. J.). The meal, 1 g, extracted with 100 ml of water at room temperature for 10 min and filtered, gave an aqueous extract with activity 2.1 S.U./ml. The average of ten preparations made in 1962 was as follows: 32% acetone extract had an activity of 20 S.U./ml; after separation of the urease precipitate the supernatant

contained 9 S.U./ml; after 4 crystallizations, the yield was 10 mg/100 g of meal and the specific activity was 160 S.U./mg. For the six preparations made in 1963, the average results were: 32% acetone extract contained 18.5 S.U./ml and the supernatant 8.5 S.U./ml; after 4 crystallizations, the yield was 10 mg/100 g of meal and the specific activity was 154 S.U./mg.

(2) Beans grown in 1963, ground in 1963. The beans were ground at Oregon State University, Corvallis, Oregon. The powder was of about 28/3 mesh. The aqueous extract contained 2.1 S.U./ml. The average of four preparations made in 1964-1965 was as follows: 32% acetone extract had 19 S.U./ml and the supernatant 6 S.U./ml; after 4 crystallizations, the yield was 15 mg/100 g meals and the specific activity was 175 S.U./mg. The average of five preparations done with 0.1% 2-mercaptoethanol in the extracting solvent was as follows: 32% acetone extract had 19 S.U./ml and the supernatant 1 S.U./ml; after 4 crystallizations, the yield was 25 mg/100 g of meal and the specific activity was 152 S.U./mg.

(3) Beans grown in 1963, ground in 1966. The beans were first chopped to pea-sized pieces in stainless-steel meal chopper (Model 5028, Globe Slicing Machine Co., Stanford, Conn.) at the Meal Laboratory of Oklahoma State University and then ground to fine powder of about 16 mesh in a Mikro-Pulverizer (Ser. No. 6067, Pulverizing Machinery Co., Roselle Park, N. J.). The aqueous extract had 2.0 S.U./ml. For the two preparations made in 1966, the average results was follows: 32% acetone extract had 19 S.U./ml and the supernatant 4 S.U./ml; after 4 crystallizations, the yield was 18 mg/100 g of meal and the specific activity was 150 S.U./ml.

(4) Beans grown in 1965, gound in 1966. A 50-lb portion of the beans were ground in a Jay-Bee Mill at Bethany-Nazarene College in Oklahoma City. The beans were ground to a fine powder of about 16 mesh in about 1 hr, without previous coarse grinding. The temperature of the powder as it issued from the grinder was higher than 70°. The 32% acetone extract of this meal contained 12 S.U./ml and the supernatant 11 S.U./ml. No urease crystals could be isolated from this extract. Another 13-lb portion of the beans was shipped to Oregon State University and ground there like the 1963 beans. The remaining beans were ground here as stated in (3). The aqueous extract of both meals had 2.0-2.1 S.U./ml. The average of five experiments was as follows: 32% acetone extract contained 16 S.U./ml and the supernatant had 13 S.U./ml; the yield of urease was only about 1-2 mg/100 g of meal.

The Protective Effect of EDTA on Urease Against the Oxidation by Air

One portion of 2 ml urease containing 10^{-3} M EDTA and another portion containing no EDTA were poured separately in two shallow dishes giving a layer 1 mm thick; the dishes were covered with a piece of parafilm with five fine holes in it. After 24 hr in the refrigerator, the samples were assayed and examined by ultracentrifuge.

The sample solution containing no EDTA gave a visible precipitate after 12 hr; after centrifuging, the supernatant was assayed. The enzymatic activity decreased from 1,000 to 392 S.U./ml during the oxidation, and the protein concentration from 5.65 to 4.72 mg/ml; i.e., the specific activity decreased from 179 to 83 S.U./mg. In the sample containing EDTA, no significant change occurred.

The ultracentrifugal patterns showed two peaks of 18s and 27s; the ratio of the areas was approximately 10:1 before exposure to air. After exposure, the sample containing no EDTA had an additional peak of 35s and the area of the 18s component was reduced to half.

It is clear from the above results that the oxidation by air can cause the polymerization of 18s urease to higher forms. The polymeric forms probably have lower activity. The mechanism of the protective effect of EDTA is still not clear; likely, EDTA reduces or prevents the catalytic effect of metal ions on the oxidation of mercapto groups to give intermolecular disulfide bonds.

Properties of 2-Mercaptoethanol Treated Urease

The urease prepared according to Mamiya and Gorin (1) by extracting jack bean meal with 32% acetone containing 2-mercaptoethanol is different in properties from that prepared without mercaptoethanol.

When 1 x 10^{-5} M urease was reacted with N-ethylmaleimide or with 5,5'-dinitrobis(2-nitrobenzoic acid) at the molar ratio of about 100, the mercaptoethanol-treated urease was found to have 17-18 mercapto groups that reacted very fast, while the untreated urease had 21-22 such groups. When mercaptoethanol-treated urease was first denatured with 4M guanidine-HCl, the reaction with N-ethylmaleimide would not be complete within a few minutes as was the case for untreated urease; about 38 mercapto groups reacted after 12 hr. Some ultracentrifugal studies indicated that mercaptoethanol-treated urease was less reactive toward oxidation.

It is very possible, therefore, that urease may contain 4-6 mercapto groups that react with 2-mercaptoethanol when it is employed in

the preparation.

Dissociation of Urease in Acetate Buffer of pH 3.1

This section supplements Chapter VII by describing some more experimental data on the dissociation of urease in acetate buffer of pH 3.1, which was conducted in the same manner as that at pH 3.5.

As shown in Fig. VII-1b, an unsymmetric peak of 4.5s was obtained after 1 hr of reaction in 0.1M acetate buffer of pH 3.1. After 6 hr, the ultracentrifugal pattern showed a single symmetric peak of 4.3s, but only 12% of the enzyme activity remained. After 24 hr, the reaction mixture showed no more change of viscosity. $s^{o}_{20,w}$ and $[\eta]$ were then measured and found to be 4.9s and 0.072 dl/g by extrapolating to infinite dilution. The molecular weight of the product was calculated to be 85,000 by the equation of Scheraga and Mendelkern as shown in Chapter VII.

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