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Interaction of Dodecyl Sulfate Anions of Low Concentration with Alkaline Bovine Serum Albumin[1]

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Interaction of dodecyl sulfate anions with bovine serum albumin at high pH was studied, in which a maximum of ten detergent anions per protein molecule were employed. Without detergent, expansion of the macromolecule occurs. Detergent reduces the expansion, affording a demonstration of the ability of the hydrocarbon section of the detergent to induce considerable conformation changes, countering unfavorable electrostatic energy changes.

Interaction of serum albumin with various ions has been the subject of considerable research in other laboratories. Much of this previous research, in the case of detergent anions,[3] has been carried out at (total detergent concentration)/(serum albumin concentration) values, y, either so large that the chemistry of the system for small y levels is obscured, or else under conditions such that no qualitative separation of forces governing the binding process can be made. The majority of the work reported in the literature has been performed with the aid of phosphate buffers, the anions of which at neutral pH bind competitively.[4]

Results reported here deal with cases in which y ranges from 0 to 10, and at pH values such that the macromolecule bears a considerable negative charge. It is probable that ionic strength, and detergent chain length and flexibility, are important factors which bear on the physical chemistry of these systems. In this paper, the case for only one detergent and one ionic strength is discussed. An assumption is made (supported, however, by some data by Scatchard and coworkers[5]) that supporting electrolyte binding does not occur at high pH.

Besides the first stage in the binding process, in which organic anions become bound somewhere in the macromolecule, there occurs even at low y-values an accompanying major conformation change by the macromolecule. This change, with low y-values and pH > 10.8, amounts to a *decrease* in hydrodynamic volume.[6]

In order to give basis to the argument (below) about decrease in volume not being due to charge repulsion between detergent anions and the macroanion, a value $y = 10$ was selected for much of the work. This should increase the negative charge on the macroanion (all at pH > 10.8) such that, ordinarily, an increase in hydrodynamic volume would be expected. Instead, the opposite occurs, emphasizing the importance of the hydrocarbon section of the detergent anion.

Experimental Methods and Calculations

Materials.—The serum albumin was the Armour Co., crystalline product; various lot numbers were used. Dintzis' method[7] for deionization was used, except that about the first third of the protein issuing from the column was discarded. Deionization was performed at room temperature; concentration was obtained by dry weight. Ultracentrifugal analysis showed that about 5% of the deionized protein was polymerized. After deionization, the stock solutions were degassed and stored in the cold under nitrogen.

Carbonate-free KOH was prepared by the resin-exchange method of Powell and Hiller[8]; KCl was A.R. grade, recrystallized. Water was glass-redistilled. The detergent, sodium dodecyl sulfate (anion abbreviation: DDS^-), was highly crystalline material, a gift of Dr. Phillip Ross. It had originally been prepared by Dreger's method.[9] Flame analysis yielded the expected value for sodium content, with experimental error. Because many workers in research on BSA have used good grades of commercial sodium dodecyl sulfate, recrystallizing it several times from ethanol and drying, some of the experiments reported here were repeated with twice-recrystallized Duponol (duPont Co. NaDDS). No significant differences were observed in the detergent–protein interaction experiment on comparing the two kinds of detergents. However, it was found that the solubility of the commercial material in aqueous salt solutions is considerably larger. Hence there is probably some chain branching, or heterogeneity, or both, present in the commercial material. Because this research emphasizes the ability of anionic substrates to interact even at low levels of substrate concentration, glassware was quite thoroughly soaked with redistilled water, and rinsed with distilled ethanol, before being allowed to dry. In working at high pH, all solution transfers were made under nitrogen, titration chambers and viscometers were filled with nitrogen, etc.

The viscometers were Cannon–Fenske viscometers, with approximately 240 sec. flow times. The solutions were forced with nitrogen through medium porosity glass filters. Flow times were taken with a precision of 0.1 sec. Cumulative times were taken, beginning with the time the base was mixed with the protein. The protein flow times increase with cumulative time at high pH—the effect is larger in the case of no detergent ($y = 0$). The effects are not due to initial temperature nonequilibria.

Because of this cumulative time dependency by the protein flow times, the flow times for each solution were extrapolated to zero cumulative time. Even if this procedure was not followed, however, the qualitative results illustrated in Fig. 4 would still certainly obtain—in fact, Fig. 4 minimizes the difference between the case for $y = 10$ DDS^-/BSA and $y = 0$. This follows from the fact that at $y = 10$ the cumulative time dependency of the protein flow times is somewhat less in the case of $y = 10$ than for $y = 0$. Hence, if the flow times at some extended cumulative time (say 2000 sec.) were used, the final result would give rise to larger differences between constant pH intrinsic viscosities, $[\eta]$, than shown in Fig. 4; this would amount to a 5 to 10% change in the difference between ordinal values. The potentiometric measurements were slightly time dependent at the beginning of each measurement, but the difference between effects by the protein and those by which the electrode comes to equilibrium was not pursued, so data were taken only for the latter case. It is unlikely that in the high pH region there is a significant delayed uptake of base by the protein, as shown by experiments on a stopped-flow titration apparatus.[10]

Hence it is assumed that the moderate time dependency of the viscosity of the alkaline protein solutions, once the large majority of reagent is used up in the initial titration of the protein molecules, is not connected with processes which would cause major shifts in the potentiometric titration curve, Fig. 3.

N.B.S. buffer salts were used for standardizing the electrode. A Beckman model GS expanded scale pH meter was used, with a

(1) Presented in part at the 140th National Meeting of tbe American Chemical Society, Chicago, Ill., Sept., 1961.

(2) Department of Biochemistry, University of Minnesota Medical School, Minneapolis 14, Minn.

(3) J. F. Foster, "The Plasma Proteins," Vol. I, Academic Press, Inc., New York, N. Y., 1960, pp. 197–202.

(4) F. Karush, *J. Am. Chem. Soc.*, **73**, 1251 (1951).

(5) G. Scatchard, J. Coleman, and A. Shen, *ibid.*, **79**, 16 (1957).

(6) A number of other workers (see ref. 3, p. 199, for principal references) either observed or postulated an "opening up," or *increase* in hydrodynamic volume, using the same detergent, but the conditions were quite different; large y-values, and pH's from the isoionic point to 10.8, were employed.

(7) H. M. Dintzis, Thesis, Harvard University, 1952.

(8) J. Powell and M. Hiller, *J. Chem. Educ.*, **34**, 330 (1957).

(9) E. Dreger, *et al.*, *Ind. Eng. Chem.*, **36**, 610 (1944).

(10) J. M. Sturtevant and R. Lovrien, unpublished observations. It is necessary to qualify this remark by noting that (on the basis of six experiments covering the pH 11.1 to 11.7 region, constant ionic strength 0.15, 25°) there is a delayed uptake of OH^- by 3 to 4 phenolic side chains, but this delay is of the order of 50 msec. All other groups, starting from isoionic BSA, titrate instantaneously (10 msec. or less). Hence the majority of the alkaline expansions of BSA occur in considerably less than a second, if the reasoning used before,[11] for acid expanded serum albumin, is employed, coupled with the data in Fig. 4 of this paper.

(11) R. Lovrien and C. Tanford, *J. Phys. Chem.*, **63**, 1025 (1959).

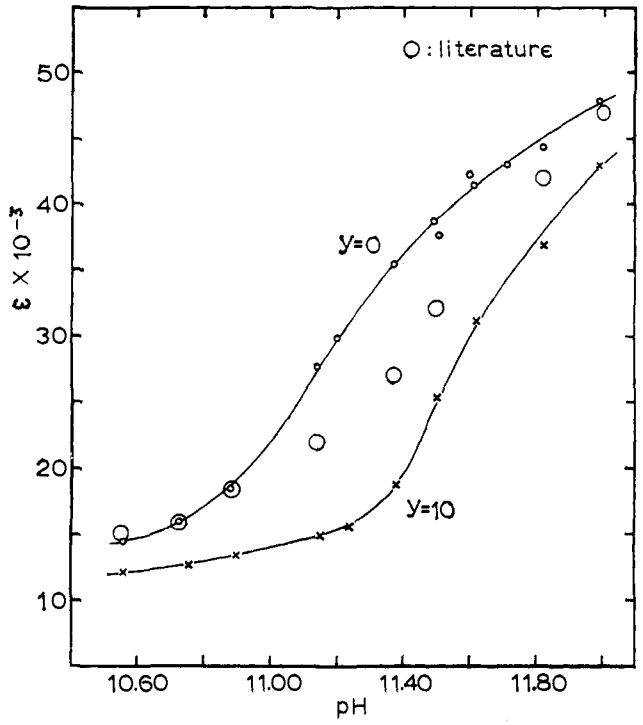

Fig. 1.—Molar extinction of BSA at 2950 Å., ionic strength 0.15, 25° based on mol. wt. = 69,000. Lower curve represents flattening of a titration curve by detergent anion. Definition of y is in the text. Literature values taken from ref. 17, Fig. 4—these are for the same temperature and ionic strength, nondeionized protein.

titration apparatus designed according to Tanford.[12] The electrode was calibrated at ionic strength 0.15, to correct for the overall apparent activity coefficient.

Temperatures were held to ±0.002° for viscosity measurements, ±0.01° for pH measurements, and ±1° for all other measurements.

Spectrophotometric measurements were made on a Cary Model 11 instrument. Fluorescence measurements were made on an instrument employing two Bausch and Lomb grating monochromators, with slit widths, measuring cell, and beam geometries, such that self-absorption phenomena, etc., in the fluorescing solutions were minimized. Experiments were performed at constant observing (fluorescence) wave length, in which exciting wave length was varied, for a series of samples in which the pH was changed. These yielded excitation spectra which were of varying amplitude, but of nearly constant wave length, 2850 Å., at the peak height. For that reason, and taking into consideration the factors mentioned above which bear on the geometry of the instrument, it seems reasonable that the fluorescence intensity observations are to some extent independent of the fact that the absorption spectra in the high pH region are changing, as the phenolic side chains become ionized. In addition, 2850 Å. is fairly close to the isosbestic region in the absorption spectra of neutral and alkali titrated serum albumin. Finally, the relative fluorescence intensity as a function of pH was covered over the pH range 2.5 to 12 and is in fair agreement with that of Steiner and Edelhoch[13] over a similar pH range.

Calculations.—The limiting kinematic viscosities follow directly from the experimental data; these were corrected, to yield the intrinsic viscosity at each pH, by Tanford's[14] method. The assumption was made that the partial specific volume of serum albumin remains constant over the whole pH range.

The ionic strength in the alkaline range was calculated on the basis of added neutral electrolyte (KCl only); reagent used to titrate the macromolecule becomes part of the neutral protein component.

Hydrogen ion titration data was used with the equation

$$\log \frac{\alpha}{1-\alpha} - \mathrm{pH} = \mathrm{p}K_{\mathrm{int}} - 0.868\,wZ \qquad (1)$$

where α is degree of hydrogen ion dissociation of the group in question; in the present case, α for the phenolic groups follows

(12) C. Tanford, in T. Shedlovsky, Ed., "Electrochemistry in Biology and Medicine," John Wiley and Sons, Inc., New York, N. Y., 1955.

(13) R. Steiner and H. Edelhoch, *Nature*, **192**, 873 (1961).

(14) C. Tanford, *J. Phys. Chem.*, **59**, 798 (1955).

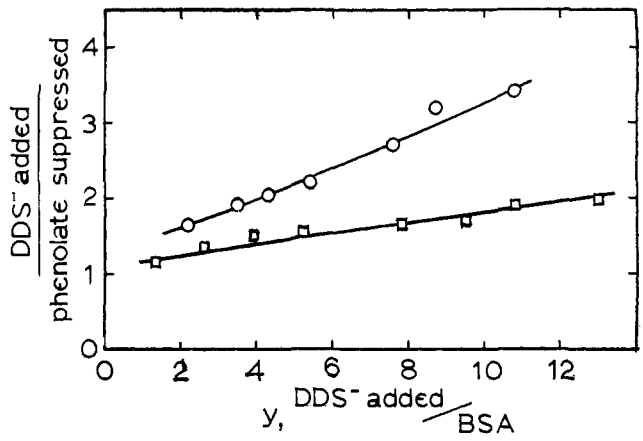

Fig. 2.—Effect of dodecyl sulfate anion on alkaline BSA, preventing ionization of phenolic groups at fixed pH. From absorption spectral data at 2950 Å.; ionic strength 0.15, 25°.

from Fig. 2. The mol. wt. was taken to be 69,000. The limiting molar extinction coefficient was found to be 57,200 in separate experiments. For phenolic groups, $\mathrm{p}K_{\mathrm{int}}$ was set equal to 10.35[15] and assumed to be the same for the case $y = 0$, and $y = 10$ DDS added per BSA; w is the electrostatic work parameter, and Z the assumed charge. The latter was obtained from the experimental titration curve (Fig. 3); in the case of $y = 10$, ten negative charges were added, assuming all the added detergent was bound. This assumption is probably not strictly correct, but is ameliorated somewhat by noting that, if all the detergent is not bound, the effects on the titration curves, etc., on a "per bound DDS^-" basis, must be even larger than those illustrated. With more dilute systems, *e.g.*, those used for spectrophotometric studies, mass action effects will cause a smaller proportion of the detergent to be bound; nevertheless, even the effects of only one or two detergents added per BSA are apparent, according to Fig. 2.

Experimental Results and Interpretation of the Data

Changes in the absorption spectrum of serum albumin at high pH are caused by ionization of the tyrosine phenolic side chains; this was quantitatively studied in 1943, by Crammer and Neuberger,[16] and again, in 1952, by Tanford and Roberts.[17] The results obtained here are plotted in Fig. 1, along with points taken from the Tanford and Roberts paper, all at ionic strength 0.15. The results are somewhat different in the pH 11.0 to 11.6 range, possibly for a number of reasons.[18] In the present work, the dodecyl sulfate is seen to cause a marked shift of the spectrophotometric titration curve to higher pH (*i.e.*, a flattening of the titration curve). This effect persists at lower added substrate levels, y, and the results for those cases are summarized by Fig. 2.

(15) C. Tanford, S. Swanson, and W. Shore, *J. Am. Chem. Soc.*, **77**, 6414 (1955).

(16) J. Crammer and A. Neuberger, *Biochem. J.*, **37**, 302 (1943).

(17) C. Tanford and G. Roberts, *J. Am. Chem. Soc.*, **74**, 2509 (1952).

(18) The difference in results is not accounted for by the small difference in the limiting extinction coefficient at high pH. The chief reasons may be: (1) The protein itself might be different; a more complete discussion of this aspect is given by Foster,[3] p. 181. (2) The protein used here was resin deionized, which would be expected to remove ionic fatty substrates (H. Dintzis, Ph.D. Thesis, Harvard Univ., Cambridge, Mass., 1952). A main point in this paper is that removal or addition of these, even in small amounts, has considerable effect on the protein behavior as shown in Fig. 1 and 2. Now in the 1952 paper by Tanford and Roberts,[17] resin deionization was not employed, but was employed for the 1955 paper by Tanford and co-workers.[15] As mentioned in the text, congruency of the potentiometric titration curve results in comparing the work here with the work by Tanford, *et al.*, in 1955, when they studied the potentiometric titration curve of serum albumin. Finally, both Tanford and co-workers and the work reported in this paper employed Armour crystalline serum albumin throughout. (3) Hydrocarbonaceous buffers, used in the earlier (1952) research, might now be expected to bind. This possibility was also mentioned by Tanford and co-workers. In any case, the results would be flattening of the titration curve, either from direct electrostatic effects due to charge neutralization by buffer cations, or from a final electrostatic effect caused by the mechanism discussed in this paper.

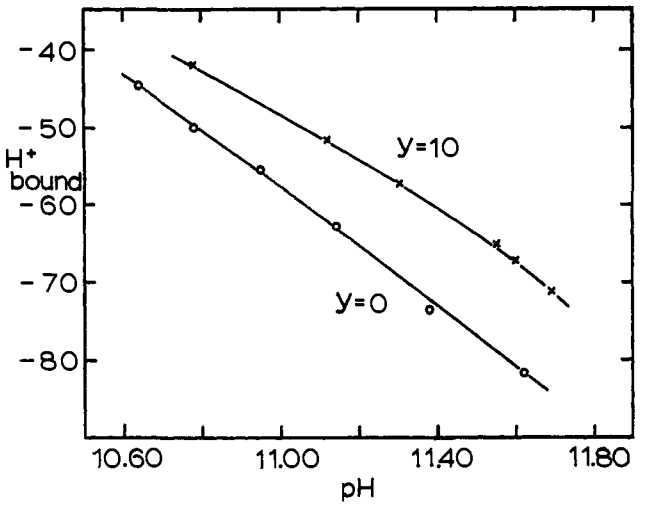

Fig. 3.—Potentiometric titration curve of BSA, ionic strength 0.15, 25°. Upper curve represents flattening of the titration curve by detergent.

Observation of flattening of titration curves by any operation gives rise to at least two possibilities: (1) There might be a change in pK_{int} of the titrating groups; (2) there could occur a change in the degree of proton dissociation if a conformation change, especially one entailing a major volume change, were forced on the macromolecule.

In order to distinguish which is the more likely possibility, the effect of dodecyl sulfate at $y = 10$, on both the potentiometric titration curve and on the intrinsic viscosity as a function of high pH, were examined. The results are plotted in Fig. 3 and 4, respectively.

Figure 3 shows that dodecyl sulfate, even in rather small amounts, affects the titration of a large number of groups, flattening the titration curve. The results for $y = 0$ yield a curve congruent with that of Tanford, Swanson, and Shore[15] (when comparison is made using the same molecular weight). Figure 4 shows that there is a marked decrease in the viscosity hydrodynamic volume of the macromolecule after it has been expanded at high pH. For that reason titration curve flattening effects by dodecyl sulfate is ascribed primarily to changes in the electrostatic interaction parameter, w, in eq. 1. Correspondingly, the w parameter was calculated for the case of phenolic group ionization with pK_{int} remaining constant. Representative values are listed in Table I.[19]

TABLE I

EFFECT OF ADDED DODECYL SULFATE, y, ON w
Ionic strength 0.15, 25°

pH	w at $y = 0$	w at $y = 10$
10.80	0.025	0.029
11.00	.020	.029
11.20	.016	.028
11.40	.015	.025
11.60	.014	.020

Near the onset of alkaline expansion, w is close to that observed by Tanford, *et al.*,[15] *ca.* 0.025. The effect of the detergent is, in the absence of gross changes in pK_{int} values of the titratable groups (see below), to keep w high. Since w depends inversely on the radius of the macroion, in whatever model might be chosen,[20] then the trends in Table I are consistent with the hypothesis that the detergent can maintain the

(19) Similar trends by w are obtained, if $-NH_2$ groups are considered, for the cases $y = 0$ and $y = 10$.

(20) C. Tanford, "Physical Chemistry of Macromolecules," John Wiley and Sons, Inc., New York, N. Y., 1961, Chapter 7.

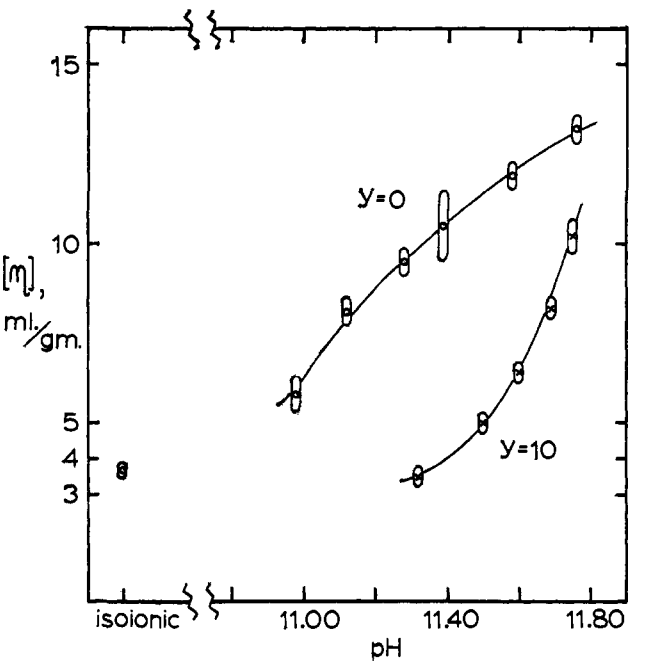

Fig. 4.—Effect of added detergent on the intrinsic viscosity of BSA at high pH, 25°, ionic strength 0.15 (excluding protein counter-ions); extrapolated to zero cumulative time after mixing base.

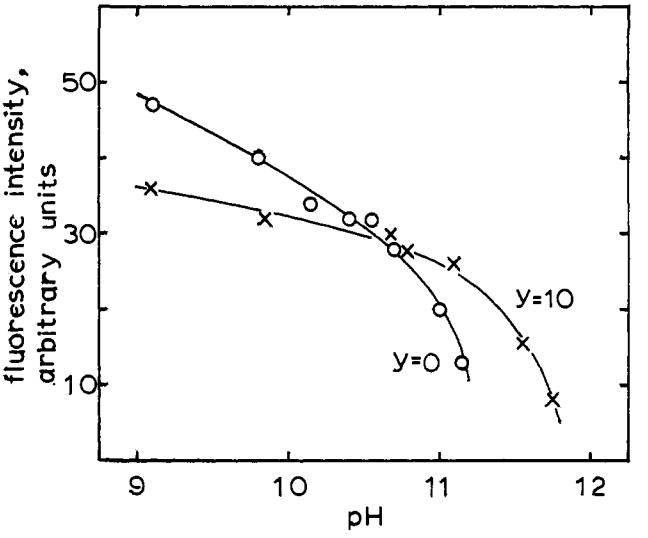

Fig. 5.—Effect of detergent on fluorescence intensity of BSA at high pH. Ionic strength 0.15, 25°; exciting wave length 2850 Å., emitting wave length 3350 Å.

macromolecule in a compact form, even at low y-values. Alternatively, if one begins with the expanded form, and adds detergent in a reversibility experiment, the detergent causes a major conformation change on binding, yielding compact macromolecules as a final product.

The dependency of fluorescence intensity for the case $y = 0$ and $y = 10$, as a function of pH, is shown in Fig. 5. For $y = 0$ the plot is very similar to that of Steiner and Edelhoch[13] who also used Armour crystalline BSA. (It was found also in this Laboratory, that at neutral pH the fluorescence of an equimolar amount of free tryptophan amino acid gave about the same fluorescence intensity as the protein, there being two tryptophans in BSA.)

Detergent causes a large change in most of the pH region. In the high alkaline branch of the plot, de-

tergent restores fluorescence, not back to that of the neutral protein molecule, but in that direction.

Fluorescing chromophores in a macromolecule are sensitive to a number of factors; among these are conformation changes which affect the surroundings of the chromophores.[21] Although the fluorescence data certainly cannot be interpreted unambiguously, it may lend some support to the thesis that detergent reverses to some extent the alkaline expansion of the BSA molecule.

Discussion

The detergent probably does not change pK_{int} of the phenolic groups directly. Interaction of the anionic end of the detergent, competing with salt ions of about 1000-fold higher concentration, to produce neutral phenol groups, seems unlikely. Stabilization of a phenolic hydroxyl group over a phenolate ion by limited amounts of hydrocarbon is a weak possibility, if one considers normal titration processes.[22-25]

The other mechanism, involving a major conformation change as part of the binding process, and in the case at hand, a volume shrinkage, seems the more simple one, with the changes in ionization of titratable groups occurring as the *final* step in the process, forced because of electrostatic interaction of the contracted macroanion with the solvent, the initial steps being shrinkage of the macromolecule induced by the detergent.[26]

The viscosity studies rather strongly reinforce this view, especially since intrinsic viscosities in the 3 to 6 cc./g. region are achieved (lower trace in Fig. 4). Such values are very characteristic of compact globular proteins, and indeed there are few alternatives for such a model either on a theoretical or experimental basis.[27,28]

Since detergent anions are being added to a flexible macroanion, an expansion should ensue, not a contraction. Hence it seems fairly certain that the hydrocarbon section of the detergent is not only somehow causing the shrinkage to ensue, but makes up for energy and entropy terms which would be expected as crude approximations to obtain: (1) positive free energy terms due to compression of the macroanion and charge neutralization of some of the titratable groups at constant pH; (2) negative entropy terms arising from side chain and backbone folding to yield a more compact macromolecule; presumably the hydrocarbon chain of the detergent also loses several degrees of freedom of rotation when it becomes bound, besides its cratic entropy.

Estimation of the entropy terms, however, is possible only for those cases where the simpler macromolecules, *e.g.*, linear vinyl polymers, change in volume. Even if it were not for the side chains, the highly cross-linked serum albumin molecule[29] would not afford a very good model for such a calculation; at best, a reasonable guess is that the sign of the chain conformation entropy term would be negative corresponding to refolding. Whether solvent structure rearrangement processes of the type proposed by Klotz and Luborsky[30] would contribute or not is a problem needing more investigation.

The free energy due to charge neutralization has a sign which is largely dependent on $(pH - pK_{dissociation})$; for protonation of five tyrosine groups at pH 11.3, $\Delta F_{T,P} = 5 \times 2.3 \times 2 \times 300\ (11.3 - 10.3) \sim +7$ kcal.

Calculation of the work necessary to shrink the macroanion to a smaller volume at constant pH depends on the model used. In the case at hand, the detergent causes a volume shrinkage of the serum albumin anion at high pH, which is assumed to be permeable to the solvent, the latter containing salt. Tanford's methods[31] for calculation of the electrostatic free energy, W_{el}, for solvent-permeable macroions were used; the macroanion radii were derived from the intrinsic viscosity data. At pH 11.40, $[\eta] = 10.6$ and 4.0 cc./g. for $y = 0$ and $y = 10$, respectively. Assuming the shape factor is 2.5 (axial ratio 1) and setting $[\eta] = (M/N)(4/3)\pi R^3$, $R = 46$ and 33 Å., respectively. From the titration curve for the case $y = 0$, $Z = -74$. For the case $y = 10$ the total charge (including the detergent contribution) is -70. These values were used in Tanford's equation (26–42).[20] In using this equation it was assumed that the other two radii, R_1 and R_0 in Tanford's notation, were both 23 Å. The results are quite insensitive to the value chosen for R_1; the value 23 Å. was picked for R_0 on the assumption that, since serum albumin seems to have a "core," this might comprise about half the molecule. Tanford's equation (26–42) yields, for $y = 0$ and $y = 10$, $W_{el} = 17{,}400$ cal./mole and $W_{el} = 39{,}800$ cal./mole, respectively, all at pH 11.40. In other words, a rather large positive electrostatic free energy must be made up by the whole system to force the volume shrinkage on the macroanion, although the charge on the molecule does decrease slightly. Even if we assume the detergent lent no negative charge to the macromolecule, but merely caused a volume decrease, W_{el} would still be about 28,000 cal./mole. Juggling R_1 and R_0 values within reasonable limits will also not change the qualitative picture.

The experiments are interpreted, then, as evidence that the hydrocarbonaceous section of the macromolecule, exerting hydrophobic forces, can compensate for some processes which should result in fair sized positive free energies of reaction.

However, there remains to be discussed the result of Strauss and Strauss.[32] They found that serum albumin did not bind *n*-heptane and isooctane (beyond a trace; one to two molecules of hydrocarbon per protein) in the neutral pH region. They concluded from this that serum albumin might lack large hydrocarbon regions under these conditions.

Nevertheless, simply examining the amino acid analysis of serum albumin would indicate that it possesses a considerable amount of nonpolar material, and it is believed[33] that, in the native (or globular)

(21) J. Longworth, Thesis, Sheffield Univ., Sheffield, England, 1962.

(22) E. Tanford, *J. Am. Chem. Soc.*, **79**, 5340 (1957).

(23) C. Tanford and J. Kirkwood, *ibid.*, **79**, 5333 (1957).

(24) K. Linderstrøm-Lang, in "Electrophoresis," M. Bier, Ed., Academic Press, Inc., New York, N. Y., 1959, Chapter 2.

(25) The material in the preceding three references discusses, *inter alia*, the energetics of burying a charged group. A shallowly buried group might well have an anomalous pK_{int} but the hydrocarbon structure would have to be rigid and tightly folded about the group in question excluding *all* water. The flexibility of saturated aliphatic hydrocarbons militates strongly against such a model, since besides the energy disadvantage there would arise an entropy disadvantage.

(26) Of course the processes may occur in concert, if the path between the initial and final thermodynamic states does not matter.

(27) J. T. Yang, in "Advances in Protein Chemistry," Vol. 16, Academic Press, New York, N. Y., 1961.

(28) C. Tanford, in "Symposium on Protein Structure," A. Neuberger, Ed., Methuen Co., London, 1958.

(29) Figure 4 illustrates an effect of the cross links in serum albumin; the upper limit of its intrinsic viscosity is much less than that of a non-cross-linked expanded coil of comparable molecular weight.

(30) I. Klotz and S. Luborsky, *J. Am. Chem. Soc.*, **81**, 5119 (1959), studied the binding of organic anions to serum albumin macroanion, comparing the effects of zero and 3.2 *M* glycine, both with low (0.02 *M*) NaCl concentration; binding was increased in the case of the latter. Because of the short dipole field of glycine it contributes weakly to the ionic strength when in low concentration, but at 3.2 *M* glycine (one charge for nine water molecules) the effective ionic strength must be enormous, much greater than 0.02, and the activity of the water markedly decreased.

(31) See ref. 20, Chapter 7.

(32) G. Strauss and U. Strauss, *J. Phys. Chem.*, **62**, 1321 (1958).

(33) C. Tanford, *J. Am. Chem. Soc.*, **84**, 4240 (1962).

state, much of this material is close enough together to form such regions.

Now it is possible that, in the case of detergent binding at *neutral* pH, an important contribution to the free energy of binding is donated by interaction of the anionic end of the detergent with the macromolecule; in fact, comparison of calorimetric studies[34] of binding inorganic anions and detergent anions yield evidence for this. In the case of the simple hydrocarbons, however, this ionic end is lacking. Also, the serum albumin molecule at neutral pH is already compact and presumably carefully folded. Hence it would perhaps have to rearrange, and make room for an incoming hydrocarbon as large as isooctane; this might be quite disadvantageous, thermodynamically. Furthermore, serum albumin at all pH values is covered to some extent with charges. In order for a hydrocarbon to bind, it might have to also get through the charged part of the exterior; in the appendix (below), some simple hypothetical models are examined to see what magnitude the electrostatic free energy contribution to such a process might be. It probably is always positive, and sometimes of a size large enough to affect the equilibria considerably.

The Conformation Change on Binding Detergent

Most interpretations of binding processes, in the absence of information about attendant conformation changes, make the assumption that pre-existing binding sites are involved. This assumption, in the absence of other information, is as reasonable as any other, but is nevertheless an assumption, especially when it is also assumed that the sites are rigid like those of a metal surface. The term "site" is convenient, however, to use for definition of the purely formal quantities which enter equations used to account for binding stoichiometry, and also to refer to those parts of the macromolecule which end up in contact with the substrate.

Karush[35] made a notable contribution by postulating that serum albumin possesses "configurational adaptability" (the term conformation is now preferably used when dealing with main chain arrangements). It is clear from Karush's discussion that there is a strong possibility that some macromolecules might engage in binding by allowing the binding site to be formed during the binding process. Karush did not discuss direct evidence to support his postulate at the time it was made.

In the case at hand, we have some evidence for distinguishing between this second process in serum albumin and the first process entailing binding to a rather rigid surface model. This follows because it is shown above that binding of detergent to alkaline expanded serum albumin leads to a major conformation change so that it is very possible that sites are formed as part of the binding process.

The quantity of greatest importance in any binding process is the free energy change. If a site can be formed as part of that process, rearrangement of the macromolecule might be fundamental to it, if that would decrease the free energy. Further, specificity of binding might not derive merely from rigid preformed sites, but from the ways in which the macromolecule can rearrange in forming complexes with some substrates. In all this, the substrate could nevertheless be finally bound in a precise way.

In connection with the idea that macromolecular rearrangement contributes to the binding process, Eyring, Lumry, and Spikes[36] have postulated "rack mechanisms," and Koshland[37] postulates an "induced-fit theory"; both pieces of work dealing with enzyme mechanism.

Appendix.—It is desirable to get some idea of what energy change is involved in transporting an uncharged molecule—preferably a hydrocarbon—from infinity to a point between a pair of charges which remain solvated by water throughout the process. Most of the refinements which probably can be made (see below) will not be dealt with here. Instead, an attempt is made to merely get an order-of-magnitude result. This will still depend sometimes in a rather sensitive way on the parameters chosen.

In one sense this problem has already been dealt with; it is the Born transport process,[38] involving transfer of ions from one solvent to another. Transfer of a pair of noncovalent ions, with radii of 1 Å., from an infinite (continuous) media of dielectric constant 78 to another with dielectric constant 5, would involve a positive free energy of transfer of about 62 kcal. per mole pair, a large quantity. If the ions remained stationary, and the solvent were transported so that water was removed and replaced with a continuous medium of hydrocarbon, the same result would obtain.

However, this model is unrealistic, because we have changed the solvent immediately surrounding each ion. A second, somewhat better, model would be the following: a very large hydrocarbon molecule is transported from infinity to a point such that it is centered between a pair of charges. The charges retain the original solvent (water) around themselves, and are far enough apart so that their coulombic interaction need not be taken into account for purposes of making approximations. The charges are spaced, however, such that on completion of the process, *i.e.*, after the hydrocarbon is inserted, the walls of the hydrocarbon are "seen" by the charges to be essentially planar, extending for a long way. The force of interaction of a charge adjacent to a plane dielectric boundary is obtained by the method of electric image charge, and is given[39] by

$$F = \frac{q^2}{4D_1b^2}\left(\frac{D_1 - D_2}{D_1 + D_2}\right) \qquad (1a)$$

D_1 = dielectric constant of the original medium (*i.e.*, H_2O, so $D_1 = 78.5$); D_2 = dielectric constant of the introduced dielectric, about 5; q is the charge of the ion (taken to be univalent, 4.8×10^{-10} e.s.u.); and b is the distance from the charge to the dielectric boundary. Since $D_1 > D_2$, the force is positive, and repels the charge, as one might expect. For two ions, the work against the force in bringing the ions up from infinity to a final distance B from their respective dielectric boundaries to assemble the configuration, would be

$$W = (-)\int_{\infty}^{B} 2F\mathrm{d}l = \frac{q^2}{2D_1B}\left(\frac{D_1 - D_2}{D_1 + D_2}\right) \qquad (2a)$$

For $B = 3$ Å., W is about 9.7 kcal.

A third model is: A pair of ions are close enough so that they engage in coulombic interaction, producing an electrostatic field, $\vec{E}_0$, distributed through the surrounding medium, with dielectric constant, D_0. Now

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a small hydrocarbonaceous body, of dielectric constant D_1, is transported up from infinity, to the equicenter on the line connecting the charges. The body has a volume, V_1, and the assumption is made that it produces no field of its own. In that case, macroscopic theory yields for the field inside the body[40]

$$\vec{E} = \frac{3D_0}{D_1 + 2D_0}\vec{E}_0 \qquad (3a)$$

If the medium is isotropic and linear and the body is small so that the field E_0 is essentially uniform around the equicenter of the charges, the work necessary to introduce the body is given by Stratton[41] as

$$W = {}^1/_2 \int_{V_1} (D_0 - D_1)\vec{E}\cdot\vec{E}_0 dv \qquad (4a)$$

Inserting 3a in 4a

$$W = \frac{3D_0(D_0 - D_1)}{2(D_1 + 2D_0)} \int_{V_1} E_0^2 dv \qquad (5a)$$

Now $E_0 = \Sigma q_i/D_0 d^2$, where q_i are the charges and d is the distance from the charge to the point at which the field has the magnitude E_0. At the equicenter, for charges of opposite sign $E_0 = 0$ and, therefore, $W = 0$.

However, for charges of like sign, $E_0 = 2q/D_0 d^2$, and since $\int_{V_1} dv = (4\pi/3)r^3$, where r is the radius of the body

$$W = \frac{8\pi(D_0 - D_1)}{D_0(D_1 + 2D_0)} q^2 \frac{r^3}{d^4} \qquad (6a)$$

For the case of a hydrocarbonaceous foreign body in H_2O, $D_0 = 78$, $D_1 = 5$. Let $r = 1$ Å., $d = 10$ Å. In this case $W = 0.005$ kcal. For $r = 2$ Å., $d = 10$ Å., $W = 0.039$ kcal. For $r = 2$ Å., $d = 8$ Å., $W = 0.095$ kcal. For $r = 2$ Å., $d = 6$ Å., $W = 0.3$ kcal. For $r = 3$ Å., $d = 6$ Å., $W = 1.0$ kcal.

These examples indicate that penetration of small hydrocarbons into regions of dispersed charges perhaps do not involve energies larger than ambient thermal energies (0.6 kcal.). For the case of penetration of a hydrocarbon of some size into more dense charge regions, such as would be expected on a compact protein molecule, where the charges would be on the average of 5 to 10 Å. apart, the energies can become considerable as W is sensitive to the factor r^3/d^4. Of course, for r^3/d^4 values which are very large, the assumptions on which eq. 4a were based begin to break down, especially the one concerning a uniform field over the volume V_1 before the hydrocarbon was inserted. Nevertheless, it may be that the numbers which (6a) yields are of the right order of magnitude, and are positive in all cases where $D_0 > D_1$.

When the hydrocarbon is inserted there would be in fact other effects which would occur, but which have been neglected here. One of these would be the polarization of the foreign body and the consequent production of a reaction field. It may be noted that even the rare gases have polarizabilities varying over a range of 0.5 to 10 cc.,[42] so that they and certain hydrocarbons, or apolar molecules with small dipole moments such as ethylenic molecules, might act quite differently from one another, apart from size factors. Another effect which might be significant in regions of high field strengths near macromolecular charges is the electrostriction effect; in this connection, O'Konski and Thacher[43] have evaluated the distortion of aerosol droplets by an electrostatic field.

A rise in energy of a macromolecule–substrate system, when the substrate is partly or entirely nonpolar, might govern either the kinetics or thermodynamics of binding reactions. Two aspects of this are of particular interest. In the equations above, the field between charges is dependent on their signs. (For interesting illustrations of the electric force patterns around pairs of charges, as contrasted for pairs of like charges with pairs of opposite charge, see ref. 41, p. 35.) Titration of proteins over a range of pH covering a series of total charge values will involve a number of changes of the charge distribution over the protein surface, even if all the side chains remain in place. Then if the models used above are valid to some extent, pH dependent binding of apolar substrates or reaction products would probably be observed, even if the region where the substrate attaches does not contain titratable groups. According to the same model, a titratable group would have to be nearby, however, to exert an effect.

A second aspect would have to do with conformation changes of macromolecule expansion processes in which production of charges near apolar side chains and larger regions would induce strain on the system. Eventually such charge–apolar group repulsion forces might aid coulombic forces in overcoming constraints in the system, leading to conformation changes and perhaps volume changes.

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