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Surfactant-selective electrode study of bile salt–protein binding

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Summary

An electrode selective to bile salt anions, has been evaluated for use in bile salt solutions and solutions containing bile salt anions and a protein. The electrode potential values obtained are a direct function of the activities of the corresponding bile salt anions. The electrode potential follows Nernstian behavior in the premicellar region of bile salt solutions. A minimum is observed in the Nernst plot corresponding to the critical micelle concentration.

Results obtained using these electrodes in solutions containing protein and bile salt have been used to construct Scatchard plots for the bile anion–protein binding. The treatment of the data with non-linear least-squares fit gave estimates of the binding constants and the number of binding sites which are comparable to previous results obtained from equilibrium dialysis measurements. Five bile salts studied bound in the following relative order: sodium deoxycholate ≃ sodium chenodeoxycholate > sodium ursodeoxycholate ≫ sodium cholate > sodium dehydrocholate. Binding studies were also carried out as a function of temperature which enabled the estimation of thermodynamic functions of binding. ΔH° of binding is positive suggesting that these biological detergents bind to hydrophobic sites on serum albumin.

Introduction

A series of papers published by Birch et al. (1972, 1973, 1974) reported the development of electrodes selective to surfactant ions based on both liquid and solid

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exchanger membranes. Recently Gilligan et al. (1977) and Kale et al. (1980) have described ion selective electrodes for conjugated bile salts and other detergents. Surfactant ion electrodes have an advantage over other methods of studying micellar solutions in that they provide a direct measure of the surfactant monomer activity alone. Above the critical micelle concentration (CMC), such measurements allow determination of the activity of the surfactant monomer in equilibrium with the micelle.

The highly reversible plasma protein binding is important for the transport and storage of endogenous substances, as well as for administered drugs. Various experimental techniques such as equilibrium dialysis, ultrafiltration, fluorescence spectroscopy (Veronese et al., 1977) have been employed to study drug protein interaction, and particularly to elucidate the extent of binding. The development of liquid membrane surfactant electrodes in principle makes possible an in situ study of the binding of surfactants to polymers and proteins. This paper examines the behavior of liquid membrane electrodes for bile salts in micellar solutions and solutions containing protein.

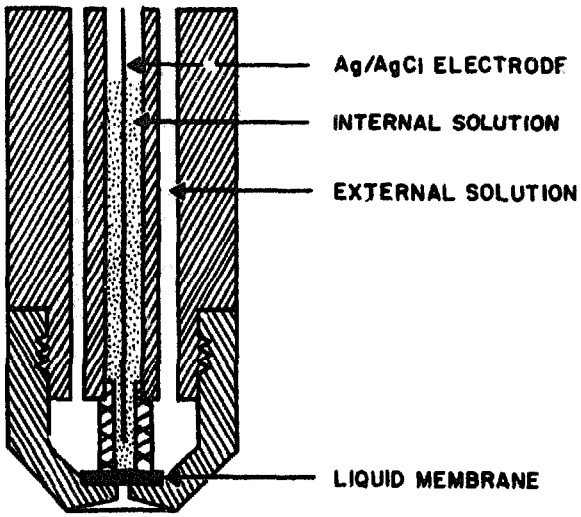
Experimental

Materials

The sources of bile salts and their purification are given elsewhere (Vadnere et al., 1980). Tributylamine and 1-octanol (Fisher Scientific), 1-bromohexadecane (Eastman Kodak) were used as obtained. Bovine serum albumin (BSA, fraction V, 99% Sigma Chemicals) was assumed to have a molecular weight of 69,000.

An electrode assembly is shown schematically in Fig. 1. The mechanical support utilizes an Orion ion-selective electrode body (92-00-00) and a porous membrane (92-20-04). The ion selective electrodes have 3 basic parts—the inner filling solution, the liquid membrane and the test solution. The inner filling solution consists of 10^{-2} M NaCl and 1×10^{-4} M of sodium salt of bile acid. A silver–silver chloride reference electrode is immersed in this solution. The outer filling solution or liquid membrane solution consists of hexadecyl tributylammonium ion exchanger in 1-octanol. The bromide salt of this ion was prepared from 1-bromohexadecane and tributylamine as described by Kale et al. (1980). Equimolar amounts of sodium bile salt and hexadecyltributylammonium bromide were then combined in 50 ml of water and extracted with 1-octanol. The organic layer was separated and used as the liquid membrane solution. The electrodes were soaked overnight in a solution identical with the inner filling solution before they were used. The reference electrode was a saturated calomel electrode.

Buffer solution (50 ml, 0.02 M Tris, pH = 9) and a small magnetic stirring bar were added to a jacketed beaker. The temperature was controlled within $\pm$ 0.1°C by circulating water from a thermostated waterbath (LAUDA RC 20, Brinkmann). The reference and surfactant electrodes were clamped in place so that the electrode tips were dipped into the aqueous solution to be studied. Care was taken to prevent trapping of small air bubbles against the membrane surface of the electrodes. The



	INTERNAL SOLUTION	EXTERNAL SOLUTION		
Ag/AgCl	0.01 M NaCl 1×10^{-4} M Na-BILE SALT AQUEOUS SOLUTION	$Bu_3(C_{16}H_{33})N^+$ BILE SALT OCTANOL SOLUTION	TEST SOLUTION	CALOMEL ELECTRODE

Fig. 1. Schematic representation of EMF cell.

solution was continuously stirred by a magnetic stirrer. After the electrodes had equilibrated, successive small aliquots of a bile salt stock solution in 0.02 M Tris were added with pipetman (Gilson, France). After each addition the electrode EMFs were measured with a Corning (Digital 110) pH meter. In dilute solution the time required to obtain a stable potential reading was approximately 3 min. At higher concentration ($> 10^{-3}$ M) stable readings were obtained almost immediately. The ion-selective electrode was dipped in a solution identical with the inner filling solution when it was not in use.

Protein binding experiments

In protein binding studies 25 ml of sodium bile salt solution in Tris buffer was placed in a jacketed beaker. The concentration of bile salt (1×10^{-3} M) solution was chosen in the concentration region where the electrode exhibited Nernstian behavior. A stock solution containing the same concentration of bile salt and 5% w/v of albumin was then added to the bile salt solution in small aliquots and EMFs were again measured. Since the total concentration of bile salt in the solution remains constant the increase in EMF is due to the binding of bile anions to protein which results in lowering of free bile salt concentration.

To calculate the thermodynamic functions $\Delta H°$, $\Delta S°$ for the binding process, binding studies were also carried out as a function of temperature.

Results and discussion

The EMF response of 5 bile salts, viz. sodium cholate (NaC), sodium deoxycholate (NaDC), sodium chenodeoxycholate (NaCDC), sodium ursodeoxycholate (NaUDC) and sodium dehydrocholate (NaDHC), was investigated as a function of bile salt concentration in 0.02 M Tris buffer at pH 9 using ion-selective electrodes. This is presented in Fig. 2. The electrode system exhibited near Nernstian response in dilute solutions giving slopes of 54, 58, 58, 53.5 and 58 mV/decade change in bile anion concentration for NaC, NaDC, NaCDC, NaUDC and NaDHC, respectively. As the concentration of bile salt increases a deviation is observed from the initial slope which suggests the reduced activity of bile anions as a result of self-association. Further increase in concentration results in a minimum which corresponds approximately to a critical micelle concentration. The cmc for various bile salts obtained from this study are compared with the literature values in Table 1. There is good agreement between the reported values obtained by other techniques and those from the present studies. If a phase separation model were exact for the aggregation process then one would expect the EMF to decrease according to Nernstian behavior until the CMC is reached and then remain constant. The fact that the EMF response

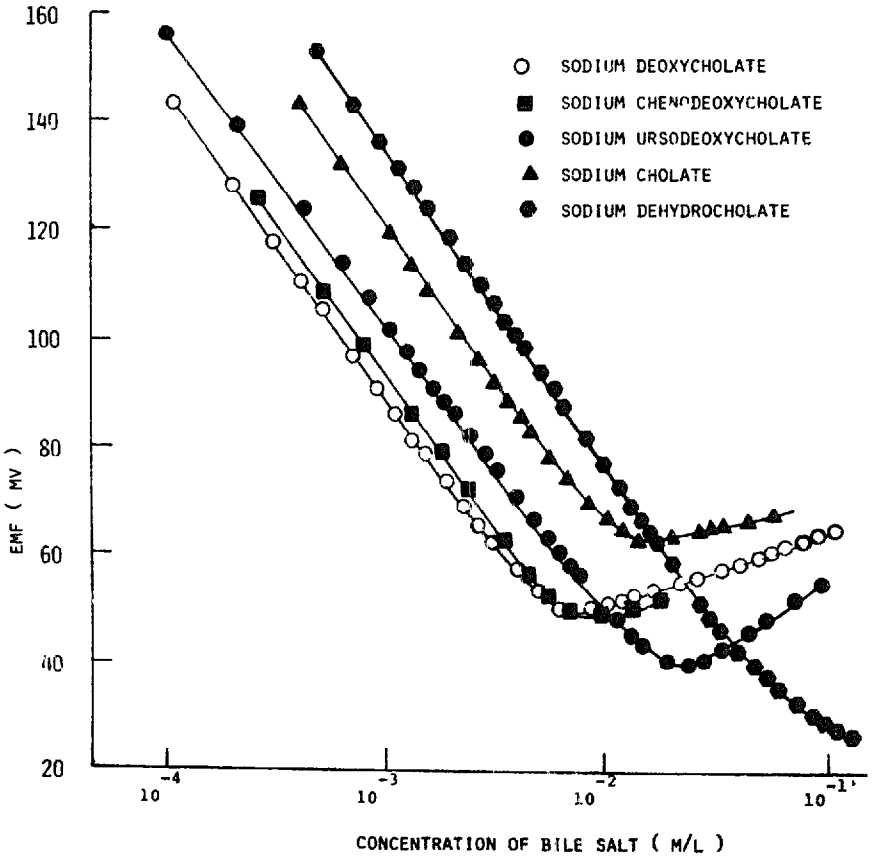


Fig. 2. EMF response obtained with bile anion electrodes vs concentration at 25°C.

deviates from near Nernstian behavior suggests that association of bile salts is continuous unlike classical detergents. A decrease in activity (or increase in EMF) after the CMC is in agreement with the results obtained by Gavach and Bertrand (1971). Similar results have been obtained for other surfactants, with an anion exchange membrane cell (Kaibara et al., 1970) and copper soap electrode (Malik et al., 1967).

Sodium dehydrocholate, a triketo bile salt exhibits no minimum in EMF–concentration relationship (Fig. 2) suggesting that it does not form micelles. This is in agreement with the observation made by Small (1968).

Gilligan et al. (1977) and Kale et al. (1980) observed a minimum followed by a maximum in the EMF vs concentration curve for sodium taurocholate and sodium taurodeoxycholate. In our present system we did not find a maximum. In their studies Gilligan et al. used *o*-dichlorobenzene, containing the water-insoluble hydrogen bonding solutes, 4-bromoacetanilide and hexachlorobenzene, as liquid membrane solvent. We used 1-octanol instead, as the liquid membrane solvent. 1-Octanol has dielectric constant ($\epsilon = 10.34$; b.p. = 195°C) comparable with that of *o*-dichlorobenzene ($\epsilon = 9.93$; b.p. = 180°C) but 1-octanol has an advantage over *o*-dichlorobenzene in that no additional hydrogen bonding solutes are needed to act as liquid membrane solvent. Our experiments on partitioning of bile salts between 1-octanol and water (Vadnere et al., 1982) suggested that 1-octanol would serve as a good extracting solvent for bile ion pairs. When we used exactly the same electrode system as employed by Gilligan et al. (1977) we also observed a minimum followed by a maximum for the above-mentioned unconjugated bile salts. However, the minimum was at slightly higher concentration than that obtained by the 1-octanol system. The reason for the maximum obtained with the *o*-dichlorobenzene as membrane solvent

TABLE I

CMC OF VARIOUS BILE SALTS

Bile salt	System	CMC (mmol/l)	Temp. (°C)	Method	Ref.
NaC	Water	13	20	Solubilization	Ekwall et al. (1957)
	Water	12	20	Solubilization	Norman (1960)
	0.1 N NaCl	20	20	Light scattering	Small (1971)
	Water (pH 9.0)	12	20	Surface tension	Small (1971)
	0.02 M Tris (pH 9.0)	16	25	ISE **	*
NaDC	Water	5.0	20	Solubilization	Ekwall et al. (1957)
	Water	5.0	–	Solubilization	Small (1971)
	–	5.0	20	Surface tension	Small (1971)
	0.02 M Tris (pH 9.0)	6.1	25	ISE **	*
NaCDC	Water	6.0	–	Solubilization	Norman (1960)
	0.02 M Tris (pH 9.0)	6.0	25	ISE **	*
NaUDC	0.02 M Tris (pH 9.0)	23	25	ISE **	*

* Results from this study.

** Ion selective electrode.

is not clear. We noticed a precipitation of crystalline needles (probably of hexachlorobenzene) from the liquid membrane solvent system employing hydrogen bonding modifiers when it was stored in a container at room temperature. The different behavior exhibited in the post-CMC region by the two different electrode systems suggests that the maximum in EMF–log CMC plot may be due to the electrode system itself and not caused by association of bile anions as suggested by Gilligan et al. (1977). Further studies are required to investigate this difference in the behavior of the two systems.

Protein binding

The binding of bile salts to protein molecules is an important factor which may influence the serum concentration and renal clearance of the bile acids, as well as their relationship to other serum constituents such as the lipids and bilirubin (Rudman and Kendall, 1956). So it is necessary to evaluate the binding parameters for these compounds.

Binding studies were performed by keeping the concentration of bile salt fixed and increasing the concentration of protein in the solution. Results of such experiments are summarized in Fig. 3. Since the total bile salt concentration is fixed, the positive change in EMF represents the decrease in bile salt anion activity due to binding. The stronger the binding the larger the change in EMF. It is clear from the figure that NaDC binds stronger than NaUDC, and NaUDC binds much stronger than NaC to albumin. Binding of NaDHC with albumin is virtually negligible.

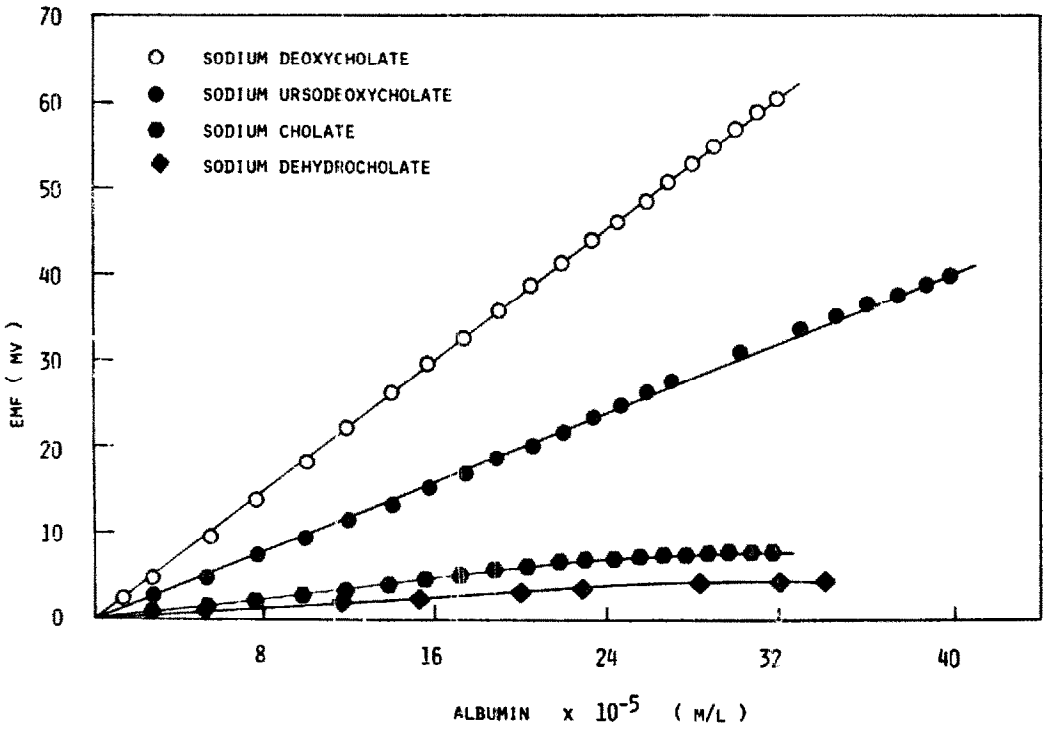


Fig. 3. EMF (mV) vs concentration of albumin at 25°C.

If the concentration of bile salt is fixed in the premicellar region where Nernstian behavior is exhibited, then the concentration of free bile anions can be determined from the relationship:

$$E = E° - N \cdot \log C_{free} \quad (1)$$

where C_{free} is concentration of unbound bile salt. E° and N are determined from the electrode behavior in the Nernstian region for pure bile salt solution. Although the electrode potential values obtained are a direct function of the activities of the corresponding ions, Eqn. 1 is expressed in terms of concentration instead of activity because values of ionic activity coefficients in solutions containing polymeric materials are not available. It will be assumed that deviations from ideality will be negligible over the concentration studied.

Scatchard plots used for studying the binding of NaCDC, NaDC and NaUDC to serum albumin are presented in Figs. 4 and 5. It is seen that these exhibit non-linear behavior. This suggests that there are more than one kind of binding site. The data were treated using non-linear least-square fits to the following equation, which

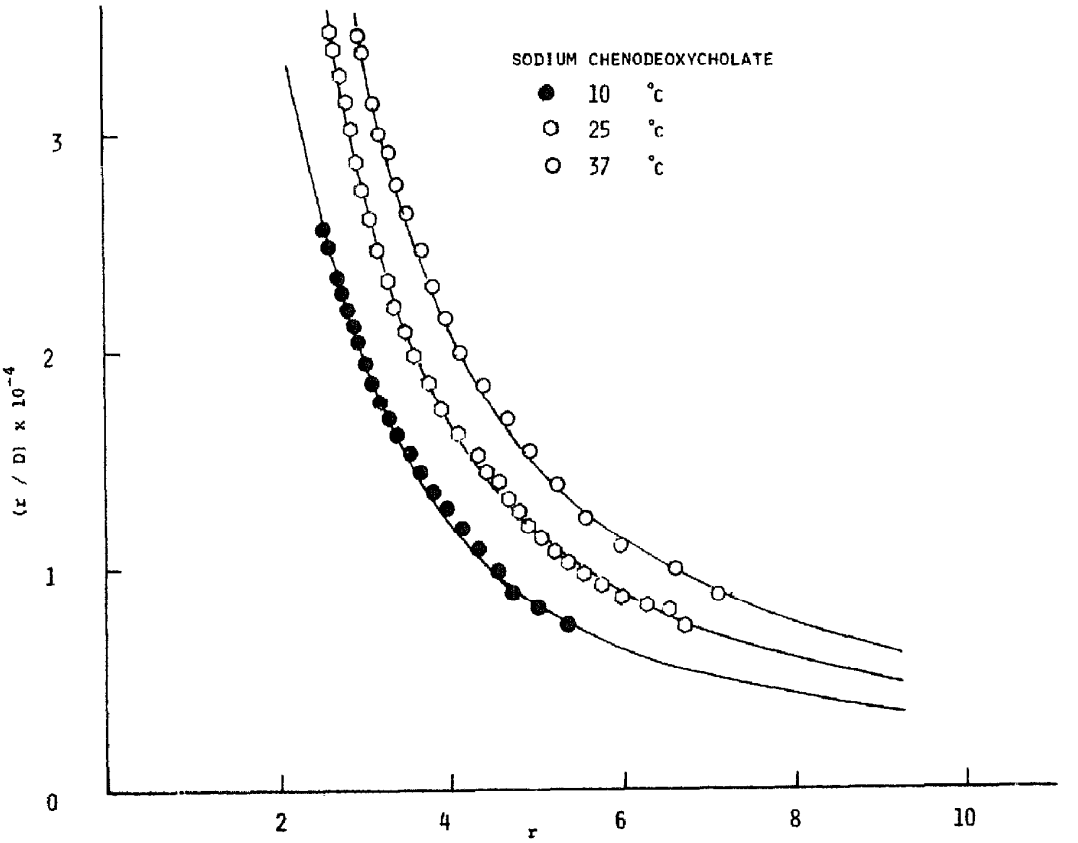


Fig. 4. Scatchard plots for the binding of NaCDC to 7.2×10^{-4} M BSA in 0.02 Tris buffer (pH 9.0) at various temperatures. (All points are experimental data while the solid lines show the values computed from the binding parameters in Table 2.)

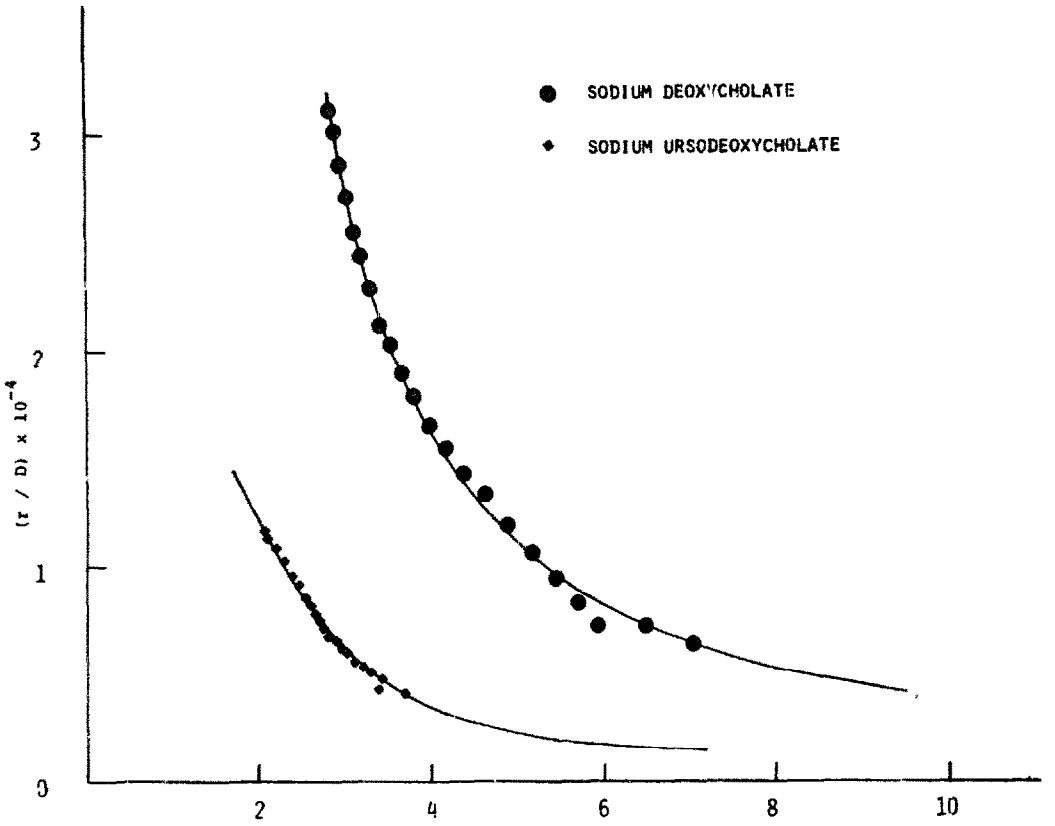


Fig. 5. Scatchard plots for the binding of NaDC and NaUDC to 7.2×10^{-4} M BSA in 0.02 M Tris buffer (pH 9.0) at 25°C. (All points are experimental data while solid lines show the values computed from the binding parameters in Table 2.)

assumes two types of binding sites.

$$r = \frac{n1 \cdot K1 \cdot [D]}{1 + K1 \cdot [D]} + \frac{n2 \cdot K2 \cdot [D]}{1 + K2 \cdot [D]}$$

where, r = conc. of bound bile salt/conc. of protein; [D] = conc. of unbound bile salt; n1 = number of primary binding sites; n2 = number of secondary binding sites; K1 = binding constant for primary binding sites; K2 = binding constant for secondary binding sites.

Binding parameters obtained by this treatment are presented in Table 2. Table 2 alsc compares the results obtained from this study with those obtained from an equilibrium dialysis study by Makino et al. (1973). The agreement between the two techniques is within expectations considering reasonable sample-to-sample variation in protein binding properties. Figs. 4 and 5 show excellent fit of observed data with the Scatchard plot constructed by using binding parameters from Table 2. The effect of temperature on binding is also seen in Fig. 4. With increasing temperature binding

TABLE 2

BILE SALT–BOVINE SERUM ALBUMIN BINDING PARAMETERS

Bile salt	Temp. (°C)	n_1	$K_1 \times 10^{-4}$ $(M/l)^{-1}$	n_2	K_2 $(M/l)^{-1}$
NaDC	25	3.19	3.35	14.0	361
	25	4.0	6.80	14.0	600 *
NaCDC	10	3.19	2.16	14.0	280
	25	3.18	3.02	14.0	396
	37	3.19	3.47	14.0	510
NaUDC	25	3.23	0.8	20.0	36

* From Makino et al. (1973).

constants increase. If $\Delta H°$ of binding is assumed to be constant, then the binding constant as a function of temperature should exhibit a van't Hoff-type relationship. Fig. 6 shows that such a relationship indeed exists enabling the calculation of the thermodynamic parameters $\Delta H°$ and $\Delta S°$ of binding. Such calculations gave the following values; $\Delta H_1^\circ = 3.5$ kcal/mol of bile salt, $\Delta S_1^\circ = 32.0$ entropy units, $\Delta H_2^\circ = 3.8$ kcal/mol of bile salt and $\Delta S_2^\circ = 24.6$ entropy units for NaCDC. Here the

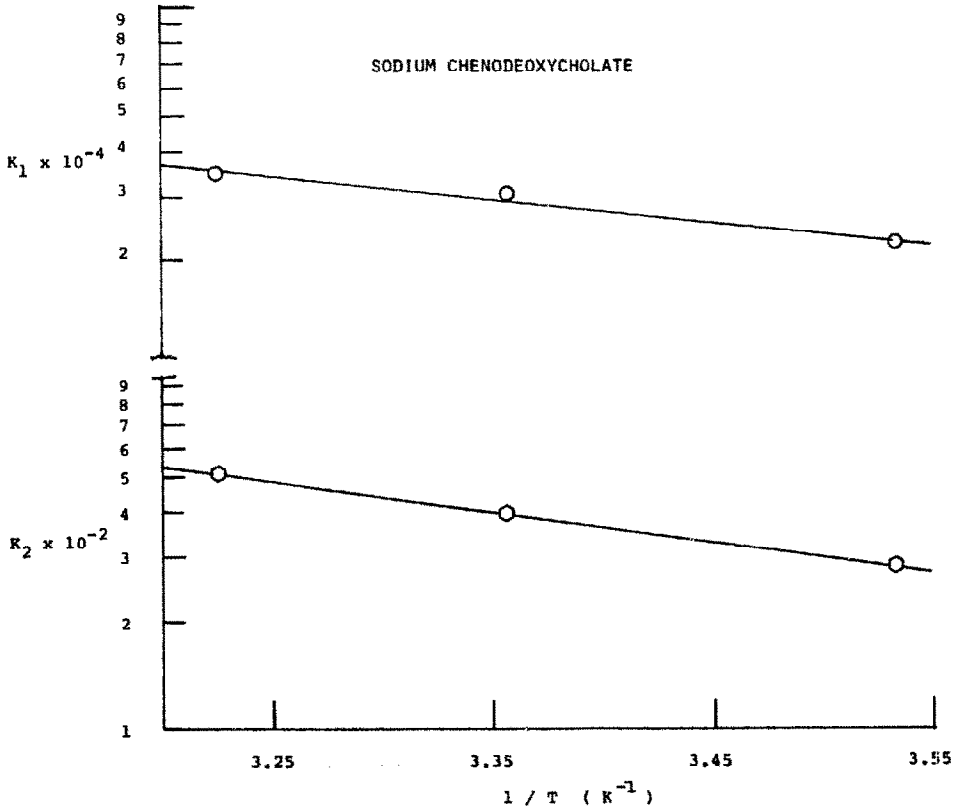


Fig. 6. van't Hoff-type plot for binding constants.

subscripts 1 and 2 refer to primary and secondary binding, respectively. The positive values of $\Delta H°$ and the large positive $\Delta S°$ values suggest that these compounds bind to hydrophobic sites on serum albumin.

In aqueous solutions, the water molecules in the vicinity of bile salt molecules are more strongly hydrogen bonded because of their non-polar nature, so that a region of higher local order exists than in pure water. When protein molecules are added to bile salt solution, bile salt molecules bind on the hydrophobic site of protein by hydrophobic interaction and the normal hydrogen bonded structure resumes accompanied by a decrease in the amount of hydrogen bonding and a less ordered state. These processes are accompanied by an increase in enthalpy (the energy required to break hydrogen bonds) and an increase in entropy as expected for a loss in order.

The chemical structure of the bile acid was found to influence the extent to which it is bound to bovine serum albumin. The extent of binding decreases as the number of hydroxyl groups on the ring system is increased. Although the position of the hydroxyl groups in the ring system has no pronounced influence on binding (e.g., NaDC and NaCDC) the stereochemical orientation of the hydroxyl group has significant effect on the binding (NaCDC and NaUDC). Replacement of a hydroxyl group by a keto group as in NaDHC suppresses the affinity for albumin. Similar observations were made by Rudman and Kendall (1956) for the binding of bile salts with human serum albumin.

In protein binding studies one is always concerned with the accompanying effects such as denaturation and conformational changes in the protein molecule. For surfactants such as dodecyl and tetradecyl sulfates large changes in viscosity and optical rotation accompany the cooperative binding, indicative of drastic conformational change (Reynolds et al., 1967; Polet and Steinhardt, 1968). A similar result is obtained for the binding of alkyl benzenesulfonates to bovine serum albumin (Decker and Foster, 1966). In contrast, the results of Makino et al. (1973) and Helenius and Simon (1972) show unambiguously that deoxycholate is not able to bind to protein in the cooperative mode characteristic of alkyl sulfate, sulfonates and benzene sulfonates. Makino et al. found no detectable change in optical rotation, for deoxycholate, which was measured from 225 to 300 nm, indicating that there is no conformational change. As a consequence of this observation, bile salts may be used to extract proteins from membranes without disruption of the native protein conformation or loss of biological activity, in contrast to common synthetic ionic detergents, which ordinarily solubilize lipid-associated protein in denatured and inactive form (Makino et al., 1973; Schwuger and Bartnik, 1980).

Binding studies were also carried out by keeping the concentration of protein fixed and increasing the concentration of bile salt. Results of such experiments for NaDC are shown in Fig. 7. It is seen from Fig. 7 that the change in EMF is much less than expected from the Nernstian slope because of the reduced activity due to binding. The presence of 5% albumin has increased the CMC from 6×10^{-3} M to 2×10^{-2} M.

Fig. 7 also shows the effect of urea on the association of NaDC. Mukerjee and Ray (1963) and Bruning and Holtzer (1961) have used dissolved urea as a probe for investigating the water structure contribution to micelle formation and hydrophobic

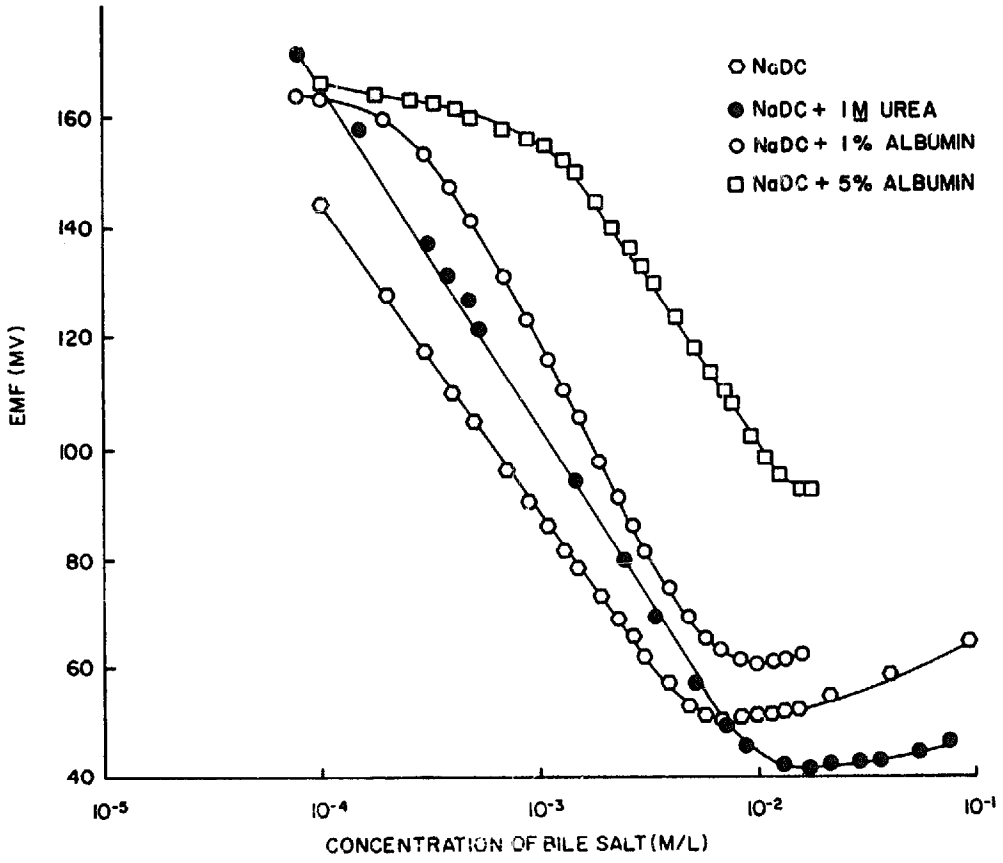


Fig. 7. Effect of 1%, 5% albumin, 1 M urea on the association of NaDC in 0.02 M Tris, pH 9 at 25°C.

bonding. In order to explain the observed anomalously low viscosities of urea solutions, Rupley (1964) suggested that urea disrupts the water structure. At high concentration urea modifies the 'iceberg' structure around solute molecules as has been inferred from micellization and protein denaturation studies without unduly affecting interfacial effects (Wetlaufer et al., 1964; Kauzman, 1959). Thus, the increased CMC of NaDC from 6×10^{-3} M to 9×10^{-3} M in the presence of 1 M urea is in agreement with the structure breaking properties of urea. Similar results were obtained by Schick (1964) for alkyl sulfate association.

Acknowledgement

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