of triethylamine in 15 cc. of tetrahydrofuran was cooled to −5° and 1.0 g. (0.007 mole) of isobutyl chlorocarbonate added with stirring. After 5 minutes at this temperature a solution of 1.67 g. (0.007 mole) of L-phenylalanylglycine monohydrate in 7 cc. of 1 *N* sodium hydroxide was added with stirring and the reaction mixture was allowed to warm to room temperature during 30 minutes. On extraction of this solution with ether, the sodium salt of the product separated immediately as a colorless, amorphous solid. This was filtered off, washed with water, resuspended in dilute hydrochloric acid and stirred for several hours at room temperature to convert the salt to the free acid. The product was again filtered off, washed with water and crystallized from a mixture of 25 cc. of glacial acetic acid and 10 cc. of water; wt. 1.45 g. (29%), m.p. about 185–188° (indefinite), $[\alpha]^{24}D$ −20.7 ± 0.5° (*c* 2.1, glacial acetic acid).

Anal. Calcd. for $C_{38}H_{47}N_5O_9$: C, 63.58; H, 6.60; N, 9.76. Found: C, 63.13; H, 6.48; N, 9.47.

B. Acid Hydrolysis of the Ethyl Ester.—A 0.30-g. (0.0004 mole) sample of ethyl dicarbobenzoxy-L-lysyl-L-valyl-L-phenylalanylglycinate (m.p. 199–200°) was dissolved in 30 cc. of dioxane containing 2 cc. of 1 *N* hydrochloric acid (0.002 mole) and the solution was heated on the steam-bath for 1 hour and then concentrated to dryness in an air stream. The colorless, solid residue was recrystallized from dilute acetic acid and twice from alcohol–water to give 0.21 g. (73%) of pure material melting at 173–175°, $[\alpha]^{24}D$ −22.0 ± 0.4° (*c* 2.4, glacial acetic acid).

Anal. Calcd. for $C_{38}H_{47}N_5O_9$: C, 63.58; H, 6.60; N, 9.76. Found: C, 63.29; H, 6.62; N, 9.55.

STAMFORD, CONNECTICUT

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, IOWA STATE COLLEGE]

Statistical and All-or-None Binding of Alkylbenzenesulfonate by Albumins[1,2]

BY JEN TSI YANG AND JOSEPH F. FOSTER

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The binding of commercial sodium dodecylbenzenesulfonate (SDBS) by bovine plasma albumin (A) and by ovalbumin (O) has been studied by equilibrium dialysis and electrophoresis. The combination of the two techniques permits further insight into the reaction than can be gained from a study by either method alone. Three regions can be distinguished in the binding curve of A. In the first (region A) the binding is essentially statistical with a limit of about twelve ions per molecule. In region B the reaction is all-or-none giving rise to a complex containing about 48 ions (approximately one per two cationic residues of the protein). In region C the binding is again of a statistical character, no upper limit being attained under the conditions employed. In the case of O the first statistical region is absent, an all-or-none reaction taking place with the binding of about 43 ions per molecule (one per cationic group). This suggests that O is more labile than A and is denatured by the first ions bound. A partial interpretation of the binding curves is undertaken in spite of the heterogeneity of the detergent. It is concluded that the free concentration of true detergent in equilibrium with the all-or-none complex is well below the critical concentration for micelle formation. It is further shown that the presence of micelles is not necessary for the all-or-none reaction (denaturation).

In recent years many investigations have been published on the interaction of proteins with ions. Of particular interest are the surface active ions which produce many diverse effects on proteins and biological systems, such as protein denaturation, dispersion and precipitation.

It is well known that proteins exhibit markedly strong affinity for long-chain alkyl sulfates and alkyl aryl sulfonates. Disagreement arises, however, as to the number of anions bound and also as to the nature of the binding process. Perhaps the most striking feature of the combination in the case of native proteins is the all-or-none character, first demonstrated in the case of ovalbumin by Lundgren, *et al.*,[3] and shown also for serum albumin by Putnam and Neurath.[4] Thus, in the electrophoretic pattern of protein–detergent mixtures of appropriate composition two distinct components are observed rather than the single broad boundary, or possibly a number of poorly resolved boundaries, which would be expected if combination were stepwise. In their classical study Lundgren and coworkers demonstrated that this all-or-none characteristic is absent if denatured ovalbumin is used.

More recently Karush and Sonenberg,[5] in studies at detergent concentration below the critical micelle level, found that binding obeys essentially a statistical law but felt it necessary to invoke the idea of heterogeneous binding sites.

It thus seemed important to reinvestigate the combination over a broad range of detergent concentration covering both the regions studied by Karush and Sonenberg and by the other workers. In this paper are presented results on the interaction of both serum albumin and ovalbumin with dodecylbenzenesulfonate. The results are in substantial agreement with those of all the abovementioned authors but lead to a different interpretation from that given by Karush and Sonenberg.

Experimental

Materials.—Crystalline bovine plasma albumin (hereafter called A) was obtained through the courtesy of Armour and Company. Ovalbumin (O) was prepared in the cold room (1–3°) from fresh egg white by a modification of the procedure of Sørensen and Høyrup.[6] Crude O was recrystallized thrice from ammonium sulfate at its isoelectric point, dialyzed free of salt against distilled water, lyophilized to dryness and kept in the cold room.

Santomerse No. 3, principally sodium dodecylbenzenesulfonate (hereafter called SDBS), was supplied by the Monsanto Chemical Company and freed of inorganic salts by dispersion in 95% ethanol. The dried SDBS was kept in an air-tight bottle. Its apparent number-average molecular weight was 354, as determined by Parr bomb sulfur analysis, the theoretical value being 348.

Partial purification of the commercial detergent was achieved by either continuous dialysis or partial precipita-

(1) Journal paper No. J-2170 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project 978. Supported in part by a grant from Swift and Company and in part by the Office of Naval Research under Contract Nonr-803(00).

(2) Taken in part from a thesis presented by Jen Tsi Yang in partial fulfillment of the requirements for the degree Doctor of Philosophy, Iowa State College, 1952. Presented at the 122nd meeting of the American Chemical Society, Atlantic City, N. J., September, 1952.

(3) H. P. Lundgren, D. W. Elam and R. A. O'Connell, *J. Biol. Chem.*, **149**, 183 (1943).

(4) F. W. Putnam and H. Neurath, *ibid.*, **159**, 195 (1945).

(5) F. Karush and M. Sonenberg, THIS JOURNAL, **71**, 1369 (1949).

(6) S. P. L. Sørensen and H. Høyrup, *Comp. rend. trav. lab. Carlsberg*, **12**, 164 (1917).

tion at low temperature. A 5% buffered SDBS solution was dialyzed against 16 liters of the same buffer which flowed at a slow rate. Most of the lower homologs could diffuse freely through the membrane, whereas the higher ones in micellar state remained non-diffusible.[7] Similar results were obtained by partial precipitation of a 1% buffered SDBS solution at 1–3°. Most of the lower homologs remained dispersed in the solution. The precipitate containing the higher ones was separated from the solution by centrifugation and redispersed in distilled water. The higher homologs from both purification schemes were employed for binding studies (see below).

Phosphate–NaCl buffer was made up with reagent-grade chemicals: 0.0321 M K_2HPO_4, 0.0036 M KH_2PO_4 and 0.100 M NaCl; pH 7.7 and ionic strength 0.20.

Electrophoresis.—Electrophoretic analyses were carried out against phosphate–NaCl buffer at 2.0° in the Tiselius-type cell, with a modified Philpot–Svensson cylindrical lens-oblique slit *schlieren* system.

Equilibrium Dialysis.—Visking cellulose casings, $^{20}/_{32}$ inches in diameter, were used for routine analyses. The bags containing phosphate–NaCl buffer were previously dialyzed against a large volume of the same buffer for about ten days, fresh buffer being used every two or three days. Twenty-ml. portions of buffered protein–SDBS solution were placed in the air-tight bags and then equilibrated against equal volumes of the buffer in test-tubes. Controls containing buffer only inside the bag were prepared in the same manner. The tubes were shaken gently in the cold room for two days following which the dialyzates were diluted, if necessary, and analyzed spectrophotometrically. All experimental data reported correspond to the average of at least two and in most cases three independent sets of data.

Protein Concentration.—The protein concentrations in solutions were determined by the micro-Kjeldahl method assuming values of 15.95% nitrogen for A and 15.78% for O.

SDBS Concentration.—A spectrophotometric method has been developed to measure quantitatively SDBS concentrations as low as $10^{-5}M$.[7] Measurements of the ultraviolet absorption were made on a Beckman model DU spectrophotometer at a wave length of 223 mμ, the apparent molar extinction coefficient, ε, being 11,100. The solutions to be analyzed were previously centrifuged at 20,000 g in the Sorvall centrifuge for 30 minutes. Random errors were observed in the second decimal of the optical densities, mostly due to impurities in the cellophane bags. Even after pretreatment as above a blank error of about 0.05 or higher in optical density was frequently obtained. Thus control runs were often made to minimize this error.

Results

Modified Equilibrium Dialysis.—In the literature equilibrium dialysis has been carried out by equilibrating the protein solution inside the cellophane bag against the outside ions studied. In the case of SDBS the anion concentration was limited by its low solubility at 1–3° in the presence of buffer. Consequently a large volume of SDBS solution at very low molar concentration was required to cover the whole range of interaction studied. Under such conditions the reaction was found to be incomplete even after one-month dialysis (see Discussion). A typical example is shown in Figs. 1 and 2. Portions of 20 ml. buffered A solution were equilibrated against a series of 2 liters of buffered SDBS solution and electrophoresis was run at definite intervals. In Fig. 1 only a slight decrease in SDBS concentration has taken place in the first several days, whereas in Fig. 2 the electrophoretic patterns clearly reveal the change of relative area between A and its SDBS complex, *i.e.*, the increase of A–SDBS complex with increasing time of dialysis.

(7) J. T. Yang and J. F. Foster, *J. Phys. Chem.*, **57**, 628 (1953).

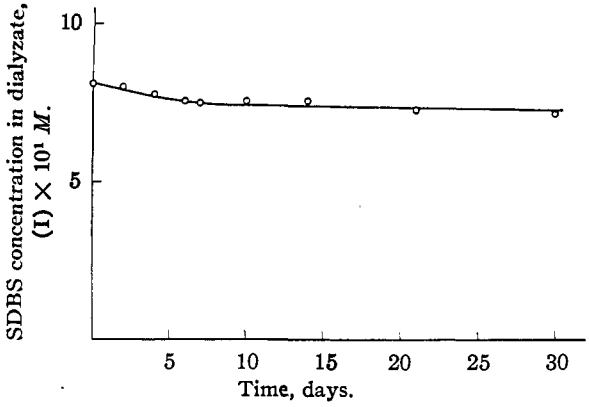

Fig. 1.—Equilibration of A (4.8×10^{-5} M) against SDBS (initial concentration 8.1×10^{-5} M).

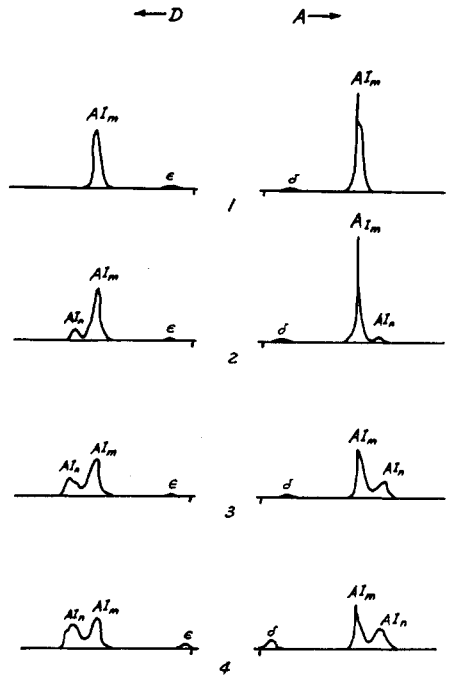

Fig. 2.—Electrophoretic analyses of A–SDBS mixtures; dialysis time: 4, 10, 20 and 30 days.

In this paper a modified procedure was adopted. Portions of A–SDBS or O–SDBS mixtures in phosphate–NaCl buffer were stored for at least two days at 1–3° and then dialyzed against equal volumes of the same buffer for another two days. The amount of free anion was finally determined in the dialyzate. This modification had two advantages over the conventional method: First, it was possible to cover a much wider range of SDBS concentration, due to the fact that most of the anion was immediately bound to the protein and thus not precipitated out. Secondly, the interaction was virtually complete after one-day dialysis.

Binding Curves of A–SDBS Mixtures.—The effect of A–SDBS mixing ratio on the binding capacity is illustrated in Fig. 3, where the average number of moles of SDBS bound per mole protein, r, is plotted against the free SDBS concentration. The protein concentration was kept constant at 5.9×10^{-5} M, the molecular weight of A being assumed to be 69,000. The initial SDBS concen-

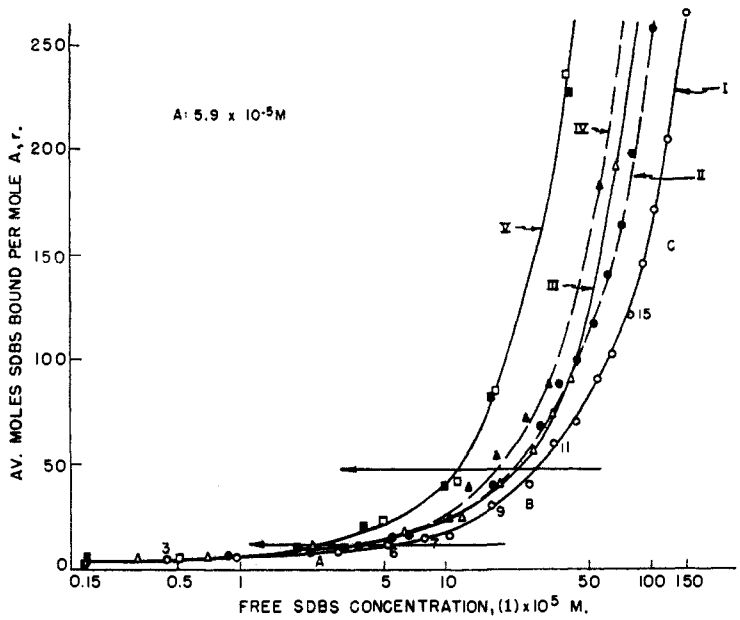

Fig. 3.—Combination of A with SDBS at 1–3°: circles, Santomerse No. 3; triangles, fractionated SDBS (by dialysis); squares, fractionated SDBS (by precipitation): solid curves, first equilibration and broken curves, second equilibration.

tration varied from 1×10^{-5} M to 5×10^{-2} M. Curves I and II represent the first and second equilibrium dialysis results, respectively, using the commercial detergent as a whole. The second dialysis was accomplished by re-equilibrating the solutions from curve I against equal volumes of fresh buffer and determining the new free anion concentration. Curves III and IV represent results of the binding by A of fractionated SDBS (by continuous dialysis). Curve V was obtained by using fractionated SDBS (by partial precipitation). Both first and second equilibration results are virtually on the same curve in this case.

Binding Curves of O–SDBS Mixtures.—For the sake of comparison similar experiments were performed on O–SDBS mixtures. The results are plotted in Fig. 4, where the protein concentration was kept constant at 9.3×10^{-5} M, assuming a molecular weight of 44,000.

Electrophoretic Analyses.—In order to determine the nature of the binding processes, electrophoretic analyses were performed on the various equilibrated protein–SDBS solutions shown in the binding curves. Some of the representative patterns for A–SDBS mixtures are given in Fig. 5 and the experimental data of curve I of Fig. 3 are listed in Table I. Inspection of the patterns clearly revealed that the binding curves underwent three different stages, which were arbitrarily labeled as regions A, B and C and separated by the two horizontal lines which intersect the binding curves. In region A only a single boundary was observed, the mobility of which was close to that of native protein but increased progressively as the number of ions bound increased. In region B an additional faster boundary migrated as a separate component. At constant protein concentration the relative area of this faster boundary increased with increasing SDBS concentration at the expense of the slower one. In region C there again appeared a single boundary, the area and mobility of which increased with increasing SDBS concentration.

Similar experiments were performed for the O–SDBS mixtures. Some of the electrophoretic patterns are shown in Fig. 6 and the experimental data for curve I of Fig. 4 are listed in Table II. The notable difference between A and O was that no region A appeared for O–SDBS mixtures.

Electrophoretic analyses were also performed on the complexes prepared with the purified detergent samples in all cases except in a few of the re-dialysis experiments. To economize on space, and since the data on the commercial detergent are most complete and represent studies with the most readily reproducible material, only those results have been included. It can be stated, however, that all results were in complete agreement with the results reported in Tables I and II. In particular, the horizontal lines drawn in Figs. 3 and 4 give a true picture of the electrophoretic behavior observed in all cases.

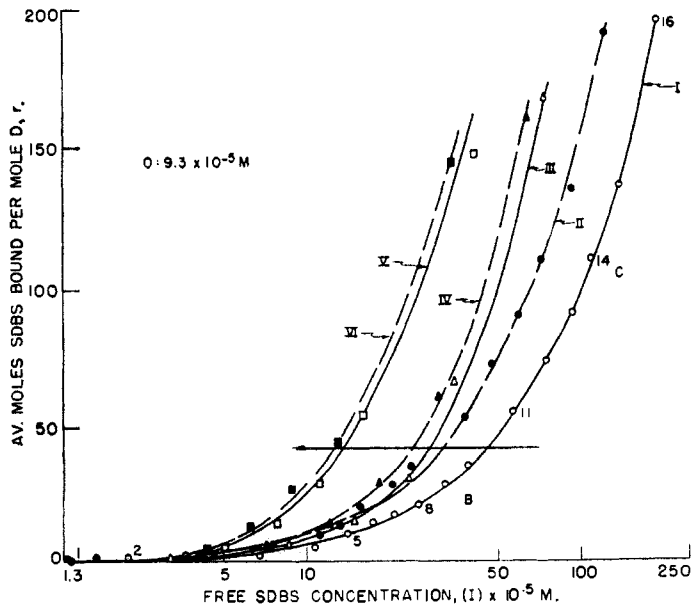

Fig. 4.—Combination of O with SDBS at 1–3°: circles, Santomerse No. 3; triangles, fractionated SDBS (by dialysis); squares, fractionated SDBS (by precipitation): solid curves, first equilibration and broken curves, second equilibration.

Discussion

Statistical Combination in Region A.—It is most significant that absolutely no sign of a separate electrophoretic component is detected in the region

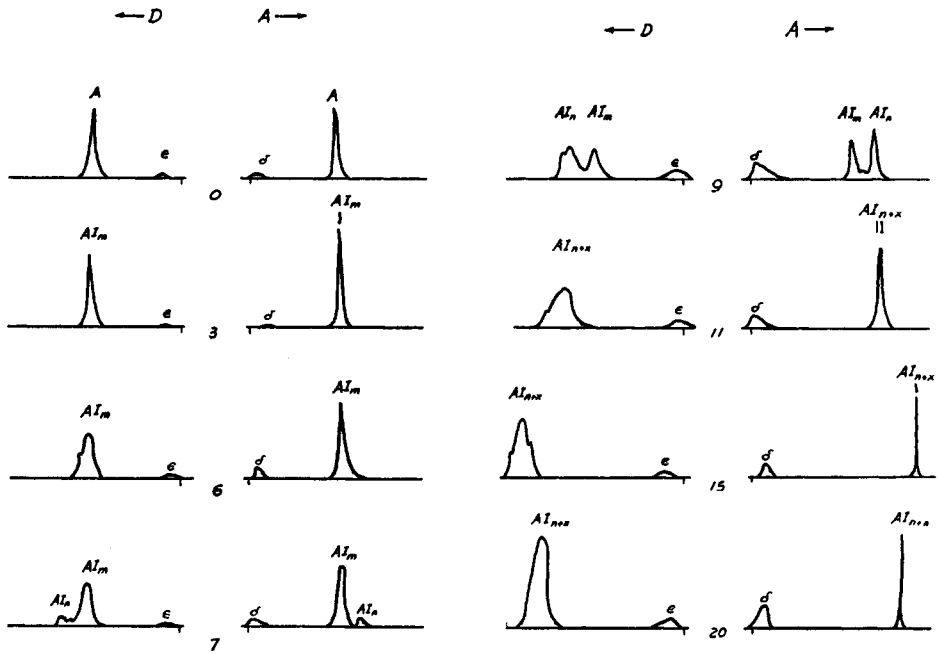

Fig. 5.—Electrophoretic analyses of A–SDBS mixtures at 4–4.5 volt cm.$^{-1}$. Time: 150 min., except No. 15, 140 min. and No. 20, 120 min. No. 0, Native A without SDBS.

TABLE I

COMBINATION OF A WITH SDBS AT 1–3° IN PHOSPHATE–NaCl BUFFER (pH 7.7, Γ/2 0.20). A: 5.9 × 10^{-6} M

Total SDBS concn. × 10^6 M	Free SDBS concn. × 10^6 M	Av. moles SDBS bound per mole protein, r	Mobilities,[a,b] $-\mu$(cm.^{2} volt^{-1} sec.$^{-1}$ × 10^5) A	AI$_m$	AI$_n$	AI$_{n+x}$	Relative area,[c] % A	AI$_m$	AI$_n$	AI$_{n+x}$	Molar ratio I/A in AI$_n$, n
0	0	0	6.6				100				
16.7	0.2	2.8		6.7				100			
30.3	0.4	5.0		7.0				100			
41.8	1.0	6.8		7.1				100			
55.8	3.0	8.5		7.3				100			
83.6	5.2	12		7.2				100			
104.4	7.9	15		7.3	9.1			87.9	12.1		48
125.4	10.5	18		7.3	9.1			71.6	28.4		43
209	17.1	30		7.4	9.3			44.7	55.3		48
293	26.1	41		7.9	9.9			15.8	84.2		48
418	34.6	60				9.8				100	
502	45.3	70				11.0				100	
649	58.0	91				11.5				100	
735	68.3	100				11.6				100	
882	83.7	120				12.4				100	
1044	95.5	150				12.3				100	
1223	109	170				12.5				100	
1457	127	200				13.0				100	
1871	159	260				13.3				100	
2050	176	290				13.6				100	

[a] The values represent the average of the descending and ascending boundaries. [b] m varies from one to about ten, n is about 48 assuming the maximum m is 12, and x is a variable. The average n was calculated from the last column. [c] Relative areas were calculated from the descending patterns.

designated A. The degree of binding at the upper limit of this region, as determined from the equilibrium dialysis results, is such that nearly 30% of the fast component should be present if the same all-or-none mechanism exists here as in region B. Such a component should be readily detected. It seems clear that the detergent is distributing itself in more or less a statistical manner over all of the available A molecules. It is further worth pointing out that there is a significant increase in the mobility of the single component with increasing mean binding ratio r as is to be expected. A slight increase in the mobility of the so-called albumin component was also reported by Putnam and Neurath[4] but the significance was not discussed. It seems clear from the combined results of the two techniques used here that this component is not pure A but a mixture of complexes containing a

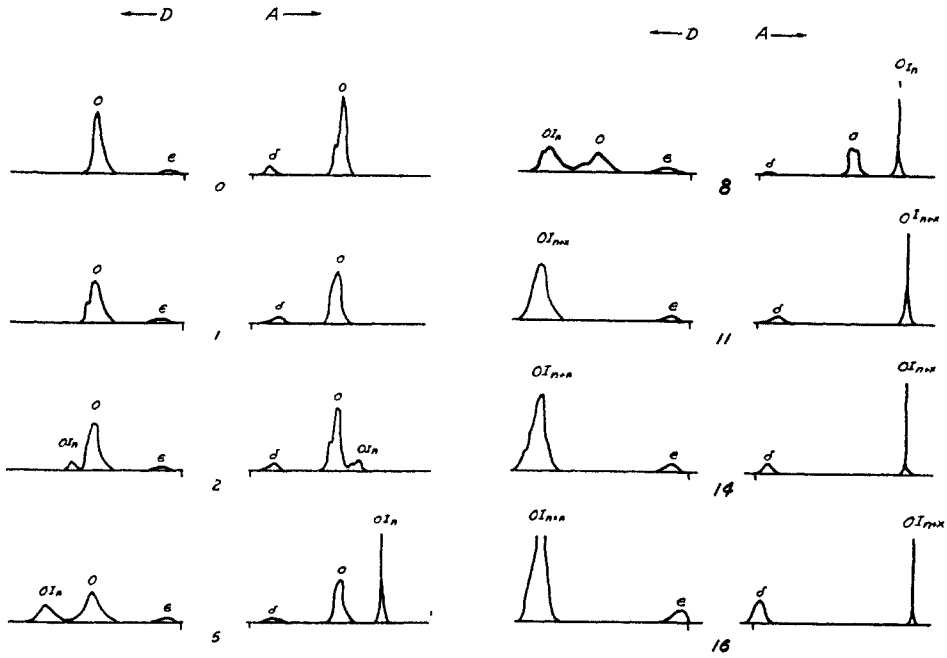

Fig. 6.—Electrophoretic analyses of O–SDBS mixtures at 4–4.5 volt cm.$^{-1}$. Time: 150 min., except No. 14, 133 min. and No. 16, 131 min. No. O, native O without SDBS

TABLE II

COMBINATION OF O WITH SDBS AT 1–3° IN PHOSPHATE–NaCl BUFFER (pH 7.7, Γ/2 0.20), O: 9.3 × 10^{-5} M

Total SDBS concn. × 10^5 M	Free SDBS concn. × 10^5 M	Av. moles SDBS bound per mole protein, r	Mobilities,[b,c] −μ(cm.^{2} volt^{-1} sec.$^{-1}$ × 10^5) O	OI_n	OI_{n+x}	Relative area,[d] % O	OI_n	OI_{n+x}	Molar ratio I/O in OI_n, n
0	0	0	6.4			100			
10.5[a]	1.4	0.8	6.8			100			
20.9[a]	2.3	1.8	6.4	8.0		94.0	6.0		40
41.8[a]	6.8	3.0	6.5	9.7		90.0	10.0		40
83.6	10.8	6.7	6.7	9.8		82.2	17.8		48
125.4	14.4	10	6.8	10.2		67.1	32.9		39
167	17.6	14	6.3	9.9		59.3	40.7		42
209	20.8	18	6.4	10.3		52.6	47.4		46
251	25.8	22	7.0	10.6		43.5	56.5		44
334	32.6	29	6.9	10.5		28.8	71.2		44
418	39.0	37	6.8	10.5		17.3	82.7		47
627	57.5	55			11.3			100	
836	75.5	74			12.1			100	
1044	94.8	91			12.5			100	
1276	112	110			12.7			100	
1580	141	140			13.0			100	
2210	194	200			13.4			100	

[a] In these runs part of the protein precipitated during equilibrium dialysis. [b] The values represent the average of the descending and ascending boundaries. [c] The average value of n is 43 on the basis of the last column and x is a variable. [d] Relative areas were calculated from the descending patterns.

relatively few detergent ions, the number increasing with amount of detergent added.

To test the possible applicability of the simple statistical theory in this region, the conventional plot of $1/r$ *versus* $1/I$ (where I is free SDBS concentration) is shown in Fig. 7. According to the theory developed by Klotz and co-workers[8] this plot should be linear in the statistical case, as indeed is found. The reciprocal of the intercept on the $1/r$ axis gives a value of about twelve, which represents the maximum number of binding sites available in region A. The corresponding equilibrium association constant for the first mole of ion bound was estimated to be about 1.9 × 10^6. The curve is qualitatively very similar to that found by Karush and Sonenberg[5] for sodium dodecyl sulfate.

In view of the deviation from the straight line seen at the end of region A it may well be questioned whether there is any justification for performing such an extrapolation. As will be seen in the next section, this deviation can be attributed to the onset of an entirely different reaction in region B. It seems reasonably certain that there is an essentially statistical reaction with a binding

(8) I. M. Klotz, F. M. Walker and R. B. Pivan, THIS JOURNAL, 68, 1486 (1946).

limit of approximately twelve ions. It is interesting to note that Duggan and Luck[9] found from viscosity measurements that about eight moles of sodium dodecyl sulfate is bound per mole of A to form a stable complex which resisted the normal changes leading to a viscosity rise of the protein in 6 *M* urea. This seems also to indicate that no pronounced structural change of the protein occurs in this first stage of binding, as would be suggested from the essentially statistical character.

All-or-None Reaction in Region B.—Toward the end of region A, the curve in Fig. 7 deviates from linearity in the direction of an increased binding. Karush and Sonenberg[5] explained this on the basis of heterogeneity of binding sites. From the electrophoretic analyses it seems clear that, at least in the present case, such a theory is inapplicable but that rather the deviation must be due to a complete change in the character of the binding reaction. This is not to say that all of the sites on the protein are equivalent; they certainly must not be. The important point is that after approximately 12 ions have been bound by A there is a change in the reaction so that approximately 36 more ions enter essentially as a unit. It seems logical to deduce that after twelve or so binding sites are occupied, further anions begin to disrupt the once tightly folded protein molecule and strive for the less accessible sites. Once the polypeptide chain starts to loosen, the potential barrier to the entrance of anions is reduced, thus resulting in the all-or-none reaction.

Our results in this region are in substantial agreement with those of Putnam and Neurath.[4] The only difference in interpretation is that the slower electrophoretic component is an AI complex rather than A. It should be noted that the mobilities of both components increase slightly but significantly with increasing r through this region. This point will be dealt with in a later section.

Analyses of the relative areas of the electrophoretic patterns under the well-defined boundaries (Table I) indicated that the composition of the complex AI_n was nearly constant and the distribution between the components depended upon the initial mixing ratio. Assuming that the first statistical complex AI_m retained twelve SDBS molecules (*i.e.*, $m = 12$) per molecule A and also, as a first approximation, neglecting the slight difference in refractive index increment between A and SDBS, the average number of binding sites for the AI_n complex was estimated to be about forty-eight, which is roughly equivalent to one half of the basic groups in the protein. Subtracting the value m from n, an additional thirty-six binding sites are available for the second (all-or-none) reaction. This seems to indicate that even in the second stage A is not fully unfolded. According to Putnam and Neurath,[4] horse serum albumin binds with sodium dodecyl sulfate to form two complexes, the so-called AD_n and AD_{2n}, in which the number of the anions bound corresponds, respectively, to one half and to the total number of the basic groups in the protein. In our work on the binding of SDBS by A the second stoichiometric complex was not detected.

(9) E. L. Duggan and J. M. Luck, *J. Biol. Chem.*, **172**, 205 (1948).

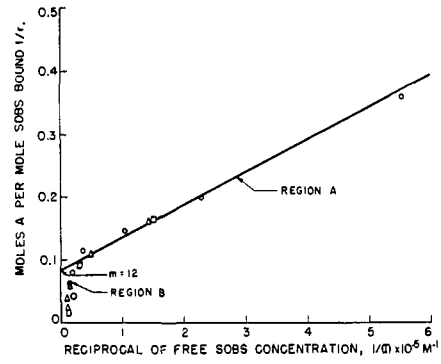

Fig. 7.—Statistical combination of A with SDBS in region A; circles, Santomerse No. 3; triangles, fractionated SDBS (by dialysis); squares, fractionated SDBS (by precipitation).

Combination in Region C.—In region C the binding ratio r is seen to increase apparently without limit, and certainly far exceeds the number of basic groups available in the protein. Presumably the binding in this region is much weaker than in region B. Such "extra bound detergent" was also found by Lundgren[3] from electrophoretic analyses in the case of ovalbumin. The mobility and area of the peak in the electrophoretic pattern both increase with increasing r value in this region as is to be expected. Plots of $1/r$ *versus* $1/I$ in this region are far from linear.

The Binding by Ovalbumin.—The general shape of the binding curves obtained with O is similar to that with A. There is, however, one striking difference and that is the absence of an initial region in which there is statistical binding (Fig. 4). Even for r values of less than two, the all-or-none complex is clearly visible in the electrophoretic patterns. This suggests that either the native protein has much less affinity for SDBS than has A, or that O is much more readily opened up by the bound detergent. Under many other conditions O does indeed seem to be a more labile protein than A.

It should be pointed out that there is one complication in the O binding studies, namely, the tendency of a small proportion of the protein to precipitate at the low SDBS:O ratios. This suggests the presence of some protein which denatures at concentrations of SDBS too low to solubilize the denatured protein.

Another significant difference between O and A is that with O the binding limit in the all-or-none step agrees rather closely with the total number of basic groups in the protein as previously shown by Lundgren and co-workers.[3] Neglecting the correction for the difference in refractive increment between protein and the detergent complex, analyses of the relative areas indicated a binding of about 43 ions per mole (or 1:3 by weight), in excellent agreement with the previously reported value.[3] It can be concluded that ovalbumin not only "opens up" more readily than A in the presence of this detergent, but also more completely.

Possible Complications Due to Micelle Formation.—In control studies without protein it was found that the detergent distributed uniformly across the membrane provided the concentration

was below the critical micelle concentration (CMC). If micelles were present, however, it was found that the distribution was not equal and indeed it has been concluded that micelles never form on the dialyzate side.[7] This raised some question as to the validity of the interpretation of the binding curves, particularly at the higher detergent levels.

Although the free detergent concentration exceeds what is thought to be the CMC in some of the experiments, it is felt that this is due in large part to lower homologs which do not form micelles (see next section). It is concluded that no appreciable concentration of detergent micelles is present in any of the studies on the following grounds:

(1) The limit between regions B and C occurs at a point on the binding curves which is in good agreement with the r values determined from relative areas of electrophoretic peaks.

(2) No detergent component was observed in any experiment.

(3) A less straightforward, but rather convincing, argument can be based on the fact that the solubility of the whole detergent was only 0.01% under the conditions used. It can be shown that at the upper limit of region C the error introduced even if micelles were present in this limiting concentration would be only of the order of 1%.

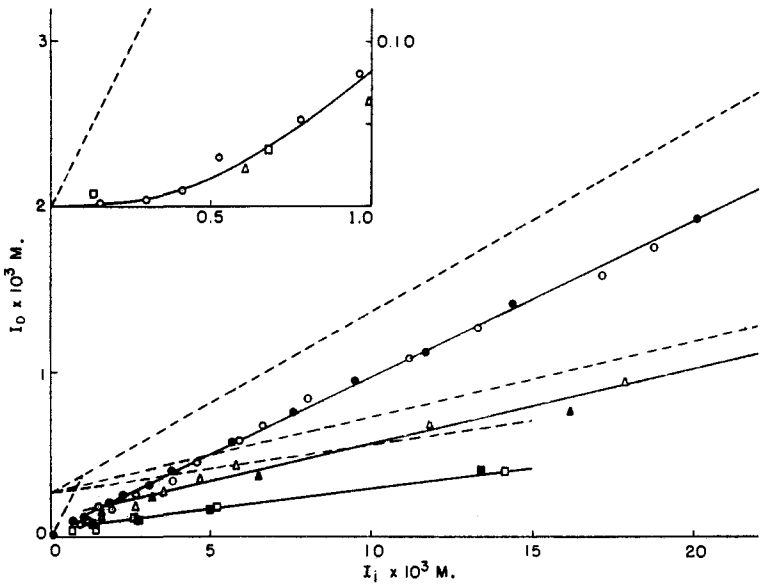

Fig. 8.—Equilibrium dialysis curves of protein–SDBS solutions: abscissa, total SDBS in the dialyzed solution (I_I) in monomeric units and ordinate, SDBS in the dialyzate (I_0); solid lines, protein–SDBS mixtures at 1–3° and broken lines, SDBS controls at 25 ± 3°; circles, Santomerse No. 3; triangles, fractionated SDBS (by dialysis); squares, fractionated SDBS (by precipitation); open symbols, A–SDBS mixtures and full symbols, O–SDBS mixtures.

Complications Due to Heterogeneity of the Detergent.—It was recognized from the beginning of these experiments that the detergents used must be heterogeneous, but it was felt that the expected type of heterogeneity, namely, some distribution in the size of alkyl residues, would not seriously affect the qualitative picture which was sought. The control experiments indicated that the heterogeneity is actually quite different, however, there being present in the commercial detergent about 20% of homologs which do not form micelles at all under the conditions employed.[7] This introduces unexpected complications into the interpretation of the binding curves and it is necessary to discuss these briefly. Before doing so, however, it should be mentioned that a supposedly more highly purified sample of SDBS[10] also was examined and found to give no better results than the technical material.

The discrepancy between the first and second dialysis curves which is so apparent in Figs. 3 and 4 was at first most puzzling and interpreted as meaning that the binding was not entirely reversible. The above-mentioned observation on the character of the heterogeneity immediately suggested the possibility that the non-micelle forming homologs also did not combine with the protein and this offered a possible explanation of the discrepancy. Thus in the first dialysis approximately half of the lower homologs should be removed so that the second equilibrium curve could not be expected to lie on the first. Attempts to purify the detergent by dialysis were not too successful although the binding curves did shift in the expected direction. The precipitation fractionation accomplished a much better purification as judged by the fact that the curves were shifted much further to the left. Further, and most important, the re-equilibration curve now almost coincided with the first curve indicating reversibility.

It seems certain, however, that even this detergent preparation is still by no means homogeneous. It has been found previously[7] that while the precipitation method is more effective than dialysis, the dependence of the free detergent concentration on total concentration is much greater than would be expected for a pure preparation. It was noted that the dependence of free detergent on total detergent in the protein binding curves is quite similar to that in the control dialysis and this is shown clearly in Fig. 8. It is concluded that the free detergent measured is thus largely composed of the lower homologs and that the concentration of the free species which is actually reacting with protein is largely indeterminant. In principle it would be possible to calculate this value by comparison with the control studies; however, in practice this resolves into attempting to determine a very small difference between two large quantities and the results would be largely worthless.

In region A it is interesting to note, however, that the points drop well below the straight line in Fig. 8. It is concluded that in region A even the lower homologs take part in the binding.

It seems clear from these considerations that the concentration of free true detergent ions is far be-

(10) Purchased from Wipaway Products Co.

low the CMC throughout at least region B. On the other hand since micelles are certainly present in this region at the time of preparation of the solutions it is not possible to rule out on this basis the possibility that micelles cause the denaturation step. In view of the fact that we have shown that micelles do not form in the dialyzate of detergent solutions, however, the results of Fig. 2 clearly show that the all-or-none step proceeds in the absence of micelles, although slowly. This result has also been obtained with O. The much faster reaction when detergent and protein are mixed directly may mean that the reaction proceeds more rapidly through a mechanism involving micelles although this cannot be definitely concluded as yet.

On the Mechanism of the Combination.—It thus appears that a conclusive study of binding of detergent ions by proteins will have to await the availability of a really pure detergent preparation, much more homogeneous than any yet available for such purposes. In the meantime it seems possible to draw some conclusions about the probable mechanism. It is suggested that there is a series of reactions of the following sort

$$\left.\begin{aligned} A + I &\rightleftarrows AI \\ AI + I &\rightleftarrows AI_2 \\ &\vdots \\ AI_{m-1} + I &\rightleftarrows AI_m \end{aligned}\right\} \quad \text{I}$$

$$AI_m + I \longrightarrow A^*I_{m+1} \quad \text{II}$$

$$\left.\begin{aligned} A^*I_{m+1} + I &\rightleftarrows A^*I_{m+2} \\ &\vdots \\ A^*I_{n-1} + I &\rightleftarrows A^*I_n \end{aligned}\right\} \quad \text{III}$$

$$\left.\begin{aligned} A^*I_n + I &\rightleftarrows A^*I_{n+1} \\ &\vdots \end{aligned}\right\} \quad \text{IV}$$

The first set of equations (I) is essentially statistical in character. When a critical number m of ions is bound the protein is opened up or denatured as indicated (II). Whether or not this step is reversible cannot be concluded but it seems probable that it is not.[11] Once the protein is opened up, however, a series of equilibria (III) is made available, the binding constants being so great that the formation of AI_n is essentially complete before further combination with AI_m takes place. Finally there are the further equilibria IV which are not important until formation of AI_n is substantially complete.

This mechanism would predict that the free detergent concentration should be substantially constant throughout region B, *i.e.*, the curves in Figs. 3 and 4 should be nearly vertical. Clearly the curves are approaching such behavior more closely with increasing purification and it seems probable that the slope is largely due to the heterogeneity. This is also the explanation, almost certainly, for the failure to find any breaks in the binding curves corresponding to the changes in binding mechanism indicated by the electrophoretic behavior.

There are other minor inconsistencies, notably the slight increase in the mobility of both components in region B in the cases of both proteins. It seems probable that these effects can be explained on the basis of the binding, to a small degree, of lower homologs but it does not seem profitable to discuss these points further at this time.

Acknowledgments.—We are indebted to Armour and Company for generous samples of the bovine plasma albumin used, and to Mr. J. L. Kucera who performed most of the electrophoretic analyses.

(11) That the all-or-none component is not observed in the electrophoretic pattern when heat denatured protein is used has been previously shown for O.[4] The authors have verified this and have shown also that this is true in the case of A.

AMES, IOWA

[CONTRIBUTION FROM THE U. S. PUBLIC HEALTH SERVICE, TUBERCULOSIS RESEARCH LABORATORY, CORNELL UNIVERSITY MEDICAL COLLEGE]

Aromatic Biosynthesis. IX. The Isolation of a Precursor of Shikimic Acid[1]

BY IVAN I. SALAMON[2] AND BERNARD D. DAVIS

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A precursor of shikimic acid in the biosynthesis of five aromatic metabolites has been isolated from culture filtrates of a mutant strain of *Escherichia coli*. It has been characterized as a previously unknown unsaturated dihydroxy-monoketo-carboxylic acid containing seven carbon atoms. Its formulation as 5-dehydro-shikimic acid is discussed.

Mutant strains of *Escherichia coli* and other bacterial species have been described which require for growth five aromatic metabolites: tyrosine, phenylalanine, tryptophan, *p*-aminobenzoic acid and *p*-hydroxybenzoic acid.[3,4] The genetic change in each of these strains has resulted in inability to carry out one of the reactions in the synthesis of a sequence of common precursors of these metabolites. Analysis of this sequence is facilitated by the fact that each mutant accumulates in its culture filtrates the substrate of the blocked reaction, and can utilize later members of the sequence to replace the required aromatic metabolites.

Strains blocked in a relatively late reaction in this biosynthetic sequence (Fig. 1) accumulate considerable amounts (up to 600 γ/ml.) of shikimic acid ($3\beta,4\alpha,5\alpha$-trihydroxy-$\Delta^{1:6}$-cyclohexene-1-carboxylic acid[5]) in their culture filtrates,[3b,6] while

(1) (a) Paper VIII of this series, B. D. Davis and U. Weiss, *Arch. exp. Path.-Pharmakol.*, **219**, 549 (1953). (b) Presented in part before the 119th Meeting Am. Chem. Soc., Boston, 1951, Abstracts of Papers, p. 21C. (c) Aided by a grant from the Squibb Institute for Medical Research.

(2) National Institutes of Health Research Fellow, 1950–1951.

(3) B. D. Davis, (a) *Experientia*, **6**, 41 (1950); (b) *J. Biol. Chem.*, **191**, 315 (1951); (c) *Nature*, **166**, 1120 (1950); (d) *J. Bact.*, **64**, 729 (1952).

(4) The requirement for *p*-hydroxybenzoic acid is in most of the mutants a relative one, and is dependent on the carbon source. A sixth, unknown aromatic factor is also required when the *p*H of the culture is raised to 7.5. *Cf.* refs. 3c and 3d.

(5) H. O. L. Fischer and G. Dangschat, *Helv. Chim. Acta*, **20**, 705 (1937); G. Dangschat and H. O. L. Fischer, *Biochim. Biophys. Acta*, **4**, 199 (1950).

(6) H. Shigeura and D. B. Sprinson, *Federation Proc.*, **11**, 286 (1952).