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Note

Adsorption of deoxycholate on Sephadex G-25

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The bile salt, sodium deoxycholate (NaDOC) has been used *in vitro* to solubilize bacterial and cell membrane proteins[1]. Deoxycholate has also been used to remove major lipids from human plasma low density lipoprotein[2]. One advantage DOC has over most detergents is that it will solubilize lipids from lipoproteins without destroying the immunological properties of the proteins[2].

Deoxycholate is used in the production of influenza virus subunit vaccines[3,4]. Once the lipid envelope of the virus has been disrupted, the DOC is removed by dialysis. Chromatography on a Sephadex G-25 column was one method investigated as a potential substitute for removal of DOC from the viral protein. It was found that DOC continued to elute even after two column volumes of buffer had been passed through the column, whether viral protein was present or not. This property of the DOC–Sephadex G-25 system should be considered when similar separations are contemplated.

Certain substances are strongly adsorbed to highly cross-linked Sephadex gels at ionic strengths above the level where ion exchange occurs. It is well known that substances having an aromatic or heterocyclic structure have an affinity for Sephadex gels[5] due to π-electron interaction and hydrogen bonding[6]. Hydrophobic adsorption of ionic complexes to Sephadex has been demonstrated[7].

It is well known that saturated amphiphilic solutes can adsorb to Sephadex LH-20 due to entropic "hydrophobic interaction"[8,9]. This paper describes a similar hydrophobic interaction between an unmodified Sephadex gel and an amphiphile containing a fully saturated hydrophobic region.

EXPERIMENTAL

All gel filtration experiments were carried out on a column containing Sephadex G-25 Fine (Pharmacia, Uppsala, Sweden). A Pharmacia K26/40 column was used with a gel bed 2.6 cm in diameter and 18.3 cm long (bed volume, $V_t = 97.2$ ml). The void volume, V_0, was determined with Blue Dextran 2000 and found to be 23.5 ml. A flow-rate of 6–7 ml/min was used throughout.

Sodium deoxycholate (A Grade) was obtained from Calbiochem (Sydney, Australia). The NaDOC solution was made up in 0.15 *M* sodium chloride and 5.0 m*M* phosphate at high pH. The pH was then adjusted to 8.2 with hydrochloric acid. Ten millilitres of each bile salt solution was applied to the column in each run.

The bile salt concentration was 2.3 m*M* except for two runs where higher NaDOC concentrations were used.

The DOC was eluted from the column in various eluants including: Dulbecco's Phosphate Buffered Saline, Ca–Mg free (PBS)[10] adjusted to pH 7.3, 8.0 or 10.0 with 1.0 *M* sodium hydroxide; distilled water adjusted similarly to pH 8.2; 6 *M* urea in PBS pH 8.2; and absolute ethanol. Column runs were performed in thermostatically controlled conditions at 4°, 40° and 60° or at room temperature (20–25°). The column was pre-equilibrated with the eluant before each run.

To determine the elution pattern of DOC, either fractions were collected and assayed by the method of Mosbach *et al.*[11], or the elution was monitored spectrophotometrically with a Varian Series 634 spectrophotometer equipped with a Hellma microflow cell (Cat. No. 186-QS) and a Varian Model 135A recorder. The absorbance due to deoxycholate was measured at 210 nm, an absorption maximum for DOC.

CALCULATIONS

Partition coefficients ($K_{av.}$) were calculated using[12] $K_{av.} = (V_e - V_0)/V_t$ where V_e is the elution volume of the solute, V_0 the void volume of the column and V_t the internal volume of the gel beads, where $V_t = V_t - V_0$. $K_{av.}$ values for the elutions in ethanol were not determined, because gel shrinkage occurred, and it was not possible to measure V_0 by conventional methods.

RESULTS AND DISCUSSION

Values of $K_{av.}$ for the various experiments with NaDOC on Sephadex G-25 are shown in Table I. $K_{av.}$ values greater than 1.0 indicate adsorption of the NaDOC to the Sephadex.

TABLE I

$K_{av.}$ VALUES FOR DEOXYCHOLATE ON SEPHADEX G-25

R.T. = Room temperature (20–25°). N.D. = Not determined.

DOC concentration of applied 10-ml sample (mM)	*Eluant*	*pH*	*Temperature (°C)*	$K_{av.}$	*No. of experiments*
2.3	PBS	8.2	4	1.8	2
2.3	PBS	8.2	37	1.9	3
2.3	PBS	8.2	40	2.0	2
2.3	PBS	8.2	60	2.0	2
2.3*	Distilled water	8.2	R.T.	1.4	2
2.3**	Distilled water	8.2	R.T.	0.8	3
2.3	6 *M* Urea in PBS	8.2	R.T.	1.3	2
2.3***	Ethanol	N.D.	R.T.	<1.0	2
2.3	PBS	7.3	R.T.	2.0	2
2.3	PBS	8.0	R.T.	1.8	3
2.3	PBS	10.0	R.T.	1.8	2
23	PBS	8.2	R.T.	1.1	2
115	PBS	8.2	R.T.	0.1	2

* Stock 115 m*M* deoxycholate solution diluted 1 in 50 in distilled water.
** NaDOC prepared in salt-free distilled water.
*** NaDOC prepared in ethanol.

The critical micelle concentration (c.m.c.) of DOC at 10° in physiological saline (pH not specified) is[13] 1.7 m*M*. At a concentration of 2.3 m*M* in PBS, NaDOC therefore exists predominantly as DOC monomers, however a small number of micelles of DOC may be present. As with other low-molecular-weight solutes, the 2.3 m*M* DOC solution becomes diluted to below 1.7 m*M* on passage through the gel bed. Therefore, most of the experiments described deal with the interaction of DOC monomers with the Sephadex gel.

Effect of temperature

Sodium deoxycholate was retained on Sephadex G-25 when the eluent used was PBS at pH 8.2, the temperature being held constant at 4°. When the temperature was increased to 40°, increased retention of DOC by the gel was observed.

Since $K_{av.}$ increases with temperature the adsorption is unlikely to be due to hydrogen bonding of the hydroxyls on the DOC molecules to the Sephadex G-25[8]. Van der Waals forces are also weakened by a rise in temperature. It therefore seems that hydrogen bonding and Van der Waals forces play a minor role in the interaction of DOC with Sephadex G-25.

Effect of ionic strength at constant pH

Sodium deoxycholate in PBS, when eluted with PBS, had a $K_{av.}$ value of 1.8. The $K_{av.}$ value was lower (1.4) when stock NaDOC solution was diluted 1 in 50 in distilled water prior to running on the column, and eluted with distilled water. When NaDOC was prepared in distilled water and the column was eluted with distilled water no adsorption to the gel occurred and DOC eluted with a symmetrical peak and a $K_{av.}$ value of 0.8.

In the presence of sodium chloride DOC interacted with Sephadex G-25. However, when NaDOC was dissolved in distilled water only, adsorption did not occur. The increased retention of DOC in the presence of sodium chloride indicates an increased preference for the DOC to leave the aqueous phase in favour of the environment of the gel, which may be compared to a salting-out effect. The increased retention is also consistent with stronger hydrophobic interaction of the DOC with the gel in the presence of electrolytes[9].

Effect of 6 M urea

With 6 *M* urea added to the NaDOC solution and the eluant the $K_{av.}$ value was 1.3. This reduced value of $K_{av.}$ relative to the $K_{av.}$ in PBS (1.8) suggests that a component of hydrogen bonding may be acting. The effect of urea however is more complex than a simple reduction of hydrogen bonding and probably involves changes in solvent structure and thus affects hydrophobic bonding, as well as the degree of hydration of the polar groups on the deoxycholate molecule[13]. It is thought that urea competes effectively with sodium chloride to re-establish hydration of the non-ionic hydroxyl groups[13]. The partial rehydration of the hydroxyls on the DOC molecule in the presence of urea is associated with a reduced interaction between the DOC and the Sephadex G-25 gel. The results indicate that the less hydrated the DOC molecule the greater the affinity the DOC has for the gel. The interaction between DOC and the gel may be through hydrophobic bonding between the cyclic hydrocarbon part of the DOC molecule and the $-CH_2-$ groups in the neighbourhood of the ether oxygen atoms of the cross links in the gel[8].

Effect of pH at constant ionic strength

At pH values of 8.0 and 10.0 the $K_{av.}$ value for DOC eluted with PBS was 1.8. When the pH was decreased to 7.3, thereby decreasing the proportion of deoxycholate anions in the solution, the $K_{av.}$ value increased to 2.0, indicating slightly stronger adsorption. The increased retention was probably due to a type of physical entrapment, as DOC at pH 7.3 has a strong tendency to form a gel[13].

Effect of solvent

In ethanol, DOC exists purely as monomers at all concentrations[13]. It was not possible to calculate the $K_{av.}$ value for DOC dissolved in ethanol and eluted with ethanol because V_0 was unknown; however, the DOC eluted at about half the column volume ($K_{av.} < 1.0$), so little or no adsorption occurred. This further suggests the hydrophobic nature of the adsorption of DOC to Sephadex G-25 in aqueous solution, because lyophobic interaction of hydrocarbons is much greater in aqueous solution than in any other solvent[14].

Effect of deoxycholate concentration

Sodium deoxycholate at concentrations much greater than the c.m.c., when eluted with PBS, produces an asymmetrical peak which trails to about two column volumes (Fig. 1). The volume at which DOC first appears depends upon the concentration of DOC applied to the column. When 23 m*M* DOC was applied to the column, the DOC peak still appeared late ($K_{av.} = 1.1$), however, when 115 m*M* DOC was applied, the DOC peak was at around the void volume ($K_{av.} = 0.1$); the results are shown in Table I. Sodium taurodeoxycholate micelles are completely excluded from Sephadex G-25 gels[15]. The above result with 115 m*M* DOC indicates that sodium deoxycholate micelles are also completely excluded from Sephadex G-25. Low levels of deoxycholate were detectable at up to two column volumes at both applied sample concentrations.

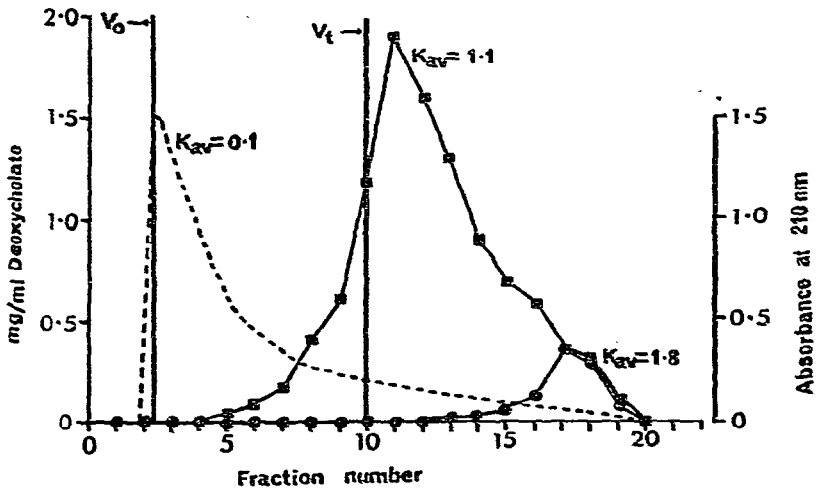

Fig. 1. Elution diagrams for 2.3 m*M* deoxycholate (●), 23 m*M* deoxycholate (■) and 115 m*M* deoxycholate (– – –) on Sephadex G-25 eluted with PBS. The elution of 115 m*M* deoxycholate was followed spectrophotometrically at a wavelength of 210 nm. The elution of 2.3 m*M* deoxycholate and 23 m*M* deoxycholate was followed by collecting 10 ml fractions and assaying for deoxycholate content by the method of Mosbach *et al.*[11].

Hydrophobic interaction is unlikely to occur between micelles and Sephadex G-25 as the hydrophobic regions of the DOC molecules are already in a non-aqueous environment. An equilibrium between micelles and monomers exists when the concentration of DOC is greater than the c.m.c. During column runs it is postulated that the deoxycholate monomers, but not micelles, adsorb onto the gel as well as partitioning into the V_i volume, so that micelles tend to move ahead of monomers and net dissociation of micelles is favoured. This occurs all the way down the column until any zone still containing micelles is eluted, followed successively by monomeric DOC and the gradual elution of adsorbed monomers. When 23 m*M* DOC was applied, all micelles had dissociated to monomers before the main peak of DOC eluted. When 115 m*M* DOC was applied the total DOC concentration was high enough to prevent breakdown of all micelles. Consequently micelles were eluted at around the void volume because they were excluded from the gel beads by size alone. These results are shown in Fig. 1.

In a further experiment, the column was pre-equilibrated with 2.3 m*M* DOC, and eluted with 2.3 m*M* DOC in PBS. When DOC was applied at 23 m*M* to the pre-equilibrated column two peaks were produced. The first peak appeared near the void volume and had a $K_{av.}$ value of 0.1. This peak was probably due to DOC micelles which were excluded from the gel. A second peak with a $K_{av.}$ value of 0.6 was also observed. The explanation for the presence of this second peak is unclear. Pre-equilibration and elution of the column with 2.3 m*M* DOC presumbly saturated the binding sites for DOC, masking the slow release of adsorbed DOC otherwise observed

CONCLUSION

The retention of DOC on Sephadex G-25 columns is probably due to hydrophobic interaction, because retention is reduced at lower temperatures, lower electrolyte concentrations, in the precence of 6 *M* urea, and in a non-aqueous solvent. The observed elution profile depends on the concentration of DOC applied to the column, and is the product of a combination of molecular sieving, the equilibrium between deoxycholate monomers and micelles, and adsorption of monomers to the Sephadex.

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