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The Role of Transport Phenomena in Ion Binding Studies of Serum Albumin*

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ABSTRACT: Two hypotheses previously suggested to explain the apparent dependency on protein concentration of equilibrium dialysis measured binding of long-chain ionic ligands to bovine serum albumin have been tested and disproved. Of greater importance, it is shown that the apparent protein concentration effect was an experimental artifact which originated in the anomalously slow approach to equilibrium with these ligands under the conditions of the earlier experiments. The effects of protein concentration, ligand size, supporting electrolyte, and temperature on the approach to dialysis equilibrium is examined. Hitherto unobserved and only partially understood kinetic anomalies occur when ionic ligands of high affinity are dialyzed *into* protein solutions which are appreciably more concentrated than 0.1%.

It has been reported from this laboratory (Ray *et al.*, 1966) that the binding isotherms of two long-chain ligands, dodecyl sulfate and dodecanol, to bovine serum albumin depend upon protein concentration. For example, the mole binding ratio, $\bar{\nu}$, in the range $\bar{\nu} < 10$ as measured by equilibrium dialysis with $\geq 1\%$ bovine serum albumin was considerably lower than that measured with 0.1% protein. This result and the observation by Klotz and Urquhart (1949) of a similar but much smaller effect in the binding of methyl orange to albumin are the only published references to such an effect.

It was suggested by Ray *et al.* (1966) that dimerization of serum albumin through a bifunctional ligand could account for the observed decrease in $\bar{\nu}$ at high protein concentration. This explanation was particularly attractive since the concentration effect was not observed with ligands containing less than 12 carbon atoms. Alternatively it was deemed possible that the ligand contained a small amount of low-affinity impurity which was not distinguishable from the ligand itself by the method of assay. Such an impurity would masquerade as a protein concentration effect.

We report tests of these two suggestions, the first by osmotic pressure measurements, and the second by measuring the binding by a triple-dialysis procedure which will eliminate any effect of ambiguous low-affinity impurities. Both hypotheses are shown to be invalid.

Of more importance the experiments reported here demonstrate that the large concentration effect observed by Ray *et al.* (1966) was an experimental artifact originating in their failure to recognize the extremely long times required for attainment of equilibrium under the conditions of their experiments. Data are presented to show that with certain ligands, and certain combinations of temperature, protein concentrations, and supporting electrolyte the approach to thermodynamic equilibrium is unexpectedly slow.

Experimental Section[1]

Materials. Crystalline BSA (Nutritional Biochemicals Corp., lot no. 5776, 7799, and 9385) was dissolved in distilled water and deionized by passage through a mixed-bed ion-exchange column (Ag 501-X8, Bio-Rad Laboratory) containing a small portion of Dowex 50W-X8 at the bottom. The protein solutions were refrigerated and used within 2 weeks.

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[1] "Certain commercial equipment, instruments, or materials are identified in this paper in order to adequately specify the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Bureau of Standards."

TABLE I: Osmotic Pressure Measurements on Bovine Serum Albumin Complexed with 0–5 moles of Dodecyl Sulfate.

Temp (°C)	$\bar{\nu}$	C (g/l.)	π (cm)	$(\pi/CRT) \times 10^4$ (mole/g)
30	0	3.33	1.18	0.138 ± 0.005
30	0	6.14	2.18	0.141 ± 0.002
30	0	10.00	3.58	0.140 ± 0.002
30	0	29.50	10.94	0.145 ± 0.001
30	1.0	20.00	7.78	0.150 ± 0.001
30	5.0	10.00	3.79	0.142 ± 0.002
30	5.0	20.00	7.97	0.156 ± 0.001
25	0	10.00	3.69	0.147 ± 0.002
25	0	29.50	10.79	0.145 ± 0.001
25	1.0	10.00	3.75	0.148 ± 0.002
25	1.3	10.00	3.55	0.142 ± 0.002
25	2.4	10.00	3.64	0.144 ± 0.002

The alkyl sulfates were a special grade made by Mann Research Laboratory and they were recrystallized from boiling ethanol before use. These materials were kept desiccated and refrigerated. Pure 4-(2-dodecyl)benzenesulfonate was a gift from Dr. Herman S. Block, Universal Oil Products, and was used as received. Picric acid was obtained from Eastman Organic Chemicals.

Soluble ultraviolet-absorbing components of the dialysis membranes (Visking) were removed by boiling the membranes in distilled water, rinsing with several changes of distilled water, and storing for several days at 2° in large volumes of distilled water.

Methods. A membrane osmometer (Mechrolab, Model 501) was used to determine osmotic pressures. Measurements were made at either 25 or 30° in phosphate buffer, 0.033 ionic strength, at pH 5.6.

Equilibrium dialysis procedures employed the familiar bags (5–15 ml) and bottles (30–100 ml). Depending upon the intent of a specific experiment, all, part, or none of the ligand was initially placed on the protein side of the membrane. By mounting bottles on a slowly rotating wheel the contents were stirred for the duration of the experiment. Temperature-controlled spaces were employed to attain the temperatures 23 and 2°.

Alkyl sulfates and dodecylbenzenesulfonate were assayed by forming a complex with methylene blue (Ray *et al.*, 1966), extracting the complex into chloroform, and measuring the optical density at 655 and 650 nm, respectively. The precision of the analysis was improved by vigorous shaking of the volumetric flask in the extraction procedure, transferral of the flask contents to test tubes and delaying for 20 min the measurement of the absorbance of the chloroform layer.

Picrate concentrations on the nonprotein side of the membrane were assayed spectrophotometrically. The absorption (optical density 0.020–0.030) contributed by residual soluble membrane components was accounted for by measuring the absorbance of a dialysate in an experiment involving only protein and buffer. Correction for adsorption of picrate on dialysis tubing was made by assuming it to be independent of the presence of the protein.

A few dialysis experiments carried out without added neutral salts required estimation of the Donnan effect, and therefore required measurements of the membrane potentials in the usual way.

Results

Effects of Protein Concentration on Equilibria Osmotic Pressure. The osmotic pressure of 0.3–3.0% solutions of BSA to which 0–5 moles of dodecyl sulfate was bound per mole of protein are given in Table I. The data in the detergent-free system are in reasonable agreement with that generally reported on this protein in that they yield by the usual extrapolation procedure a molecular weight of approximately 71,000. Without knowledge of how the second virial coefficient varies with $\bar{\nu}$, it is inappropriate to derive molecular weight estimates of the BSA–dodecyl sulfate complexes. However the data of Table I do not give evidence of the large degree of dimerization *via* ligand required for an explanation of the concentration effect noted by Ray *et al.* (1966).

Multiple Dialysis. Figure 1 gives the results of an experiment to evaluate the possibility that the protein concentration effect in the binding of dodecyl sulfate reported by Ray *et al.* (1966) was caused by the presence in the dodecyl sulfate sample of lower affinity analogs which were not distinguishable from ligand itself by the method of assay. A 1% BSA solution containing sodium dodecyl sulfate was dialyzed 28 hr against buffer. The external solution (the nonprotein side) was removed and analyzed. The result is plotted as $\bar{\nu}$, the mole ratio of bound ligand, against the log of the equilibrium ligand concentration, C. At the concentration of ligand present in the first dialysis, nearly all of the dodecyl sulfate in the system was bound. If the ligand sample does not contain a low-affinity component, replacement of the dialysate by pure buffer and continuing dialysis to a new equilibrium will have no effect other than to establish an additional point on the more dilute part of the binding

isotherm. If there is a low-affinity component present, it will be present predominantly as free ligand and hence, its total concentration will be reduced by a factor of approximately two[2] (while the total concentration of dodecyl sulfate on the protein side is only slightly reduced) as a result of the second dialysis step. The data of Figure 1 show, however, that the second dialysis step data fall on the same isotherm established by the first and, hence, eliminates the possibility that the original ligand sample contained lower affinity components, the analysis for which is not distinguishable from that of the ligand itself.

The third dialysis step of the experiment described by Figure 1 was performed by tenfold dilution of the dialyzing protein solution and subsequent dialysis *vs.* pure buffer. The slight increase observed in measured binding with 0.1% protein is only barely in excess of experimental error. It is negligibly small relative to the effect noted by Ray *et al.* (1966) and establishes that the binding of dodecyl sulfate is not significantly dependent upon albumin concentration, at least after removal of parts of the original ligand material.[3]

Kinetic Aspects of Dialysis Experiments with High-Affinity Ligands. We have examined the effect of protein concentration on the relative ease of attaining equilibrium in the dialysis of several ligands. The rate-determining step in these dialysis measurements is that of transport across the membrane and not that of the reaction between BSA and ligand. Our experiments show that, depending upon the size of the ligand, the rate of dialysis across the membrane is not only affected by protein concentration but is also dependent upon the nature of the supporting electrolyte and temperature. As a result of these experiments we suggest that the apparent contradiction between the results of Figure 1 and those of Ray *et al.* (1966) arises because of failure in the earlier work to allow experimentally for the unusually low rate of transport of the long-chain ligand across the membrane.

In Figure 2, the dialysis of dodecyl sulfate to albumin is shown to be first order over most of the process. Since at relatively low ligand concentrations essentially all ligand transported to the protein side is bound to the protein, even when the concentration of the latter is only 0.1%, the rate is approximately proportional to the ligand concentration on the nonprotein side over a large portion of the dialysis period. The slopes of the initial portions of the semilog plots of Figure 2 should be, and are, independent of protein concentration.

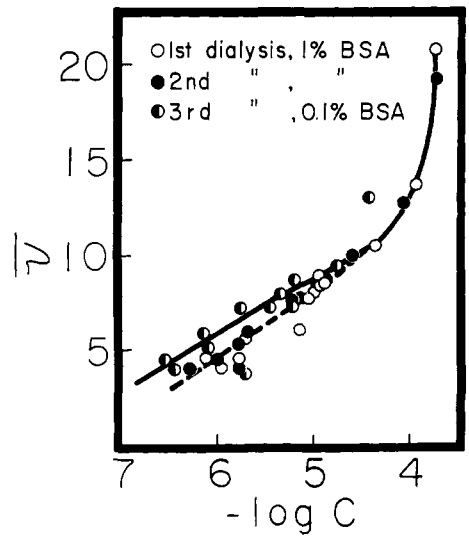

FIGURE 1: Binding of dodecyl sulfate in 0.1 and 1.0% bovine serum albumin solution at 2°, measured by dialysis. $\bar{\nu}$ is the mole binding ratio, C the equilibrium ligand concentration.

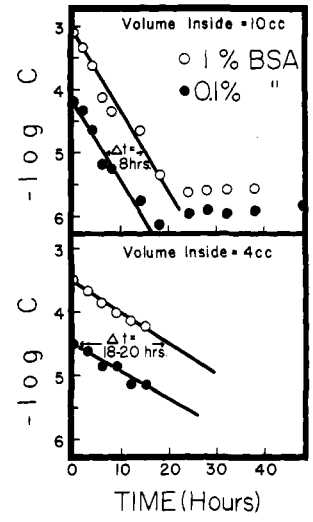

FIGURE 2: First-order diffusion of dodecyl sulfate across the membrane in 0.1 and 1.0% bovine serum albumin at 2°.

If the straight lines in Figure 2 represented the entire process of the approach to equilibrium, the dialysis against the more concentrated protein solution would reach equilibrium in an additional 8 or 18 hr, depending upon the geometry, beyond that required in the dilute protein dialysis where the mass transport of ligand across the membrane is only one-tenth as great. However, the process depicted in Figure 2 is a poor representation of the final approach to equilibrium, where the amounts of free ligand on both sides of the membrane approach equality. Analysis of the entire course of the approach to equilibrium is a difficult exercise, but we can examine the initial state (as above) and the terminal stage, as follows, without difficulty.

[2] Assumes equal volumes on the two sides of the membrane.

[3] Since the highly purified preparation of dodecyl sulfate used by Ray *et al.* (1966) was no longer available for test by the triple-dialysis method just described, the possibility must be considered that the concentration dependence reported by these authors was due to low-affinity impurities in the preparation used by them. This possibility appears to be excluded by (a) the high purity of the Ray preparation; and (b) most importantly by the fact that the apparent concentration dependence reported by Ray *et al.* can be reproduced with the present preparation if the experiments are performed as in the earlier research.

As the final equilibrium is approached we can write

$$\frac{d(a - x)}{dt} = -k(a - x) + k(1 - p)x \qquad (1)$$

where a is the ligand concentration on the nonprotein side at the beginning of the experiment, x and $a - x$ are the concentration at time t on the protein and nonprotein sides, respectively, k is a rate constant dependent upon a number of factors, including the area of the membrane, and p is the fraction of total ligand on the protein side which is bound to protein. With anions of high affinity, p tends to approach unity, especially when $\bar{\nu}$ is small; it is nearer unity at high protein concentrations than at low ones. Although p is a function of time, starting at 1.0 and gradually declining to a slightly lower value such as 0.98, we have assumed it to be a constant in the following. Integration of eq 1 and introduction of initial conditions gives

$$t = \frac{x_\infty}{ka} \log \frac{x_\infty}{x_\infty - x} \qquad (2)$$

in which x_∞ is the equilibrium concentration of x (bound plus free), and $1 - p$ has been replaced by $(a/x_\infty) - 1$. The equilibrium concentration of free ligand is $(1 - p)x_\infty$.

It will be noticed that if log $(x_\infty - x)$ is plotted against time, the limiting slope as x_∞ is reached in either dilute or concentrated protein solution will contain the same factor x_∞/a. When $\bar{\nu}$ is small, this factor is near unity with both protein concentrations, and the rate of final approach to equilibrium ($x = x_\infty$) should not differ greatly in systems which differ only in protein concentration and a.

Thus a simple kinetic picture does not include any factors which would lead one to expect a much slower approach to equilibrium with more concentrated protein. However when 99% of the ligand transport required for a given equilibrium has occurred, the displacement of $a - x$ from $a - x_\infty$ is relatively small in a 0.1% BSA experiment compared with that using 1.0% BSA. At the latter concentration more than 99.7% of a may have to be transported in a dialysis experiment with a high-affinity ligand in the low $\bar{\nu}$ range.

In Figure 3 a pronounced delay in reaching equilibrium is shown with dodecylbenzene sulfate, for which x_∞/a at low $\bar{\nu}$ is even higher than 0.997. The magnitude of the effect is particularly noteworthy in view of the fact that the experiments were carried out at room temperature rather than at 2°. Again it is apparent that an experimental design which places all ligand initially on the nonprotein side of the membrane imposes an unusually long equilibration period which if not provided leads erroneously to the conclusion that a protein concentration effect on the equilibrium exists. The position of the true isotherm is given when the equilibrium is approached from both sides. From such data in Figure 3 no concentration effect appears at $\bar{\nu} < 10$. At $\bar{\nu} > 10$ the data obtained in the more concentrated BSA solution are slightly but significantly displaced in the direction of lower binding relative to that obtained with 0.1% protein. Since these latter

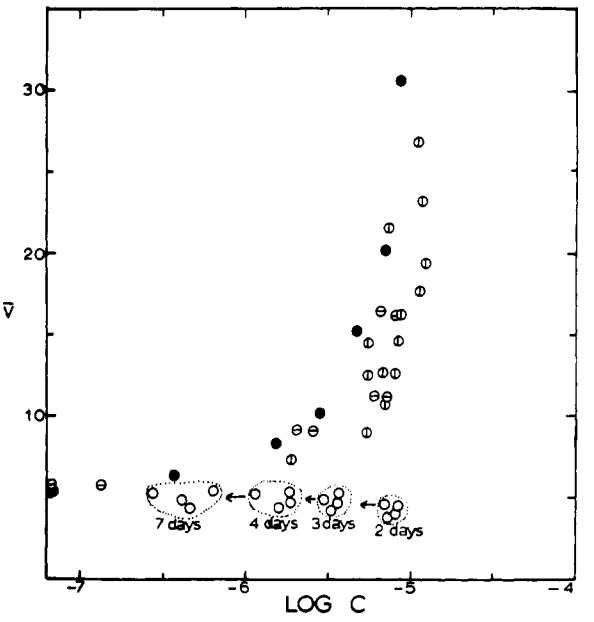

FIGURE 3: Binding of dodecylbenzenesulfonate to 0.1 and 1.0% bovine serum albumin at 23° in phosphate (pH = 5.6, μ = 0.033). Experimental conditions for dialysis: (○) 1% bovine serum albumin, ligand initially on nonprotein side; (Φ) 1% bovine serum albumin, ligand initially both sides of membrane, *net* diffusion toward protein; (⊖) 1% bovine serum albumin, ligand initially both sides of membrane, net diffusion away from protein; (●) 0.1% bovine serum albumin.

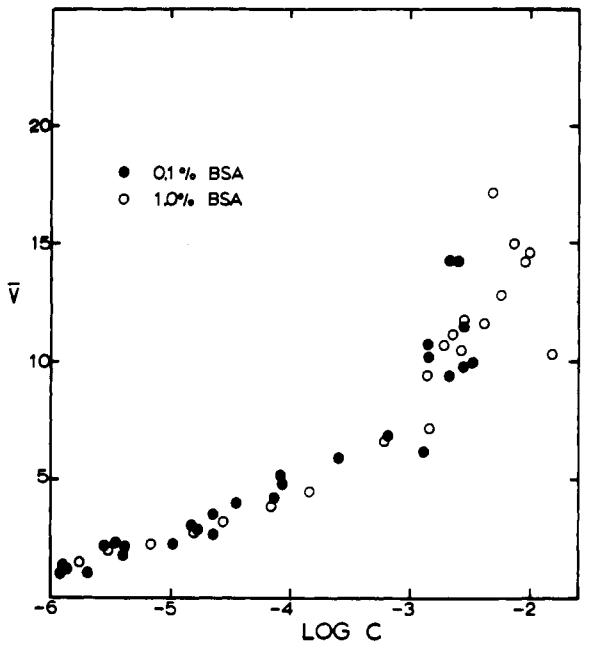

FIGURE 4: Binding of picrate to 0.1 and 1.0% bovine serum albumin at 23° in phosphate (pH 5.6, μ = 0.033).

data represent experiments with net diffusion in some cases toward and in others away from the protein, the effect is not kinetic, *i.e.*, is a valid equilibrium effect. At $\bar{\nu} > 10$, however, unfolded protein is present

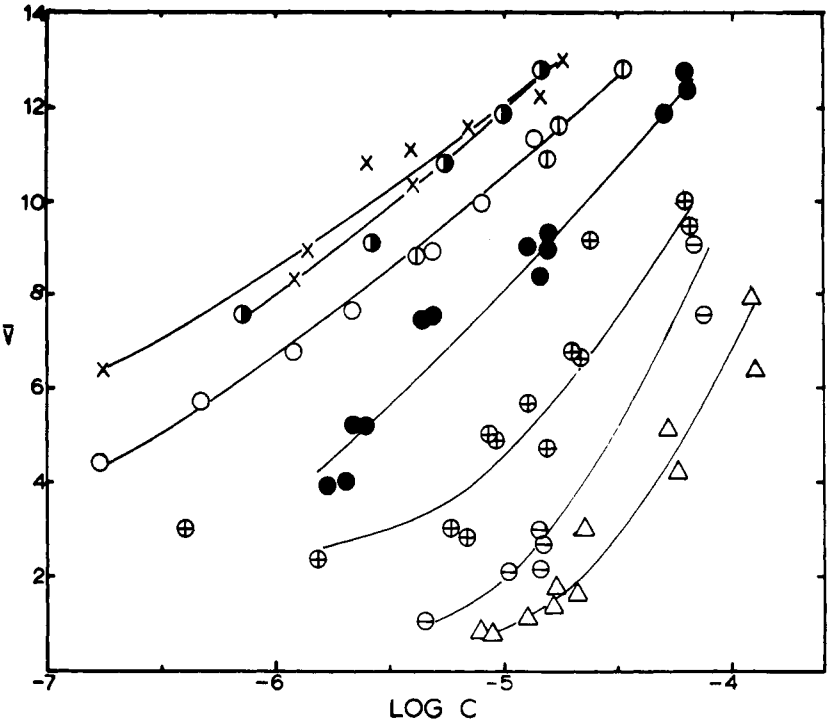

FIGURE 5: Effect of supporting electrolyte concentration on "apparent" equilibrium achieved in dialysis (17–24 hr) of dodecyl sulfate with 0.1% bovine serum albumin at 23°. Experimental conditions: Ligand initially on protein side; (○) 0.033 μ phosphate buffer, pH 5.6; (◑) 0.0005 M NaCl; (×) H_2O.Ligand initially on nonprotein side; (⊕) 0.033 phosphate buffer, pH 5.6; (●) 0.005 M NaCl, (⊕) 0.001 M NaCl, (⊖) 0.005 M NaCl, (△) H_2O.

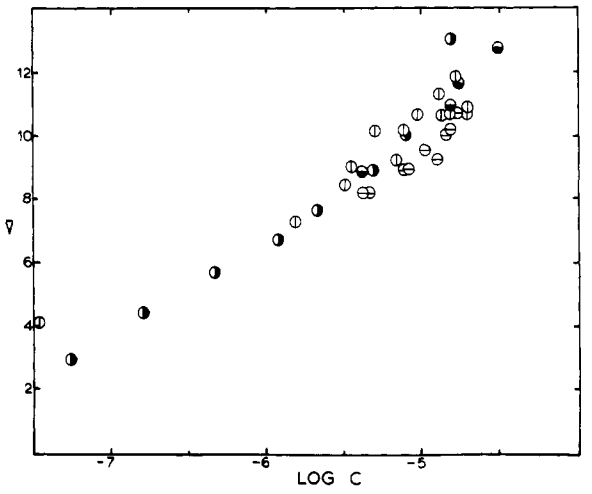

FIGURE 6: Equilibrium binding of dodecyl sulfate to 0.1% bovine serum albumin in water alone, and in buffered solutions at 23°. *Net* diffusion away from protein; (◑) 0.033 μ phosphate buffer, pH 5.6; (⊕) water. *Net* diffusion toward protein; (◒) 0.033 μ phosphate buffer, pH 5.6; (⊖) water.

(Reynolds *et al.*, 1967) and a different situation may prevail.

The binding of a less asymmetric ligand, picrate, is clearly independent of protein concentration (Figure 4).

Kinetics. THE EFFECT OF MEDIUM, LIGAND SIZE, AND TEMPERATURE ON KINETICS. Figure 5 demonstrates the effect of supporting electrolyte on the speed with which equilibrium across the membrane is achieved in a dodecyl sulfate dialysis experiment at 23°, with 0.1% BSA. The measured binding in 0.033 μ phosphate buffer is independent of the initial distribution of ligand on the two sides of the membrane and therefore represents equilibrium. In the total absence of salt, or at all NaCl concentrations, the results depend markedly upon the design of the experiment, the apparent dependence being increasingly marked as the electrolyte concentration is reduced. Ligand has difficulty crossing the membrane when no buffer is present and hence the no-salt and low-salt isotherms of Figure 5 do not represent equilibria.

In Figure 6 additional binding measurements without salt, but with ligand initially on both sides of the membrane, are, in essential agreement with those measured in buffer when corrected for Donnan inequalities. The close agreement in the low $\bar{\nu}$ range between the data obtained in the presence and in the absence of salt suggests that the usual exponential correction terms ($e^{-2\omega z}$) applied in calculation of binding constants from isotherms of ionic ligands (Klotz *et al.*, 1946; Scatchard, 1949) are unnecessary in this case, as has been noted earlier (Ray *et al.*, 1966; Reynolds *et al.*, 1967). It is also clear that the buffer anions do not compete effectively with these ligands, even though the concentration of the former at equilibrium is 10^3–10^4 times the concentration of ligand.

Figure 5 makes clear that equilibrium is approached

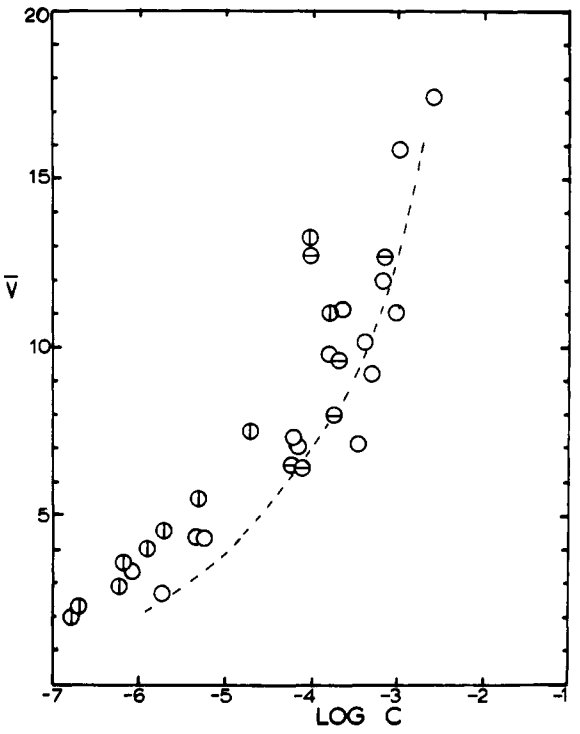

FIGURE 7: Equilibrium binding of octyl sulfate to 0.1% bovine serum albumin in water alone and in buffer (μ = 0.033, pH 5.6; Reynolds *et al.*, 1967). No-salt experiments: (○) ligand initially on both sides of membrane; (Φ) ligand initially on protein side; (⊖) ligand initially on nonprotein side.

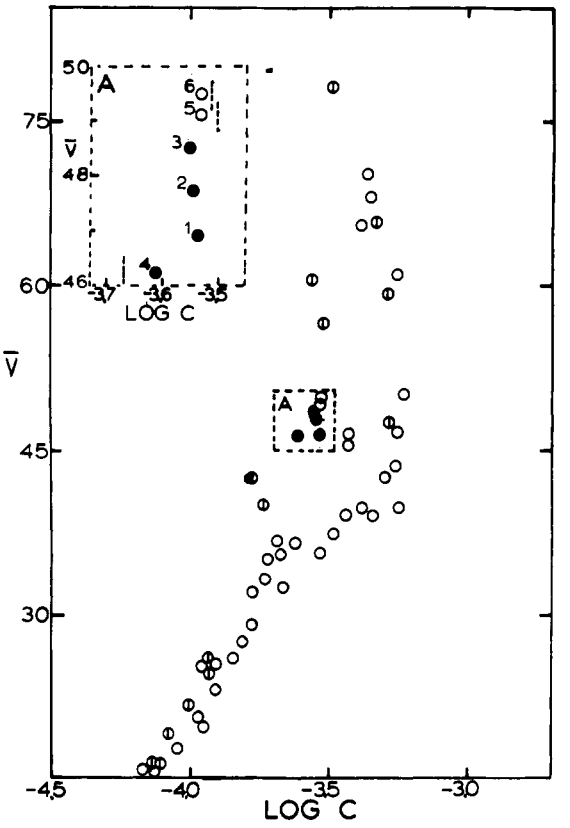

FIGURE 8: Effect of supporting electrolyte on "apparent" equilibrium at higher $\bar{v}$ binding of dodecyl sulfate to 0.1% bovine serum albumin at 2°. *Net* diffusion toward protein; (○) 0.033 M NaCl and (Φ) 0.033 μ phosphate buffer (pH 5.6) *Net* diffusion away from protein; (●) 0.033 M NaCl. See text for explanation of insert A.

more closely in a given time interval when all ligand is initially added to the protein rather than nonprotein side of the membrane. This is undoubtedly related to the fact that very much less transport is required; very little ligand is free and transportable.

The effect of electrolyte on the apparent binding isotherms of Figure 5 may be due to the shielding by electrolyte ions of the electrical field of ligand anions adsorbed on membrane pore surfaces. Adsorption of even very small amounts of dodecyl sulfate may result in significant electrical repulsion of ligand (Kushner and Parker, 1957). At higher electrolyte concentration the extent of adsorption to the membrane may be reduced. Dialysis measurements of the binding of a shorter chain ligand, octyl sulfate (Figure 7), are less sensitive to the initial experimental distribution of ligand on the two sides of the membrane. The measured binding is only slightly less in buffer than in water (corrected for Donnan effects) for $\bar{v}$ below 7 and not significantly different at higher $\bar{v}$. Again the inorganic anions appear to offer only limited competition.

THE EFFECT OF SUPPORTING ELECTROLYTE AND TEMPERATURE ON THE KINETICS OF DIALYSIS EXPERIMENTS AT $\bar{v} > 10$. The higher $\bar{v}$ range of dodecyl and myristyl sulfate binding depicted in Figures 8 and 9 demonstrates an unexpected kinetic effect observed at 2° when NaCl is substituted for phosphate buffer (μ = 0.033, pH 5.6) of equivalent ionic strength. Most of these experiments were initiated with ligand on both sides of the membrane. In Figure 8, except for the data in the area defined by the dashed lines, all points represent experiments with *net* diffusion of ligand toward the protein. At $\bar{v} > 37$, most of the results in the sodium chloride medium show less binding relative to that found with phosphate buffer. The data included within the dashed lines of Figure 8, an area enlarged in the insert, show that the apparent difference in the binding in the two media is a kinetic effect and does not represent a difference in equilibrium binding. Points 1–3 are from an experiment in triplicate carried out with constant agitation at 2° for 72 hr. Aliquots from the nonprotein side were then removed for analysis and replaced by salt solution with or without ligand so that the ligand concentration external to the protein became that represented by the three vertical dashed lines of the Figure 8 (insert). After 24-hr additional dialysis, points 4–6 were obtained. Two of these three points represent experiments in which net diffusion during the final 24-hr period was toward the protein. The six points clearly define the isotherm in the $\bar{v}$ range 45–50 as falling between $-\log C = 3.5$ and 3.6 and the maximum difference (10–15%) in equilibrium $\bar{v}$ values in buffer over that in salt is much reduced from the apparent difference indicated by some of the data of Figure 8.

This unexpected kinetic difference between chloride

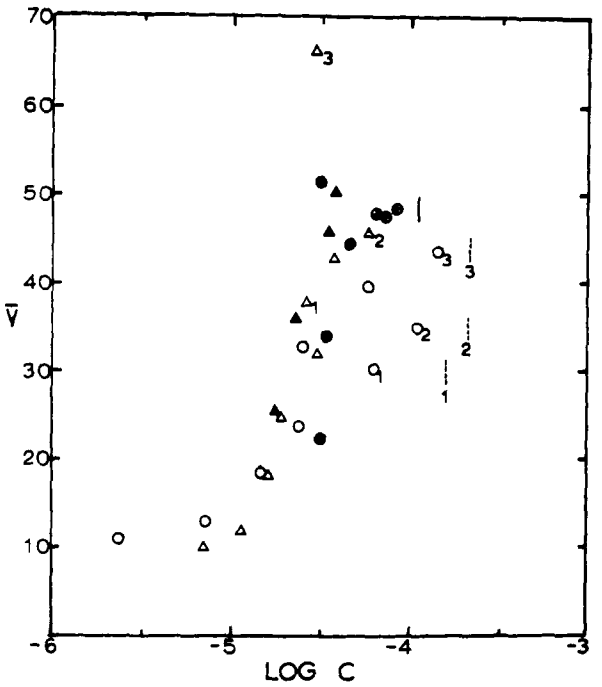

FIGURE 9: Effect of supporting electrolyte on "apparent" equilibrium at higher $\bar{\nu}$ binding of myristyl sulfate to 0.1% bovine serum albumin at 2°. Filled symbols ((●) 0.033 M NaCl, (▲) 0.033 μ buffer, pH 5.6) represent *net* diffusion away from protein, unfilled symbols net diffusion toward protein. Vertical dash lines represent initial ligand concentration on nonprotein side for points labeled 1–3. Cross-hatched circles represent experiments started with ligand concentrations on nonprotein side equal to that indicated by solid vertical line but with 7, 10, and 17% greater total ligand concentration on the protein side than that calculated for the experiment in chloride labeled 2. Dialysis in all experiments was for 72 hr.

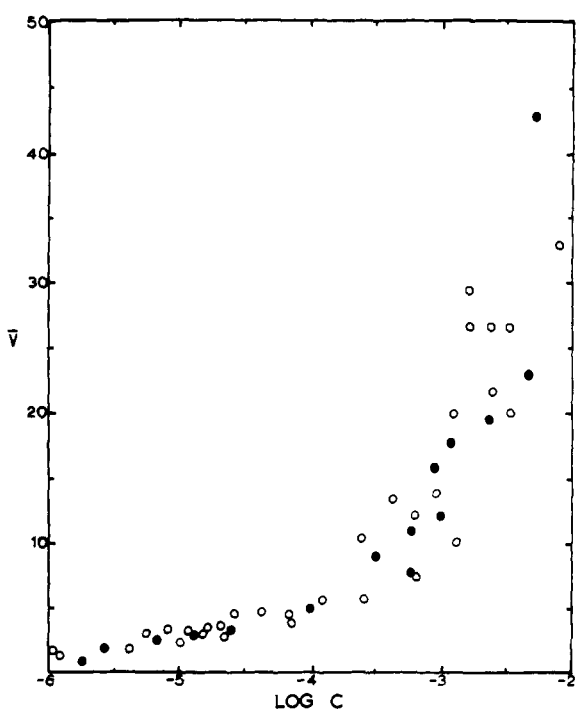

FIGURE 10: Binding of picrate to 0.1% bovine serum albumin at 23° in salt and buffer mediums. (●) 0.033 M NaCl and (○) 0.033 μ phosphate buffer (pH 5.6).

and phosphate observed with dodecyl sulfate was also found with myristyl sulfate at 2°. In Figure 9, the numbered points represent duplicate experiments in which only the supporting electrolyte was different. In each of the pairs the apparent binding is greater in the buffered solution than in chloride. The experiments represented by the cross-hatched circles show that the open circle point labeled 2 (and hence open circle points 1 and 3) which designates an experiment in the NaCl medium, cannot represent an equilibrium result. Additional evidence that the 2° results of Figure 9 are rate determined rather than equilibrium is provided by binding data obtained at 23°. At this temperature, with myristyl sulfate initially distributed on both sides of the membrane and dialysis limited to 17 hr to avoid complications due to ester hydrolysis, the binding isotherms obtained in buffer and sodium chloride are superimposable from $\bar{\nu} = 20$ to 55. We have also observed kinetic effects with dodecylbenzene sulfonate similar to those reported with dodecyl and myristyl sulfates. The importance of ligand size in these experiments is indicated by the fact (Figure 10) that the measured binding of picrate is independent of whether the support electrolyte is neutral salt or buffer ion.

Discussion of Kinetic Aspects of Dialysis Measurements. Equilibrium dialysis has long been considered the method of choice for binding studies with macromolecules. However, application of the method to a particular ligand and substrate requires assurances that the process of dialysis has proceeded sufficiently close to the equilibrium state to permit thermodynamic conclusions to be drawn. The work just described shows that equilibrium may at times be unexpectedly difficult to achieve and this may result in grossly misleading conclusions. With serum albumin and large detergent ligands of high affinity, several cases have been presented in which significant delays in achievement of equilibrium occur under certain conditions. The first involved dialysis with a relatively high protein concentration (*e.g.*, 1% BSA). Although the delay has not been explained, it is likely to be due in part to the difference in amount of ligand (over that in 0.1% BSA experiments) which must be transported where ligand is initially separated from protein. This is not the complete answer since equilibrium in the absence of protein, even at fairly high ligand concentrations is achieved fairly rapidly. High protein concentrations, with or without ligand, may coat the membrane in such a manner as to reduce permeability to ligand. The permeability remains high enough to allow fairly rapid attainment of equilibrium when ligand is initially on the protein side, and only a small fraction need pass to the exterior solution.

A second case of delayed attainment of equilibrium occurs when high-affinity detergent anions are used without salt or with neutral salt instead of the usual phosphate buffer medium.

When no salt is present the rate is particularly slow because charge conservation requires that ions cross

the membrane in pairs of unlike charge, *i.e.*

$$\text{rate } (H_2O) = k_1C^2$$

where C is the concentration of ligand. With neutral salt present, the exponent is reduced to unity since exchange of ions of like charge across the membrane may take the place of travel in pairs, *i.e.*

$$\text{rate (neutral salt)} = k_2(C)\ \text{(support anion)}$$

If k_1 and k_2 are not greatly different, the rate in neutral salt solutions will exceed that in water, since $C > C^2$ (C being <1) and [support anion] $> C$. However, the movement of support anion is against a slight gradient as a result of some binding to the protein.

In both cases the terminal stages will be greatly retarded by the gradual disappearance of the *ligand gradient*, as shown in the kinetic equation given earlier. The ion pair mechanism is likely to prevail in the terminal stages.

When buffer is substituted for neutral salt, the ion-exchange mechanism is facilitated because the unfavorable support anion gradient is mitigated by the following series of events: (a) ligand binding results in an increased binding of hydrogen ions (Scatchard and Black, 1949); (b) the ratio $HPO_4^{2-}/H_2PO_4^-$ is increased slightly; and (c) the supply of divalent anions available for exchange tends to remain constant. The supply of doubly charged HPO_4^{2-} permits exchange of two ligand anions for one HPO_4^{2-} ion, and the building up of unfavorable gradients is minimized by normal buffer action.

Quite aside from the mechanisms proposed, it has been clearly established that with anions of high affinity the rate of migration toward the protein side of the membrane is extremely slow when no other electrolyte is present, somewhat faster when neutral salt is provided, and only fast enough to achieve equilibrium within experimentally feasible times when a buffer is used. Even in the latter case, equilibrium is not readily achieved unless the protein solution is as dilute as 0.1%. No mechanism has been offered to explain the last effect.

It has long been a truism that an equilibrium result can only be distinguished from a kinetic anomaly when the position of equilibrium has been established by approach from both sides. This is clearly true in the present case. Empirically, however, it appears that with anions of high-affinity equilibrium is quickly attained when all of the ligand is placed initially on the protein side of the dialysis membrane. This fact has been observed by other investigators (Yang and Foster, 1953). This recourse is not without risk when ligands are used in concentrations which cause unfolding of the protein, since there may be an element of irreversibility or hysteresis in such cases (Hill and Briggs, 1956). Therefore reliance on approach to equilibrium with movement of ligand across the membrane in both directions appears mandatory in experiments such as have been described here.

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