The Nonspecific Large Binding of Amphiphiles by Proteins†

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ABSTRACT: Alkyl sulfates, at concentrations well below their critical micelle concentrations, form complexes with human serum albumin and bovine serum albumin which solubilize the water-insoluble dye dimethylaminoazobenzene as effectively as micelles of alkyl sulfates themselves. This solubilization occurs only at concentrations of detergent sufficient to initiate unfolding of the native protein molecules. The concentration of dispersed dye is proportional to the amount of detergent bound to the unfolded protein. The available binding sites in individual unfolded molecules are completely or largely filled, but the proportion of molecules which are unfolded increases with the concentration of detergent. These conclusions are supported by results obtained at several concentrations of the proteins and at several ionic strengths. Solubilization experiments with high concentrations of decyl sulfate show that the latter unfolds human serum albumin at 20°, but that the affinity of decyl sulfate to the *unfolded* protein is much lower than that of dodecyl sulfate, although both detergents have the same affinity for native protein. When human serum albumin is unfolded in 6 M guanidine hydrochloride, and reduced with β-mercaptoethanol, large amounts of detergent (sodium dodecyl sulfate) are bound at low concentrations only when compared with initially native protein. The largest amounts demonstrated to be bound at 0.033 ionic strength (phosphate) are below the 1.4 g of detergent/g of protein reported by Reynolds and Tanford, but the latter figure is reached at low protein concentration at lower ionic strength. There is no sharp transition between the binding of 0.4 g/g and 1.4 g/g under the conditions of our experiments. Ovalbumin, which in the native state lacks high-affinity binding sites for dodecyl sulfate, binds almost as much (as grams per gram) at high concentrations as do serum albumins, with approximately similar free energies of binding. Thus the binding of large amounts of alkyl sulfates to proteins involves sites common to the unfolded forms of numerous proteins.

Substances which are sparingly soluble in water, or those having a partially lipid character, have very high affinities for the albumins, and appear to be bound to a definite number of sites in the native molecule. Certain long-chain anionic detergents, with solubilities over about 5×10^{-4} M, transform the albumins to another conformer; and well over 100 equiv are bound. The recent work of Reynolds and Tanford (1970a,b) which indicates that as many as 325 equiv of dodecyl sulfate are bound to bovine serum albumin which has had its disulfide bonds reduced makes it highly desirable to investigate the mechanism of this large binding. It is also important to develop methods for measuring it which are easier and less susceptible to experimental error than is equilibrium dialysis at the high equilibrium concentrations required to achieve saturation. At these high concentrations micelles of the ligand are commonly present and the attainment of equilibrium (with unreduced protein) is exceedingly slow (Pitt-Rivers and Impiombato, 1968).

Certain dyes which are highly insoluble in dilute aqueous buffer solutions not far from neutrality are dispersed in detergent micelles when the latter are formed at concentrations above the critical micelle concentrations (cmc).[1] In fact this phenomenon is somethimes used to determine the cmc under various conditions since the concentration of dissolved dye is readily determined spectrophotometrically (filled circles in Figure 1a). The values of the cmc are affected by the ionic strength and by the nature of the counterion present. Most other influences on the cmc of ionic detergents, such as that of temperature, are small although the latter has an influence on micelle size (Tanford, 1973).

The presence of serum albumins, however, and many other proteins, even at low concentrations (*ca.* 10^{-5} M), has a profound effect on the solubility of the dye at concentrations of detergent below the cmc (see, among others, Blei (1960), Breuer and Strauss (1960)). This effect is illustrated in Figure 1a. At low detergent concentrations the dye dissolved does not depend on detergent concentration (region 1); at slightly higher concentration the amount of dye dissolved rises rapidly as the detergent concentration is increased (region 2). At still higher concentrations, the dissolved dye increases only slowly as more and more detergent is added (region 3), until finally the same concentration of detergent dissolves the same amount of dye when protein is present as in its absence (crossing point, region 4). Finally, the dependence on detergent concentration of dye dissolved becomes large again and the lines for protein absent or present become parallel (region 5) with *less* dye dissolved at a given detergent concentration when protein is present.

The present communication describes quantitatively the foregoing phenomenon for bovine and human serum albumins, and ovalbumin, and shows how it depends on the nature of the detergent, the pH, the ionic strength, and the amounts of detergent bound by the protein; and relates the binding properties of the protein to the changes induced by binding ligand. New information as to the nature of the

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[1] Abbreviations used are: SDS, sodium dodecyl sulfate; cmc, critical micelle concentration.

protein-detergent in these and other protein complexes is also deduced.

Experimental Section

Materials: Crystalline bovine serum albumin (B.D.H. Chem. Ltd. (England), lot 1715632) was used without deionizing. Experiments were performed with 0.1, 0.2, and 0.05% solutions. Phosphate buffers at pH 7.4 and ionic strength 0.033 were used (Ray *et al.*, 1966). Concentrations were determined spectrophotometrically, using $E_{1cm}(1\%)$ 6.67.

Crystalline human serum albumin (Pentex lot 31) was used without deionizing (see above), using $E_{1cm}(1\%)$ 6.4. Since earlier work was performed with different lot numbers (Steinhardt *et al.*, 1971), new long-time (10 day) equilibrium dialysis measurements were carried out at pH 5.6 at 10 and 20° at concentrations of sodium dodecyl sulfate up to 0.004 M and $\bar{v}$ over 250 (Pitt-Rivers and Impiobato, 1968). The results at lower concentrations are very close to those previously published (Steinhardt *et al.*, 1971) when allowance is made for the inadvertent use in the earlier experiments of 0.1117% protein instead of 0.1%.[2] With the lowest ionic strength (0.005) the pH rose gradually to 6.2 as $\bar{v}$ exceeded 40. The concentrations of protein were 0.1, 0.2, and 0.05%. A few experiments were performed at pH 7.4 at 0.033 ionic strength.

Ovalbumin was a five times crystallized preparation from Sigma.

The dimethylaminoazobenzene, from Fluka, used in the bovine serum albumin and ovalbumin experiments was recrystallized from hot ethanol. It obeyed Beer's law and had a molar extinction coefficient in ethanol of 26,000. The dye used with human serum albumin, also from Fluka, was recrystallized from acetone by adding water. The "absorbance" in 1 g/l. of dodecyl sulfate micelles (1 g/l. above cmc) was about 0.80 at 412–415 mm at pH 5.6. The dye used with bovine serum albumin had an absorbance *in ethanol* of 1.14 at pH 7.4. At pH 5, a second maximum appeared near 540 nm which grew as the pH fell below 5, but was without appreciable effect on the optical density at the first maximum. The extinction at or near 415 has therefore been used as a measure of the amount of dye solubilized. The presence of protein was without noticeable effect on either extinction or wavelength of the maximum.

Alkyl sulfates were a high purity custom synthesis prepared for us by Mann Research (now Schwartz-Mann).

Methods. The equilibrium dialysis experiments referred to above have already been described (Ray *et al.*, 1966; Reynolds *et al.*, 1967). The solubilization of dimethylaminoazobenzene was measured by placing approximately 10 mg of crystals in small Teflon-capped vials, and adding buffer solution plus various quantities of sodium dodecyl sulfate (from 0.24 to 40 mg) in a total volume of 10 ml in each vial. One set contained no protein and a duplicate set contained the specified concentration of bovine serum albumin or human serum albumin, usually 0.1%. The vials were rotated at ~2 rpm in an air-bath at 20° (human serum albumin) or a water bath at 25° (bovine serum albumin and ovalbumin) for 24–48 hr, and optical densities of the filtrates were measured in Cary spectrophotometers (Models 14 or 16).

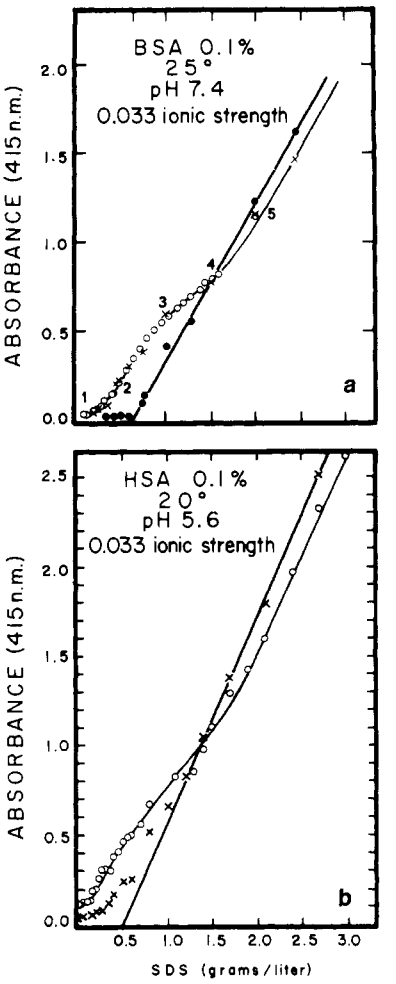

FIGURE 1: (a) The effect of 0.1% bovine serum albumin (BSA) on the solubilization of dimethylaminoazobenzene by SDS in pH 7.4 phosphate buffers of 0.033 ionic strength at 25°. (O and X) Protein present (two experiments); (●) protein absent. (b) The effect of 0.112% human serum albumin (HSA) on the solubilization of dimethylaminoazobenzene by SDS in pH 5.6 phosphate of ionic strength 0.033 at 20°. The intersection of the straight line with the abscissa indicates the critical micelle concentration (cmc) in the absence of protein. (O) Protein present; (X) protein absent.

The experiment with human serum albumin unfolded by 6 M guanidine hydrochloride was performed on protein treated with the unfolder and β-mercaptoethanol as described by Reynolds and Tanford (1970a,b). The water-insoluble product was dissolved by prolonged stirring in buffer (pH 5.6) which contained 0.3 g of SDS/l. (1.04×10^{-3} M) and the protein concentration was adjusted to 1.117 g/l.

Results

Representative data from experiments with both human and bovine serum albumins with the insoluble dye dimethylaminoazobenzene in phosphate buffers of 0.033 ionic strengths are shown in Figure 1a and b. Although the ionic strengths are the same, different pH values and temperatures, and slightly different concentrations of protein, have been used.[3]

The five regions of Figure 1a described above, as well as the general characteristics of the curve for SDS without protein, are readily recognized also in Figure 1b. The indicated cmc is 2.15×10^{-3} M in Figure 1a and 1.67×10^{-3} M in Figure 1b. The slopes of the two "micellar" curves (no

[2] This correction is also required (but is inconsequential) for the data published by Steinhardt and Stocker (1973a,b). The error arose from the use of an incorrect value of $E_{1cm}(1\%)$ in these papers. The correct value is 6.4.

protein) are about 1.08 (pH 5.6) and 0.89 (pH 7.4). Both proteins appear to disperse little or no dye (region 1) when only small amounts of detergent are present (up to 0.16 g/l. in the case of human serum albumin, about 0.2 g/l. in the case of bovine serum albumin). Thus the first stage of unfolding of the proteins should be almost complete although no solubilization has taken place (Decker and Foster, 1966; Steinhardt *et al.*, 1971). The protein curves cross the micellar curve at 1.6 and 1.3 g/l. respectively (region 4), and enter into rapidly rising regions of dye dispersion just beyond, parallel to the micellar curve (region 5). The crossing point is at higher absorbance (1.0) with human serum albumin than with bovine serum albumin (0.8) because regions 2 and 3 are steeper with the former protein.[3]

Analysis of these data requires distinction between total detergent concentrations, as shown above, and the amounts bound to protein. Binding isotherms for these two proteins at 0.033 ionic strength at a number of pH values and temperatures have been measured in our laboratory. With bovine serum albumin reliable equilibrium dialysis binding data are available up to binding ratios ($\bar{\nu}$) slightly above 100. With human serum albumin the data extend to $\bar{\nu} = 200$. In the interest of conserving space only the data for the latter are given here.

Figure 2 gives $\bar{\nu}$ as a function of C_T/P, the ratio of total molal detergent concentration to human serum albumin (or as grams of detergent per liter, at three concentrations of protein used here) and at two different pH values. The relation between $\bar{\nu}$ and C_T/P is linear at values of C_T/P over about 20–30. The data shown in Figure 2 were obtained with the same batch of human serum albumin used in the solubilization experiments. Similar isotherms for bovine serum albumin at 0.033 ionic strength are available. The linear relations shown in Figure 2 prevail outside the region of "statistical" (law of mass action) binding at $\bar{\nu}$ < about 15 and have proved reliable to $\bar{\nu} > 165$, at which point a slight falling off in slope sets in.

The data of Figure 1a and b have been replotted in Figure 3a and b in terms of dye dissolved or dispersed per liter (in absorbance units at 412–415 nm) as a function of equivalents of SDS bound per mole of protein. A second abscissa scale, on the face of the figure, shows the total SDS *present* per liter of solution or per gram of protein.

The results are simply described; with bovine serum albumin (A) little if any solubilization of the dye occurs until about 16 equiv of SDS are bound; (B) the absorbance then increases sharply, until about 29 equiv are bound; (C) thereafter the absorbance increases *linearly and uniformly* at a lower rate to over 110 equiv of SDS bound. A comparison of the slopes obtained with and without protein shows that the albumin SDS complexes are about as effective in solubilizing dye as simple SDS micelles up to the $\bar{\nu}$ limits shown. The initial sharper rise may result from the fact that new binding sites become available and occupied as a detergent-induced conformation change occurs (Reynolds *et al.*, 1967). It will be noted *that the slopes of the "micellar" curves* (no protein present) *in Figure 3a and b are very similar* in spite of the large difference of pH and temperature

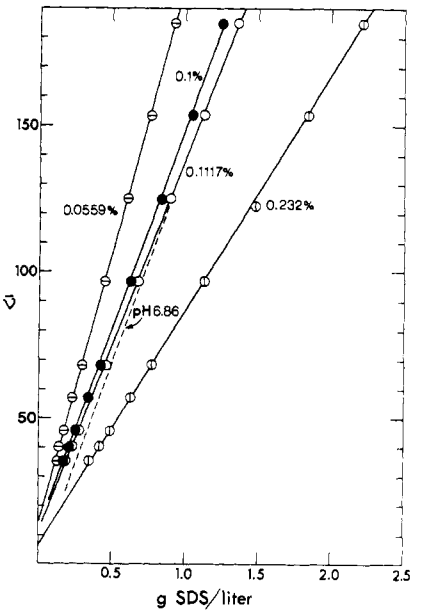

FIGURE 2: The molal ratios ($\bar{\nu}$) of dodecyl sulfate bound to human serum albumin as functions of detergent concentrations at pH 5.6; at four concentrations of human serum albumin at 25° and 0.033 ionic strength. Data are also given for 0.112% human serum albumin at pH 6.86.

and the differences in dye extinction (see Methods). The results with human serum albumin (Figure 3b) are very similar to those with bovine serum albumin (Figure 3a): (a) no dye is solubilized when less than about 20 equiv of SDS are bound, but then (b) the absorbance increases rapidly up to about 50 equiv bound,[4] and then (c) increases more gradually at about the same rate as prevails when dye is dispersed in SDS micelles in the absence of protein.

The steeper slope (Figure 3b) between 20 and 50 equiv of SDS bound is, as with bovine serum albumin, directly related to the fact that no dye is solubilized when less than about 20 equiv are bound. The doubling of this slope in this region (in both human serum albumin and bovine serum albumin) implies that SDS binds first to discrete sites which do not permit the merging of bound detergent molecules into a contiguous domain; when the detergent concentrations are high enough to bring about the first unfolding transition in the albumins, *all* of the bound detergent in some complexes (including the part previously bound in isolated discrete sites) becomes available in something loosely akin to the micellar form which causes solubilization.

There are only small differences between Figure 3a and b although they show the results of experiments with different proteins at widely different pH values. Experiments have shown that part of this difference is due to pH. Another part of the difference is due to the better buffering of phosphate at pH 6.86 than at 5.6; at the latter nominal pH and at *lower buffer concentrations* (<0.01), the well known Scatchard-Black ΔpH effect causes the pH to rise from 5.6 to almost 6.2, at relatively small $\bar{\nu}$.

Thus, we describe the enhancement of detergent dye solubilization by protein as follows: (a) the detergent initial-

[3] The absorbances are higher in Figure 1a than in Figure 1b. A small part of the difference is due to the 11.2% higher concentration of protein in Figure 2. A greater part is due to the difference in pH. More dye is solubilized at pH 5.6 than at pH 7.4 (Reynolds *et al.*, 1970). This statement is also valid when no protein is present and the dye is dispersed in pure SDS micelles.

[4] The sharper transition region with bovine serum albumin may be related to the fact that there is a sharp drop in the mean residue optical rotation of bovine serum albumin which is practically complete at $\bar{\nu}$ = 20, whereas the corresponding drop for human serum albumin (which is larger) is not attained until $\bar{\nu}$ reaches 40–50 (Steinhardt *et al.*, 1971).

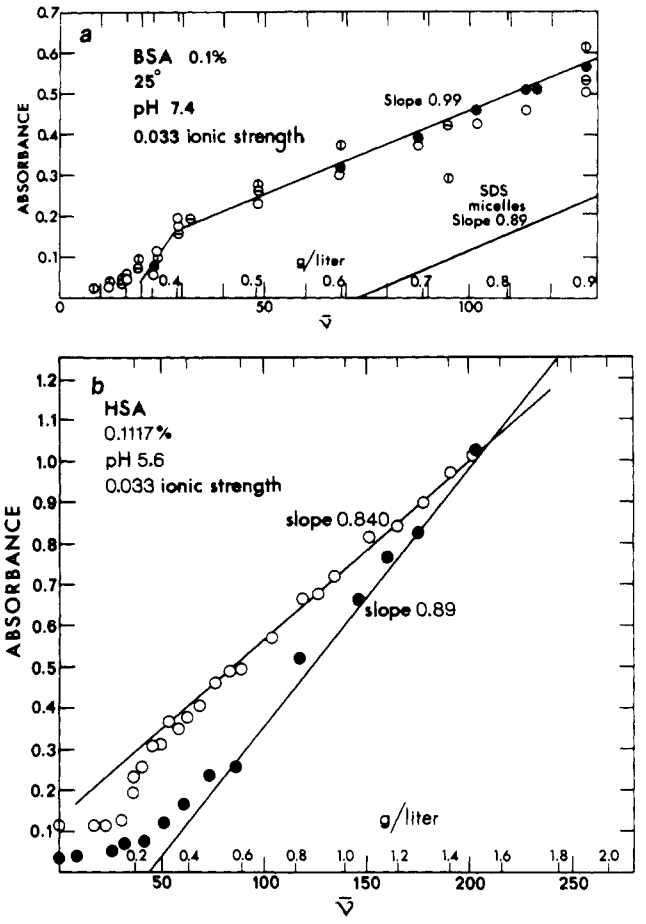

FIGURE 3: (a) Amounts of dye dispersed (absorbance units) as affected by the relative amounts of detergent bound to protein ($\bar{\nu}$). 0.1% bovine serum albumin at pH 7.4 and 25°. Two scales are given for the abscissa: (a) $\bar{\nu}$, the molal ratio of bound detergent to protein and (b) the concentration of total detergent (grams per liter). The lowest portion of the curve obtained without protein (dye dissolved in detergent micelles) is shown in the lower left-hand corner, plotted against the total detergent concentration scale. The results of four different experiments are indicated by the symbols, O, Φ, Θ, X. (b) The same as (a) for 0.112% human serum albumin at pH 5.6 and 20° at 0.033 ionic strength.

ly bound to isolated sites on the native protein is ineffective in dispersing dye, but (b) detergent bound to unfolded protein (which is present at $\bar{\nu}$ above about 20) is about as effective as the detergent micelles which form at higher detergent concentrations (>cmc) in the absence of protein.

There is no binding of micelles (which solubilize dye): in the presence of 0.1% protein, at this ionic strength, the concentration of uncombined monomers does not exceed the cmc until substantially more than 1 g/l. of detergent is present at the crossing point, provided micelles and protein-SDS complexes are equally effective in dispersing dye.

Detergent micelles and protein-detergent complexes cannot compete for detergent except at sharply defined concentrations represented on our figures by a zone immediately to the right of the crossing points. The very high cooperativity of micelle formation (Tanford, 1973) ensures that at higher concentrations only micelles will be formed. The protein-detergent complexes may be equally cooperative once each protein molecule is unfolded, but unfolded molecules appear over a wide concentration range characteristic of the unfolding process. The data of Figure 1, and others described later, show that the concentration of *free* detergent at the crossing point is slightly below the cmc. Thus, in Figure 1a, at the crossing point 0.54 g of SDS are present, and the cmc is 0.62; the absorbance is equal to that produced by dye solubilized in 0.92 g of micelles, almost identical with the figure 0.93 reported by Pitt-Rivers and Impiombato (1968). In addition, the separation of the parallel curves (0.14 g/g) reflects a shift of the micellar curve to higher SDS concentrations due to additional SDS bound by the protein which is therefore not available for micelle formation. This additional amount does not solubilize dye. Thus, the total bound is 1.06 g/g of proteins. The free concentration at the crossing point is thus about 0.48 g/l., and the free concentration at which the curves become parallel is 0.62 g/l., exactly the same as the cmc. Similar calculations can be made for the human serum albumin data in Figure 1b and elsewhere in this paper. The small nonsolubilizing binding is equivalent to attributing 87% solubilization effectiveness to the protein-detergent complexes as compared with pure micelles. The "equal effectiveness" of protein-detergent complexes and of micelles is no longer exact.

Effects of Protein Concentration. These conclusions may be tested by conducting similar experiments in which the conditions (protein concentration, ionic strength, pH, and temperature) *are varied widely.* The raw data from experiments in which the human serum albumin concentration was varied from 0.0559 to 0.2234% are shown in Figure 4. Data points for 0.1117% protein only are given; the results for the other two concentrations are represented by continuous traces (the actual data are shown in Figure 5). All the data have well-defined regions 1–5 (Figure 1b), but the data for 0.223% show a dip at the lowest concentration of SDS. All three sets differ only slightly in regions 2 and 5, but show large differences in the slopes of region 3 and the level at which the crossing points with the micellar curve occur (with 0.0559% protein lowest, with 0.223% protein highest).[5] In the parallel region 5, the separation is smallest with low protein and largest with high protein.[6] The "micellar" curves for all three are the same, as they should be, and similar to those already displayed.

By assuming that no concentration-dependent protein aggregation occurs, which might also affect the detergent binding, the binding isotherms at 0.0559 and 0.223% human serum albumin (Figure 3) may be applied to the data shown in Figure 4; Figure 5 shows the results of such calculations in terms of the dye dissolved (absorbance units) against the calculated SDS bound per liter, $\bar{\nu}$[P], at the three protein concentrations.

At concentrations below the crossing points in Figure 4 (at vertical arrows on Figure 5) the amounts of dye dispersed are directly proportional to the amounts of detergent bound ($\bar{\nu}$[P]). With the two lowest concentrations there is practically complete identity of the data obtained, within narrow limits of experimental error and of estimates of amounts bound. The proportionality of absorbance to grams of bound SDS is about 0.95 for all three curves, up to the crossing points in Figure 5. With the highest concentration

[5] The small discrepancy, at low concentrations, of the data obtained with 0.223% protein may be attributed to a number of alternative explanations which have one feature in common, that the amounts of detergent actually combined at higher concentrations of protein differ from those calculated for this concentration from the 0.1117% starting data. Self-aggregation could produce such an effect.

[6] The separation of the parallel linear regions is a measure of the amounts of detergent bound which do not solubilize, or of a lesser effectiveness of protein-bound detergent compared to simple micelles (see Discussion).

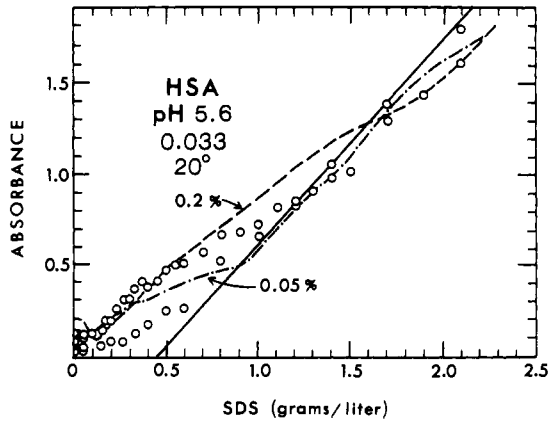

FIGURE 4: The effect of human serum albumin concentration on the amounts of dye dispersed in solutions of various concentrations of sodium dodecyl sulfate. (○) Data for 0.1117% protein (Figure 1a). Data for 0.0559% and 0.223% human serum albumin are represented by labeled continuous traces. The heavy straight line represents dye dissolved in SDS micelles containing no protein.

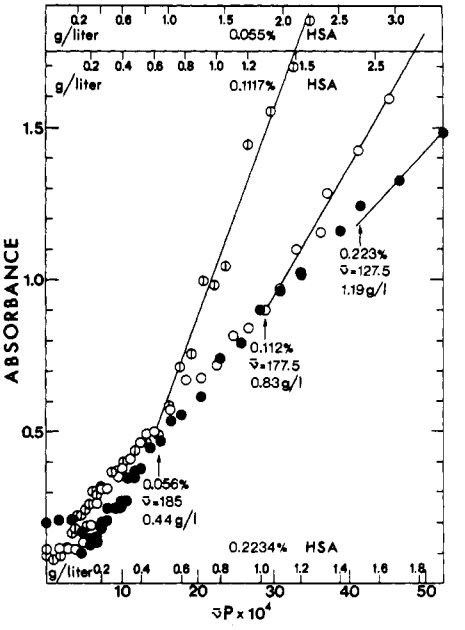

FIGURE 5: The effect of three human serum albumin concentrations on the relation between dye dissolved and SDS bound to protein per liter. At high molal ratios, all three concentrations of protein produce SDS complexes which dissolve dye equally effectively. The abscissa is the product of $\bar{\nu}$, the molal ratio, multiplied by the protein concentration. Three alternative abscissa scales are provided in terms of grams of SDS per liter, corresponding to the same $\bar{\nu}[P]$ values at the three concentrations of protein.

of protein (two sets of data shown) at low concentrations of detergent (<0.4 g/l.) slightly smaller amounts of dye are dispersed. The data at all three protein concentrations are in excellent agreement as they approach the respective intersections with the micellar curve.

At concentrations beyond the intersections of the curves for protein complexes with the "micellar" curves, beyond which dye dispersion by micelles makes a large contribution, the curves appear to diverge. The diverging lines beyond the vertical arrows are a clear indication that in this range of concentrations dye is solubilized by protein-free micelles rather than by protein-detergent micelles. The slopes of the two upper divergent lines represent a ratio of dye solubilized (absorbance units) to grams of micelles per liter of 1.00. The lower line, representing the highest protein concentration, has a slope of only 0.73, but inspection of Figure 4 will show that the change in slope in the vicinity of the crossing point is more gradual at this higher concentration of protein. The slope becomes higher (actually very close to 1.0) beyond the limits of Figure 5. Thus, all the data up to close to the crossing point are consistent with the concept that solubilization is proportional to the amount of detergent bound to partially unfolded protein; and that the effectiveness in solubilization of protein-detergent complexes is initially the same as that of ordinary detergent micelles.

A question remains as to whether any additional detergent is bound to protein at concentrations beyond the crossing point with the micellar curve. The high cooperativity of micelle formation makes it unlikely that any monomer binding occurs beyond the concentration at which the lines with and without protein become parallel. The sharpness of the change of slope at the crossing point with 0.056% protein (Figure 4) also suggests that almost no further binding occurs. The number of equivalents bound per gram of protein (185), at the crossing point at this protein concentration, is equivalent to about 0.79 g/g. At higher concentrations of protein the change in slope is more gradual, and it is conceivable that both micelles and protein complexes contribute to the solubilization over a short range beyond the crossing point. Thus, there is about 0.74 g/g bound at the 0.1117% crossing point. At the much more gradual crossing point for twice this concentration, only about 0.53 g/g are bound. Thus, the more gradual the crossing, the more binding must occur "behind" the micellar curve, and the larger spread between the parallel portions of the corresponding curves confirms this conclusion. No more occurs beyond the concentration at which the protein curve runs *fully parallel* to the micellar curve.

Although it has been shown that protein-detergent complexes are quantitatively as effective as detergent micelles in solubilizing dimethylaminoazobenzene, when $\bar{\nu} < 150$ nevertheless at high concentrations less dye is solubilized by detergent solutions containing protein. Thus, at high $\bar{\nu}$, some detergent is bound which does not solubilize dye as effectively as pure micelles. Experiments with numerous other proteins and other dyes are required to further define this distinction between solubilizing and nonsolubilizing bound detergent, and are now in progress.

Effects of Ionic Strength. Changes in ionic strength have profound effects on micelle formation, expecially at low ionic strengths. The cmc falls drastically as the ionic strength rises; the precise effect depends on the nature of the counterion (see, for example, Goddard *et al.*, 1953; Emerson and Holtzer, 1967). The cmc values indicated by the dye solubilization method in these experiments are given in column 2 of Table I.[7] Only the cmc seems to be affected. The solubilizing effectiveness of the detergent micelles, as measured by the slope of the micellar curve (third column of Table I), is not changed.

Figure 6 shows that variations in ionic strength are almost without effect on the solubilization of dimethylaminoazobenzene by 0.223% human serum albumin unless the ionic strength is below 0.01. At 0.005 ionic strength substantially less solubilization occurs at a given total concen-

[7] The cmc values are lower than those given by other methods. It is well known that dye solubilization often results in the determination of cmc values that are lower than those determined by purely physical means.

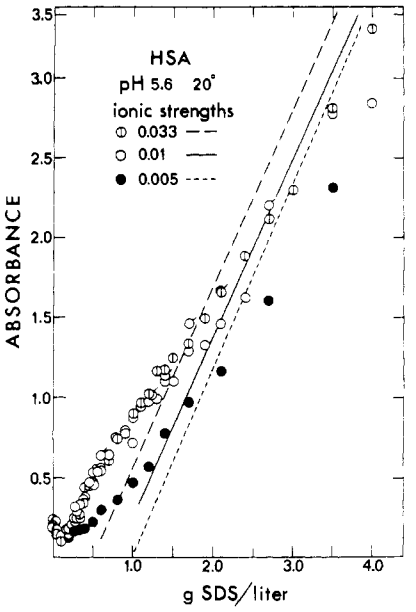

FIGURE 6: The effect of three ionic strengths on the amounts of dye dispersed by SDS and SDS–human serum albumin complexes at pH 5.6 and 20°. The binding by micelles containing no protein is represented by the three straight lines to the left of the data obtained in the presence of protein.

tration of SDS than at an ionic strength at or above 0.01. This would be expected only if the formation of protein–detergent complexes is *reduced* by low ionic strength. Thus, the large effect of ionic strength on the cmc of pure detergent micelles does not apply to the detergent–protein complexes, *i.e.*, the protein does more than merely lower the cmc by participating in the micelle.

At the lowest ionic strength, less dye dispersion occurs at a given concentration of detergent than is found at the two higher ionic strengths. The protein–detergent complexes appear to require some shielding by other ions to accommodate a dye molecule. The effectiveness, per gram of bound detergent, is nevertheless the same at all the ionic strengths (Table II). The need to use three different concentration scales in Figure 6, for the three ionic strengths, shows clearly that the effectiveness is not the same in terms of *total* detergent present.

Effects of Guanidine Hydrochloride and Disulfide Reduction. Reynolds and Tanford (1970a,b) reported maximum binding by unfolded reduced proteins of 1.4 g of

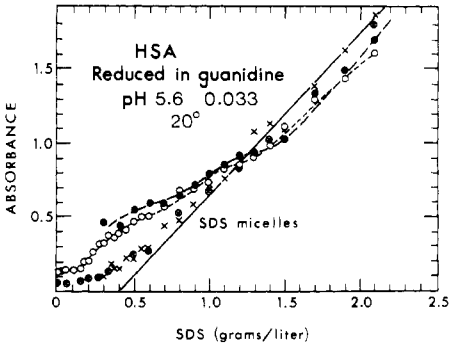

FIGURE 7: The effect of disorganization of human serum albumin by reduction by β-mercaptoethanol in 6 M guanidine hydrochloride, on the amounts of dimethylaminoazobenzene dispersed in human serum albumin–SDS complexes. (●) Data obtained with reduced unfolded protein; (○) data obtained with initially native human serum albumin (Figure 1b); (X and ⊗) data obtained without protein.

TABLE I: Effect of Ionic Strength on Solubilization by Micelles at pH 5.6.

Ionic Strength (phosphate)	Cmc	Δ Absorbance/ Δ grams of micelles
0.005	3.65×10^{-3}	1.00
0.01	2.80	1.03
0.033	1.75	0.98

SDS/g of protein, equivalent with bovine serum albumin to $\bar{v}$ = 335. Similar results were obtained with reduced proteins by Pitt-Rivers and Impiombato (1968) who also demonstrated binding of 0.93 g/g if the proteins were not reduced. We have applied the present method to the use of guanidine-unfolded reduced human serum albumin prepared by the method of Reynolds and Tanford. The results with 0.117% protein are shown in Figure 7 together with the data on initially native protein (from Figure 1b). At 0.3 g/l. of SDS about 20–25% more dye is solubilized by the unfolded protein–SDS complex than in the experiment starting with the native protein. The amount dissolved rises only slightly (from 0.45 to 0.6 absorbance unit) as the total detergent concentration rises to 0.7 g/l. From this point on, there is no difference in the amounts of dye solubilization effected by initially native and by unfolded reduced protein, *at the same total SDS concentration.* Thus these high concentrations appear to unfold the untreated protein to the same extent as the guanidine salt and reduction, insofar as its ability to bind detergent (*ca.* 0.8 g of SDS/g) is concerned.

Results Obtained with Another Ligand (Decyl Sulfate). Decyl sulfate micelles are almost as effective as dodecyl sulfate micelles in dispersing dimethylaminoazobenzene: 0.86 absorbance unit per g of micelles per liter (0.77 absorbance unit/g of protein) instead of 1.0–1.1 (Figure 8). This figure indicates large binding of decyl sulfate to human serum albumin unless the protein–decyl sulfate complexes are substantially more effective in dispersing dimethylaminoazobenzene than the decyl sulfate micelles themselves. Subject to this proviso, 0.7 g of decyl sulfate/1.112 g of protein is bound at the crossing point ($\bar{v}$ = 166). This figure is unex-

TABLE II: Molal ratios ($\bar{v}$) of SDS Bound to Protein at the Crossing Points (Figure 7).

Protein Concentration (%)	$\bar{v}$	g/g
Ionic Strength 0.033, pH 5.6		
0.056	185	0.77
0.112	177.5	0.74
0.223	127.5[a]	0.53
Protein Concentration 0.2234%, pH 5.6		
Ionic Strength	$\bar{v}$	Total g of SDS/g of Proteins
0.005	169	0.79
0.010	152	0.71
0.033	144[a]	0.79

[a] These data are from two different experiments.

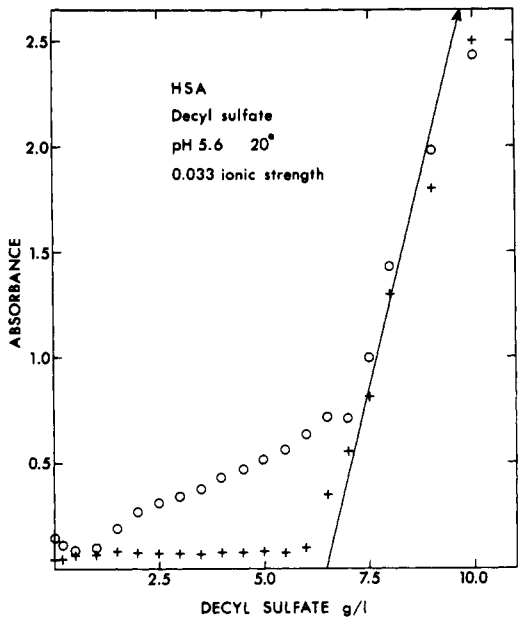

FIGURE 8: Dye solubilization data similar to that in Figure 1a, obtained with sodium decyl sulfate instead of sodium dodecyl sulfate.

pected since the highest value of $\bar{\nu}$ found with decyl sulfate binding to human serum albumin in equilibrium dialysis experiments has been barely over 25 (much higher values have been found with bovine serum albumin (Reynolds *et al.*, 1967)). The equilibrium dialysis experiments were not extended to total decyl sulfate concentrations over about 0.002 M; and the present dye solubilization experiments find $\bar{\nu} \sim 166$ at a concentration of about 0.029 M, nearly 15 times as high. Thus, the dye solubilization experiments yield important new information about the shorter chain detergent, namely that at sufficiently high concentrations, it binds to human serum albumin to extents similar to those attained with dodeyl sulfate. Thus, decyl sulfate at sufficiently high concentrations unfolds human serum albumin at 20° as does dodecyl sulfate at lower concentrations. Therefore the affinity of decyl sulfate *for unfolded protein* is much lower than that of dodecyl sulfate (Steinhardt and Reynolds, 1969), although the affinities of these two alkyl sulfates for *native* albumins are almost identical. This finding is consistent with our previous observation that longer chain detergents such as myristyl and dodecyl benzenesulfonate have much higher affinities than dodecyl sulfate for *unfolded* proteins, although their affinities for native albumins do not differ greatly (Steinhardt and Reynolds, 1969).

Results with Ovalbumin. We have investigated other proteins by the dye solubilization method. The data for one of these, hen's ovalbumin, are reported here (others will be reported later). The data (Figure 9) show that ovalbumin forms SDS complexes which solubilize dimethylaminoazobenzene in the same range of detergent concentrations (below the cmc) as the serum albumins. In the absence of equilibrium dialysis experiments with pure detergent, only a cursory analysis is presented. As with the albumins the initial binding at the lowest detergent concentrations does not solubilize dye. Dye is effectively dispersed with detergent concentrations between 0.3 and 0.9 g/g. In region 5 (steep parallel curves) the protein curve is at about 0.2 g/g higher detergent concentrations than the nonprotein micellar curve.

If protein complex and micelles are equally effective it

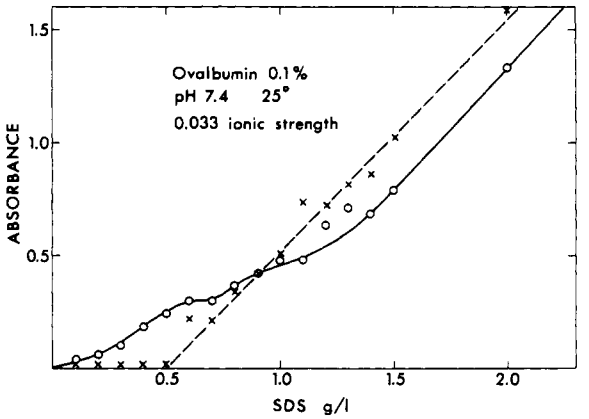

FIGURE 9: The solubilization of dimethylaminoazobenzene by ovalbumin–SDS complexes in pH 7.4 (0.033 ionic strength phosphate buffers) at 25°. The slopes of the straight line regions are determined by data which extend to absorbances above 10.

appears that about 0.6 g of SDS are bound per gram of ovalbumin at this ionic strength ($\bar{\nu} > 144$). Approximately another 0.2–0.3 g/g appear to be bound without solubilizing. The tentative total, ~0.8 g/g ($\bar{\nu}$ *ca.* 192), is just below that of Pitt-Rivers and Impiombato (1968). Higher binding ratios may be revealed by experiments at lower ionic strengths; such experiments are in progress. Yang and Foster (1954) have shown that the protein appears to combine with a constant fraction of the detergent present from the lowest concentrations, *i.e.*, as does *unfolded* serum albumins.[8]

Work in progress, to be reported elsewhere, shows that the nonspecific high binding is not accompanied in all cases by the phenomenon of dye solubilization. The results also depend on the dye used. The existence of the solubilization effect, therefore, in particular proteins, constitutes new and nonredundant information about these proteins.

Discussion

Ordinary SDS micelles under the conditions of these experiments contain about 100 SDS anions (Emerson and Holtzer, 1967). Our results appear to show that such micelles and the albumin–SDS complexes are equally effective in solubilizing dimethylaminoazobenzene. This suggests that the protein–SDS complexes may contain approximately the same number of SDS anions as do the SDS micelles, *i.e.*, enough SDS combines with individual partly unfolded albumin molecules to form complexes which combine with as much dye as the ordinary micelles. The ratio moles of SDS/moles of dimethylaminoazobenzene is about 80 in both.[9] $\bar{\nu}$ is an average then of the large amounts combined by certain molecules (those which have been "cooperatively" unfolded) and the much smaller amounts by the remaining protein molecules. Since $\bar{\nu}$ may exceed 300 at high SDS concentrations, each protein–SDS complex may contain from two to four times as much SDS as an ordinary micelle, and disperse two to four times as much dye. Analysis of electrophoretic and equilibrium dialysis data on bovine serum albumin by Decker and Foster (1966, 1967) and of detergent binding isotherms by Reynolds *et al.* (1967)

[8] Yang and Foster (1953) published extensive data with a somewhat heterogeneous sample of detergent.

[9] The ratio of dye to detergent is more significant than the ratio dye/micelle. Thus the latter varies widely (Jacobs *et al.*, 1972). Raising the ionic strength increases the micelle number (Coll, 1970) but the ratio dye/SDS (micellar) remains more nearly constant.

leads to similar conclusions as to the high cooperativity of high binding, and the wide range of averaging in $\bar{\nu}$.

Goddard *et al.* (1953) have shown that while inorganic cations depress the cmc in an inverse order relative to their hydrated size, the effect of organic cations, which is much larger, is greater the greater their ionic radius, which increases with their molecular weight. However the conception that the protein cation has its effect by thus lowering the cmc is untenable: the complexing of SDS with proteins has effects other than a mere lowering of the cmc: this is shown by the absence of ionic strength effects above 0.01, and by the fact that this view would lead to a micelle number (with bovine serum albumin and human serum albumin complexes) of some 300. The micelle number would also have to depend on the molecular weight of the *protein* if a fixed ratio of maximum binding (*i.e.*, 1.4 g/g) prevails. There is ample evidence that SDS micelles do not bind to these proteins.

To estimate the upper limits of SDS binding which can be measured by the dye solubilization method, we refer to the crossing points of the curves obtained with and without protein under six different sets of conditions (protein concentration and ionic strength). These data are given in Figures 5 and 6. The $\bar{\nu}$ values at these six crossing points are summarized in Table II. The highest $\bar{\nu}$ values are realized at low protein concentrations and at low ionic strength. An experiment at 0.005 ionic strength and 0.0559% human serum albumin (not included in the figures) gave a crossing point at about 1.15 g of SDS/l., which at these concentrations corresponds to $\bar{\nu}$ close to 250.

This is appreciably greater than the figure 0.93 g/g found by Pitt-Rivers and Impiombato with unreduced bovine serum albumin; 0.9 g/g corresponds to $\bar{\nu} = 215$ on a molecular weight basis of 69,000 and 203 if the molecular weight is 65,000. We have shown that reduced human serum albumin treated by guanidine hydrochloride (Figure 7) solubilizes more dye at lower SDS concentration than does the untreated protein, but that the difference does not persist to higher concentrations; there is an almost negligible difference in the crossing points obtained with and without the guanidine-mercaptoethanol treatment. It is difficult, therefore, to see why our solubilization data should indicate $\bar{\nu}$ values as high as 250 even without the drastic unfolding treatment by guanidine. However, the figure 0.93 g/g was obtained at ten times higher protein concentration at a concentration of free detergent, 0.93 mM, set by the cmc. It is likely that higher amounts bound would have been found at lower ionic strengths, possibly approaching those for reduced protein under our conditions.

Thus, the apparent limit of binding to proteins is necessarily set by the cmc, *i.e.*, the highest attainable concentration of SDS monomer. If the cmc is large enough at the ionic strength used, the true maximum will be attained. Low ionic strengths favor a higher cmc, therefore higher binding. However, binding is itself reduced by very low ionic strengths. In the range of the present experiments, the former effect outweighs the latter, at all ionic strengths above 0.01.

The results with decyl sulfate and with ovalbumin taken together have wide implications. Reynolds and Tanford (1970a,b) and Pitt-Rivers and Impiombato (1968) have already called attention to the quantitative uniformity of many proteins in binding large quantities of dodecyl sulfate. We find now that decyl sulfate, which has approximately the same affinity for human serum albumin as dodecyl sulfate, but which has not previously been observed to unfold proteins at ordinary temperatures, does so at high concentrations, studied conveniently and accurately by the solubilization method. The decyl sulfate ion has thus been shown to have a much lower affinity for unfolded protein than does dodecyl sulfate although the affinities of the two ions for the native proteins are sensibly equal.

The results obtained after unfolding the reduced protein in guanidine hydrochloride (Figure 7) show that solubilization increases with degree of unfolding. Since high concentrations of SDS unfold, there is no increase in solubilization due to treatment with guanidine salts at these SDS concentrations; the protein is already unfolded. Thus, the slope of the absorbance data against SDS concentration, with protein present, could be determined by two factors: (a) an increasing proportion of unfolded molecules; and (b) an increase in the amounts of monomeric SDS bound by individual unfolded molecules. Since the *slopes* (against bound SDS) with protein present are the same as the slopes of absorbance against amount of SDS micelles in the "micellar" (no protein) data, one must conclude that the second factor (b) above is practically constant, *i.e.*, available sites in unfolded protein molecules are always filled at high SDS concentrations. In this way, the protein-SDS complexes can be as "effective" as ordinary SDS micelles in solubilizing dye.

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