Differences in the Solubilizing Effectiveness of the Sodium Dodecyl Sulfate Complexes of Various Proteins†

Jacinto Steinhardt,* Joan R. Scott, and Kulbir S. Birdi

ABSTRACT: The detailed reversible binding isotherms of sodium dodecyl sulfate ($NaDodSO_4$) with 13 different initially native proteins are reported; the data were obtained at 20 °C and pH 7.1, ionic strength 0.033, with amounts bound with some proteins up to 1.1 g per g of protein. Although the isotherms of some of the proteins do not vary widely, extreme variations between certain classes are found. Thus, for example, hemoglobin and myoglobin both have high affinities and high binding capacities, while γ-globulin, apoferritin, and transferrin have low initial affinities, and change drastically at higher concentrations. The protein-$NaDodSO_4$ complexes solubilize the water-insoluble dye dimethylaminoazobenzene (DMAB) as effectively as micelles of pure $NaDodSO_4$ when only small amounts (0.2 to 0.5 g/g of $NaDodSO_4$) are bound. In most cases this effectiveness falls progressively as larger amounts are bound, and may even cease altogether at limits characteristic of the individual protein. With some of the latter, a second region of renewed solubilization occurs when substantially higher amounts of $NaDodSO_4$ are present. In all cases, solubilization by ordinary micelles in normal amount occurs when the *free* $NaDodSO_4$ concentration exceeds the critical micelle concentration, but the binding of $NaDodSO_4$ to the protein also increases, in competition with formation of micelles. With some, but not all, proteins the $NaDodSO_4$ bound at concentrations above the cmc also solubilizes DMAB. In such cases the solubilizations by the protein-$NaDodSO_4$ complexes and by the simple micelles are additive. The significance of the differences in binding and solubilizing encountered among these proteins is discussed in terms of surface structure, cooperativity of binding, and protein composition. No certain correlations with content of most amino acids, subunit structure, solubility, and hydrophobicity have been found, but there is a weak inverse dependence of solubilizing effectiveness on molecular size and indications of a strong dependence on content of cationic groups.

A water-insoluble dye, dimethylaminoazobenzene (DMAB), dissolves in aqueous solutions of sodium dodecyl sulfate ($NaDodSO_4$[1]) and other detergents when the concentrations of the latter exceed their critical micelle concentrations (cmc). The amount of dye dissolved is proportional to the concentration of micelles, which is zero below the cmc. One mole of dye is dispersed per 70 mol of $NaDodSO_4$ (as micelles) at all ionic strengths. In a previous paper (Steinhardt et al., 1974) we have shown that, in the presence of certain proteins (human and bovine serum albumins and ovalbumin), the dye is dispersed at concentrations of anionic detergents far below the cmc. A number of different kinds of evidence were presented which established that the solubilization by the complex (the amount dissolved over and above any amount which might be dissolved by any $NaDodSO_4$ micelles present) was very clearly the same as the amount of dye solubilized in an equivalent quantity of pure micelles, up to about 200 equiv bound. The disulfide bonds of the proteins used in the experiments just described were intact. However, denaturation of serum albumins by guanidine hydrochloride and reduction by dithiothreitol had only small enhancing effects on the binding.

The significance of this behavior of $NaDodSO_4$ bound to protein in equilibrium with dissolved monomers rather than with micelles required explanation. We have now proceeded to show by examining the interactions of ten other proteins with $NaDodSO_4$, in both the presence and absence of DMAB, that the same generalization does not apply fully to all of them, not only because there are significant differences in the $NaDodSO_4$ binding isotherms of the various unreduced proteins but also because these differences are accompanied by differences in their respective effects on the solubilization of DMAB. In those few cases in which the dependence of dye dispersion on bound $NaDodSO_4$ follows very nearly the same law of proportionality as in the earlier work, the coefficient of proportionality varies from protein to protein. These variations are, in some cases, clearly outside the range of experimental variability established in our work. These findings bear on both the theory of micellar structure and intercalation into micelles, and on the surface features of dissolved unreduced protein. The latter may be arranged in dye-solubilizing clusters of bound detergent, although the equilibria of such clusters with free monomeric $NaDodSO_4$ and their efficacy in solubilizing dye vary substantially from one protein to another.

We have shown elsewhere (Reynolds et al., 1967; Steinhardt and Reynolds 1969; Steinhardt et al., 1974) that the binding equilibrium involving protein and detergent owes its appearance of high cooperativity (up to about 0.4 g/g bound) to the fact that large amounts of detergent are bound by proteins only after they have undergone a drastic unfolding which exposes many binding sites inaccessible to the ligand before unfolding. This results in isotherms that are almost literally perpendicular at unfolding concentrations of free detergent. Thus, it appears that most of the sites on any given molecule are either largely vacant or almost entirely filled; the apparent binding ratio is an average (from which the proportion of unfolded protein molecules may be deduced). In terms of this model the ap-

† From the Department of Chemistry, Georgetown University, Washington, D.C. 20057, and the Institute of Physical Chemistry, Danish Technical University, Lyngby, Denmark. *Received March 2, 1976.* Supported by a National Science Foundation grant. Expenses of collaboration were defrayed by a NATO grant and by a grant from Statens Naturvidenskabelige Forsk ningsrad (Denmark). Parts of the material in this paper were presented at the 59th Annual FASEB Meeting at Atlantic City, New Jersey, in April 1975.

[1] Abbreviations used: BSA, bovine serum albumin; HSA, human serum albumin; cmc, critical micelle concentration; $NaDodSO_4$, sodium dodecyl sulfate; DMAB, dimethylaminoazobenzene.

parent varying affinities of proteins to $NaDodSO_4$ in the high concentration range reflect differences in the concentrations at which unfolding occurs. However, it is shown below that the cooperative effects at amounts bound over 0.4 g/g are of a more complicated nature.

Experimental Section

Materials. The sodium dodecyl sulfate used was a high purity sample obtained from Theo. Schuchardt. It was analyzed by gas chromatography after hydrolysis with 1.2 N HCl for 1 h at 80 °C. The results showed that over 97% was dodecyl sulfate with less than 3% tetradecyl sulfate and decyl sulfate. This compares with only 69% purity for the "high-purity" material supplied by Schwarz/Mann used in much of our earlier work (for which a purity of 99.5% was claimed by the manufacturer; the principal impurity was tetradecyl sulfate) (Birdi, 1976). The Schuchardt material used in this investigation is no longer available, but the pure $NaDodSO_4$ supplied by British Drug Houses is approximately equally pure and readily available at a low price. ^{35}S-labeled sodium dodecyl sulfate (lots N14024 and J28015/2) was obtained from Amersham/Searle. Gas chromatographic assay demonstrated that this $NaDodSO_4$ was approximately as pure as Schuchardt or British Drug House material. All other reagents used have been previously described (Steinhardt et al., 1974).

The proteins came from the following sources: from Nutritional Biochemicals, bovine serum albumin (crystalline, lots 8167 and 3441), ovalbumin (lot 8728, 5× crystallized), transferrin (lot 5773, horse, salt, free), human γ-globulin (lot 6014, fraction II), β-lactoglobulin (lot 1268, 3× crystallized, bovine), lysozyme (lots 6157 and 6447, 3× crystallized), conalbumin (lot 8075), and aldolase (lot 2026, rabbit muscle, 5× crystallized); from Pentex, human serum albumin (lot 32) and apoferritin (lots 11-2 and 13, horse spleen, 2× crystallized); from Sigma, myoglobin (lots 14C-0710 and 14C-0720, whale skeletal muscle type II salt free, lyophilized) and catalase (lot 85C-8135, 2× crystallized, aqueous suspension, beef liver). They were used without further purification except for dialysis when supplied as crystals in concentrated salt solution. The human hemoglobins were purified chromatographically on DEAE-Sephadex A-50 columns in this laboratory and were free of A_2 and F fractions.

Methods. All the proteins except Hb^+ and Mb^+ were used at a concentration of 0.1% in 0.033 ionic strength phosphate buffer at pH 7.1. The two exceptions were used at 0.167%. Sodium azide (0.02%) was present to retard bacterial growth. The measurement of solubilization has been described (Steinhardt et al., 1974). Two to four days of slow rotation at 20 °C was allowed for equilibration before assay of the dispersed dye at 20 °C in a Cary 14 spectrophotometer at 405–415 nm. Binding isotherms were obtained by equilibrium dialysis of 1 ml of solutions containing buffered protein against 1 mL of solutions containing no protein. To assure attainment of equilibrium, periods of up to 14 days were employed; furthermore, pairs of experiments were done in one of which the $NaDodSO_4$ was initially all on the protein side of the membrane and, in the other, the $NaDodSO_4$ was all on the protein-free side. The degree of attainment of equilibrium and the reversibility of the data were therefore always apparent. Duplicate determinations of binding (when made) which did not agree within 5% were not included, even as averages. Such cases were rare.[2] Duplicate determinations of solubilization seldom disagreed by more than this amount. Assay was by means of added small amounts of radioactive $NaDod^{35}SO_4$ (added to the "cold" stock solution of Schuchardt $NaDodSO_4$, kept cold, and used within a week) on the basis of calibrations at the time of assay. All counts were made on 20-μL samples withdrawn from both sides of the membrane, added to 10-mL aliquots of Aquasol, and counted in a Beckman Model LS-150 scintillation counter. They required no adjustment for quenching. Calibrations were made with 20-μL of three dilutions of stock solution within the 2% reproducibility of the counting procedure. In some experiments assays were also made by the methylene blue technique (Reynolds and Tanford, 1970). The amounts apparently bound were consistently slightly higher when the methylene blue technique was used. This discrepancy may be related to the effects of azide and protein on the color extracted by chloroform in the methylene blue technique. Gas chromatography showed that the radioactive ligand was about as pure as the "cold" ligand; therefore only the results obtained by means of radioactive tracers have been adopted in this paper.

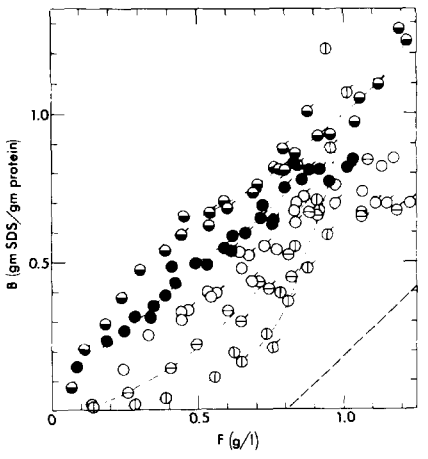

FIGURE 1: The amount of $NaDodSO_4$ bound (B) in grams per gram at 20 °C by five proteins (all except Mb^+ at 0.1%; Mb^+ at 0.167%) as a function of the free $NaDodSO_4$ (F) at equilibrium. Tags on data points represent data obtained with reversed direction of dialysis. The diagonal line at lower right represents the dye solubilized when no protein is present. Symbols: HSA (○); apoferritin (Φ); IgG (Θ); metmyoglobin (◒); aldolase (●).

Protein concentrations on both sides of the membranes were measured at the conclusion of every experiment by the Lowry method (Lowry et al., 1951). Volumes were also measured to detect leaks or persisting osmotic effects. Overall material balances that were not within 5% of the theoretical value (amounts originally present) resulted in the rejection of particular points. Most of the experiments had material balances within 3%.

Results

The results are presented in three principal sections: (1) binding isotherms at 20 °C for various proteins; (2) solubilization of DMAB by the same proteins; (3) the relation between dye dispersed and the amounts of $NaDodSO_4$ bound by each protein.

Isotherms. Figure 1 shows the data obtained with five proteins in terms of $NaDodSO_4$ bound (gram per gram of protein) against *free* $NaDodSO_4$ present at equilibrium (grams per liter). The sums of the ordinate and abscissa for each point represent the total $NaDodSO_4$ present (grams per gram or per liter). The five proteins represented in Figure 1 have been selected because they differ widely in their $NaDodSO_4$ binding.

[2] The reproducibility attained can be judged by inspecting the data for aldolase (Figure 5). In this figure the experimental points come from four different equilibration experiments.

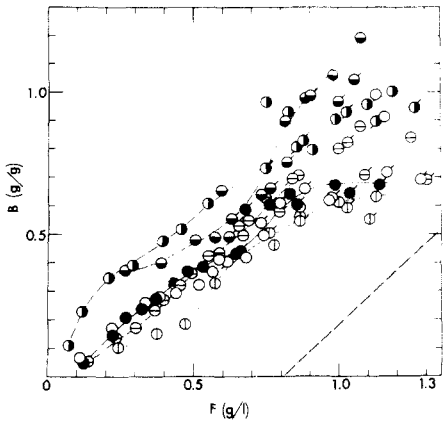

FIGURE 2: The same as Figure 1; data obtained with six additional proteins (all except Hb^+ at 0.1%; Hb^+ at 0.167%). Symbols: conalbumin (○); ovalbumin (●); β-lactoglobulin (⊖); transferrin (⊘); lysozyme (◒); methemoglobin S (◑).

The close agreement of circles and flagged circles (data obtained with diffusion away from or toward the protein side of the dialysis membrane) indicate that equilibrium has been obtained.

An empirical description of these differences follows:

(1) Although proteins which are at least partly unfolded in detergent solutions might be expected to show strong resemblances, the differences in affinity of the proteins to $NaDodSO_4$ are large. Thus at 0.2 g of $NaDodSO_4$ bound per gram of metmyoglobin the concentration of free $NaDodSO_4$ is approximately 0.1 g/L. With aldolase 0.15 g/L are free, with HSA (and catalase, not shown) about 0.29, with human γ-globulin 0.475, and with apoferritin almost 0.7—an overall span of about sevenfold.

(2) These ratios are larger at lower levels of binding, and lower at high levels, especially at the highest shown. Thus they can not be interpreted simply in terms of ratios of association constants, without further information. The curves of least binding (γ-globulin and apoferritin) are steeply concave upward, a strong indication that there are few if any high affinity sites on these proteins in the native state, and that a large number of binding sites become exposed and almost quantitatively occupied at higher free concentrations (Steinhardt and Reynolds, 1969; Steinhardt et al., 1974). There are only slight indications of similar upward concavity when the proteins show a higher binding tendency—in fact there are signs of leveling off after a long linear or almost linear region. It would appear, therefore, that three of these proteins have at moderate $NaDodSO_4$ concentrations numerous binding sites of higher affinity than those possessed by γ-globulin or apoferritin in the same range of concentration.

Neither the limits of the concavity upward nor any consistent signs of discontinuity occur at the cmc in any of the curves (under the conditions of these experiments the cmc of $NaDodSO_4$ is about 0.80 g/L, when measured by the solubilization of DMAB by pure micelles).

(3) There is also no consistent sign of a discontinuity in binding at about 0.4 g/g at the ionic strength of our experiments. Reynolds and Tanford reported 0.4 g/g as a maximum value for bovine serum albumin at the ionic strength of our experiments, and the same figure for other proteins at higher ionic strengths.[3]

(4) The two strongly binding proteins are devoid of disulfide bridges and have a low subunit molecular weight; the two weakest binders have a high molecular weight and have numerous disulfides, a constraint against complete unfolding. The protein in the middle, HSA, has many disulfides localized in several separate domains. When these groups are reduced and carboxyamidomethylated, according to the procedure of Shirahama et al. (1975), the results (not shown) obtained with BSA, which are very close to those obtained with HSA, show somewhat increased binding, and follow closely the aldolase curve.

To avoid crowding Figure 1, binding data for a number of other initially native proteins are shown in Figure 2. Human methemoglobin strongly resembles whale metmyoglobin but binds slightly smaller amounts (per gram) at amounts bound over 0.35 g/g. There are signs that this difference is due to a minor inflection region at amounts bound between about 0.4 g/g and 0.6 g/g. The smallest binding is shown by transferrin which, like two of the proteins in Figure 1, has an initial region which is concave upward. Transferrin may therefore undergo a similar cooperative transition (here at about 0.5 g/L free) to the one postulated for γ-globulin and apoferritin. The curves for the other proteins (lysozyme, ovalbumin, and β-lactoglobulin) lie close together, with lysozyme highest, at about the level of aldolase in Figure 1. As with the data in Figure 1 none of the data represented here approach or merge with the micellar boundary (dotted line on lower right). However the total amounts of $NaDodSO_4$ present (bound plus free) are above the cmc along most of the curves for all the proteins.

The second column of Table I lists the amounts bound in equilibrium with the cmc (free concentration). The third column of Table I shows that there is a weak inverse correlation between the amounts bound per gram of protein at the cmc and the size of the protein molecule (with catalase and aldolase the substitution of subunit size appears to strengthen the correlation).

Solubilization of DMAB by $NaDodSO_4$–Protein Complexes. Figures 4 to 8 which present data similar to the curves for the serum albumins and egg albumin already published (Steinhardt et al., 1974) have as abscissa the total $NaDodSO_4$ present (bound plus free) instead of the free $NaDodSO_4$ only. When protein is not present, solubilization by micelles occurs above the cmc, shown by the rising straight line which begins on the abscissa at the cmc. Their position on the abscissa when protein is present is affected by the binding of $NaDodSO_4$ monomers by the protein, by being shifted to the right (to higher concentrations).

All of these figures illustrate what is actually found with DMAB at total concentrations of $NaDodSO_4$ up to 2 to 4 g/L.[4] The presence of any of the proteins causes dye to be dispersed at concentrations far below the cmc (the effect is very small with catalase, and nearly zero with pepsin which is not shown). Beyond certain amounts of dye dissolved (which depend on the protein), there is a much lower dependence on $NaDodSO_4$ concentration—or even a close approach to a flat plateau in a few cases as the data cross the micellar curve. At still higher concentrations of detergent the presence of protein depresses rather than enhances the dispersal curve in the absence of protein. Between the crossing points and the parallel slope regions at higher $NaDodSO_4$ concentrations, additional detergent is bound by proteins as shown by the binding data, but the powers of dispersal of the additional complexes formed are

[3] Reynolds and Tanford varied the cmc by varying the ionic strength in order to demonstrate that only monomeric $NaDodSO_4$ was bound to unfolded reduced protein. There are other effects of ionic strength on detergent binding, at least when the proteins are not reduced.

[4] Solubilization data for the other proteins are given in composite curves for the individual proteins, which follow.

TABLE I: Summary of Certain Features of the Binding and Solubilization Data.

Protein	$NaDodSO_4$ Bound at cmc/free $NaDodSO_4$ (g/g)	Size or Subunit Size	Solubilization at cmc (absorbance units per g)[c]	Sol. Effective Complexes Formed above cmc[b]	$\Delta(B+F)$ of Regions Parallel to Micellar Curve
Apoferritin	0.40	480 000 (18 500)	0.48	+	−0.15
Human γ-globulin	0.50	160 000	0.48	−	+0.21
Transferrin	0.51	77 000	0.39	−	0.25
Conalbumin	0.55	86 180	0.53	−	0.20
BSA	0.58	65 000	0.63	−	
HSA	0.61	65 000	0.58	−	0.26
Ovalbumin	0.63	44 000	0.60	−	0.10
β-Lactoglobulin	0.65	35 000 (18 000)	0.58	−	0.10
Catalase	0.66	250 000 (62 000)	0.42	+	0.49
Aldolase	0.72	149 000 (50 000)	0.60	?	0.22
Lysozyme	0.75	14 100	0.56	+	0.35
RCAM-BSA	0.86	65 000	0.64	?	0.25
Met-HbS^+ (1.28/1.67) =	0.78[a]	68 000 (16 500)	0.46[a] (0.76/1.67 g)	−	0.90
Met-myoglobin (1.41/1.67) =	0.84[a]	16 500	0.42[a] (0.70/1.67 g)	−	1.18

[a] Corrected to g/g. [b] Minus signs signify little or no solubilization by $NaDodSO_4$ bound at $F >$ cmc. [c] The ratios $\Delta A/\Delta B$ differ somewhat more widely than the absolute value of ΔA in this column; they are not in the same order.

much lower than when the initial amounts (<0.2 g/g) are bound.

As later figures show, the curves for the various proteins show considerable differences at the smallest amounts of $NaDodSO_4$ present. The data for all of them lie closest together between 0.9 and 1.3 g/L present, and they diverge strongly at over 1.5 g/L, but disperse *less* dye in this region than if protein were absent.

Particular interest attaches to the solubilization data for two of the heme proteins, metmyoglobin and methemoglobin (Figure 6), shown in Figures 1 and 2 to have the highest affinity for the detergent. As the result of this high affinity, and the higher concentrations of protein used (0.167%), unusually large amounts of $NaDodSO_4$ must be present for micelles to exist (at least 0.80 g/L above the amounts bound at a concentration of total $NaDodSO_4$ equal to the cmc; 1.67 times the entry in Table I). Since the amount bound is about 1.5 g/L, one should not expect to see with the heme proteins, at $NaDodSO_4$ concentrations $(B + F)$ below about 2.3 g/L (1.5 + 0.8), the sharp rise in dye dispersion parallel to the micellar curve, which is shown by the solubilization data for most of the proteins. Actually such a rise does not appear with Mb^+ up to a concentration of about 2.5 g/L, and then the slope is less steep than that of the micellar curve (indication that not all the added $NaDodSO_4$ is going into micelles). With Hb^+, the value lies closer to the minimum 2.3 g/L. In view of the binding data shown in Figure 1, it appears that binding of $NaDodSO_4$ by Mb^+ continues at free concentrations above the cmc *in competition with the formation of normal micelles.* Hb^+ and Mb^+ are discussed further in a later section.

The extent of solubilization at the cmc for all proteins is listed in the fourth column of Table I. The fifth and sixth columns of the table list respectively whether protein $NaDodSO_4$ complexes formed above the cmc solubilize dye and the displacement of the micellar (no protein) curve to parallel linear regions found at high $NaDodSO_4$ concentrations.

Relation of Dye Dispersion to Amounts of $NaDodSO_4$ Bound by the Proteins. We have shown elsewhere (Steinhardt et al., 1974) that the amounts of DMAB dispersed by the serum albumins and ovalbumin in solutions are directly proportional to the amounts of these protein-$NaDodSO_4$ complexes formed. With this larger group of proteins the simple relationship turns out to be subject to certain complications.

For purposes of description we divide the 13 proteins investigated into two principal classes, without implying underlying resemblances. The first group is very similar to the serum albumins. We therefore present our new data for the latter here because they are more accurate than those previously published (due to impurities in the $NaDodSO_4$ used earlier), and because the method of analysis used for all 13 proteins may be introduced in this way. The second class is characterized by the existence of more than one step in the binding and solubilization isotherm.

Figure 3 shows a composite plot of the way in which the absorbance depends on the amounts of detergent bound for both serum albumins. 0.04, Absorbance, has been subtracted from all the solubilization data to adjust for the average baseline absorption. In this and in most of the figures which follow data obtained at absorbances or binding (g/g) greater than 1.0 are omitted, as are data for high total $NaDodSO_4$ concentrations, usually over 2.4. The omitted binding data may be inspected in Figures 1 and 2. The initial high degree of dependence ($\Delta A/\Delta B$ = 1.2 or 1 mol of dye per 58 mol of $NaDodSO_4$) found with small amounts of $NaDodSO_4$ with both proteins diminishes when larger amounts are bound. $\Delta A/\Delta B$ increases slightly again at the highest amounts shown, where solubilization by micelles plays a part. The data for BSA solubilization are slightly higher than those for HSA when over 0.5 g of $NaDodSO_4$ per gram is bound.

To understand why these changes occur, we superimpose in Figure 4 the data for *binding* (B, g/g), and for *solubilizing* (Δabsorbance) against total $NaDodSO_4$ (B, + F, g/L), and add a curve (dotted line) for free $NaDodSO_4$ (F, g/L). In order to avoid complicating the plot data are shown here only for

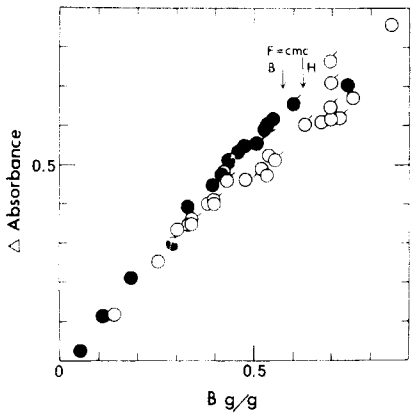

FIGURE 3: The dependence of dye dispersed (absorbance units) on amounts of $NaDodSO_4$ bound by 0.1% solutions of HSA and BSA. Arrows indicate the points at which F = cmc for the two proteins. Symbols: HSA (○); BSA (●).

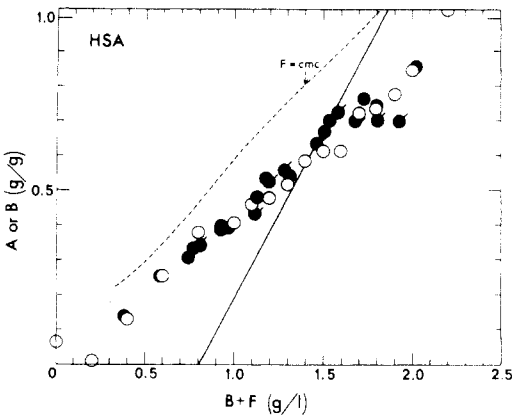

FIGURE 4: The relation of total $NaDodSO_4$ concentration ($B + F$) of dye dispersed (A) and of $NaDodSO_4$ bound (B) by 0.1% HSA. The solid diagonal represents the solubilization by micelles without protein. The broken line shows the free SDS concentration (F). Symbols: (○) dye solubilization; (●) binding.

HSA. The numerical values for the absorbances (which depend on the dye used) are by chance about the same as the numerical values of the amounts bound in the units shown; the ratio of these two quantities therefore remains near unity.

The free $NaDodSO_4$ (F) is larger than the bound (B) at all values of $(B + F) < 2.0$. F reaches the cmc at $(B + F) \cong 1.40$ g/L where the absorbance and the binding are both about 0.60. Beyond this value of $B + F$, micelles must be present. They grow slowly as B (filled in circles) climbs to 0.85, increasing by about 0.38 (to 1.18) after exceeding the cmc (beyond the range of the figure). This increase (as micelles) should account for an increase in absorbance due to micellar solubilization of about 0.36 unit. An increment of only 0.27 absorbance unit is actually observed in the interval between $(B + F) = 1.40$ and 2.0. It appears therefore that the binding of $NaDodSO_4$ to protein above the cmc, although it occurs, does not form additional complex capable of solubilizing DMAB. The absorbance and binding still run parallel above the cmc, due to micellar dispersal of the dye.

The same conclusions result from the experiments with BSA (plot against $[B + F]$ not shown), although there are no available binding data above 0.8 g/g at $(B + F) = 1.18$. The solubilization effected by BSA is slightly more pronounced than that effected by HSA—the numerical values for absorbance are always greater than for binding, up to the point at which $F \cong$ cmc.

The fact that the binding by micellar complexes is parallel to the no-protein micellar curve but at *higher* values of $B + F$ (Figures 2 and 4) supports the observation that some of the $NaDodSO_4$ in protein complex, above the initial amounts bound, is *less* effective at solubilizing than normal (no-protein) micelles.

With reduced and carboxyamidomethylated BSA (not shown), the binding is increased by about 0.08 g/g below the cmc, but the solubilizing effectiveness is unchanged except for a large increase at low total $NaDodSO_4$ as previously reported (Steinhardt et al., 1974) for reduced BSA.

We may summarize as follows: When free $NaDodSO_4$ is below the cmc, dye solubilization appears to be proportional to amounts of $NaDodSO_4$ bound, in the molar ratio of 1 dye to an average of 70 $NaDodSO_4$ as in pure $NaDodSO_4$ micelles. When F exceeds the cmc, the additional solubilization no longer depends on B, but is proportional to the micelle concentration (F − cmc). Bound $NaDodSO_4$ is fully as effective as micellar $NaDodSO_4$ in solubilizing dye only when small amounts are bound; it drops in effectiveness gradually as B increases. Thus, when solubilization finally becomes proportional to the free *micellar* $NaDodSO_4$, the curve obtained with protein is to the right of the curve obtained with $NaDodSO_4$ micelles alone.

If all the proteins behaved as do the serum albumins, it would always be possible to measure the maximum binding of $NaDodSO_4$ at least to the cmc by measuring the solubilization of DMAB. This is not always the case.

A number of the other proteins resemble the serum albumins in showing additional binding when F exceeds the cmc, and in having this additional binding essentially ineffective in solubilizing DMAB. These include conalbumin and β-lactoglobulin (not shown), two heme proteins (metmyoglobin and methemoglobin S), γ-globulin and transferrin. The paragraphs that follow show that, within this group, affinities for $NaDodSO_4$ and solubilizing effectiveness may otherwise differ widely without violating the criterion that additional binding of $NaDodSO_4$ occurs above the cmc, and that this additional binding makes little or no contribution to the dispersal of dye.

With conalbumin (not shown) the $NaDodSO_4$ bound and the DMAB solubilization follow almost exactly the same course at all $NaDodSO_4$ concentrations, showing similar changes of slope and points of inflection below, at, and above the cmc. As with most proteins the coefficient $\Delta A/\Delta B$ falls as B increases, and it is not unreasonable to find it as low as 0.38 when B is high.

With β-lactoglobulin (data not shown) the absorbance curve follows the parallel binding curve very closely. At the cmc the absorbance begins to rise somewhat more rapidly than the amounts of $NaDodSO_4$ bound since solubilizing micelles begin to be present. The ratio $\Delta A = 0.03/\Delta B = 0.15$ is even lower than the value which characterizes conalbumin and may actually be zero. Thus, in these two proteins, above the cmc there is little solubilizing effectiveness of the $NaDodSO_4$ complex. Micelles which form, in competition with binding, solubilize dye with close to characteristic effectiveness.

The remaining two proteins of this set (γ-globulin and transferrin) are characterized by very low binding at low $NaDodSO_4$ concentrations. γ-Globulin has both a low binding affinity and a low solubilizing effectiveness. Figure 7A shows that the binding curve is superimposed on the absorbance (solubilization) curve to beyond the cmc, which here is at $B +$ F = 1.29. Between $B + F = 1.33$ and 1.73 ($\Delta = 0.40$), B in-

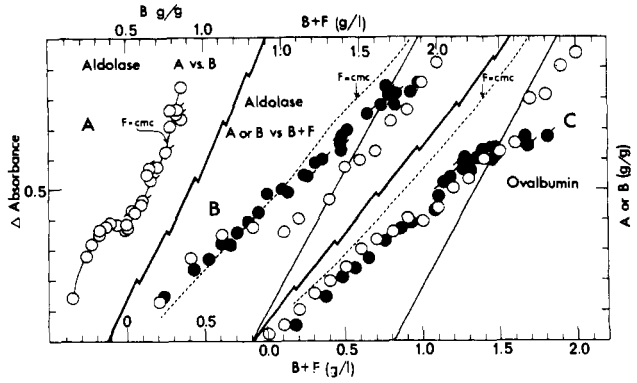

FIGURE 5: (A) Dependence of dye dispersed by aldolase on amounts of $NaDodSO_4$ bound by 0.1% protein. See Figure 3. (B) Relation of dye dispersed and of $NaDodSO_4$ bound by aldolase to total $NaDodSO_4$ concentration. Quantities as in Figure 4. (C) Quantities represented are as in Figure 4. Symbols (B and C): (○) dye solubilization; (●) binding; (- - -) free $NaDodSO_4$.

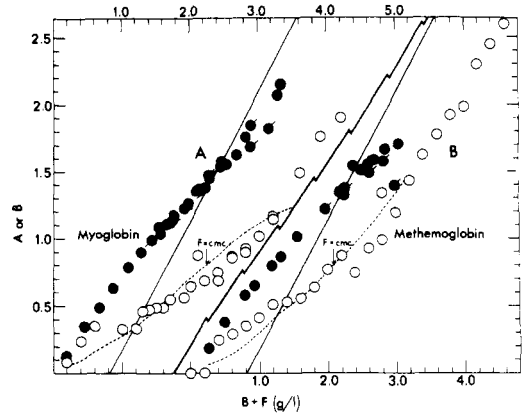

FIGURE 6: Relation of dye dispersed and of $NaDodSO_4$ bound to total $NaDodSO_4$ concentration, for (A) metmyoglobin and (B) methemoglobin S (both at 0.167%). Quantities represented are as in Figure 5 except that A and B are in units of g per 1.67 g of protein. Note the reduced scale. Symbols: (○) dye solubilization; (●) binding; (- - -) free $NaDodSO_4$.

creases by 0.14; F increases by 0.25, and the absorbance increases by 0.21. Since all of the increase in F is above the cmc, ΔF accounts for an increase in absorbance of about 0.24, close to the observed rise of 0.20. Thus there is no indication that $NaDodSO_4$-protein complex formed above the cmc solubilizes, i.e., the solubilizing effectiveness is zero above about 0.6 g/g $NaDodSO_4$ bound.

Transferrin has the same low initial binding tendency as γ-globulin, but the binding curve is less steep at higher $NaDodSO_4$ concentrations and levels off as B rises beyond 0.6 g/g. The solubilizing ability of its $NaDodSO_4$ complex is remarkably low; Figure 7B shows that the ratio of absorbance (corrected for a slight color in the protein solutions) to bound $NaDodSO_4$ is hardly over 0.5 and only rises beyond 0.75 as the cmc is approached ($B + F = 1.3$ g/L). Above the cmc, B increases by only 0.13 g/g, so the entire 0.24 increase in absorbance between 1.3 and 1.76 total $NaDodSO_4$ may easily be attributed to the 0.46 g/L increase in micelles in the same interval.

Two of the proteins differ from the foregoing in showing two distinct steps in the binding of $NaDodSO_4$ separated by a plateau (metmyoglobin showed such a plateau only in the solubilization data). Some of the binding occurs at $F >$ cmc, but the contributions to solubilization of this binding increment are small at best.

The affinity of *aldolase* for $NaDodSO_4$ is high. It contains no disulfide groups which might restrict the amounts of unfolding. The data (four experiments) shown in Figure 5A show two distinct regions of binding and solubilization below the cmc. Up to about 0.35 g/g bound dispersed and $NaDodSO_4$ bound are proportional. The next 0.2 g/g bound causes no further dye dispersal. Above about 0.55 g/g bound dye dispersion increases at a somewhat lower ratio to $NaDodSO_4$ bound than at the outset.

The nature of the discontinuities at 0.35 and 0.55 g/g bound is revealed in Figure 5B. Clearly the extent of solubilization is initially proportional to the amount of $NaDodSO_4$ *bound* but ceases to rise when the amount bound reaches 0.36 g/g, at which point ΔA (dye dispersed) levels off also at about 0.36–0.39 absorbance unit. The total $NaDodSO_4$ concentration at this point is about 0.70 g/L and no more dye is dispersed until this figure increases beyond 1.05 g/L. Thus an increase in bound $NaDodSO_4$ of 0.15 g/g has no solubilizing effect. However, dye is again dispersed when the total $NaDodSO_4$ exceeds 1.05 g/L. At this latter value of $B + F$, F is still below the cmc. Thus, the second rise in absorbance is not due to solubilization by micelles. However, beyond $B + F = 1.50$, micelles are present, and add to whatever solubilization is brought about by further binding of $NaDodSO_4$ by the protein (not more than 0.11 g/g within the range of the available binding data). The absorbance rises steeply, predominantly because of the micellar curve at levels above those shown in Figure 5B.

Thus the aldolase-detergent complex solubilizes dye to an extent proportional to the bound detergent, in two distinct steps, separated by a plateau in which bound detergent has no solubilizing effect. The second step is different from, but merges into, normal solubilization by $NaDodSO_4$ micelles.

Ovalbumin falls into the same pattern as aldolase, although it binds less $NaDodSO_4$. Figure 5C shows that solubilization occurs in two distinct steps, the second of which ends near the cmc (1.40 g/L on the abscissa). Solubilization increases sharply at $NaDodSO_4$ concentrations higher than this. Binding also occurs in two steps, but within the range of the data only about 0.05 g/g bound is added at $NaDodSO_4$ concentrations above the cmc. Solubilization and binding run parallel, but at a higher ratio in the first step than in the second. Most if not all of the increase in absorbance above the cmc is accounted for by solubilization by micelles.

The remaining three proteins include another heme protein which behaves quite differently from metmyoglobin and methemoglobin, plus two other proteins, lysozyme and apoferritin. All three show solubilization by $NaDodSO_4$-protein complexes formed above the cmc.

Catalase data (Figure 7C) show that, like γ-globulin and transferrin, this protein, although a heme protein, binds only a small fraction of the $NaDodSO_4$ present when the total $NaDodSO_4$ is below about 0.5 g/L. At higher concentrations the increments in binding are larger—from one-third to nearly one-half of the $NaDodSO_4$ present is bound as the total $NaDodSO_4$ approaches 1.7 g/L.

The solubilizing effectiveness of the complex is also low: $\Delta A/\Delta B$ is only about 0.7 between total $NaDodSO_4$ concentrations of 0.5 to 1.8 g/L. The binding of $NaDodSO_4$ continues when the cmc is exceeded at $B + F > 1.45$ g/L. The slope of the absorbance curve never becomes quite parallel with that of the micellar (no-protein) solubilization curve since about one-quarter of each increment of $NaDodSO_4$ concentration goes into forming the protein complexes. It appears, therefore,

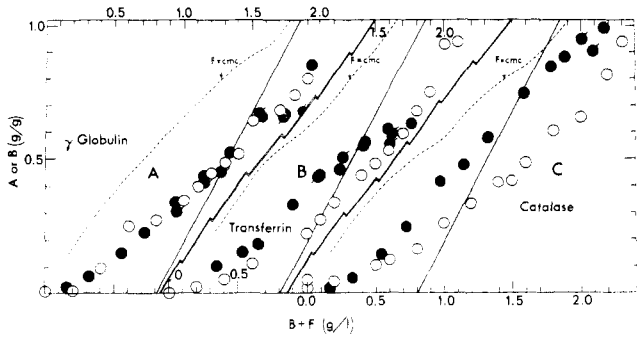

FIGURE 7: Relation of dye dispersed and of $NaDodSO_4$ bound to total $NaDodSO_4$ concentration, for (A) human γ-globulin; (B) transferrin; and (C) catalase. Quantities represented are as in Figure 4. Symbols: (○) dye solubilization; (●) binding; (- - -) free $NaDodSO_4$.

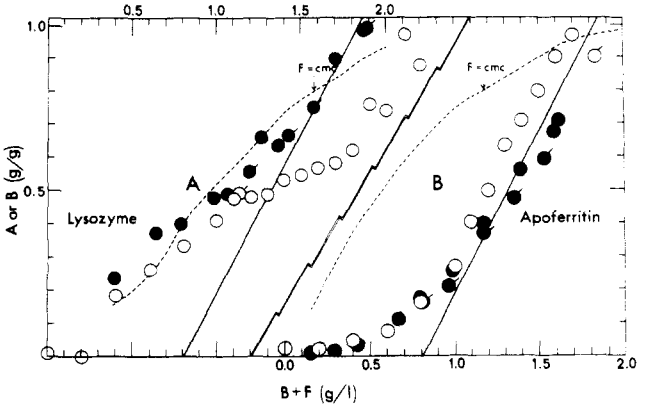

FIGURE 8: Relation of dye dispersed and of $NaDodSO_4$ bound to total $NaDodSO_4$ concentration, for (A) lysozyme and (B) apoferritin. Quantities are as in Figure 4. Symbols: (○) solubilization; (●) binding; (- - -) free $NaDodSO_4$.

that the protein-$NaDodSO_4$ complexes formed above the cmc make a small contribution to the dye solubilization at concentrations of $NaDodSO_4$ greater than 1.5 g/L.

Lysozyme and apoferritin belong in the same miscellaneous group. Figure 8A shows a delayed rapid rise in ΔA with *lysozyme*, followed by a more gradual rise between $B + F = 0.4$ and 1.1. After a still more gradual rise of another 0.2 g/L between 1.1 and 1.8 g/L the absorbance rises rapidly, almost parallel to the micellar curve but shifted 0.4 g/L to the right. The curve for $NaDodSO_4$ binding follows closely along the absorbance curve to 0.47 g/g bound, then shows little if any increase over an interval of about 0.15 g/L, and then increases sharply, nearly parallel to, but almost on the micellar curve. Further binding (0.2 g/g) occurs at values of $F >$ cmc. The $NaDodSO_4$ complexes formed in competition with micelles (about 0.2 g/g) must be as effective in dispersing dye as the lowest increments of $NaDodSO_4$ bound since the increase in absorbance in this region is about 0.4 and micelles can contribute only about 0.13 to this figure (see F curve).

The unusual results with lysozyme below 1 g/L may be due to the partial precipitation and redissolution of the protein.

Apoferritin resembles lysozyme in retaining fairly high solubilizing effectiveness of the $NaDodSO_4$–protein complexes formed at concentrations above the cmc but it is characterized by a much lower initial affinity.

Figure 8B shows that the smallest amounts of $NaDodSO_4$ bound are highly effective in solubilizing dye; thereafter the absorbance increases as the $NaDodSO_4$ bound increases but the first $NaDodSO_4$ bound, up to an absorbance of about 0.4, is slightly more effective ($\Delta A/\Delta B = 1.5$) than successive portions. Above 0.4 the effectiveness appears to stay constant at about 1.4 absorbance units per gram bound. Beyond 0.9 g bound (not shown) the effectiveness appears to increase again. Between the cmc (1.18 g/L on the $B + F$ scale, where $B = 0.38$ g/g) and the highest point plotted, the amount bound increases by 0.52 g/g. The free $NaDodSO_4$ increases by only 0.16 g/L, presumably entirely in the form of micelles. This 0.16 g accounts for 0.15 absorbance unit out of the total increase in absorbance above the cmc of 0.60 unit (part of it off the figure). Thus an increase of 0.45 absorbance unit is attributable to $NaDodSO_4$-protein complex equivalent to the 0.52 g/g of bound $NaDodSO_4$. The ratio 0.45/0.52 = 0.86 absorbance unit per g/g bound is lower than the initial value (near 1.4) below the cmc.

The relative binding affinities for $NaDodSO_4$ of the proteins investigated are readily inferred from examination of Figures 1 and 2. The average solubilizing effectiveness (below the cmc) relative to that of BSA is given as follows: apoferritin (1.2); conalbumin (1.0); γ-globulin (1.0); ovalbumin (0.95); β-lactoglobulin (0.89); human serum albumin (0.88); lysozyme (0.80); aldolase second step (0.80); transferrin (0.78); catalase (0.62); methemoglobin S (0.62); metmyoglobin (0.49). Other experiments have shown that the relative effectiveness for crystalline pepsin is near zero.

The solubilizing effectiveness of the $NaDodSO_4$ complexes at $F >$ cmc is small or, in many cases, equal to zero.

Discussion

The original purpose in undertaking these experiments was (a) to elucidate the nature of the large "nonspecific" binding of detergents to proteins (Steinhardt et al., 1974); (b) to explore and explain differences in solubilizing behavior between individual proteins; and (c) to find, if possible, relationships between the binding, manifestation of change in conformation, and other indications of protein unfolding.

The experiments reported here have demonstrated first how varied the detergent binding isotherms may be for individual initially native proteins. They also all agree in demonstrating that, up to certain specific limits, $NaDodSO_4$ bound to protein can disperse a water-insoluble dye, DMAB, and that this solubilization occurs at total concentrations of $NaDodSO_4$ at which no free micelles are normally present. The experiments also show that proteins differ greatly in the extent to which they form *solubilizing* complexes of $NaDodSO_4$ and that the effectiveness of such complexes can also vary. Some proteins (e.g., aldolase and ovalbumin) form successive solubilizing complexes at two different levels of $NaDodSO_4$ binding.

It has not proved possible to correlate this detergent behavior quantitatively with such other properties as shape of protein, subunit structure, solubility, helicity, acid–base behavior, or amino acid composition. There is, however, a weak inverse relation to size (or subunit size) which is not unexpected if the events we have studied occur on the surface of molecules that are not fully unfolded. It may also be noted that two heme proteins characterized by especially high affinity for $NaDodSO_4$ have hydrophobicities (Tanford, 1973) about 50% higher than the other heme protein in our set, catalase, which has a low affinity. It will be shown elsewhere that other proteins, containing particularly high amounts of lysine or arginine (such as histones), are characterized by higher binding and higher solubilization.

The proteins which have the highest affinities and the highest maximum binding capacities for our detergent permit a much more extensive view of their solubilizing behavior in

the absence of micelles (the best examples of this in our data are the higher concentrations of $NaDodSO_4$ with metmyoglobin and methemoglobin) than do the others. A possibility exists that the second solubilizing region for high amounts bound observed with aldolase might be found with other proteins if its appearance were not masked by solubilization by micelles.

The existence of limits to the amounts of solubilization, particularly (as in aldolase) where regions of plateau interrupt regions of increasing B, modify our earlier conclusion (Steinhardt et al., 1974) that a very high degree of "cooperativity" characterizes the binding equilibria. We had suggested that apparent intermediate extents of binding (values of B) represented *mixtures* of predominantly uncombined protein molecules with protein molecules in which the binding sites are all, or nearly all, occupied. The existence of regions of plateau is difficult to reconcile with such a view. However, this consideration does not exclude the existence of isolated regions of cooperativity as, for example, above and below a plateau.

All of the proteins are capable of binding large amounts of $NaDodSO_4$, as reported by other investigators (Reynolds and Tanford, 1970; Pitt-Rivers and Impiombato, 1968; Nelson, 1971; Allen, 1974; Takagi et al., 1975). Our own experiments using initially native proteins (not subjected to treatment with denaturants or reductants) do not support a uniform limiting value of 1.4 g/g for all proteins, nor do they show as high affinities for $NaDodSO_4$ as are implied by the data for *reduced* proteins of Reynolds and Tanford. The effectiveness of the bound detergent in solubilizing dye is very high when only small amounts of $NaDodSO_4$ are bound but falls off to an extent which varies from protein to protein when larger amounts are bound. It is obvious from the results taken as a whole that the bound detergent solubilizes very effectively only when it is bound in one of several different possible ways.[5] The mode which solubilizes best is obviously the mode that has the highest free energy of binding.[6] Since $NaDodSO_4$ can bind even when the free $NaDodSO_4$ exceeds the cmc, and at concentrations at which micelles form, the free energy of binding at this concentration must approach that of micelle formation. Since the free energy of binding of the smallest amounts bound is demonstrably larger, it must therefore equal or exceed the free energy change for micelle formation. Under these conditions, the solubilizing effectiveness is highest. It is possible therefore that the first portion of the $NaDodSO_4$ bound is in many respects organized similarly to ordinary micelles. The organization of the larger amounts of bound $NaDodSO_4$, which usually have a lower solubilizing effect, is not known, nor can we now explain the great differences in affinity between, for example, methemoglobin and γ-globulin, or in the shapes of their binding isotherms. However, effects of size and of hydrophobicity have been noted.

It can not be assumed that identical results will be obtained with all water-insoluble dyes. Work already reported (Birdi and Steinhardt, 1975) shows that the behavior of Orange OT is entirely different from that of DMAB. Certain other insoluble substances, including naphthalene, anthracene, azobenzene, and Sudan II, are now under study.

[5] The portions of the solubilization curves which are parallel to the no-protein micellar curve (always found if enough $NaDodSO_4$ is present) are always to the right of the micellar curve. The size of the shift is a measure of the amount of nonsolubilizing $NaDodSO_4$ bound; it is proportional to the sum of the product of all the distinct categories, by the effectiveness of each. Thus parallel portions are never found until no more $NaDodSO_4$ is bound, except in the unlikely case that the incremental $NaDodSO_4$ bound is as effective as protein-free micelles.

[6] Except for the small amount of binding to the proteins which occurs before any unfolding takes place.

References

Allen, G. (1974), *Biochem. J. 137,* 575.

Birdi, K. S. (1976), *Anal. Chem. 74* (in press).

Birdi, K. S., and Steinhardt, J. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 598.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Nelson, D. A. (1971), *J. Biol. Chem. 246,* 3895.

Pitt-Rivers, R., and Impiombato, F. S. A. (1968), *Biochem. J. 109,* 825.

Reynolds, J. A., Herbert, S., Polet, H., and Steinhardt, J. (1967), *Biochemistry 6,* 737.

Reynolds, J. A., and Tanford, C. (1970), *Proc. Natl. Acad. Sci. U.S.A. 66,* 1002.

Shirahama, K., Tsujii, K., and Takagi, T. (1974), *J. Biochem. (Tokyo) 75,* 309.

Steinhardt, J., Stocker, N., Carroll, D., and Birdi, K. S. (1974), *Biochemistry 13,* 4461.

Steinhardt, J., and Reynolds, J. A. (1969), Multiple Equilibria in Proteins, New York, N.Y., Academic Press.

Takagi, T., Tsujii, K., and Shirahama, K. (1975), *J. Biochem. (Tokyo) 77,* 939.

Tanford, C. (1973), The Hydrophobic Effect, New York, N.Y., Wiley.