Biochem. J. (1973) **135**, 231–236
Printed in Great Britain

The Interaction between Ribonuclease A and Surfactants

By MALCOLM N. JONES,* HENRY A. SKINNER,† EDWARD TIPPING† and ALAN WILKINSON*
* *Department of Biological Chemistry and*
† *Department of Chemistry, University of Manchester, Manchester M13 9PL, U.K.*

(*Received* 6 *April* 1973)

1. U.v. difference spectra show that the anionic surfactant sodium *n*-dodecyl sulphate unfolds ribonuclease A at pH 7.3 and 10.3, but that the cationic surfactant *n*-dodecyltrimethylammonium bromide does not affect the conformation of the enzyme. 2. Equilibrium-dialysis experiments show that sodium *n*-dodecyl sulphate binds to ribonuclease A, but no binding of *n*-dodecyltrimethylammonium bromide could be detected at pH 7.3. 3. The enzymic activity of ribonuclease A is unaffected by *n*-dodecyltrimethylammonium bromide up to a concentration of 0.03 M at 25°C. 4. Ultracentrifuge studies support the conclusion that *n*-dodecyltrimethylammonium bromide does not interact significantly with ribonuclease A. 5. The enthalpy change as measured by microcalorimetry on binding of sodium *n*-dodecyl sulphate to ribonuclease A is consistent with an exothermic enthalpy of binding occurring simultaneously with an endothermic enthalpy of chain unfolding.

The interaction of surfactants with proteins has been the subject of numerous investigations over several decades and the topic has been reviewed by Steinhardt & Reynolds (1969). Protein–surfactant systems may serve as models for the study of the interactions between membrane proteins and lipids; additionally, the widespread use of the anionic surfactant sodium *n*-dodecyl sulphate for the solubilization of membrane proteins and in polyacrylamide-gel electrophoresis has stimulated interest in the nature of the interaction between this surfactant and globular proteins. It has been shown by Reynolds & Tanford (1970*a*,*b*) that sodium *n*-dodecyl sulphate binds to a range of reduced proteins to form complexes which probably have a rod-like conformation. The binding forces are not fully understood, but it is clear that both ionic interactions and hydrophobic bonding are involved.

In the present paper we report a comparative study of the interaction between pancreatic RNAase A‡ [ribonucleate pyrimidine nucleotido-2′-transferase (cyclizing), EC 2.7.7.16] and the two surfactants sodium *n*-dodecyl sulphate and *n*-dodecyltrimethylammonium bromide. Both surfactants contain the same hydrocarbon chain and their hydrophobic bonding to RNAase A might be expected to be similar. A variety of experimental techniques was applied in this investigation, including u.v. difference spectroscopy, ultracentrifugation, enzymic-activity measurements, equilibrium dialysis and microcalorimetry. It was found that whereas *n*-dodecyltrimethylammonium bromide does not interact significantly with ribonuclease A, sodium *n*-dodecyl sulphate binds to the protein and induces concomitant conformational changes.

‡ Abbreviation: RNAase A; ribonuclease A.

Experimental

Materials

Sodium n-dodecyl sulphate. This was prepared from *n*-dodecanol (*puriss* grade; Fluka A.G. Chemische Fabrik, Buchs, Switzerland) by the method of Dreger *et al.* (1944). The purity of the final product was confirmed by the absence of a minimum in a plot of surface tension versus concentration measured by using a Du Noüy tensiometer. The critical micelle concentration in water at 25°C was 0.0082 M in good agreement with literature values (Mukerjee & Mysels, 1971).

n-Dodecyltrimethylammonium bromide. This was prepared from *n*-dodecyl bromide (Fluka; *puriss* grade) and trimethylamine (BDH Chemicals Ltd., Poole, Dorset, U.K.) by refluxing in ethanol with a cold-finger condenser. Purification by repeated crystallization from an acetone–ethanol mixture containing 5% (v/v) ethanol gave a product that showed no minimum in the surface tension versus concentration curve and the critical micelle concentration in water at 25°C was 0.0148 M, in good agreement with literature values (Mukerjee & Mysels, 1971).

RNAase A. This was type 1A (5 × crystallized) obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., and was used as supplied.

Cytidine 2′:3′-cyclic monophosphate (sodium salt). This was obtained from Sigma (London) Chemical Co. Ltd.

Buffers. Three buffers were used in the investigation. Two Tris buffers (each 10mM-Tris) were adjusted to pH7.3 and pH10.3 by the addition of 0.1M-HCl and 3M-NH_3 respectively; the phosphate buffer (pH7.1) contained 1.17mM-Na_2HPO_4 and 1.48mM-NaH_2PO_4.

Water. Deionized water was distilled from alkaline $KMnO_4$ and then redistilled.

Methods

U.v. difference spectra. Measurements were made in the range 230–310nm with a Perkin–Elmer model 402 double-beam recording spectrophotometer with a times five expansion setting. The instrument reading was adjusted to zero with RNAase A solutions in both cuvettes and difference spectra were obtained by adding portions of surfactant solutions to one cuvette. The final RNAase A concentration was 3.5g/l.

RNAase activity. The enzymic activity of RNAase A in the absence and presence of surfactants was measured with cytidine 2′:3′-cyclic monophosphate as substrate by the method of Crook *et al.* (1960). A standard solution of the substrate at a concentration of 3.0mg/ml was made up in dimethylformamide containing just sufficient water for dissolution. The substrate solution (0.1ml) was added to the two spectrophotometer cuvettes containing 2.9ml and $(2.9-x)$ml of the appropriate buffer solution respectively. The reaction was started by the addition of $x$ml of an enzyme solution (0.30g/l) in the same buffer. The extinction was continuously recorded at 284nm with a Perkin–Elmer model 402 spectrophotometer.

Sedimentation analysis. Measurements of sedimentation velocity were made in 12mm standard cells and in synthetic boundary cells by using an AnD rotor in a Spinco model E analytical ultracentrifuge operating at 56100rev./min (229100g, $r_{av.}$ 6.51cm) at 20°C. Schlieren optics were used and photographs were taken at 8-min intervals.

Equilibrium dialysis. Glass bicompartment cells were made from two Quickfit F15 Pyrex flanges. Each compartment had a volume of approx. 5ml and the dialysis membranes were placed between thin Neoprene gaskets and clamped between the two compartments by means of brass frames. Samples of standard solutions of RNAase A and the surfactants in buffer were added to one compartment of the cell together with sufficient buffer to give a final volume of 5ml. Buffer (5ml) was added to the other compartment. The assembled cells were placed in a thermostat bath at 25°C and gently shaken. Equilibration times of 48h and 96h gave identical results for the sodium *n*-dodecyl sulphate systems and a period of 144h was allowed for equilibration in the *n*-dodecyltrimethylammonium bromide systems. In all experiments, the RNAase A concentration in the cells was 1g/l. The dialysis membranes were cut from Visking tubing. Those used for the sodium *n*-dodecyl sulphate experiments were washed with a sodium *n*-dodecyl sulphate solution at 60°C, and then with water; those for the *n*-dodecyltrimethylammonium bromide experiments were acetylated by soaking in an acetic anhydride solution (100ml/l) in pyridine for 12h at room temperature, washed with a cetyltrimethylammonium bromide solution and finally washed with water.

Analysis of the free surfactant concentrations at equilibrium in the cell compartment not containing the protein was carried out by removing samples and by using the Rosaniline hydrochloride method of Karush & Sonenberg (1950). Knowing the total amount of surfactant, the amount of free surfactant and the amount of protein in the dialysis cells the amount of surfactant bound to the protein can be calculated. For sodium *n*-dodecyl sulphate analysis the procedure was essentially the same as that described by Karush & Sonenberg (1950), modified to cover the concentration range of interest. For *n*-dodecyltrimethylammonium bromide analysis, samples of the *n*-dodecyltrimethylammonium bromide solutions were removed from the cells and added to a sodium *n*-dodecyl sulphate solution at a concentration in excess of the *n*-dodecyltrimethylammonium bromide concentration. The precipitate of the anionic–cationic surfactant complex was filtered off and the filtrate analysed for sodium *n*-dodecyl sulphate as described above. This 'back-titration' method worked very satisfactorily and gave good linear calibration plots. In the analyses the extinctions of the final chloroform–ethyl acetate extracts were measured on a Unicam SP.500 spectrophotometer at 540nm.

Microcalorimetry. A Beckman 190B microcalorimeter was used. The instrument, a twin-cell conduction calorimeter, has been described in detail elsewhere (Skinner, 1969), and was calibrated as in previous work (Jones *et al.*, 1971).

One of the annular cells was charged with 0.1500 (±0.0004)g of RNAase A solution (concentration 2.193±0.007mmol/kg of solution) in the drop well, and 15.0 (±0.01)g of surfactant solution of known concentration in the annular space. The reference twin cell was charged similarly with the RNAase A solution and with 15.0 (±0.01)g of buffer solution. The dilution of protein after mixing in both cells is the same, so that the enthalpy of dilution of RNAase A in both cells was presumed to cancel.

Results and Discussion

RNAase A–sodium n-dodecyl sulphate interaction

Fig. 1 shows the number of *n*-dodecyl sulphate ions bound per molecule of RNAase A ($\bar{v}$) as a function of the free sodium *n*-dodecyl sulphate concentration in solution, measured by equilibrium dialysis. The curve initially rises very steeply, indicating that there are

probably between 10 and 20 strongly binding sites. It is significant that at pH 7.3 the number of positively charged groups on the protein is approximately 18. Over the concentration range investigated binding increases without a limit and no specific complexes are formed, as have been reported for reduced proteins (Reynolds & Tanford, 1970*a*). At the higher values of $\bar{v}$ the total sodium *n*-dodecyl sulphate concentration in the solutions is in excess of the critical micelle concentration of the pure surfactant solution (0.006M at 25°C in this medium, as measured by us, by the conductance method), but the free sodium *n*-dodecyl sulphate concentration is below this concentration. This fact reflects the greater stability of the surfactant–protein complex compared with the ionic micelle. It should be noted that the equilibration times used in the experiments exceeded the time required for a pure micellar solution to equilibrate across the dialysis membranes, and hence the possibility that the high values of $\bar{v}$ are due to retention of micelles in the protein solution is eliminated (Abu-Hamdiyyah & Mysels, 1967). Blank experiments in which the protein was omitted also confirmed that equilibration had been achieved.

To clarify the presentation of the subsequent results the binding curve of Fig. 1 has been used to calculate $\bar{v}$ in solutions of known total sodium *n*-dodecyl sulphate concentration and RNAase A concentration. The material balance equation requires that:

$$\bar{v} = \frac{[\text{surfactant}]_{\text{total}} - [\text{surfactant}]_{\text{free}}}{[\text{RNAase A}]} \qquad (1)$$

Values of $\bar{v}$ in a given solution were chosen initially as reasonable estimates on the basis of Fig. 1. From eqn. (1) the corresponding values of $[\text{surfactant}]_{\text{free}}$ were calculated. This procedure was continued on a trial-and-error basis until the chosen values of $\bar{v}$ were self-consistent both with Fig. 1 and with eqn. (1).

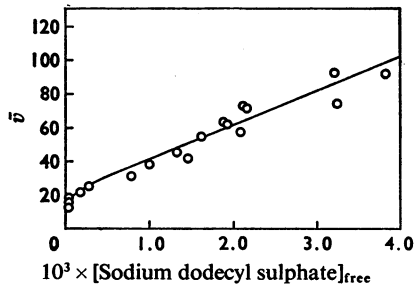

Fig. 1. *Binding of sodium n-dodecyl sulphate to RNAase A as a function of free surfactant concentration*

$\bar{v}$ = number of surfactant molecules bound/RNAase A molecule. All measurements were made in 10mM-Tris buffer, pH 7.3, at 25°C as described in the Experimental section.

It is implicit in the use of eqn. (1) that $\bar{v}$ is not a function of protein concentration. For the protein concentrations used here (1–3.5g/l) this assumption is reasonable (Cassel *et al.*, 1969).

The u.v. difference spectra of RNAase A in sodium *n*-dodecyl sulphate solutions show two peaks at approx. 243 and 287nm. The peak at 287nm has been attributed to a change in the environment of the three abnormal tyrosine residues when sodium *n*-dodecyl sulphate initiates chain unfolding (Bigelow & Sonenberg, 1962; Pittz & Bello, 1971). The peak at 243nm appears not to have been reported previously. It is most probably due to the environmental change of the phenylalanine residues on chain unfolding. Fig. 2 shows the molar difference extinction coefficient for the 287nm peak as a function of the number of sodium *n*-dodecyl sulphate molecules bound per protein molecule. Note that $\Delta\epsilon_{287}$ is negative because the surfactant plus protein solution has a smaller extinction than the protein solution. Below a value of $\bar{v}$ of approx. 16 the surfactant–protein complex was insoluble at the protein concentration used. The steep rise in $\Delta\epsilon_{287}$ between $\bar{v} \simeq 16$ and $\bar{v} \simeq 30$ can be attributed to chain unfolding. Above $\bar{v} = 30$, $\Delta\epsilon_{287}$ falls abruptly. Although the maximum in the curve is not large relative to the scatter of the points a similar maximum was observed when the experiment was done at pH 10.3 and we believe that it is a real effect. Very similar results were observed when the ionic strength of the solution was increased by the addition of 0.1M-NaCl. The maximum and the slight increase in $\Delta\epsilon_{287}$ with $\bar{v}$ probably reflect small changes in the environment of the tyrosine residues as more sodium *n*-dodecyl sulphate molecules are bound to the protein.

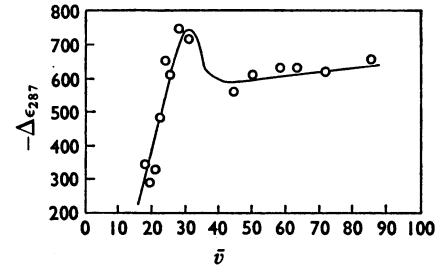

Fig. 2. *Molar difference extinction coefficients at 287nm for RNAase A plus sodium n-dodecyl sulphate as a function of the number of sodium n-dodecyl sulphate molecules bound per RNAase A molecule* ($\bar{v}$)

Both spectrophotometer cuvettes contained 3ml of a solution of 3.5g of RNAase A/l in 10mM-Tris buffer, pH 7.3. One cuvette also contained sodium *n*-dodecyl sulphate in the concentration range 4–15mM.

Fig. 3 shows the sedimentation coefficients at 20°C of mixtures of sodium *n*-dodecyl sulphate plus RNAase A as a function of the concentration of RNAase A. In these measurements the sodium *n*-dodecyl sulphate concentration was kept constant at 0.05M so that the ratio of surfactant to protein increases as the protein concentration decreases. In both standard cells and synthetic-boundary cells a single slightly skewed peak was observed on the schlieren photographs. The *s* values obtained by using synthetic-boundary cells decrease with decreasing RNAase A concentration and can be extrapolated to the *s* values of sodium *n*-dodecyl sulphate micelles. The *s* values obtained in standard cells are similar to the synthetic-boundary-cell measurements at 0.75mmol/kg of RNAase A but diverge at lower RNAase A concentrations. Diffusion of free sodium *n*-dodecyl sulphate across the solution-buffer boundary in the synthetic-boundary experiments could be responsible for this divergence at low RNAase A concentrations. The *s* values for the sodium *n*-dodecyl sulphate + RNAase A solutions are considerably lower than those of RNAase A itself and clearly illustrate complex-formation.

Fig. 4 shows the enthalpy of interaction between RNAase A and sodium *n*-dodecyl sulphate at 25°C as a function of $\bar{v}$. The enthalpy of interaction is exothermic and the shape of the curve can be explained in terms of binding and chain unfolding as illustrated in the inset. The sequence of thermograms (Fig. 5) strongly suggests that the interaction of sodium *n*-dodecyl sulphate with RNAase A involves two processes. At low values of $\bar{v}$ (Fig. 5*a*) a rapid exothermic binding process occurs. At intermediate values of $\bar{v}$ (Fig. 5*b*) the initial rapid exothermic binding process is followed by a slower endothermic process, which

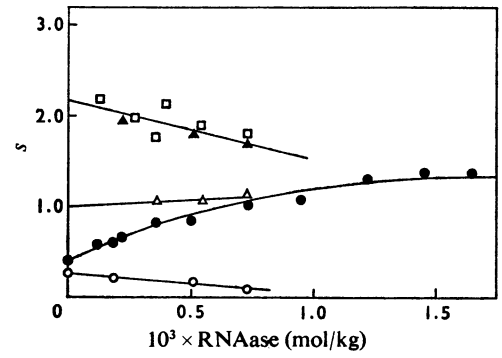

Fig. 3. *Sedimentation coefficients (s) for the systems RNAase A plus sodium n-dodecyl sulphate and RNAase A plus n-dodecyltrimethylammonium bromide at* 20°C

All the measurements were made in 10mM-Tris buffer, pH7.3. ▲, RNAase A (standard cell); □, RNAase A + 0.05M-*n*-dodecyltrimethylammonium bromide (standard cell); ●, RNAase A + 0.05M-sodium *n*-dodecyl sulphate (synthetic-boundary cell); △, RNAase A + 0.05M-sodium *n*-dodecyl sulphate (standard cell); ○, RNAase A + 0.05M-*n*-dodecyltrimethylammonium bromide (synthetic-boundary cell) micelle peak.

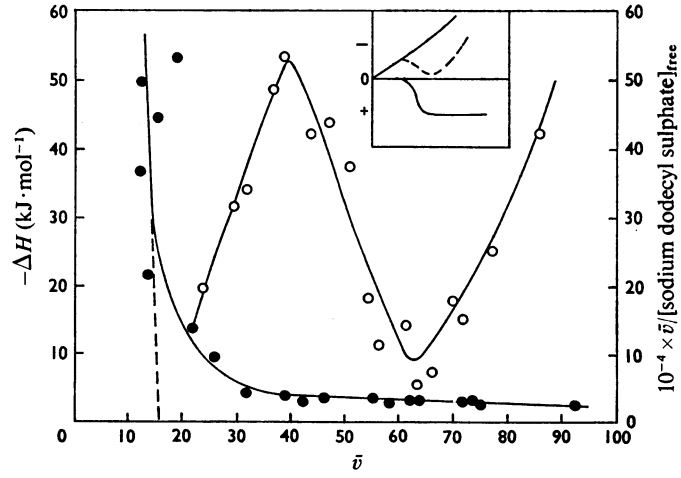

Fig. 4. *Enthalpy of binding of sodium n-dodecyl sulphate to RNAase A as a function of the number of sodium n-dodecyl sulphate molecules bound per RNAase A molecule* ($\bar{v}$), *and Scatchard plot for binding*

Left-hand axis, ○, enthalpy of binding measured in phosphate buffer, pH 7.1 at 25°C. Right-hand axis, ●, Scatchard plot of binding data of Fig. 1. The inset diagram is a qualitative resolution of the enthalpy of binding into two components, where the negative sign indicates binding and the positive sign, unfolding.

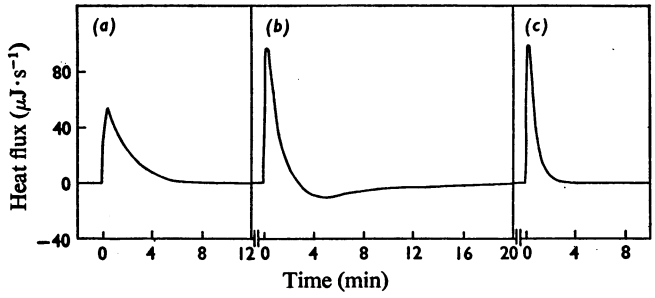

Fig. 5. *Thermograms for the system of sodium n-dodecyl sulphate–RNAase A interaction*

(*a*) Heat flux obtained with a Beckman 190B microcalorimeter on mixing 0.1503g of RNAase A solution (2.186mmol/kg of solution) with 15.00g of sodium *n*-dodecyl sulphate solution (1.03mmol/kg of solution); on mixing $\bar{v} = 29.4$ and the enthalpy change, $\Delta H = -39.5\,kJ \cdot mol^{-1}$ of RNAase A. (*b*) Heat flux obtained on mixing 0.1497g of RNAase A solution (2.193mmol/kg of solution) with 15.00g of sodium *n*-dodecyl sulphate solution (3.43mmol/kg of solution); $\bar{v} = 63.0$, $\Delta H = -6.9\,kJ \cdot mol^{-1}$ of RNAase A. (*c*) Heat flux on mixing 0.1499g of RNAase A solution (2.193mmol/kg of solution) with 15.00g of sodium *n*-dodecyl sulphate (5.05mmol/kg of solution); $\bar{v} = 85.5$, $\Delta H = -52.7\,kJ \cdot mol^{-1}$ of RNAase A.

might be attributed to chain unfolding. At high values of $\bar{v}$ both binding and unfolding are possibly occurring more rapidly so that the endotherm becomes absorbed in the overall recorded exothermic process. It must be stressed that the thermograms do not directly reflect the rate of the process occurring because of the finite time-constant of the calorimeter. But a thermogram of the type shown in Fig. 5(*b*) can only arise where a second slower process is superimposed on the initial reaction.

Also shown on Fig. 4 are the binding data replotted according to the Scatchard equation (Scatchard *et al.*, 1957):

$$\frac{\bar{v}}{[\text{surfactant}]_{\text{free}}} = K(n - \bar{v}) \qquad (2)$$

In eqn. (2) K is a constant and n the number of binding sites. This equation applies to identical independent binding sites but it is easily shown that for non-identical interacting sites the intercept on the $\bar{v}$ axis is equal to n. At low values of $\bar{v}$ the Scatchard plot can be extrapolated to a value of n of approx. 15. At high values of $\bar{v}$ the curve is almost parallel to the abscissa, which implies that n is very large. The results indicate that chain unfolding probably continues until about 60 sodium *n*-dodecyl sulphate molecules are bound per molecule of enzyme, after which the unfolded chain binds more surfactant molecules until saturated. The point of saturation cannot be determined from these data.

RNAase A–n-dodecyltrimethylammonium bromide interaction

No binding of *n*-dodecyltrimethylammonium bromide to RNAase A could be detected at pH7.3 and no u.v. difference spectra were obtained at pH7.3 or 10.3 under conditions comparable with those used in the sodium *n*-dodecyl sulphate plus RNAase A experiments. The enthalpy of interaction between *n*-dodecyltrimethylammonium bromide and RNAase A was athermal within experimental error at 25°C. The *s* values of RNAase A measured in standard cells are unaffected by *n*-dodecyltrimethylammonium bromide at a concentration of 0.05M (Fig. 3). When synthetic-boundary cells were used two peaks were distinguishable on the schlieren photographs, one attributable to *n*-dodecyltrimethylammonium bromide micelles and one corresponding to RNAase A. The *s* values of the *n*-dodecyltrimethylammonium bromide-micelle peak were slightly decreased on increasing the RNAase A concentration (Fig. 3).

We deduce from these observations that there is no significant interaction between *n*-dodecyltrimethylammonium bromide and RNAase A. To investigate this system further the enzymic activity of RNAase A was measured with cytidine 2′:3′-cyclic monophosphate as substrate (Crook *et al.*, 1960). Fig. 6 shows the initial rate of change of extinction as a function of RNAase A concentration for a constant substrate concentration. The reaction is first-order in RNAase A and substrate concentration. The initial rate of reaction is unchanged within experimental error when the solution contains 0.03M-*n*-dodecyltrimethylammonium bromide. In contrast similar experiments showed that RNAase A was completely deactivated by 0.03M-sodium *n*-dodecyl sulphate. Deactivation was complete at 0.003M-sodium *n*-dodecyl sulphate when the RNAase A concentration was 1.5μM-RNAase A. These kinetic experiments firmly support the conclusion that *n*-dodecyltrimethylammonium

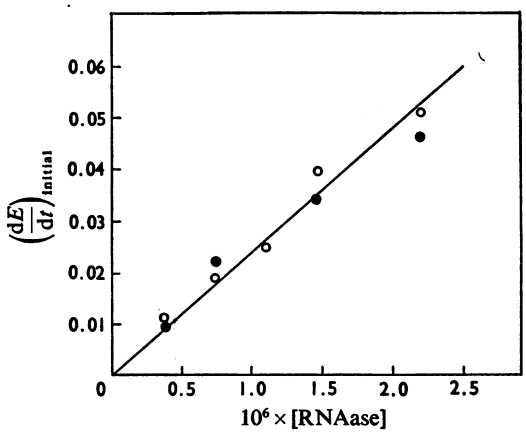

Fig. 6. *Initial rate of change of extinction for the reaction between RNAase A and cytidine 2′:3′-cyclic monophosphate at 25°C*

○, 10mM-Tris buffer, pH7.3; ●, 10mM-Tris buffer, pH7.3, 0.03mol of *n*-dodecyltrimethylammonium bromide. In all experiments the cytidine 2′:3′-cyclic monophosphate concentration was 0.1mg/ml.

bromide does not interact with RNAase A under the conditions reported here.

The difference between the action of sodium *n*-dodecyl sulphate and *n*-dodecyltrimethylammonium bromide on RNAase A cannot be explained in terms of the sign of the charge on the surfactants, relative to the net charge of RNAase A. The isoelectric point of RNAase A is 9.7. The u.v. difference spectra for sodium *n*-dodecyl sulphate plus RNAase A are very similar at pH7.3 and pH10.3; difference spectra are not observed at either pH value for *n*-dodecyltrimethylammonium bromide plus RNAase A. The surfactant chain length is the same for both surfactants, so that hydrophobic interactions should be similar. It can be concluded that the chemistry of the surfactant head group must be a determining factor in surfactant–protein interactions of the type discussed here. It is unlikely that strong ionic links between the trimethylammonium head group and negative sites on the protein are formed, because of the shielding effect and the steric hindrance of the methyl groups. In sodium *n*-dodecyl sulphate the negative charge is spread over the exposed sulphate oxygen atoms and much stronger ionic links can be envisaged. Further, it appears from the present work that the formation of ionic links between the surfactant and the protein are necessary for interaction to occur at all. In the native state the more hydrophilic amino acid residues are located on the surface of the protein molecules. The exposure of the internal hydrophobic amino acid residues is not possible without chain unfolding. The latter will be induced provided that prior and relatively strong ionic interactions take place with exposed specific sites on the surface of the protein.

We thank the University of Manchester Colloid Fund for a grant to E. T. and Miss Linda Shawcross for experimental assistance.

References

Abu-Hamdiyyah, M. & Mysels, K. J. (1967) *J. Phys. Chem.* **71**, 418–426

Bigelow, C. C. & Sonenberg, M. (1962) *Biochemistry* **1**, 197–204

Cassel, J., Gallagher, J., Reynolds, J. & Steinhardt, J. (1969) *Biochemistry* **8**, 1706–1713

Crook, E. M., Mathias, A. P. & Rabin, B. R. (1960) *Biochem. J.* **74**, 234–238

Dreger, E. E., Keim, G. I., Miles, G. A. & Shedlovsky (1944) *J. Ind. Eng. Chem.* **36**, 610–617

Jones, M. N., Agg, G. & Pilcher, G. (1971) *J. Chem. Thermodyn.* **3**, 801–809

Karush, F. & Sonenberg, M. (1950) *Anal. Chem.* **22**, 175–177

Mukerjee, P. & Mysels, K. J. (1971) *Nat. Stand. Ref. Data Ser. Nat. Bur. Stand. (U.S.)* **36**, 51

Pittz, E. P. & Bello, J. (1971) *Arch. Biochem. Biophys.* **147**, 284–298

Reynolds, J. A. & Tanford, C. (1970*a*) *Proc. Nat. Acad. Sci.* U.S. **66**, 1002–1007

Reynolds, J. A. & Tanford, C. (1970*b*) *J. Biol. Chem.* **245**, 5161–5165

Scatchard, G., Coleman, J. S. & Shen, A. L. (1957) *J. Amer. Chem. Soc.* **79**, 12–20

Skinner, H. A. (1969) in *Biochemical Microcalorimetry* (Brown, H. D., ed.), p. 11, Academic Press, New York

Steinhardt, J. & Reynolds, J. A. (1969) *Multiple Equilibria in Proteins*, Academic Press, New York