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# Structural Effects of the Interaction of Human Serum Albumin with Sodium Decyl Sulfate[1]

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The optical rotatory and viscosity behavior of human serum albumin was investigated as a function of sodium decyl sulfate concentration. At concentrations up to $5 \times 10^{-3}$ *M*, *i.e.*, in the region where 10–14 molecules of detergent are bound per albumin molecule, the levorotation drops from 64 to 58°. A similar change is observed when the *p*H is raised from 8 to 9.4 in the absence of detergent. In both cases the reduced viscosity remains constant. Acetylated albumin fails to show a decrease in levorotation upon the addition of small amounts of detergent. It is concluded that the observed drop is associated with the loss of charge from $\epsilon$-ammonium groups and arises from a small change in the secondary structure of the protein. Upon further increase in the detergent concentration the levorotation rises and reaches its maximum of 70° at $3.4 \times 10^{-2}$ *M* decyl sulfate. A similar rise in levorotation (from 61 to 70°) can be brought about by raising the *p*H from 2 to 8 at a constant detergent concentration (0.1 *M*). In both cases these changes are interpreted as a result of the increase in the net charge of the protein. In the first case, the binding of the detergent anions neutralizes the cationic charges of the protein, in the second case, the ionization of the carboxyl groups increases the net charge. The rise in reduced viscosity which accompanies the rotatory change in both instances and the reversal of these changes in high salt concentrations support the interpretation that high detergent concentrations effect an increase in the hydrodynamic volume of the protein molecules due to the intramolecular electrostatic repulsion within the protein–detergent complex. The observed changes in the physical properties are discussed in reference to the secondary and tertiary structures of serum albumin.

Among the substances used in the study of protein denaturation detergents have lately received particular attention due to the relatively mild physical changes caused by them. Unlike urea and similar substances, the detergents are unable to compete for the peptide hydrogen bonds that stabilize the secondary structure of proteins. Their interaction must, therefore, be largely restricted to the side chains of the protein including both the charged groups and the non-polar groups. It should be noted, however, that the binding of ionic detergents may disrupt peptide hydrogen bonds indirectly. This effect would result from intramolecular electrostatic repulsion or from the competitive breaking by the detergent of non-polar side chain interactions whose integrity is required for the stabilization of the helical structure. The physical changes proteins undergo in the course of their interaction with detergents can, therefore, to a large degree, be evaluated in terms of changes in their tertiary structure. That some effects are also exerted on the helical organization of the peptide chain is indicated by changes in optical rotation, which regularly accompany the binding of detergent molecules. Since the measurement of viscosity yields information on changes in molecular volume and asymmetry, the combined measurement of these two physical properties greatly facilitates the characterization of a given structural state.

The interaction between the alkyl sulfate homologs and serum albumin has been the subject of several studies. Putnam and Neurath,[2] studying the interaction of dodecyl sulfate and horse serum albumin found that the uptake of detergent occurred in several successive steps involving distinct electrophoretic species with fixed numbers of detergent molecules attached. The number of detergent anions bound by the second new component ($AD_{2n}$) was found equivalent to the sum of the cationic groups of the protein. Karush and Sonenberg[3] working at low detergent concentrations measured the association constants for three alkyl sulfate homologs ($C_8$, $C_{10}$, $C_{12}$) with bovine serum albumin and found that the affinity increased with increasing chain length. Pallansch and Briggs[4] confirmed and extended the findings of these two groups of workers. As the result of these studies it has become apparent that combination with detergents involves interaction between the charged and the non-polar portions of both detergent and protein, and as a result of this interaction the protein undergoes structural changes. The character and the extent of these changes depends on the number of detergent molecules bound for each homolog.

The purpose of the present study was to evaluate the structural changes that accompany detergent binding by human serum albumin largely by the measurement of optical rotation and viscosity as a function of decyl sulfate concentration.

## Experimental

Human serum albumin (HSA) was contributed by Cutter Laboratories and was 99% pure by electrophoretic analysis. A 9% stock solution was prepared in distilled water, adjusted to *p*H 7.4 with NaOH and stored in the frozen state. Its concentration was determined by micro-Kjeldahl analysis. Acetylated serum albumin was prepared according to the method described by Fraenkel-Conrat[5] with some modifications. It was found that unless the *p*H is kept near 8 by the dropwise addition of NaOH while the acetic anhydride is added, only 80% acetylation can be achieved, as against 97% when this modification is introduced. The acetylated protein was dialyzed at 5° against running distilled water for 5 days. The degree of acetylation was determined by the ninhydrin method of Harding and MacLean.[6] Sodium decyl sulfate (SDeS) was kindly prepared by Mr. Robert Marks according to the method of Dreger, *et al.*[7] It was twice recrystallized from *n*-butanol. Optical rotations were measured at room temperature with a Bellingham and Stanley polarimeter, which could be read to 0.01 of a degree, using a sodium vapor lamp and a 100 mm. cell. The specific rotations are accurate to ±1°. Viscosity measurements were made in Ostwald–Fenske viscosimeters in a 25° water-

(1) These studies were aided by a grant from the National Science Foundation and by a research grant (H-869) from the National Heart Institute of the National Institutes of Health, Public Health Service.

(2) F. W. Putnam and H. Neurath, *J. Biol. Chem.*, **159**, 195 (1945).

(3) F. Karush and M. Sonenberg, THIS JOURNAL, **71**, 1369 (1949).

(4) M. J. Pallansch and D. R. Briggs, *ibid.*, **76**, 1396 (1954).

(5) H. Fraenkel-Conrat in D. M. Greenberg, ed., "Amino Acids and Proteins," Charles C Thomas, Springfield, Ill., 1951, p. 547.

(6) Ref. 5, p. 574.

(7) E. E. Dreger, G. I. Keim, G. D. Miles, L. Shedlovsky and J. Ross, *Ind. Eng. Chem.*, **36**, 610 (1944).

bath. The blanks used for the determination of reduced viscosities differed from the samples only by the absence of protein. The uncertainty in the reduced viscosities is ±0.005. The titration of HSA was carried out in 20 ml. of a 0.89% solution of HSA in 0.1 *M* SDeS by addition of standard 1 *N* HCl and NaOH from a micro-buret.

The binding of sodium decyl sulfate to HSA was determined by equilibrium dialysis. The dialysis was carried out at 25° for a period of 14 days. This interval was necessary since the effective rate of diffusion at high concentrations is considerably reduced by the micellar structure of the detergent. Ten ml. of 0.89% HSA containing the detergent was dialyzed against 10 ml. of distilled water. Since no salt was added, it was possible to calculate the amount of bound SDeS from the Donnan distribution. For this purpose the determination of the equilibrium concentrations of detergent on both sides of the cellophane membrane were required. These were obtained by the determination of the $Na^+$ concentration on both sides by flame-photometric analysis. The outside concentration of $Na^+$ was assumed to be equal to the detergent anion concentration outside, and the concentration of free detergent inside the bag was then calculated from the Donnan equation

$$[DeS^-]_i = \frac{[Na^+]_o^2}{[Na^+]_i}$$

Correction was made for the small amount of $NaHCO_3$ contained in the SDeS stock solution. The quantities of detergent in each compartment were calculated by multiplication of the concentrations by the respective volumes. These were determined by the dilution the known original concentration of the protein had undergone, as measured by the biuret method we have previously employed.[8] The molecular weight of HSA was taken to be 65,000.

## Results and Discussion

When a 1% solution of human serum albumin (HSA) containing 0.15 *M* NaCl and 0.02 *M* phosphate (*p*H 7.4), is treated with sodium decyl sulfate in concentrations ranging from $1 \times 10^{-3}$ *M* to $2 \times 10^{-1}$ *M* the optical rotation of the protein undergoes a characteristic series of changes (Fig. 1).

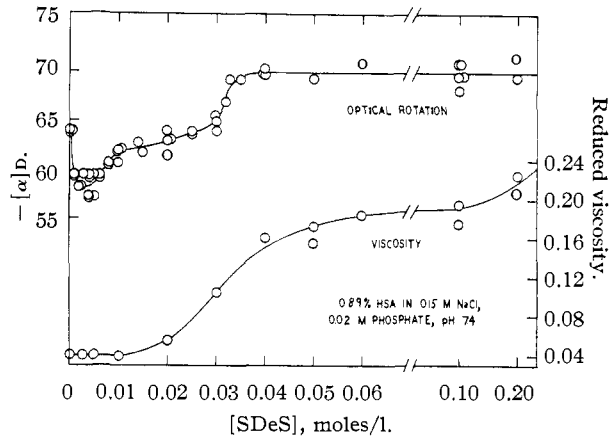


Fig. 1.—Specific rotation and reduced viscosity of HSA as a function of SdES concentration.

From 0 to $4 \times 10^{-3}$ *M* sodium decyl sulfate the levorotation drops from the native value of 64° to about 58°. It then gradually increases to reach the value for the native protein at $3 \times 10^{-2}$ *M* sodium decyl sulfate. Above this concentration the levorotation rises steeply and at $3.3 \times 10^{-2}$ *M* reaches the value of 70°. Beyond this concentration the rotation stays at 70° up to $2 \times 10^{-1}$ *M*, the highest concentration used.

**Effect of Low Detergent Concentrations on the Optical Rotation.**—It will be convenient to consider first the initial phase of the rotation *versus* concentration curve, the one characterized by the decrease in levorotation. Figure 2 shows that this

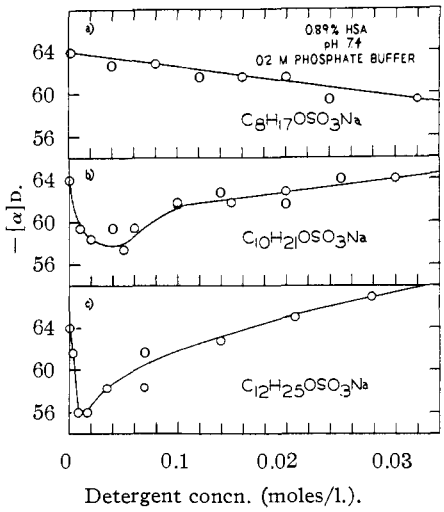


Fig. 2.—Specific rotation of HSA at low concentrations of three alkyl sulfate homologs.

drop is not restricted to sodium decyl sulfate, since both sodium octyl sulfate and sodium dodecyl sulfate cause a similar depression in the levorotation. The difference in their effectiveness is indicated by the variation of the detergent concentration at which the minimum occurs. The lowest homolog, octyl sulfate, reduces the rotation to 59° at a concentration between $2 \times 10^{-2}$ to $3 \times 10^{-2}$ *M*, whereas the highest homolog, dodecyl sulfate, causes the minimum of 56° to occur at $1 \times 10^{-3}$ *M*. The order of effectiveness in evoking this decrease in levorotation corresponds to the order of the affinities of these detergents for bovine serum albumin as determined by Karush and Sonenberg.[3] From the data of these authors it can be calculated that for decyl sulfate at $4 \times 10^{-3}$ *M* total concentration, there would be 11 moles of detergent bound per mole of protein. For dodecyl sulfate the binding of 10 molecules occurs at $1.9 \times 10^{-3}$ *M*. In the above-mentioned study the cited values represent the highest ones measured and are close to the extrapolated maximum binding, *n*, which is taken to be 14. This *n* refers to the group of sites that are first to combine with detergent. Pallansch and Briggs[4] arrive at an *n* value of 10 for dodecyl sulfate under somewhat different conditions. Their work indicates that beyond this value the protein changes its binding properties. A new set of binding sites opens up accompanied by the appearance of a faster migrating electrophoretic component. This indicates that at this point some configurational change has occurred. Our results show that this change is associated with a transition from decreasing to increasing levorotation.

Since anionic detergents appear to interact with the cationic sites of the protein, the question may be asked whether the decrease in levorotation might not be associated with the charge neutralization of some of these cationic sites. It has been ob-

(8) G. Markus and F. Karush, THIS JOURNAL, **79**, 134 (1957).

served by Jirgensons[9] that the optical rotation of native serum albumin decreases between *p*H 7 and 9.5 just before the steep rise at still higher *p*H values. We have confirmed this finding and found a drop of 6° in the optical rotation in that *p*H range (Fig. 3). In the *p*H range from 7.2 to 9.4 albumin

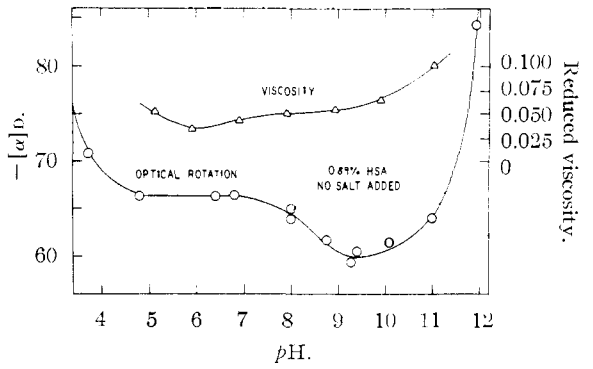


Fig. 3.—Specific rotation and reduced viscosity of HSA as a function of *p*H. (The viscosity values are not corrected for the electroviscous effect.)

dissociates about 15 $H^+$ ions,[10] some of which must come from the imidazolium groups (intrinsic $pK$ 6.9), others from the $\epsilon$-ammonium groups ($pK$ 9.8). Since the dissociation of the former groups starts well below *p*H 6.9, where there is no change observed in the optical rotation, it is likely that the configurational change indicated by the decreased levorotation is associated with the loss of charge of the $\epsilon$-ammonium groups.

Further evidence of the role of the $\epsilon$-ammonium groups is provided by experiments with acetylated HSA in which these groups are masked. Figure 4a

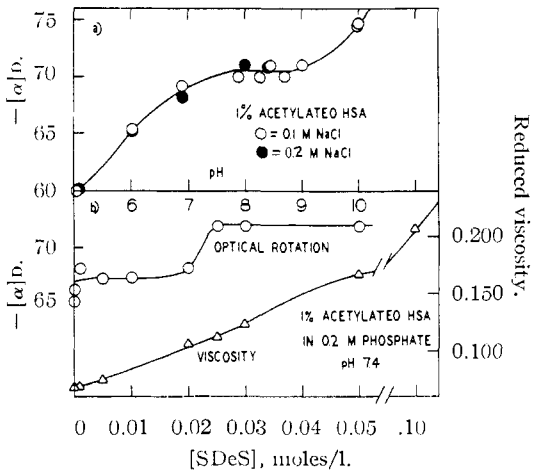


Fig. 4.—(a) Specific rotation of acetylated HSA as a function of *p*H; (b) specific rotation and reduced viscosity of acetylated HSA as a function of SDeS concentration.

shows the optical rotation of this protein as a function of *p*H. The dip observed in the case of the native albumin is absent from this curve at *p*H 9.4, corresponding to the absence of lysine residues that could be titrated in this *p*H region. The effect of the absence of these cationic groups, however, is implicit in this curve, since extrapolation to the region of insolubility, *i.e.*, to *p*H values below 5, would give a specific rotation of about −57°. Similarly, addition of SDeS to acetylated HSA at *p*H 7.4 (Fig. 4b) fails to cause the drop in levorotation observed in the native albumin at this *p*H. Since it can be shown (see below) that for the native HSA this effect is independent of the net charge within wide limits, as demonstrated by the same rotatory

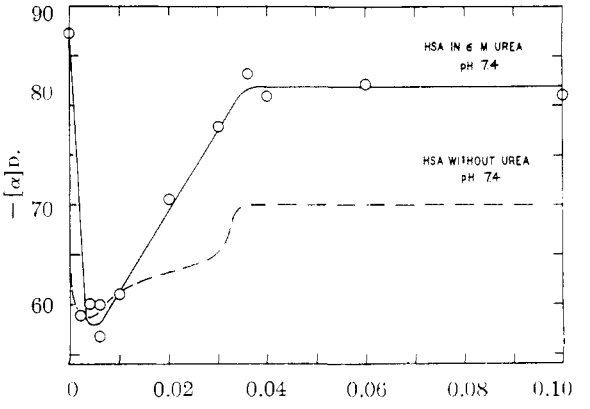


Fig. 5.—Specific rotation of HSA in 6 $M$ urea as a function of SDeS concentration. (The broken line representing the same relation in the absence of urea is taken from Fig. 1.)

decrease upon the addition of detergent at *p*H 3 and *p*H 5 when 0.1 $M$ NaCl is present (Table I), the elimination of the effect is not likely to be due to the higher net charge of the acetylated HSA at *p*H 7.4, than that of the native HSA at the same *p*H.

TABLE I

EFFECT OF *p*H AND NaCl ON $[\alpha]_D$ IN LOW SDeS CONCENTRATION

| *p*H | Salt added | SDeS, moles/l. 0 | 0.001 | 0.002 | 0.003 | 0.004 | Max. Δ $[\alpha]_D$ |
|---|---|---|---|---|---|---|---|
| 3.0 | 0 | −72 | −70 | −67 | −64 | −62 | 10 |
| | 0.1 $M$ NaCl | −66 | −65 | .. | −62 | −60 | 6 |
| 5.0 | 0 | −66 | 64 | −61 | 61 | .. | 5 |
| | 0.1 $M$ NaCl | −65 | 65 | −58 | −58 | −58 | 7 |
| 7.4 | 0.04 $M$ NaCl | −65 | .. | −58 | .. | −57 | 9 |
| | 0.3 $M$ NaCl | −64 | −60 | −58 | −60 | −60 | 6 |
| 8.0 | 0 | −65 | −60 | 58 | −60 | .. | 7 |
| | 0.1 $M$ NaCl | −62 | −62 | 58 | −60 | −58 | 4 |
| 9.4 | 0 | −62 | −60 | −59 | −59 | −59 | 3 |
| | 0.1 $M$ NaCl | −61 | −62 | −60 | −60 | −59 | 2 |

The intimate connection between the drop caused by titration with $OH^-$ ions and with detergent anions is further revealed when HSA is treated with increasing concentrations of decyl sulfate at *p*H values between 3 and 9.4. Table I shows that the maximum levorotatory decrease in 0.1 $M$ NaCl between *p*H 3 and 8 is 7°; at *p*H 9.4, however, it is only 2°. (The small value observed on the addition of the detergent at this *p*H indicates that the effective charge neutralization of only a fraction of the lysine residues is sufficient to suppress the effect. At high concentrations of detergent there is binding at the cationic sites as suggested by the high

(9) B. Jirgensons, *Arch. Biophys. Biochem.*, **59**, 420 (1955).

(10) C. Tanford, S. A. Swanson and W. S. Shore, THIS JOURNAL, **77**, 6414 (1955).

levorotation (−70°) in 0.1 *M* SDeS at *p*H 9.4 (Fig. 6).) The influence which the partial neutralization of lysine residues exerts on the structure of the protein is a subtle one, since it is not accompanied by measurable changes in viscosity (Fig. 1, 3, 4). The effect probably is not due to the increase in net negative charge that occurs between *p*H 7 and 9.4, since increase in net charge tends to increase the levorotation rather than decrease it and usually causes a corresponding rise in the viscosity (see below). The observed phenomenon may be due to a *local* charge effect, whereby loss of the positive charge from neighboring residues permits a portion of the protein molecule to acquire additional secondary structure.

**Protective Action of SDeS against Urea.**—In 1948 the significant observation was made by Duggan and Luck[11] that certain organic anions at appropriate concentrations prevented the rise in viscosity of bovine serum albumin in 6 *M* urea. Among the anions studied, dodecyl sulfate was found most effective. Figure 5 shows the effect of sodium decyl sulfate concentration on the optical rotation of HSA in 6 *M* urea at *p*H 7.4. The most striking feature of this curve is that about 0.005 *M* SDeS completely prevents the rise in optical rotation caused by 6 *M* urea. The maximal protective effect thus occurs in the region of the minimal rotation caused by the decyl sulfate alone (Fig. 5, dotted line), *i.e.*, where 10–14 molecules of detergent are bound per molecule of HSA. The effect is completely reversible: the same rotation is obtained whether the detergent or the urea is added first. Though at this point it is not possible to describe unequivocally the mechanism of protection, it is quite clear that the binding of the first group of detergent molecules causes a very significant change in the configurational stability of serum albumin. It should be noted that the configuration assumed by HSA at *p*H 9.4 in the absence of detergent is in itself not protective against 6 *M* urea. This indicates that the structural change giving rise to the low rotation is not sufficient to provide the protective effect.

**Effect of High Concentrations at Constant *p*H.**—As indicated above, sodium decyl sulfate in concentrations above $4 \times 10^{-3}$ *M* increases the levorotation of HSA to yield the native value of −64° at $3 \times 10^{-2}$ *M*. Binding data are difficult to obtain in this region due to micelle formation by the detergent, but the best data available (Table II) indicate that it is between $2.7 \times 10^{-2}$ and $3.4 \times 10^{-2}$ *M* SDeS that the binding of 96 moles of detergent per mole of HSA occurs. This range includes the steep rise in optical rotation between $3 \times 10^{-2}$ and $3.4 \times 10^{-2}$ *M* SDeS (Fig. 1). The maximum observed rotation of −70° is reached at $3.4 \times 10^{-2}$ *M* SDeS, *i.e.*, in the concentration range where all the 96 cationic sites are probably combined with detergent.

(11) E. L. Duggan and J. M. Luck, *J. Biol. Chem.*, **172**, 205 (1948).

Subtracting the 10–14 molecules involved in the first phase of the interaction, we note that there are about 80 additional molecules bound when the levorotation of 70° is reached. We know from both binding and viscosity data that combination with the detergent continues beyond $3.4 \times 10^{-2}$ *M* detergent. This concentration then is a significant one, for it marks the end of one aspect of the structural transformation that is caused by the interaction with decyl sulfate. The binding beyond this concentration is probably entirely hydrophobic in character, *i.e.*, it involves the interaction of the non-polar moieties of detergent molecules, either with non-polar residues of the protein, or with the non-polar portion of detergent molecules already bound. The change of −12° in the optical rotation from $4 \times 10^{-3}$ *M* to $3.4 \times 10^{-2}$ *M* SDeS is then the result of the attachment of approximately 80 additional molecules of detergent to one molecule of HSA.

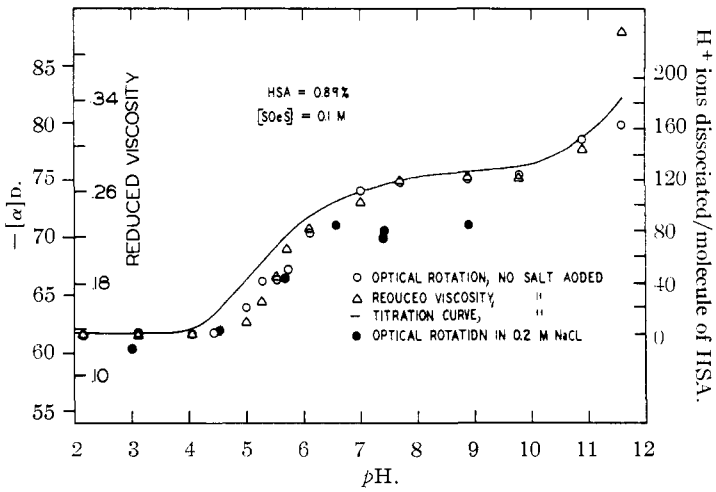


Fig. 6.—Specific rotation, reduced viscosity and $H^+$ ion dissociation of HSA in 0.1 *M* SDeS as a function of *p*H.

TABLE II

BINDING OF SODIUM DECYL SULFATE BY HSA AT *p*H 7.4, 25° BY EQUILIBRIUM DIALYSIS

HSA = $1.34 \times 10^{-6}$ mole; no salts added

| SDeS total, moles × $10^4$ | SDeS in outside soln., moles × $10^4$ | SDeS in inside soln., moles × $10^4$ | SDeS bound, moles × $10^4$ | Total concn. of SDeS inside, moles/l. | Moles SDeS bound/ mole HSA |
|---|---|---|---|---|---|
| 4.32 | 2.03 | 1.68 | 0.61 | 0.023 | 46 |
| 5.19 | 2.54 | 1.82 | .83 | .027 | 62 |
| 6.93 | 3.21 | 2.05 | 1.67 | .034 | 125 |
| 8.65 | 3.81 | 2.91 | 1.93 | .049 | 144 |
| 17.30 | 7.66 | 7.52 | 2.12 | .099 | 158 |

The value of the maximum attainable levorotation depends on the alkyl sulfate homolog used. This value for octyl sulfate is 64° and for dodecyl sulfate 68°. These variations must be due to the effect of the non-polar portions of these detergents. A rotation of −80° obtained in a saturated solution of octadecyl sulfate indicates that the effect on the levorotation increases with increasing chain length.

**Effect of High Detergent Concentrations as a Function of *p*H.**—High values of levorotation and

viscosity caused by interaction with detergents make a partial unfolding or swelling of the protein molecule very probable, but the cause of this transformation has not been clearly defined. Table III shows the effect of salt concentration on the optical rotation of HSA in 0.1 *M* SDeS at

TABLE III

EFFECT OF NaCl ON $-[\alpha]_D$ OF HSA IN 0.1 *M* SDeS AT *p*H 7.4, HSA = 0.89%

| NaCl, moles/l. | $-[\alpha]_D$ |
|---|---|
| 0 | 73-75 |
| 0.1 | 70 |
| .2 | 70 |
| .5 | 67 |
| 1.0 | 66 |

*p*H 7.4 and room temperature. These results demonstrate that increasing ionic strength depresses the levorotation, an effect best attributed to a decrease of electrostatic repulsion between neighboring portions of the protein molecule. Recent work by Yang and Foster[12] and by Tanford[13] has called attention to the large increase in levorotation and viscosity of serum albumin on the acid side of the isoelectric point. These changes were also shown to be highly sensitive to ionic strength and were interpreted as the result of swelling due to the high net charge on the protein. (The similar behavior at high *p*H values has not been studied in detail.) Since the binding of decyl sulfate by albumin increases the net negative charge (or decreases the net positive charge), the question can be asked, what is the contribution of the charge effect itself to the structural change brought about by the detergent. If the high levorotation at neutral *p*H and decyl sulfate concentrations above $3.4 \times 10^{-2}$ *M* were due to the high negative net charge on the protein, then at low *p*H values, where the 100 carboxyl groups carry no charge, the net charge on the protein would be much less, and the levorotation should be markedly reduced, whereas in the *p*H range where the carboxyl groups dissociate the levorotation should increase. Figure 6 shows that this analysis is essentially correct. In this experiment 0.89% HSA in 0.1 *M* SDeS was adjusted to *p*H values ranging from 2.2 to 12.0 without added salt and in 0.2 *M* NaCl. When the ionic strength is high the flat portion of the curve on the acid side is at $-60°$ and the plateau in the neutral region at $-70°$. In the absence of detergent the optical rotation at *p*H 3 at high ionic strength is $-66°$. The difference of 6° between this value and the $-60°$ in 0.1 *M* dodecyl sulfate at the same *p*H corresponds to the specific effect of the $\epsilon$-amino groups described in the first part of this paper. This difference is established at a concentration as low as $4 \times 10^{-3}$ *M* SDeS, beyond which no further change in the optical rotation is observed.[14] Thus, beside this specific one, SDeS has no other effect on the optical rotation in this *p*H range.

However, the acquisition of negative charges by the dissociation of the carboxyl groups in the presence of 0.1 *M* SDeS increases the levorotation from 62 to 75° in the absence of added salt, and from 60 to 70° in 0.2 *M* added salt. This effect is associated with an increase in apparent hydrodynamic volume as indicated by the proportionally rising reduced viscosity. It is improbable that this effect would be due to further binding of SDeS molecules, since with higher *p*H values the negative net charge on the protein would reduce the binding rather than increase it.

In order to establish the correlation between the measured physical changes and the change in charge of the albumin molecule, the protein was titrated from *p*H 2 to 12 in the presence of 0.1 *M* SDeS and no added salt. In calculating the number of $H^+$ ions dissociated from HSA, correction was made for the titration curve of 0.1 *M* SDeS alone, since it was found that the detergent micelles strongly buffer in the region from *p*H 3 to 6.

The $H^+$ ion dissociation curve (Fig. 6) is seen to correspond fairly well to the *p*H-dependence of both optical rotation and viscosity. The agreement is certainly close enough to suggest that the physical changes are brought about by the ionization of the carboxyl groups. The shift of the buffering zone in the titration curve toward higher *p*H values, as compared to the corresponding zone in the native protein is due to the well-known effect of anion binding on the $H^+$ ion equilibria of protein side chains.[15] The rise of $-10°$ in optical rotation as a result of raising the *p*H from 2 to 7.4 in 0.1 *M* SDeS and 0.2 *M* NaCl can be compared with the rise of $-12°$ when the concentration of SDeS is increased from $4 \times 10^{-3}$ to $3.4 \times 10^{-2}$ *M* at *p*H 7.4 and 0.2 *M* phosphate buffer. In the latter case we concluded that within this concentration range there was an uptake of about 80 moles of detergent or the neutralization of about 80 positive charges.

**Effects of High Concentrations on Acetylated HSA.**—The findings on the acetylated serum albumin are in qualitative agreement with the above considerations. Here the maximum rotatory increase brought about by the addition of SDeS at *p*H 7.4 (Fig. 4b) is less than half of the corresponding change in the unmodified protein, in agreement with the 60% reduction in the number of available cationic sites. The data on the *p*H dependence of acetylated HSA in the absence of SDeS (Fig. 4a) indicates again that the increase in charge increases the levorotation. The protein when de-ionized with a mixed ion-exchange resin is insoluble and the supernatant has a *p*H of 4.5; at *p*H 5.0 the protein will stay in solution. Extrapolation of the curve to *p*H 4.5 yields $-57°$ for the isoelectric rotation. The difference of $-7°$ between this value and the $-64°$ characteristic for the unmodified protein at the isoelectric point shows, as mentioned above, the effect of the blocking of the $\epsilon$-amino groups.

**Unmasking of Disulfide Bonds.**—A striking property of detergents, shared by other "denaturing agents," is their ability to make available groups for chemical reactions. In 0.2 *M* SDeS, *e.g.*,

(12) J. T. Yang and J. F. Foster, THIS JOURNAL, **76**, 1588 (1954).

(13) C. Tanford, J. G. Buzzell, D. G. Rands and S. A. Swanson, *ibid.*, **77**, 6421 (1955).

(14) In bovine $\gamma$-globulin, where the specific effect on the lysine residues is absent, the addition of 0.1 *M* SDeS at *p*H 2 gives a $-[\alpha]_D$ of 45°, in agreement with the rotation of the native, isoelectric protein.

(15) I. M. Klotz and J. M. Urquhart, THIS JOURNAL, **73**, 3182 (1951).

all of the 17 disulfide bonds of HSA are reduced by 0.1 $M$ $\beta$-mercaptoethylamine–HCl, whereas only 1 disulfide bond is split in the absence of the detergent.[8] Furthermore, the facilitation of the reduction of disulfide bonds in HSA in 0.1 $M$ reducing agent increases as the maximum initial rotation of $-70°$ has been attained at 0.03$M$. The relative viscosity increases further and binding data indicate that the number of detergent molecules combined has not reached its maximum even at 0.1 $M$ concentration.

**Structural Considerations.**—What structural change of the protein is reflected in the constant optical rotation of $-70°$? Since optical rotation in polypeptides is regarded as a function of the degree of secondary organization of the chain, it is reasonable to assume that this value expresses the maximal transformation of the secondary structure caused by the combination with decyl sulfate. The continuing increase in viscosity and in the availability of disulfide bonds for reduction reflects further loosening of the molecule on the level of its tertiary structure. Electrostatic repulsion between neighboring polypeptide segments would be expected to separate these segments and thus to contribute to both viscosity and accessibility of bonds situated in the interior of the molecule. The latter function is more indicative of such a process than is viscosity because of the difficulty of ascertaining how much of the viscosity rise is due just to the increase in molecular volume and asymmetry caused by the attachment of a large number of detergent molecules. Since detergents do not compete for the H-bonds of the secondary structure, the rotation of $-70°$ probably represents the extent to which the secondary structure of HSA can be distorted by charge effects. In this connection it is significant that in preliminary studies on the rotatory dispersion of HSA we found that $\lambda_0$, the extrapolated intercept of the curve with the wave length axis in linear Drude plots, was 2660 Å. for both native HSA and the same protein in 8% sodium "lorol" sulfate ($-[\alpha]_D$ 72.0), whereas 10 $M$ urea shifted $\lambda_0$ to 2340 Å. If the value of $\lambda_0$ is indicative of the degree of secondary structure,[16] this finding would indicate that, unlike urea, the detergent did not interfere extensively with the helical organization.

In this connection it is significant that the optical rotation of HSA in 6 $M$ urea and increasing concentrations of SDeS reaches the plateau of $-81°$ at the same SDeS concentration at which $-70°$ was reached in the absence of urea (Fig. 5). As mentioned before, the binding of detergent measured in the absence of urea continues beyond this concentration; the extent of disruption caused by 6$M$ urea is probably limited therefore not by the number of detergent molecules bound, but by the structural state which is brought about by the amount of detergent bound at the molarity of 0.034.

(16) K. Linderstrøm-Lang and J. A. Schellman, *Biochim. Biophys. Acta*, **15**, 156 (1954).

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# The Acetylcholinesterase Surface. VIII. Further Observations on Bifunctional Inhibition of the Enzyme[1]

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It has been observed that the bifunctional aminoalcohol *cis*-2-dimethylaminocyclohexanol (I) at *p*H 7.4 acts as a mild inhibitor of the acetylcholinesterase system, with an unexpected strength about one order of magnitude greater than that of its methiodide derivative or the natural inhibitor choline. This inhibition appears to be of the reversible, non-competitive type at substrate concentration levels below $4 \times 10^{-3}$ $M$, leading to the possibility that the Michaelis–Menten constant at these levels is a true equilibrium constant. This possibility is strengthened by the observation of Jenden, *et al.*, that a bis-quaternary salt III also displays non-competitive inhibition of AChE at the same substrate levels. The order of strength of I has been interpreted in terms of a zwitterion as the effective inhibitory species. Esterase inhibition by I is also found with the frog *rectus abdominis* preparation, with a discrepancy factor between *rectus* and *in vitro* estimations of strength approximating that found for the more potent inhibitor eserine. Further study of the inhibition produced by the bifunctional diamine N-($\beta$-dimethylamino)-ethylpiperidine (II) in the $10^{-4}$–$10^{-7}$ $M$ concentration range has revealed a decrease in effectiveness on incubation in glass which appears to depend largely on reaction volume and incubation time. This has been interpreted in terms of sorption on protein and on glass at these low levels, and is in accord with previous observations that appreciable amounts of II readily can be absorbed from solution by such proteins as gelatin and serum components. A further test of the steric requirements of the enzymatic surface between those loci capable of adsorbing the polar functions of inhibitors or substrates has been made with the compound 1,4-diazabicyclo[2,2,2]octane. Kinetic tests indicate that the size of the cage structure of this molecule exceeds the sterically acceptable limits of the surface.

## Introduction

In continuation of previous studies designed to probe into chemical and stereochemical aspects of the catalytic regions on the surface of acetylcholinesterase (AChE), with aminoalcohols[2] and diamines[3] being used as bifunctional inhibitors to reveal ele-

(1) The opinions in this paper are those of the author and do not necessarily reflect the views of the Navy Department.

(2) (a) H. D. Baldridge, W. J. McCarville and S. L. Friess, THIS JOURNAL, **77**, 739 (1955); (b) S. L. Friess and H. D. Baldridge, *ibid.*, **78**, 2482 (1956).

(3) (a) S. L. Friess and W. J. McCarville, *ibid.*, **76**, 1363 (1954); (b) S. L. Friess and H. D. Baldridge, *ibid.*, **78**, 199 (1956).