A sample of the dimer (285 mg) was reduced and alkylated as described above. After purification on Sephadex G-75, additional monomer (95 mg) was obtained. Dimer (103 mg) was also recovered in this experiment. When this reaction was run using [^{14}C]acetamidomethanol (13.7 μCi/mmol) prepared from ^{14}C-labeled acetamide, the monomer was found to have a specific activity of 110 μCi/mmol corresponding to the incorporation of eight residues of Acm. The dimer had a specific activity of 167 μCi/mmol or 14 residues of Acm.

Enzymatic Degradation. (a) A sample of octa-*S*-Acm-octahydro-*S*-protein was hydrolyzed with papain followed by aminopeptidase-M according to the method of Brewer, *et al.*[27] The results are given under column a in Table II. The sodium citrate column[6] was used only for the analysis. (b) A sample was also hydrolyzed using pronase followed by aminopeptidase-M according to the method described by Bennett, *et al.*[28] The results are given under column b in Table II.

Reconversion to Ribonuclease-*S*′. Octa-*S*-Acm-octahydro-*S*-protein (12 mg) was dissolved in 0.75 ml of 50% aqueous acetic acid and a solution of mercuric acetate (15.1 mg) in 0.17 ml of 50% acetic acid was added. After standing for 70 min at room temperature, β-mercaptoethanol (0.9 ml) was added. The mixture was stirred in a N_2 atmosphere for 19 hr. The mercuric ion and excess β-mercaptoethanol were then removed by chromatography on a 3 × 29 cm column of Sephadex G-25 (fine) using 0.1 *N* acetic acid as eluent. The fractions containing protein were combined and diluted to 25 ml with 0.1 *N* acetic acid. The solution was shown to contain 9 mg of *S*-protein by the method of Lowry[29] using a solution of *S*-protein as standard. A solution of *S*-peptide (5 mg), $NaH_2PO_4 \cdot H_2O$ (1.24 g) and Na_2HPO_4 (0.852 g) in 25 ml followed by β-mercaptoethanol (10 μl) was added and the pH adjusted to 6.5 with 40% aqueous methylamine. The total volume was brought to 75 ml with H_2O, and four drops of $CHCl_3$ were added to prevent bacterial contamination. After standing 14 days in the presence of air, the yield of ribonuclease-*S* activity was determined essentially according to the method of Anfinsen, *et al.*[30] The yield of enzymatic activity was equal to 3.2 mg of ribonuclease-*S* (30%). A precipitate (4.6 mg) was also present. Copper sulfate (75 μg) was added to increase the rate of oxidation of the remaining thiol and the solution was allowed to stand to complete disappearance of thiol.[26] No change in enzymatic activity occurred during this time. EDTA solution (1 ml of 0.1 *M*) was added. The solution was lyophilized and the salts removed by passage through a 2.5 × 28 cm Bio-Gel P-6 (200–400 mesh) column using 1 *M* NH_4OAc (pH 7.5) as eluent. The protein-containing fractions were combined and lyophilized (3.7 mg having enzymatic activity equal to 1.6 mg of ribonuclease-*S*). This material was purified by chromatography on a 1.5 × 43 cm carboxymethyl Sephadex C-50 column using 0.2 *M* sodium phosphate buffer of pH 6.47 as eluent. The product was eluted at 168 ml, the same elution volume as ribonuclease-*S*. This material (1.3 mg) had a specific activity of 90%. A by-product of lower specific activity was eluted first and overlapped slightly with the product (0.6 mg, 45% active).

Acknowledgment. We wish to thank Mr. Richard N. Boos and his associates for elemental analyses and Mr. Carl Homnick for amino acid analyses. We are greatly indebted to Mr. S. M. Miller and Mr. R. M. Pospolita for the large-scale preparation of Acm-Cys and the *N*-carboxyanhydrides used in this work. It is a pleasure to acknowledge the excellent technical assistance of Mr. James Deak and Mr. John Sondey.

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(28) C. Bennett, W. H. Konigsberg, and G. M. Edelman, *Biochemistry*, **9**, 3181 (1970).
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(30) C. B. Anfinsen, R. R. Redfield, W. L. Choate, J. Page, and W. R. Carroll, *ibid.*, **207**, 201 (1954).

A Chlorine-35 Nuclear Magnetic Resonance Study of Dodecyl Sulfate Binding to Bovine Serum Albumin[1]

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Abstract: The nuclear magnetic resonance spectrum of chlorine-35 ions in the presence of bovine serum albumin is broadened considerably. The chloride interaction is presumably at centers of positive charge with no metal ion being necessary. Binding of dodecyl sulfate and other anions to albumin decreases this interaction as shown by a decrease in the chlorine resonance line width. By monitoring the chlorine-35 line width when adding detergent, a titration curve is obtained which shows that most of the chloride binding is eliminated in the first 5–10 equiv of added detergent. This decrease occurs when the dodecyl sulfate is binding at the eight–ten strong binding sites. Presumably a center of positive charge on the protein where chloride binds is being neutralized. Higher concentrations of dodecyl sulfate are needed to remove all chloride interaction. Titration of albumin labeled at the free cysteine with a mercuric ion is different from the titration of free albumin, demonstrating that the titration curve is sensitive to changes in the protein. Acetimidation of the lysines in bovine serum albumin, which does not change the net charge, does little to change the detergent binding to the protein. Anions other than dodecyl sulfate also give titration curves. Hexyl, octyl, and decyl sulfate are not as effective in reducing chloride binding at similar concentrations.

Nuclear magnetic resonance spectroscopy of quadrupolar ions is rapidly becoming a valuable tool in the study of biological systems. Chlorine-35,[2,3] calcium-43,[4] sodium-23,[5] and magnesium-25[6] are some

(1) This investigation was supported in part by funds provided for biological and medical research by the State of Washington Initiative Measure No. 171. A preliminary account of this work was delivered at the Pacific Slope Biochemical Conference, Seattle, Wash., June 1969.

(2) T. R. Stengle and J. Baldeschwieler, *J. Amer. Chem. Soc.*, **89**, 3045 (1967).
(3) R. G. Bryant, *ibid.*, **91**, 976 (1969).
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(6) J. A. Magnuson and A. A. Bothner-By, "Magnetic Resonances in Biological Research," C. Franconi, Ed., Gordon and Breach, London, 1971, p 365.

of the nuclei which have been used.[7] In the early studies using chlorine ion, complexing of chlorine ion with mercury attached to a sulfhydryl group on a protein was used. In this way, free sulfhydryl groups in hemoglobin[2] and sulfhydryl groups in albumin were the sites probed in this nmr technique.[3] Later chloride interaction with the zinc in carbonic anhydrase was used in studying the active site of this enzyme.[8,9]

The above mentioned studies used a metal ion to interact with chlorine ion, but this is not a requirement. Ellis, Dunford, and Martin[10] have demonstrated a chloride interaction with ferric horseradish peroxidase which may be attributed to the chloride binding to a nonmetal positively charged binding site. Zeppezauer and coworkers[11] have studied the interaction of bromine ions with a variety of proteins by observing the bromine-81 resonance of bromide ion in aqueous solution of the proteins. The interaction which they report is attributed mainly to nonspecific electrostatic interactions. In the study reported below, we have used the chlorine ion interaction with bovine serum albumin as a probe for studying the interaction of detergents with this protein.

The theory of the nuclear magnetic resonance technique used here is explained in the references already cited. When the chlorine ion is bound to a site through covalent bonds as with mercury or by ionic bonds as may be the case in horseradish peroxidase,[10] a principal factor contributing to the observed line width is the contribution from the quadrupole moment.

$$C(e^2qQ)^2\tau_r$$

The quantity e^2qQ is the quadrupole coupling constant in the complex, and τ_r is the rotational correlation time. C is a constant determined by the quadrupolar nucleus being used. Under conditions of rapid exchange and little contribution from the chemical shifts, the observed line width, $\Delta\nu$, is given by

$$\Delta\nu = \Delta\nu_{\text{free}}P_{\text{free}} + \sum_i P_{ci}\Delta\nu_{ci}$$

where P_{free} and P_c represent the fraction of free and bound ions, $\Delta\nu_{\text{free}}$ is the line width of the free hydrated chlorine ion, and $\Delta\nu_c$ represents the line width of the complexed ions. The summation over i indicates the possibilities of many different sites. In most cases P_{free} is close to 1.0 so that the contribution from the bound species can be determined by simply subtracting the line width of the chlorine standard from the observed line width. It is important to note that the line width increase which is observed is dependent not only on the fraction of bound molecules P_{ci}, a parameter which can be controlled by varying concentrations of protein or chlorine ion, but also on the quadrupole coupling constant where eq, the electric field gradient, is a measure of the type of bond formed, and on τ_r which would be expected to depend on the nature of the protein. In the work reported below, ionic interaction of chlorine ion with positive sites on the albumin molecule seems likely. Much previous work[12,13] has demonstrated the existence of multiple binding sites.

The binding of detergents to proteins, and in particular, to bovine serum albumin, has been reported by several research groups. In general, the number of binding sites with large association constants is approximately 10, with the number for sodium dodecyl sulfate usually being reported as 8–9.[14–18] When observing the chlorine-35 resonance in a solution of albumin containing sodium chloride, changes in the line width are produced by changes in the nature of the protein, since the line width is a function of the rotational correlation time, the number of the binding sites, and the character of the binding sites. The binding of sodium dodecyl sulfate and other detergents to albumin reduces the chlorine ion interaction with the protein causing the marked decrease in line width. The greatest decrease occurs for the first 1–10 equiv of detergent. The results reported here examine a technique which may be useful when employed in conjunction with other methods for studying binding.[19] A brief study of one application has already been reported.[20]

Experimental Section

The chloride spectra were obtained using a modified Varian DP-60 nuclear magnetic resonance spectrometer. The V-4210 radiofrequency unit was locked to a General Radio GR-1164 frequency synthesizer which was swept with a voltage ramp.[21,22] Calibration of each spectrum was carried out by direct reading of the radiofrequency at any point in the spectral scan. The radiofrequency was swept near 5.868 MHz which corresponds to a magnetic field strength of approximately 14,000 G. Sweeps to both high and low frequency were always taken to minimize effects of field drift. For base-line stabilization, a Princeton Applied Research Model 121 lock-in amplifier was used in conjunction with audiomodulation of 2000 Hz. Error in the line width is probably $\pm 5\%$.[23]

Protein concentration was checked by comparing the OD at 280 mμ on a Cary 14 spectrophotometer. Concentrations of all samples at a given value were found to be the same within $\pm 1\%$. This is true for the various commercially available samples or treated samples. The 2 mg/ml of mercaptalbumin III gave an OD about 5% less than the albumin samples. Calculations for SDS/BSA[24] ratios were determined by assuming a molecular weight for albumin of 67,000. The SDS/BSA ratios reported are based on total SDS added. From previously determined binding constants and also equilibrium dialysis experiments from our laboratory, this number is almost identical with the bound SDS/BSA ratio. Indeed,

(7) For a recent review on the use of quadrupolar ions, see: O. Jardetzky and N. G. Wade-Jardetzky, *Annu. Rev. Biochem.*, **40**, 605 (1971).

(8) R. L. Ward, *Biochemistry*, **8**, 1879 (1969).

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(10) W. D. Ellis, H. B. Dunford, and J. S. Martin, *Can. J. Biochem.*, **47**, 157 (1969).

(11) M. Zeppezauer, B. Lindman, S. Forsen, and I. Lindqvist, *Biochem. Biophys. Res. Commun.*, **37**, 137 (1970).

(12) C. W. Carr, *Arch. Biochem. Biophys.*, **46**, 417 (1953).

(13) G. Scatchard, W. L. Hughes, F. R. N. Gurd, and P. E. Wilcox in "Chemical Specificity in Biological Interactions," F. R. N. Gurd, Ed., Academic Press, New York, N. Y., 1954, p 163.

(14) J. F. Foster and K. Aoki, *J. Amer. Chem. Soc.*, **80**, 5215 (1958).

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(16) J. A. Reynolds, S. Herbert, H. Polet, and J. Steinhardt, *ibid.*, **6**, 937 (1967).

(17) J. M. Cassel and J. Steinhardt, *ibid.*, **8**, 2603 (1969).

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(19) For a complete discussion of other techniques, see: J. Steinhardt and J. Reynolds, "Multiple Equilibria in Proteins," Academic Press, New York, N. Y., 1969.

(20) J. A. Magnuson, D. S. Shelton, and N. S. Magnuson, *Biochem. Biophys. Res. Commun.*, **39**, 279 (1970).

(21) D. E. Wisnosky, *Rev. Sci. Instrum.*, **40**, 499 (1969).

(22) Instrument based on that reported by D. J. Davis and D. E. Wisnosky at the Ninth Experimental NMR Conference, Mellon Institute, Pittsburgh, Pa., 1968.

(23) This is an error assumed for this type of experiment by many workers. Our variation of each sample in multiple spectra was usually smaller than $\pm 5\%$.

(24) Abbreviations used are: SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.

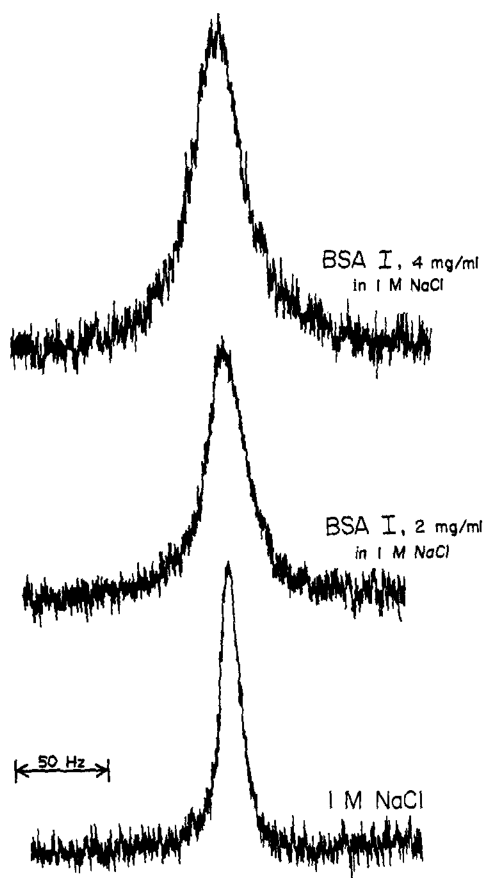

Figure 1. Spectra of the chlorine-35 resonance in 1 *M* sodium chloride, in 1 *M* sodium chloride with 2 mg/ml of albumin I, in 1 *M* sodium chloride with 4 mg/ml of albumin I.

the small amount of free SDS causes difficulty in some binding studies.[25]

Viscosity measurements were made with a Cannon Instrument Model M-1 temperature bath and Cannon-Ubbelohde semimicro dilution viscometers. The temperature was maintained at 37.00 ± 0.01°, the same temperature as in the nmr experiments.

Bovine albumin I from Sigma is a purified form of Cohn's fraction V.[26] It was prepared by method IV of that publication.[27] Bovine albumin II fraction V powder was obtained from Sigma. Bovine mercaptalbumin III, twice crystallized, from Mann Research Laboratories was used without further purification. To remove the mercury from the sample, dialysis was conducted against 2-mercaptoethanol (5 g/l.) and then distilled water. The dialyzed protein was then lyophilized.

Lysozyme, egg white, three times recrystallized, grade I, from Sigma, and α-chymotrypsin, three times recrystallized, from Worthington, were used without further purification. To detect chlorine line-width changes, it was necessary to use 10 mg/ml in 1 *M* sodium chloride.

Sodium dodecyl sulfate from Matheson Coleman and Bell was dissolved in distilled water in various concentrations. During the titrations reported herein, aliquots of dodecyl sulfate solution were added to 5 ml of the albumin solution. Volume changes were always less than 1%. These additions produced no change in the relative proportions of chlorine ion and protein. Sodium dodecyl sulfate (lauryl sodium sulfate, M.A.) from Mann Research Laboratories was also used. Within the limits of the nmr experiment, this purified form gave the same results as the detergent from Matheson Coleman and Bell. Sodium hexyl, octyl, and decyl sulfate and dodecyl sulfonate were best grades obtainable from Mann Research Laboratories.

(25) C. J. Halfman and J. Steinhardt, *Biochemistry*, **10**, 3564 (1971).
(26) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, *J. Amer. Chem. Soc.*, **69**, 1753 (1947).
(27) We thank Mr. Louis Berger from Sigma for information on this matter.

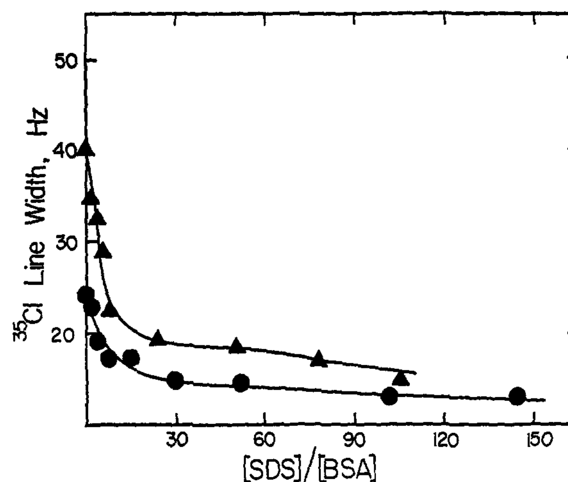

Figure 2. Titration with SDS of 2 mg/ml of albumin I (●) and 4 mg/ml of albumin I (▲), in 1 *M* sodium chloride; chlorine-35 line width *vs.* SDS/BSA.

Bovine albumin I was acetimidated by the procedure of Hunter and Ludwig.[28] Reynolds has previously shown that 57 of the ε amino groups had been acetimidated.[29]

Results

Bovine serum albumin was dissolved in 1 *M* sodium chloride solutions and spectra of the chlorine ion were taken. Figure 1 shows the effect of 2 and 4 mg/ml solutions on the chloride line width. The line broadening produced is easily detected. Upon adding sodium dodecyl sulfate to the solution, the chlorine line width narrowed until it approached that of the 1 *M* sodium chloride standard. The increase over the standard with 2 mg/ml of albumin is about 14 Hz for the various samples making the observed line width close to 26 Hz. The 4 mg/ml albumin solutions exhibit an increase of another 14 Hz to approximately 40 Hz for the observed line width. This corresponds closely to what is expected from the equation for the observed line width given earlier.

The titration curve for albumin I is presented in Figure 2. At molecular ratios less than 10 for detergent to protein, the line width decreases rapidly. The decrease is linear with an increase of SDS/BSA ratio within the limits of the nmr experiment. At greater values, the line width slowly decreases until it reaches that of the standard. The lines drawn in the titration curves are used solely to correlate the data. Although the error is assumed to be approximately ±5% in line-width measurements, the precision among the various samples of similar or closely related albumins is quite good. The sharp decrease in line widths for the first ten detergent molecules can be seen clearly with 2 or 4 mg/ml solutions. The additional subsequent decrease in chloride interaction is most apparent in titration at higher albumin concentration.

To a certain extent the interaction of chlorine ion with albumin is independent of purity of protein. After extensive dialysis of albumin I against 1 *M* sodium chloride, no change in the titration curve was visible. Fraction V and a sample of albumin I defatted ac-

(28) M. J. Hunter and M. L. Ludwig, *Methods Enzymol.*, **11**, 595 (1967).
(29) J. A. Reynolds, *Biochemistry*, **7**, 3131 (1968).

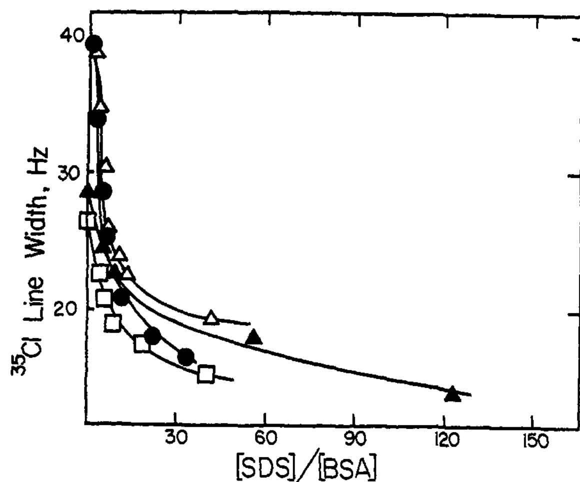

Figure 3. Titrations in 1 *M* sodium chloride with SDS of 4 mg/ml of albumin II (Cohn's fraction V) (△); 4 mg/ml of albumin I dialyzed against 1 *M* sodium chloride (●); 2 mg/ml of albumin I defatted (□); and 2 mg/ml of albumin I in acetate buffer, pH 4.75 (▲); chlorine-35 line width *vs.* SDS/BSA.

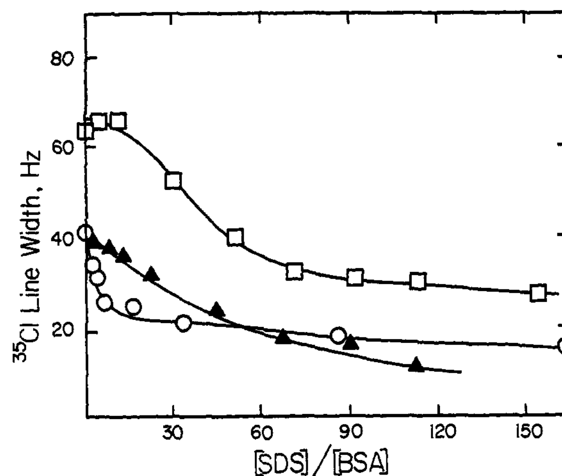

Figure 5. Titration in 1 *M* sodium chloride with SDS of 2 mg/ml of mercaptalbumin III, obtained commercially (▲); 2 mg/ml of mercaptalbumin III with all dimers converted to mercury–albumin monomers (□); 4 mg/ml of mercaptalbumin III freed of mercury with mercaptoethanol (○); chlorine-35 line width *vs.* SDS/BSA.

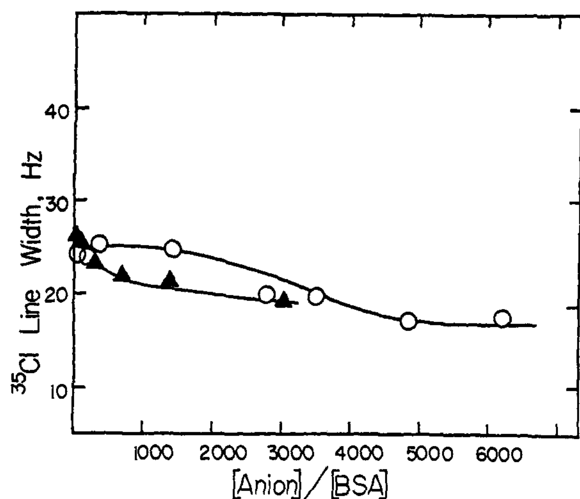

Figure 4. Titration in 1 *M* sodium chloride with sodium thiocyanate (▲) and sodium trichloroacetate (○), of 2 mg/ml of albumin I; chlorine-35 line width *vs.* thiocyanate/BSA and trichloroacetate/BSA.

cording to the procedure of Chen[30] gave similar titration curves. In general the solutions of albumin were not buffered so that no interference from anions would occur. The solutions varied from pH 5.6 to 6.5 in the course of a titration. However, a titration of 2 mg/ml of albumin I in 0.05 *M* sodium acetate buffer, pH 4.75, showed the same sharp drop in chlorine line width as found in the unbuffered samples. These results are shown in Figure 3.

The effect of various cations was examined by carrying out the dodecyl sulfate titration in 1 *M* potassium chloride and 1 *M* lithium chloride. Titration of albumin I in these salt solutions produced curves similar to those obtained in 1 *M* sodium chloride. Therefore, the effect of the cations appears to be negligible. The potassium chloride solution did produce a small amount of precipitate at higher dodecyl sulfate concentration. The precipitate is probably the potassium salt of the detergent. It should be noted that the albumin definitely promotes solubilization of the detergent in these high concentration salt solutions.

A 2 mg/ml albumin I sample in 1 *M* sodium chloride was titrated with trichloroacetate and thiocyanate, two anions known to bind well with albumin.[12] The results are shown in Figure 4. Throughout the titration, the chlorine line width slowly decreases, but the decrease is small. The ratio of ion concentration to albumin I concentration is much larger than shown in the dodecyl sulfate titrations. The binding constant of these ions with albumin is too low to overcome the 1 *M* chloride concentration. At higher concentrations of trichloroacetate, some of the chlorine line width is due to trichloroacetate–chloride interaction, so the trichloroacetate may inhibit chloride interaction better than indicated by the titration curve.

Figure 5 shows the titration curves for a variety of samples of mercaptalbumin III. Curves for the commercial sample, for the commercial sample freed of mercury by 2-mercaptoethanol treatment, and for the commercial sample to which has been added enough mercuric chloride to convert all dimers to monomers are shown. Analysis of the commercial sample by chlorine resonance titration showed that in our sample, unlike that reported by Bryant,[3] approximately two-thirds of the albumin was albumin–mercury dimer and one-third was a monomer consisting of one mercury per albumin chain. It is apparent that the titration of this sample is quite different from the mercury-free sample, which corresponds to that of albumin I. The sample containing all mercury–albumin monomers reacts like the commercial sample. Evidently, in the mercury derivatives, dodecyl sulfate does not bind as strongly, and higher concentrations of detergent are necessary to block chloride interaction as detected in the magnetic resonance experiment. Titration of mercury-free albumin III which had been labeled at the free sulfhydryl with *N*-ethylmaleimide produced a curve similar to those for the unlabeled mercury-free albumin III and the albumin I–detergent titrations reported above. Evidently the mercury atom has a large effect on detergent bind-

(30) R. F. Chen, *J. Biol. Chem.*, **242**, 173 (1967).

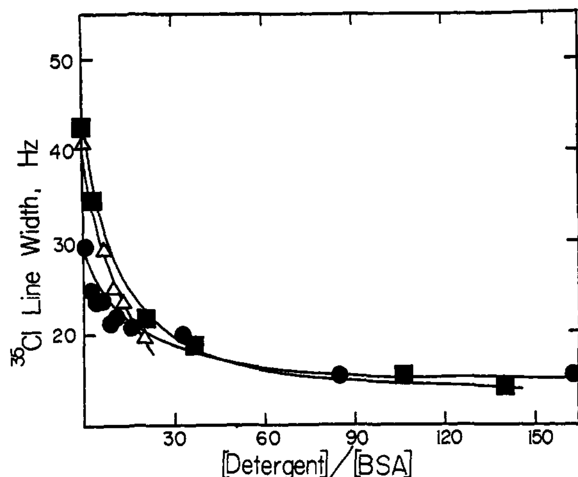

Figure 6. Titration in 1 *M* sodium chloride with SDS of 4 mg/ml of acetimidated albumin I (●); with sodium dodecyl sulfonate of 4 mg/ml of albumin I (Δ); and with SDS of 4 mg/ml of albumin I (■); chlorine-35 line width *vs.* detergent/BSA.

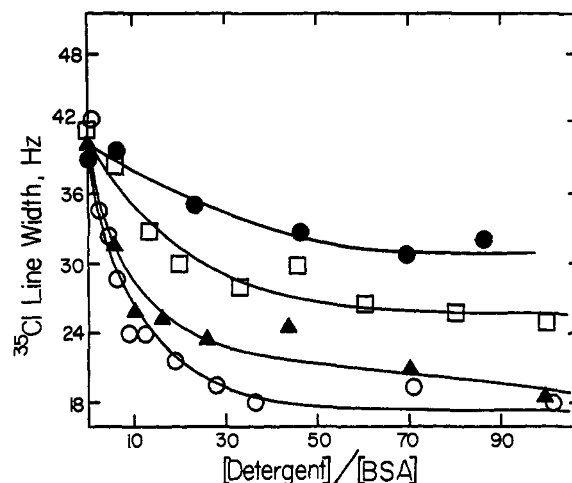

Figure 7. Titration in 1 *M* sodium chloride of 4 mg/ml of albumin I: with hexyl sulfate (●); with octyl sulfate (□); with decyl sulfate (▲); with SDS (○); chlorine-35 line width *vs.* detergent/BSA.

ing. It should be noted that the chlorine line width of mercury–albumin solutions is greater than the corresponding solutions with no mercury, because of the contribution from the chloride interaction with the mercury ion bound to albumin.[2] With the sample containing a large excess of mercuric ion the line width is always considerably greater, but the shape of the curve is the same as that of the commercial mercaptalbumin.

Titrations with Methyl Orange and sodium dodecyl sulfonate have been carried out. The sulfonate is like the sulfate, but Methyl Orange, another hydrophobic anion, is not effective in reducing chloride interactions at similar concentrations. Cetyltrimethylammonium bromide, a cationic detergent, and Triton X-100, a nonionic detergent, do not influence the chlorine ion interaction with albumin. The sulfonate titration is shown in Figure 6. At sulfonate albumin ratios near 20, a slight amount of precipitate forms.

To investigate the nature of the charge interaction, albumin I was acetimidated. The titration curve for this sample is also shown in Figure 6. Where the charge interaction has been preserved, the detergent binding is similar to that of the unreacted albumin. The line width is reduced for the 4 mg/ml of solution when compared with that of 4 mg/ml of albumin I. The pH of the acetimidated protein solution is approximately 6.45. When compared with unreacted albumins at the same pH, the line width is noticeably less. This may indicate less chloride interaction with the acetimidated derivatives.

Because dodecyl sulfate causes unfolding of albumin at high concentrations,[16] several nonunfolding detergents were examined. Figure 7 presents the titration curves for *n*-hexyl, *n*-octyl, and *n*-decyl sulfate compared with dodecyl sulfate. Effectiveness in reducing the chloride interaction is proportional to increasing alkyl chain length. Although the decyl sulfate has been shown not to cause unfolding, it does inhibit all chloride interaction at higher concentrations.

Since the correlation time influencing chlorine ion relaxation is a function of viscosity, viscosity measurements were carried out on the solutions. During the course of the experiment, the viscosity change is so small as to be almost unnoticeable (±1%). At low SDS/BSA ratios where the chlorine line width narrows appreciably, no change in viscosity was detected. The viscosity of a 4 mg/ml albumin I solution is less than 1% greater than that of the solvent 1 *M* sodium chloride; therefore, the contribution from macroscopic viscosity to the line width in albumin solutions is negligible. This reasoning assumes that the microscopic and macroscopic viscosity are somewhat related in influencing the correlation time.[31,32] An intrinsic viscosity might be a better indication of change in the microscopic viscosity or viscosity at the chlorine ion binding site. Intrinsic viscosities were determined for albumin I solutions in 1 *M* sodium chloride. Concentrations greater than those of the nmr experiment were necessary, since 2 and 4 mg/ml solutions produced little or no change in viscosity. Intrinsic viscosities at 37° with SDS/BSA ratios of 0, 3, and 15 were 2.7, 3.1, and 2.6 cc/g, respectively.[33] The reduced viscosities for given concentrations of BSA were constant for the above SDS/BSA ratios. This is in agreement with work of Reynolds, Gallagher, and Steinhardt[34] who demonstrated that reduced viscosities do not increase until bound SDS/BSA is greater than 20 at pH 5.6. Thus, it appears unlikely that changes in the chlorine line width are related to gross changes in the correlation time τ_r.

We have examined the effect of lysozyme and α-chymotrypsin on the chloride line width for sodium chloride solutions of these enzymes. The broadening produced is small compared to that of the albumin. A

(31) R. A. Craig and R. E. Richards, *Trans. Faraday Soc.*, **59**, 4406 (1963).

(32) C. Deverell and R. E. Richards, *Mol. Phys.*, **9**, 551 (1966).

(33) Density corrections have not been applied for these values. This is relatively unimportant compared with changes in these numbers which might be reflected in the rotational correlation time. As they vary only slightly, they may indicate no drastic change in the correlation time for the protein. These values correspond quite closely to the 3.7 cc/g usually quoted for albumin, a value usually determined at lower ionic strength and lower temperature: C. Tanford, "Physical Chemistry of Macromolecules," Wiley, New York, N. Y., 1961, p 394. The anomalous viscosity region reported by Reynolds and coworkers in ref 16 was not investigated; extrapolation from higher concentration to zero concentration was done to determine the intrinsic viscosities.

(34) J. A. Reynolds, J. P. Gallagher, and J. Steinhardt, *Biochemistry*, **9**, 1232 (1970).

10 mg/ml solution of these proteins was used to obtain line widths of 21.2 Hz for lysozyme and 18.8 Hz for α-chymotrypsin. Lysozyme was chosen for investigation because it has many sites for chlorine ion binding.[12] The small increase in broadening may be a reflection of the reduced correlation time for the smaller proteins. Dodecyl sulfate titration of the lysozyme was attempted, but precipitation of the detergent resulted. With 10 mg/ml of α-chymotrypsin, SDS/protein ratios of 25/1 produced no decrease in chloride interaction as detected by the nuclear magnetic resonance technique. Albumin binds the detergent quite well and, as already noted, promotes the solubility of the detergent in 1 *M* sodium chloride. Dodecyl sulfate interaction with albumin is quite characteristic. These other proteins may require much higher concentrations of SDS before chloride interaction is blocked. Because of the small line broadening produced by these smaller molecular weight proteins, it will be necessary to increase the fraction of bound chloride. One way is to increase the protein concentration. Another way is to reduce the sodium chloride concentration. The extension of this technique to lower salt concentrations is desirable for both increasing the bound chloride fraction and also for the purpose of reducing the ionic strength. Because of the low sensitivity of the nmr method, time averaging techniques for signal enhancement are necessary. By using a time averaging computer, 0.1 *M* sodium chloride solutions could be investigated.[35] This will increase the contribution to the line width from the bound chloride and also eliminate some detergent solubility problems which occur at high salt concentration.

Discussion

Chlorine-35 nmr spectroscopy is already established as a good tool for probing biological systems.[36] Obviously, to be of value, sites for chloride interaction must exist. This may be through a bound metal ion or to some positively charged site on the protein itself, as with bovine serum albumin. Changes in the sites are then reflected by changes in the line width, as when dodecyl sulfate binds to the protein. Unfortunately, the technique does not allow one to determine thermodynamic parameters, as the relaxation mechanism producing line broadening involves several terms which are not explicitly known. However, one can obtain information about the ionic interaction of the protein with its environment. The results are most meaningful when interpreted in conjunction with other techniques for studying protein–ligand binding.

The binding of dodecyl sulfate to albumin reduces chloride binding, but it cannot be definitely established, although it seems likely, that the sulfate binds at a chloride site. Ray and coworkers,[15] in order to explain the greater binding of octyl sulfate over octanol, have proposed that binding exists at or near a cationic group or cluster, presumably composed of lysines or arginine.[15,37] In this case the reduction in chloride line width would result from competition of the sulfate for the chloride site. The binding constants for the two ions may be related in no major way as the chloride binding is Coulombic while the detergent binding is primarily hydrophobic.

A possibility for having different sulfate and chloride binding sites or a mixture of several similar and several distinct sites exists. Binding of dodecyl sulfate at one site might reduce the affinity for chloride at another site through electrostatic interaction. However, binding of detergent anions is relatively independent of protein charge implying that the detergent produces few electrostatic perturbations.[38] Indeed Cassel and Steinhardt[17] have demonstrated that in ΔpH measurements electrostatic correction factors calculated from Debye-Hückel theory are too large. In unbuffered solutions and in solutions buffered at pH 4.75, the nmr binding results are similar. Therefore, over the narrow pH region examined, the chloride–detergent titrations are also insensitive to pH and thus to charge variations.

The effectiveness of various alkyl sulfates in reducing chloride interaction increases with the binding affinity and number of sites, parameters which are known to correlate with chain length. Using previously reported values for binding constants and site numbers,[16] it can be shown that even where high- and low-energy sites are filled with octyl sulfate, all sites for chloride binding are not removed. However, decyl sulfate is effective in removing all chloride interaction, albeit the concentration must be higher than for dodecyl sulfate. It has been hypothesized that the decyl detergent is just the right length to interact with both hydrophobic and specific ionic sites. The reduced ability to block chloride binding compared with the dodecyl sulfate may reflect the lower number of high energy sites. Apparently neutralization of the chloride binding site is not a primary factor in determining the detergent's ability to produce massive unfolding. Both decyl sulfate and dodecyl sulfonate, two nonunfolding detergents, prevent chloride binding at low concentrations of detergent. Indeed, the sulfonate is as effective as dodecyl sulfate. Change in the electrostatic nature of the protein at the chloride binding sites may be a necessary condition for unfolding but certainly is not sufficient. This agrees with earlier suggestions[16] attaching little importance to electrostatic repulsion as a denaturing force.

(35) Magnesium-25 cations have been examined in our laboratory. With time averaging, we have been able to reduce the magnesium concentration tenfold from those concentrations required for adequate signals from single scans.

(36) Other nmr techniques for studying detergent binding have been developed: (a) T. W. Johnson and N. Muller, *Biochemistry*, **9**, 1943 (1970); (b) R. M. Rosenberg, H. L. Crespi, and J. J. Katz, *Biochim. Biophys. Acta*, **175**, 31 (1969).

(37) J. Markus, R. L. Love, and F. C. Wissler, *J. Biol. Chem.*, **239**, 3687 (1964).

(38) J. R. Reynolds, J. P. Gallagher, and J. Steinhardt, *Biochemistry*, **9**, 1232 (1970).