THE EFFECTS OF SODIUM DODECYL SULFATE ON THE STRUCTURE OF LYSOZYME

by

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I. INTRODUCTION

Sodium dodecyl sulfate (SDS) strongly binds to a variety of proteins at high binding ratios and subsequently produces conformational changes (Reynolds and Tanford, 1970a,b). In aqueous solutions SDS can exist in monomeric and micellar forms. The concentration of each depends upon the total SDS concentration, the ionic strength, and the temperature. Apparently, only the monomer form binds (Reynolds and Tanford, 1970 a,b). The way detergents bring about the observed conformational changes is not entirely clear. Tanford, (1968) suggested that proteins are partially unfolded by the detergent, after which existing isolated hydrophobic side chains interact with the hydrophobic tails of detergent molecules to form micelle-like ordered regions. However, recent studies suggest that there is a combination of hydrophobic and hydrophillic interactions with proteins upon SDS binding (Van Musiwinkel-Voetberg and Veeger, 1973). Regardless of which is the predominating interaction the net result produces an unfolding of the protein molecule.

It is the purpose of this study to investigate the conformational changes produced by SDS using the method of hydrogen-deuterium exchange and comparing the results with those obtained with native lysozyme using the same method. Hydrogen-deuterium (H-D) exchange has been used extensively to study conformational changes in proteins mainly because

it offers the following advantages: (1) sensitivity to small conformational changes and (2) specificity for the peptide group. It follows then that, in principle, a conformational change in any region of the protein can be detected since peptide groups are present throughout all regions of the molecule. Thus, a complete kinetic exchange curve (degree of exchange-vs-time) for a protein can reflect the conformation of the entire molecule and not just parts of it (Hvidt and Neilsen, 1966). With the preceeding advantages in mind, it would seem reasonable that H-D exchange is indeed a suitable technique to study the changes in the lysozyme molecule produced by SDS. In other words, the resulting conformational changes due to SDS perturbation should modify the rate of exchange of the peptide hydrogens with the solvent D_2O.

Before attempting the use of H-D exchange to study the extent of denaturation of lysozyme by SDS the following questions had to be answered. 1. How does the degree of denaturation vary with the ratio of SDS to lysozyme? 2. How fast is the denatured state reached upon dissolution of native protein in SDS solutions? Differential ultraviolet spectroscopy was selected to obtain measurements related to the above questions since changes in the absorption of aromatic residues are known to occur upon denaturation and since the method can readily be adapted to time dependent studies.

II. REVIEW OF THE LITERATURE

A. Hydrogen-Deuterium Exchange As a Conformational Probe

Historically, hydrogen-deuterium exchange had its origins in the middle thirties when Fenger-Eriksen, et al. (1936) and Linderstrom-Lang, et al. (1938) developed methods for measuring deuterium content of D_2O and mixtures of H_2O and D_2O. These studies led to an attempt by Krogh and Ussing (1936) and Ussing (1938) to determine the incorporation of deuterium in various tissue proteins of animals which had received subcutaneous injections of D_2O. In addition, Ussing (1938) showed that the treated tissue proteins contained deuterium which in part, was bound by stable linkages directly to carbon as a result of the protein synthesis during the experiment. Subsequently, Ussing studied hydrogen isotope exchange in horse serum incubated with D_2O at room temperature. His observations showed that exchange increased as a function of time and there existed a correlation between the degree of exchange and the content of nitrogen and oxygen bound hydrogen atoms in the serum proteins.

However, it was not until the early fifties that the Linderstrom-Lang group attempted to use hydrogen-deuterium exchange quantatively as a possible tool for probing conformational changes in proteins. The most definitive results that followed showed that simple peptides, randomly coiled

peptides and denatured proteins in aqueous solutions exchange their labile hydrogens with solvent hydrogens within a matter of a few minutes. Labile hydrogens are defined to be those hydrogens attached to oxygen, nitrogen and sulfur. Non-labile hydrogens, on the other hand, are defined to be those hydrogens attached to carbon. Interestingly, non-labile hydrogens do not exchange under normal experimental conditions (Hvidt and Linderstrom-Lang, 1955; Schildkraut and Scheraga, 1960). Native proteins exhibit a slower exchange rate than denatured proteins. This slowness of exchange is closely related to the conformation of the protein molecule in aqueous solution (Englander, 1967).

Since labile hydrogens are the only hydrogens capable of detection and interpretation, it is necessary therefore to understand which types contribute to the exchange curves. The labile side chain hydrogens, with the exception of serine hydroxyl and primary amide groups, are bound to ionizable groups (e.g. COOH, SH, NH-C(NH)-NH_2-,imidazole, etc.). These labile hydrogens exchange readily when completely exposed to aqueous solvents and the rate of exchange can be monitored using relaxation kinetic techniques and/or proton magnetic resonance methods. Thus, Grunwald, et al. (1957) reported that methylammonium ions exchange with second order rate constants of 6×10^8 M^{-1} sec^{-1}; Luz and Meiboom (1963) observed rate constants of 4.8×10^7 sec^{-1} for the carboxyl proton of acetic acid at 25°C, Eigen (1963) measured rates on the

order of 10^{10}- 10^{11} M^{-1} sec^{-1} for phenol, guanidine and sulfhydryl groups. Additionally, there are buried groups such as tyrosine OH, which are few (there are 3 tyrosines in lysozyme) and even if their rates were reduced by 10^4, exchange would be instantaneous compared to amide rates.

The most important group of labile hydrogens to structural studies are those hydrogens bound in the amide group. These hydrogens exchange at rates slower than labile side chain hydrogen atoms and have half-lives on the order of minutes to hours (Blout, et al., 1961; Bryan and Nielsen, 1960; Nielsen, et al., 1960). Recently, McBride-Warren and Mueller (1972) reported the exchange behavior of peptide hydrogens of 3% lysozyme in 0.2M NaCl using the near infrared region rather than the fundamental infrared region, which was previously used to follow H-D exchange. Peptide hydrogens in 3% lysozyme had half-lives on the order of approximately 8 minutes for fast, approximately 80 minutes for slow, and up to hours for the very slow classes of peptide hydrogens. Interestingly, this was the first time the near infrared region was used in hydrogen-deuterium studies for a native protein.

In proteins amide hydrogens can be classified into fast, slow, and very slow classes of peptide hydrogens. The first two classes probably represent hydrogens which are normally accessible to solvent. The very slow class represents core hydrogens which are generally excluded from solvent and

do not exchange at a detectable rate unless subjected to extremes of pH and temperature. Finally, the presence of more than one class of slowly exchanging peptide hydrogens reflects the entirety of the protein conformation. Thus, such conformations that result from degree of helicity, amount of anti-parallel β-structure, amount and nature of hydrophobic regions inaccessible to solvent and interactions of side chains with each other or with the solvent will exhibit different exchange behavior. Each of these variations will therefore produce different exchange rates and hence different classes of exchanging peptide hydrogens.

B. The Mechanism of Hydrogen-Deuterium Exchange

The general model of hydrogen exchange in proteins was proposed by Linderstrom-Lang and co-workers (Linderstrom-Lang, 1955; Linderstrom-Lang and Schellman, 1959; Hvidt and Nielsen, 1966). It proposes that a protein molecule in aqueous solution will be in a dynamic state with fluctuations occurring between various conformations of the molecule around an average conformation. The basic assumption in the exchange mechanism is that an arbitrary peptide hydrogen in a segment of the protein molecule is dependent on the local conformation and is either completely buried within the protein matrix, and thereby inaccessible to solvent, or is totally exposed to the aqueous solvent. It further assumes the exchange rate for the individual peptide hydrogen to be

independent of the exchange of the other peptide hydrogens in the molecule. Therefore, for each peptide hydrogen there exists an equilibrium between a buried and open conformation of a segment of the protein molecule and this equilibrium controls the exchange of the individual peptide hydrogen.

The reaction scheme proposed by Hvidt and Nielsen (1966) which covers these assumptions is formulated by equation 1:

$$\text{Non-exchanging} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Open} \xrightarrow{k_3} \text{Exchange} \qquad (1)$$

In the non-exchanging conformation, the peptide hydrogen is completely inaccessible to the bulk solvent and exchange is prevented. On the other hand, in the open conformation, the peptide hydrogen can exchange with a hydrogen atom from a solvent molecule, and the peptide hydrogen in this conformation is freely exposed to the bulk solvent. Then, the exchange rate constant, k_3, will be the same as for peptide hydrogen atoms in a random coil polypeptide.

Further assuming equilibrium to be undisturbed by the exchange reaction, an expression for the exchange of an arbitrary peptide hydrogen (m) in a protein can be given based on equation 1 by the following rate constants (Berger and Linderstrom-Lang, 1957; Hvidt and Nielsen, 1966):

$$k_m = \frac{k_1 \cdot k_3}{k_1 + k_2 + k_3} \qquad (2)$$

The exchange rate for the m_{th} peptide hydrogen is thus determined by the mutual relationships between k_1, k_2, and k_3. Independent information as to what transconformational reactions k_1 and k_2 refer is not easily obtainable. Also in equation 2 such peptide hydrogens are ignored which lie so freely exposed on the surface of the protein molecule that they spend a great part of their time in the open conformation. If $k_2 >> k_1$, then the rate constant for the m_{th} peptide hydrogen reduces to:

$$k_m = \frac{k_1 k_3}{k_2 + k_3} \qquad (3)$$

Depending on the mutual magnitude of k_2 and k_3, Hvidt and Nielsen (1966) defined two limiting cases for the exchange constant for the m_{th} peptide hydrogen:

EX_1: $k_m = k_1$, provided $k_2 << k_3$, and

EX_2: $k_m = \frac{k_1 k_3}{k_2}$, provided $k_2 >> k_3$

Thus equation 3 represents an analysis of the non-exchanging N-conformation ($k_1 << k_2$) of a slowly exchanging hydrogen atom and offers the two exchange mechanisms to account for the

exchange behavior. For example, in the EX_1 mechanism, the opening reaction (Buried ——→ Open) determines the rate limiting step, $k_m = k_1$. In the EX_2 mechanism, on the other hand, k_1/k_2 enters the expression for the observed rate constant, $k_m = \frac{k_1}{k_2} k_3$. Interestingly, the EX_2 mechanism is influenced by specific acid-base catalysis as well as other parameters that influence hydrogen exchange. All model compounds, polypeptides, and most proteins have exhibited an EX_2 type of exchange. Willmusen (1971) has employed a graphical method to test hydrogen exchange data to see if it is indicative of the EX_2 mechanism over a given pH range. In the method, exchange data for a protein is plotted as a function of log ($[OH^-]$ x time). On such a plot, data gathered at a series of pH values should superimpose, after correcting for possible charge effects (McBride-Warren and Mueller, 1972), if EX_2 behavior is followed. This was found to be the case for a number of proteins up to pH 9 and beyond.

Berger, et al. (1959) used nuclear magnetic resonance to study the model amide, N-methylacetamide, at pH < 2 (acid range) and pH > 8 (basic range). They found the rate of exchange depended linearly on the hydrogen and hydroxyl ion concentrations. They further showed that the base-catalyzed mechanism involves direct deprotonation of the amide nitrogen. Nielsen and co-workers, using infrared spectroscopy, showed that acid and base catalysis could be used to explain the

results obtained with oligopeptides and poly-D,L-alanine (Nielsen, 1960; Nielsen, et al., 1960; Bryan and Nielsen, 1960). A more recent and thorough analysis of catalysis of the exchange of N-methylacetamide using the near infrared region was observed by Klotz and Frank (1965).

The implications of these studies on model amides and polypeptides show that pH can alter exchange rates of biopolymers through its effect on k_3 (see equation 1). Generally, if the observed rate constant appears pH dependent, an EX_2 mechanism is assumed to prevail. On the other hand, the observed rate constant of EX_1 exchange would be independent of pH if the Buried $\longrightarrow$ Open reaction is not pH dependent.

The basis for the effect of charge on hydrogen exchange had its origin from the extensive work of Berger, et al. (1959). Basically, they showed that specific acid-base catalysis of N-methylacetamide required the formation of charged intermediates, positive for acid catalysis and negative for base catalysis. These charged intermediates are formed by the catalytic action of deuterium (D^+) and deuteroxide (OD^-) ions in D_2O as seen in equations 4 and 5.

Specific acid catalysis:

$$\underset{\text{H}}{\overset{\text{O}}{\text{C-N-}}} + D_3O^+ \xrightarrow{k_D} \underset{\text{H}}{\overset{\text{O D}}{\text{-C-N}^+}} + D_2O \xrightarrow{\text{fast}} \underset{\text{D}}{\overset{\text{O}}{\text{-C-N-}}} + HD_2^+O \qquad (4)$$

Specific base catalysis:

$$\underset{\underset{H}{}}{\overset{O}{-C-N-}} + OD^- + D_2O \xrightarrow{k_{OD}} \overset{O}{-C-N^-} + HOD + D_2O \xrightarrow{fast} \underset{\underset{D}{}}{\overset{O}{-C-N-}} + HOD + OD^- \quad (5)$$

Therefore, based upon charge repulsion in the transition state, a rate reduction is expected for the acid catalyzed reaction as a macromolecule builds up a positive charge. However, there should be a corresponding enhancement of the base catalyzed reaction due to the stabilization of the negatively charged intermediate. Praisman and Rupley (1968) found that the hydrogen-tritium exchange of lysozyme was slowed by increasing the ionic strength. Fewer hydrogens were exchanged at zero time as the concentration of NaCl was increased from 0 to 3%. These results also could be explained if the positive charge on lysozyme is reduced by the higher salt concentrations. Thus, by increasing the ionic strength the electrostatic forces are reduced and the increased catalytic effect seen with the base catalyzed reaction is also reduced.

The need for an electrostatic factor, much like that used for titration data, to correct hydrogen exchange data, was originally proposed by Hvidt and Nielsen (1966). This term would incorporate a w factor (Linderstrom-Lang's electrostatic interaction factor) which can be determined from titration data in the absence of additional binding by other ions. Their suggestion was that e^{-2wZ} and e^{+2wZ} factors

could be used to estimate the magnitude of the correction factor needed to correct both the acid and base rate constants. Coleman and Willumsen (1969) found that exchange data for insulin followed OD^- catalysis expected for model compound behavior only after correcting for charge effects as measured from titration studies.

The direct effect of a net positive charge on the exchange of a polymeric amide group was reported by Kakuda, et al. (1971). Their results showed typical acid-base catalysis but a shift in pD_{min} to the acid region due to the reduced acid catalysis. The interpretation of these results were explained by either the electrostatic interactions raising the energy of the transition state or a lowering of the effective concentration of deuterium ions in the vicinity of the polymer. McBride-Warren and Mueller (1972) reported the hydrogen-deuterium exchange of native lysozyme and postulated an effect of net charge on the exchange data. Using the polymer data of Kakuda, et al. (1971) they calculated the effect of a net positive charge on the lysozyme molecule would be to reduce pD_{min} by 0.72 units relative to the pD_{min} of an uncharged polypeptide. Recently, Kakuda (1973), using a charged polymeric amide poly (ε-aminomethylacrylyl-L-lysine) and desalted lysozyme, showed that in both cases, the acid catalyzed rates were slower and a shift of pD_{min} toward the acid region occurred with increasing net positive charge.

C. The Effect of Detergents on Proteins

It has been known for quite some time that detergents bind to proteins and in most cases tightly bound complexes result. For instance, dodecyl sulfate can have an intrinsic binding constants on the order of 10^5 to 10^6 M^{-1} (Steinhardt and Reynolds, 1969). Detergents frequently show their denaturing ability, as evidenced by the increase in viscosity, changes in optical properties and changes in X-ray diffraction patterns. Lundgren (1949) has characterized the interaction between proteins and detergents as consisting of two forces: electrostatic, involving acidic and basic groups of proteins, and hydrophobic, involving apolar regions of proteins to form ordered micelles with detergent molecules. Relatively low concentrations of detergents exert a higher denaturing action compared with urea or guanidine hydrochloride. For instance, Putnam and Neurath (1943) observed an increase in the intrinsic viscosity of serum albumin of 4.3 to 22 ml/g by 8M urea, but to 25 ml/g by 0.17M sodium dodecyl sulfate. In homologous alkylsulfate compounds the chain length is an important factor in the denaturing ability. Thus, Karush and Sonenberg (1949), Pallansch and Briggs (1954), Ray, et al. (1966), Reynolds, et al. (1967) observed that the affinity at low concentrations of sodium alkylsulfates (C_8, C_{10}, C_{12}, C_{14}) for bovine serum albumin increased with increasing chain lengths and

that the intrinsic binding constant K is dependent upon pH. It should be noted that above a critical concentration, some detergents can have a stabilizing effect on proteins (Gibbs, et al., 1952). These authors reported that egg albumin is denatured by four long-chain aliphatic amines below their critical micelle concentrations and in the region above the critical micelle concentration the amines tend to stabilize the denatured protein, thus preventing precipitation. Therefore, Gibbs, et al., (1952) proposed that the mechanism of solubilization of the denatured protein occurs in two phases as supported by titration studies. The first phase involves an amine-amine interaction, i.e., the formation of micelles, which corresponds to a amine:protein weight ratio of about 0.35:1. The second phase involves a combination of amine cations with protein carboxylate ions, which corresponds to an amine:protein weight ratio of about 0.63:1. The findings in their study are consistent with the results obtained by Luck (1947) who found that soaps and detergents can, at times, stabilize proteins against various forms of denaturation such as precipitation.

Markus, et al. (1964) reported protection of human serum by SDS against unfolding by 6M urea. Fourteen equivalents of dodecyl sulfate which are bound very tightly fully reversed the effect of urea as measured by increased levorotation. Stabilization is attributed to the availability of free ε-amino groups on the protein because if they are acetylated no stabilization occurs. Furthermore, the protective effect gradually diminishes at pH values between 7.5 and 11.5.

Finally, at high urea concentrations (7 to 8M) the protection also diminishes and is fully lost at 10M.

Cationic detergents, such as dodecyldimethylbenzyl-ammonium chloride, dodecyltrimethyl and dodecylammonium bromide, denature ovalbumin rapidly at room temperatures yielding solutions which show marked streaming birefringence. The denatured protein aggregates but the rotary diffusion constant decreases only slowly with increasing degree of aggregation. Hanna and Foster (1953) interpreted this phenomena as due to either lateral aggregation of the elongated rods that result from denaturation of the protein or that denaturation produces a loosening or swelling of the protein which leads to a reduction of solubility. The swollen units might then aggregate in such a manner that unfolding is forced. Another cationic detergent, dodecylpryidinium bromide, was observed by Yang and Foster (1954) to be capable of converting the N form of bovine serum albumin to the F form in the region of pH 3.5 to 4.5.

Although cationic and nonionic detergents interact with proteins, most of the experimental data have revolved around arylsulfonates and alkylsulfates, especially the anionic detergent sodium dodecyl sulfate. Lundgren, et al. (1943) observed that SDS was bound to ovalbumin in an "all-or-none" fashion. A further observation depicted a complex with ovalbumin containing a full complement of detergent equivalent to the number of cationic groups in the protein.

The binding of SDS with horse serum albumin occurs in several successive steps corresponding to distinct electrophoretic species as observed by Putnam and Neurath (1945). At least two discrete protein-dodecyl sulfate complexes corresponding to 55 and 110 detergent ions bound has been confirmed by light scattering measurements which have also given evidence of highly uncoiled albumin molecules. SDS binds in a cooperative manner with bovine serum albumin. Thus, there is first a quantative reaction in which detergent ions are bound one by one up to a limit of about 10; there is then a complete change in the nature of the reaction and approximately 80 additional detergent ions are taken up (Pallansch and Briggs, 1954). Recently, Burkhard and Stolzenberg (1972) observed that the binding of dodecyl sulfate to cytochrome c also occurs in two phases. Namely, the result of the initial phase involves the binding of relatively low number of anions to cytochrome c, a conformation change occurs, which allows binding of a much larger number of detergent ions.

Finally, the effect of pH upon the binding of SDS and subsequent unfolding is of utmost importance. Thus, Reynolds, et al. (1970) using SDS and bovine serum albumin in a pH range of 3.8 to 7.4 observed some significant findings. At a low pH range (4.8 to 5.6) unfolding is not as pronounced as in the region of higher pH's. The extent of unfolding was substantiated by viscosity studies. They further surmised that at high pH's unfolding does not occur until substantial

equilibrium concentrations of detergent are present and that in the absence of unfolding very small equilibrium concentrations of detergent are bound. Additionally, they observed that at a pH of 3.8 with two ionic strengths (0.004 and 0.033), the higher ionic strength hinders unfolding.

D. Lysozyme--Characterization and Properties

Hen egg white lysozyme consists of a single polypeptide chain of 129 amino acid residues, cross linked by four disulfide bonds. It's primary sequence has been reported by Jolles, et al. (1963) and Canfield (1963). The molecular weight determined by Sophianopoulus, et al. (1962) is 14,400 as compared to a theoretical molecular weight of 14,300 from the amino acid sequence. The isoelectric point is between 10.5 and 11.0 (Alterton, et al., 1945) and an overall crystalline dimension of 30 x 30 x 45 Å (Krigbaum and Kugler, 1970).

The molecule can be looked at as consisting of two parts. The first 40 residues form a right hand wing in which the peptide chain is coiled twice around a core of nonpolar residues. Residues 41 through 95 branch out to form a left hand wing which appears to have a less rigid conformation and contains a high proportion of polar residues. The remainder of the chain partially closes the gap between the two wings, leaving a cleft between the two parts of the molecule (Phillips, 1967). Lysozyme contains about 30% helicity which is divided among three runs of helix, each approximately ten residues

long (Blake, et al., 1965; Blake, et al., 1967). It also contains a region of beta structure: residues 41 through 48 run in a straight line and the residues 49 through 54 double back to form hydrogen bonds to the first one. Thus, an antiparallel pleated sheet structure is formed. The charged or polar groups and the terminal groups are distributed over the surface, with the exception of glutamic acid 57, serine 36, 60 and 91, threonine 40 and tyrosine 20, 23 and 53. Finally, it is believed that two carboxylic acids glutamic acid 35 and aspartic acid 52, lie in the vicinity of the active site.

Lysozyme undergoes a dimerization in solutions of pH 5.0 to 9.0. Sophianopoulos and Van Holde (1964) proposed that dimerization occurs primarily between 2 monomers, each of which loses a proton from a group with a pKa of 6.2. Upon dimerization, the pKa of this group is shifted below pH 5.4. Viscosity data indicates no change in tertiary structure throughout this region in which dimerization occurs. Using the method of sedimentation equilibrium they obtained apparent molecular weights, which were unfortunately not corrected for non-ideality. Additionally, conditions were not varied enough to help elucidate the nature of the groups involved and the forces affecting the association of lysozyme.

The action of SDS on lysozyme can manifest itself basically in two ways: inhibition of activity and a conformational transition. Inhibition studies by Meyer, et al. (1947)

observed that lysozyme activity was greatly reduced by 10^{-3}M dodecyl sulfate. However, Smith and Stocker (1949) found that a concentration of 5.0×10^{-4}M SDS was required for 100% inhibition, which corresponds to a SDS:lysozyme weight ratio of 0.72:1. The conformational transitions induced by SDS can be followed by the usual optical and hydrodynamic methods but these methods cannot unequivocally identify which conformations are present. For instance, Jirgensons (1963, 1966) investigated the optical rotary dispersion properties of lysozyme after SDS induced conformations. Ultraviolet Cotton effect studies on solutions of lysozyme showed no significant changes in b_o values upon treatment with detergent in the pH region of 4.9 to 6.3 at a weight ratio of SDS:lysozyme of 0.36/1. In later experiments the treatment of lysozyme with SDS and a weight ratio of SDS:lysozyme of 2.88/1 at pH 4.8 to 7.4, even at 50-60^{o}C, did not result in large changes in the Cotton effects. However, strong acid solutions of lysozyme (pH 2.7) yielded broadened positive maxima at 198 to 203 nm. Upon treating the acid solution with detergent for 2 hours at 50^{o}C, the positive peak increased in intensity and broadened, splitting into 2 maxima, one at 197-200 nm and another at 202-204 nm. The conclusions reached in these experiments were that lysozyme was resistant to the action of SDS (i.e., insignificant changes in the Cotton effects) or that the amount of helicity did not change, or was compensated by changes in beta structure. Nevertheless, in order to account

for the conformational changes induced by SDS, he further postulated that an α-helical structure with its intramolecular hydrogen bonds would not have a high affinity for a nonpolar detergent "tail". On the other hand, the hydrophobic interior or proteins (without much helical structure) may be an attractive site in which nonpolar "tails" can become buried, subsequently leading to the observed conformational change. Finally, Glazer and Simmons (1965) also reported that the optical rotary dispersion of lysozyme near 233 nm is not affected by sodium dodecyl sulfate up to a weight ratio of SDS:lysozyme of 16:1. However, an aromatic Cotton effect was observed at 280 nm (attributable to tryptophan and possibly to tyrosine), which disappeared upon exposure to SDS. The magnitude of the change he concluded, was due to a conformational change involving exposed tryptophan residues to the solvent.

III. EXPERIMENTAL

A. Materials

Hen egg white lysozyme (3.2.1.17) (Lot Ly OEA) was purchased from Worthington Biochemical Corporation as a 2X recrystallized electrophoretically pure powder, activity assayed to be 9000 units per milligram by the Shugar method (Shugar, 1952). D_2O with an assay of 99.8% purity was purchased from Bio-Rad Laboratories. Sodium dodecyl sulfate, Lot No. X3057 and Lot No. X3477, purchased from Schwarz/Man was recrystallized from hot 95% ethyl alcohol and lyophillized. Phosphoric acid (85% H_3PO_4) and all other salts used in this study were of reagent grade and were used without further purification.

B. Methods

1. Absorbance Measurements

Light absorption spectra were measured on a Cary 14R recording spectrophotometer. The temperature of the cell compartment was controlled with a Haake Model FE-constant temperature circulating bath. The temperature was monitored with a thermistor probe attached to a Yellow Springs Instrument Company Tele-Thermometer, Model 42.

2. pH Measurements

All pH measurements were made using a Beckman Century SS pH meter equipped with a combination electrode (Beckman No. 39030). For D_2O solutions the measured pH readings were corrected to pD by the equation of Glasoe and Long (1960), pD = pH (meter reading) + 0.4.

3. Calculation of the Critical Micelle Concentration of Sodium Dodecyl Sulfate

Sodium dodecyl sulfate forms micelles above a critical micelle concentration (cmc) and the extent of micelle formation is a function of temperature and ionic strength. It seemed desirable, therefore, to calculate the cmc of SDS under the conditions employed in these studies. The cmc of SDS for a given experimental condition was calculated by the equation of Emerson and Holtzer (1967).

$$\text{Log cmc} = -0.666 \log (c_o + c_3) - 3.491 \qquad (6)$$

where c_o is the cmc at zero ionic strength and c_3 is the molar concentration of added salt ions having the same charge as the monomer ions. The cmc's obtained using this equation compared favorably with the selected values of critical micelle concentrations as presented in Mukerjee and Mysels (1971).

4. Differential Ultraviolet Spectroscopy of Denatured Lysozyme

All solutions for differential ultraviolet (UV) spectroscopy were prepared in buffered deionized water. A

pH of 6.0 was obtained by mixing solutions of monobasic and dibasic sodium phosphate to give a total phosphate concentration of 0.05M. A 0.01% solution of lysozyme was used in all experiments. In 5 cm cells this concentration will give an absorbance of approximately 1.25 at 280 nm. Sodium chloride was added to all solutions to maintain a constant ionic strength of 0.15. To obtain the concentration of sodium chloride necessary to obtain an ionic strength of 0.15, equation 7 was utilized:

$$I_{Total} = I_{Na_XPO_4} + I_{NaCl} + I_{cmc} \qquad (7)$$

where I_{Total} is 0.15, $I_{Na_xPO_4}$ is the calculated ionic strength of 0.05M total phosphates and I_{cmc} is the ionic strength of the monomer concentration of SDS calculated by equation 6.

Identically prepared lysozyme solutions were added to 5 cm reference and sample cells and a base line determined. Next, amounts of SDS were added to the sample cell corresponding to ratios between 2.25/1 and 6/1 of SDS/lysozyme and the spectra recorded. Attempts to obtain a ratio below 2.25/1 were not possible because of the extreme insolubility of the lysozyme-SDS complex. All concentrations of sodium dodecyl sulfate except the 5/1 and 6/1 ratios were below the critical micelle concentration as calculated by the equation of Emerson

and Holtzer alluded to earlier. Nevertheless, those spectra did not differ from the spectra observed for the other ratios. Probably this reflects the fact that sufficient SDS was bound to lysozyme to reduce the free SDS concentration to below the critical micelle concentration. Therefore, the problem of light scattering caused by micelle formation of detergent molecules was not contributing to the absorbance changes noted. The temperature of the cell compartment was thermostatically controlled and maintained at 25°C.

Ultraviolet difference spectra were obtained between 400 and 250 nm using a 0 to 0.1 slide wire and matched cells. The absorbance changes at 270, 278, 285, 291.5, 297.5 and 300 nm were selected in order to ascertain the conformational changes exerted by the various ratios of SDS to lysozyme. To obtain the corrected or net absorbance change due to the detergent binding the absorbance at 400 nm was chosen as the internal reference point.

The net change in absorbance at any peak and with any SDS:lysozyme ratio was determined in the following manner. First, as noted earlier, a baseline spectrum in the range of 400 to 250 nm is recorded which contained enzyme but no SDS. Differences between $A_{400\ nm}$ and A_{λ} are noted and designated $(A_{400\ nm} - A_{\lambda})$. This value corrects for any mismatches in the strongly absorbing lysozyme solutions. Second, a spectrum is recorded after the addition of the required amount of solid SDS, noting also the $A_{400\ nm}$ and A_{λ}

values. Third, the ($A_{400\ nm} - A_{\lambda}$) of the baseline spectrum is added or subtracted, as needed, to the A_{λ} of the spectrum that contains added SDS to give $A_{\lambda(corrected)}$. Thus, the true spectrum of denatured-vs-native lysozyme is obtained. Hence, the net change at any wavelength reflects conformational changes. Figure I shows a typical difference spectrum of a weight ratio SDS/lysozyme of 2.75/1. This difference spectrum can be compared with the ultraviolet spectrum of native lysozyme which is illustrated in figure II.

In order to observe the effect of time upon the difference spectra of denatured lysozyme, the following study was undertaken. The ultraviolet difference spectrum of a typical run was obtained as described above and the absorbance changes at 270, 280, 290 and 300 nm were monitored at times of 9 and 17 minutes and 24 hours. The same correction factor using 400 nm as the internal reference point was applied to the appropriate wave lengths.

5. Hydrogen-Deuterium Exchange of Denatured Lysozyme

After obtaining the extent of denaturation of lysozyme by SDS with various weight ratios of SDS/lysozyme in the preliminary differential UV spectroscopy, it seemed desirable, therefore, to do the exchange work. A pD range of 3.76 to 5.91 was selected for this study. A weight ratio of SDS/lysozyme of 3/1 was chosen because this was shown to be adequate enough for lysozyme denaturation. Furthermore, it has been reported

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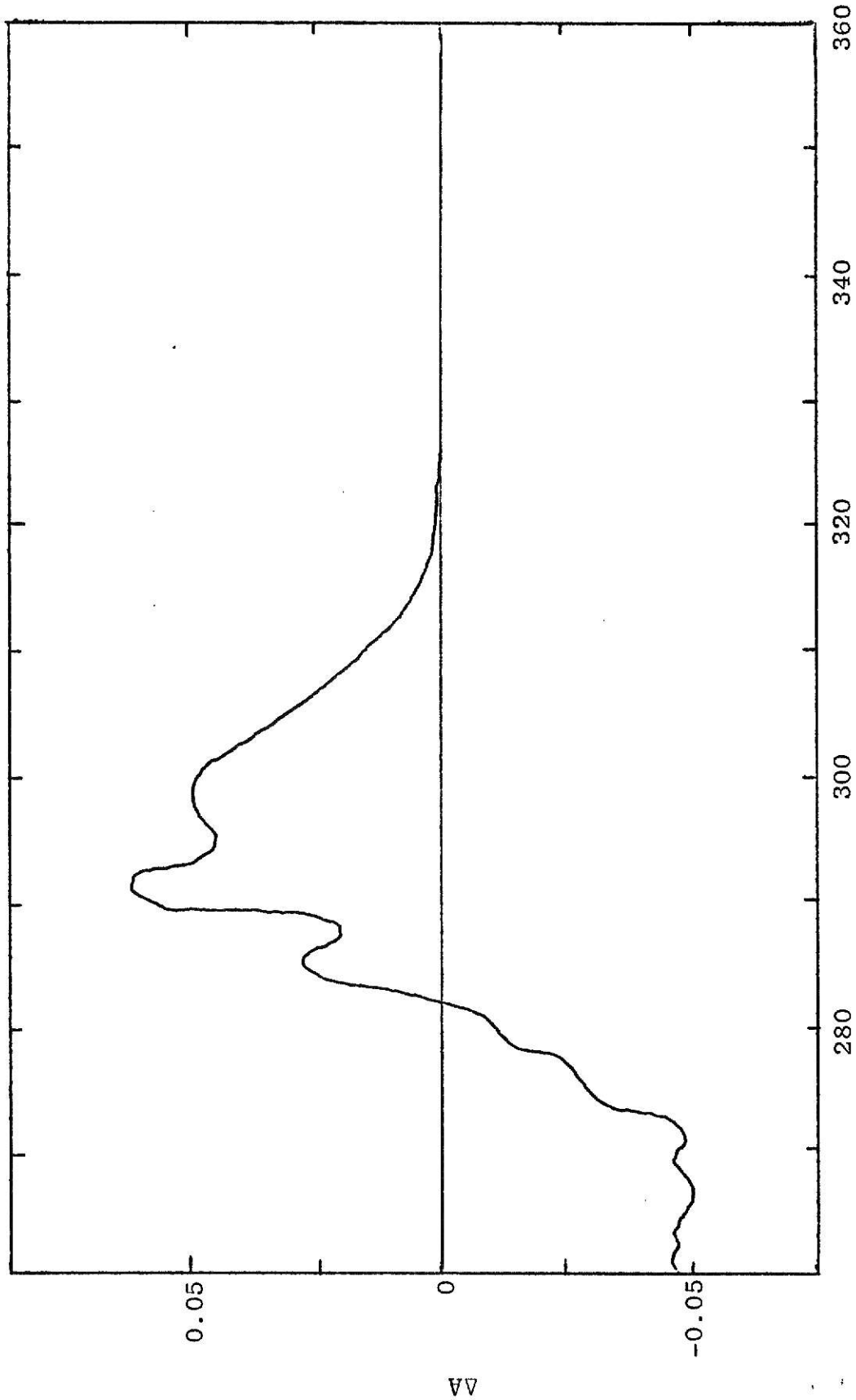
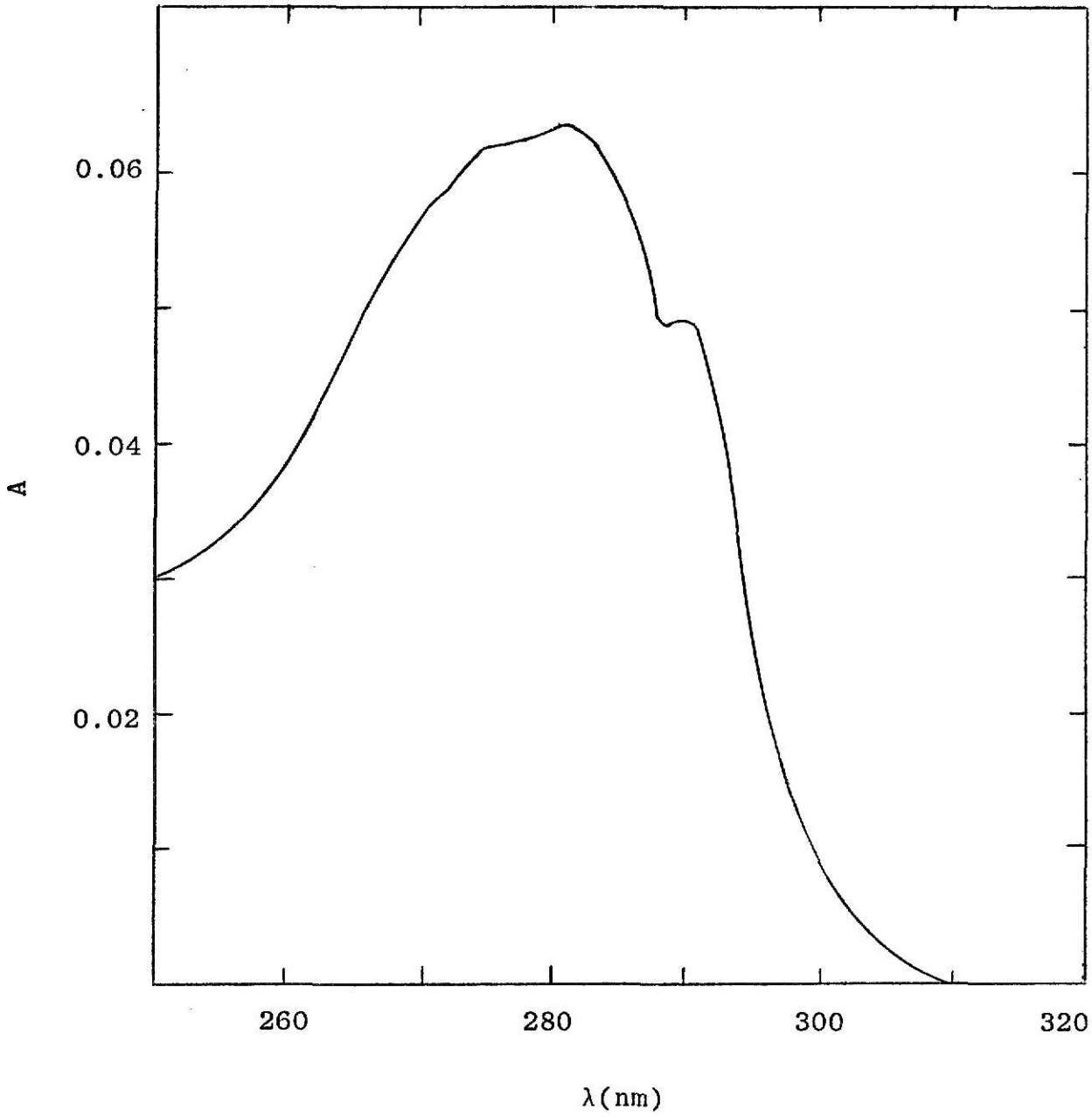


Figure I: Difference spectrum of SDS denatured lysozyme, 25°C. SDS/lysozyme 2.75/1 (w/w), pH 6.0, ionic strength, 0.15. Reference cell, native lysozyme (0.01%), sample cell, denatured lysozyme, 5 cm cells.

Figure II: Ultraviolet spectrum of native lysozyme, 25°C. Lysozyme, 0.025%, pH 6.0; ionic strength, 0.15; cell path, 1 cm.



that SDS induces conformational changes in proteins at a monomer contration of 1.0×10^{-4}M or a weight ratio of SDS/protein of 0.4/1 (Steinhardt and Reynolds, 1969; Reynolds and Tanford, 1970b; Nelson, 1970).

At the beginning of each experiment a spectrum of air-vs-air was run to establish a basis for any instrumental changes affecting a typical run. The final concentration of all components in the exchange studies are: SDS = 9%, lysozyme = 3%, phosphate buffer = 0.05M, and sodium chloride = 0.05M. The desired pH for any experiment was obtained by adjusting with concentrated phosphoric acid. The reference solution was prepared by mixing equal volumes of 18% SDS in D_2O with 0.1M phosphate buffer containing 0.1M sodium chloride also in D_2O. Thus, the resulting concentrations are halved. In a similar manner the sample solution was prepared by mixing vigorously equal volumes of 6% lysozyme in 0.1M phosphate buffer containing 0.1M sodium chloride in D_2O with 18% SDS also in D_2O. This sample solution is then centrifuged for at least 3 minutes to minimize the foam that resulted from dissolution. Finally, the sample was poured into a 5 cm cell and along with the reference cell was placed into the instrument for the commencement of the run.

Approximately 8-10 minutes are required from the initial mixing of lysozyme and the actual start of a run. The exchange data were gathered at 1.410 μm and 1.515 μm by alternatively recording each wavelength continuously with

occasional breaks to record the absorbance at 1.185 μm which was used as the internal baseline. At the beginning of the run, a chart speed of 1 inch per minute was used. The decrease at 1.515 μm was followed continuously for the first minute, then the change in absorbance at 1.410 μm was followed for the next minute. In between, baseline readings were taken by scanning at 1.185 μm for 30 seconds. This procedure was repeated for the first 30 minutes but later in the run longer time intervals were required to see a measurable change. After the rate of exchange had slowed considerably the chart speed was reduced to $\frac{1}{4}$ inch per minute. When it appeared that the exchange process was very slow and, in fact, too slow to follow, the run was terminated. The average time an exchange reaction was followed was approximately 3 hours. The infinity reading or time of complete exchange varied from 24 hours for pD's having fast exchanging processes to 72 hours for pD's having slow exchanging processes. The pH and thus pD of the sample and reference cells was measured after the infinity reading was recorded.

6. The Near-Infrared Spectrum of Denatured Lysozyme

In this region, three peaks are noted: one representing a decrease in absorbance due to NH disappearance with it's maximum at 1.515 μm; one representing increase in absorbance due to HOD production at 1.410 um; and a reference peak at 1.185 um which should be independent of the degree

of exchange since it represents the CH overtone. Furthermore, since SDS contains CH groups which could also absorb at 1.185 μm, it's effect is nullified by being present in both the reference and the sample cell. Figure III shows the near infrared spectra of denatured lysozyme both at an early time of exchange, 10 minutes after the dissolution of the lysozyme where NH absorption is high and HOD absorption is low, and at the infinity reading, where HOD absorption is high and NH absorption is low. The spectra represent exchange data taken at pD = 5.91.

7. Extinction Coefficient of HOD

The molar extinction coefficient for HOD under the experimental conditions of this study was determined in the near-infrared region by adding small amounts of H_2O with a microburet to a weighed D_2O solution in a 2 cm spectrophotometer cell. The composition of the solution was the same as that used in the H-D exchange studies except no lysozyme was added. Initially, a spectrum of 3% SDS in D_2O-vs-3% SDS in D_2O was scanned to establish a baseline spectrum. After each addition of H_2O an entire spectrum was scanned from 1.60 μm to 1.115 μm. The absorbance readings at 1.410 um, corrected for baseline at 1.185 um, were plotted against molar HOD. The molar extinction coefficient for HOD was calculated from the slope of this plot. Lambert-Beer's law was obeyed up to 1.6M HOD. The value of 0.0489 1/mole-cm at 1.410 μm was used in the subsequent determination of the number of

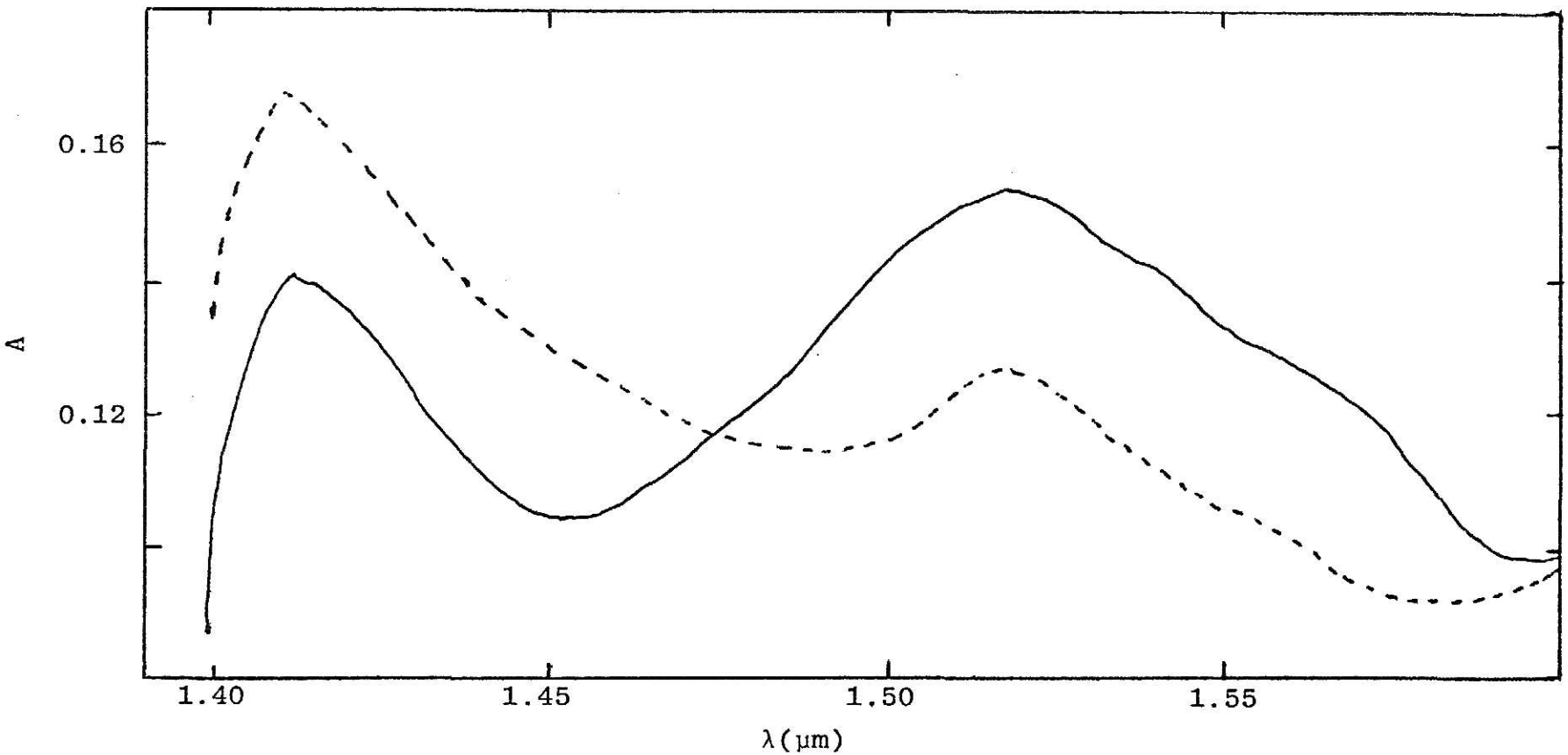


Figure III: Near-infrared spectra of SDS denatured lysozyme at 10 minutes and at infinite time, pD, 5.91, 25°C. SDS/lysozyme, 3/1 (w/w). Dashed line shows spectrum after complete exchange and solid line gives absorbance at 10 minutes.

exchangeable hydrogens.

8. Calculation of Rate Constants

Representing the exchange process as

$$\underset{\text{H}}{\overset{\text{O}}{\text{-C-N-}}} + D_2O \longrightarrow \underset{\text{D}}{\overset{\text{O}}{\text{-C-N-}}} + HOD$$

then the reaction is driven to the right for dilute solution an excess of 99.7% D_2O. The reaction rate becomes psuedo-first order in the forward direction and the rate expression is formulated as:

$$-d(NH)/dt = d(OH)/dt = k(NH)$$

Therefore, the rate of disappearance of NH will equal the rate of formation of HOD. Next, an estimate of the rate constants was made from plots of log $(A_\infty - A_{OH})$ or log $(A_{NH} - A_\infty)$-vs-time. Graphical results revealed a curve characterized by more than one rate constant. Linderstrom-Lang (1955) has shown hydrogen exchange in proteins can be described by a sum of pseudo-first-order terms, which can be written as

$$(A_\infty - A_{OH}) = \sum_i A_i e^{-kit} \qquad (8)$$

where A_{OH} is the 1.41-μ absorbance reading at any time, t; A is the absorbance associated with each group i; and k_i is the first-order rate constant for that group. As a general rule, sufficient points were taken over a given time period of the run; usually 3-4 hours for each analysis. In all the

experimental runs with SDS denatured lysozyme, two or three rate constants were adequate to fit the data.

A computer program was devised to solve the above equation for the best A_i and k_i values to fit the data by the method of least squares analysis (McBride-Warren, 1971). Additionally, the criteria applied to determine the number of parameters needed to adequately fit a set of data were based on a comparison between the overall root-mean-square deviation, RMSD, and the estimated precision of the absorbance measurements ± 0.0004 unit. RMSD was calculated according to the equation:

$$\text{RMSD} = \left[\frac{\sum_{i}^{n}(\Delta A_i - \Delta A_{calcd})^2}{(n - p)}\right]^{\frac{1}{2}} \qquad (9)$$

where $\Delta A_i = (A_\infty - A_{OH})$ are the experimental values at any time, t; ΔA_{calcd}, the corresponding theoretical changes, n the number of data, and p the number of parameters.

Hydrogen-deuterium exchange is pH dependent as a result of specific acid and base catalysis (Klotz and Frank, 1965; Leichtling and Klotz, 1966). After determining the k_i's for all of the exchange data, the parabolic rate pD profile was computed from a non-linear least squares (Mueller, 1966) fit to the equation:

$$k_{obs} = k_D(D^+) + k_{OD}(OD^-) \qquad (10)$$

or since $(OD^-) = K_{D_2O}/(D^+)$

$$k_{obs} = k_D(D^+) + k_{OD}K_{D_2O}/(D^+) \quad (11)$$

where k_D and k_{OD} are the specific acid and base catalytic constants, respectively. (D^+) and (OD^-) are the activities of the deuterium and deuteroxide ions and k_{D_2O} is the self-dissociation constant for D_2O. The catalytic constants were also estimated by plotting k_D-vs-(D^+) and k_{OD}-vs-(OD^-), the slopes of these lines were used as the initial k_i values for the computer fit. The catalytic constants obtained made it possible to calculate the pD of minimum exchange (pD_{min}) using the following equation (Leichtling and Klotz, 1966)

$$(D^+_{min}) = (k_{OD}K_{D_2O}/k_D)^{\frac{1}{2}} \quad (12)$$

Additionally, k_{min} was calculated from the following equation (Leichtling and Klotz, 1966)

$$k_{min} = 2k_D(D^+_{min}) \quad (13)$$

9. Calculation of Exchangeable Hydrogens in Denatured Lysozyme

The number of hydrogens in each class can be calculated from the A_i values by the following equation:

$$h_i = \frac{A_i}{A_{theoretical}} \times 128 \quad (14)$$

where h_i is the number of hydrogens in class i, A_i is the calculated A value for class i, $A_{theoretical}$ is the total absorbance change and 128 is the number of peptide hydrogens in lysozyme. The theoretical absorbance change was calculated from the residue molar concentration of lysozyme (2.48×10^{-1}M) (corrected for 7% water) and the molar extinction coefficient of HOD (0.0489 1/mole-cm) at 25°C. Thus, $A_{theoretical}$ = (HOD)lc = 0.06063, where l is the path length of the cell (5 cm) and c is the residue molar concentration of lysozyme.

IV. RESULTS

A. Differential Ultraviolet Spectroscopy of Denatured Lysozyme

The UV difference spectrum of denatured lysozyme with varying ratios of SDS to lysozyme was carried out at a pH = 6.00. Accordingly, ΔA, the net absorbance change, is plotted against selected wavelengths. Figure IV shows the results for ratios of 2.25/1 and 6.0/1. Only these two extremes are shown because the other results are comparable. These results show that a conformational change has occurred as evidenced by the perturbation of the spectra of the aromatic residues. Figure I clearly shows that these peaks occur at 286, 292 and approximately 300 nm and are principally due to perturbations of tyrosine and tryptophan residues.

Table I summarizes these changes and it can be concluded that within experimental error the absorbance differences are essentially the same for all the ratios studied. However, it must be remembered that ratios below 2.25/1 were unobtainable. Furthermore, the process of SDS binding to lysozyme occurred in less than 20 minutes and the changes subsequently produced were not substantially altered even after 24 hours. This fact is illustrated in Table II.

Figure IV: Difference spectra of SDS denatured lysozyme, pH 6.0; 25°C; ionic strength, 0.15; lysozyme, 0.01%. Filled circles, SDS/lysozyme = 2.25/1; open circles, SDS/lysozyme = 6/1.

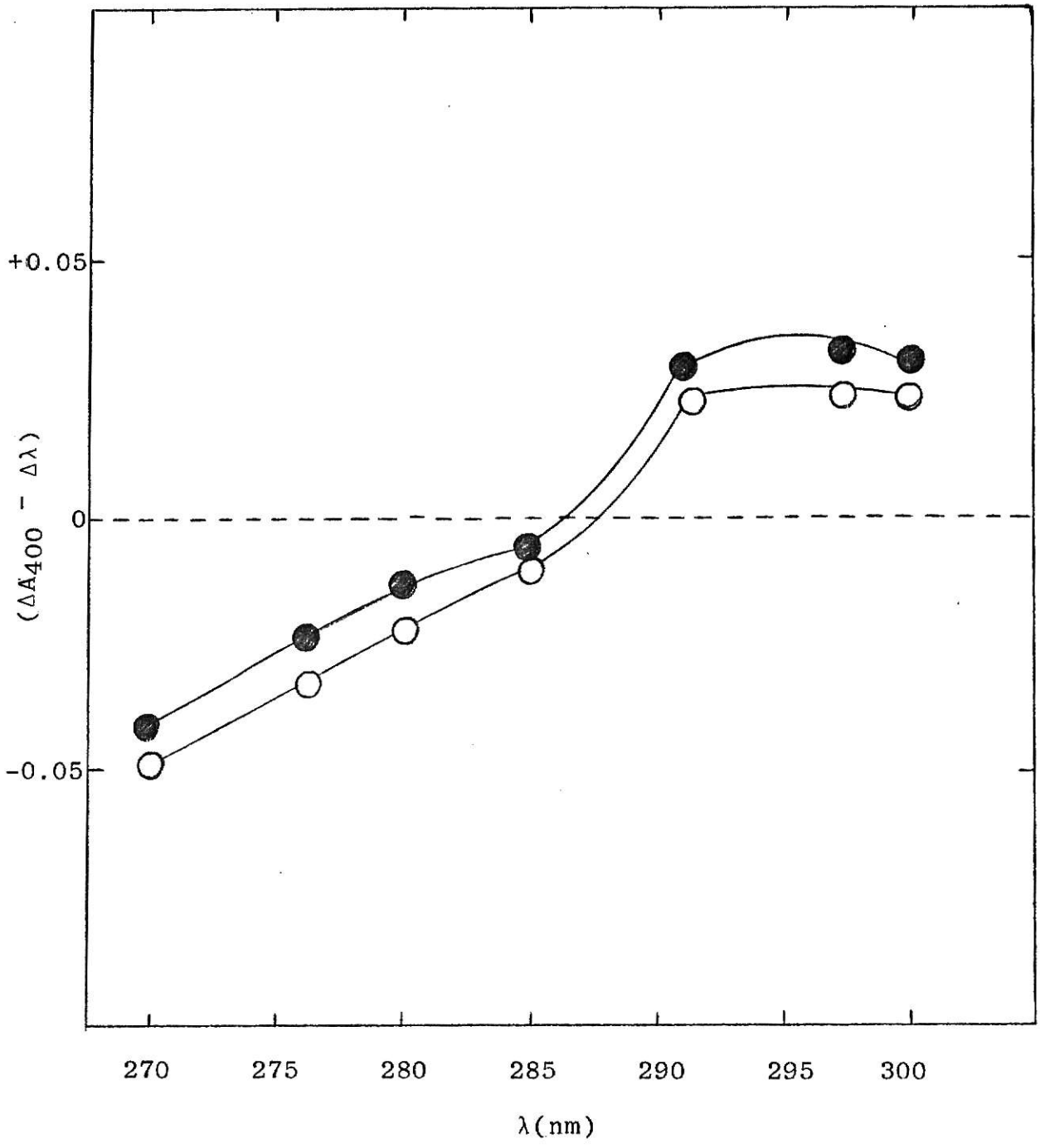


Table I

Ultraviolet Difference Absorbances of SDS Denatured-vs-Native Lysozyme

λ(nm)	2.25/1	2.5/1	2.75/1	4/1	5/1	6/1	Average
270	-0.043	-0.054	-0.047	-0.046	-0.047	-0.051	-0.048±0.004
278	-0.025	-0.037	-0.030	-0.028	-0.028	-0.034	-0.030±0.004
280	-0.015	-0.025	-0.010	-0.017	-0.016	-0.016	-0.016±0.006
285	0.008	0.000	0.003	0.004	0.006	0.000	0.004±0.007
291.2	0.030	0.022	0.026	0.027	0.027	0.027	0.026±0.003
297.5	0.032	0.025	0.025	0.024	0.024	0.024	0.026±0.004
300	0.028	0.022	0.026	0.023	0.023	0.023	0.024±0.003

Table II

Time Study of Ultraviolet Difference Absorption of Denatured Lysozyme. SDS/Lysozyme = 3/1 (w/w), I = 0.15, Sample = Denatured Lysozyme, Reference = Native Lysozyme.

Time	ΔA_{270}	ΔA_{280}	ΔA_{290}	ΔA_{300}
9 min.	-0.0375	-0.0335	-0.0190	-0.0020
17 min.	-0.0375	-0.0335	-0.0190	-0.0015
24 hrs.	-0.0400	-0.0345	-0.0220	-0.0035

B. Hydrogen-Deuterium Exchange of Denatured Lysozyme

After convincing ourselves that a conformational change had occurred, and occurred sufficiently fast not to affect exchange rates, it was felt that hydrogen-deuterium exchange studies could better describe the denaturation process. Amide H-D exchange of a weight ratio of SDS/lysozyme of 3/1 was followed by measuring HOD production at 1.410 μm and also NH decrease at 1.515 μm. The pD range studied was from pD = 3.76 to pD = 5.91.

The exchange process of the denatured protein is presented on a plot of log $(A_\infty - A_{OH})$-vs-time as shown for pD = 3.88 in Figure V. This plot is typical of the results obtained throughout the pD range investigated. The curvature of this plot clearly shows that more than one first-order rate process is involved. Rate constants were estimated graphically and calculated by computer fit to the equation 8 which was utilized for two or three rate constants. The smooth line in Figure V gives the best computer fit to the experimental data represented as open circles. The computer program was used to calculate the best A_i and k_i values to fit the data. A complete list of A_i and k_i values for denatured lysozyme is shown in Table III and IV together with the corresponding overall root-mean-square deviation, RMSD, which was found from equation 9.

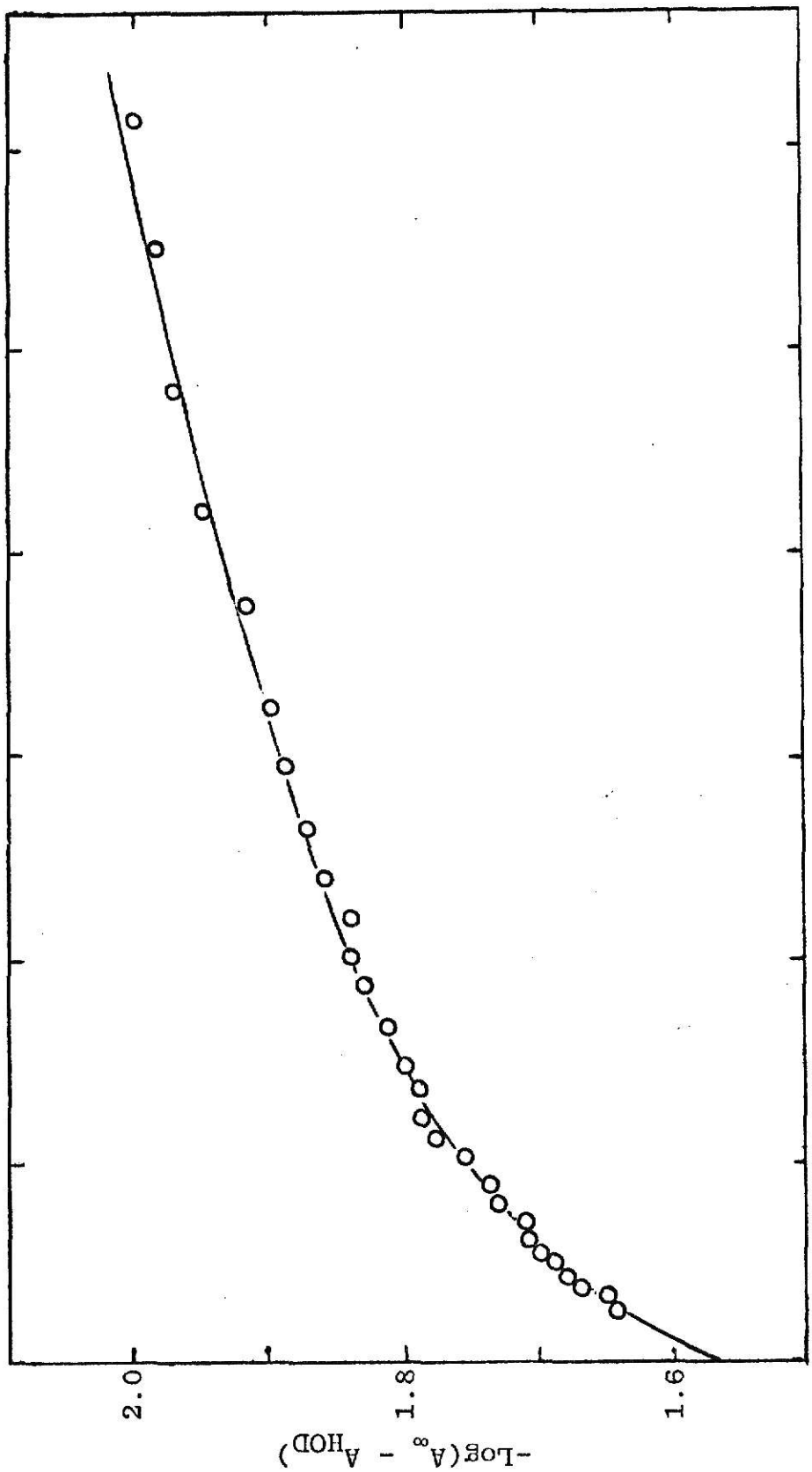


Figure V: First-order plot of HOD absorbance for SDS denatured lysozyme in D_2O, pD, 3.88. The solid line is calculated fit for experimental points (open circles), 25°C.

Table III

Calculated Absorbance Values for SDS-Denatured Lysozyme, 25°C, SDS:Lysozyme = 3/1 (w/w).

pD	A_1	A_2	A_3	A_{Total}
3.76	0.0127	0.0094	0.0084	0.0305
3.795	0.0076	0.0128	0.0068	0.0272
3.88	0.0085	0.0093	0.0099	0.0549
4.385	0.0069	0.0123	0.0049	0.0241
4.75	0.0133	0.0023	0.0099	0.0255
4.95	0.0141	0.0066	0.0108	0.0315
5.18	0.0100	0.0113	0.0065	0.0278
5.82	------	0.0134	0.0062	0.0196
5.89	------	0.0089	0.0079	0.0168
5.91	------	0.0140	0.0044	0.0184

Table IV

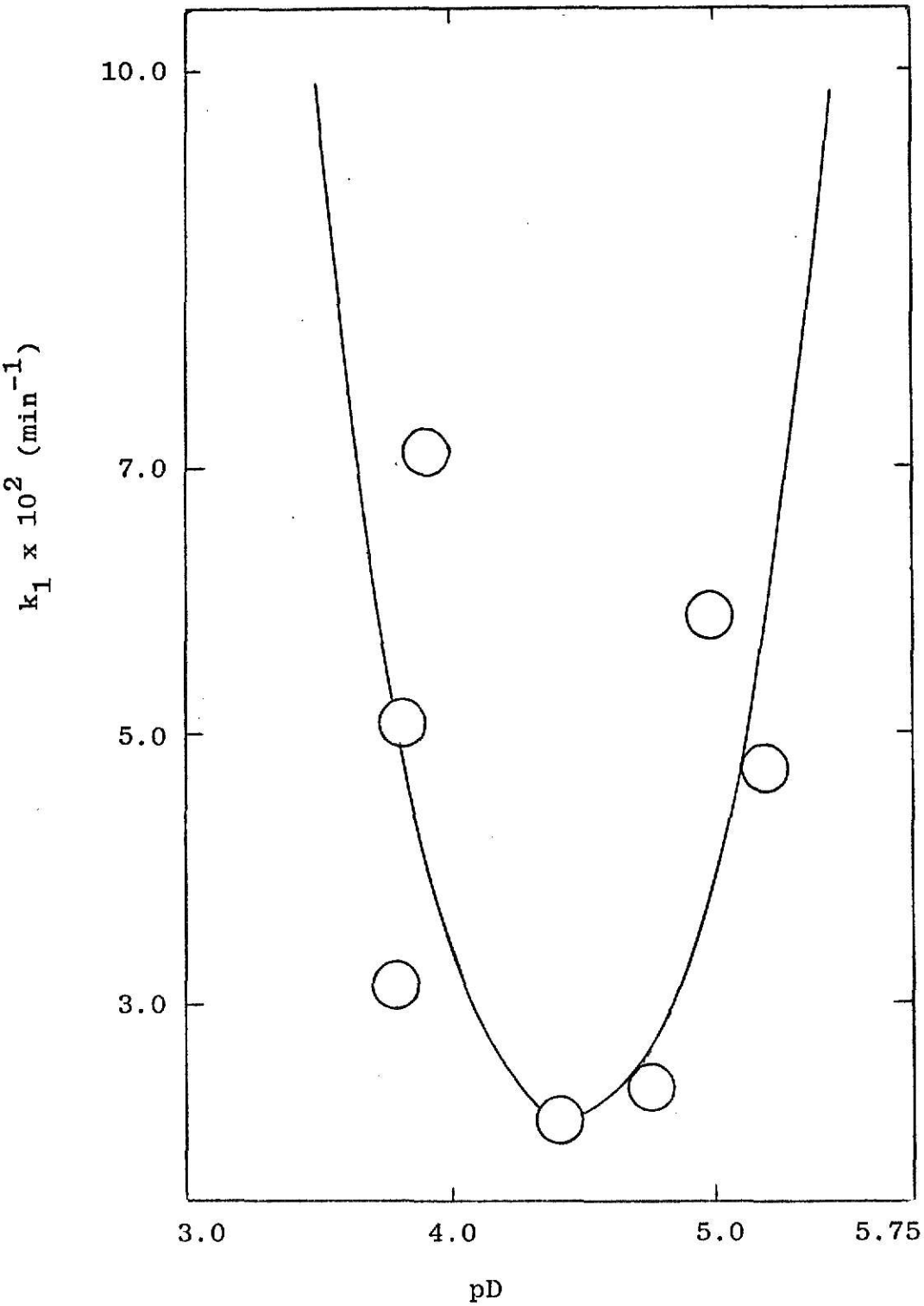
Calculated Rate Constants for SDS-Denatured Lysozyme, 25°C, SDS:Lysozyme = 3/1 (w/w).

pD	$k_1 \times 10^2$	$k_2 \times 10^2$	$k_3 \times 10^3$	RMSD $\times 10^4$
3.76	3.11	1.348	0.0084	2.97
3.795	5.10	1.190	0.0090	1.53
3.88	7.13	0.992	0.996	1.71
4.385	2.09	0.427	0.0075	2.22
4.75	2.31	0.914	0.0091	2.09
4.95	5.91	1.437	1.22	2.98
5.18	4.73	1.15	0.0075	3.48
5.82	----	6.14	7.60	2.11
5.89	----	7.33	10.80	1.55
5.91	----	8.40	7.50	1.77

C. Rate Constants and pH Dependence of Denatured Lysozyme

To illustrate the effect of pH on the exchange studies, figures VI and VII were constructed to present graphically the data contained in Table IV, as k_1 and k_2-vs-pD. The rate constants found at each pD value were subjected to a non-linear least squares program which gave a fit to equation 11 describing specific acid and base catalysis. The fit of the data to this equation gave the calculated curves which are shown as a solid line in figures VI and VII. Three k_i and A_i pairs were necessary to fit all of the data except in cases where the pD was greater than 5.20. Above this pD only k_2 and k_3 are observable because k_1 became too fast to follow as a result of base catalysis. The other parameters related to pH dependence, pD_{min} and k_{min}, were estimated from the calculated fit of the experimental data. The values thus obtained were pD_{min} = 4.43 and $0.053 min^{-1}$ for k_2; pD_{min} = 4.45 and $k_{min} = 0.021 min^{-1}$ for k_1. The dashed line in figure VII represents the k_2 data obtained by McBride-Warren and Mueller (1972) of 3% lysozyme in 0.2M NaCl. Clearly, denatured lysozyme shows a pD_{min} shifted toward a higher pD. Comparisons of k_1 and k_3 of native and denatured lysozyme were not possible because of insufficient data (k_1 of native lysozyme) and a good calculated fit (k_3 of denatured lysozyme). Additionally, the pH dependence in denatured lysozyme is not as pronounced for the k_3 data as

Figure VI: Rate-pD profile for k_1 of SDS denatured lysozyme SDS/lysozyme = 3/1 (w/w). Solid line gives the computed fit to experimental data (open circles).



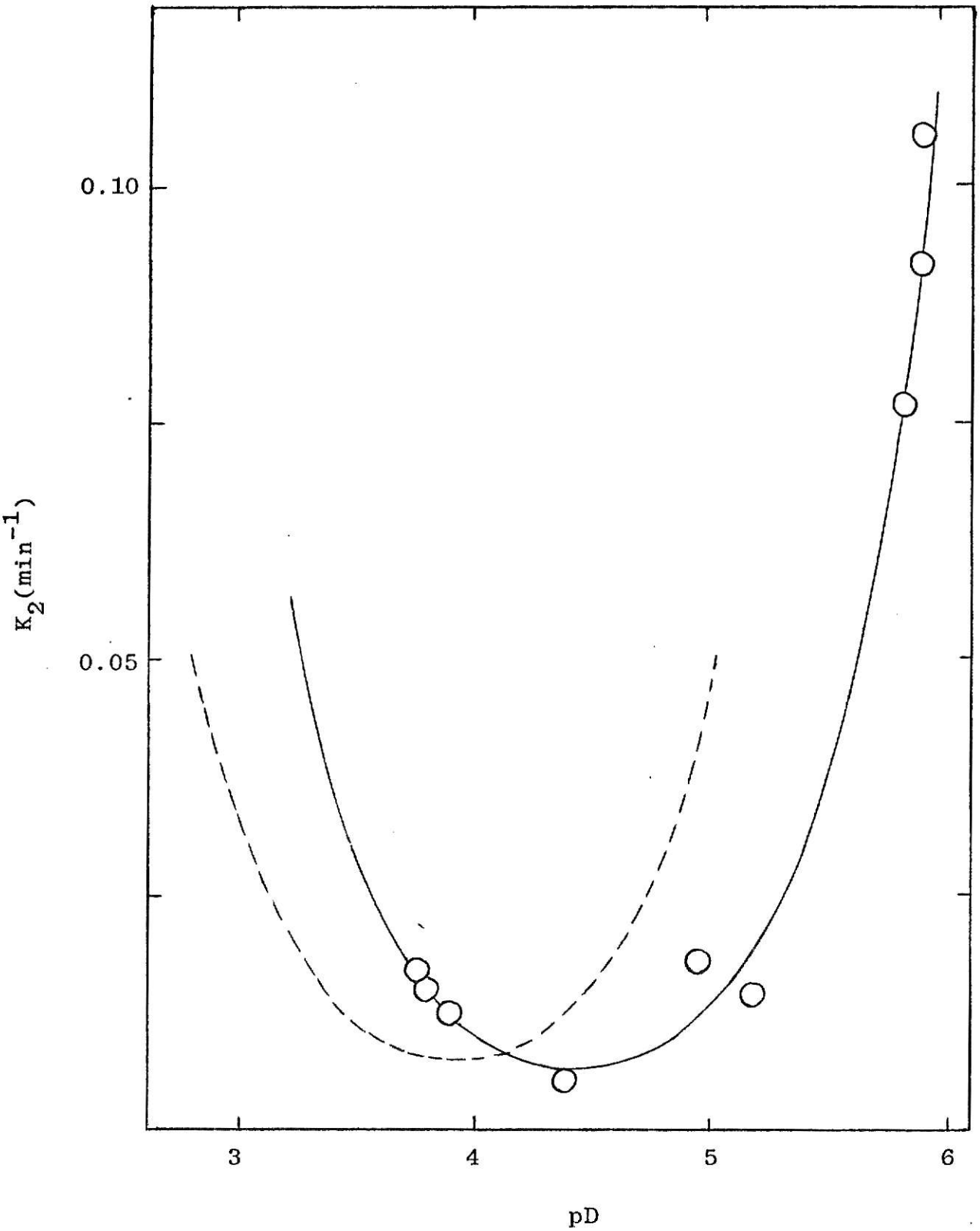


Figure VII: Rate-pD profile for k_2 of SDS denatured lysozyme, SDS/lysozyme, 3/1 (w/w). Solid line gives the computed fit to experimental data (open circles) dashed line is native lysozyme in 0.2M NaCl.
Ref: McBride-Warren and Mueller. Biochem., 11, 1785, (1972).

with k_1 and k_2. Nevertheless, the fact that a k_3 term is needed indicates that there are some hydrogens exchanging at a very slow rate even in SDS denatured lysozyme.

D. Exchangeable Hydrogens in Denatured Lysozyme

Based upon a theoretical absorbance of 0.06063 for complete exchange of all amide hydrogens in lysozyme at at concentration of 1.93×10^{-3}M, the number of hydrogens involved in each class were calculated as outlined in the experimental section. Lysozyme concentrations were corrected for the 7% water in the commercial preparation (McBride-Warren, 1971). The results are shown in Table V. The h_o values represent those peptide hydrogens which exchange too rapidly to be observed at 25°C and are defined as $128 - h_{Total}$, where $h_{Total} = h_1 + h_2 + h_3$.

Table V

Number of Peptide Hydrogens in Each Exchanging Class in SDS-Denatured Lysozyme.

pD	h_o	h_1	h_2	h_3	h_{Total}
3.76	63	27	20	18	65
3.795	71	16	27	14	57
3.88	69	18	20	21	59
4.385	77	15	26	10	51
4.75	74	28	5	21	54
4.95	61	30	14	23	67
5.18	69	21	24	14	59
5.82	--	--	28	13	(41)
5.89	--	--	19	17	(36)
5.91	--	--	30	9	(36)
Average	69±6	22±6	21±8	16±5	59±6

V. DISCUSSION

A. Differential Ultraviolet Spectroscopy

Changes in the UV spectra of proteins associated with denaturation have been extensively studied in recent years and has been used as criteria for the disruption of secondary and tertiary structure of proteins. These studies have been restricted mainly to the spectra of proteins and model compounds in the region between 270 and 300 nm. The changes have been assigned almost entirely to the alterations in the environment of the aromatic residues (Bevan and Holiday, 1952; Donovan, 1969). Peaks in the difference spectra of proteins in the 292-294 nm region are assigned to tryptophan, in the 285-288 nm region to tyrosine (plus a small contribution by tryptophan), and below 270 nm to phenylalanine. The position and intensity of these peaks are largely dictated by a given protein (Yanari and Bovey, 1960).

One of the aims of this study was to determine to what extent denaturation varies with the weight ratio of SDS/lysozyme and how fast was the denaturation process. It was felt that the technique of differential UV spectroscopy would answer these questions. As shown in figure I the binding of SDS to lysozyme produces positive peaks in the difference spectrum of tyrosine and tryptophan, whereas with

phenylalanine a negative trough results. The shape of the difference spectrum and the behavior of the aromatic residues is consistent with the results obtained by Hiyashi, et al. (1968a,b), using a cationic detergent dimethylbenzylmyristyl-ammonium chloride at a weight ratio of detergent to lysozyme of 1/1. Thus, in the case of SDS, the inference from the difference spectra suggests that tyrosine and tryptophan residues are perhaps embedded or partially embedded in an apolar region of the native protein and upon unfolding these residues are transferred to a more polar environment and hence absorbs more in the denatured protein. Or put a better way, upon the unfolding of the lysozyme molecule these residues can be more fully exposed to solvent in the denatured state than in the native state. An examination of stereoscopic photographs of the three dimensional model proposed for this enzyme from X-ray analysis by Blake, et al. (1967), Phillips (1967) illustrates that of the three tyrosine residues, two (Tyr-20 and Tyr-23) are located within the interior of the molecule, whereas Tyr-53 is part of the pleated sheet structure. Three of the six tryptophan residues (Trp-62, Trp-63, and Trp-123) are located on the surface and the remaining three (Trp-28, Trp-108, and Trp-111) are partially embedded within the protein interior.

In an attempt to explain the appearance of the positive difference spectrum in the 300 nm region of lysozyme as a result of denaturing action, Ananthanarayanan and Bigelow,

(1969) proposed that four of the tryptophan residues are located in a positively charged environment in the native structure of lysozyme due to the close proximity of guanidino groups of arginine and the lysyl side groups (personal communication with D. C. Phillips). Then, upon unfolding these residues are probably transferred to a less positive environment. Thus, changes in the polarity of the environment of the tryptophan residues could account for the observed difference spectra.

Phenylalanine, on the other hand, displays a negative trough in the difference spectrum suggesting that upon unfolding the transfer is to a less polar environment. Again, the X-ray analysis illustrates that the phenylalanine residues, Phe-3, Phe-34 and Phe-38, are located on the surface of the lysozyme molecule.

Of course the interpretations presented above are probably oversimplified mainly because it fails to take into account the changes produced by other groups in the protein which might affect the ground and excited state of the absorbing chromophores. Furthermore, the interpretation of difference spectra many times are also complicated by the fact that the absorbing chromophores can produce anomolous difference spectra. For instance, Pitt and Bello (1971) and Bello and Bello (1972), using model tyrosine compounds, ribonuclease and reduced carboxymethylated ribonuclease, proposed that SDS (30×10^{-3}M) exposes nearly all of the protein tyrosine residues to the

solvent but that they are partially remasked by the bound SDS molecules. This behavior is in contrast to Bigelow and Sonenberg (1962) who reported that SDS (greater than 10^{-3}M) exposes all tyrosine residues in ribonuclease to solvent without remasking.

Figure IV illustrates the extent of denaturation upon increasing the weight ratio of SDS to lysozyme. Note that within experimental error the changes were not significantly altered thus indicating that binding of SDS by increasing its concentration does not produce further conformational changes. Furthermore, it should be remembered that in these studies 5 cm cells are used and measurements made on a 0-0.1 slide wire. As indicated in the experimental section the weight ratios of 5/1 and 6/1 are above the cmc of SDS. Reynolds and Tanford (1970a) reported that SDS binds to a number of reduced proteins at high binding ratios (0.4 g SDS/g of protein and 1.4 g SDS/g of protein) producing gross conformational changes. Various studies have shown unreduced proteins do not bind as much SDS as reduced proteins. For instance, Pitt-Rivers and Impionbata (1968) reported SDS binds 1.4 times more to reduced ovalbumin, ribonuclease, ovomucoid and β-lactoglobulin than to the native counterparts of these proteins. To the contrary, Nelson (1970) reported that for some proteins with disulfides intact bind 1.2-2.2 g of SDS per g of protein at an ionic strength of 0.1. Even if unreduced lysozyme bound less SDS,

the effect of the SDS binding could result in a decrease of the free SDS concentration available for micelle formation. Thus, it is probable in these studies that the binding by SDS is to such an extent that micelles are never formed because the unbound concentration of SDS is below that of the cmc.

The other question of time involvement in the binding of SDS to lysozyme was answered by observing the spectrum immediately after dissolution and at times up to 24 hours. The spectra were basically the same. Therefore, the binding of SDS was essentially complete in less than 20 minutes. These results are presented in Table III. The fact that the denaturation of lysozyme by SDS occurs rapidly at room temperature is consistent with the results obtained with other denaturants such as urea and guanidine hydrochloride on proteins (Smillie, 1959; Glazer and Smith, 1961; Nelson and Hummel, 1962). For instance, Glazer and Smith (1961) reported that the kinetics of denaturation of 0.047% ovalbumin by 3M guanidine hydrochloride at pH 5.6, 23^{o}C, preceeded with a half time of 2 minutes. In addition, most of the other proteins examined, the changes in spectrum produced by denaturation, all occurred so rapidly that changes in the difference spectrum as a function of time was not possible. Thus, we were reasonably sure that H-D exchange studies could be initiated soon after dissolution and that the rates would reflect a definite denatured state.

B. Hydrogen-Deuterium Exchange of Denatured Lysozyme

A variety of information can be obtained from H-D exchange studies of conformational changes in proteins in the near-infrared region. The method has as a major advantage the stimultaneous analysis of peptide hydrogens produced by HOD absorbance increase and NH decrease. Additionally, the nature of the exchanging group can be inferred from the comparison of rate constants of the exchange data obtained by these two spectral regions. Thus, McBride-Warren and Mueller (1972) utilized the near-infrared region for rate constant calculations, class size analysis of exchanging hydrogens and pH dependence of hydrogen-deuterium exchange for lysozyme. The results achieved indicated the pD_{min} was nearly constant for all of the rate constants (pD = 3.95) and the number of hydrogens for kinetic classes was approximately constant (± 5 peptide hydrogens) over the pH range investigated for the two most rapidly exchanging groups.

The nature of the environment of the exchanging amide group produced by SDS binding was investigated by Klotz and Mueller (1969). The authors observed the effect of the binding of SDS to the polymer poly-N-isopropylacrylamide. The major finding of this study was a shift in pD_{min} (1.5 pD units) and an enhancement of k_{min} (3-fold increase), both of which probably result from the change in the acid-base character of the amide group upon SDS binding. Although

the polymer used was a model compound and very much unlike a protein, native or denatured, the results achieved were qualitatively similar to the results obtained in this study. In each case a shift in pD_{min} was noted. Therefore, an examination of the rate-pD profile and pD_{min} of denatured lysozyme is necessary. Additionally, a comparison between the number of observable hydrogens in native and SDS denatured lysozyme is required and will be discussed in the subsequent section.

The proposed mechanism for specific acid-base catalysis of hydrogen-deuterium exchange was given by equations 4 and 5 in the introduction. The rate controlling steps were k_D due to the weak basicity of the NH group, and k_{OD} due to the weak acidity of the NH group. Additionally, a charged intermediate was formed in either reaction. Therefore, let us examine the possibility of using the charged intermediate mechanism to account for the shift in pD_{min} in figure VII. Upon binding of detergent ions to lysozyme the resulting net negative charge should stabilize the positive intermediate in equation 4 and destabilize the negative intermediate in equation 5. Then, the result of this weakening of the basicity of the NH group is manifested in increased base catalysis. Thus, the entire parabolic rate-pD profile is shifted toward higher pD values. It should be noted that at higher pD's (greater than 5.2) the data could be fit to only two rate constants. k_1 was not observable above this pD (Table VI).

The pD_{min} shift is illustrated in figure VII which shows 3% lysozyme in 0.2M NaCl with a pD_{min} of 3.95 and SDS denatured 3% lysozyme with a pD_{min} of about 4.40; a shift of almost 0.40 pD units. This shift is not as pronounced as the results with poly-N-isopropylacrylamide but clearly the pD_{min} of native lysozyme and denatured lysozyme is different. The difference in the magnitude of the shift could perhaps be explained by noting that lysozyme has four intact disulfide bonds and hence may not bind as much SDS as the polymer. Therefore, it appears, that the charged intermediate is having a moderate effect on the overall parabolic rate-pD profile, simply by modifying (D^+) and (OD^-) catalysis and hence k_D and k_{OD} are changed accordingly.

Interestingly, in a similar study of hydrogen-deuterium exchange of chick lactate dehydrogenase in the presence and absence of SDS, DiSabato and Ottensen (1965) observed a rather significant finding. The authors utilized the Amide II band (1552 cm^{-1}) of the fundamental region of the infrared and monitored the decrease in absorbance of the Amide II as a function of time. The action of SDS (SDS/CHLDH = 4/1 w/w) was shown to cause a large increase in both the rate (30-fold) and the extent of exchange of the amide hydrogens (2-fold) of the enzyme in approximately one hour for a given pD. In addition, there was a displacement of 10 cm^{-1} toward longer wavelength of the Amide I band (1658 cm^{-1}) which is characteristic of the infrared spectra of denatured proteins in the

fundamental region. One serious drawback to this experiment must be noted, however; only a pH of 6.9 was used. Therefore, the apparent increase in rates may be due to a shift in pD_{min}.

C. The Number of Observable Hydrogens in Denatured Lysozyme

An additional item of interest related to the rate constants was investigated in the hydrogen exchange process. That item was the various classes of peptide hydrogens and how they were affected by SDS denaturation. Therefore, one of the significant findings arising from this study was a decrease in the total number of observable hydrogens as compared to native lysozyme. Table IV shows the results of the number of observable hydrogens, in terms of h_1, h_2, h_3, and h_{Total}. Class size analysis indicates that generally h_1 and h_2 can be determined within ± 7 peptide units with one notable exception (pD = 4.75). An explanation for this anomaly is not apparent, especially since the RMSD for this run was the lowest in the pD range of 4.385 to 4.95. The third class showed a slight variation from ± 5 peptide units which is expected since this is the slowest class of hydrogens. However, the results for h_3 indicate that although lysozyme is denatured by SDS there are some hydrogens (core) that are resistant to exchange. Further analysis of the various classes indicate that h_1 cannot be detected above pD of 5.2 because base catalysis renders the exchange process to fast

to be observed. Additionally, there is a variation in the h's related to each rate constant. However, where three rate constants are observed there is an average total of 59 hydrogens. When two rate constants (k_2 and k_3) are adequate, i.e., above pD 5.2, the average total amounts to 39 hydrogens. Now the difference between these total averages corresponds to 20 hydrogens. Therefore, in looking at h_{Total} beyond pD 5.2 the reduction of 20 hydrogens could represent the loss of k_1 because it is the rate constant for the fastest exchanging class.

It is now of interest to compare the observable hydrogens (h_{Total}) of native lysozyme (102) with those of denatured lysozyme (59). A comment should be made at this point concerning a true comparison of native and denatured lysozyme. It is very difficult to make absolute comparisons and interpretations of the individual class sizes. Thus, h_{Total} should be used as comparisons and hence subsequent conclusions. The reason for this difficulty can be understood if we point out that in the data of McBride-Warren and Mueller (1972) h_1 means one distinct class in the native state whereas h_1 in the denatured state could represent yet another discrete class. Furthermore, upon a conformational change hydrogens are shifted from one class to another upon the unfolding of the molecule and subsequent exposure to solvent. The decrease in the number of observable hydrogens then is consistent with the concomitant increase in the rate constants: that is, more

hydrogens are exchanging at a rate too fast for observation in the denatured state than in the native state.

McBride-Warren and Mueller reported a total of 102 observable hydrogens and 26 hydrogens that exchanged too rapid to be detected for 3% native lysozyme. In this study we observe only 59 hydrogens (with the 3 rate constants). This represents a difference of 43 hydrogens that exchanged too rapidly in the denatured state. Therefore, if we add the h_o's for both native and denatured lysozyme, that is, 26 and 43, the resultant total number is 69 hydrogens which exchanged too fast. Thus, it seems that upon a conformational change induced by SDS approximately 43 additional hydrogens are available to solvent for rapid exchange.

It is thus apparent that sodium dodecyl sulfate has a noteworthy denaturing action on lysozyme. The results from H-D exchange studies in the near-infrared region indicates a shift in the pD_{min} towards basic pD's. This shift is relative to native lysozyme. In addition, there is a decrease in the number of observable hydrogens upon the binding of SDS to native lysozyme. Finally, it appears that even with four disulfides intact lysozyme is substantially unfolded by SDS.

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THE EFFECTS OF SODIUM DODECYL SULFATE ON THE STRUCTURE OF LYSOZYME

by

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The binding of sodium dodecyl sulfate (SDS) to proteins and the subsequent changes in the secondary and tertiary structure has been the subject of many investigations. Various optical methods such as optical rotary dispersion and circular dichroism suggest that, in the case of lysozyme, changes in the secondary and tertiary structure do not vary significantly. However, hydrodynamic studies suggest unfolding of the molecule does occur. Therefore, the purpose of this study was to investigate the conformational changes in lysozyme produced by SDS binding using the method of hydrogen-deuterium (H-D) exchange. The results obtained from denatured lysozyme could then be compared with the results from native lysozyme using the same method.

Effects of the detergent, sodium dodecyl sulfate, on the structure of lysozyme were investigated by differential ultraviolet (UV) spectroscopy and by hydrogen-deuterium exchange. In the UV studies native lysozyme was compared to denatured at SDS/lysozyme ratios from 2.25/1 to 6/1 (w/w) using 0.01% enzyme in 0.05 M phosphate (total), pH 6.0, in 5 cm cells. Difference spectra were recorded at 25^{o}C on a Cary 14R spectrophotometer equipped with a 0-0.1 slide wire. Although the spectra indicated substantial alteration of the structure of lysozyme, no significant differences were noted for any of the ratios. Furthermore, no significant change

in the difference spectrum at 3/1 could be detected between 10 minutes and 24 hours. It was decided, therefore, that H-D exchange rates could be performed on a stable SDS:lysozyme complex. The exchange rates were determined in the pD range of 3.88 to 5.91. The rate data at each pD were analyzed in terms of two or three classes of exchangeable hydrogens and rate constants. The analysis of the pD dependence of the rate constants showed the position of minimum exchange rate shifted from pD 3.95 for native lysozyme to about 4.4 for SDS denatured lysozyme. On the average 43 more hydrogens exchanged in 6 hours than for the native enzyme. Therefore, it appears that even with the four disulfides intact lysozyme is substantially unfolded by SDS.