Fluorine Magnetic Resonance Study of the Interaction of Serum Albumin with 8,8,8-Trifluorooctylbenzene-*p*-sulfonate Ions*

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ABSTRACT: Sodium 8,8,8-trifluorooctylbenzene-*p*-sulfonate (NaF_3OBS) has been prepared and shown by fluorine nuclear magnetic resonance to behave as a typical anionic detergent with a critical micelle concentration of 0.0218 M. Equilibrium dialysis and ultraviolet spectrophotometry were used to find the binding isotherm for the interaction of F_3OBS^- and bovine serum albumin at a pH of 6.8 and 30°. From nuclear magnetic resonance measurements on these solutions the fluorine chemical shift of the bound detergent ions was calculated as a function of $\bar{n}$, the average number of ions bound per molecule of protein. The results are discussed in terms of a model according to which up to 17 F_3OBS^- ions can be bound at initial binding sites where their chemical shift is 3.475 (parts per million upfield from the external reference 1,1,2-trichlorotrifluoro-1-propene). These binding sites apparently persist when the number of bound ions is $r \geq 17$, and then the additional $(r - 17)$ ions are in a micelle-like environment where their chemical shift is near 4.83.

Exchange among all bound sites and the free state is rapid on the nuclear magnetic resonance time scale. Observed variations in the fluorine and proton line widths are also consistent with the simple model. No evidence was found that would show whether or not AD_r complexes with one or a few specific values of r are formed preferentially in these solutions.

The binding of detergent ions by proteins has been studied by a variety of methods most of which involve the determination either of a bulk property of the solution or of a spectroscopic variable associated with the protein molecule. An alternative approach is to use a detergent containing a suitable chromophore and to observe changes in the corresponding spectrum brought about by the binding process. It is becoming increasingly recognized that physical interactions of organic molecules can conveniently be studied by nuclear magnetic resonance spectroscopy using a fluorine substituent as the chromophore, because of the pronounced solvent dependence of fluorine chemical shifts. Micellization of detergents has been studied in our laboratory (Muller and Birkhahn, 1967, 1968; Muller and Johnson, 1969a) by a fluorine nuclear magnetic resonance method, and others have used this technique to investigate the binding of small molecules by enzymes (Spotswood *et al.*, 1967; Zeffren and Reaville, 1968; Sykes, 1969). We have undertaken to explore the utility of this method for studies of protein–detergent interactions and report here some results for bovine serum albumin and F_3OBS^-.[1]

We have shown previously that the physical properties of soaps or detergents with terminally trifluorinated alkyl chains are closely parallel to those of the analogous unfluorinated materials, the major difference being that the critical micelle concentration is approximately doubled when the fluorine atoms are introduced. It thus appeared likely that interactions between bovine serum albumin and such fluorinated detergents would be similar to those between this protein and ordinary detergents (Decker and Foster, 1966), though one might also expect differences such as those found (Reynolds *et al.*, 1967) among detergents of the same family when the length of the hydrocarbon chain is varied.

F_3OBS^- was chosen as the first detergent ion to be studied because it is fairly easy to make, its critical micelle concentration is high enough to permit the use of solutions which produce moderately strong nuclear magnetic resonance signals without encountering complications from micelle formation, and its absorption spectrum makes it readily possible to determine free-detergent concentrations by equilibrium dialysis and ultraviolet spectrophotometry.

Experimental Section

Materials. The fluorinated detergent was prepared as the sodium salt, NaF_3OBS, starting with 8-phenyloctanoic acid (Sapon Laboratories) and using the sequence of reactions

$$C_6H_5(CH_2)_7COOH + H_2SO_4\ (20\%\ \text{fuming}) \longrightarrow HO_3SC_6H_4(CH_2)_7COOH + H_2O \quad (1)$$

$$HO_3SC_6H_4(CH_2)_7COOH + 3SF_4 \longrightarrow CF_3(CH_2)_7C_6H_4SO_2F + 2HF + 3SOF_2 \quad (2)$$

$$CF_3(CH_2)_7C_6H_4SO_2F + 2NaOH\ (\text{aqueous}) \longrightarrow CF_3(CH_2)_7C_6H_4SO_3Na + NaF + H_2O \quad (3)$$

Details of the synthetic procedures are available elsewhere (Johnson, 1970). The product was recrystallized four times from water, extracted with hexane for 11 hr, and then

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[1] Abbreviations used are: F_3OBS^-, 8,8,8-trifluorooctylbenzene-*p*-sulfonate; AD_r, a complex containing one molecule of albumin and r detergent ions; $\bar{n}$, average number of detergent ions bound per bovine serum albumin molecule.

recrystallized twice more, first from water and then from slightly wet 1-butanol. *Anal.* Calcd: C, 48.55; H, 5.20; F, 16.47; Na, 6.65; S, 9.25. Found: C, 48.60; H, 5.23; F, 16.30; Na, 6.51; S, 9.50. The proton nuclear magnetic resonance spectrum showed only the peaks anticipated for the *para* isomer of sodium 8,8,8-trifluorooctylbenzenesulfonate.

Crystalline bovine serum albumin (Nutritional Biochemicals Corporation, control number 8161) was used without further purification.[2] Buffer salts and other electrolytes were reagent grade. Water from the laboratory distilled-water supply was redistilled in glass apparatus, collected, and capped while hot to exclude gases.

Methods. For the dialysis experiments, the tubing (Union Carbide Corp. no. 36, flat width 1.73 in.) was cut along one edge, opened, and washed first with dilute detergent and then with hot water. It was stored under water with a small amount of dissolved benzoic acid to retard biodegradation. Immediately before use it was washed with water and then with the appropriate electrolyte solution. The buffer solution used in most of this work contained 0.048 M NaH_2PO_4 and 0.021 M Na_2HPO_4, giving a nominal pH of 6.8. Addition of protein reduces the pH slightly, but in no case should this depression exceed 0.3 pH unit.

Standard solutions of NaF_3OBS were used in silica microcells with a 1-cm path length in a Beckman DU spectrophotometer, to determine the extinction coefficient at 223 mμ. The resulting value, 1.35×10^4, is in good agreement with the values for octyl- and dodecylbenzenesulfonate (1.32×10^4 and 1.35×10^4, respectively) reported by Decker and Foster (1966). Bovine serum albumin concentrations were obtained spectrophotometrically at 279 mμ using an $\epsilon_{1\text{ cm}}^{1\%}$ value of 6.67 and assuming a protein molecular weight of 69,000.

Solutions containing known weights of detergent and protein in buffer were dialyzed against buffer solution in a multicavity dialysis cell (Chemical Rubber Co. no. 0611/71) at 30° for 8–16 hr, the cell being continuously turned about a horizontal axis at 10 rpm. In a trial run it was found that equilibrium was more than half-complete in 1 hr. The detergent concentration in each protein-free compartment was determined spectrophotometrically, as was the protein concentration in a portion of the solution from the opposite compartment. The remainder of each protein-containing solution was placed in a nuclear magnetic resonance tube with a sealed capillary tube containing the reference compounds. A mixture of 1,1,2-trichlorotrifluoro-1-propene with 2% 1,2-difluorotetrachloroethane was used to provide the reference peak (trichlorotrifluoropropene) and to verify that the temperature during each nuclear magnetic resonance spectral run was 30.5 + 1.0° (Muller and Johnson, 1969b). A few spectra were obtained with trifluoroacetic acid as the reference.[3] Fluorine nuclear magnetic resonance spectra were recorded with a Varian HA spectrometer operated at 56.445 MHz, with a Varian C-1024 signal-enhancing computer. About 50 scans were sufficient to provide adequate spectra for chemical shift determinations, made by setting the recorder pen on the estimated center of the resonance and then finding the difference between the manual and swept oscillator frequencies. All shifts are reported in parts per million, a positive sign being used when the shift is to higher field. The precision varies with the signal strength and shape, but in no case should the uncertainty exceed ±0.02. In some of the spectra, the fine structure due to spin–spin coupling of the fluorine nuclei with nearby protons was removed by double irradiation with an NMR Specialties SD-60B heteronuclear spin decoupler. This was found to have no effect on the fluorine chemical shift, and it makes the signal somewhat easier to detect.

A few proton nuclear magnetic resonance spectra were obtained with a Varian A-60A spectrometer using solutions in D_2O prepared with bovine serum albumin which had been twice dissolved in 99.77% D_2O and twice freeze dried.

No protein-containing solutions were used that had stood at room temperature for more than 1.5 days, or that had been stored under refrigeration (0–4°) for over 1 week.

Results and Discussion

Properties of NaF_3OBS. Since NaF_3OBS is a new material we characterized it by finding its fluorine chemical shift as a function of concentration in water and also in buffer solutions like those used to prepare the protein-containing samples. The monomer shift, micelle shift, and cmc were evaluated from plots of the shifts against the reciprocal of the total detergent concentration (Muller and Birkhahn, 1967). The monomer shift is 3.65 at 36.8° and 3.745 at 30.5°, almost exactly the same as values for previously examined trifluorodetergents. The micelle shift, 4.63 at 36.8°, is about 0.2 ppm lower than those found for detergents with no aromatic ring in the hydrophobic portion. The critical micelle concentration (cmc) is 0.0218 M, very nearly twice the value, 0.011 M, reported for ordinary octylbenzenesulfonate (Paquette *et al.*, 1943). The monomer and micelle shifts are not significantly affected by the buffer, but the critical micelle concentration is reduced to 0.0082 M. This lowering conforms to the empirical rule that the critical micelle concentration of an ionic detergent follows a relation of the type

$$\log cmc = a + b \log (M^+) \qquad (4)$$

where (M^+) is the total counterion concentration and b is in the neighborhood of −0.6 (Corrin, 1948). The free-detergent concentrations, D_f, in the protein–detergent samples were below 0.004 M except for the three solutions at the highest $\bar{n}$,

[2] A referee has raised the possibility that our results might be affected by unsuspected traces of paramagnetic metal ions introduced with the bovine serum albumin. However, when proton magnetic resonance spectra were examined for solutions of the protein in D_2O–HDO we observed that such impurities were present, if at all, at too low a level to cause appreciable broadening of the HDO peak. In separate experiments using aqueous detergent solutions doped with Mn^{2+} at concentrations high enough to broaden the water peak severely, we could detect no changes in the position or width of the CF_3 signal. As we had expected, the covalently bound fluorine atoms do not interact efficiently with the metal ion, and therefore impurities of this sort should be without effect.

[3] In the temperature range $30° < T < 60°$ shifts measured using trifluoroacetic acid can be converted into the trichlorotrifluoropropene scale using $\delta (Cl_3F_3C_3) = \delta (F_3CCOOH) + 16.855 - 5.5 \times 10^{-3}T$. Older data on trifluorodetergents (Muller and Birkhahn, 1967, 1968), measured using $C_6H_5CF_3$ as the reference, can be converted into the trichlorotrifluoropropene scale using: $\delta (Cl_3F_3C_3) = \delta (C_6H_5CF_3) + 2.253 + 2.9 \times 10^{-4}T$.

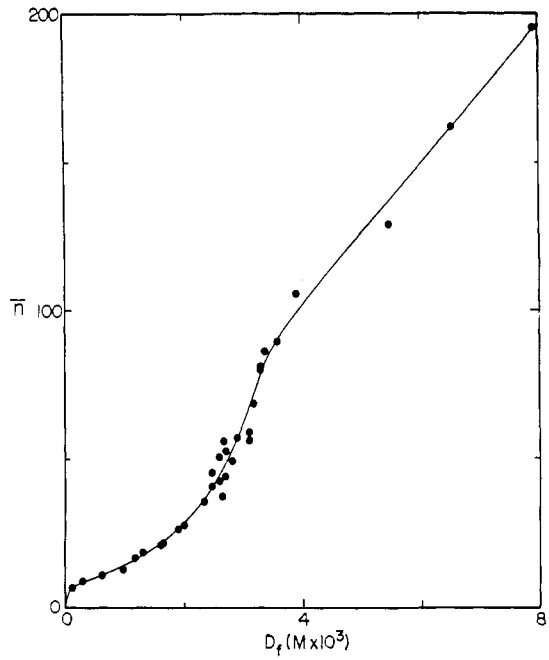

FIGURE 1: Binding of F_3OBS^- by bovine serum albumin at pH 6.8 and 30° shown as a plot of $\bar{n}$, the average number of detergent ions bound per molecule of protein, against the free-detergent concentration, D_f.

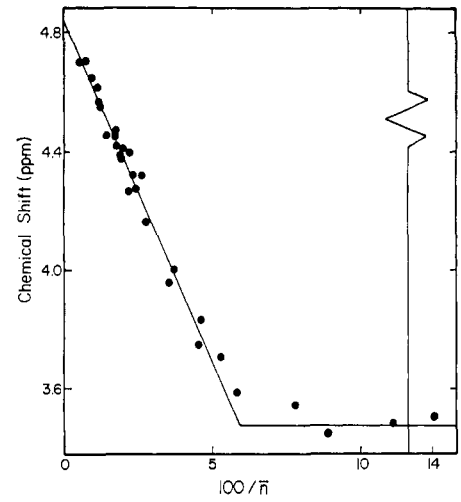

FIGURE 2: Fluorine chemical shifts (parts per million to higher field from the external reference, 1,1,2-trichlorotrifluoro-1-propene) for bound F_3OBS^- as a function of $100/\bar{n}$. The solid line for $100/\bar{n} < 5.9$ corresponds to eq 7.

which had D_f = 0.00546, 0.00652, and 0.00792 M, respectively. It is therefore highly unlikely that enough ordinary micelles are present in these solutions to exert a significant effect on the chemical shifts.

Equilibrium Binding Data for Bovine Serum Albumin–F_3OBS^-. From the spectrophotometric analysis of each solution obtained in the equilibrium dialysis experiments, the free-detergent concentration and the average number of ions bound per bovine serum albumin molecule, $\bar{n}$, were calculated. The results are presented graphically in Figure 1. Apparently F_3OBS^- is somewhat less tightly bound by the protein than unfluorinated octylbenzenesulfonate, as shown, for example, by the fact that for F_3OBS^- $\bar{n}$ is approximately 20 when $D_f = 1.5 \times 10^{-3}$ M, while for OBS^- the same value of $\bar{n}$ is reached when D_f is only 0.6×10^{-3} M (Decker and Foster, 1966). This difference parallels the difference in critical micelle concentrations for the two detergents, mentioned above. A plot of $\bar{n}/D_f$ against $\bar{n}$ in the region of low binding numbers (Scatchard *et al.*, 1957; Johnson, 1970) suggests that the protein has about 11 equivalent and noninteracting binding sites for F_3OBS^-. Similar results were reported by Decker and Foster (1966) for octyl- and dodecylbenzenesulfonates.

Chemical Shift Data. The fluorine magnetic resonance of F_3OBS^- in solution is a triplet because of spin–spin coupling between the fluorine nuclei and the protons of the adjacent methylene group. When bovine serum albumin is present, the position of this triplet and the widths of its components depend on the protein and detergent concentrations, but in no case is an additional signal observed. This shows that exchange of the detergent ions between the free state and all possible bound states is rapid on the nmr time scale, that is, the residence time of a trifluoromethyl group in any one environment is no longer than 10^{-2} sec (indeed probably no longer than 10^{-3} sec, see below.) Since the chemical shift, δ_f, of the free detergent is known, and the D_f was measured for each solution, the average chemical shift for bound detergent ions, δ_b, could be calculated with the formula

$$\delta_b = \delta_{obsd} + (D_f/D_b)(\delta_{obsd} - \delta_f) \quad (5)$$

where D_b is the concentration of bound F_3OBS^- and δ_{obsd} is the measured chemical shift. The values so obtained are presented in Figure 2, where δ_b is plotted as a function of $1/\bar{n}$. There was no *a priori* reason to plot the data in this way, but it is noteworthy that when this is done the points for $\bar{n} > 17$ fall very nearly on a straight line, while for $\bar{n} < 17$ the shift is approximately constant.

Region $\bar{n} < 17$. Using the symbol $[AD_r]$ to represent the concentration of the species formed when one molecule of albumin binds r detergent ions, and δ_r for the detergent chemical shift for this species, the average bound detergent shift is given by

$$\delta_b = \sum_r r\delta_r[AD_r]/\sum_r r[AD_r] \quad (6)$$

Then the near constancy of δ_b for $\bar{n} < 17$ could most easily be explained by supposing that in this range only species with $r < 17$ are present in significant amounts and that all of these have the same or nearly the same δ_r, equal approximately to 3.475. It is striking that this value is smaller than δ_f, whereas the micelle shift is considerably larger than δ_f. Similar shifts to lower field have been reported for other small, fluorine-containing molecules bound by dissolved proteins (Spotswood *et al.*, 1967; Zeffren and Reaville, 1968; Sykes, 1969). Evidently the environment of bound detergent at low binding numbers is quite unlike that in the micelle. We have found that other solvent systems which yield fluorine shifts near 3.47 include aqueous electrolyte solutions, aqueous solutions of certain organic compounds including glycine,

glycerol, and urea, and plain water at elevated temperatures (Johnson, 1970). Although no firm conclusions can be drawn at this stage, it seems most likely that the CF_3 groups in complexes with $r < 17$ are in a highly aqueous environment.

To test this hypothesis we determined the chemical shift for several solutions with $\bar{n} \cong 8$ in which the solvent was replaced either by D_2O and buffer or by solutions of water, buffer, and an additive (1 M urea, 1 M ethylene glycol, 0.38 M LiCl, or 0.32 M NaCl, see Johnson, 1970). The changes in δ_b are small and, because the lines are quite broad, difficult to measure precisely. However, in each case, the effect is in the same direction as the effect of a similar solvent change upon δ_f measured in protein-free solutions. The only possible exception is ethylene glycol where, because the effects are less than 0.01 ppm, even the signs cannot be reliably established. The possibility cannot be excluded that the solvent changes may be influencing δ_b indirectly by causing small changes in the protein conformation. Nevertheless the results again suggest that CF_3 groups of bound F_3OBS^- are near the "surface" of the protein–detergent complex where they can be directly affected by changes in solvent composition.

In a further effort to elucidate the nature of the binding, we measured the chemical shift of $CF_3(CH_2)_9OH$ (prepared from trifluorocapric acid by a lithium aluminum hydride reduction, see Johnson, 1970) solubilized by bovine serum albumin and by sodium dodecyl sulfate. The alcohol is virtually insoluble in water, and $\bar{n}$ values were calculated assuming that in the alcohol–bovine serum albumin solutions all of the dissolved alcohol is protein bound. For three solutions with $\bar{n} = 6$, 13, and 21, the chemical shift is nearly constant at 3.61 ppm. In contrast to this, when the alcohol is solubilized in detergent micelles its shift is about 4.5 ppm. The neutral alcohol thus behaves rather like the charged detergent ion, and it would seem that the proton can bind either species at rather similar (but by no means necessarily identical) sites.

Region $\bar{n} > 17$. For dodecyl sulfate and dodecylbenzenesulfonate it has been shown that solutions with $11 < \bar{n} < 80$ behave as if they contained only three AD_r species, with $r = 11$, n, or $2n$, where n is a number in the neighborhood of 40, the exact value depending on the nature of the detergent ion and perhaps on the experimental conditions. For detergents with shorter hydrocarbon chains, evidence for the formation of complexes with preferred r values was not obtained (Pallansch and Briggs, 1954). This raises the question of whether or not such behavior can be deduced from the nuclear magnetic resonance data for bovine serum albumin–F_3OBS^-.

When $n > 17$, the data points fall close to the straight line

$$\delta_b = 4.83 - 23.0/\bar{n} \quad (7)$$

With the help of eq 6 it is readily verified that a sufficient (but no necessary) condition to assure that eq 7 will be obeyed is that only species with $r > 17$ be present and that, for any of these

$$\delta_f = 4.83 - 23.0/r \quad (8)$$

How many different AD_r species actually occur becomes irrelevant if this condition is satisfied, and therefore the nuclear magnetic resonance results can be used neither to prove nor to rule out the existence of complexes with preferred values of r. They do, however, rule out the possibility that there is a range of binding numbers such as $n < \bar{n} < 2n$ in which there are only two species, AD_n and AD_{2n}, with the same chemical shift, as this would require a plateau in the plot of δ_b *vs.* $1/\bar{n}$.

Although the data allow more than one interpretation it is worth noting that eq 8 could arise in the following appealingly simple way. Let it be supposed that the protein has 17 sites at which F_3OBS can be tightly bound, which are not necessarily equivalent but which all cause the detergent resonance to fall at or near 3.475. When $r > 17$, the first 17 ions occupy these preferred binding sites, and the remaining $(r - 17)$ find themselves in a micelle-like environment with $\delta = 4.83$. This could occur either because the protein has numerous hydrophobic sites where the ions can be bound with $\delta = 4.83$ or because enough additional ions are bound in one step to create a micelle-like aggregate, attached to the bovine serum albumin molecule and stabilized by it. On the basis of the earlier work on bovine serum albumin–detergent interactions we feel that the latter possibility is the more likely. In either case, with rapid exchange of detergent ions among all binding sites, such species would have $\delta_r = [17(3.475) + (r - 17)(4.83)]/r$, which is identical with eq 8.

There appear to be no compelling reasons to reject such a simple model, though we emphasize again that it is not proved by the available data. It is at first glance somewhat disturbing that for dodecyl sulfate the sites which account for binding when $r < 10$ appear to be destroyed when r becomes much larger (Pallansch and Briggs, 1954). However, Reynolds *et al.* (1967) have found that this destruction of binding sites is accompanied by a gross disorganization of the protein structure, and that, if the detergent is replaced by decyl sulfate or other species with relatively short hydrocarbon chains, 100 or more ions can be bound *without* such a major structural change. It is quite possible that F_3OBS^- falls into the latter category and that for these materials the initial binding sites are preserved over the whole range of binding numbers. If this is so, then the dependence of δ_b on $\bar{n}$ may be strikingly different when a larger detergent ion is used, and this possibility will be explored in future work in this laboratory.

Another discordant note is that while the nuclear magnetic resonance data suggest that there are 17 initial binding sites, the number obtained from the Scatchard plot is 11. We believe that the difference is real, but offer no interpretation for it except to note that the Scatchard procedure yields the number of equivalent and noninteracting binding sites, while the chemical shifts suggest only that 17 sites, which may not be equivalent or noninteracting, can bind detergent with $\delta \cong 3.475$ and that most of these sites are filled before others are used. Thus the two results are not necessarily in conflict.

Line Widths. The detergent fluorine signals in the absence of bovine serum albumin are about 2.2 Hz wide at half-height because of unresolved long-range spin–spin couplings. When protein is present they are broadened, the greatest widths being reached when $\bar{n}$ is small. The line width, $\Delta\nu$, is related to the relaxation time, T_2, by the equation $\pi\Delta\nu = 1/T_2$, and in rationalizing the changes in $\Delta\nu$ it is convenient to recall

the equation (Pople *et al.*, 1959) for the relaxation time of a nucleus exchanging between two sites, a and b

$$1/T_2 = P_a/T_{2a} + P_b/T_{2b} + 4\pi^2(\nu_a - \nu_b)^2P_a^2P_b^2(\tau_a + \tau_b) \quad (9)$$

Here P_a, ν_a, τ_a, and T_{2a} are, respectively, the probability of finding the nucleus at site a, its chemical shift in frequency units at that site, its mean residence time there, and its relaxation time there, and the symbols with b subscripts represent the corresponding quantities for site b. Strictly speaking, the present situation demands a more general equation allowing for more than two sites, since not all bound detergent molecules are in equivalent positions, but some semiquantitative conclusions can be drawn from eq 9 by letting the a site be the free detergent and assigning to the b site properties which represent average values for bound detergent.

The observed line broadening could then arise in two ways. Since the motional freedom of the detergent ion diminishes when it is bound, T_{2b} should be significantly smaller than T_{2a}, and then the term P_b/T_{2b} alone could account for the changes in line width. Alternatively, if exchange between the free and bound states is not too fast, the broadening could be ascribed to the last term in eq 9. The quantity $4\pi^2(\nu_a - \nu_b)^2P_a^2P_b^2$ varies with $\bar{n}$ and for the lowest $\bar{n}$ values, where the lines are broadest, is of the order of 10^2 sec^{-2}. This term could then contribute appreciably to the line width if the sum of the mean residence times, $(\tau_a + \tau_b)$, is larger than about 10^{-2} sec. At much higher binding numbers, $(\nu_a - \nu_b)^2$ is much larger, and a significant contribution to the line width could arise even with τ's in the range 10^{-3}–10^{-2} sec.

A tentative choice between these two possibilities may be made on the basis of line-width measurements in the proton magnetic resonance spectra of bovine serum albumin–F_3OBS^- solutions in D_2O. The signals from the protein are very broad (Kowalsky, 1962). We found that they are essentially unchanged by the presence of the detergent, which supports the view that F_3OBS^- causes no extensive unfolding of the protein. The detergent–proton resonances are a little broader than the fluorine signals. Binding by the bovine serum albumin induces only very small changes in the detergent–proton chemical shifts, so that the quantity $4\pi^2(\nu_a - \nu_b)^2P_a^2P_b^2$ is here almost certainly less than 1.0. Therefore the broadening could be attributed to the τ-dependent term only by assuming residues times greater than 1 sec, and this would lead to fluorine signals much broader than those actually found. Thus the width of the proton signals must be ascribed to the P_b/T_{2b} term, and then the similarity between the proton and fluorine line widths very strongly suggests that the latter also arise predominantly in this way and that the residence times are probably less than 10^{-3} sec.

Assuming then that the last term in eq 9 is negligible, it becomes possible to calculate T_{2b} from the observed fluorine line widths. The values so obtained (Figure 3) are not very precise because of the low signal-to-noise ratios but clearly decrease as $\bar{n}$ decreases. Such a trend is quite consistent with the simple model described above. It is to be expected that the motional freedom of the detergent ions would be most restricted when they are bound at one of the postulated 17 initial binding sites, and the relaxation time for such ions

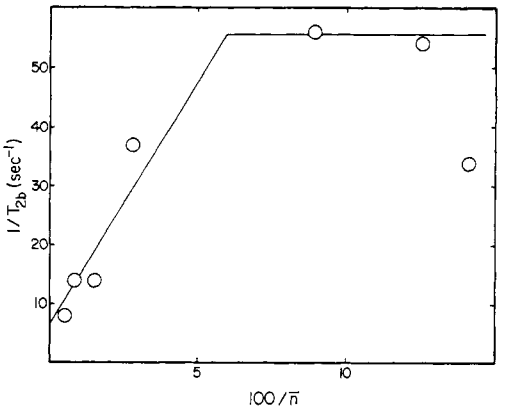

FIGURE 3: Fluorine nuclear relaxation times for bound F_3OBS^- at several values of $\bar{n}$, shown as a plot of $1/T_{2b}$ *vs.* $100/\bar{n}$. The solid line for $100/\bar{n} < 5.9$ corresponds to eq 11.

may be assigned the symbol T_{2i}. Since micellization normally causes no appreciable change in the fluorine line widths, the $(r - 17)$ ions supposedly bound in a micelle-like environment should have relaxation times essentially equal to T_{2a}. The average relaxation time for an AD_r species with $r > 17$ should then obey

$$(1/T_{2b})_r = [17(1/T_{2i}) + (r - 17)(1/T_{2a})]/r \quad (10)$$

which yields after averaging over all species

$$1/T_{2b} = 1/T_{2a} + (17/\bar{n})(1/T_{2i} - 1/T_{2a}). \quad (11)$$

Using $T_{2a} = 0.14$ sec and $T_{2i} = 0.018$ sec, eq 11 reproduces the observed variation of T_{2b} with $\bar{n}$ reasonably well, as shown in Figure 3. Since it is difficult to estimate reliably the errors in the experimental values of T_{2b}, especially for the weakest signals, it is not clear what significance, if any, should be attached to the point at the extreme right. We hope eventually to reinvestigate the region of lowest $\bar{n}$ with the improved sensitivity afforded by a spectrometer operated at 94.1 MHz.

It is interesting that the required value of T_{2i} is about two orders of magnitude larger than that calculated by Fischer and Jardetzky (1965) for protons of penicillin G bound to serum albumin. If the interpretation offered for the present data is correct, this indicates that even for the most tightly bound detergent ions the CF_3 group has considerably more motional freedom than any part of the bound penicillin molecule.

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Multioscillator Fluorescence Depolarization. Anisotropy of Dye Binding*

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ABSTRACT: Fluorescence polarization measurements can be used to determine whether dyes bind to proteins with specific or random orientations. Polarization, as a function of temperature and viscosity, is measured at several exciting wavelengths corresponding to various absorption oscillators. In this way there is a photoselection for different protein–dye ensembles whose emission will be observed. A theoretical analysis indicates that it is possible to distinguish a specific orientation of the bound dye relative to the protein, from a random orientation, provided that the protein is not a sphere. Examples of specific as well as random binding have been observed with dyes bound to liver alcohol dehydrogenase and bovine serum albumin. Situations where a dye can rotate independently of the protein are also discussed.

The interaction between small molecules and proteins is usually described in terms of stoichiometry, binding affinity, and the relationships between the various binding sites (Weber, 1965). While such data are basic to an understanding of protein–ligand interactions, additional information about binding can be obtained. For example, it is of interest to obtain an estimate of the extent of orientation of a small molecule relative to a protein. A study of the orientational anisotropy as a function of temperature, pH, or number of moles of a small molecule bound to a protein would complement other binding data to yield an improved picture of the molecular interaction.

One approach for evaluating the orientational anisotropy of bound dyes is that of Weber and Anderson (1969) who examined the energy transfer between several 1-anilinonaphthalene-8-sulfonate molecules bound to bovine serum albumin. They concluded that there is orientational anisotropy in this system, *i.e.*, that the binding is not random. This method requires that more than 1 molecule of dye be bound per protein molecule since otherwise energy transfer will not occur.

This paper describes another approach, based on depolarization of fluorescence at different exciting wavelengths, which allows us to discriminate between random and specific orientation of a single ligand bound to a prolate ellipsoid. Using this method we have compared the extent of orientational specificity of several complexes and conjugates. Only a few of these will be illlustrated here as examples of the applicability of the method.

We also measured the degree of orientational anisotropy, σ, of anilinonaphthalenesulfonate adsorbed to bovine serum albumin as a function of $\bar{n}$, the average number of anilinonaphthalenesulfonate molecules bound per bovine serum albumin molecule.

Theory

Binding Classes. It is convenient to postulate three general binding classes. Class I encompasses those protein–dye adducts in which the dye always maintains a constant orientation relative to the protein axes. The dye absorption and emission oscillators can thus be regarded as intrinsic protein oscillators, uniquely fixed relative to the protein. Class II adducts are those in which the dye is fixed relative to the protein, but in a

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