Binding of Large Organic Anions and Neutral Molecules by Native Bovine Serum Albumin*

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ABSTRACT: Measurements are reported of the binding, by the highest affinity sites on bovine serum albumin (BSA), of *n*-octane, and four octane derivatives (1-octanol, octyl sulfate ion, octyl sulfonate ion), and of two *n*-dodecane derivatives (1-dodecanol and dodecyl sulfate). These measurements constitute the first part of an investigation of the nature of the binding sites and the source of the binding energy involved in the binding of neutral molecules and ions, other than hydrogen ion, by proteins. The results show that the hydrocarbon moiety of all of these substances contributes the major share of the binding energy, and that the binding energy increases markedly with the length of the hydrocarbon chain. In the case of the anions, coulomb forces appear to make a subsidiary contribution of 1000–2000 cal; these forces are exerted by cationic sites, without regard to the net negative charge. Although this study has been limited to the region of relatively small amounts of binding, it is clear that there are more than a single set of sites in the native protein which differ in their affinity for a particular ligand, although this effect can be masked, as with dodecyl sulfate, by an unfolding of the molecule and the consequent release of new sites, at concentrations below those at which lower affinity sites of the native protein can be engaged.

Only the binding on sites of the highest affinity, whether in native or unfolded protein, has an important effect on the ultraviolet difference spectrum in the 270- to 295-mμ region; this effect (*i.e.*, a blue shift) does not necessarily indicate unfolding. A pronounced dependence of binding on protein concentration has been found with the longer chain ligands, and has been interpreted as an effect due to the binding of a single ligand to two protein molecules, when the ligand concentration is low and the protein concentration high. pH (within the stability region) affects the binding of neutral ligands but not of anions; possible explanations of this anomalous pH effect are offered.

Studies of the prototropic (acid–base) equilibria of proteins have benefited importantly from a knowledge of the identity of the sites at which hydrogen ions are dissociated or combined, and from information gleaned from model compounds in which the characteristic dissociations may be studied in isolation. It is known, for example, that seven such *kinds of sites* contribute to prototropic phenomena involving proteins between pH 2 and 14. Distinguishing between these sites and their widely different affinities for protons has made possible a fairly complete understanding of the over-all prototropic behavior of proteins, and the effects on it of temperature, solvent, and various chemical and physical interactions (Tanford, 1962; Steinhardt and Beychok, 1964).

It is now well known that many proteins combine reversibly with ions other than hydrogen ion, and with certain neutral molecules, to extents which do not differ greatly in magnitude from the extents of their combination with hydrogen ion. Except for the combination of some proteins with certain metal ions, almost nothing is known with certainty as to the chemical nature of the binding sites, whether the same sites bind both ions and neutral molecules, or whether specific sites for particular ligands are involved. Furthermore, and perhaps more important, there are no model compounds. The binding of most ligands other than H^+ seems to depend on fairly high molecular weight or on charge or site constellations which are unlikely to be found without high molecular weight.[1]

There is no clear-cut evidence to support any broad theory which can be used to predict which proteins, such as serum albumin, will bind most substances, or which will bind them most tightly; while others, such as lysozyme, bind little if at all; nor is there an established theoretical basis for predicting which substances are most tightly bound. We do not refer here to those substances, such as the substrates of enzymes, which are bound with high specificity to an extent of 1 or 2 equiv, and we also exclude the highly specific interactions of antigens and their antibodies. Finally, there have been no general explanations of why binding ligands sometimes protect (stabilize) protein, but also,

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[1] The formation of micelles by some ligands may be regarded, to a limited extent, as a model system for the binding of the same ligand by proteins.

with the same ligand, are sometimes the direct cause of denaturation. With hydrogen ion, on the other hand, we know only of the destructive action that accompanies combination with protons beyond some threshold quantity that varies from protein to protein, and which seems to be related to the accumulation of a net charge.

Some years ago, one of the present authors presented evidence, for both a fibrous and a dissolved globular protein, that binding energy increases with the over-all linear dimensions of the ligand (Steinhardt *et al.*, 1941, 1942; Steinhardt and Zaiser, 1950) and that when an anion was bound very tightly, the binding was accompanied by an increased velocity of hydrolysis in weakly acid solutions of both amide and peptide bonds (Steinhardt and Fugitt, 1942). Later work with serum albumin and β-lactoglobulin (Karush and Sonenberg, 1949; Yang and Foster, 1953; Pallansch and Briggs, 1954; Hill and Briggs, 1956) has produced results which tend to confirm the first generalization; the last three of these papers have also demonstrated that combination with large quantities of detergent anions is always accompanied by changes in molecular parameters that are most concisely described as "unfolding," and which bear some resemblance to the changes that accompany the binding of large amounts of hydrogen ions. Most of the recent work with ligands that are effective in "unfolding" has assigned a single association constant to binding by the native molecule (prior to unfolding), and has appeared to assume that the somewhat weaker binding that occurs where unfolding ensues is due to sites that were not accessible before unfolding, while the native sites are abolished when unfolding occurs. With anions that do not manifestly unfold, the prevailing interpretation (Scatchard, 1949; Scatchard *et al.*, 1957) has been in terms of specified numbers of different kinds of sites which differ in affinity, with no explicit account taken of the possibility of unfolding, or of the effect of the latter on the numbers of sites or of their affinities for the ligand under investigation.

It has appeared to the present authors that a general understanding of binding by proteins, and its consequent effect upon their stability, might be enhanced by a systematic investigation and comparison involving a single protein and a series of homologous ligands, such as alkanes, the corresponding alcohols, and the corresponding acids (sulfate half-esters and sulfonates), rather than by a choice of ions and molecules that have less obvious common features. Such an investigation might hopefully lead to distinguishing between the contributions of coulomb forces on the one hand and effects due to hydrophobic interactions, hydrogen bonding, and polarizability of the ligands. If such an investigation were then repeated with a number of other proteins which differ in their propensity to bind the same ligands, and which differ widely in such structural features as stabilization by disulfide bonds, helical content, and interactions with nonaqueous solvents, a pattern of correlations between structural features and tendency to bind, as well as of effects of binding on stability, might begin to emerge.

The present paper therefore describes an early stage of these investigations with crystalline bovine serum albumin.[2] It is largely confined to the binding isotherms of the *native* molecule, prior to unfolding, with two sets of homologs containing 8 and 12 carbon atoms, respectively, and consisting of the normal alkanes, alcohols, and sulfate half-esters, or sulfonates. Extensions of the analysis to other substances, and to the region of unfolded protein, will be published elsewhere.

Experimental Procedures

Materials. Labeled *n*-octane (1-^{14}C) supplied by Baird Atomics was diluted with the unlabeled compound ("purissima") supplied by Fluka (ICN), stated to be 99.83 mole % pure. The dilution employed yielded a corrected count rate in the scintillation counter process described below of 10^{10} cps/mole.

Labeled 1-octanol (1-^{14}C) supplied by Atomic Accessories, Inc. (Lot 20) was diluted with the unlabeled compound, supplied by Mann Research Laboratories, stated to be 99.8% pure. It was employed at a count rate of about 10^{10} cps/mole.

Labeled 1-dodecanol (1-^{14}C) obtained from Baird Atomics (Lot 2168) was diluted with highly purified material obtained from the Applied Science Laboratories of Pennsylvania State University having a purity of better than 99.9%. Because of the low solubility of this material in water, the labeled material could not be used in greater than a threefold dilution.

Sodium octyl sulfate and sodium octyl sulfonate were prepared by Mann Research Laboratories; since they were free from visible evidence of contamination by the method of thin layer chromatography, they are believed to be 99.9% pure. In all but the earliest experiments the material used was recrystallized from hot alcohol.

The sodium dodecyl sulfate used in the early experiments was a highly purified sample kindly provided by Professor K. J. Mysels of the Department of Chemistry, University of Southern California. Later experiments were performed with material obtained from Mann Research Laboratories, labeled "purified by the method of Crestfield, Smith, and Allen." This material assayed 85% before further purification when compared to the original highly purified sample in the assay described below. A single recrystallization from boiling alcohol sufficed to bring the purity above 99%. A ^{35}S-labeled sample obtained from Volk assayed only 41% pure but was subsequently purified to the same level by two recrystallizations from boiling alcohol. The impurities initially present in this sample not only gave misleading results but also affected the porosity of the Visking membranes used in the equilibrium dialysis, and thus prevented the attainment of equilibrium when high concentrations were used.

The crystalline protein used, bovine serum albumin,

[2] Abbreviations used: BSA, bovine serum albumin; cmc, critical micelle concentration.

was obtained from Nutritional Biochemicals Inc. Stock solutions (5%) were deionized on a column of a mixed-bed resin (Biorad) above 2 in. of an acid resin (Dowex 50W-x4). The stock solutions were stored at 2° and discarded after 2 weeks if not exhausted earlier. No effort was made to remove the traces of decanol which may have been present as a result of the use of about 1 mole of decanol/mole of protein in crystallizing it. No effort was made to remove any residual fatty acid which may have been present in the protein, since there is reason to believe that the acid–heptane–isooctane treatment of Goodman (1957) may either modify the protein, or leave it combined with small amounts of hydrocarbon (see, among others, Alfsén, 1963). Wishnia and Pinder (1964) and Scatchard *et al.* (1959) (*cf.* Tables I and II) have found that protein deionized by the methods used here give binding data which are not significantly different from the acid-treated material. No differences were observed between the several batches of protein used in the present work, but very small differences in binding would not have been detected.

Methods

1. Equilibrium Dialysis. Bags made of Visking tubing were filled with 5 ml of protein solution in buffer and were suspended in 10 ml of the same buffer, initially containing all of the ligand under investigation. In later experiments shallow plastic cells of equal volume, separated by a Visking membrane and clamped together, replaced the bags. In both cases the solutions were gently agitated at 2° by slow rotation on a motor-driven disk which inverted their contents several times a minute. The membranes were soaked in distilled water for some hours before being used. It was found that, *with pure materials*, and in the range of the amounts of binding reported here, 12–17 hr sufficed for the attainment of equilibrium. Longer equilibrium times appear to be required with certain pure detergents when they were present in higher concentrations. Membranes have been encountered (Matheson) which alter the protein drastically in a fashion which reduces the binding at low equilibrium concentrations and increases it at high equilibrium concentrations, an effect similar to that produced by unfolding (J. A. Reynolds, S. Herbert, H. Polet, and J. Steinhardt, unpublished data).

The amounts bound were calculated by either of two methods: (A) When labeled compounds were used, concentrations of the ligand on both sides of the membrane were determined in a KCl scintillation counter (usually 100 sec) after cooling in the dark. Approximately 0.5-ml portions were added to 10 ml of the Bray scintillation solution (Bray, 1960). The results were corrected for quenching (about 10%) and for background (2–3 cps); the latter correction was unimportant except at the lowest equilibrium concentrations. The differences in counts per unit volume on the two sides of the membrane were converted into concentration differences, and are given in the graphs and tables, expressed as $\bar{\nu}$, the average molal ratio of moles of ligand/mole of protein. Since the total radioactivity on both sides of the membranes always agreed within 1–2% with the radioactivity initially present in the "outside" solution, it appears that no correction is required for adsorption of either protein or ligand on the membrane. (B) With unlabeled compounds only the "outside" (protein-free) solutions were analyzed. A methylene blue complex of the anions (dodecyl and octyl sulfates, octyl sulfonate) was extracted in chloroform (Mukerjee, 1956) and the optical density of the dry chloroform solution read in a Cary 14 spectrophotometer at 655 (dodecyl sulfate) or 653 mμ (octyl compounds). Sample (5 ml) was added to a mixture of 5 ml of 0.03 N HCl solution, 5 ml of methylene blue solution (24 mg/l.), and 5 ml of $CHCl_3$. A blank correction was determined for the dye extracted when no complex is present. The HCl concentration was initially such as to bring the pH of the aqueous phase to about 2. In later work with the octyl compounds, the acid was omitted; a much more efficient extraction resulted and the blank correction was decreased. When this method is used it is not possible to estimate the detergent concentration in the protein solution directly since the absorption spectrum of the dye is altered by protein. The calculation of amounts bound depends on the initial and final "outside" concentrations only, the volumes of "inside" solutions, and the protein concentration. Thus, no direct estimates can be made in the actual experiments of the effects of adsorption on membranes or cell walls. However, blank experiments were run with (1) only protein present and (2) only detergent present. In the range of concentrations and equilibration times reported here, no adsorption of detergent or protein was observed.

2. Solubility. With both octanol and dodecanol (labeled) some determinations were made with the equilibrium ("outside") concentration (4 ml) kept at saturation by the presence of excess undissolved material. Much higher amounts of binding were achieved in this way than would otherwise have been possible (up to $\bar{\nu}$ 20, in the case of octanol). Only the protein solution was analyzed in the scintillation counter since the counts for saturated "outside" solutions were always the same. In one experiment with dodecanol, no dialysis membrane was employed; in effect the "solubility" of dodecanol in solutions containing varying amounts of protein was measured after separation of the excess solid (dodecanol) phase.

Results and Discussion

Octanol. The amounts of 1-octanol bound by BSA (1% solutions) in 0.033 ionic strength phosphate buffers at a number of pH values and at two temperatures are shown in Figure 1. The reciprocals of the average molal ratio, $\bar{\nu}$, are plotted *vs.* the reciprocal of the equilibrium concentration, in accordance with the familiar Klotz equation

$$\frac{1}{\bar{\nu}} = \frac{1}{n} - \frac{1}{nK}\frac{1}{C_A} \qquad (1)$$

in which n represents the number of binding sites having the same intrinsic binding constant, K, for the ligand, here 1-octanol, and C_A is the equilibrium concentration of the octanol. The intercept at $1/C_A = 0$ is then the reciprocal of the number of sites, and the slope of the straight lines is the reciprocal of nK. Equation 1 is a direct consequence of the law of mass action *if it is assumed* that all n sites have the same affinities for the ligand, and if the derivation takes into account the proper combinatorial factors that separate the successive titration constants from one another and from the intrinsic binding constant (Steinhardt and Beychok, 1964). When there exist more than one set of identical binding sites, and two or more values of K, there is a departure from the linear relation between $1/\bar{\nu}$ and $1/C_A$ at low $1/C_A$, *i.e.*, $1/\bar{\nu}$ falls below the extrapolated straight line. Changes in certain molecular parameters, such as viscosity, do not occur within the linear regions, but invariably occur somewhere within the region of the negative deviation from the Klotz plot.

It will be noted for each set of data included in the figure that the linear relation stated in eq 1 is observed for the most dilute equilibrium concentrations and the lowest amount of binding (values of $\bar{\nu}$ up to 4 or 5). The data obtained for each set of conditions at the higher concentrations are all compressed into the lower left-hand corner of the figure where they fall well below the extrapolated linear region. This is always found when binding experiments with BSA are extended to higher molal ratios, and has been variously interpreted as indicating the existence of additional sets of sites which bind more weakly than the sites to which eq 1 applies (Scatchard *et al.*, 1957) or a continuous distribution of values of the binding constant K (Karush, 1950; Karush and Sonenberg, 1949; Schellman *et al.*, 1954); and to changes in the conformation of the protein brought about by the initial binding, which leads to the appearance of new binding sites as the result of an unfolding process. The appearance of new sites is possibly accompanied by the disappearance of the original set of sites in the folded (native) protein (Hill and Briggs, 1956). In the present paper, only a preliminary attempt is made to choose between these alternatives (it will be shown elsewhere that the first and third explanations actually apply), since this paper is concerned solely with binding by the set of sites in the native protein distinguished by having the highest affinity for the ligand.

The following conclusions can be drawn from the linear portions of the data in Figure 1: (1) Within the region of high affinity binding the *number* of available sites (five) is independent of both temperature and pH, except at the highest pH where it may be as great as 7. (2) The binding constants, however, are rather surprisingly pH dependent although the ligand is not an ion; the association constant rises as the pH increases over most of the range shown from 3370 to 7140. (3) The affinity is also slightly higher as the temperature rises, when the results are compared at the same pH (5.6). This rise seems to establish that ΔH for the binding reaction is positive; thus, a substantial

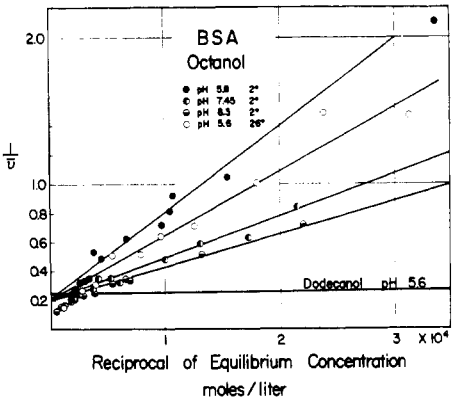

FIGURE 1: Reciprocal plot of 1-octanol binding by BSA in 0.033 ionic strength phosphate buffers. The nearly horizontal line at the bottom of the figure represents the data for 1-dodecanol.

entropy effect must be responsible for the fairly high negative $\Delta F°$ (−4460 to −5050 cal, depending on the temperature). If ΔH were 0, the entropy change per site must be at least 17 eu; since ΔH is slightly positive, the entropy change must be even greater. Such ΔS values are quite consistent with hydrophobic interactions (Kauzmann, 1959). (4) The phosphate ions do not appear to compete with octanol. Experiments with 0.1 M phosphate show almost the same amounts of octanol binding as do experiments with 0.033 ionic strength phosphate.

In connection with the unexpected effect of pH on the association constants we note that K seems to be inversely proportional to the 5th root of H^+ between pH 5.6 and 7.45. We have confirmed by pH measurements that the binding of octanol and other alcohols in unbuffered solutions makes BSA a slightly stronger acid in this pH range (the initial change is followed by a slow reverse change with octanol but not with hexanol). It may be recalled that Wishnia and Pinder (1964), working with butane, found an increase in binding with pH. The change, however, appeared to leve off at pH values not far above those characteristic of the N–F transition, to which it was ascribed. In the present case, if an additional transition is invoked to explain the effect, it appears to occur well above the N–F region. It is well to note, also, that butane behaves very differently than does octanol; Wishnia's experiments with the former do not indicate a limiting number of sites (n for butane must be greater than 40 and may be infinity).

Since the experiments with longer chain alkane derivatives, described in later parts of this paper, were performed with more dilute solutions of protein, some of the measurements with octanol were repeated with 0.1% BSA. The results of these measurements were the same within the experimental error as those shown in Figure 1, obtained with 1% protein; the scatter is somewhat greater with the more dilute solutions.

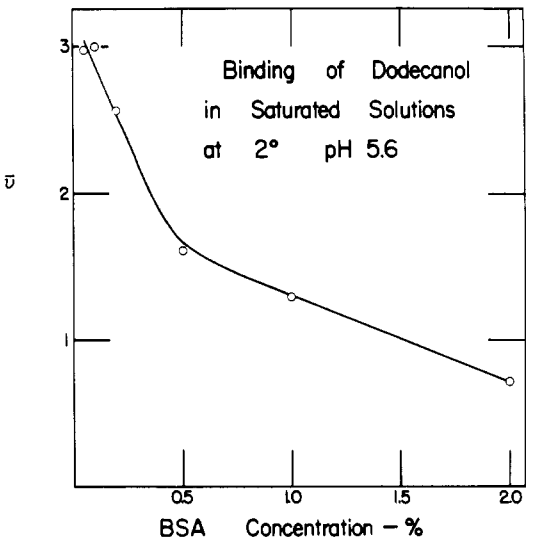

FIGURE 2: Amounts of *n*-dodecanol bound by BSA as affected by BSA concentration. The free dodecanol concentration is 2.4×10^{-5} M.

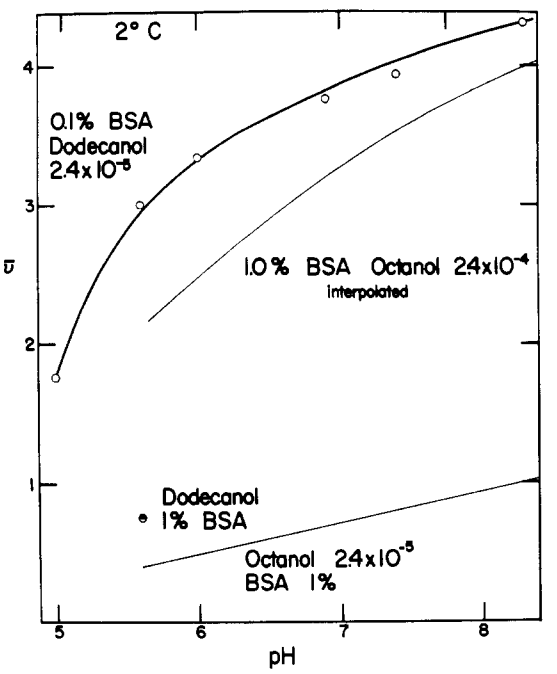

FIGURE 3: The effect of pH on the binding of 1-dodecanol by 0.1% BSA in phosphate buffers. The lighter lines, included to permit comparisons, show the results with octanol at two BSA concentrations. A single point (lower left corner of figure) shows how the binding of dodecanol falls at higher protein concentrations.

Octane. It has been difficult to get data for the binding of octane, principally because the results have varied from one lot of radioactive octane to another. We have had a single-labeled octane sample which, when measured isotopically, gave the accepted value for the solubility of *n*-octane in water, verified experimentally with our sample of pure unlabeled material (see Materials in Experimental Procedures). Another sample, almost certainly mislabeled, or grossly impure as to labeling, gave a solubility barely one-tenth as high as the first. The first sample was assumed here to be sufficiently pure for use in these studies, but sufficed only for two measurements in saturated solutions, one at pH 5.6 and one at 8.3. Both of the $\bar{v}$ values obtained (1.44 at pH 5.6, equilibrium concentration 1.43×10^{-4}, and 2.0 at pH 8.3, equilibrium concentration as before) were approximately 70–90% as great as the values interpolated on the corresponding 1-octanol isotherms.

In the absence of a large amount of data these results may indicate that the binding constants (or *n* values) of octane are somewhat smaller than those of octanol, but not greatly so. The low solubility of octane would, in any case, prevent direct observation of the entire isotherm even for the set of sites of highest affinity.

Dodecanol. Before proceeding to the ionic homologs of octanol and octane, the results with the remaining nonionic ligand, dodecanol, will be given. They illustrate an additional characteristic of the results obtained with all the 12-carbon ligands which is absent with octane and octanol just described, namely dependence of binding on protein concentration. A line representing the results obtained with 0.1% protein is given in Figure 1, for comparison with the results with octanol. It is clear that dodecanol binds on the same number of sites as octanol, or possibly on four rather than five, but it is also clear that the association constant for the longer chain alcohol is a great deal higher. The association constant represented by the almost horizontal slope in Figure 1 (and by the six points shown at the top of Figure 3) represents an association constant of 150,000 if $n = 5$, a value about 45 times higher than the value of octanol.

Since the solubility of dodecanol in the phosphate buffer is only 2.4×10^{-5} M, the method used initially for measuring the binding made use only of saturated solutions (dodecanol is a solid at 2°, and the excess after equilibration with protein solutions can be easily separated). Figure 2 shows how the solubility of the alcohol is affected by the concentration of the protein. If the solubility increment per mole of protein in the solution were a constant, the results, calculated as $\bar{v}$, could have been represented by a horizontal line. It is obvious, however, that the molal ratio falls with increasing concentration of protein, and does not level off at concentrations up to 2%. The value plotted in Figure 1 is based on the results shown in Figure 2 for 0.1% protein, close to the slightly higher value which would result from extrapolation to infinitely dilute protein.

The same pronounced effect of pH on the binding which was found with octanol appears again with dodecanol. Figure 3 shows how the molal ratio, $\bar{v}$, for 0.1% protein rises steadily at pH values between

5 and 8.2, as the equilibrium concentration of the ligand is held constant by the presence of saturating body, at 2.4×10^{-4} M. A single point in the lower left-hand corner, obtained with ten times the protein concentration, is included as a reminder of the large dependence on protein concentration. The dotted lines, included for comparison, show the pH trend in the binding for octanol at two different equilibrium concentrations of the ligand both of which are below saturation. The lower concentration is the same as that of the dodecanol data, the other ten times higher. The data for octanol are at 1% protein concentration. It should be noticed that, since the dodecanol data depend strongly on protein concentration and the octanol data do not, the very much greater difference in affinity for BSA does not appear when the comparison is made at 1% protein concentration; $\bar{\nu}$ for dodecanol at 1% is only slightly higher than $\bar{\nu}$ for octanol when they are compared at an equilibrium concentration of 2.4×10^{-5} M.

Sodium Dodecyl Sulfate. In going to the ionic ligands, results with the twelve-carbon homolog will be described first, because the effects of concentration of protein and pH have been studied more fully with dodecyl sulfate than with the eight carbon anions. The results on the effects of protein concentrations are more revealing than those described for dodecanol because it has been possible to make measurements with the more soluble dodecyl sulfate over a wide equilibrium concentration range of ligand rather than with saturated solutions alone.

Figure 4, which includes measurements of higher binding than either Figures 1 or 2, gives the binding isotherms for protein concentrations between 0.05 and 2.0%. The representation is in the form usually used for hydrogen ion titration curves, amounts bound per unit of protein *vs.* the negative logarithm of the equilibrium ligand concentration. The highest concentration represented is much lower than the critical micelle concentration for this ligand; 0.008 is the cmc in water at 25° (Williams *et al.*, 1955). Although there is a wealth of data at the lowest concentrations, and some scatter, which is due to the difficulty of measuring low amounts of binding when the protein is very dilute, careful inspection will show that: (1) there is very little difference between the data for the two lowest protein concentrations. As in the case of dodecanol there will be very little difference between the experimental points for 0.1% protein and any extrapolations to infinitely dilute protein solutions. (2) The differences between the data for the two highest protein concentrations are also small, but large differences occur between 0.2 and 1.0%. Whatever process is responsible for the effect of protein concentration on binding has its principal effect between these limits. (3) The similarities and differences just described are very marked for $\bar{\nu}$ values below 8–10. There are smaller differences above this $\bar{\nu}$ value for 0.1, 0.2, and 0.48% protein, the only concentrations at which measurements were made in this upper range. (4) Data obtained at 0.1% protein at pH 6.8 are, unlike the case of

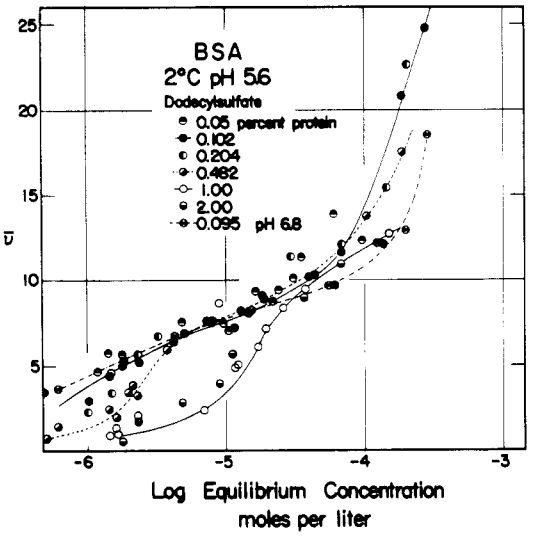

FIGURE 4: Binding of dodecyl sulfate ion by BSA at 2° at a number of protein concentrations and at two pH values (phosphate buffers). The curves shown are for guidance only.

octanol or dodecanol, in good agreement with data obtained at pH 5.6, in the region of low binding ($\bar{\nu}$ 8–10) referred to in (3). There is, however, a reduction in the binding at the higher pH value when $\bar{\nu}$ exceeds these limits. (5) Just as in the case of octanol, there is a second stage of binding, which in the present case sets in at equilibrium concentrations of ligand just below 10^{-4} M. In the experiments with dodecyl sulfate the greater affinity permits measurement up to much higher $\bar{\nu}$ values than was done with octanol. The steepness of the rise at $-\log c$ values between 3.6 and 4.0 shows that a great many more binding sites than have been "titrated" in this region must exist. In fact values of $\bar{\nu}$ considerably over 100 have been observed, in keeping with the earlier results of others with this and other detergents (Pallansch and Briggs, 1954; Yang and Foster, 1953), and will be reported and analyzed in another communication.

However, the abruptness with which this secondary rise in $\bar{\nu}$ begins makes clear that the steepness does not result solely from a large number of binding sites, but must partake of the character of a "cooperative" phenomenon; this is usually interpreted as meaning that its binding of additional equivalents is facilitated by the binding that has already occurred, but it may also result from an unfolding of one conformation of the protein, in which only a few groups are exposed, to another form in which many more are accessible. This transition may be an all-or-none phenomenon so that only two molecular species are present at any equilibrium concentration, each in a limited number of stages of binding. The electrophoretic patterns for BSA and dodecyl sulfate reported by Putnam and Neurath (1945) and by Pallansch and Briggs (1954) support, and are fully consistent with, this interpreta-

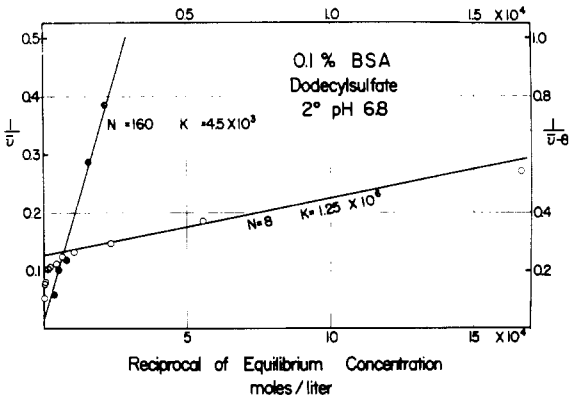

FIGURE 5: Reciprocal plot of the binding of dodecyl sulfate ion by BSA at pH 6.8 (phosphate buffer) at 2°. The filled circles on the steep line at the left are explained in the text.

tion. The important characteristic of such models is that the secondary rise in the neighborhood of 10^{-4} M is not a simple titration curve, but a combination of the "titration" of newly accessible groups, plus the effect of an increase in the number of accessible groups over a narrow region of pH. The analogy with the steepness of the titration curve of ferrihemoglobin in the region in which histidines are "unmasked" by the addition of acid at pH values just below 4 (Beychok and Steinhardt, 1959) is a very suggestive one. An extension and analysis of this portion of the binding isotherm will be reported elsewhere.

It should be noted that at the pH of all of these experiments, the protein bears a net negative charge; thus there can be no *net* or over-all coulombic contribution to the binding energy of anions. Nevertheless binding of very large numbers of these anions occurs in the case of those which have a high affinity, such as dodecyl sulfate. The high affinity, which will be shown to be greater than that of the homologous alcohol, may nevertheless have a coulombic contribution from binding near local positive charges or charge constellations. Efforts to estimate this coulombic contribution are made in a later section.

The peculiar effect of protein concentration can be provisionally accounted for by the assumption that long-chain aliphatic molecules can adhere to more than one protein molecule; this effect, which will be most noticeable at high ratios of protein to ligand, will result in a lower average molal ratio of ligand bound to protein present. The consequent partial dimerization of protein should be verified by molecular weight measurements, possibly by the sedimentation–velocity method; until then, the dimerization remains a hypothesis, but one which appears more plausible than the alternative: competition of dodecyl sulfate with residual bound fatty acid anions. The latter alternative will produce the observed concentration effect, but only if at least 5 equiv of fatty acid is associated with the deionized crystalline protein employed.

The measurements with dodecyl sulfate at pH 6.8, which include the highest $\bar{\nu}$ values obtained in these experiments, are shown again as a reciprocal plot in Figure 5. The intercept indicates a set of eight strongly binding sites, with an intrinsic binding constant of about 1.3×10^6. This value is roughly ten times higher than the binding constant for the uncharged homolog, dodecanol, and there are eight binding sites rather than five when the charge is present.

The steep line through the filled circles in Figure 5 would represent binding by all the remaining sites if these sites were to exist in the native molecule. The filled circles were obtained by subtracting 8 from all the values of $\bar{\nu}$ over that value. About 160 sites are indicated from the intercept, each having a binding constant of 4500. The number of sites, 160, agrees with the value of Strauss and Strauss (1958) in equilibrium with the critical micelle concentration. This agreement is not to be taken very seriously since it is known that the protein unfolds at $\bar{\nu}$ values only slightly greater than 8 (*cf.* Pallansch and Briggs, 1954, among others). Thus, the second set of sites may not exist in the folded protein, and the original eight sites may not exist in the unfolded form; furthermore, the unfolding occurs over a fairly wide range of values of $\bar{\nu}$, so that it should not be expected that the data should fit a simple reciprocal plot except in the range of $\bar{\nu}$ beyond the value at which unfolding is complete, a range which is, in fact, beyond that represented in Figure 5. If the data were to be replotted without subtracting 8 (*i.e.*, on the assumption that none of the original sites survived unfolding), N would be much greater than 160, in fact well over 300, but again there is no reason to expect that a straight line should represent the data within the region of unfolding. An analysis of this region, based on much higher $\bar{\nu}$ values, and on criteria of unfolding, will be published elsewhere. It is clear that if both sets of sites are present in the native molecule their binding ranges of concentration overlap; *i.e.*, the binding of the first equivalent in the "second" set occurs at lower concentrations than the last equivalent of the "first" set: 1.25×10^6 divided by eight is less than 4500 multiplied by 160.

The data of Pallansch and Briggs (1954), although internally consistent, and supported by unfolding estimates based on electrophoresis, are difficult to reconcile with the data shown in Figures 4 and 5, or in Table I. These investigators report a binding constant of 20,000, rather than the value 1,300,000 reported here. Binding constants measured with closely related compounds, such as dodecyl benzenesulfonate (not entirely pure) (Yang and Foster, 1953) and octyl benzenesulfonate (J. D. Mullen, unpublished data), are considerably closer to the present values. The fact that Pallansch and Briggs used higher protein concentrations accounts for part of the difference, but is inadequate to account for the extreme discrepancy noted here. Karush's data (1950) are too scanty in the low $\bar{\nu}$ region to furnish any additional standard of comparison.

It will have been noted that the same abscissa was used in plotting Figure 5 as in Figure 3 (octanol),

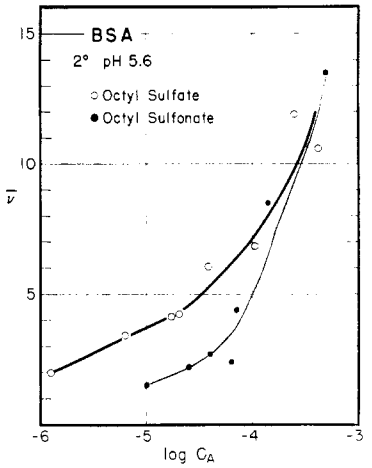

FIGURE 6: Binding of octyl sulfate and octyl sulfonate ions by 0.1% BSA at 2° in 0.033 ionic strength phosphate buffer.

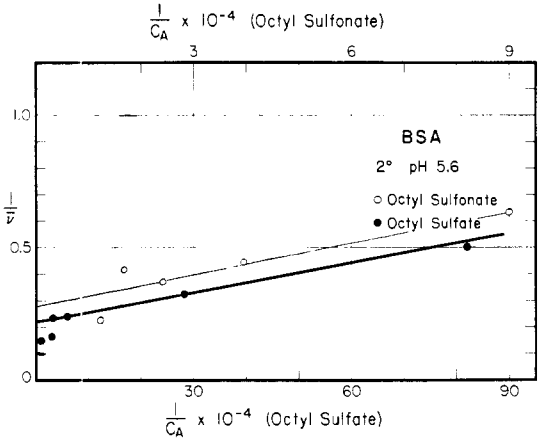

FIGURE 7: Reciprocal plots of the binding of octyl sulfate and octyl sulfonate ions by 0.1% BSA at 2° in 0.033 ionic strength phosphate.

TABLE I: Binding Constants for Native Serum Albumin (2°, 0.033 Ionic Strength Phosphate Buffers).

Ligand	% Protein Concn	pH	n	$K \times 10^{-4}$	$-\Delta F°$ (cal)
Octane	1.00	5.6	If $n = 5$, about the same as octanol		
Octanol	1.00	5.6	5	0.34	4500
		5.6[a]	5	0.37	4500
		5.6[b]	5	0.46	5100
		7.45	5	0.71	4900
		8.3	5	0.55	4700
	0.10	5.6	5	About the same as 1%	
Octyl sulfate	0.10	5.6	4–5[d]	70	7100
Octyl sulfonate	0.10	5.6	3[d]	10	6300
Dodecanol	0.10	5.6	4–5	15	6600
Dodecyl sulfate	0.05	5.6	8	150	7900
	0.10	5.6	8	100	7700
	0.20	5.6	8	(70)	(7900)[c]
	0.48	5.6	8	(34)	(8000)
	1.00	5.6	8	(11)	(6500)
	2.00	5.6	8	(10)	(6300)
	0.10	6.8	8	125	7800

[a] 0.10 M phosphate. [b] 26°. [c] Bracketed values have only nominal significance. [d] n could be the same for both octyl sulfate and octyl sulfonate, within the experimental error of the data in Figure 7. However additional experiments indicate that n is invariably smaller for octyl sulfonate.

although Figure 5 deals with the binding of an anion rather than a neutral molecule. Scatchard (1949) has shown that eq 1 should be replaced by the following

$$\frac{1}{\bar{\nu}} = \frac{1}{nKC_Ae^{-2wZ}} + \frac{1}{n} \tag{2}$$

or

$$\bar{\nu} = \frac{nKC_Ae^{-2wZ}}{1+KC_Ae^{-2wZ}} \tag{21}$$

in which w is the well-known electrostatic interaction factor (Tanford, 1962; Steinhardt and Beychok, 1964). w depends on ionic and solvent parameters, including ionic strength. The exponential term takes into account the reduction in the activity coefficient of the central ion, as it accumulates charges. The difficulty in using eq 2 is that w is unknown. If it is calculated from the Debye–Hückel theory for a compact spherical ion the size of native BSA, and for the ionic strength of the phosphate buffer, the correction introduced up to $\bar{\nu} = n = 8$ is rather small (w is less than 0.05). At larger $\bar{\nu}$ values, unfolding sets in and the ion is far from spherical and compact. w therefore becomes even smaller, certainly not over 0.02. There are grave reasons to question whether the activity coefficients vary in the manner expressed by the exponential term when the dipolar nature of the protein ion, its size, and its large charge at high $\bar{\nu}$ are taken into account. Its applicability to ligands having large apolar tails is also in question. In this paper, therefore, eq 1 rather than eq 2 has been employed; the exact nature of the proper activity coefficient correction is still under investigation.

Octyl Sulfate and Octyl Sulfonate. Binding data for both of these substances in the region of low $\bar{\nu}$ are shown in Figure 6. This figure reveals, rather unexpectedly, that the sulfate half-ester and the sulfonate behave very differently. Smaller amounts of sulfonate are bound at every equilibrium concentration, but the discrepancy decreases at higher concentrations. Reciprocal plots of these data (Figure 7) suggest that at lower equilibrium concentration the sulfonate is bound at fewer sites (three) than is the sulfate (four or five),

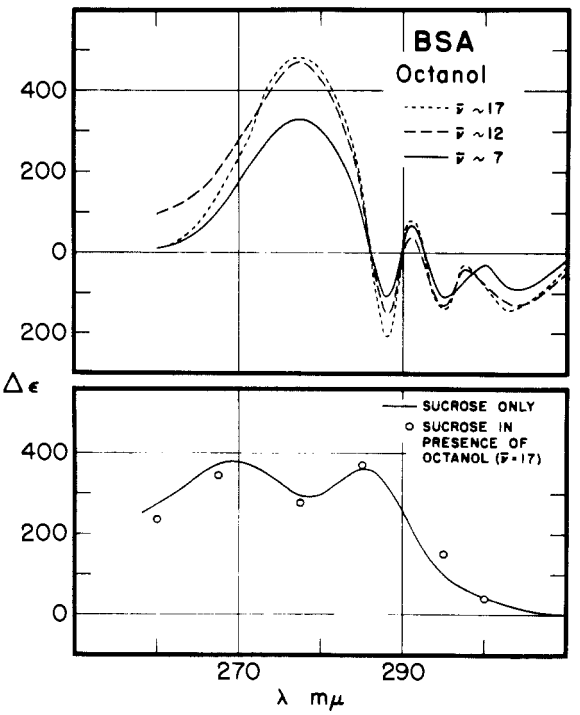

FIGURE 8: Difference spectra. Upper graph: ultraviolet difference spectra obtained with 1-octanol and 0.1% BSA at three different degrees of binding. Lower graph: the difference spectrum due to 20% sucrose alone. The points, superimposed on curve 2, were obtained with 17 moles of bound octanol/mole of protein. Since the values were measured at 2° and the spectra at room temperature, the binding may be slightly higher than indicated above.

and that there is a difference in the association constants (approximately 700,000 for the sulfate, and only 100,000 for the sulfonate). This suggestion is supported by work with other pairs of sulfates and sulfonates, to be reported elsewhere. Deviations from the linear region of the reciprocal plots set in at lower concentrations with the sulfonate than with the sulfate. Additional evidence for these differences by the ΔpH methods of Scatchard and Black (1949) will be published elsewhere. The possibility that there may be more than one intrinsic binding constant for the sulfate (two sets of sites in the native molecule) cannot be excluded on the basis of the data presented. An analysis of higher $\bar{\nu}$ data for *both* substances, in terms of criteria of unfolding, is underway and will be presented elsewhere.

Effects of Binding on Ultraviolet Spectra. The fact that with some ligands, *e.g.*, dodecyl sulfate, "unfolding" occurs at $\bar{\nu}$ values above certain threshold values, which depend on the ligand, has been cited in the foregoing as a reason for limiting the analysis in this paper to low $\bar{\nu}$ values. The concept of unfolding by detergent molecules goes back to early work by Anson (1939) and Putnam and Neurath (1945) who worked with mixtures of sulfate half-esters, and Pallansch and Briggs (1954) who used purified dodecyl sulfate. Both used electrophoretic criteria of the existence of two distinct ionic complexes when $\bar{\nu}$ exceeded about 10–12. Yang and Foster (1953) obtained very similar results with another impure detergent containing aryl as well as alkyl substituents, and Hill and Briggs (1956) have described similar effects with β-lactoglobulin. There have been numerous other observations that the formation of complexes of BSA containing large numbers of equivalents of some of these detergents is accompanied by partial disorganization of the molecule (Bigelow and Sonenberg, 1962; Herskovitz and Laskowski, 1962 (with concentrations above the cmc); Leonard and Foster, 1961; Marcus and Karush, 1957; J. D. Mullen, unpublished data) as evidenced by changes in precipitability, viscosity, ultraviolet difference spectra, and optical rotation, although it has also been shown that detergent ions at low concentration protect proteins from the disorganizing effects of high pH (Lovrien, 1963) and of urea (Meyer and Kauzmann, 1962; Green, 1963).

We have therefore examined the effects of the ligands included in this paper in the low $\bar{\nu}$ region reported here by means of measurements of specific viscosity and of difference spectrophotometry in the ultraviolet region. As will be evident, our examination has led to the conclusion that the difference spectra have only a very limited usefulness as a criterion of unfolding, and that recourse must be had to less ambiguous criteria such as viscosity. The results will be summarized here.

Curve 1 in Figure 8 shows ultraviolet difference spectra obtained with octanol at three different $\bar{\nu}$ values, 6.7, 12, and 17 (the latter two are practically identical). The results can be interpreted as showing small perturbations of the tryptophyl and tyrosyl absorptions. Both of these effects can be interpreted as "blue shifts" in the absorption curves of these two chromophores. The tyrosyl blue shift, present at all $\bar{\nu}$ values, has been considered a common feature of denaturation (Wetlaufer, 1962). Herskovitz and Laskowski, (1962) have developed a "solvent perturbation" method for detecting changes in the accessibility of chromophores to solvent by testing the effect of a "perturbant" solute, in high concentrations, on the difference spectra produced by the agent under investigation. Thus, for example, curve 2 in Figure 8 shows the effect of 20% sucrose alone on the BSA spectrum. The superimposed points represent the effect of 20% sucrose when octanol ($\bar{\nu}$ = 17) is present in both solutions, but with sugar again only in one. The results are indistinguishable from the effects of sugar when octanol is absent; thus whatever is the effect on BSA which is produced by binding large amounts of octanol it does not alter the accessibility of otherwise masked chromophores (tyrosyls and tryptophyls) to the solvent or perturbant. One must conclude either that (1) no masked chromophores are present, which is contrary to the findings of Hertzkovitz and Laskowski, or that (2) no unfolding occurs, or (3) the chromophores exposed by unfolding are masked by the bound ligand.

Leonard and Foster (1961), working with dodecyl sulfate, suggest partial masking by bound ligand to explain their data. A fourth possibility, that binding does not occur when the perturbant is present, is of too *ad hoc* a nature to deserve further consideration at this time.

Somewhat similar but more marked effects are found with dodecyl sulfate (Figure 9) at $\bar{\nu} = 3$ and 5.3 where no unfolding is believed to occur. These results are quite similar to more detailed results published earlier without designation of the amount of binding (Bigelow and Sonenberg, 1962), and to data of J. D. Mullen (unpublished data) with an incorrect designation of the extent of binding $\bar{\nu}$, based on the results of Pallansch and Briggs. Dodecyl sulfate produces even more obvious blue shifts than with octanol; but again *the sucrose perturbation spectrum is not altered by the presence of dodecyl sulfate.*[3] Another perturbant, ethylene glycol, gives the same results. The similarity to the octanol results suggests that unfolding may not occur with octanol at $\bar{\nu}$ values as high as 17, in spite of the blue shift at this value.[4]

It will be shown elsewhere that blue shifts, such as have been described here, accompany binding of alkanes, alcohol, and detergents to the first set of sites, *i.e.*, the sites which have the highest association constants, and are not necessarily a diagnostic feature of unfolding. When unfolding occurs, such as with dodecyl sulfate at $\bar{\nu}$ values over about 8, the amplitude of the difference spectra grows, probably at least partially as the result of binding on other sites which were previously masked. The fact that the solvent perturbation technique shows no change in the accessibility of chromophores to sucrose appears to show that this unmasking is not the result of destruction of the secondary structure. When unfolding does not occur, as with the eight carbon ligands included in this paper, the increase in $\bar{\nu}$ beyond the number of high-affinity sites (indicated by intercepts in the reciprocal plots) does not cause further augmentation of the main features of the difference spectra, produced by smaller binding. Here the larger binding (*e.g.*, $\bar{\nu} = 17$ with octanol) must involve other sets of preformed sites, *i.e.*, not liberated by unfolding. We conclude, therefore, that only the sites of highest affinity in either folded or unfolded protein have large effects on the aromatic chromophores in the protein. It is only in this special sense that the blue shifts can be used as a diagnostic feature of unfolding, *i.e.*, unfolding causes *additional* sites affecting these chromophores to become accessible for binding. Viscosity measurements to be described elsewhere appear to confirm that the eight-carbon compounds investigated do not unfold in the concentration range studied here, but that dodecyl sulfate does at $\bar{\nu}$ values just over 8.

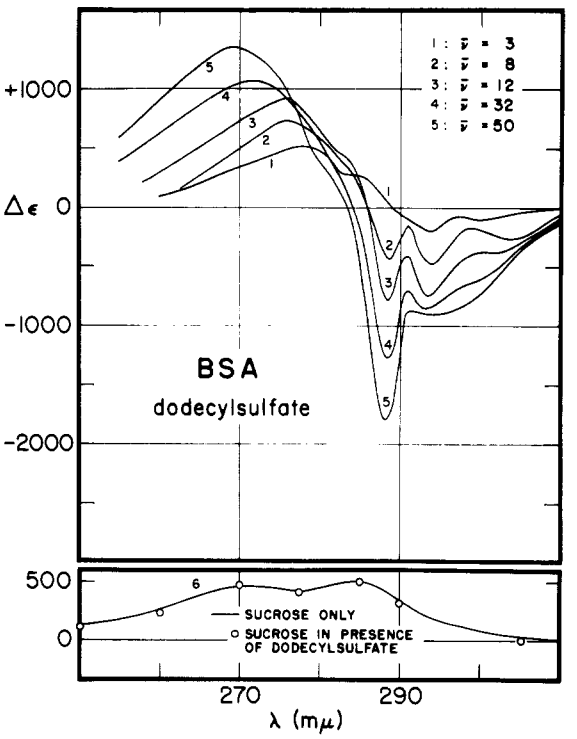

FIGURE 9: Ultraviolet difference spectra. Curves 1–5: ultraviolet difference spectra obtained with dodecyl sulfate and 0.1% BSA at five different degrees of binding. Curve 6: the difference spectrum due to 20% sucrose. The points superimposed on curve 6 were obtained with 12 moles of bound dodecyl sulfate/mole of protein. The same result is obtained with $\bar{\nu} = 50$, or when ethylene glycol is used as perturbant.

Relation of Binding Energy to Molecular Parameters. No simple relationship of affinity to molecular size or chain length, or the presence of charges, can be expected when the number of sites alters from one ligand to another. However, a few simple conclusions can be drawn from the data in Table I for the normal alkane derivatives included in this study, all of which bind far more strongly than aromatic sulfonates in the same range of molecular weights, or than single branched-chain alkane derivatives (J. Cassel and J. Steinhardt, unpublished data).

Because of the low solubility of octane, its affinity may be compared with that of octanol only at very low $\bar{\nu}$ values. In this range, the number of sites and the binding energy ($-\Delta F^\circ \approx 4500$ cal) are so nearly the same as those of octanol that the binding characteristic must be attributed to the hydrocarbon chain, rather than to the presence of polarizable groups. One of the ionic compounds, having the same hydrocarbon tail (octyl sulfate), binds more strongly, but not spectacularly so. $-\Delta F^\circ$ increases only by about 2600 cal. The other ionic compound with a C_8 tail (octyl sul-

[3] The latter findings are contrary to those of Leonard and Foster (1961), who found small effects, smaller than those characteristic of the N–F transformation.

[4] However, octanol has been shown by other criteria of unfolding, such as changes in spectra and w (J. Steinhardt, unpublished data), to disorganize another protein, ferrihemoglobin.

fonate) binds with only about 1600 cal more energy than the alcohol, and on even fewer sites! It is, of course, by no means clear why the negatively charged group should increase the binding energy, since at pH 5.6 the protein itself bears a small negative charge. The effect of charge suggests strongly that the binding is to, or in the neighborhood of, particular cationic groups, or clusters of such groups such as Loeb and Saroff (1964) have invoked for the binding of small anions by ribonuclease. The greater effectiveness of the sulfate half-ester in enhancing the free energy may be tentatively attributed to its less localized charge, or to its greater length. The reduction in the number of sites for the sulfonate is less easily explained.[5]

When C_{10} compounds are compared with the corresponding C_8 compounds, the very great effect of chain length is immediately apparent. Dodecanol differs from octanol by as great a factor as does octyl sulfate. Dodecyl sulfate, however, differs from dodecanol by only 1200 cal rather than by more than 2000, as in the corresponding C_8 pair. Charge may be less important when the affinity due to the length of hydrocarbon chain is already very high, as it is here. For example, the affinity of dodecyl sulfate for BSA is as high as the affinity of the hydrogen ion for the conjugate bases of moderately weak acids in water, in spite of the much greater coulomb force contribution to the latter attraction, and even though the fact that the driving force for detergent binding is mostly entropic in origin rather than enthalpic, as it is in the acid–base reaction cited.

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[5] When the pH is raised from 5.6 to 6.8 the number of cationic side chains in the protein is slightly reduced, but there appears to be no change in n or $-\Delta F°$. It must be concluded that these cationic groups (histidines and possibly the α-amino group) cannot be involved in the high affinity binding. The ϵ-amino groups of lysines and the guanido groups of arginine would therefore seem to be involved. Studies at higher pH are required to resolve this possibility, which is consistent, in part, with the suggestion of Markus *et al.* (1964).