Makinen, M. W., and Kon, H. (1971), *Biochemistry 10*, 43.
Malchy, B., and Dixon, G. H. (1969), *Can. J. Biochem. 47*, 1205.
Malchy, B., and Dixon, G. H. (1970), *Can. J. Biochem. 48*, 192.
McConnell, H. M., and Hamilton, C. L. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 776.
Moffat, J. K. (1971), *J. Mol. Biol. 55*, 135.
Nagel, R. L., and Gibson, Q. H. (1967), *J. Biol. Chem. 242*, 3428.
Ofosu, F. (1971), Ph.D. Thesis, University of Toronto.
Ofosu, G., Campbell, D. W., and Connell, G. E. (1971), *Can. J. Biochem. 49*, 90.
Ofosu, F., and Connell, G. E. (1971), *Can. J. Biochem. 49*, 637.
Ogawa, S., and McConnell, H. M. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 19.
Ogawa, S., McConnell, H. M., and Horwitz, A. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 401.
Smith, I. C. P. (1972), *in* Biological Applications of Electron Spin Resonance Spectroscopy, Bolton, J. R., Borg, D., and Swartz, H., Ed., New York, N. Y., John Wiley-Interscience (in press).
Smithies, O. (1959), *Biochem. J. 71*, 585.
Smithies, O., Connell, G. E., and Dixon, G. H. (1966), *J. Mol. Biol. 21*, 213.

Interaction between Sodium Dodecyl Sulfate and Ferricytochrome *c*†

R. K. Burkhard* and Gary E. Stolzenberg‡

ABSTRACT: The interaction of sodium dodecyl sulfate with ferricytochrome *c* from horse heart was studied at pH 7.35. The interaction appears to occur in two phases. The first of these is suggested to involve binding of the dodecyl sulfate anions to the cationic sites of the protein causing an unfolding of the protein, loss of its absorption band at 695 nm, and hyperchromic blue shift of its Soret peak to approximately 408 nm. The second phase is suggested to involve binding of the hydrophobic portions of the dodecyl sulfate to the hydrophobic amino acid residues of the protein and to be associated with a partial loss of the initial hyperchromicity of the Soret peak and a further shifting to approximately 406 nm.

Detergents have a variety of uses in biochemical laboratories. Two examples are the use of sodium dodecyl sulfate to solubilize mitochondrial proteins (see Morton, 1955, for review) and gel electrophoresis of sodium dodecyl sulfate–protein complexes to determine molecular weights (Shapiro *et al.*, 1967; Weber and Osborn, 1969). It has long been known, however, that detergents can denature proteins and considerable effort has been made by numerous investigators to elucidate this type of denaturation. Much of this effort has been directed toward explaining the denaturation of bovine serum albumin in terms of protein–ion interactions (see Putnam, 1948; Foster, 1960; Steinhardt and Reynolds, 1969; Tanford, 1968, 1970, for reviews). We thus thought it appropriate to study the interaction between the anionic detergent dodecyl sulfate and the mitochondrial protein cytochrome *c* since these two substances often encounter each other during biochemical manipulations and little has been done toward explaining the effects of this detergent on cytochrome *c* in terms of protein–ion interactions. This paper reports some of our observations and it is hoped they will be of interest to those involved in fractionating mitochondrial proteins or studying detergent–protein interactions.

The fact that dodecyl sulfate affects cytochrome *c* has been known since 1940 when Keilin and Hartree reported that it altered the visible absorption bands of this protein in both of its oxidation states (Keilin and Hartree, 1940). Further studies of this observation have been made since then (Rabinovitz and Boyer, 1950; Tsushima and Miyajima, 1956), but to date these spectral alterations have not been related to the interaction that occurs between these substances. Accordingly, we reexamined the spectral changes caused by adding dodecyl sulfate to ferricytochrome *c* and determined the extent of interaction between these two substances so that a correlation between these could be made and hopefully provide further information on how cytochrome *c* is affected by this detergent.

Experimental Section

Dodecyl Sulfate Samples. Sodium dodecyl sulfate was obtained from two sources: Matheson Coleman & Bell (Norwood, Ohio) and the Mann Research Laboratories (New York, N. Y.).

The detergent from the first source was found to contain dodecyl sulfate and tetradecyl sulfate, in a ratio of approximately 2:1 by weight, plus small amounts of unesterified alcohols and inorganic matter. The purity of this detergent could be improved by extraction of the unesterified alcohols with ether, extraction of the detergent from the inorganic matter by use of hot *n*-butyl alcohol, and precipitation from this solvent by adding ether.

The detergent supplied by Mann was a special grade recommended by Dr. J. Steinhardt of Georgetown University for use in equilibrium dialysis (personal communication). It was used without further purification.

† Contribution No. 130, Department of Biochemistry, Kansas Agricultural Experiment Station, Kansas State University, Manhattan, Kansas 66502. *Received November 18, 1971.*

‡ Current address: Metabolism and Radiation Research Laboratory, U. S. Department of Agriculture, Fargo, N. D. 58103.

Spectral studies made use of both detergent samples, but only the Mann sample was used in the equilibrium dialysis studies.

Determination of Dodecyl Sulfate. Dodecyl sulfate was routinely determined by a colorimetric procedure. To accomplish this a dodecyl sulfate–Methylene Blue complex was first formed in the aqueous system to be analyzed. It was then extracted with *o*-dichlorobenzene and the absorbance of the extract determined at 665 nm. Blanks for the assay were prepared in an analogous manner substituting buffer solution for the dodecyl sulfate solution. A new standard curve was prepared for each series of assays. The Beckman DU-2 spectrophotometer was used to perform these assays.

This method had two advantages over previously published colorimetric methods employing Methylene Blue (*e.g.*, Ray *et al.*, 1966). First, it was found that the dodecyl sulfate–Methylene Blue complex was more stable in *o*-dichlorobenzene than in chloroform. Secondly, the high boiling point of *o*-dichlorobenzene made it possible to extract the colored complex in stoppered test tubes without having the stoppers pushed out, and decreased the possibility of errors that might arise from solvent evaporation. Because of these advantages, we could analyze a large number of samples simultaneously.

Determination of the Critical Micelle Concentration of Dodecyl Sulfate. The literature contains many reports of the critical micelle concentration (cmc)[1] of dodecyl sulfate, but due to the fact that this value will vary with temperature and solvent it was thought desirable to determine the cmc for dodecyl sulfate under the conditions employed in these studies. Solubilization of *p*-dimethylaminoazobenzene (DAB) was used to determine this quantity.

For systems not containing cytochrome *c* small samples of DAB were placed in test tubes and to each was added 5 ml of dodecyl sulfate in 0.1 M buffer. The tubes were capped and shaken at 20–22° for 2 hr. The solid dye that remained was removed and the absorbance of the resulting solution was determined at 405 nm. The cmc was obtained by examination of a plot of absorbance *vs.* log dodecyl sulfate concentration. The concentration at which the break in this plot appeared (between 0.7×10^{-3} and 0.9×10^{-3} M) was selected as the cmc in our system. This is in good agreement with the values obtained by conductivity methods for the cmc of dodecyl sulfate at 25° in salt solutions of comparable ionic strength (Mysels and Mysels, 1965).

In an effort to relate changes in the cytochrome *c* spectrum to the degree of aggregation of surfactant an analogous study was performed with systems containing cytochrome *c*. After removal of the excess solid dye absorbances were determined at 497 nm. For each system containing dye, cytochrome *c* and dodecyl sulfate, a corresponding blank was prepared containing cytochrome *c* and dodecyl sulfate so that any spectral changes which might be due to the interaction of cytochrome *c* with the detergent could be taken into account. The wavelength 497 nm was selected for these studies since it is one of the minima in the cytochrome *c* spectrum and the molar absorbancy index of DAB is still appreciably large.

Spectra of Cytochrome c–Dodecyl Sulfate Mixtures. Most spectral studies of cytochrome *c*–dodecyl sulfate mixtures were performed by using a Cary Model 11 spectrophotometer, but a few were made using the Cary Model 14 or Beckman Model DU-2 spectrophotometer. The concentrations of cytochrome *c* solutions were varied over 100-fold range (8.5×10^{-7} to 8.5×10^{-5} M), and concentrations of dodecyl sulfate were used as appropriate to obtain molar ratios of detergent to cytochrome *c* as high as several thousand to one. Because of the wide range of concentrations involved, spectrophotometer cells of various path lengths were used ranging from 0.1 to 10 cm. The cytochrome *c* used for these studies was horse heart, Type III, as supplied by Sigma Chemical Co. (St. Louis, Mo.). It was used without further purification.

Binding of Dodecyl Sulfate to Cytochrome c. Equilibrium dialysis was used to study the binding of dodecyl sulfate to cytochrome *c* (Rosenberg and Klotz, 1960). As for the spectral studies the concentrations of cytochrome *c* and dodecyl sulfate employed varied considerably; however, in none of the binding experiments was the equilibrium concentration of dodecyl sulfate above its cmc. The dialysis tubing used for these studies was obtained from the Visking Division of Union Carbide Co. (Chicago, Ill.). Prior to use it was extracted for 2 hr with boiling water by use of a Soxhlet extractor and then soaked in buffer. Since it has been reported that cytochrome *c* can diffuse through this type of membrane under certain conditions (Margoliash and Walasek, 1967), it was necessary to establish whether we could use this type of membrane for our equilibrium dialysis studies. Using spectrophotometric measurements in the Soret region as a means of detecting cytochrome *c*, we were unable to detect any diffusion of this protein through our membranes under the conditions we employed for dialysis studies.

The cytochrome *c* used for binding studies was identical with that used for the spectral studies. All binding studies were performed at 20–22°.

Buffer Systems. All studies employed a sodium phosphate buffer (pH 7.35) which was prepared from solutions of reagent grade sodium phosphates. Potassium-containing salts were not used due to the low solubility of dodecyl sulfate in the presence of potassium ion. The buffer used for spectral studies was generally 0.1 M; the buffer used for equilibrium dialysis was 0.033 M.

Results

Preliminary experiments to determine the relative effects of sodium dodecyl sulfate, sodium cholate, sodium deoxycholate, and Triton X-100 (Rohm and Haas, Philadelphia) on the absorption spectrum of ferricytochrome *c* revealed that only sodium dodecyl sulfate caused significant alteration. When this surfactant was added to solutions of ferricytochrome *c* at pH 7.35, the Soret peak shifted to shorter wavelengths and the absorbance of the solution increased. The changes elicited by dodecyl sulfate further appeared to occur in two steps. The first of these, when completed, was characterized by a shift of the Soret peak to approximately 408 nm and an increase in the absorbance to approximately 125% of that at the original Soret peak. This was then followed by a second step which, when completed, was characterized by a partial loss of the initial hyperchromicity to give an absorbance value approximately 110% of that at the original Soret peak and a further shift of the wavelength of maximum absorption to approximately 406 nm.

These effects were observed over 100-fold range of ferricytochrome *c* concentrations, but the concentrations of surfactant required to produce equal effects differed for each protein concentration. Also these effects were not related in a

[1] Abbreviations used are: cmc, critical micelle concentration; DAB, *p*-dimethylaminoazobenzene; *r*, average number of moles of sodium dodecyl sulfate bound per mole of cytochrome *c*; [A], concentration of the free sodium dodecyl sulfate in equilibrium with bound sodium dodecyl sulfate.

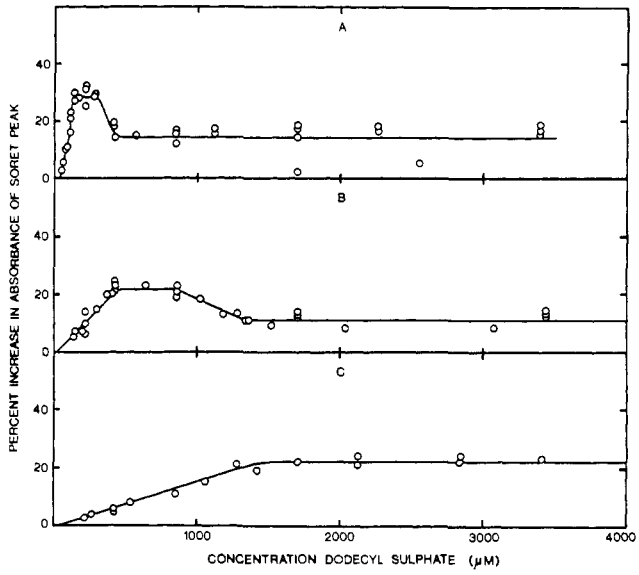

FIGURE 1: Per cent increase in absorbance of Soret peak as a function of concentration of sodium dodecyl sulfate. Concentration of cytochrome *c*: 8.5×10^{-7} M (A), 8.5×10^{-6} M (B), and 8.5×10^{-5} M (C). Data beyond 4000 μM dodecyl sulfate (B and C) were taken but are not plotted in this figure.

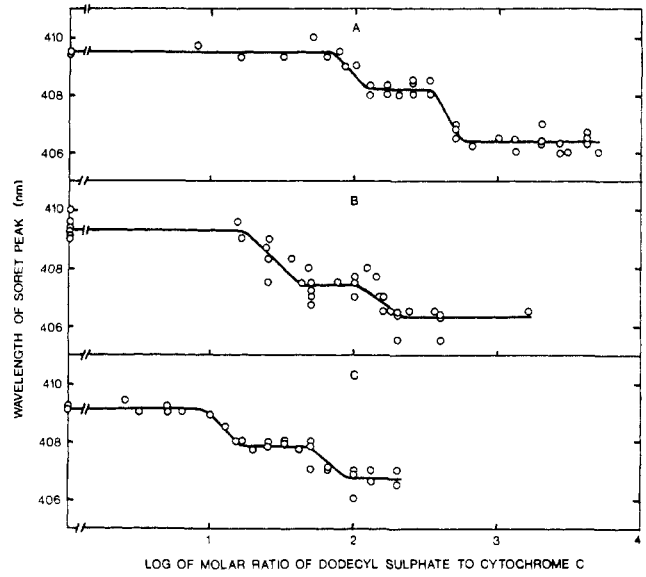

FIGURE 3: Wavelength of Soret peak as a function of the logarithm of the molar ratio of sodium dodecyl sulfate to cytochrome *c*. Concentration of cytochrome *c*: 8.5×10^{-7} M (A), 8.5×10^{-6} M (B), and 8.5×10^{-5} M (C).

simple manner to either the concentration of the surfactant (Figure 1) or the molar ratio of surfactant to protein (Figures 2 and 3). When cytochrome *c* was 8.5×10^{-7} M the highest absorbance occured in the region of 200–300 μM dodecyl sulfate. With 8.5×10^{-6} M protein this occured between 400 and 600 μM and with 8.5×10^{-5} M cytochrome *c* between 1300 and 4000 μM dodecyl sulfate. Expressed in terms of molar ratios of surfactant to protein, the highest absorbance occurred in the region of 200–300:1 for the most dilute solution of cytochrome *c*, at 50–80:1 for the intermediate solution, and at 15–50:1 when the cytochrome *c* was 8.5×10^{-5} M.

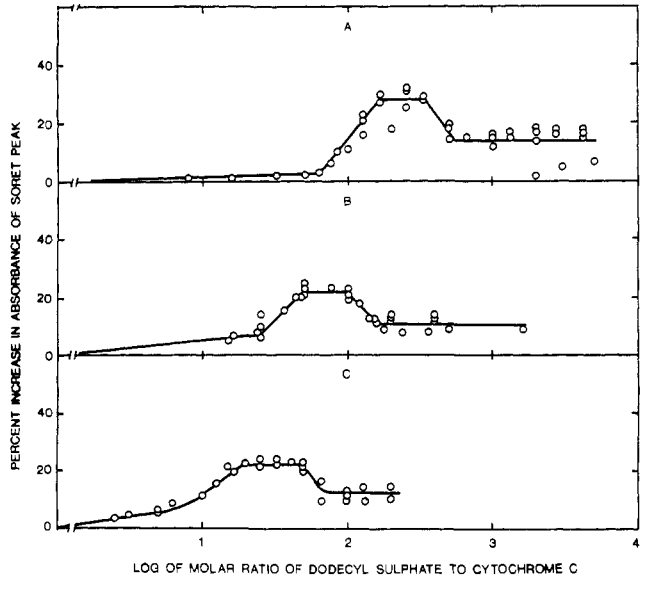

FIGURE 2: Per cent increase in absorbance of Soret peak as a function of the logarithm of the molar ratio of sodium dodecyl sulfate to cytochrome *c*. Concentration of cytochrome *c*: 8.5×10^{-7} M (A), 8.5×10^{-6} M (B), and 8.5×10^{-5} M (C).

Results from binding experiments involving 8.7×10^{-5} M cytochrome *c* are shown in Figure 4. In the top part of this figure (part A) the data are presented in the manner suggested by Scatchard (1949). The same data are presented in part B but in terms of the average number of moles of dodecyl sulfate bound per mole of protein (*r*) *vs.* the total amount of dodecyl sulfate present (free and bound dodecyl sulfate). The straight line is the calculated least-squares line relating these two quantities over the range studied and was used to calculate

TABLE 1: Calculated Extents of Interaction at Selected Molar Ratios of Sodium Dodecyl Sulfate to Cytochrome *c*.

Concn of Cytochrome *c* (M)	Molar Ratio of Sodium Dodecyl Sulfate to Cytochrome *c* and Its Logarithm		*r*[a]
8.75×10^{-6}	20	1.3	8.0 ± 2.1
	25	1.4	10.2 ± 1.9
	32	1.5	12.9 ± 1.6
	40	1.6	16.4 ± 1.5
	50	1.7	20.7 ± 1.6
	63	1.8	26.2 ± 2.1
8.75×10^{-5}	4	0.6	2.6 ± 0.3
	5	0.7	3.5 ± 0.3
	6	0.8	4.6 ± 0.3
	8	0.9	6.0 ± 0.2
	10	1.0	7.8 ± 0.2
	13	1.1	10.0 ± 0.2
	16	1.2	12.9 ± 0.2
	20	1.3	16.4 ± 0.2
	25	1.4	20.8 ± 0.3

[a] *r* is the average number of moles of sodium dodecyl sulfate bound per mole of cytochrome *c*. The deviations shown are standard deviations.

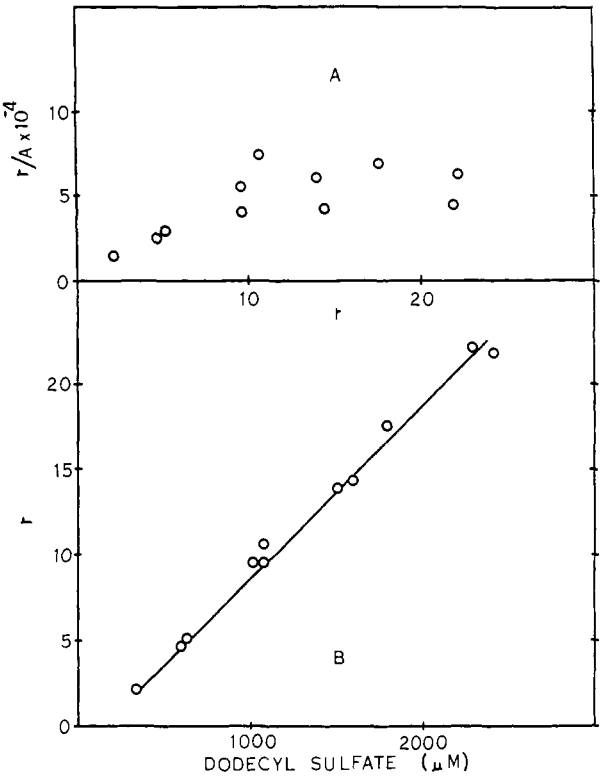

FIGURE 4: Quantitative binding data for the sodium dodecyl sulfate–cytochrome *c* interaction. *r* is the average number of moles of dodecyl sulfate bound per mole of protein, [A] is the concentration the free dodecyl sulfate in equilibrium with the bound dodecyl sulfate. In the top portion of this figure (part A) the data are plotted as *r*/[A] *vs. r* (Scatchard, 1949). In the bottom portion (part B) the data are plotted as *r vs.* total dodecyl sulfate (free and bound). The solid line is the calculated least squares line relating these quantities. Concentration of cytochrome *c* was 8.75×10^{-5} M.

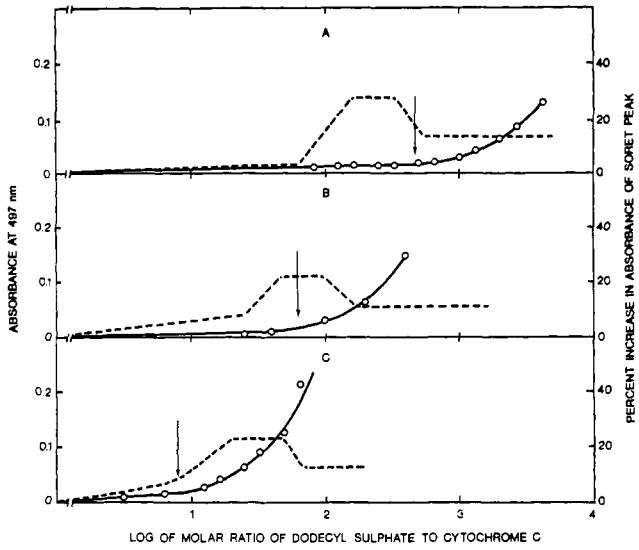

FIGURE 5: Solubilization of *p*-dimethylaminoazobenzene (as determined from absorbance af 497 nm) and per cent increase in absorbance of Soret peak of cytochrome *c* as functions of the logarithm of the molar ratio of sodium dodecyl sulfate to cytochrome *c*. The solid lines represent the solubilization data; the dashed lines represent the Soret data. The latter were taken from Figure 3. The vertical arrows indicate where marked increases in the absorbance at 497 nm and hence solubilization were considered to begin. Concentration of cytochrome *c*: 8.5×10^{-7} M (A), 8.5×10^{-6} M (B), and 8.5×10^{-5} M (C).

values for *r* at selected molar ratios of dodecyl sulfate to cytochrome *c* (Table I).

Binding studies were also performed with 8.7×10^{-6} M cytochrome *c*. When the data obtained were plotted the results were similar in appearance to those for the more concentrated system and thus were treated in a similar manner. Attempts to obtain reliable data with 8.7×10^{-7} M cytochrome *c* were not successful due to the fact that in these very dilute solutions the differences between free and total dodecyl sulfate were very small and the relative errors correspondingly very large.

Discussion

One of the goals of this study was to correlate changes in the Soret region of the ferricytochrome *c* spectrum to the extent of interaction between this protein and sodium dodecyl sulfate. Since detergents can undergo micellization, it was first necessary to determine the role, if any, that micellization of dodecyl sulfate *per se* had in the observed spectral changes.

Solutions of an impure surfactant may exhibit turbidity at concentrations below the cmc and this turbidity will disappear as micellization occurs. If there were differences in turbidity between the solutions in the blank and sample spectrophotometer cells, these differences could affect the observed absorbances. We were able to show, however, that this factor was not responsible for our observed changes in absorbance. The spectral results obtained with an impure dodecyl sulfate (Matheson Coleman & Bell), which did give rise to varying degrees of turbidity below the cmc, and a more carefully purified dodecyl sulfate (Mann Research Laboratories), which did not exhibit turbidity below the cmc, were essentially the same. Even in cases where the impure surfactant preparation was used and turbidity did develop, centrifugation, which removed the turbidity, did not alter the sequence of absorbance changes.

The formation of dodecyl sulfate micelles, *per se*, was likewise ruled out as a cause of the observed spectral changes by experiments which related these changes to solubilization of DAB. For instance, solubilization of DAB begins at about 400 μM dodecyl sulfate when in the presence of 8.5×10^{-7} M cytochrome *c*. At this particular concentration of cytochrome *c* the appearance and loss of hyperchromicity are almost complete before solubilization of the azo dye begins. At the intermediate concentration of cytochrome *c* (8.5×10^{-6} M) solubilization of the dye begins as the initial hyperchromicity develops, and at the highest concentration of protein solubilization of DAB begins before the hyperchromic maximum occurs (Figure 5).

Thus, although micellization of dodecyl sulfate may occur in certain of these systems, the spectral effects observed are unrelated to whether this detergent is present in the monomeric or micellar form. This is consistent with the observations of numerous other investigators who have observed similar hyperchromic effects and shifts of maximum absorption in the Soret region of the ferricytochrome *c* spectrum when it was denatured by urea, organic solvents, and amides, none of which undergo micellization (Stellwagen, 1967; Myer, 1968; Herskovits *et al.*, 1970a–c; Kaminsky and Davison, 1969).

Quantitative studies of the dodecyl sulfate–cytochrome *c* interaction indicated that this is not a simple interaction and made it possible to calculate the amounts of dodecyl sulfate bound at selected molar ratios of dodecyl sulfate to cytochrome *c*.

The conclusion that this interaction is not a simple one is

supported by the argument that if it were (*i.e.*, if it involved a single set of binding sites with identical intrinsic binding constants) then a plot of $r/[A]$ *vs.* r would result in a straight line with a negative slope. Our plots, however, showed an initial increase in $r/[A]$ as r is increased (Figure 4A). Many years ago Karush observed a similar anomalous plot in his study of the binding of an azo dye to bovine serum albumin (Karush, 1952). At that time he pointed out that plots such as this would result if the binding of an ion increased the number of sites available for the binding of additional ions. It thus seems reasonable as a result of these binding studies to propose that the interaction of dodecyl sulfate with cytochrome *c* involves an unfolding of the protein and exposure of previously unexposed sites. This is consistent with information gained from many previous studies involving protein denaturation and protein–ion interactions (see Putnam, 1948, Foster, 1960, Steinhardt and Reynolds, 1969, and Tanford, 1968, 1970, for reviews).

Stellwagen had shown by both physical and chemical methods that concentrations of urea which cause a maximum loss of the 695-nm band of ferricytochrome *c* also cause unfolding of this protein (Stellwagen, 1968). We found that with 8.5×10^{-5} M cytochrome *c* the hyperchromic blue shift in the Soret region was accompanied by a concomitant loss of absorbance at 695 nm, and that increases in the molar ratio of dodecyl sulfate to cytochrome *c* beyond that which was necessary to produce maximum hyperchromicity in the Soret region did not cause further loss of absorbance at 695 nm. This observation lends support to the suggestion made above to account for the unusual binding data, namely, that the interaction of dodecyl sulfate with cytochrome *c* involves an unfolding of the protein, and further suggests that the protein may be completely unfolded in experimental systems which produce maximum hyperchromicity in the Soret region and concomitant shifting of the Soret peak to *ca.* 408 nm.

Table I lists the calculated amounts of dodecyl sulfate bound to cytochrome *c* at selected molar ratios. If these are compared to the spectral changes elicited by adding dodecyl sulfate to this protein (Figures 1–3, parts B and C) it appears that the initial increases in absorbances in the Soret region and concomitant shifts of the Soret maxima to *ca.* 408 nm are complete when approximately 20 moles of dodecyl sulfate is bound in the case of 8.5×10^{-6} M cytochrome *c* and when approximately 14 moles of dodecyl sulfate is bound in the case of 8.5×10^{-5} M cytochrome *c*.

Analysis of horse heart cytochrome *c* has shown that it contains 19 lysyl residues, 2 arginyl residues, and 3 histidyl residues, and that the N-terminal glycyl residue is acetylated (see Margoliash and Schejter, 1966 for review). If one assumes, as Margoliash and Schejter have, that at neutral pH all of the lysyl and arginyl residues are fully charged and that half of the histidyl residues are charged, then the polypeptide portion of the cytochrome *c* molecule would have 22.5 positive charges at neutral pH. Subtracting one of the three histidyl residues in this polypeptide due to its involvement in coordination to the iron moeity of cytochrome *c*, and again assuming that only one of the remaining histidyl residues is charged, cytochrome *c* would possess 22 cationic amino acid residues which might be available for interaction with the dodecyl sulfate anion. Dickerson *et al.* (1971) have indicated that all of these 22 residues are on the outside of the crystalline substance. It seems reasonable, therefore, to propose that the initial phase of the dodecyl sulfate–cytochrome *c* interaction (the hyperchromic blue shift) involves reaction of dodecyl sulfate anions with cationic sites on the surface of the protein. It is of interest to note that the number of these sites is sufficient to accommodate the number of dodecyl sulfate anions that must be bound to produce maximum hyperchromicity and the concomitant shifting of the Soret maximum to *ca.* 408 nm.

It has also been suggested that the interior of native cytochrome *c* is a region of packed hydrophobic chains (Dickerson *et al.*, 1968). We have argued above that the initial phase of the dodecyl sulfate–cytochrome *c* interaction involves an unfolding of the protein and thus it seems reasonable to propose that the number of hydrophobic side chains which might be available for interaction with the hydrophobic portion of dodecyl sulfate anions would increase during the first phase of this interaction. This then leads to the further proposal that the second phase of the dodecyl sulfate–cytochrome *c* interaction involves interaction between these hydrophobic groups. If this were true then one would expect this phase of the interaction to be adversely affected by lowering the ionic strength of the system. Experiments designed to check this point showed that indeed this was the case. When the ionic strength was lowered tenfold the first phase of the dodecyl sulfate–cytochrome *c* interaction was not affected noticeably but the second phase was with higher molar ratios of detergent to protein being required to produce equivalent loses of hyperchromicity and shifting of the wavelength of maximum absorption to *ca.* 406 nm. Binding studies were not performed with concentrations of dodecyl sulfate necessary to produce this second spectral change and thus we are not able to relate this change to the extent of binding.

Finally, extensive studies have been made of the interactions between anionic detergents and bovine serum albumin (see Putnam, 1948; Foster, 1960; Steinhardt and Reynolds, 1969; and Tanford, 1968, 1970, for reviews). In more than one case, it appears that as the result of an initial phase of the interaction, which involves the binding of a relatively low number of anions, a conformational change occurs, which allows binding of a much larger number of detergent anions. Our suggestion for the dodecyl sulfate–cytochrome *c* interaction is patterned after this.

Acknowledgments

We thank Drs. G. Dana Johnson, Delbert D. Mueller, and William E. Klopfenstein for allowing us to use their spectrophotometric and chromatographic apparatus.

References

Dickerson, R. E., Kopka, M. L., Weinzierl, J. E., Varnum, J. C., Eisenberg, D., and Margoliash, E. (1968), *in* Structure and Function of Cytochromes, Okunuki, K., Kamen, M. D., and Sekuzu, I., Ed., Baltimore, Md., University Park Press, p 225.

Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O. B., Samson, L., Cooper, A., and Margoliash, E. (1971), *J. Biol. Chem. 246*, 1511.

Foster, J. F. (1960), *in* The Plasma Proteins, Putnam, F. W., Ed., Vol. I, New York, N. Y., Academic Press, p 179.

Herskovits, T. T., Gadegbeku, B., and Jaillet, H. (1970a), *J. Biol. Chem. 245*, 2588.

Herskovits, T. T., Jaillet, H., and DeSena, A. T. (1970c), *J. Biol. Chem. 245*, 6511.

Herskovits, T. T., Jaillet, H., and Gadegbeku, B. (1970b), *J. Biol. Chem. 245*, 4544.

Kaminsky, L. S., and Davison, A. J. (1969), *Biochemistry 8*, 4631.
Karush, F. (1952), *J. Phys. Chem. 56*, 70.
Keilin, D., and Hartree, E. F. (1940), *Nature (London) 145*, 934.
Margoliash, E., and Schejter, A. (1966), *Advan. Protein Chem. 21*, 113.
Margoliash, E., and Walasek, O. F. (1967), *Methods Enzymol. 9*, 339.
Morton, R. K. (1955), *Methods Enzymol. 1*, 25.
Myer, Y. P. (1968), *Biochemistry 7*, 765.
Mysels, E. K., and Mysels, K. J. (1965), *J. Colloid Sci. 20*, 315.
Putnam, F. W. (1948), *Advan. Protein Chem. 4*, 79.
Ray, A., Reynolds, J. A., Polet, H., and Steinhardt, J. (1966), *Biochemistry 5*, 2606.
Rabinovitz, M., and Boyer, P. D. (1950), *J. Biol. Chem. 183*, 111.
Rosenberg, R. M., and Klotz, I. M. (1960), *in* Analytical Methods of Protein Chemistry, Alexander, P., and Block, R. J., Ed., Vol. II, New York, N. Y., Pergamon Press, p 133.
Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51*, 660.
Schejter, A., and George, P. (1964), *Biochemistry 3*, 1045.
Shapiro, A. L., Viñuela, E., and Maizel, J. V., Jr. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Steinhardt, J. and Reynolds, J. A. (1969), Multiple Equilibria in Proteins, New York, N. Y., Academic Press.
Stellwagen, E. (1967), *J. Biol. Chem. 242*, 602.
Stellwagen, E. (1968), *Biochemistry 7*, 2893.
Tanford, C. (1968), *Advan. Protein Chem. 23*, 121.
Tanford, C. (1970), *Advan. Protein Chem. 24*, 1.
Tsushima, K., and Miyajima, T. (1956), *J. Biochem. (Tokyo) 43*, 761.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Nuclear Magnetic Resonance Studies of Hemoglobins. VII. Tertiary Structure around Ligand Binding Site in Carbonmonoxyhemoglobin†

Ted R. Lindstrom, Ingrid B. E. Norén, Samuel Charache, Hermann Lehmann, and Chien Ho*

ABSTRACT: Proton nuclear magnetic resonance (250 MHz) (nmr) has been used to study the ring-current-shifted resonances that appear in the nmr spectra of adult and other variant human carbonmonoxyhemoglobins. Intensity measurements indicate that the three highest field resonances are methyl groups. The spectra of two β-chain mutants, Hb Sydney (E11β67 Val→Ala) and Hb Zürich (E7β63 His→Arg), having single amino acid substitutions in the heme pocket, are used to show that two of these resonances can be assigned to the βE11 valine methyls. The spectra of isolated α and β chains indicate the α- and β-chain contribution to the tetrameric hemoglobin spectrum and clearly show that the tertiary structure of the heme pocket is not significantly affected by the tetrameric association of liganded chains. These nmr studies demonstrate that the heme environments of the α and β chains in carbonmonoxyhemoglobin A are not equivalent.

The presence of ring-current-shifted resonances in the proton nuclear magnetic resonance (nmr) spectra of proteins enhances the value of nmr in the study of protein structure. Ring-current-shifted resonances occur when a proton is positioned above or below the plane of an aromatic ring so as to be influenced by the diamagnetic anisotropy of the conjugated system. This effect shifts the resonance position of the proton to higher field and the magnitude of the shift is quite dependent on the geometrical relationship between the aromatic ring and the affected proton. The greatest shifts occur at very close distances near the center of the ring and decrease as the vertical distance and the distance from the center of the ring increase as described quantitatively for benzene by Johnson and Bovey (1958) and recently for porphyrins by Shulman *et al.* (1970). The utility of these effects is well established for protein spectra in which the ring-current-shifted resonances appear outside the normal spectral region, allowing direct observation of individual residues in the protein (McDonald and Phillips, 1967; McDonald *et al.*, 1969; Sternlicht and Wilson, 1967; Ho *et al.*, 1970).

The study of the resonances in hemoproteins is uniquely propitious because the porphyrin rings in hemoproteins have large ring-current fields and the affected resonance peaks are shifted to a greater extent than can be realized by the ring-current field of aromatic amino acids (Abraham, 1961;

† From the Department of Biophysics and Microbiology and the Department of Biochemistry, Faculty of Arts and Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania (T. R. L., I. B. E. N., and C. H.), the Hematology Division, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland (S. C.), and MRC Abnormal Hemoglobin Research Unit, Department of Biochemistry, University of Cambridge, Cambridge, England (H. L.). *Received November 18, 1971.* Supported in part by research grants from the National Science Foundation (GB-25053), the National Institutes of Health (HE-10383, HE-02799, and RR-00292), and the American Heart Association, Inc. This is publication No. 189 of the Department of Biophysics and Microbiology, University of Pittsburgh.

* To whom communications should be addressed: Department of Biophysics and Microbiology, University of Pittsburgh, Pittsburgh, Pa. 15213.