ence of the vinyl group and appears to a small extent in PVP samples which exhibit an absorption peak in the ultraviolet at 235 mμ.

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A Study of the Interaction of Dodecyl Sulfate with Bovine Serum Albumin[1,2]

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The interaction of dodecyl sulfate with bovine serum albumin, in phosphate–sodium chloride buffer of *p*H 6.8 and ionic strength 0.2, was determined at 22° by the dialysis equilibrium method through a range of equilibrium free detergent concentrations of 0–80 × 10^{-5} *M*. The detergent was dissolved in the external buffer solution and allowed to equilibrate with protein in buffer solution inside a cellophane sac. The equilibrium detergent concentration in no case exceeded the critical micelle concentration of the detergent. Each equilibrated system was subjected to electrophoretic analysis at 20° using the external solution as the overlying solution and the protein solution as the underlying solution in the electrophoresis cell. Moles of bound detergent per mole of protein (*r*) covered a range from 0 to 40. At values of $r > 10$, the course of the interaction isotherm deviated greatly from its initial course, reflecting a greater binding capacity of the protein for the detergent than would be expected on the basis of the simple mass action interaction exhibited at *r*-values less than 10. Concomitantly there appeared a second, faster moving, boundary in the electrophoresis patterns. It is pictured that, as the *r*-values exceed 8–10, the protein is caused to undergo a change in physical organization in which form it exhibits a greater binding capacity for the detergent. The over-all binding of the detergent is the summation of the binding by the two forms of the protein and can be adequately described in terms of two simple mass action functions applying, respectively and in degree proportional, to the two forms of protein present in a given equilibrium system. Evidence is presented that the protein molecules are not equally susceptible to the action of initially bound detergent in causing the change in physical form which is accompanied by the change in interaction capacity.

The capacity for and intensity of the interactions of a protein with small molecular weight substances of known chemical structure, together with any recognizable effects upon the protein molecule which occur as a consequence of such interactions, must reflect important features of the physicochemical structure of the protein. Through the description and interpretation of these interaction properties, a clearer understanding of the complex molecular configuration of the protein itself may eventually be attained.

The interactions of proteins with anionic detergents, particularly with the alkyl and alkyl-aryl sulfates and sulfonates, have received considerable study.[3–7] At low concentrations, dodecyl sulfate has been observed to enhance the resistance of serum albumin in solution to the denaturation effects of heat[3] while at higher concentrations such detergents promote denaturation to such a degree as to allow fiber formation from native corpuscular proteins.[4]

Putnam and Neurath, from studies of the precipitating action of dodecyl sulfate on proteins at *p*H values acid to their isoelectric points[5] and from electrophoresis studies of the complex formed in a phosphate–sodium chloride buffer of *p*H 6.8, ionic strength 0.2,[6] concluded that the interaction involved was of the nature of a stoichiometric binding which occurs to an extent determined by the number of available ionized cationic groups on the protein molecule. With horse serum albumin (P) they reported the formation of two distinct complexes, PD_n and PD_{2n}, where *n* was approximately 55. As increasing proportions of detergents were mixed with the protein, the stepwise formation of PD_n from P occurred in increasing degree, followed by a second stepwise conversion of PD_n to PD_{2n}. From their interpretation of the process it would appear that, at all stages, practically all of the detergent is bound in the complexes and that, starting with the native protein, the individual protein molecules react with *n* molecules of detergent or not at all. When sufficient detergent is present to convert all of the native protein to the PD_n complex an all or none process is repeated to form the PD_{2n} complex. Electrophoresis patterns, obtained with the protein–detergent–buffer as underlying solution and with buffer alone as the overlying solution in the cell, showed peaks for P, PD_n and PD_{2n} and no intermediates, emphasizing the stepwise and apparent all or none nature of the process. When an individual protein molecule is capable of combining with a large number of detergent molecules, it would appear from mass action considerations that such an all or none type of reaction is improbable. However, the sequential and stepwise conversion of the complex into distinct electrophoretic entities of uniform mobility requires explanation.

Karush and Sonenberg[7] used differential dialysis techniques to follow the binding of dodecyl sulfate by bovine serum albumin in 0.025 *M* phosphate buffer, *p*H 6.1. Their studies were restricted to fairly low mixing ratios of detergent and protein where the moles of detergent bound per mole of protein did not exceed approximately 10. The bind-

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(2) This study was supported in part by a research grant from the National Institutes of Health, Public Health Services.

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

(4) H. P. Lundgren, THIS JOURNAL, **63**, 2854 (1941); *Textile Res. J.*, **15**, 335 (1945).

(5) F. W. Putnam and H. Neurath, THIS JOURNAL, **66**, 692 (1944).

(6) F. W. Putnam and H. Neurath, *J. Biol. Chem.*, **159**, 1952 (1945).

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

ing process was shown to be one of a reversible equilibrium existing between bound and free detergent in the system but was not describable in terms of the assumption of a definite number of identical reacting sites on the protein and the simple mass action theory described by Klotz, *et al.*,[8] for interaction under this condition. Deviation from this theory occurred most significantly in the region of higher amounts of detergent bound. Karush and Sonenberg proposed a heterogeneity theory based on a particular distribution of intrinsic binding constants to account for their results.

In the present study the interaction of dodecyl sulfate and bovine serum albumin has been investigated by differential dialysis and electrophoresis methods, identical equilibrium systems being employed in both measurements. A more comprehensive description of the interaction process has resulted.

Experimental

The protein used in this work was from a single lot of Armour's crystallized bovine serum albumin and was shown to be electrophoretically homogeneous in the buffer employed. The stock protein was found to contain 9.1% contaminants, volatile at 105° and atmospheric pressure, adjudged to be H_2O. This factor and a molecular weight of 69,000 were used in the calculation of the molarity of the protein concentrations in the solutions employed.

Sodium dodecyl sulfate was synthesized from Eastman Highest Purity grade lauryl alcohol using the procedure of Dreger, *et al.*[9] The twice recrystallized product was considered to be 100% pure since a comparison of its action on the protein with the action of a pure sample of dodecyl sulfate prepared by the Fine Chem. Div. of du Pont de Nemours and Co. revealed no detectable interaction differences.

Visking cellulose casing, found to have no detectable interaction with the alkyl sulfate employed, was used in all dialysis experiments.

The protein–detergent interaction was studied in a buffer having a calculated ionic strength of 0.20 and a measured *p*H of 6.8 containing 0.025 mole of Na_2HPO_4, 0.025 mole of KH_2PO_4 and 0.100 mole of NaCl per liter. Reagent grade salts were used without further purification in the buffer preparation. Preliminary studies showed that the solubility of the alkyl sulfate in this buffer dropped sharply with temperature below 18°. Above this temperature dilute solutions of the detergent on standing for periods longer than 36 hours underwent detectable hydrolysis. These facts were kept in mind in the design and execution of the experimental work. In no case did more than 24 hours elapse between the start of any dialysis experiment and the completion of the subsequent electrophoretic analysis.

Points determining the shape of the adsorption isotherm were obtained using the equilibrium dialysis technique.[8] The protein solutions in the equilibrated dialysis systems used for each determination were then further analyzed by standard electrophoresis methods employing a Klett–Longsworth–Tiselius apparatus.

Fresh solutions, prepared by dissolving accurately weighed amounts of protein or alkyl sulfate in buffer were used in each dialysis experiment, all of which were carried out in a room maintained at 22°. The electrophoretic analyses were performed in a water-bath at 20 ± 1°. Since Karush[7] has shown the binding of sodium dodecyl sulfate by serum albumin to be practically independent of temperature, the temperature differential between the two types of experiments is considered inconsequential. Trial runs showed that in this temperature range the dialysis systems attained equilibrium within a ten-hour period when the solutions on both sides of the dialysis membrane were stirred continuously. In practice the dialysis was extended somewhat beyond this period as a precautionary measure.

The analytical method used for determining the concentrations of detergent in the equilibrium solutions was that described by Karush and Sonenberg[10] modified slightly to obtain lower blank values by the use of a 25% chloroform–75% carbon tetrachloride mixture in the organic phase. The rosaniline hydrochloride employed was a product of Eastman Kodak Co., recrystallized twice from water and vacuum dried before use. Absorption values at 5450 Å. were measured with a Beckman spectrophotometer.

An initial series of experiments was performed with systems in which 25 ml. of the buffer solution containing 3.30×10^{-6} mole (1%) of protein was sealed into a length of cellulose tubing and dialyzed against 1975 ml. of buffer–detergent solutions. The initial concentration of the detergent solution in each system was chosen so as to obtain a comparatively uniform distribution of experimental points along the measurable isotherm. The maximum amount of detergent that could be attained in any system was limited only by the solubility of the detergent in the external volume of buffer employed. In all these experiments the detergent reached the protein only by diffusion from the external solution. After dialysis with continuous stirring for a period of 14 hours, the equilibrium detergent concentration in the solution outside of the dialysis sack was determined and the protein solution inside the sack was examined electrophoretically immediately thereafter using the equilibrium solution against which it had dialyzed as the overlying solution in the Tiselius cell. An 11-ml. cell equipped with standard high capacity electrode vessels was routinely used in this series of experiments. Calculations of the amounts of detergent bound by the protein in these systems involved the observation of small differences between two relatively large numbers. A consequent low degree of accuracy was attainable, particularly at low detergent concentrations.

Improvement in analytical accuracy was obtained when the entire series of experiments was repeated using systems in which 25 ml. of the protein–buffer solutions was equilibrated against only 225 ml. of buffer–detergent solution. The general procedures and the conditions employed were as described above. To utilize the smaller available volume of equilibrated detergent solution in the electrophoresis experiments, the Tiselius cell was equipped with the modified electrode vessel described by Alberty.[11] Mobility values of higher accuracy were also obtainable with this modification.

The electrophoresis patterns obtained at 20° were kept free of anomalous convection peaks by employing low field strengths over extended periods of time. In all runs field strengths were held at values below 1 volt per cm. The time duration of individual analyses varied between 20,000 and 30,000 sec. These optimal conditions were empirically arrived at in preliminary studies. Analysis of whole blood serum samples under these conditions revealed no unusual pattern formation as compared to that obtained at 4°. The absence of δ and ϵ boundaries in the electrophoresis patterns however, could mean that these boundaries were normally very weak and were destroyed by the weak convection currents not avoidable at this temperature.

The enlarged image of each Tiselius pattern was projected onto graph paper and traced by hand. The curves so obtained were resolved into their individual peaks, where necessary, by the method of Tiselius and Kabat.[12] The areas under the curves were measured with a planimeter.

Specific refractive increments of the protein and of the detergent, in the buffer solution, were determined with a differential refractometer (Phoenix Precision Instrument Company, Philadelphia). These were found to be 0.00178 per gram for the protein and 0.00112 per gram for the dodecyl sulfate. In calculating the relative amounts of the total protein distributed in the various boundaries corresponding to the peaks in the electrophoresis patterns, these specific refractive increments were employed as described below.

Results

The Interaction Isotherm.—The quantities measured in the two series of differential dialysis and electrophoresis experiments are summarized in Table I. The moles of detergent bound per mole

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

(9) E. E. Dreger, G. I. Keim, G. D. Miles, L. Shedlovsky and J. Ross, *Ind. Eng. Chem.*, **36**, 610 (1944).

(10) F. Karush and M. Sonenberg, *Anal. Chem.*, **22**, 175 (1950).

(11) R. A. Alberty, *J. Phys. Colloid Chem.*, **54**, 47 (1950).

(12) A. Tiselius and E. A. Kabat, *J. Exp. Med.*, **69**, 119 (1939).

of the protein, $D_B/P = r$, at equilibrium were calculated by the relationship

$$r = \frac{D_T - [D]_F V}{P} \quad (1)$$

where

D_T = total moles of detergent initially added to the system
$[D]_F$ = concn. of detergent in the equil. dialysate (moles/l.)
P = moles of protein present in system
V = vol. of the total fluid in system (l.)

In Fig. 1 the plot of r *versus* $[D]_F$ illustrates the adsorption or binding isotherm characteristic of the interaction for r-values varying from $r = 0.85$ (expt. 51) to $r = 40$ (expt. 25). Experimental data for the two series are given as points on the graph. Included in this figure, also, are two theoretical curves showing the forms that adsorption isotherms would take if the simple theory of the mass action law were applicable and described by the relationship $r = n[D]_F/(K + [D]_F)$ where n is the number of equivalent sites per molecule of protein at which interaction could occur, and K is the intrinsic dissociation constant of the interaction. In the two curves shown $n = 10$ and $K = 5 \times 10^{-5}$ for the lower curve, and $n = 80$ and $K = 2 \times 10^{-4}$ for the upper. The significance of these curves will become apparent later. In Fig. 1 there are also plotted the results obtained by Karush and Sonenberg[7] from differential dialysis studies of the interaction of bovine serum albumin and dodecyl sulfate. The ionic strength and the pH of their system were lower than those used in the present experiments, which may account for the divergence in the positions of the isotherms in the two cases.

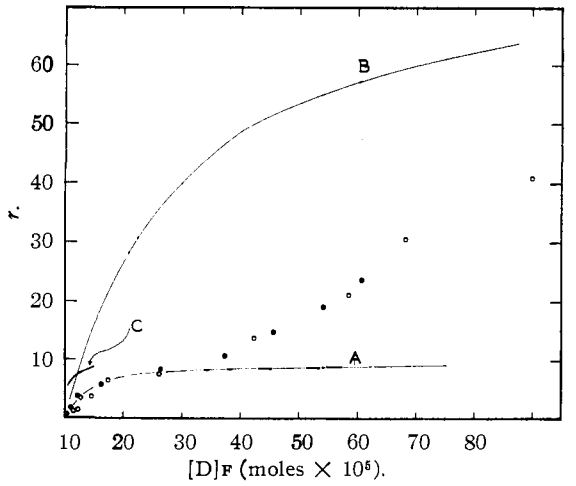

Fig. 1.—Interaction isotherm—moles bound detergent per mole protein (r) *versus* equilibrium free detergent concentration $[D]_F$. Points are experimental values (open circles, 2000 ml. systems; closed circles, 250 ml. systems): A = theoretical isotherm for $n = 10$ and $k = 5 \times 10^{-5}$; B = theoretical isotherm for $n = 80$ and $k = 2 \times 10^{-4}$; C = experimental data for Karush and Sonenberg.[7]

In Fig. 2, the binding isotherm is plotted in the form suggested by Scatchard[13] as that rendering the most dependable extrapolation for the purposes of estimating n and K values in the case of simple mass action law application. Here the equation for the isotherm assumes the form

$$r/[D]_F = \frac{n}{K} - \frac{r}{K}$$

and when $r/[D]_F$ is plotted against r, extrapolation to intersection on the r-axis yields the value of n and extrapolation to the $r/[D]_F$ axis yields n/K. In Fig. 2 the experimental values of $r/[D]_F$ *versus* r, while not as consistent as would be desired at low values of r, nevertheless indicate that the *initial* binding of detergent by the protein may satisfactorily be assumed to agree with that described by the above equation, when $n = 10$ and $K = 5 \times 10^{-5}$. A line is drawn on the figure, based on these values of n and K. The experimental points, for values of $r < 8$–10, scatter fairly uniformly about this line. Above the value of $r = 10$ however the experimental points diverge radically from this simple isotherm.

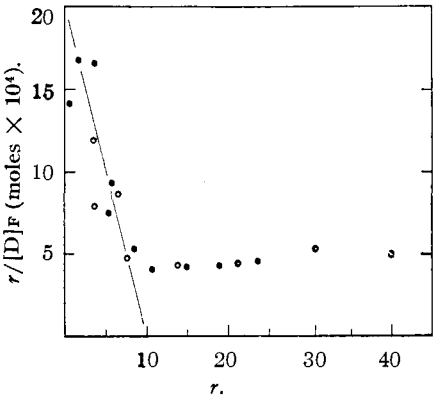

Fig. 2.—Interaction isotherm—$r/[D]_F$ *versus* r. Points are experimental data (denoted as in Fig. 1). Solid line drawn for theoretical isotherm where $n = 10$ and $k = 5 \times 10^5$.

The Electrophoresis Analyses.—In the electrophoresis experiments the dialysate containing the equilibrium concentration of detergent was employed as the overlying solution. The protein detergent complex dispersed in the equilibrium solution formed the underlying solution in the electrophoresis cell. Under these conditions, if only one electrophoretic species of protein exists in the solution and this protein exhibits a uniform binding tendency for the detergent, only one migrating peak should be observed in each leg of the cell and these should migrate with equal mobility (δ and ϵ boundary effects neglected). This mobility would be the constituent mobility of the protein ($\bar{u}_p$) in the equilibrium detergent solution. At the pH of these experiments both the protein and the detergent ion bear a net negative charge and the value of $\bar{u}_p$ should increase linearly with increase in r if each bound detergent ion increases the charge on the complex in an equivalent degree.

In Fig. 3 are shown the electrophoresis patterns of a series of experiments in which r was varied from zero to approximately 25. All these patterns were obtained at 20°, after 26,600 seconds electrophoresis time at a field strength of 0.955 volt per cm. At r-values below 8–10, a single boundary, A, migrated. As the value of r increased above this

(13) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

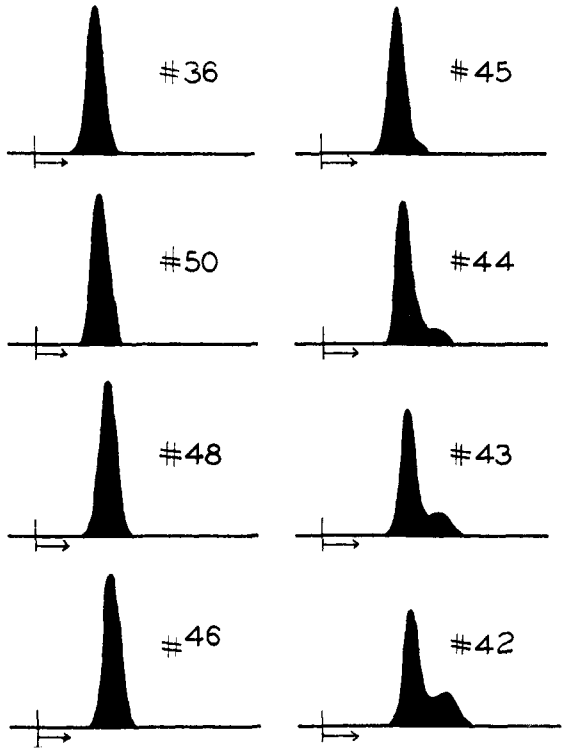

Fig. 3.—Electrophoresis patterns of descending boundaries, after 26,600 seconds at 0.95 volt/cm. field strength and 20°, for experiments of corresponding numbers given in Tables I and II.

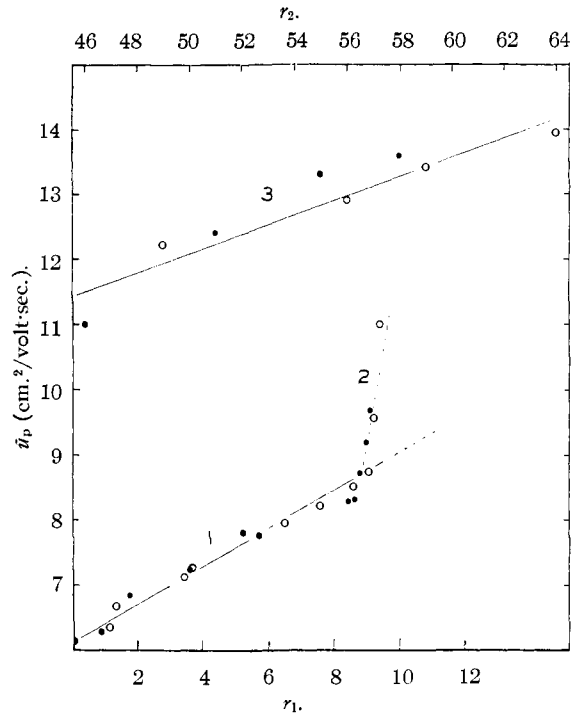

Fig. 4.—Mobilities of A and B peaks *versus* the r-values of the complexes migrating in the corresponding boundaries. In curve 1, r_1 (= r) is calculated according to equation 1. In curves 2 and 3, r_1 and r_2 are obtained by use of equation 5a from values of r calculated according the equation 1 in those experiments where both A and B peaks are present in the electrophoresis patterns. Open circles, 2000 ml. systems; closed circles, 250 ml. systems.

range there appeared a second boundary, B, of higher mobility and of progressively higher relative area in the electrophoresis patterns. From a plot of r *versus* mobility of the A boundary (as measured in the descending leg of the cell) it is shown in Fig. 4 that $\bar{u}_p$ increases proportionally with r throughout the range where $r < 9$ according to the relationship

$$\bar{u}_p = u_p + (0.27 \times 10^{-5} \times r)$$

each molecule of detergent bound by the protein increases the mobility of the complex by 0.27×10^{-5} cm.²/volt second. Other features of Fig. 4 will be identified below.

The appearance of a second, faster moving peak in the electrophoresis patterns, as the value of r exceeds 8–10, is interpreted to mean that some of the protein molecules had undergone a change in physical structure which resulted in a marked increase in their binding capacity for the detergent. This change in physical structure of the protein molecule must have *resulted* from a disrupting action by the detergent molecules initially bound and may be pictured as a step in the process of "opening up" of the native protein molecules—an initial step in the denaturation of the protein. The idea that a true stepwise change in the configuration of the protein, from its native form, is involved finds support from the observations (a) that the process is reversible insofar as electrophoretic evidence is concerned (*vide infra*) and (b) that such a stepwise change in the binding capacity of the protein for detergent does not appear if the protein is previously denatured by heat. It is pictured, therefore, that the detergent molecules which are initially bound by the native protein molecules act as "molecular wedges" in some fashion such as to eventually "open" the protein molecule and thus expose new surfaces or sites upon which a greater degree of binding will occur in equilibrium with a given free detergent concentration. That the protein molecules in our preparation of crystallized bovine serum albumin are not all equivalent to each other in their capacity to withstand the "wedge" action of the detergent is indicated by the observation that the process of conversion of native protein to the modified form does not occur at a single critical value of $[D]_F$.

The relative amounts of the protein existing in the two forms P_1 and P_2, can be estimated from the areas under peaks A and B in the patterns of the descending boundary as follows. The specific refractive increment of the protein in the buffer employed was determined to be 0.00178 while that of the detergent was 0.00112. The area in the electrophoresis pattern of a boundary of 1% protein in buffer solution against the pure buffer was found to be 430 (= C_P) planimeter units. That of a 1% dodecyl sulfuric acid solution in buffer against buffer was calculated to be 0.00112/0.00178 × 430 = 270 (= C_D) planimeter units. The molecular weight of the detergent acid is 266 (= M_D), that of the protein is taken to be 69,000

TABLE I

Expt. no.	D_T, moles $\times 10^5$	Dialysis equilibrium $[D]_F$, moles/l. $\times 10^5$	Dialysis equilibrium r (from eq. 1)	r (from eq. 5a)	Electrophoresis (desc. boundary) Σ_A, planimeter units	Σ_B, planimeter units	μ_A, cm.^{2}/v.sec. $\times 10^5$	μ_B, cm.^{2}/v.sec. $\times 10^5$
			V = 2.0 liters, P = 0.33 $\times 10^{-5}$ mole					
20	3.56	1.59	1.15	2.4	430	0	6.36	...
21	5.20	2.38	1.33	3.2	435	0	6.69	...
22	6.94	2.90	3.45	3.8	430	0	7.22	...
23	10.40	4.60	3.64	4.8	435	0	7.36	...
29	17.36	7.60	6.54	6.0	435	0	7.95	...
28	34.60	16.1	7.58	7.6	440	0	8.20	...
30	69.40	32.4	13.90	13.5	385	55	8.51	12.20
24	104.00	48.5	21.20	19.6	345	105	8.72	12.88
26	126.50	58.2	30.60	28.3	270	185	9.58	13.42
25	173.60	80.2	40.00	43.9	165	310	11.00	13.82
			V = 0.25 liter, P = 0.33 $\times 10^{-5}$ mole					
51	0.43	0.60	0.85	1.1	430	0	6.29	...
50	0.86	1.07	1.80	1.8	425	0	6.83	...
49	1.73	2.16	3.60	3.0	430	0	7.23	...
48	3.45	6.20	5.76	5.5	435	0	7.77	...
47	3.45	6.94	5.22	5.8	440	0	7.80	...
46	6.92	16.35	8.62	7.7	435	0	8.30	...
45	10.40	27.30	10.85	10.3	415	25	8.27	11.00
44	13.87	35.75	14.95	15.2	375	70	8.72	12.30
43	17.35	44.25	19.07	20.0	335	115	9.20	13.30
42	20.81	51.75	23.95	25.1	295	160	9.68	13.60
36	0	0	0		430	0	6.15	...

(= M_P). The area, Σ_A, in planimeter units under peak A will be the sum of the areas due to P_1 and D_1, disappearing in that peak. The weight % of D disappearing in peak A will be equal to $r_1 \times M_D/M_P \times$ wt. % $P_1 = 0.00385 r_1 \times$ wt. % P_1, where r_1 is the moles of detergent bound per mole of the native form of the protein, P_1. Assuming that the protein, P_1, interacts with detergent according to the relationship (see Fig. 2)

$$r_1 = \frac{10[D]_F}{5 \times 10^{-5} + [D]_F} \qquad (2)$$

the r_1 values for the complex corresponding to the A peak can be calculated. The weight % of P_1 in the A peak may then be calculated from the area under this peak according to the equation

$$\text{wt. \% } P_1 = \frac{\Sigma_A}{C_P + 0.00385 r_1 C_D} \qquad (3)$$

Since the total weight per cent. of protein in each experiment was unity (1%) the weight % of protein, P_2, disappearing in peak B is given as 1 − wt. % P_1 = wt.% P_2. Further, r_2, the ratio of moles of detergent bound per mole of P_2 may be approximated from the area under peak B, Σ_B, in the patterns by the analogous equation

$$r_2 = \frac{\Sigma_B - \text{wt. \% } P_2 \times C_P}{\text{wt. \% } P_2 \times 0.00385 \times C_D} \qquad (4)$$

For those experiments in which two peaks are present in the electrophoresis patterns, Table II gives for each the value of r_1 as calculated from equation 2, the percentage of total protein which disappears in peak A as calculated from equation 3 and the value of r_2 calculated according to equation 4, using the measured values of Σ_A and Σ_B (Table I).

Explanation of the Interaction Isotherm in the Light of Electrophoresis Data.—At equilibrium detergent concentrations, $[D]_F$, below approximately 15 $\times 10^{-5}$ mole per liter, where the values of r are less than approximately 9, the interaction isotherm is assumed to be satisfactorily described by equation 2. Through this initial range of interaction, electrophoretic analysis reveals only one migrating boundary when the protein solution is run against its equilibrium dialysate. The mobility, $\bar{u}_p$, of this boundary (A) increases proportionally with increase in r. Above a value of r = 8–10 and $[D]_F$ = 15–20 $\times 10^{-5}$ the interaction isotherm (Figs. 1 and 2) deviates strongly from its initial course and a second, faster, boundary (B) becomes detectable in the electrophoresis pattern. As r increases, the percentage of total protein decreases in peak A and increases in peak B in a manner illustrated in Fig. 5, a plot of r *versus* $P_2/P_0 (= 1 - P_1/P_0)$. It can be assumed that the native protein, P_1, is changing to a modified form, P_2, in which the capacity to bind detergent is distinctly different from that of the native form of the molecule. Each native protein molecule which undergoes this change does so in an all or none manner, *i.e.*, no intermediate stages of change are evident. However, among the native molecules there exists a distinct variation in their capacity to withstand the disrupting forces, exerted by the adsorbed detergent, which cause the change to P_2.

Insofar as the course of the interaction isotherm is concerned, it can be pictured that when a molecule of P_1 changes to a molecule of P_2, its original interaction tendencies with the detergent are effectively erased and a new set of constants will be required to describe its further interaction with the detergent. If it is assumed that the interaction of form P_2 with detergent is also describable in terms of the simple theory of the mass action law, as in the case of the interaction with form P_1, an equa-

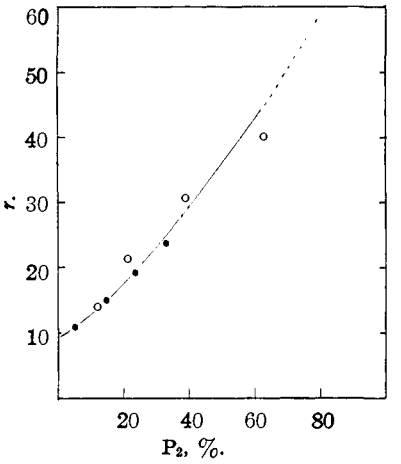

Fig. 5.—Relationship between r (total moles of detergent bound per mole of total protein) and the percentage of P_2 form of the protein present in the equilibrium system: open circles, 2000 ml. systems; closed circles, 250 ml. systems.

tion describing the entire isotherm would take the form

$$r = r_1 \frac{P_1}{P_0} + r_2 \frac{P_2}{P_0} = \left(\frac{n_1[D]_F}{K_1 + [D]_F} \times \frac{P_1}{P_0}\right) + \left(\frac{n_2[D]_F}{K_2 + [D]_F} \times \frac{P_2}{P_0}\right) \quad (5)$$

where r equals the total moles of detergent bound per mole of total protein (P_0) (calculated from analytical values of D_T and $[D]_F$, *cf.* Table I), r_1 equals the moles of detergent bound per mole of protein in the form P_1 (calculated on the basis of $n_1 = 10$ and $K_1 = 5 \times 10^{-5}$, *cf.* Fig. 2) and r_2 equals the moles of detergent bound per mole of protein in form P_2. Values of P_1/P_0 and P_2/P_0 are obtained from electrophoresis patterns (*cf.* Table II). By introducing these values of r, r_1, P_1/P_0, P_2/P_0 and $[D]_F$ into equation 5, the values of r_2 can be calculated for the various experiments in which both A and B peaks occur in the electrophoresis patterns. Values of r_2 obtained in this procedure are shown in Table II.

TABLE II

Expt. no.	r_1 (from eq. 2)	Wt. % P_1 = P_1/P_0	r_2 (from eq. 4)	r_2 (from eq. 5)	r_2 (from eq. 5a)
30	8.6	0.880	36	52	49
24	9.1	.785	56	65	56
26	9.2	.615	50	64	59
25	9.4	.375	63	58	64
45	8.5	.947	40	54	46
44	8.8	.855	50	51	51
43	9.0	.762	50	51	55
42	9.1	.670	53	54	58

A plot of the calculated values of r_2 *versus* $r_2/[D]_F$ should yield, by extrapolation, values of n_2 and K_2. In Fig. 6 the values of r_2 as obtained from both methods of estimation described above are plotted against the corresponding values of $r_2/[D]_F$. All of the experimental errors are cumulative in the calculated values of r_2 and $r_2/[D]_F$. The scatter of the points on Fig. 6 is broad and the r_2 range is small. However, if the relationship $r_2 = n_2[D]_F/(K_2 + [D]_F)$, is assumed to apply, the data can yield values of n_2 and K_2 which are approximately correct. The best straight line drawn through the area of the scattered points of Fig. 6, guided by the assumption that the simple theory of the mass action law holds, places the value of n_2 (intercept on r_2-axis) at approximately 80 and the value of K_2 (slope equals $-1/K_2$) at approximately 2×10^{-4}.

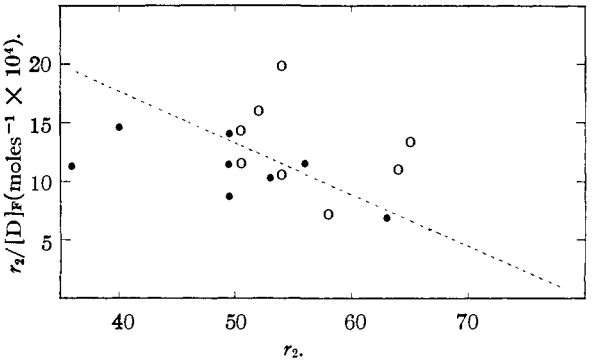

Fig. 6.—Plot of r_2, the moles of detergent bound per mole of P_2 form of protein *versus* $\gamma_2/[D]_F$ (for open circles values of r_2 were obtained from equation 4); for closed circles values of r_2 were obtained from equation 5. Dotted line corresponds to theoretical isotherm where $n = 80$ and $k = 2 \times 10^{-4}$.

The equation, then, which should adequately describe the entire interaction isotherm, up to values of $r = 40$, is

$$r = \left(\frac{10[D]_F}{5 \times 10^{-5} + [D]_F} \times \frac{P_1}{P_0}\right) + \left(\frac{80[D]_F}{2 \times 10^{-4} + [D]_F} \times \frac{P_2}{P_0}\right) \quad (5a)$$

Values of r calculated from equation 5a are given in Table I.

Mobilities of the B Peak.—As is illustrated in Fig. 4, the A peak in the electrophoresis patterns exhibits a mobility increment, for r-values below 8–10, of about 0.27×10^{-5} cm.^{2}/volt second. Above values of $r = 10$, where the B peak appears in the patterns, the mobility of the A peak continues to increase, and with an increment which is higher than that at the lower values of r. This observation is difficult to explain on the basis of the assumption that for the native protein, P_1, the limiting value of r is $n_1 = 10$. Eventual explanation of this apparent anomaly must await studies using a detergent which will be more susceptible to accurate analysis at the low equilibrium concentrations encountered in this region of the interaction isotherm.

From the calculated values of r_2, in those systems where a B peak appears in the electrophoresis patterns, it would be expected that the mobility of this peak would be considerably higher than is observed, if the mobility increment per anion adsorbed were the same as that for the A peak. The values of r_2 obtained by the above methods of calculation contain so many accumulated errors inherent in the experimental measurements involved, that a plot of such r_2-values *versus* the mobilities of the corresponding B peaks show no accurate

trend. If, however, it is assumed that the true and effective values of r_2 are given by the relationship $r_2 = 80[D]_F/(2 \times 10^{-4} + [D]_F)$, these r_2-values can be calculated (*cf.* Table II) for the various values of $[D]_F$ obtained from the analyses of the equilibrium dialysates in the experiments where both A and B peaks are present. When these r_2-values are plotted against the corresponding mobilities of the B peaks, the relationship shown at the top of Fig. 4 is found. The mobility increment per anion bound is indicated to be approximately one half that for the complex in the A peak. The fairly close approximation of the experimental points obtained in this manner to a straight line affords additional evidence that the interaction of detergent with the P_2 form of the protein is indeed described by the mass action relationship given above. The lower value of the mobility increment per anion bound by form P_2, compared to form P_1, may be related to a change in symmetry of the protein molecule during the process of conversion of P_1 to P_2 forms.

Reversibility of the $P_1 \rightarrow P_2$ Process.—When sufficient detergent has been added to a sample of native protein to bring about a conversion of a considerable fraction to the P_2 form, and the mixture is allowed to dialyze against water until no further detergent can be removed, electrophoretic analysis of the protein (after equilibration against buffer) indicates the presence only of the native or P_1 form. Equilibration of this regenerated P_1 protein against detergent, in an amount required to yield a complex of an r-value of 8 with the original protein, still yields only one peak (A) in the electrophoresis pattern. From these observations it would appear that the process $P_1 \rightarrow P_2$ is entirely reversible and that the P_1 form regenerated from the P_2 form is identical with the native protein. That such may not be entirely the case is indicated by the fact that the isoelectric point of bovine serum albumin to which even a small amount of detergent has been added (actually less than that required to form any P_2 protein), upon exhaustive dialysis and electrodialysis was found to be at a *p*H of 4.78 as compared to *p*H 5.2 for the native protein. Either a small amount of the detergent is not removable by dialysis or the detergent has mobilized some small amount of dialysable substance originally bound to the native protein and which had a small determining effect on its isoelectric point.

While the $P_1 \rightarrow P_2$ process appears to be reversible upon the removal of the detergent through extended dialysis, the process is not smoothly dependent upon the equilibrium concentration of detergent. There is a hysteresis effect; when a mixture of high P_2 content and one of low P_2 content are dialyzed to equilibrium against a detergent solution of intermediate equilibrium concentration the P_2/P_0 ratios for the two solutions do not reach the same value, that with the original higher P_2 content remaining somewhat higher at the new equilibrium state. This does not in any way invalidate the description given for the interaction isotherm but means that the ratio of P_1 to P_2 may vary somewhat at a given value of $[D]_F$ depending upon the direction from which the equilibrium is approached.

That the reaction $P_1 \rightarrow P_2$, for a given equilibrium value of $[D]_F$, was completed within the dialysis time and under the other conditions employed in these experiments is indicated by the fact that the relative areas of the A and B peaks in the electrophoresis patterns remain the same whether run after 12, 24 or 36 hours of dialysis. The time required for complete conversion of those molecules of the protein that are susceptible to conversion to the P_2 form at a given value of $[D]_F$ is short. The remaining (non-susceptible to conversion at that value of $[D]_F$) molecules of the protein are stable indefinitely. These facts emphasize the concept that a heterogeneity exists in the resistance of the protein molecules in this preparation to the $P_1 \rightarrow P_2$ change. Because of the long time period required for the development of the electrophoresis patterns, the kinetics of this reaction could not be followed by this method.

Dependence of the Stepwise Complex Formation on the Length of the Hydrocarbon Chain of the Detergent.—Qualitative electrophoresis experiments, employing the pure C_8 and C_{10} homologs of dodecyl sulfate, indicate that, while these substances interact with bovine serum albumin and cause an increase in $\bar{u}_p$ with increase in the detergent composition of the mixture, only one peak appears in the electrophoresis patterns even at the highest detergent concentrations attainable. The C_{14} homolog gave rise to a second peak in the electrophoresis pattern even at the low mixing ratio possible within its limited solubility range. Dialysis equilibrium experiments have not been made with these homologs of dodecyl sulfate and quantitative relationships are not therefore available but it is apparent from these qualitative studies that the chain length of the hydrocarbon residue in the detergent plays a very determinant part in the process by which the protein molecules are modified from the P_1 to the P_2 state.

Discussion

Within the accuracies of the chemical and electrophoretic analyses attainable in this study, the isotherm defining the interaction between dodecyl sulfate and bovine serum albumin, within the range of $r = 0$ to $r = 40$, may be described by an equation based upon the assumed applicability of the simple theory of the mass action law and the idea that the physical structure of the native protein molecule is caused to undergo a change to a new physical form as a consequence of a "disrupting action" by the initially bound detergent. A tentative picture of the sequence of events taking place with regard to the individual protein molecules, as the detergent content of their environment increases, may be drawn as follows. At low ranges of detergent concentration (and in the buffer system employed in the present experiments) the protein interacts with the detergent according to the mass action law where approximately 10 equivalent sites on the protein molecule exhibit an intrinsic dissociation constant for interaction of about 5×10^{-5}. As the average r-value exceeds 8–9 some of the protein molecules undergo a change which is accompanied by a marked increase in the capacity

of the molecule to react with detergent. The interaction of this modified protein with the detergent is again described as a mass action process but the number of equivalent sites, n, upon which interaction occurs is now approximately 80 with an intrinsic dissociation constant of $K = 2 \times 10^{-4}$. The r-value for the total protein is the sum of the r-values for each of the two species of the protein present times their relative amounts.

As the initial number of detergent molecules bound exceeds some statistically critical value for a particular native protein molecule, the bound detergent appears to trigger a physical rearrangement, an "opening up," of the protein molecule. That a change in the asymmetry of the protein molecule accompanies this process is substantiated by the findings of Duggan and Luck[3] that the relative viscosity of bovine serum albumin–sodium dodecyl sulfate solutions increases progressively with increase in detergent concentrations only after the total detergent content of the mixture reaches a value equivalent to that at which r = 8–10. Since this process, from our experiments, is shown to be roughly reversible, it would seem that no extensive denaturation of the protein molecule is involved. Perhaps "opening up" occurs along one plane of the original folded molecule, which, when the detergent is later removed by dialysis, can refold into the original state. This "opening up" process is accompanied by an elimination of the original binding properties of the native form of the protein molecule and the substitution of a new set of binding properties. For the individual protein molecule the process appears to be an "all or none" reaction since no intermediate or transitional state is indicated in the electrophoresis patterns. This could mean that the initial binding of the detergent occurs along or within the protein molecule in the plane of folding which, as the anions of the detergent accumulate and the forces which were originally responsible for maintenance of the fold are neutralized by their presence, opens up. In this way the region in which initial interaction takes place will be erased and the opened up region will replace it as the site of interaction. Subsequent removal of the adsorbed detergent would allow for a refolding of the protein molecule to its native configuration. The hysteresis effect observed in this reversible procedure might be expected.

The albumin molecules in our sample (composite from many animals) were not all equally susceptible to this "opening up" action of the detergent. Some molecules suffered modification when the average r-value was only 8–9 and the $[D]_F$ value was *ca.* 20×10^{-5}, while others had not yet been modified at the highest $[D]_F$ values reached in this series (80×10^{-5}). There is evidence, from the mobilities of the P_1 peak remaining at these higher $[D]_F$ values, that more than the "limiting" number of 10 detergent molecules were adsorbed by the protein that remained unmodified under this condition. In any case there existed a distinct variability among the protein molecules in their capacity to withstand the "opening up" forces exerted by the initially bound detergent. Since the conversion of P_1 to P_2 is an equilibrium process (even though a definite hysteresis is shown), this may be taken as evidence that a heterogeneity exists among the protein molecules with regard to the intensity of the forces (H bond, etc.) knitting them together in the native form.

That the conversion of P_1 to P_2 and the over-all form of the interaction isotherm bears no relationship to the possibility of micelle formation by the detergent is indicated by the fact that the highest $[D]_F$ value reached in this study is well below the probable critical concentration of this detergent in salt solutions of the ionic strength of the buffer employed.[14]

Indications are that, when the detergent content is forced into a higher range than that covered in the present study (by adding higher ratios of detergent directly to the protein), a second stepwise change in the binding capacity of the protein will occur. A P_3 modification of the protein is then detected as a new peak in the electrophoresis pattern showing a mobility greater than that of the P_2 form. These P_2 and P_3 forms of bovine serum albumin probably correspond to the PD_n and PD_{2n} complexes reported by Putnam and Neurath for horse serum albumin but any relation between their values of n (= 55) and that found here for the P_2 form (= 80) is problematic.

In the light of the present studies, it seems reasonable to expect that an extension of such experiments to a variety of proteins may yield interesting and instructive information concerning the gross physical organization of the proteins.

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(14) M. L. Corrin and W. D. Harkins, THIS JOURNAL, **69**, 683 (1947).