Biochemical Pharmacology, Vol. 23, pp. 3139–3145. Pergamon Press, 1974. Printed in Great Britain.

DISPLACEMENT OF SULFAETHIDOLE FROM BOVINE SERUM ALBUMIN BY SOME ALKYLDIMETHYLBENZYLAMMONIUM CHLORIDES

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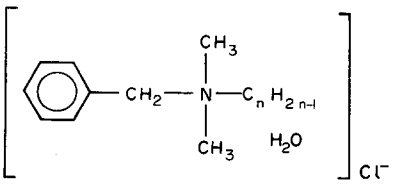
(*Received* 26 *February* 1974; *accepted* 29 *March* 1974)

Abstract—Alkyldimethylbenzylammonium chlorides have been shown to displace sulfaethidole, a strongly bound sulfonamide, from bovine serum albumin, using measurements of the optical activity induced into the drug after the binding reaction. The higher homologues modify the circular dichroism spectrum of the albumin in the region associated with tryptophan and tyrosine residues. Measurements at lower wavelengths suggest that the higher homologues may cause small conformational changes in the protein.

IN PREVIOUS investigations, circular dichroism (CD) has been used to determine the binding parameters after the interaction of sulfaethidole, a long-acting sulfonamide, with crystalline bovine serum albumin (BSA).[1] Although dialysis showed a single site of high affinity ($K = 1{\cdot}2 \times 10^5$ liter mole^{-1}) and three secondary sites of lower affinity ($K = 1{\cdot}0 \times 10^3$ liter mole^{-1}), quantitative investigations of the induced CD curves showed a single binding site ($K = 2{\cdot}1 \times 10^5$ liter mole^{-1}). A clearly defined isobestic point was seen throughout the titration range. The fact that only the primary binding site on the BSA was capable of inducing optical activity into the sulfaethidole enabled the displacement of the sulfa drug from its primary binding site by other acidic drugs to be quantitatively investigated.[2] These investigations showed that these drugs shared a primary binding site on BSA. At this time it was noticed that the germicide, benzalkonium chloride, displaced sulfaethidole from BSA. Benzalkonium chloride is a mixture of alkyldimethylbenzylammonium chlorides of general formula



where n is predominantly 12 or 14 but varies from 8 to 18. The ability of the pure homologues to displace sulfaethidole has now been investigated by CD, together with the effect of the quaternary ammonium compounds on the CD of the BSA from

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200 to 300 nm. Assuming that the reduced binding is due to a displacement phenomenon, an estimate of the binding constants of the quaternary compounds for the primary site of the acidic drug can be made.

MATERIALS

Sulfaethidole *N'*-(5-ethyl-1,3,4-thiazol-2-yl) sulfanilamide (Smith, Kline & French) was recrystallized twice from water to give a melting point of 185–186°. The crystallized BSA was obtained from Sigma Chemical Co. (batch number 10C-8080). The benzalkonium chloride U.S.P. was commercial Zephiran (Winthrop Laboratories), and the C_8–C_{19} alkyldimethylbenzylammonium chlorides were a gift from the Sterling–Winthrop Research Institute. All other chemicals were reagent grade, and deionized water was used throughout.

METHOD

All CD spectra were obtained using a 6002 attachment to a Cary 60 spectropolarimeter, using a slit programmed for a half-band width of 15 Å. All solutions were prepared in deionized water containing a 0·054 M sodium phosphate buffer, pH 7·4, made isotonic with sodium chloride at 22°. The concentration of BSA was $1{\cdot}45 \times 10^{-5}$ M and of sulfaethidole $2{\cdot}523 \times 10^{-5}$ M throughout the investigations. For the displacement investigations, the solutions were scanned in 10-mm cells from 350 to 245 nm. The effect of the quaternary ammonium compounds on the CD spectra of the albumin was also investigated through the same wavelength region and any correction necessary for the small effect of the compounds on the ellipticity was made. A 100-fold dilution of these solutions was scanned from 250 to 200 nm in 10-mm cells. The induced ellipticity is defined as the ellipticity observed from the drug plus albumin minus the ellipticity due to the albumin alone. The signal-to-noise ratio was never less than 10 to 1.

RESULTS AND DISCUSSION

The ellipticity induced into sulfaethidole after the binding to BSA was reduced in the presence of all the analogues from n-8 to n-19. The effect of various concentrations of the n-17 compound is shown in Fig. 1. The induced curves are shown uncorrected for the small effect of the quaternary compound on the BSA spectrum alone, but when corrected, the curves are all of the same shape having a positive peak near 257 nm and a negative peak near 279 nm with an isobestic point at 267 nm. These characteristics are similar to those observed after the displacement of sulfaethidole from BSA by various acidic drugs.[2] The displacement of bound sulfaethidole by the various homologues is shown as a function of concentration in Fig. 2. The effect rises to a maximum with the n-14 compound, the higher homologues showing some decrease in displacing ability. It is interesting to note that the n-14 compound is the most active germicide of the homologues.

The reduced binding of sulfaethidole may be caused by direct competition by the quaternary ammonium compounds for the same binding site or by conformational changes in the albumin caused by the homologues. Even the n-8 causes lowered binding of sulfaethidole, and such short-chain compounds are unlikely to cause conformational changes. Also if the reduced binding of the drug were caused by a conformational change altering the nature of the interaction or of the binding site, then

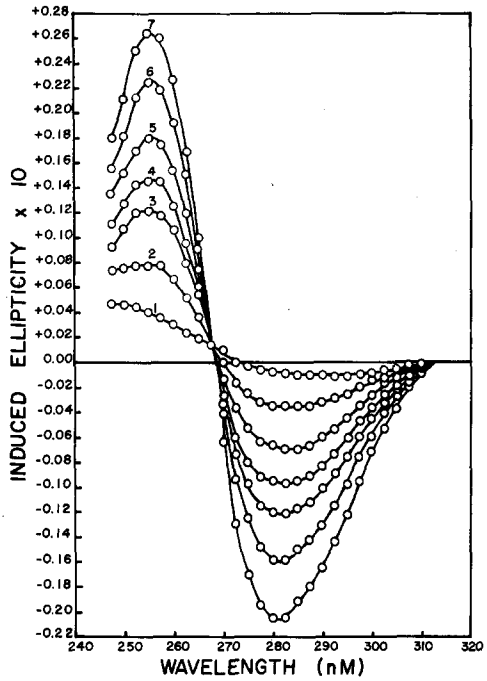

FIG. 1. Extrinsic Cotton effect curves for sulfaethidole in the presence of BSA and the n-17 homologue. Concentrations: BSA $1{\cdot}45 \times 10^{-5}$ M, sulfaethidole $2{\cdot}52 \times 10^{-5}$ M; n-17 (1) $4{\cdot}88 \times 10^{-4}$ M, (2) $3{\cdot}51 \times 10^{-4}$ M, (3) $1{\cdot}95 \times 10^{-4}$ M, (4) $1{\cdot}46 \times 10^{-4}$ M, (5) $9{\cdot}76 \times 10^{-5}$ M, (6) $4{\cdot}88 \times 10^{-5}$ M and (7) 0.

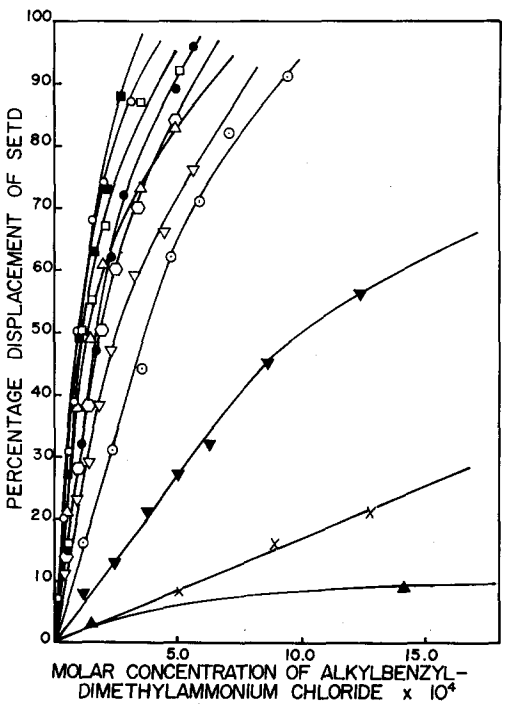

FIG. 2. Displacement of sulfaethidole (SETD) by the homologues as a function of concentration. The n values: ▲ = 8, × = 10, ▼ = 11, ⊙ = 12, ● = 13, ■ = 14, ○ = 15, □ = 16, △ = 17, ⬡ = 18 and ▽ = 19.

TABLE 1. CRITICAL MICELLE CONCENTRATIONS

Chain length	CMC's × 10^3
8	220
10	37
11	14·0
12	6·9
13	2·7
14	1·2
15	0·60
16	0·24
17	0·10
18	0·033
19	0·018

some change in the characteristics of the induced CD curves would probably be noticed. No such changes were observed with any of the antagonists, and the reduced binding seems to be the result of competition at the binding site. This may be a direct competition, or may indicate that the same area of the binding site is shared by the competing ligands. Tanford[3] has suggested that cationic amphiphiles may share hydrophobic areas of high affinity binding sites with anionic amphiphiles.

The homologues form micelles, and micelle formation is a competitive phenomena to their binding to BSA. The critical micelle concentrations (CMC's) measured in water at 25° are shown in Table 1.[4] These CMC's can be expected to be lowered in the presence of the buffer salts, and in the current investigations, micelle formation

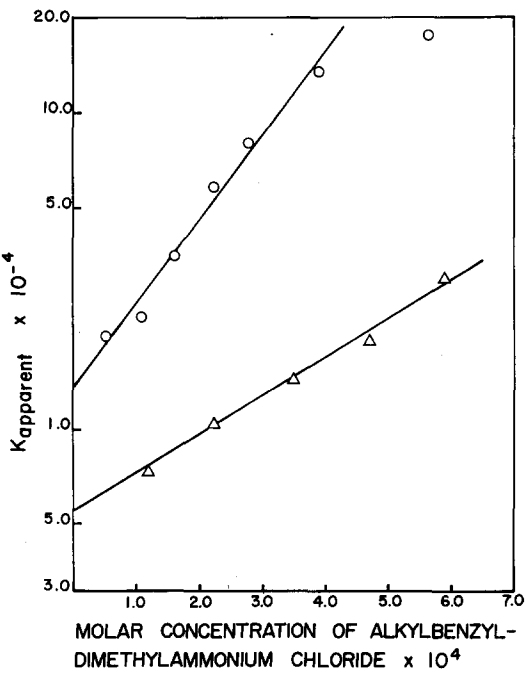

FIG. 3. Apparent binding constant of quaternary homologue for the primary sulfaethidole binding site on BSA as a function of concentration: ○ = n-13; △ = n-12.

TABLE 2. ESTIMATED BINDING CONSTANTS

Chain length (n)	$K \times 10^{-4}$
8*	(0·15)
10	0·099
11	0·28
12	0·55
13	1·4
14	3·1
15	2·7
16	3·2
17	2·8
18	2·2
19	1·6

* Only two measurements were made on the n = 8 compound and no extrapolation is possible.

can be expected to be a competitive phenomena to binding of the higher concentrations of n-16 and above homologues.[5]

Reynolds and Tanford[6] have shown that dodecyl sulfate micelles do not bind to a number of proteins, and no micellar binding is to be expected in this present case. The comparatively low binding constant of 10^4 (see below) for these cationic detergents suggests that the equilibrium will be shifted further toward micelle formation than has been noticed with anionic detergents.

In a previous paper,[2] attempts were made to calculate the binding constant for antagonists displacing sulfaethidole from its primary binding site. In that investigation as well as the current, concentrations of sulfaethidole and BSA were chosen so that 75 per cent of the available primary binding site on albumin is filled with sulfaethidole, whereas only 1·5 per cent of the total drug concentration is attached to the secondary sites. This low binding to the secondary sites together with the fact

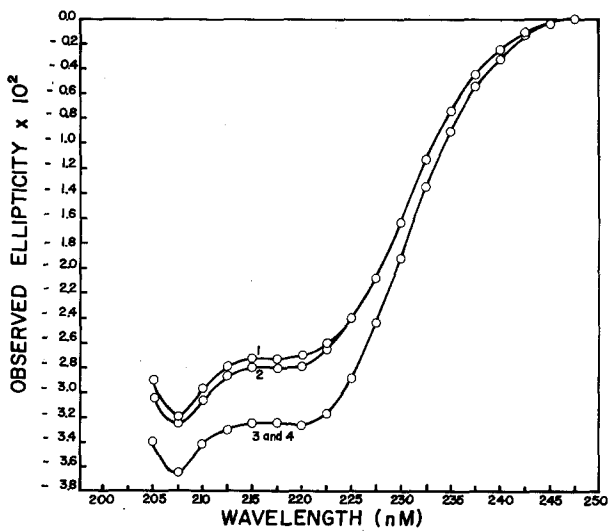

FIG. 4. Effect of quaternary homologues on the CD curves of BSA. (1) Sulfaethidole, BSA and $4{\cdot}57 \times 10^{-6}$ M n-19; (2) sulfaethidole, BSA and $4{\cdot}83 \times 10^{-6}$ M n-18; (3) $1{\cdot}45 \times 10^{-7}$ M BSA alone; and (4) BSA and $2{\cdot}53 \times 10^{-7}$ M sulfaethidole.

that secondary sites do not give any induced ellipticity allows binding to these sites to be ignored in any calculation of binding constants. Using the method previously described,[2] apparent binding constants as a function of ligand concentration can be calculated for the alkylbenzyldimethylammonium chloride homologues; extrapolation to zero concentration, as shown in Fig. 3, eliminated problems due to micelle formation and binding to sites of lower affinity. Binding to higher energy sites would, however, be a major complication in this method of estimating binding constants. The binding constants for the homologues are shown in Table 2. The values of 10^4 are in agreement with those quoted for the binding of other long-chain quaternary ammonium compounds to BSA,[7–9] and this again suggests that sulfaethidole, an anionic drug, shares the same primary binding site as the quaternary ammonium compounds. These binding constants are significantly lower than those found for the highest affinity sites on BSA for anionic detergents, such as the much investigated sodium dodecyl sulfate which has a binding constant of $1{\cdot}4 \times 10^6$ for 5–6 primary sites.[10–12] The binding constants appear to be substantially independent of chain length from n-13 to n-19. This is in agreement with the observations of the binding of anionic amphiphiles to BSA where little or no free energy change is observed for chain lengths greater than 12 carbon atoms.[10,13–15].

The effect of the amines on the CD curve of BSA alone was investigated at several concentrations through the 300–250 nm region associated with the aromatic amino acid residues of the protein. Below n-12, the spectrum of the BSA was not modified; however, the n-13 compound modified the spectrum in the 260 nm region. The higher analogues also modified the spectrum at higher wavelengths. These changes were always small, positive in sign, and are probably due to modification of the environment for the tryptophan or tyrosine residues of the albumin. These changes will be reinvestigated with the more sensitive CD equipment becoming available.

Figure 4 shows the effect of the cationic amphiphiles on CD spectrum of BSA at lower wavelengths. Sulfaethidole itself had no effect on the spectrum; however, all the homologues above n-12 did, as summarized by the changes in ellipticities at 220 nm shown in Table 3. The homologues did not alter the shape of the curve associated with the predominantly helical BSA (Fig. 4) but did reduce the magnitude of the negative ellipticity throughout the region. These changes in ellipticities are very similar to those reported for the binding of dodecyl sulfonate to BSA.[16] Although

TABLE 3. EFFECT OF CATION ON CD SPECTRA OF BSA AT 220 nm

Chain length	% Decrease in ellipticity	Concn of cation (Moles liter^{-1} $\times$ 10^3)
8		1·41
10		1·28
11		1·23
12	4·5	1·18
13	4·7	0·564
14	9·4	0·543
15	13·9	1·05
16	12·6	0·504
17	15·1	0·488
18	14·2	0·483
19	17·3	0·457

this lower region of the CD spectrum is usually regarded as indicative of the secondary structure of the protein, interpretation of changes in terms of conformation are not unambiguous, and it has been suggested that histidine as well as aromatic amino acid residues in the protein may contribute to the spectrum in this region.[16,17] Foster and Yang[9] have reported a reversible structural change in BSA after the binding of the n-12 homologue; however, more recently Nozaki *et al.*[7] have suggested no conformational changes after the binding of tetradecyltrimethylammonium chloride to BSA at low ligand-to-protein ratios. At higher detergent concentrations, they did observe gross denaturation accompanied by large changes in CD and optical rotatory dispersion. It seems probable that the longer chain dialkylbenzylammonium chlorides investigated here do cause some conformational change on binding to albumin even at low concentrations; however, the change is small in comparison to that caused by similar chain length anionic detergents. The commercial benzalkonium chloride U.S.P. germicide product, containing predominantly n-12 and n-14 homologues, displaces sulfaethidole with changes in chiral properties of BSA expected for this mixture.

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