and chemical relationship of the abnormal protein to the virus. However, it must not yet be assumed that the abnormal protein and the virus protein are necessarily identical. Unlike intact tobacco mosaic virus, the abnormal protein cannot be heated to 60° without being denatured.[12] Even at 0–5° the abnormal protein does not appear to be as stable as either intact virus[12] or nucleic acid-free virus protein.[17] Preliminary experiments by Dr. H. Fraenkel-Conrat and Mrs. B. Singer of this Laboratory[12] showed that the abnormal protein, like the intact virus or nucleic acid-free virus protein, contained no detectable N-terminal amino acids. However, unlike the intact virus or virus protein, treatment of the abnormal protein with carboxypeptidase yielded glutamic acid and traces of several other amino acids in addition to the C-terminal threonine. Finally, the abnormal protein contained only $^2/_3$ as much of the masked sulfhydryl group as did the nucleic acid-free virus protein or intact virus.[18]

Comparisons of particle sizes of the unpolymerized abnormal protein with the unpolymerized nucleic acid-free virus protein are contained in another report.[19] The possible biological relationships of the abnormal protein to the virus, based on isotope studies, have been considered elsewhere.[10,20]

Acknowledgments.—We are indebted to Mrs. Terry Andrews, Miss Mary K. Brown and Mr. Don de Fremery for valuable technical assistance.

(17) P. Newmark, unpublished experiments.

(18) H. Fraenkel-Conrat and R. C. Williams, *Proc. Nat. Acad. Sci.*, **41**, 690 (1955); H. Fraenkel-Conrat, *J. Biol. Chem.*, **217**, 373 (1955).

(19) R. T. Hersh, Ph.D. Thesis, University of California, Berkeley, 1955; P. Newmark, R. T. Hersh and R. W. Myers, in preparation.

(20) C. Van Rysselberge and R. Jeener, *Biochim. Biophys. Acta*, **17**, 158 (1955); B. Commoner and S. D. Rodenberg, *J. Gen. Physiol.*, **38**, 475 (1955).

LAWRENCE, KANSAS

[CONTRIBUTION FROM DEPARTMENT OF AGRICULTURAL BIOCHEMISTRY, UNIVERSITY OF MINNESOTA]

A Study of the Interaction of *n*-Octylbenzene-*p*-sulfonate with β-Lactoglobulin[1,2]

BY ROBERT M. HILL AND D. R. BRIGGS

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The binding of *n*-octylbenzene-*p*-sulfonate by β-lactoglobulin in 0.1 ionic strength phosphate buffer, *p*H 6.8, occurs in three distinct stages as the equilibrium concentration of the detergent is increased. At low detergent concentrations the protein molecule interacts according to the mass action law to bind 2 or 3 molecules of detergent, probably in a tail first manner, the intrinsic dissociation constant being 1.59×10^{-5}. At this point the protein molecule undergoes a change in which its ability to bind detergent is greatly increased. This increased binding is believed to take place in a head first manner and also proceeds according to the mass action law, the intrinsic dissociation constant being 2.32×10^{-4} and the number of available sites being approximately 22. Simultaneously, however, a third type of binding occurs which is micellar in nature and involves interaction between the detergent molecules already bound to the protein and the detergent in solution. A limit to this binding occurs when the free detergent concentration reaches its critical micelle concentration. This micellar binding does not produce any physical change in the protein structure and the equilibrium is the same when approached from either direction. Binding at the lower levels, dependent upon an "opening-up" of the protein molecule, shows a hysteresis effect upon reversal indicating that the new protein species formed is stabilized by different conditions than are required in its formation. The fact that the "opening-up" of all of the protein molecules does not occur at a single free detergent concentration is taken to indicate that the native molecules vary in their ability to withstand this action of the detergent. An equation is proposed which adequately describes the experimentally determined interaction isotherm.

During recent years a number of investigators have reported on the interaction of proteins with detergent ions. Such studies have been directed toward obtaining information about the nature of the reactive sites of protein molecules in solution. It has been recognized that protein–ion interaction is dependent upon both the charge and the chemical nature of the ion involved. Anionic detergents have been of particular interest because of their bactericidal activity and because of the changes they produce on the physical properties of various proteins. The organic sulfates and sulfonates, because of their favorable solubilities, have received considerable attention. Since the measurement of light absorption in the ultraviolet region has become a routine operation, the use of detergents containing aromatic groups has become preferred because of the ease and accuracy with which their concentrations can be determined by this method.

(1) Paper No. 3385, Scientific Journal Series, Minnesota Agricultural Experiment Station.

(2) A part of a thesis submitted by Robert M. Hill to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the Ph.D. degree. This study was aided by a research grant from the National Institute of Health, Public Health Services.

At low equilibrium concentrations of detergent, it has been found[4,5] that an interaction occurs, describable in terms of a simple mass action relationship,[3] based on the concept of a homogeneity of interaction sites. However, upon increasing the equilibrium concentration of detergent, deviations from this relationship are readily observed. Karush and Sonenberg[4] explained these as being due to a heterogeneity of binding sites. The combined use of equilibrium dialysis and electrophoretic methods has provided a means for a more complete study of such interactions and, subsequently, a more complete description of the interaction process has evolved.

Pallansch and Briggs,[5] studying the interaction of highly purified sodium dodecyl sulfate with bovine serum albumin at free detergent concentrations well below that required for micelle formation, observed that when the moles of anion bound per mole of protein exceeded 10, a faster moving protein component appeared in the electrophoretic patterns.

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

(4) F. Karush and M. Sonenberg, *ibid.*, **71**, 1369 (1949).

(5) M. J. Pallansch and D. R. Briggs, *ibid.*, **76**, 1396 (1954).

They demonstrated the total binding of the detergent to be the sum of the binding by the two forms of the protein, the native and the "opened-up" form. Two simple mass action equations adequately described the binding. It was suggested that when approximately 10 moles of detergent is bound per mole of the protein, the latter undergoes a physical transition to a form which exhibits entirely new binding properties. This transition appeared to be an all or none reaction for any single protein molecule and was shown to be reversible. However, due to the low solubility of their detergent, they were not able to realize a complete conversion of the electrophoretically slow to the fast form of serum albumin. They observed, also, that the shorter-chain detergents, while adsorbed by the proteins, were not able to cause the appearance of the second boundary in the electrophoretic pattern. Yang and Foster[6] reported a similar study using serum albumin and a mixture of alkylbenzene sulfonates. Their results appeared to be influenced by the heterogeneity of chain lengths of the detergent employed.

Preliminary experiments indicated that β-lactoglobulin undergoes similar electrophoretic changes with either sodium dodecyl sulfate or sodium *n*-octylbenzene-*p*-sulfonate and that it was possible to obtain a complete conversion to the fast form. Since the latter detergent is considerably more soluble and has a higher critical micelle concentration than the former, it appeared possible through its use to determine the maximum binding capacity of the protein directly and to test over an extended range the equation proposed by Pallansch and Briggs.

Experimental

β-Lactoglobulin was isolated from raw skim milk according to a modification of the method of Palmer.[7] The milk was first warmed to room temperature and the *p*H adjusted to 4.6 with hydrochloric acid to precipitate the casein, which was then removed by filtration. The filtrate was adjusted to *p*H 7.0 with sodium hydroxide and the globulins and albumins were precipitated by addition of ammonium sulfate to 83% saturation.[8] After filtration, the precipitate was dissolved in a quantity of water to yield a salt concentration of 14% and the *p*H was adjusted to 7. A saturated solution of ammonium sulfate was added until the salt concentration was 50% saturated to precipitate globulins, which were then removed by filtration. The salt concentration of the filtrate was adjusted to 83% saturation to precipitate the β-lactoglobulin which was removed by filtration and transferred to Visking cellulose casings. These were dialyzed in the cold against 75% saturated ammonium sulfate. The salt concentration of the outer solution was gradually decreased by dilution. After extended dialysis all the salt was removed. The contents of the dialysis sacks were concentrated to one-third of the original volume by per-evaporation, the *p*H adjusted to 5.2, seed crystals were added and dialysis was continued in the cold against distilled water. After crystallization was complete the supernatant liquid was separated by centrifugation. The crystalline protein was washed once with cold water and dissolved in 0.1 *N* potassium chloride and dialyzed against distilled water until crystallization was complete. The product so obtained was crystallized thrice more from potassium chloride. The final crystals were suspended in water, lyophilized and dried in a vacuum desiccator.

Protein concentrations of the solutions used were determined where required, by obtaining the dry weight of an aliquot and correcting for any salts which might be present. The values so obtained agreed with those checked by the micro Kjeldahl method.

Phosphate buffers were prepared according to a graph plotted from the calculations of Green[9] using anhydrous analytical reagent grade potassium mono- and dibasic phosphates. In all experiments the ionic strength was kept at 0.1. No ionic strength correction was made for added detergent.

Sodium *n*-octylbenzene-*p*-sulfonate was prepared from Eastman Kodak Co. highest purity caprylic acid according to the method of Paquette, *et al.*,[10] with the exception of the step for the reduction of caprylylphenone. In this step the modified Wolf–Kishner method was substituted[23] for that of Clemmensen suggested by these authors.

The critical micelle concentration of the detergent in the buffer was determined to be 2.8×10^{-3} molar by the method of Corrin, Klevens and Harkins,[11] 4.9×10^{-3} molar according to the procedure of Kolthoff and Stricks[12] and 3.4×10^{-3} molar when determined by the method of Yang and Foster.[13]

The concentration of the detergent was determined by utilizing the ultraviolet absorption of the aromatic nucleus at wave lengths of 223 and 260 mμ, the apparent molar extinction coefficients being 13,800 and 415, respectively.

Dialysis casings used in these studies were of regenerated cellulose tubing manufactured by the Visking Corporation for experimental purposes. These were washed as suggested by Yang and Foster,[13] by filling with distilled water and boiling for two hours after which they were rinsed with water. This washing procedure was repeated twice more. Finally the casings were washed with flowing distilled water for six hours. They were then stored under distilled water, which was frequently renewed, until used. The casings were handled with rubber gloves during all operations. The protein solutions were carefully introduced into a section of the tubing which had been tied with dental floss at one end. The sacks were then tightly tied with floss and washed with distilled water to remove any external contamination.

Detergent uptake studies were made by equilibrating 10 ml. of a 0.5% solution of protein in buffer sealed inside the dialysis sack, against 10 ml. of a solution containing buffer and detergent. The vials used for this study were closed with rubber stoppers covered with aluminum foil. These were maintained at 20° and rotated rapidly end over end once every eight minutes. The detergent bound to the protein was determined from the difference between the initial and final detergent concentrations in the outer solution after correcting for dilution.

For electrophoretic analysis 25 ml. of 0.5% protein in buffer was equilibrated at 20° against 460 ml. of detergent–buffer solution. Standard electrophoresis techniques were employed with a Klett–Longsworth–Tiselius apparatus equipped with electrode vessels described by Alberty,[14] the equilibration dialysate being layered over the protein solution in the cell. The temperature was maintained at 20° for all determinations. To eliminate convection currents in the cell, the potential gradient was maintained at the low value of approximately 0.9 volt/cm. The duration of the electrophoresis runs was 18,000 seconds in all experiments. The image of the scanned photograph of each pattern was enlarged by projection and manually traced on ruled cross section paper. All measurements of distance as well as of area were obtained from these tracings. In the case of mobility (that of the descending boundary is reported), the distance measured on the tracing was corrected for enlargement. The areas under the curves were measured with a planimeter, and since only relative areas are of significance, the values were not corrected for enlargement. Where needed, the patterns were resolved into individual peaks by the method of Tiselius and Kabat.[15] The conductivities of the solutions

(6) J. T. Yang and J. F. Foster, THIS JOURNAL, **75**, 5561 (1953).

(7) A. H. Palmer, *J. Biol. Chem.*, **104**, 359 (1934).

(8) S. P. L. Sorensen, *Compt. rend. trav. Lab. Carlsberg Ser. Chim.*, **12**, 158 (1917); **23**, 63 (1939).

(9) A. A. Green, THIS JOURNAL, **55**, 2331 (1933).

(10) R. G. Paquette, E. C. Lingafelter and H. V. Tarter, *ibid.*, **65**, 686 (1943).

(11) M. L. Corrin, H. B. Klevens and W. D. Harkins, *J. Chem. Phys.*, **14**, 480 (1946).

(12) I. M. Kolthoff and W. Stricks, *J. Phys. Colloid Chem.*, **52**, 915 (1948).

(13) J. T. Yang and J. F. Foster, *ibid.*, **57**, 21 (1953).

(14) R. A. Alberty, *ibid.*, **53**, 114 (1949).

(15) A. Tiselius and E. A. Kabat, *J. Exptl. Med.*, **69**, 119 (1939).

were measured in a cell having a constant of 14.85 using the conventional wheatstone bridge equipped with oscillator and headphone.

Results

Equilibrium-Dialysis Studies.—The points in Fig. 1 show the relationship between the experimentally determined values of r, the moles of detergent bound to each mole of protein, and D_f, the equilibrium free detergent concentration, for low values of D_f. It is possible to determine whether this isotherm follows the simple mass action law, $r = nD_f/(K + D_f)$ . . . (1) where n is the number of sites per protein molecule available for binding and K is the intrinsic dissociation constant. If the above equation is transformed, as suggested by Scatchard,[16] to $r(K/D_f) = n - r$ and r/D_f is plotted against r, the intercept on the r/D_f axis is equal to n/K and on the r axis the intercept is equal to n. The linear relationship shown as curve A in Fig. 2 indicates that the initial portion of the curve follows the mass law. The maximum number of sites for this step in the binding curve is indicated to be 2.35 and the value for K is 1.59×10^{-5}. The curve shown in Fig. 1 is the isotherm calculated by substitution of these values into equation 1. Good agreement is observed with the experimentally determined points up to an r value of about 2.5.

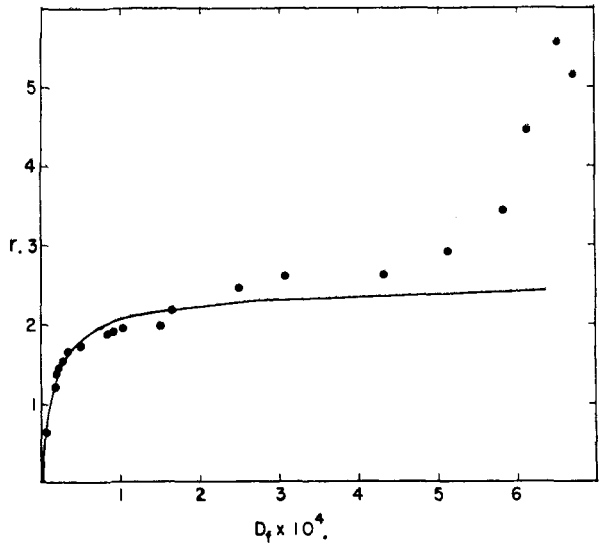


Fig. 1.—Theoretical isotherm for low detergent level interaction compared with experimentally determined points.

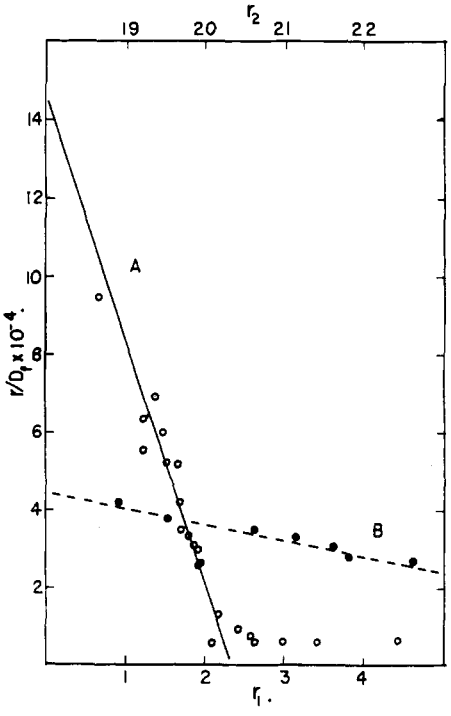


Fig. 2.—Interaction isotherm plotted according to Scatchard. r_1/D_f *vs.* r_1 is shown by curve A and the open points. r_2/D_f *vs.* r_2 is shown by curve B and solid points.

The points plotted in Fig. 3 show the relationship between experimentally determined values of r and D_f over the entire range studied. There does not appear to be any limit to this curve at high values for D_f. It is apparent, however, that the isotherm may be divided into three distinct regions, the initial region which was mentioned above, a second between $r = 2.5$ and $r = 30$, and a third at r values greater than 30.

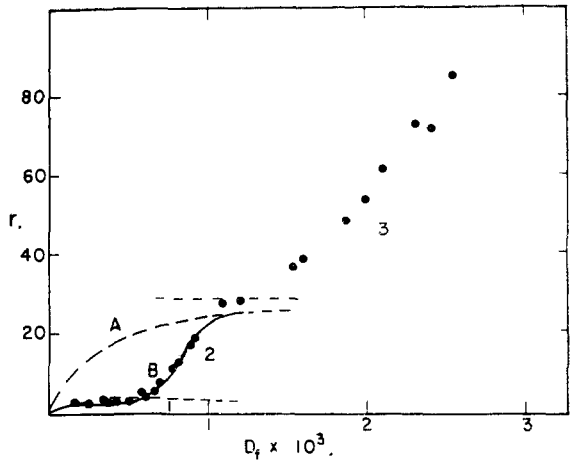


Fig. 3.—Complete interaction isotherm showing regions 1, 2 and 3. Curve A represents the theoretical binding isotherm for the fast component using the constants obtained from Fig. 2, curve B. Curve B is the theoretical isotherm calculated according to the equation 2.

Electrophoretic Analysis.—Inspection of the electrophoretic patterns in Fig. 4 indicates that the preliminary division of the binding isotherm into regions 1 and 2 is justified. In patterns corresponding to region 1 (patterns 127, 183, 184) only a single boundary is observed while in those corresponding to region 2 (patterns 185–191 inclusive) an additional component appears and migrates as a faster peak. As the free detergent concentration is increased the amount of the faster component increases at the expense of the slower one. Thus at the lower levels of interaction when r is less than 2.5 only one constituent protein–detergent complex exists in solution. The mobility of this complex increases from that of the protein alone as r increases but at $r = 2$ it is only slightly greater

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

TABLE I

ANALYSIS OF ELECTROPHORESIS PATTERNS[a]

Pattern no.	Mobility (cm.²/sec. volt) slow × 10⁵	fast × 10⁵	*Df* (mole/l.) × 10⁴	Total area	% Area slow	Mmoles protein slow × 10⁴	fast × 10⁴	Mmoles detergent bound by slow × 10⁴	fast × 10⁴	r_2, moles/mole
127	9.3	..	0.0	2.90	100	..	...	..	....	..
184	9.6	12.0	3.6	2.76	99+	31.9	...	84.7	....	..
185	9.8	14.6	4.46	2.92	97.4	31.1	0.83	70.3	15.7	18.9
186	10.0	15.1	5.15	2.86	95.0	31.5	1.66	71.6	32.4	19.5
187	9.8	15.3	5.85	2.86	91.0	30.2	3.00	68.9	62.1	20.6
188	9.8	16.9	6.40	2.80	86.5	28.7	4.50	66.0	95.0	21.1
189	10.9	17.5	7.05	2.82	73.2	23.3	8.55	54.0	185	21.6
190	10.1	17.4	7.45	2.90	50.0	15.9	15.9	36.9	343	21.8
198	10.8	17.5	8.15	3.06	50.3	16.1	15.9	37.8	382	23.9
191	11.2	18.2	8.45	3.03	34.5	11.0	20.9	25.9	474	22.6
192	..	19.0	12.2	3.11	...	..	33.1	..	960	29.0
193	..	19.6	16.3	3.48	...	..	33.1	..	1300	39.3
194	..	21.3	19.8	3.72	...	..	33.1	..	1800	54.4
195	..	21.3	27.7	4.16	...	..	30.5	..	2700	88.0
197	..	23.8	32.0	4.14	...	..	30.5	..	2900	95.0

[a] Areas are in planimeter units and mobilities are for the descending boundaries.

than that of the native protein not previously exposed to detergent.

The appearance of the faster moving complex has been interpreted by Pallansch and Briggs to mean that some of the protein molecules have undergone a physical structural change which has resulted in a marked increase in binding capacity of the protein. These modified protein molecules form a different constituent complex with the detergent. These authors suggest that the initially bound detergent acts as a "molecular wedge" to "open-up" the protein molecule to expose new sites having different binding characteristics. The relative amounts of protein present in each of the two forms may be estimated from the areas under the slow and fast peaks in the electrophoresis patterns. From the constants for the initial binding previously obtained, as plotted in Fig. 2, curve A, the r values for the slow form (r_1) are obtained and the total detergent bound by this form may be determined from the relationship $D_1 = r_1P(A_1/A_t)$, where D_1 is the total number of moles of detergent bound by the slow form, P is the total moles of protein represented by the total area, A_t, under both the peaks and A_1 is the area enclosed under the slow peak. The moles of detergent bound, D_2, by the fast form may then be calculated as $D_2 = D_r - D_1$, where D_r is the total moles of detergent bound as determined by the dialysis experiments. Then $r_2 = D_2A_t/PA_2$. Table I contains an analysis of these experiments.

When the isotherm for the second step is plotted according to the method of Scatchard as in curve B, Fig. 2, and the constants determined, it is found that $n_2 = 32$ and $K_2 = 7.1 \times 10^{-4}$. Curve A, Fig. 3, is the theoretical isotherm for the second step. The first two regions of the binding curve may then be expressed as

$$r_1 + r_2 = r_{1,2} = A_1/A_t\left(\frac{2.35D_f}{1.59 \times 10^{-5} + D_f}\right) + \frac{A_2}{A_t}\left(\frac{32D_f}{7.1 \times 10^{-4} + D_f}\right) \quad (2)$$

This is the form of equation proposed by Pallansch and Briggs and this isotherm is indicated in Fig. 3 by curve B. It is seen to be in good agreement with the experimentally determined values in regions 1 and 2.

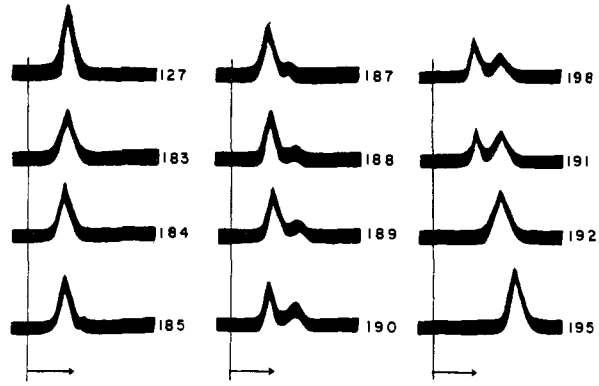


Fig. 4.—Electrophoresis patterns of descending boundaries after 18,000 seconds at a potential gradient of 0.9 volt/cm. at 20° in 0.1 ionic strength, phosphate buffer *p*H 6.8. Free detergent concentrations in molarity × 10⁴ are as follows: 127, none; 183, 2.90; 184, 3.6; 185, 4.46; 187, 5.85; 188, 6.4; 189, 7.05; 190, 7.75; 198, 8.15; 191, 8.45; 192, 12.2; and 195, 23.9.

Reversibility of the Binding Isotherm.—Observations by Putnam and Neurath,[17] by Lundgren,[18] by Karush,[19] and by Pallansch and Briggs[5] all indicate that the interaction of detergent with any of a variety of proteins is purely physical in nature and that the detergent may be quantitatively removed from the protein by exhaustive dialysis or otherwise reduction of the equilibrium free detergent concentration in the protein solution to zero. The experiments of Pallansch and Briggs with dodecyl sulfate and serum albumin and the present experiments with octyl benzenesulfonate and β-lactoglobulin demonstrate that an action of the detergent is to cause a change in the protein, presumably a change in its physical structure, which allows for an increase in its capacity to interact with the detergent. While the interaction of detergent with

(17) F. W. Putnam and H. Neurath, THIS JOURNAL, **66**, 692 (1944).
(18) H. P. Lundgren, *Textile Research J.*, **15**, 335 (1945).
(19) F. Karush, THIS JOURNAL, **72**, 2705 (1950).

the protein is smoothly reversible with changes in D_f so long as the protein remains in a given state of physical organization, it appears that the process involving the change in such organization of the protein is not smoothly reversible with change in D_f. As a result a hysteresis effect is observed in the binding isotherm in the range of D_f through which the change in protein organization occurs.

If a sample of protein in buffer is first equilibrated against a high concentration of detergent in buffer (*i.e.*, brought to some point high on the initial or forward interaction isotherm) and then is allowed to equilibrate against buffer alone or against a buffer–detergent solution of lower detergent concentration, and the final r and D_f values determined, the hysteresis in reversibility of the isotherm can be demonstrated. Two series of such determinations were made, in which the initial equilibrium values of D_f were 32×10^{-4} and 20×10^{-4} molar, respectively. In Fig. 5 the values of r at various final levels of D_f are compared with those of the initially determined isotherm. The binding in regions 1 and 2 and in region 3 are not reversible in the same manner. Reversal within region 3 follows the forward adsorption isotherm very closely and thus the same equilibrium is attained regardless of the direction from which it is approached. However, when the concentration of free detergent is reduced so that regions 2 or 1 are entered, the hysteresis effect is observed. In this case the nature of the equilibrium at any given value of D_f depends on the direction from which it is approached. From electrophoresis patterns obtained on "reversed" samples in the lower regions, it was found that the fraction of fast protein component upon "reversal" was greater than that obtained at the same free detergent concentration when equilibrium is approached in the forward direction. The value of r calculated according to equation 2 agreed, however, with the observed r in all such experiments. The region of hysteresis was due, therefore, not to the lack of a smooth reversibility of the binding process proper, but to a lack of smooth reversibility in the "opening up" and "folding up" processes in the protein molecules involved.

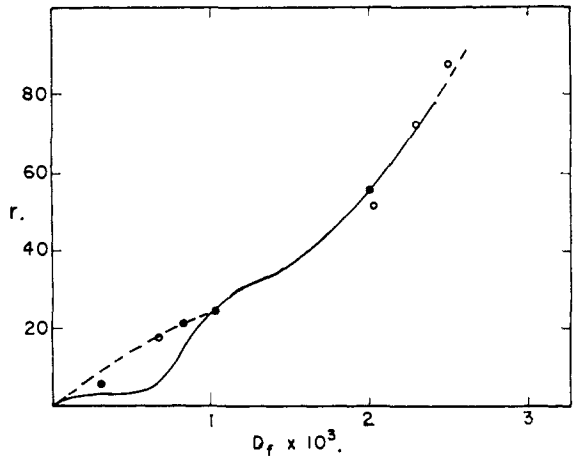


Fig. 5.—Reversal of detergent–protein interaction. Solid line is initial binding isotherm. Open and closed points obtained after partial removal of detergent by dialysis from an initial free detergent concentration of 32×10^{-4} molar and 20×10^{-4} molar, respectively.

Samples of protein, in each of the two high initial detergent concentrations mentioned, were exhaustively dialyzed against buffer to remove *all* of the detergent. Electrophoretic analyses of the resulting protein solutions showed only one component, indistinguishable in mobility from that of the native protein. When a 25-ml. sample of this detergent free protein solution was again equilibrated against 460 ml. of 1.12×10^{-3} molar detergent in buffer, the final concentration of free detergent was 9.3×10^{-4} molar and two components were observed in the electrophoretic patterns. Their relative areas and values for r fell directly on the isotherm obtained initially for the native protein. To this extent, the "refolded" protein obtained after complete removal of the detergent appeared to be identical with native protein. It has not been possible, however, to crystallize the protein after it has been treated with any amount of detergent. Ultracentrifugal studies show that only one component, with respect to sedimentation characteristics, is present regardless of the detergent concentration in which the protein is placed. Thus no aggregation or fragmentation of the protein molecules occurs as a result of the interaction or of the postulated change in the physical structure of the protein.

Discussion

It has been observed by previous investigators[20–22] that both the charged group and the hydrocarbon chain influence the manner in which a detergent molecule interacts with a protein. Two general types of binding are thus suggested, one being through an electrostatic link, as proposed by Putnam and Neurath, in which the anionic group of the detergent is attracted to a cationic group of the protein. The necessity of having a large hydrocarbon tail on the molecule indicates that non-ionic binding of a van der Waals type must also be involved. Whether both factors are involved in each of the observed binding steps has not yet been determined. In the electrophoresis studies no second component appears below an r value of approximately 2.5, the detergent being bound in accordance with the mass action law. The fact that the limiting value for r_1 is not an integer may indicate that not all of the native protein molecules are alike in their ability to bind detergent. The second component which appears in the electrophoresis patterns possesses a much higher mobility than the slow component, indicating a considerable increase in detergent binding capacity for those protein molecules appearing as the fast component. Again the mass action law appears to be followed in which the calculating limiting value for r_2 is 32.

The observation that there are no intermediate peaks appearing in the transition from the slow to the fast form in any electrophoretic pattern is taken to indicate that the initial binding process must somehow induce an all or none change in the pro-

(20) J. Steinhardt, C. H. Fugitt and M. Harris, *J. Research Natl. Bur. Standards*, **26**, 293 (1941).

(21) J. Steinhardt and C. H. Fugitt, *ibid.*, **29**, 315 (1942).

(22) P. D. Boyer, G. A. Ballou and J. M. Luck, *J. Biol. Chem.*, **167**, 407 (1947).

tein molecule with respect to its binding characteristics. An "opening-up" of the protein molecule by the detergent along some line of cleavage where there is a tendency for the detergent to accumulate could be involved. Pallansch[23] found that ionic strength of the buffer did not affect the initial binding, but did influence the second binding step. This could mean that the initially bound detergent molecules were solubilized in the protein, tail first, by van der Waals forces. Approximately two to three molecules would be sufficient to induce rupture of the physical bonds responsible for maintaining the native configuration of the β-lactoglobulin molecule and cause it to "open-up" to expose new sites which would possess the different binding characteristics observed. It is assumed that each molecule undergoes this unfolding in an all or none fashion and that these new sites would then bind according to a new mass action expression. Since ionic strength appears to be influential in this binding, it may be assumed that these molecules bind head first by electrostatic links.

The observation that the desorption curve is not identical with the adsorption curve in this lower region does not mean that the actual binding of the detergent by either "form" of the protein is not a smoothly reversible process. Rather, it means that some higher equilibrium concentration of detergent is required to trigger the "opening-up" process than is required to maintain the protein in the "opened up" condition. Once the configuration of the protein has been modified, it appears to be stabilized in this configuration by the detergent subsequently bound. However, once "all" the detergent is removed again, the molecule apparently reverts to a form very nearly like that of the native, and so the "opening-up" process must be repeated and the binding isotherm for this reverted protein follows the same course as that for the native protein.

The region 3 portion of the curve does not approach any recognizable limit, and reversal studies indicate that no configurational change in the protein molecule is involved in this region of the isotherm. Only one electrophoretically homogeneous component is present in this region, and yet an entirely different mechanism from that in the second stage of binding seems to be operative. When the theoretical binding isotherm for the fast form (second stage) is combined with that experimentally determined for region 3, the "S" shaped curve (solid line) shown in Fig. 6 is obtained. This curve resembles some of the isotherms reported in the literature from studies of the adsorption of vapors on solids for which Brunauer, Emmett and Teller[24] have formulated an equation, based on an extension of Langmuir's theory, to include both monolayer and multilayer adsorption. These isotherms also show two regions, the curve at low pressures being concave to the pressure axis (abscissa) while at high pressures, in the region approaching the condensation point, it is convex to this axis. These authors considered the multilayer adsorption as being due to the same forces that are responsible for

(23) M. J. Pallansch, thesis, University of Minnesota, 1953.

(24) S. Brunauer, P. H. Emmett and E. Teller, THIS JOURNAL, **60**, 308 (1938).

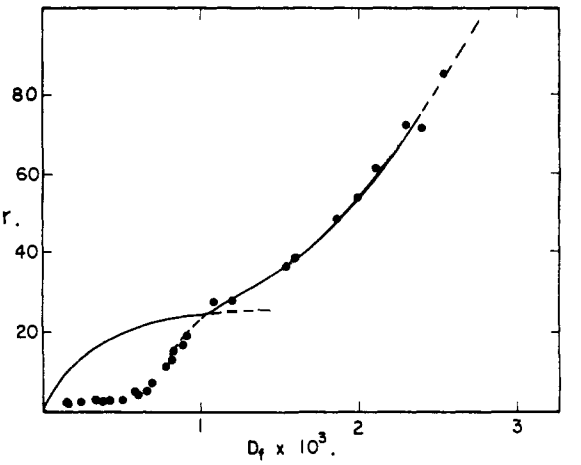


Fig. 6.—Apparent interaction isotherm for fast component (solid line) compared to experimental isotherm (points).

condensation. Their final equation for the isotherm takes the form

$$v = \frac{v_m CP}{(P_0 - P)\,1 + (C - 1)P/P_0}$$

where v is the total volume of gas adsorbed, v_m the volume of gas required for a complete monolayer, P_0 is the saturation pressure of a gas and P is any lower pressure. C is a measure of the adsorption tendency and $\ln C = (E_1 - E_L)/RT$ where E_1 is the heat of adsorption for the monolayer and E_L the heat of liquefaction of the gas. This idea may be applied to the problem at hand by making the following modifications and assumptions. It is assumed that in this system the free detergent molecules in a liquid medium may be treated in a manner analogous to gas molecules in a gaseous medium and that all assumptions made for the vapor system will hold equally well for the liquid system. This has been found to be a valid assumption at low levels of binding. As the pressure on the gas is increased, liquefaction will finally occur at P_0. The detergent in solution undergoes an analogous rather abrupt transition, as the concentration is increased, from simple ions to micelles. The concentration at which the detergent ions aggregate to form micelles, the critical micelle concentration (CMC), may be designated as D_c and is analogous to P_0. The free detergent concentration D_f is analogous to any lower pressure, P. From statistical considerations Dole[25] has determined that the BET equation gives, as one of its parameters, the number of adsorption sites and not necessarily the total surface of the adsorbant. Thus v_m could be replaced by n_m, the total number of molecules adsorbed in the first layer, each at an equivalent site. If it is assumed that multiple adsorption may occur at these sites, then the total number of detergent molecules adsorbed per mole of protein, at any value of D_f, replaces v and this is identical with r used in previous equations. The constant C may be replaced by K^* so that $\ln K^* = (E_1 - E_m)/RT$ where E_1 is the heat of binding for the formation of a monolayer on the sites of the "opened-up" protein and E_m is the heat of micelle formation. The modified equation becomes

(25) M. Dole, *J. Chem. Phys.*, **16**, 25 (1948).

$$r_2 + r_3 = r_{2,3} = \frac{n_m K^* D_f}{(D_c - D_f)\,1 + (K^* - 1)(D_f/D_c)} \quad (3)$$

Transforming this equation to

$$\frac{D_f}{r_{2,3}(D_c - D_f)} = \frac{1}{n_m K^*} + \left(\frac{K^* - 1}{n_m K^*} \times \frac{D_f}{D_c}\right)$$

and plotting $D_f/r_{2,3}(D_c - D_f)$ against D_f/D_c, a straight line should be obtained whose intercept is $1/n_m K^*$ and whose slope is $(K^* - 1)/n_m K^*$. Thus the values for n_m and for K^* may be determined from experimental data. Using a value for D_c of 3.3×10^{-3} (this value gave the best straight line and agrees reasonably well with values of D_c experimentally determined for this detergent) and data from the isotherm constructed in Fig. 6, such a plot was found to yield a straight line. From the slope and the intercept values of $n_m = 22$ and $K^* = 14.2$ were found.

The entire binding isotherm can then be expressed by the relationship

$$r_1 + r_2 + r_3 = r = \frac{A_1\, 2.35 D_f}{A_t\, 1.59 \times 10^{-5} + D_f} + \frac{A_2\, 22 \times 14.2 \times D_f}{A_t(D_c - D_f)(1 + 13.2\, D_f/D_c)} \quad (4)$$

This isotherm is compared with the experimental points in Fig. 7. It appears, then, that the binding at high free detergent concentrations is of micellar type only, due to mutual tail to tail attraction of the dissolved detergent molecules for the detergent already bound onto the fast form of the protein. At free detergent concentrations below the CMC the number of multimolecular aggregates of detergent in the solution proper is negligible as compared to those existing on the surface of the protein. Kolthoff and Stricks[12] have observed indications of the existence of micelles below the CMC in dye solubilization studies. The micellar bound detergent is in equilibrium with simple detergent ions. The isotherm for reversal of binding in region 3 is identical with the initial adsorption curve since no structural change in the protein molecule occurs in this region. A limiting value of r would be reached at the CMC when micelles form in the solution, thereby maintaining the molecular free detergent concentration constant.

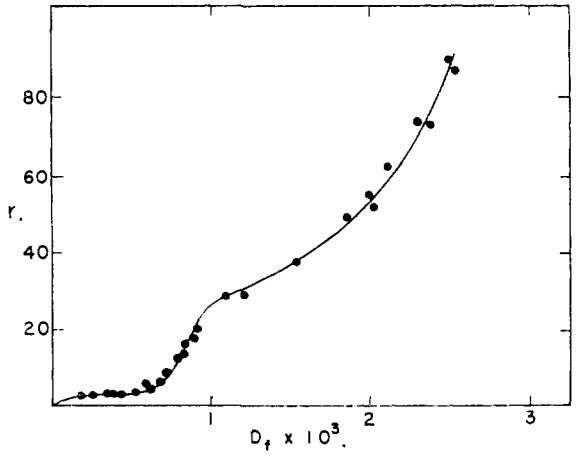


Fig. 7.—Theoretical interaction isotherm (according to equation 4) for β-lactoglobulin and octylbenzene-*p*-sulfonate compared with experimental points

This concept is in agreement with the observation that no new peak appears in the electrophoretic patterns for this region. The area under the one peak progressively increases, however, because the increase in binding of detergent brings about an increase in the weight concentration of the detergent–protein complex.

The maximum number of 22 sites on the fast form of the protein available to bind detergent calculated according to the BET equation (Fig. 8, curve A). is considerably less than the 32 sites obtained earlier from the mass action calculations for the second binding step. It appears that micelle

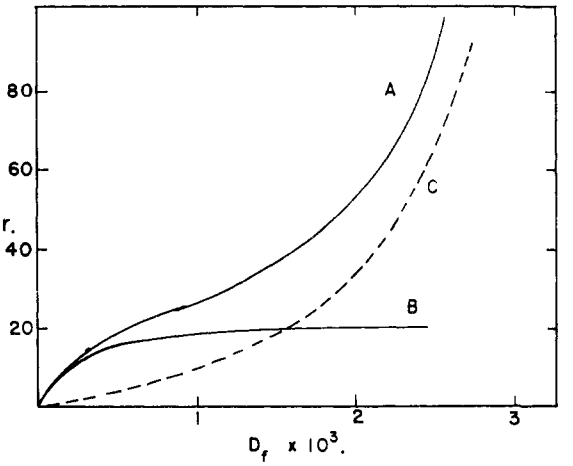


Fig. 8.—Curve A is a complete theoretical isotherm (according to equation 3) for the fast component as calculated by the BET equation. Curve B is the theoretical isotherm for the fast component according to the simple mass action equation using constants obtained from the BET equation (from equation 6). Curve C is the isotherm for micellar binding.

type interaction begins to occur before all the sites are covered with a monolayer. A new binding isotherm may be calculated for the second step using the mass action equation and the constants obtained from the BET equation. For this calculation $n = 22$ and, at low levels of interaction, D_c/K^* is equal to K in the mass action equation. Thus

$$r_2 = 22D_f/2.32 \times 10^{-4} + D_f \quad (5)$$

This isotherm is constructed in Fig. 8—Curve B. The difference, $r - r_2 = r_3$, is represented by curve C of Fig. 8, which is the isotherm for micellar type binding. This type of binding is appreciable even at low D_f levels. This curve follows that of an infinite series of the type $y = x/(1 - x)$ and may be written as $r_3 = BD_f/(D_c - D_f)$. Plotting r_3 against $D_f(D_c - D_f)$ a straight line is obtained having a slope of 22.4. Using this constant the interaction isotherm for micellar binding is

$$r_3 = 22.4D_f/3.3 \times 10^{-3} - D_f$$

Therefore, the isotherm obtained according to the BET equation may be equally well represented in this case by

$$r_2 + r_3 = r_{2,3} = \frac{22D_f}{2.32 \times 10^{-4} + D_f} + \frac{22.4D_f}{3.3 \times 10^{-3} - D_f}$$

Analogous to the BET theory, $\ln K^* = (E_1 - E_m)/RT$, and the assumption can be made that E_2

$= E_3 = \ldots . E_i = E_m$. Therefore once the sites are covered by a monolayer of detergent, K^* should be equal to 1 and the BET equation for this type of binding reduces to $r_3 = nD_f/(D_c - D_f)$ which is identical in form with the empirical equation obtained above.

It is seen therefore that the values of r_2 obtained experimentally represent the sum of the detergent bound according to the manner of the second mass action step plus a small amount due to micellar binding. When the latter is neglected and a simple mass action analysis is made of the isotherm in region 2, an incorrect value for the maximum number of sites would be obtained. It is only because the CMC value for octylbenzenesulfonate greatly exceeds the D_f value at which the conversion of β-lactoglobulin to the "opened-up" form is complete that the importance of micellar type binding is recognizable.

ST. PAUL, MINN.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, STATE UNIVERSITY OF IOWA]

Formation of Peptide Bonds by Aminolysis of Homocysteine Thiolactones[1]

BY REINHOLD BENESCH[2] AND RUTH E. BENESCH[2]

RECEIVED JULY 11, 1955

It has been found that N-acylhomocysteine thiolactones can react with amines or amino acids with the formation of a peptide bond. The hydrolytic opening of the thiolactone ring can be suppressed in favor of aminolysis by using low temperatures, since the temperature coefficient of the rate of aminolysis was found to be exceptionally small. The preparation of some representative peptides in the form of their S-(phenylmercuri) derivatives is described and the possible application of the aminolytic reaction to the introduction of sulfhydryl groups into proteins is discussed.

Our interest in this field arose through a search for a method which would permit the *de novo* introduction of –SH groups into proteins under as mild conditions as possible. The only previous attempt in this direction seems to have been Schoeberl's[3] use of polythioglycolides, a rather ill-defined group of compounds. Furthermore, the data reported leave the claim that direct thiolation of protein amino groups has been accomplished by this method in serious doubt.

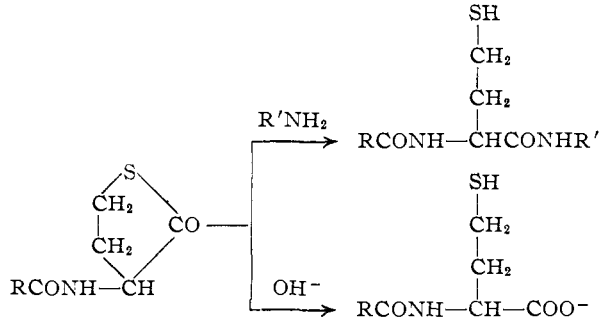
N-Substituted homocysteine thiolactones appeared to be more suitable starting materials for this purpose.[4,5]

In order to utilize these compounds for the formation of peptide bonds between homocysteine residues and the amino groups of other amino acids, peptides or proteins, several requirements should be met.

(1) The coupling should proceed under as mild conditions as possible, *i.e.*, in aqueous solution, at low temperature and as near neutrality as possible.

(2) Formation of homocysteinylhomocysteine bonds must be prevented. This was done by acylation of the amino group. The earlier experiments were performed with N-benzoylhomocysteine thiolactone.[4] This compound is, however, rather insoluble in water, particularly at lower temperatures. Therefore N-acetylhomocysteine thiolactone, which is extremely water soluble, was prepared so that the reactions could be carried out in completely aqueous solution.

(3) Hydrolytic splitting of the thiolactone ring should be avoided as far as possible, since this ring can be opened either by hydrolysis or by aminolysis



Since only the RNH_2 but not the RNH_3^+ form of an amino acid can react with the thiolactone, the solution must be sufficiently alkaline to produce an appreciable concentration of the RNH_2 species. In this *p*H range the hydrolytic opening of the ring therefore becomes a serious competitive side reaction. Kinetic studies provided a clue to this dilemma. It was found that, whereas the rate of hydrolysis has a normal temperature coefficient ($k_{t+10}/k_t \sim 2.5$), the influence of temperature on the rate of aminolysis is very much smaller ($k_{t+10}/k_t \sim 1.15$). This is illustrated by Tables I and II.

The results were obtained by following the disappearance of thiolactone at constant *p*H and with a 10-fold excess of the amine in the RNH_2 form. The half times for the disappearance of thiolactone therefore represent a comparison between the rate of hydrolysis in borate buffer and the rate of aminolysis plus hydrolysis in ammonia and glycine buffer, respectively. They clearly show that, although the rates of the two competing reactions are comparable or even identical (Table I) at elevated temperatures, the rate of hydrolysis—but not that of the aminolysis—becomes negligible at 0° (Table II). The small temperature coefficient of the rate of aminolysis may be due to the formation of an intermediate complex, the dissociation of which increases with increasing temperature (*cf.* Conant and Bartlett[6]).

(1) A preliminary account of this work was presented at the 74th Meeting of the American Society of Biological Chemists, San Francisco, California, April, 1955 (*Federation Proc.*, **14**, 487 (1955)).

(2) Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin.

(3) A. Schoeberl, *Angew. Chem.*, **60**, 7 (1948).

(4) V. du Vigneaud, W. I. Patterson and M. Hunt, *J. Biol. Chem.*, **126**, 217 (1938).

(5) J. S. Fruton, *Adv. Protein Chem.*, **5**, 1 (1949).

(6) J. B. Conant and P. D. Bartlett, THIS JOURNAL, **54**, 2893 (1932).