The Interaction of Bovine Plasma Albumin with Detergent Anions. Stoichiometry and Mechanism of Binding of Alkylbenzenesulfonates*

Rolan V. Decker† and Joseph F. Foster

ABSTRACT: The mode of binding of alkylbenzenesulfonates to bovine plasma albumin (BPA) has been examined by means of equilibrium dialysis and moving boundary electrophoresis experiments using two highly purified anions, octylbenzenesulfonate (OBS^-) and dodecylbenzenesulfonate (DBS^-). In each case there is an initial binding involving 11 ± 1 sites. In accord with previous conclusions this binding appears to involve equivalent sites which do not interact. In this binding range only a single electrophoretic boundary is observed, as expected. At higher levels of binding, at pH 6.5, three electrophoretic components can be distinguished having the compositions, respectively, AD_{11}, AD_{38}, and AD_{76}. The latter two components correspond to the AD_n and AD_{2n} complexes observed 20 years earlier by Putnam and Neurath [*J. Biol. Chem. 159*, 195 (1945)] though the value of n is significantly lower in the present case. Several lines of evidence show that the composition of each of the three complexes is remarkably constant throughout the range of their coexistence. Formation of the higher complexes from AD_{11} is accompanied by some loss of helix content in the protein as judged from optical rotatory dispersion experiments. Two theoretical mechanisms are considered which will account for the constancy of composition of the various complexes and for the detailed binding isotherms. Both mechanisms presuppose two cooperative conformational changes in the protein. In one case, the binding to the two isomerized forms is assumed to be fully cooperative with strong stabilizing forces (micellization forces) between bound anions. In the other mechanism no such interactions are invoked; rather it is assumed that all binding reactions are purely statistical in character. The resultant equations for the two mechanisms are shown to be equivalent for the conditions of the experiments so that no basis is provided for distinguishing which is operative. At pH 9.5, AD_n does not exist as a stable intermediate. A progressive disproportionation of AD_n into two complexes (presumably AD_{11} and AD_{76}) is shown to take place as the pH is raised from *ca.* 6 to 9. Some implications of the binding pattern are discussed.

The propensity of plasma albumin for binding anions of all sorts has evoked the interest of numerous investigators. Consequently, a wealth of information has been accumulated concerning the interaction of plasma albumin with organic and inorganic anions of various types. Although the very strong binding of detergent anions is not a unique property of plasma albumin, the intriguing experimental manifestations of this interaction have been viewed by many workers as an important tool in studies of the three-dimensional structure of this protein molecule.

From the early results of Putnam and Neurath (1945) it was clear that discrete complexes form between albumin and anionic detergents. They suggested, from electrophoretic studies with horse plasma albumin and dodecyl sulfate (DS^-),[1] that these complexes had the composition AD_n and AD_{2n}, where n is *ca.* 55. From the close correspondence of the value of $2n$ to the total number of cationic groups on the protein at neutral pH they concluded that the formation of this complex is a result of the binding of one detergent anion to each cationic site. One of the present authors (Foster, 1960) has suggested that this close correspondence is probably only fortuitous. He pointed to the well-known tendency of detergents to form micelles because of the lower free energy which results when the hydrocarbon portion of the anion is forced out of the aqueous phase into close association with the hydrophobic R groups of other such ions. In addition, he cited results of experiments (Foster, 1949, and Aoki, 1956) in which the extent of binding of anionic detergents to proteins far exceeds the number of available cationic groups. It seems clear that not only in the case of organic dye

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† Permanent address: Division of Mathematics and Physical Sciences, Southwestern (Oklahoma) State College, Weatherford, Okla.

[1] The following abbreviations are used: DS^-, dodecyl sulfate; OBS^-, octylbenzenesulfonate; DBS^-, dodecylbenzenesulfonate; AD_m, AD_n, and AD_{2n} are stoichiometric complexes containing m, n, and $2n$ detergent anions, respectively; BPA, bovine plasma albumin.

anions (Fredericq, 1956) and fatty acid anions (Teresi and Luck, 1948, 1949) but also in the case of detergent anions the hydrophobic nature of the anion plays an important role in the binding to proteins.

Results obtained by other investigators have been in general agreement with the conclusion of Putnam and Neurath that one or more albumin–detergent complexes are formed but there is no agreement as to the stoichiometry nor even as to the number of complexes formed. The values obtained for n (or $2n$) have varied from the 48 of Yang and Foster (1953a) to the 80 of Pallansch and Briggs (1954), both groups of workers finding only a single stoichiometric complex. On the other hand both of those studies demonstrated that the cooperative steps giving rise to the stoichiometric complexes are preceded by a statistical binding of 10–12 detergent ions to strong-binding sites which evidently preexist in the native protein. Karush and Sonenberg (1949) had earlier studied the binding behavior in the region of relatively low detergent activity and had concluded these sites to be heterogeneous as to their affinity for detergent ions. The results of Yang and Foster (1953a) and of Pallansch and Briggs (1954), however, indicated these 10–12 sites to be essentially homogeneous and noninteracting. The curvature of the binding plots of Karush and Sonenberg, which led them to conclude heterogeneity of the sites, is almost certainly due to the onset, at higher detergent levels, of a cooperative binding with formation of the higher stoichiometric complexes.

Both Yang and Foster (1953a) and Pallansch and Briggs (1954) were led to the conclusion that formation of the higher complexes results from a cooperative alteration of the protein structure with exposure of many new, but weaker binding sites. In addition Pallansch and Briggs reached the important conclusion that the few strong-binding sites existing in the native protein must be destroyed in this cooperative structural change. Discovery of the N–F transformation in plasma albumins led to the suggestion (Foster and Aoki, 1958) that this might be the structural alteration responsible for destruction of 10–12 strong-binding sites and formation (or exposure) of the large number of weaker binding sites. Strong supporting evidence for this suggestion has been presented by Leonard and Foster (1961).

One of the most complete studies to date of the interaction of plasma albumins with detergents is that of Aoki (1958). That study involved the interaction of horse serum albumin with both anionic (dodecyl sulfate) and cationic (dodecylpyridinium) detergents at various pH values. With the anionic detergent, studies at pH 5.6 and 6.8 confirmed formation of three complexes AD_m, AD_n, and AD_{2n}, where $m = 12$ and $n = 105/2$. At pH 8.9, only one discrete complex in addition to AD_m was formed, presumably but not definitely AD_{2n}. At pH 10.6, no discrete complexes were formed. Aoki suggested that the alteration of the binding pattern with increasing pH reflects a pH-dependent change in the internal structure of the protein. Such a transition was reported later by Leonard *et al.* (1963) to occur between pH 7 and 9.

In quantitative studies of the binding of detergent ions to proteins use of an aromatic detergent offers the distinct advantage that the concentration of free detergent can be measured, in dialysis experiments, spectrophotometrically. For this reason Yang and Foster (1953a) employed dodecylbenzenesulfonate (DBS^-). Unfortunately the sample of detergent available at that time was rather heterogeneous with respect to the nature of the alkyl chain, and they concluded that a definitive study of this system would have to await the preparation of a homogeneous sample of the detergent. Several alkylbenzenesulfonates have now been synthesized (Goyal, 1961) and shown, after extensive purification, to be homogeneous as judged by several chemical and physical criteria. Thus a more conclusive reexamination of the interaction of detergent ions of this type with plasma albumins became feasible.

Experimental Section

Materials. Bovine plasma albumin (Armour and Co., Lot W69312) was stored at pH 2 and 2–3° for 48 hr and then centrifuged and filtered to remove lipid contaminants (Williams and Foster, 1959, 1960). Bovine plasma albumin obtained from Pentex, Inc. (Lot 9), did not require this treatment since it had been observed to release no lipid material upon standing in the cold at low pH. The lipid-free preparations were deionized (Dintzis, 1952), lyophilized, and stored at 2–3°. The Pentex preparation dissolved with great difficulty in neutral salt solution but with facility at pH values below the range of the N–F transformation; thus, solutions of this bovine plasma albumin were routinely prepared by dissolving the lyophilized protein quickly (5–10 min) in 0.1 M HCl followed by neutralization with an equivalent quantity of 0.1 M LiOH. Both preparations exhibited the usual 5–10% of faster sedimenting component (dimer) in the ultracentrifuge. The Armour albumin was used in the experiments with octylbenzenesulfonate (OBS^-), and the experiments using dodecylbenzenesulfonate (DBS^-) were performed with the Pentex preparation exclusively.

The alkylbenzenesulfonates employed in this study were those previously synthesized by Goyal (1961). The synthetic procedure involved Friedel–Crafts acylation of benzene followed by a Clemmensen reduction of the product to the corresponding alkylbenzene. The isolated alkylbenzene was sulfonated using fuming sulfuric acid, and the salt of the sulfonic acid was precipitated from the ether extract of the reaction mixture with saturated aqueous NaCl. The final product was purified by recrystallization from 50% alcohol. The purified sodium *p*-alkylbenzenesulfonates were subjected to ultraviolet spectrophotometric, ion exchange, and elemental analyses. In addition, the volatile methyl ester of dodecylbenzenesulfonic acid was subjected to gas chromatography at 250°. All these methods gave evidence of the purity and homogeneity of the preparations. Thus, no further purification was undertaken for this study.

Lithium chloride was used as the supporting electro-

lyte throughout most of these studies for reasons which will be discussed later. This salt was Analytical Reagent grade but was found to require treatment with Dowex chelating resin A-1 to remove trace amounts of di- and trivalent cations which reduced considerably the solubility of the detergents. Stock solutions were treated with the resin and found by Mohr's titrimetric method to be 3.94 M and 3.99 M in Cl^-.

Dialysis tubing (cellulose casing with inflated diameter of $^{20}/_{32}$ in. from Visking Corp.) was cut to uniform lengths of 20.0 cm, heated three times in saturated $NaHCO_3$ near the boiling point for 2 hr, and rinsed thoroughly with deionized water to remove impurities. The treated tubing was stored in deionized water at 2–3°.

All other reagents were Analytical Reagent grade and were used without further purification. Deionized water (Barnstead Bantam demineralizer, Model BD-1, column 0808) was used for preparing all solutions.

Methods. Detergent binding measurements were carried out by the classical method of equilibrium dialysis (Klotz *et al.*, 1946). The detergent was placed inside the dialysis bag with the BPA, since Yang and Foster (1953a) found that equilibration is much more rapid when this method is used than when the detergent and BPA are initially separated by the dialysis membrane. For experiments at low total detergent concentrations, stock solutions were prepared from carefully weighed samples of the detergent and 0.1 M salt solution. For the higher detergent concentrations, the carefully weighed detergent was dissolved in *ca.* 10 ml of 0.1 M salt solutions. In all cases, 10.0 ml of *ca.* 1% (w/v) BPA was added either to the dissolved sample or to appropriate volumes of the stock detergent solution, and the mixtures were diluted to 25.0 ml with 0.1 M salt solution. Rapid solution of the detergent was effected by warming the suspension. The solutions were then cooled before further dilution or addition of the BPA solution. Detergent solutions and BPA solutions were freshly prepared for each experiment.

The pH of the BPA–detergent solutions was adjusted to the desired value by addition of known volumes of 0.1 M OH^- to 20.0 ml of the BPA–detergent solution so that correction for the dilution could be made. For each experimental point, 10.0 or 15.0 ml of the BPA–detergent solution was pipetted into a section of dialysis tubing knotted at one end; the open end was then knotted and the filled bag was placed into 25.0 ml of 0.1 M Cl^- solution in a screw cap Pyrex tube (*ca.* 50 ml). The space above the solution was flushed with N_2, and the tube was sealed with the screw cap which had been equipped with a Parafilm gasket. Also, a strip of Parafilm was wrapped tightly around the base of the cap as a further precaution against possible leakage.

The OBS^- dialyses were carried out in a water bath thermostated at 7.0 ± 0.10°. The tubes were clamped to a vertical wheel which rotated (*ca.* 10 rpm) to provide constant stirring of the solutions being dialyzed and of the bath itself. The DBS^- dialyses were carried out at ambient temperature (24 ± 2°), the tubes being mechanically rocked end to end at a rate of *ca.* 22 oscillations/min. The period of dialysis was 18–22 hr, after which time the pH of the BPA–detergent solutions and of the dialysates was determined as well as the concentration of the detergent in the dialysate.

Optical rotatory dispersion measurements were carried out using the Polarmatic recording spectropolarimeter (Bendix Corp.). A 1-cm cell was used and the measurements were carried out at ambient temperature (24 ± 2°). Three stock solutions were prepared from a stock solution of 3.15% (w/v) BPA in 0.1 M LiCl. One stock solution contained only BPA at a concentration of 0.315%. The two other stock solutions contained, in addition to 0.315% BPA, 55.6 and 111 moles of DBS^-/mole of BPA. These solutions were prepared in volumetric flasks and were adjusted to pH 6.58 with 0.1 M LiOH before adding the last few milliliters of 0.1 M LiCl to bring the level of the solution up to the mark. Solutions were then prepared from various volumes of the three stock solutions to produce the desired BPA–DBS^- ratio while maintaining the BPA concentration at 0.315%. The pH of the solutions was measured after they were analyzed using the spectropolarimeter.

Electrophoresis experiments were performed using the Perkin-Elmer electrophoretic apparatus, Model 38, equipped with a Schlieren scanning optical system, or with the Spinco Model H electrophoresis–diffusion instrument, equipped with Schlieren cylindrical lens and Rayleigh interference optical systems. With the former instrument, a 2.0-ml cell with cross-sectional area 0.900 cm^2 was used; with the latter instrument a 2.0-ml cell with cross-sectional area 0.125 cm^2 was used. In all cases the water bath was thermostated at 22.0 ± 0.1°, and low field strengths (1.0–2.3 v/cm) were used to prevent excessive heat convection.

In all experiments 15.0 ml of BPA–DBS^- solution (*ca.* 0.4% BPA) was dialyzed overnight *vs.* 25.0 ml of 0.1 M LiCl at 24 ± 2° just prior to the electrophoresis run. The pH of both the BPA–DBS^- solution and the dialysate was checked before filling the electrophoresis cell, and the pH of the dialysate was adjusted to the pH of the BPA–DBS^- solution when necessary. The small volume of dialysate was necessitated by the limited amount of NaDBS available. In order to maintain contact between the BPA–DBS^- complex and the proper free detergent concentration, the cell was filled as usual using a portion of the dialysate and the BPA–DBS^- solution. The remainder of the cell and the electrode vessels were filled with 0.1 M LiCl. Then, the remainder of the dialysate was carefully layered immediately above the descending arm of the center section. Thus, when the boundaries were formed, the column of BPA–DBS^- solution was bounded at both ends by dialysate. The high degree of enantiography of the ascending and descending patterns thus obtained gave evidence for the validity of this technique.

Photographs of the Schlieren patterns were enlarged 5- to 10-fold for determination of areas under the peaks. A curve was sketched through the midpoints of vertical lines spanning the wide tracing of the Schlieren pattern obtained with the Perkin-Elmer instrument. When

appropriate, the curves were resolved into component peaks assuming that each peak was Gaussian in shape. The areas of these peaks were then measured with a compensating polar planimeter. The velocities of the components were taken from slopes of plots of displacement of the maximum ordinates *vs.* time, which were generally quite linear as can be seen in Figure 1. The conductivities of BPA–DBS^- solutions, needed for calculating mobilities, were determined at 22° with a Model RC-16 conductivity bridge (Industrial Instruments, Inc.).

Measurements of pH made use of a Radiometer, Model PH 22, glass electrode pH meter; a Beckman, Model G, pH meter equipped with external Beckman general-purpose glass and fiber junction calomel electrodes; or a Beckman research pH meter fitted with a Beckman general-purpose glass electrode and a silver–silver chloride reference electrode with a ground glass sleeve-type junction. For reasons discussed below, 0.1 M LiCl was used as the salt bridge between the solution under study and the reference electrode. All pH measurements were made at 24 ± 2°, and Beckman or Sargent standard buffers were used to standardize the pH meters. A combination glass and fiber junction calomel electrode was used with the Radiometer pH meter for the pH adjustment of the solutions used in the rotatory dispersion measurements.

The concentrations of BPA solutions were determined from their absorbance at 279 mμ using either the Beckman DU spectrophotometer or the Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer; $E_{1cm}^{1\%}$ was assumed to be 6.67 for BPA at this wavelength (Sterman, 1955). Molar BPA concentrations were calculated assuming the molecular weight of BPA to be 66,000 (Spahr and Edsall, 1964).

Free detergent concentrations in the dialysates were calculated from the absorbance at 223 mμ and the molar extinction coefficients for OBS^- and DBS^- which were determined to be 1.32×10^4 and 1.35×10^4, respectively. The accuracy of the spectrophotometric method suffered somewhat from the fact that at the low levels of detergent concentration employed, the presence in the dialysate of minute quantities of BPA and/or ultraviolet absorbing impurities from the dialysis tubing produced random errors which were at times appreciable. Attempts were made to correct for the errors due to impurities in the dialysis tubing, but the amount of such material appearing in the dialysate in the absence of BPA and detergent was negligible in most cases. It is felt that errors resulting from the presence of traces of BPA were the most serious since BPA absorbs strongly at 223 mμ. Occasional samples of dialysate which exhibited an absorption much higher than expected were rejected as probably in error due to contamination by protein.

Results

The initial goal of this work was to reexamine the binding behavior of the BPA–DBS^- system using the highly purified NaDBS and an unbuffered salt medium commonly used in studies of protein molecules, *i.e.*, 0.1 M NaCl or 0.1 M KCl. Preliminary experiments indicated, however, that the extent of binding of DBS^- attainable in either of these media would be severely limited by the low solubility of the pure NaDBS. This difficulty was not encountered by Yang and Foster

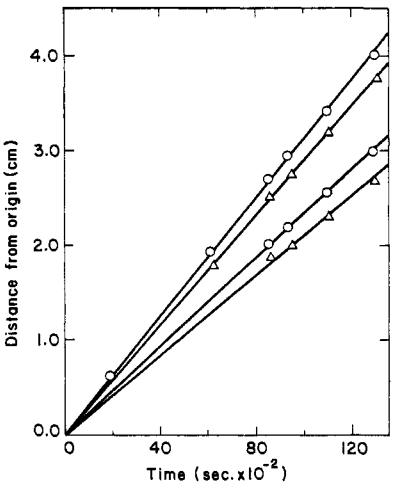

FIGURE 1: Boundary displacements *vs.* time for a representative electrophoresis run: 0.4% BPA in 0.1 M LiCl; $\bar{\nu}$ = 61.9 moles of DBS^-/mole of BPA; temperature, 22.0 ± 0.1°; pH = 9.55; field strength = 1.33 v/cm. Ascending and descending displacements are denoted by circles and triangles, respectively, for the slower and faster components.

TABLE I: Equilibrium Dialysis Data for the Octylbenzenesulfonate–BPA System.

D_T[a] ($M \times 10^5$)	D_f[b] ($M \times 10^5$)	BPA (moles $\times 10^7$)	$\bar{\nu}$	pH
Solvent: 0.1 M KCl				
104	59.0	8.59	20.7	6.29
52.1	23.0	8.59	13.3	6.32
10.4	0.49	8.59	4.6	6.36
5.21	0.17	8.59	2.4	6.28
Solvent: 0.1 M NaCl				
234	123	7.65	58.2	6.70
179	114	5.11	44.2	6.52
93.4	59.5	7.62	17.8	6.60
71.2	46.2	5.08	17.3	6.40
46.6	23.5	7.59	12.0	6.60
18.8	2.55	7.59	8.6	6.72
14.4	2.18	5.07	8.4	6.61
7.51	0.31	7.59	3.7	6.62
5.73	0.30	5.07	3.7	6.68

[a] D_T = total detergent concentration. [b] D_f = concentration of free detergent.

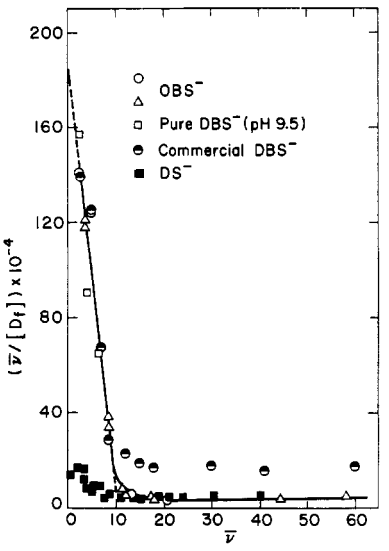

FIGURE 2: Scatchard plot of binding data for BPA and several anionic detergents: OBS^- data designated by ○ and Δ were obtained using 0.1 M KCl at pH 6.32 ± 0.20 and 0.1 M NaCl at pH 6.71 ± 0.10, respectively, all at 7.0 ± 0.1°; DBS^- (pure) data were obtained using 0.1 M LiCl at pH 9.52 ± 0.07 and 24 ± 2°; commercial DBS^- data are those of Yang and Foster (1953a) in NaCl–phosphate buffer, pH 7.7, Γ/2 = 0.20 at 1–3°; DS^- data are those of Pallansch and Briggs (1954) in NaCl–phosphate buffer, pH 6.8, Γ/2 = 0.20, at 22°. The curve is drawn to fit the OBS^- data.

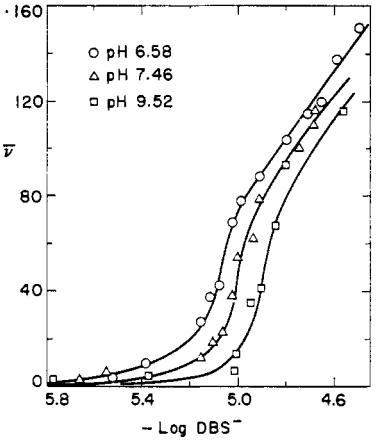

FIGURE 3: The interaction of BPA and DBS^- at several values of pH, plotted as $\bar{\nu}$, the average number of moles of DBS^- bound per mole of BPA, *vs.* the logarithm of the free detergent concentration; solvent, 0.1 M LiCl; temperature, 24 ± 2°.

(1953a), undoubtedly because of the heterogeneity of the commercially available NaDBS used in their studies. Since several lower molecular weight (and more soluble) alkylbenzenesulfonates were available in this laboratory, OBS^- was chosen as a possible substitute for DBS^-.

The Binding of OBS^- by BPA. The results of equilibrium dialysis experiments using OBS^- and BPA in two different media are summarized in Table I. It should be pointed out that the maximum value of $\bar{\nu}$ reported in the table is the maximum value obtainable under the experimental conditions employed. The principal difficulty here was the decreased solubility of the detergent at 7°.

The binding of ligands by sites on a protein which are equivalent and noninteracting obeys the equation (Scatchard *et al.*, 1957)

$$\bar{\nu}/D_f = K(m - \bar{\nu}) \qquad (1)$$

where k is the equilibrium constant for the association of the anion with a single site, $\bar{\nu}$ is the average number of moles of detergent anion bound per mole of protein, m is the total number of sites having an association constant k, and D_f is the free detergent concentration. A Scatchard plot of the binding data obtained with the BPS–OBS^- system is presented in Figure 2. Also included in this figure are the data Pallansch and Briggs (1954) obtained using DS^-, those of Yang and Foster (1953a) from their BPA–DBS^- system, and points obtained in the present study at pH 9.52 using pure DBS^-. From Figure 2 it can be seen that m for the four detergent systems is *ca.* 11 in all cases but that k depends on the type of detergent used.

Other workers have observed that the cooperative uptake of detergent anions by BPA continues to a value of $\bar{\nu} \sim 100$. The results of the experiments with OBS^- made it clear that a complete study of the detergent binding characteristics of BPA could not be carried out under the conditions employed thus far because of the limited solubility of the detergent. It had been noted that the solubility of NaOBS is higher in 0.1 M NaCl than in 0.1 M KCl, suggesting that decreasing the size of the cation of the supporting electrolyte increases the solubility of the detergent. Results obtained with 0.1 M LiCl indicated that not only is this true but that experiments could be carried out using DBS^- if they were performed at ambient laboratory temperature (20–25°).

The Binding of DBS^- by BPA. Binding data plotted as $\bar{\nu}$ *vs.* log D_f are presented in Figure 3 for the BPA–DBS^- system in 0.1 M LiCl at three different pH values and 25°. Because of the difficulties encountered in determining D_f, as discussed above, the values of D_f are not highly accurate but are probably good to at least ±5% over most of the range covered in Figure 3. The relative errors are probably significantly greater than this for D_f below about 4×10^{-6} M so that little significance can be attached to the binding curves in neutral solution in the initial binding region, *i.e.*, up to $\bar{\nu} \sim 10$. Except at very low binding (*i.e.*, $\bar{\nu} \sim 3$), the values of $\bar{\nu}$ are considered to be quite accurate since at higher binding levels this parameter is relatively insensitive to errors in D_f.

Scatchard plots of the DBS^- binding data were con-

structed in an attempt to determine k and m for the initial binding stage in this system. The inaccuracy of D_f in the range of $\bar{\nu} < 10$ for experiments near neutral pH precluded accurate determinations of the value of the constants for this initial binding. However, the data obtained at pH 9.52 for $\bar{\nu} < 12$ were included in Figure 2, and it appears that these data fall very close to the curve drawn for OBS^-, suggesting that m for DBS^- is also *ca.* 11, at least at this pH. For $\bar{\nu}$ values between 10 and 80 the slopes of the Scatchard plots were in all cases positive, clearly a result of the highly cooperative character of the binding reactions.

It should be mentioned that the picture of detergent binding obtained here does not seem to be complicated by the formation of detergent micelles. The values of D_f observed in these experiments were in all cases below the critical micelle concentration of 8.1×10^{-5} M as determined in 0.1 M LiCl by the method of Yang and Foster (1953b).

Electrophoretic Behavior of the BPA–DBS^- Complexes at pH 6.5 and 9.5. Electrophoretic experiments were performed at a number of $\bar{\nu}$ values at pH 6.55 ± 0.05. The results of these experiments are summarized in Table II. The patterns obtained were similar in most respects to those obtained by other workers (Pallansch and Briggs, 1954; Yang and Foster, 1953a; Aoki, 1958). Experiments performed below $\bar{\nu} = 12$ and above $\bar{\nu} = 80$ exhibited only one boundary. Two components were observed between $\bar{\nu}$ values of 12 and 36 as well as between 40 and 76, in both instances the amount of faster component increasing with $\bar{\nu}$ at the expense of the slower component. In all, three complexes were observed which will be designated as AD_m, AD_n, and AD_{2n} in accordance with the convention followed by the other workers in this field. (That the number of detergents bound in the second cooperative step is twice the number bound in the first cooperative step is verified by results to be discussed below.) An unexpected result was the observation of three components at $\bar{\nu} = 38.4$. Under these conditions, small peaks resulting from the presence of small amounts of AD_m and AD_{2n} trail and precede, respectively, the major component, AD_n.

TABLE II: Electrophoresis Data for the BPA–DBS^- System in 0.1 M LiCl at pH 6.55 ± 0.05 and 22.0°.

	Relative Area (%)[a]			Descending Mobility ($-\mu$) ($cm^2\ v^{-1}\ sec^{-1} \times 10^4$)		
ν	1	2	3	1	2	3
0	100	..	...	0.87	...	...
2.8	100	..	...	0.96	...	...
5.6	100	..	...	0.95	...	...
8.3	100	..	...	0.97	...	...
12.5	95	5	...	1.01	1.34[b]	...
22.2	52	48	...	1.07	1.61	...
31.9	20	80	...	1.05	1.56	...
38.4	10	82	8	1.09	1.60	1.94
46.2	...	83	17	...	1.69	1.72[b,c]
58.0	...	46	54	...	1.69	1.98
66.9	...	21	79	...	1.69	2.00
75.5	...	5	95	...	1.63	1.97
86.1	...	..	100	...	...	2.12
89.2	...	..	100	...	...	2.14
94.0	...	..	100	...	...	2.16

[a] Obtained from ascending patterns; component 1 is the slowest; 3 is the fastest. [b] Subject to some uncertainty because the component appeared as only a shoulder on the main peak. [c] Only ascending value was available.

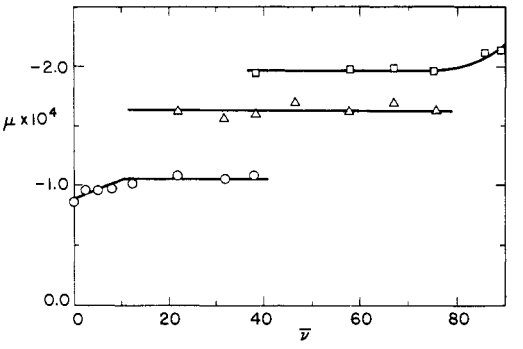

FIGURE 4: Mobility (μ, $cm^2\ v^{-1}\ sec^{-1}$) of the BPA–DBS^- complexes *vs.* $\bar{\nu}$: pH 6.55 ± 0.05: 0.1 M LiCl; temperature, 22.0 ± 0.1°; field strength, 1.4 v/cm.

The mobilities of the peaks observed in the descending patterns are plotted in Figure 4. Since these experiments were performed using an unbuffered supporting electrolyte, it seemed that the mobility of the boundary moving into the more buffered environment (*i.e.*, the descending boundary) would yield the more accurate mobility value at the measured pH. Nevertheless, the descending velocity seldom varied by more than 3% from the average of the ascending and descending velocities.

The results of the electrophoresis of various mixtures of BPA and DBS^- at pH 9.55 ± 0.01 are presented in Table III. These data are in qualitative agreement with those obtained by Aoki (1958) with horse plasma albumin and DS^- at pH 8.9. Below $\bar{\nu} = 11$ only one component was observed. Two components were observed, however, at all values of $\bar{\nu}$ between 12 and 70, the amount of faster component increasing with a concomitant decrease in the amount of slower component.

Optical Rotatory Dispersion Experiments. The results of optical rotatory dispersion experiments using BPA and DBS^- in 0.1 M LiCl at pH 6.6 are summarized in Figure 5 as $-b_0$ *vs.* $-\log D_f$. The b_0 values were obtained from Moffitt–Yang plots (Moffitt and Yang, 1956) of the data based on the equation

$$[m']_\lambda = a_0\left(\frac{\lambda_0^2}{\lambda^2 - \lambda_0^2}\right) + b_0\left(\frac{\lambda_0^2}{\lambda^2 - \lambda_0^2}\right)^2 \quad (2)$$

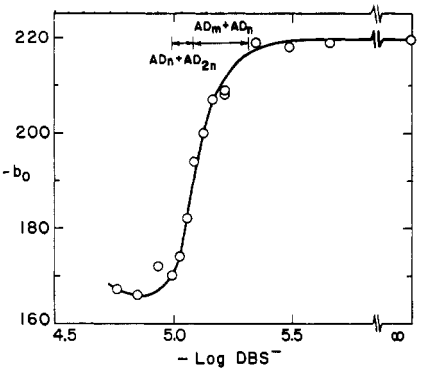

FIGURE 5: Dependence of the b_0 parameter of the Moffitt equation on the negative logarithm of the free DBS^- concentration, $pDBS^-$, for the BPA–DBS^- system; 0.1 M LiCl; pH 6.6; temperature, 24 ± 2°. The regions of coexistence of two forms in the electrophoresis studies are designated by arrows at the top of the figure.

TABLE III: Electrophoresis Data for the BPA–DBS^- System in 0.1 M LiCl at pH 9.55 ± 0.01 and 22.0°.

	Relative Area (%)[a]		Descending Mobility ($-\mu$) ($cm^2\ v^{-1}\ sec^{-1} \times 10^4$)	
$\bar{\nu}$	1	2	1	2
0	100	..	1.37	...
5.5	100	..	1.45	...
8.4	100	..	1.43	...
11.5	96	4	1.49	?
17.4	89	11	1.47	?
22.2	70	30	1.52	1.86
24.1	68	32	1.56	1.98
31.2	55	45	1.51	2.00
39.0	43	57	1.59	2.13
47.4	22	78	1.63	2.23
59.2	9	91	1.59	2.20
64.1	5	95	1.47	2.09

[a] Obtained from ascending patterns; component 1 is slower.

where a_0, b_0, and λ_0 are dispersion constants and λ is the wavelength of light. The value of λ_0 was taken as 218 mμ (Leonard *et al.*, 1963) and other details of calculation were the same as employed by Leonard and Foster (1961).

Assuming any change in b_0 to be due to change in helix content of the protein, AD_{2n} contains about 75% of the amount of helix present in native BPA.

Discussion

General. The Scatchard plots shown in Figure 2 support the conclusion of Yang and Foster (1953a) and of Pallansch and Briggs (1954) that the initial binding involves 10 to 12 protein sites which are virtually equivalent and noninteracting. These results also demonstrate the binding affinity in the initial range to be much stronger for OBS^- than for DS^- (intrinsic binding constants *ca.* 1.8×10^5 and 1.8×10^4, respectively). This emphasizes the importance of the aromatic ring in this initial binding reaction, in agreement with earlier conclusions of Fredericq (1954). The binding of DBS^- in this range is evidently much stronger still, as indicated by the great difficulty of measuring the free detergent concentration in the experiments at neutral pH. As indicated previously, the binding of this latter ion at pH 9.5 is similar in strength to the binding of OBS^- at neutral pH. An attempt to estimate the intrinsic constant for the binding of DBS^- to the initial 11 sites at neutral pH will be found later in this discussion.

The total length of the hydrocarbon portion of the anion is of importance in the cooperative binding steps. It can be seen that OBS^- and DS^- exhibit similar binding behavior above $\bar{\nu} = 15$ (Figure 2). However, the data obtained by Yang and Foster (1953a) show that DBS^- (commercial preparation) is bound more strongly at $\bar{\nu}$ values >15 than is OBS^-. The contrast is even more striking in the case of pure DBS^- as is seen by comparison of the data of Figure 3 with those of Table I.

Stoichiometry of the Binding Reactions. A superficial analysis of the electrophoretic patterns together with the analysis of the binding data leads to the conclusion that for this system m is *ca.* 11, n, 38, and $2n$, 76. A plot of the fraction of BPA existing in each of the complexes *vs.* $\bar{\nu}$ should give more definitive information concerning the stoichiometry of the cooperative binding steps. However, determination of the fraction of BPA existing in each of the complexes requires accurate knowledge of the refractive increments of BPA and DBS^- in the complex. Since this information was not available, another mode of attack was attempted.

If f_m is the fraction of BPA present as AD_m, then when only two components are present, the fraction of BPA existing as AD_n, f_n, is equal to $1 - f_m$. Thus

$$\bar{\nu} = m(f_m) + n(1 - f_m) \tag{3}$$

It then follows that

$$f_m = -\frac{1}{n-m}\bar{\nu} + \frac{n}{n-m} \tag{4}$$

and

$$f_n = \frac{1}{n-m}\bar{\nu} - \frac{m}{n-m} \tag{5}$$

However

$$f_m = \frac{b_1 A_m}{(\mathrm{BPA})_T} \tag{6}$$

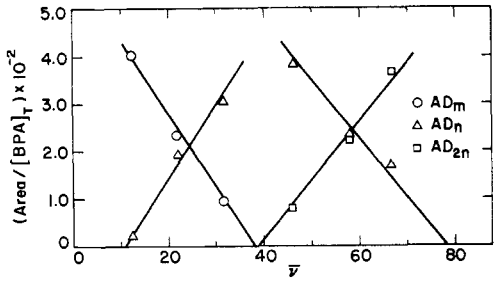

FIGURE 6: Composition of BPA–DBS$^-$ solutions at various values of $\bar{\nu}$ at pH 6.55 ± 0.05. Data are plotted as areas of individual peaks (resolved as Gaussian peaks from electrophoresis patterns) divided by the total BPA concentration (w/v); temperature, 22.0 ± 0.1°; solvent, 0.1 M LiCl; field strength, 1.4 v/cm.

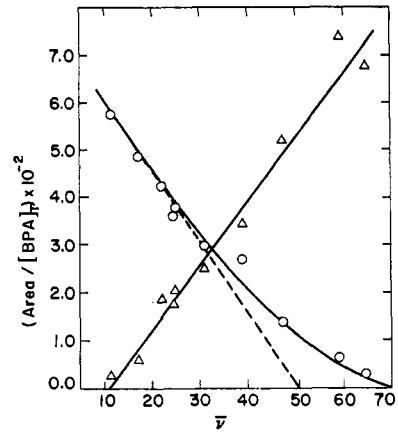

FIGURE 7: Composition of BPA–DBS$^-$ solutions at various values of $\bar{\nu}$, at pH 9.55 (○, slower component; △, faster component). Data are plotted as areas of individual peaks (resolved as Gaussian peaks from electrophoresis patterns) divided by the total % BPA concentration. Temperature, 22.0 ± 0.1°; solvent, 0.1 M LiCl; field strength, 1.3 v/cm. Extrapolated line is based on the relative linearity of the points obtained at $\bar{\nu} < 30$.

and

$$f_n = \frac{b_2 A_n}{(\mathrm{BPA})_T} \tag{7}$$

where A_m and A_n are the areas under the AD$_m$ and AD$_n$ peaks, respectively; b_1 and b_2 are factors which convert the total areas A_m and A_n, respectively, to concentrations of BPA existing as the corresponding component; and (BPA)$_T$ is the total BPA concentration in the sample.

Making the appropriate substitutions

$$\frac{A_m}{(\mathrm{BPA})_T} = -\frac{1}{b_1(n-m)}\bar{\nu} + \frac{n}{b_1(n-m)} \tag{8}$$

and

$$\frac{A_n}{(\mathrm{BPA})_T} = \frac{1}{b_2(n-m)}\bar{\nu} - \frac{m}{b_2(n-m)} \tag{9}$$

Thus, if $A_m/(\mathrm{BPA})_T$ is plotted *vs.* $\bar{\nu}$, a straight line with slope $-1/b_1(n-m)$ and intercept of the abscissa equal to n should result if b_1, n, and m are constant. Also, $A_n/(\mathrm{BPA})_T$ *vs.* $\bar{\nu}$ will give a straight line whose slope and intercept of the abscissa are $1/b_2(n-m)$ and m, respectively, if b_2, n, and m are constant. Similar equations result from consideration of samples containing only AD$_n$ and AD$_{2n}$. Variation in either n or m or both with increasing $\bar{\nu}$ would be expected to result in curvature of such plots.[2]

Plots of area/(BPA)$_T$ *vs.* $\bar{\nu}$ are presented in Figure 6 for the electrophoretic experiments conducted at pH 6.5. The number of data points is small and there is obviously error in the determination of areas, but reasonably straight lines can be drawn which indicate (by the intercepts on the abscissa) that for this system $m = 11 \pm 1$, $n = 38 \pm 1$, and $2n = 76 \pm 2$. Apparently m, n, and $2n$ do not change significantly with $\bar{\nu}$. This conclusion is supported by the data in Figure 4 which show that the mobilities of all three complexes remain virtually constant as $\bar{\nu}$ increases from 12 to 76. This constancy cannot be attributed to a neutralization of charge effects due to changes in binding of other ions accompanying detergent binding. The differences in mobility of the three complexes AD$_m$, AD$_n$, and AD$_{2n}$ together with the increase in mobility of the highest complex upon binding about 10 additional detergent anions/BPA beyond $\bar{\nu} = 76$ strongly argue against such a neutralization. It seems safe to conclude that all three complexes possess virtually constant composition throughout the range of their coexistence.

The evidence that the BPA–DBS$^-$ complexes which form at pH 6.55 are AD$_{11}$, AD$_{38}$, and AD$_{76}$ indicates that in this system the number of detergent anions bound in AD$_{2n}$ is indeed twice the number bound in AD$_n$, as suggested first by Putnam and Neurath (1945). However, those workers found n to be *ca.* 55. Although Pallansch and Briggs (1954) observed no AD$_{38}$ complex, they did observe one whose approximate composition was AD$_{80}$. It is conceivable that the conditions employed by Pallansch and Briggs were such that only AD$_m$ and AD$_{2n}$ existed, though their conditions were not drastically different from those of Yang and Foster (1953a) and Putnam and Neurath (1945). Yang and Foster observed only an AD$_{48}$ complex, but concluded that this corresponded to AD$_n$.

As has been observed by Aoki (1958), the electrophoretic behavior of BPA–detergent systems near pH 9 differs markedly from that at neutral pH. The data

[2] The possibility of an adventitious cancellation of variations in n and m and the associated b_1 and b_2 cannot be eliminated but seems remote.

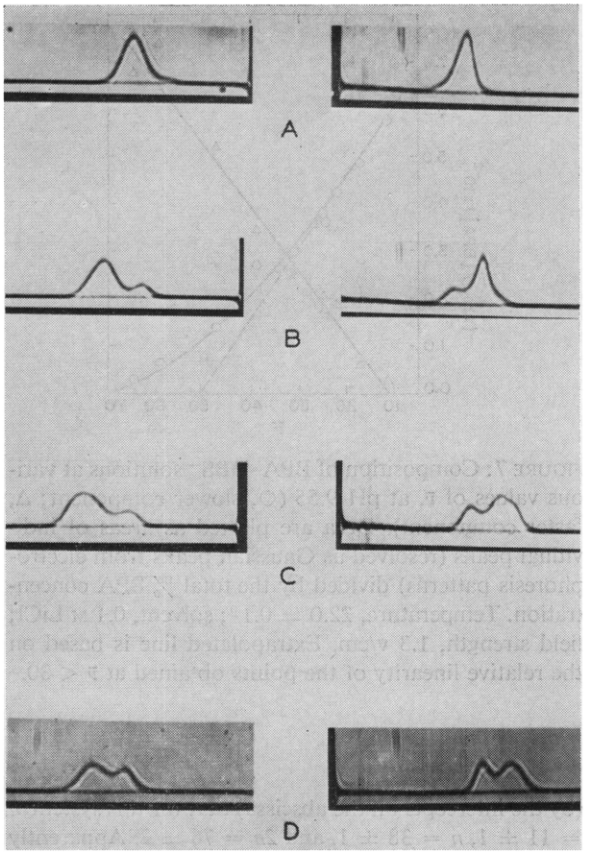

FIGURE 8: Representative electrophoresis patterns for the BPA–DBS^- system at $\bar{\nu} = 38$ and various pH values: solvent, 0.1 M LiCl; temperature, 22.0 ± 0.1°; field strength = 1.5 v/cm. Ascending patterns are on the right, and the corresponding descending patterns are on the left; the direction of boundary movement is to the right and left, respectively. Photographs taken 12,000 sec after initiating run: (A) pH 5.70; (B) pH 6.71; (C) pH 7.64; and (D) pH 9.68.

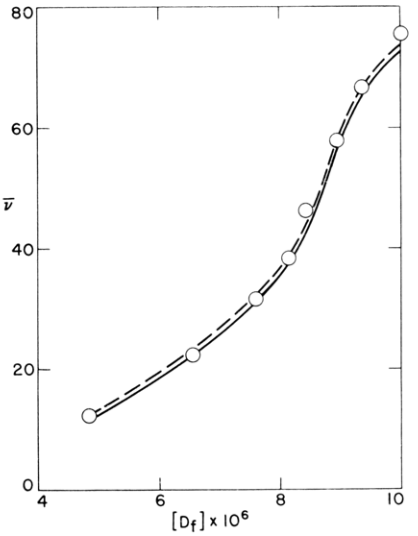

FIGURE 9: Theoretical binding isotherms as calculated for the mechanisms presented. Experimental values are given by the circles; broken curve, fully cooperative mechanism with constant composition for the three complexes; solid curve, statistical binding to equivalent and noninteracting sites on the three isomeric forms of BPA.

obtained in the present work at pH 9.55 were plotted as area/$(BPA)_T$ *vs.* $\bar{\nu}$ according to eq 8 and 9. As can be seen in Figure 7, the slope of the curve for the slower component decreases, indicating that the average number of moles of DBS^- bound per mole of BPA in the faster component increases with $\bar{\nu}$ from an extrapolated value of *ca.* 50 to the intercept value near 70. Evidently the AD_{38} component does not exist at this pH. The increase in mobility of the faster moving peak with increasing $\bar{\nu}$ (shown in Table III) is also suggestive of an increase in the average number of detergent anions bound in this component. It should be noted that the area/$(BPA)_T$ *vs.* $\bar{\nu}$ curve for the faster component intercepts the abscissa at a value of $m = 11$, demonstrating the constancy of m over the pH range 6.5–9.5.

An attempt was made to gain some insight into the disproportionation of AD_n, which apparently takes place between pH 6 and 9, by performing electrophoretic experiments using BPA–DBS^- mixtures with $\bar{\nu}$ *ca.* 38 at various pH values. Some representative patterns from this series of experiments are shown in Figure 8. The disproportionation was expected to take place over the pH range of the alkaline transition (pH 7.5–9.0) observed by Leonard *et al.* (1963). The pattern at pH 5.7 shows essentially a single component with only a trace of slower moving material (presumably AD_{11}). Patterns B–D show increasing amounts of AD_{11} plus a faster migrating peak AD_x, where x is clearly unknown but must be >38. These experiments indicate that the disproportionation begins at a pH below 6.7 and is probably complete prior to pH 9.7.

A Postulated Mechanism for the Detergent Binding. The most remarkable feature of the binding of detergent ions by plasma albumins is the formation of stoichiometric complexes AD_n and AD_{2n}. Complexes of intermediate composition are not seen in the electrophoretic patterns near pH 7. As suggested by Yang and Foster (1953a) and Pallansch and Briggs (1954), AD_n and AD_{2n} must result from structural rearrangements of the protein molecule which expose new binding sites. Even so it is not clear why all newly exposed sites would bind anions without formation of partially saturated complexes.

We assume, as a minimal requirement for explaining the observed results, that there exist three isomeric forms of the protein, A–C. The complete binding curve from $\bar{\nu} = 11$ to $\bar{\nu} = 76$ can be calculated in this case from the electrophoretic composition, f_i, since

$$\bar{\nu} = \Sigma \bar{\nu}_i f_i = 11 f_A + 38 f_B + 76 f_C \qquad (10)$$

The second equality holds if, and only if, the three forms are each saturated with detergent throughout the range of their coexistence. To obtain f_A–f_C from the areas under the respective electrophoretic peaks requires knowledge of the contribution of the protein moiety to the total refractive increment of each component. Making the assumption that the contribution in each case is simply proportional to the weight fraction of protein in the complex (*i.e.*, that the relative refractive increments of protein and detergent are independent of composition of the complex) the f values are given by

f_A = 0.95 area of component 1/total area

f_B = 0.84 area of component 2/total area

f_C = 0.73 area of component 3/total area

The theoretical binding isotherm so calculated is shown in Figure 9 as the dashed curve. The excellent agreement with the experimental binding data shows the internal consistency of the results and gives additional evidence for the near constancy of composition of the various electrophoretic components.

The most obvious way in which this constancy of composition could be explained is by invoking some cooperative interaction between the bound detergent ions. In a sense this case would be analogous to micelle formation, the protein in some way acting as a nucleus for micellization and dictating the size (stoichiometry) of the micelle. This possibility has been considered by Arvidsson (1965). Since the process occurs at free detergent levels well below the critical micelle concentration of the detergent *per se*, the protein would have to stabilize the micelle in some manner. Assuming that the binding to the A form is purely statistical, as seems essentially to be the case from the results presented previously, and that the strong statistical sites in A are destroyed on isomerization to either B or C (as was concluded by Pallansch and Briggs, 1954), this mechanism can be represented as

$$\begin{array}{ccccc} A & \rightleftharpoons & B & \rightleftharpoons & C \\ \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow \\ AD & & BD_n & & CD_{2n} \\ \downarrow\uparrow & & & & \\ AD_2 & & & & \\ \downarrow\uparrow & & & & \\ \downarrow\uparrow & & & & \\ AD_m & & & & \end{array} \qquad (11)$$

Define two intrinsic isomerization constants

$$K_{AB} = [B]/[A] \qquad (12a)$$

$$K_{BC} = [C]/[B] \qquad (12b)$$

Further let the intrinsic constant characterizing the statistical binding to the m sites of A be K_m and define a constant K_n which characterizes the cooperative binding of n detergent ions to B. Assume that binding to C is characterized by a very similar standard free energy change per mole of D so that the cooperative binding constant for this step is K_n^2. The apparent equilibrium constants K_{AB}' and K_{BC}' are then given by

$$K_{AB}' = \frac{[B] + [BD_n]}{[A] + [AD] + \ldots [AD_n]} = K_{AB}\frac{1 + K_n[D]^n}{(1 + K_m[D]^m} \qquad (13a)$$

$$K_{BC}' = \frac{[C] + [CD_{2n}]}{[B] + [BD_n]} = K_{BC}\frac{1 + K_n^2[D]^{2n}}{1 + K_n[D]^n} \qquad (13b)$$

Also

$$\bar{\nu}_A = \frac{mK_m[D]}{1 + K_m[D]} \qquad (14a)$$

$$\bar{\nu}_B = \frac{nK_n[D]^n}{1 + K_n[D]^n} \qquad (14b)$$

$$\bar{\nu}_C = \frac{2nK_n^2[D]^{2n}}{1 + K_n[D]^{2n}} \qquad (14c)$$

Yang and Foster (1953a) suggested, alternatively, that the newly exposed sites bind detergent statistically but with intrinsic binding constants so strong that formation of AD_n and AD_{2n} are essentially complete at detergent levels where the appropriate isomerized forms of the protein exist in significant concentration. It is desirable, in view of the improved data now available, to analyze in more detail the possibility that all of the binding steps could be essentially statistical in character without interaction between binding sites or bound detergent anions. Assuming again three isomeric forms to exist and that the initial m strong binding sites are destroyed on isomerization, this mechanism may be summarized as

$$\begin{array}{ccccc} A & \rightleftharpoons & B & \rightleftharpoons & C \\ \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow \\ AD & & BD & & CD \\ \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow \\ \| & & \| & & \| \\ \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow \\ AD_m & & BD_n & & CD_{2n} \end{array} \qquad (15)$$

Defining K_{AB}, K_{BC}, and K_m as before and defining K_n as the intrinsic binding constant for both forms B and C, it can be shown that

$$K_{AB}' = K_{AB}\frac{(1 + K_n[D])^n}{(1 + K_m[D])^m} \qquad (16a)$$

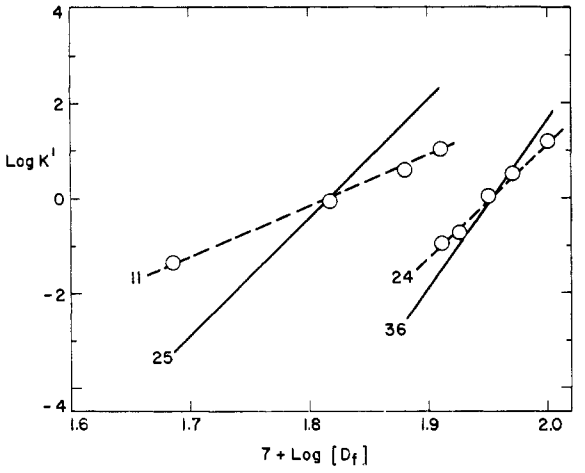

FIGURE 10: Experimental and theoretical dependence of log K_{AB}' and log K_{BC}' on log D_f. The solid lines were calculated for the statistical binding mechanism; circles and dashed lines are experimental values.

and

$$K_{BC}' = K_{BC}\frac{(1 + K_n[D])^{2n}}{(1 + K_n[D])^n} = K_{BC}(1 + K_n[D])^n \quad (16b)$$

Also, in this case

$$\bar{\nu}_A = \frac{mK_m[D]}{1 + K_m[D]} \quad (17a)$$

$$\bar{\nu}_B = \frac{nK_n[D]}{1 + K_n[D]} \quad (17b)$$

$$\bar{\nu}_C = \frac{2nK_n[D]}{1 + K_n[D]} \quad (17c)$$

Assuming m, n, and $2n$ to be fixed by the experimental data as 11, 38, and 76, respectively, there remains the task of finding best values for four parameters: the two isomerization constants K_{AB} and K_{BC} and the two intrinsic binding constants K_m and K_n. To accomplish this a trial and error procedure has been followed. Values for f_A–f_C were taken from the observed electrophoretic boundary areas as before and K_m and K_n were then chosen to yield a good fit of the binding data. Following selection of these values, K_{AB} and K_{BC} were chosen to give best fit of the electrophoretic composition data. The values finally chosen are as follows

$$K_m = 5.0 \times 10^6 \text{ l. mole}^{-1} \quad \log K_{AB} = -27.0$$

$$K_n = 2.0 \times 10^6 \text{ l. mole}^{-1} \quad \log K_{BC} = -48.5$$

In Figure 9 the calculated binding curve (solid curve) is compared with the binding data observed experimentally for free detergent concentrations above 4 $\times 10^{-6}$ M. The fit is seen to be quite satisfactory and indeed virtually as good as is obtained with the assumption of constant composition (dashed curve). In Figure 10 the values for log K_{AB}' and log K_{BC}' as calculated from eq 16a and 16b are compared with the values calculated from the electrophoretic boundary areas. Log K_{AB} and log K_{BC}' are seen to be substantially linear in log D_f. The slopes for the theoretical lines are, respectively, 25 and 36. The corresponding plots for the case of constant composition are not shown but clearly would be linear with slopes of 27 (38 − 11) and 38 (76 − 38), respectively. Not unexpectedly, the experimentally observed slopes are less steep than those calculated for either theoretical mechanism. It has been suggested previously that resolution of the various electrophoretic boundaries implies a microheterogencity of the protein with respect to the detergent binding reactions (Sogami and Foster, 1963). Pallansch and Briggs (1954) concluded that heterogeneity exists with respect to the conversion to the isomeric form responsible for formation of the AD_{80} complex. Such heterogeneity would lead to an artificial reduction in the apparent order of the reaction in detergent. Indeed, it is surprising that the theoretical and observed slopes for the second isomerization reaction are so similar (*ca.* 36 as compared to 24). This implies that microheterogeneity is manifested much more strongly with respect to the first isomerization reaction than with respect to the second.

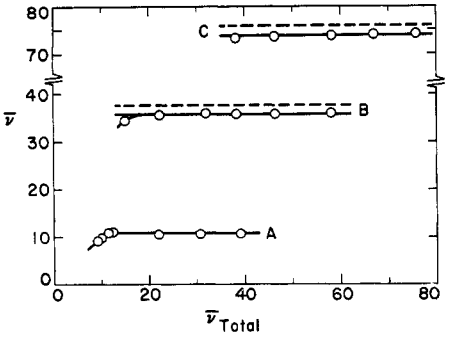

FIGURE 11: Values for $\bar{\nu}_A$, $\bar{\nu}_B$, and $\bar{\nu}_C$, calculated for the statistical binding model, presented as a function of total binding $\bar{\nu}_{total}$. Dashed lines are for the cooperative model.

A more stringent test of the model is the question of whether it can account for the virtual constancy of composition of the various forms over the range of binding in which they are observed. Figure 11 shows the calculated values of $\bar{\nu}_A$–$\bar{\nu}_C$ plotted as a function of total number of detergent ions bound per molecule of protein. The resemblance between these curves and those for mobilities *vs.* $\bar{\nu}$ (Figure 4) is striking. Clearly this model does account quite adequately for the observed constancy of composition of the AD_n and AD_{2n} complexes, and micellization forces or other cooperative interactions are not needed to account for this important feature of the detergent binding behavior.

Although no detailed calculations are given for the model represented by eq 11–14, it can be demonstrated readily that it is equally capable of describing the experimental results of this paper. Equations 13 and 14 for the cooperative model are strikingly similar to those for the purely statistical model, namely eq 16 and 17. In fact it can be shown that under the conditions of these experiments for the first mechanisms to apply eq 14 requires

$$K_m[D] \gg 1$$

and

$$K_n[D]^n \gg 1$$

and for the second mechanism to apply, eq 17 requires

$$K_m[D] \gg 1$$

and

$$K_n[D] \gg 1$$

With these restrictions, it can be seen that eq 13 and 16 are equivalent. It follows that a choice between these models and any of numerous other related and intermediate models must depend on other types of experiments. In many such studies the microheterogeneity of the protein will be a complicating factor. It is ironic that the factor which permits electrophoretic resolution and thereby enabled discovery (by Putnam and Neurath) of the cooperative formation of the various stoichiometric complexes should at the same time mitigate against a fully quantitative understanding of the reactions involved.

It is tempting to suppose that the isomerization reactions involved are the same as the two known steps of the N–F transition, as was concluded by Leonard and Foster (1961). Thus both K_{AB} and K_{BC} are presumed to be pH-dependent parameters and are undoubtedly much smaller at neutral pH than in acid solution.[3] It seems probable that the change in binding behavior at higher pH has its origin in the pH dependence of these constants. At pH 6.5, K_{AB} and K_{BC} are such that formation of C is detectable prior to complete destruction of A.[4] As the pH is raised above neutrality, through the range of the alkaline transition (Leonard *et al.*, 1963), both isomerization constants probably increase.[5] It is suggested that the failure to resolve the intermediate AD_n form at pH 9.5, and the results on the disproportionation of AD_n with increasing pH, described above, result from the fact that K_{BC} increases relatively faster with increasing pH in the alkaline range than does K_{AB}.

Finally, some additional remarks are in order with respect to the assumption that the m sites of the native form of the protein (A in the representation used here) are destroyed on isomerization. This was inferred by Pallansch and Briggs (1954), but further evidence would be desirable. It is probable that this assumption is not critical insofar as the above discussion and conclusions are concerned. If these sites were not destroyed, some of the n sites in B and C would be replaced by m sites. The present binding data could undoubtedly be fitted with no difficulty. However, the form of eq 13, 14 and 16, 17 would be altered substantially and one of the most interesting features of either of the present models would be lost. If the m sites are destroyed on isomerization and replaced by more numerous weaker sites, it follows that under suitable conditions detergent could shift the equilibrium between A and B either way depending on its concentration.[6] This can be seen by inspection of either eq 13a or 16a. This possibility was pointed out by Foster and Aoki (1958) who showed that in fact detergent at low levels does shift the N–F equilibrium toward the N or native form. Various observations that low levels of detergent ions protect albumins toward denaturation have been attributed to this effect (Foster, 1960). More recent observations show that low levels of DS^- repress the high pH expansion of BPA (Lovrien, 1963) and the urea denaturation of human plasma albumin (Markus *et al.*, 1964). In the latter case the detergent binding isotherms actually exhibited a sigmoidal character in the presence of 6 M urea at alkaline pH. It seems quite clear from these results that the m strong sites are destroyed in isomerization. As a consequence the albumin–detergent interaction exhibits some of the properties of an allosteric system. A subsequent paper will describe results of pH titration studies of

[3] With chloride as the only anion, the two steps of the N–F transition appear to merge with a midpoint of *ca.* pH 4. The inability to separate the two steps under these conditions, together with the fact that the exact pH dependence of the transition is not known, prevents meaningful calculations of the expected values of the isomerization constants at the pH values used in the present studies.

[4] The vastly different order of magnitude of the estimated values for K_{AB} and K_{BC} is misleading and results from the high order of the reactions in detergent. Thus at D_f corresponding to the binding of 38 detergent ions K_{AB}' and K_{BC}' differ by only 10^3 theoretically and 10^2 as observed experimentally for the microheterogeneous system. It is interesting that the present system showed no plateau between the first and second cooperative binding steps in the optical rotation experiments (Figure 5) such as was seen by Leonard and Foster (1961) in experiments with DS^-. On the basis of several criteria, the BPA used in the present work appears to be relatively homogeneous when compared with other preparations. If the BPA used in the present study is more homogeneous than that used by Leonard and Foster, the plateau would be expected to be more pronounced and the transitions sharper than in the earlier experiments.

[5] Increases in these constants would lead to an increase in binding affinity at elevated pH. This tendency would be counteracted by the increased electrostatic repulsion due to increasing negative charge on the protein. Evidently the latter effect dominates as shown by Figure 3.

[6] For this to occur in the fully statistical case of binding it is required that $mK_m > nK_n$. This requirement is not quite met by the binding constants derived for the present case but might be met under other conditions. The requirements are less stringent for the fully cooperative case and this might be taken as evidence that the latter mechanism is more nearly correct.

AD_m and AD_{2n} and will lend further support for a stabilization of the native form by binding to the first (m) sites.

Acknowledgment

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