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Further Studies of the Isomerization of Bovine Plasma Albumin. The Effect of Detergent Ions at Low *p*H and Preliminary Observations at High *p*H[1]

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The isomerization (N–F) equilibrium of bovine plasma albumin was studied in the presence of sodium dodecyl sulfate (SDS) at *p*H values below the isoelectric *p*H. This detergent has a pronounced effect in displacing the equilibrium toward the native (N) form. The *p*H dependence corresponds to the uptake of three hydrogen ions in the conversion of N to F as was previously found in absence of detergent. The dependence of composition on detergent ion concentration can be fitted most accurately by assuming that the N form possesses 10 strong binding sites of intrinsic dissociation constant 7.5×10^{-6} whereas in the F form these sites are destroyed and replaced by 100 weaker binding sites of intrinsic constant 2.7×10^{-4}. This change in binding behavior corresponds very closely to that previously deduced by Pallansch and Briggs to explain the all-or-none binding of SDS by plasma albumin at alkaline *p*H. It is concluded that the N–F transition is responsible for such cooperative detergent binding. Over most of the binding range electrophoretic mobilities of both forms were independent of detergent concentration indicating detergent binding under these conditions to be competitive with chloride binding. The cationic detergents dodecyltrimethylammonium bromide and dodecylpyridinium bromide were found to be without appreciable effect on the N–F equilibrium under the conditions employed. Some preliminary studies are reported which indicate the N–F transition to occur in absence of detergent at *p*H values above 10. Under such conditions, however, the transformation appears not to be completely reversed on lowering the *p*H.

In previous studies in this Laboratory it has been shown that bovine plasma albumin (BPA) undergoes an "isomerization" equilibrium in the *p*H range 3.5–4.5 in presence of chloride ion.[3] It has also been found that this equilibrium is influenced by the species of anions coexisting in the system. Thus the position of the equilibrium is shifted in the acid direction in the order $SCN^- > Cl^- >$ acetate, this sequence being the same as the order of relative binding affinities of the anions. It was thought of interest to study the effect of the strongly bound detergent ions on the equilibrium. In this study the effect of sodium dodecyl sulfate (SDS), which is one of the most commonly employed detergent anions in protein studies, has been examined. Limited studies have been conducted with cationic detergents dodecyltrimethylammonium bromide (DTAB) and dodecylpyridinium bromide (DPB).

This study seemed of particular importance in view of the repeated observations showing the cooperative nature of protein–detergent binding.[4–8] Thus it would be of great interest if it could be shown that the N–F isomerization were responsible for coöperative binding. The results indicate strongly that this is the case.

Experimental

Pentex bovine plasma albumin, Lot No. A1201, was used. Protein solutions were de-ionized by means of the Dintzis ion-exchange column.[9] Protein concentrations were determined in a Beckman Model DU spectrophotometer assuming a value of $E^{1\%}_{1\,cm.}$ of 6.67 at 279 mμ. All reagents were of C.P. grade. The SDS had been synthesized previously.[10] The cationic detergents dodecyltrimethylammonium bromide (DTAB) and dodecylpyridinium bromide (DPB) also were synthesized.[11] Cellophane bags for dialysis were boiled in sodium carbonate solution prior to use.

Electrophoresis of BPA in presence of SDS was carried out at ionic strength 0.02 chloride ion and at 0° using a Perkin–Elmer electrophoresis apparatus Model 38 equipped with Schlieren scanning optical system. Sedimentation measurements were conducted at room temperature (25–30°) at 59780 r.p.m. using a Spinco Model E. ultracentrifuge equipped with phase-plate Schlieren optical system.

In preparing electrophoresis solutions 9 cc. of protein solution was dialyzed overnight against 391 cc. of a mixture of HCl and NaCl in a cold room at 1–2° using mechanical agitation. On the following day the SDS solution of desired concentration was made up using 10 ml. of the outer solution from the dialysis. One ml. of this detergent solution was added to the protein solution to yield 10 ml. of protein solution of the desired protein and detergent concentration. After adding the detergent the protein solution was again permitted to stand overnight in the cold room. In all studies the BPA concentration was held constant at 0.2% (20 mg. in 10 ml.) and that of SDS was varied from 0 to 10 mg./10 ml. In this procedure no detergent was added to the supporting electrolyte. In earlier experiments where detergent was added to the supporting electrolyte difficulties were encountered in the form of lack of enantiography in the electrophoresis patterns. It is felt that this probably resulted from incomplete equilibration of detergent across the dialysis membrane. In some cases at high detergent concentration there was even an appearance of precipitate in the ascending limb. It is calculated below that the free detergent concentration in the protein solutions was of the order of 10^{-6} to 10^{-5} molar. Hence omission of detergent from the supporting electrolyte seems justifiable.

The *p*H of protein solutions was measured using a Beckman Model G *p*H meter, just prior to electrophoresis, at room temperature. The conductivity, however, was measured at 0°. Mobility values reported are mean values obtained from ascending and descending patterns. Per cent. compositions were calculated from ascending patterns only.

Results

Some typical electrophoretic patterns are reproduced in Fig. 1. It is seen that enantiography is reasonably good in most cases. In Fig. 2 is shown the effect of variation of total detergent concentration on the percentage composition of the system at a single *p*H, 3.91, ionic strength 0.02. The

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(3) K. Aoki and J. F. Foster, THIS JOURNAL, **78**, 3538 (1956); **79**, 3385, 3393 (1957); J. F. Foster and K. Aoki, *J. Phys. Chem.*, **61**, 1369 (1957).

(4) H. P. Lundgren, THIS JOURNAL, **63**, 2854 (1941).

(5) F. W. Putnam and H. Neurath, *J. Biol. Chem.*, **159**, 195 (1945).

(6) (a) J. T. Yang and J. F. Foster, THIS JOURNAL, **75**, 5560 (1953); (b) J. F. Foster and J. T. Yang, *ibid.*, **76**, 1015 (1954).

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

(8) K. Aoki and J. Hori, *Bull. Chem. Soc. Japan*, **29**, 104 (1956).

(9) H. M. Dintzis, Ph.D. Thesis, Harvard University, 1952.

(10) K. Aoki, H. Shimosato and Y. Kamino, *Bull. Nagoya City Univ.*, **1**, 67 (1955).

(11) K. Aoki, to be published.

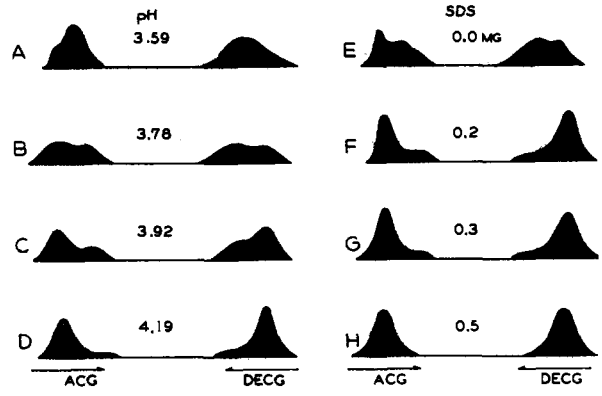

Fig. 1.—Electrophoretic patterns of BPA in presence of SDS at 0.02 Cl^-, 0° and 5.0 volt cm.$^{-1}$: A, *p*H 3.59, BPA/SDS = 20/0.1, 8000 sec.; B, *p*H 3.78, BPA/SDS = 20/0.1, 8000 sec.; C, *p*H 3.92, BPA/SDS = 20/0.1, 6000 sec.; D, *p*H 4.19, BPA/SDS = 20/0.1, 6000 sec.; E, *p*H 3.93 BPA/SDS = 20/0, 6000 sec.; F, *p*H 3.93, BPA/SDS = 20/0.2, 6000 sec.; G, *p*H 3.90 BPA/SDS = 20/0.3, 6000 sec.; H, *p*H 3.91, BPA/SDS = 20/0.5, 6000 sec.

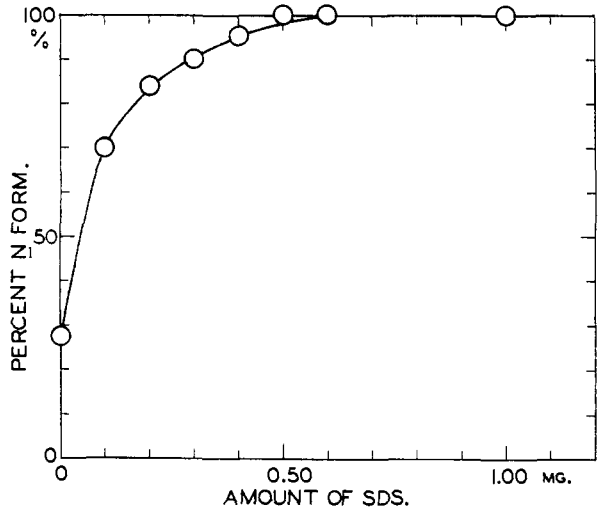

Fig. 2.—Per cent. N form *vs.* amount of SDS added to 20 mg. of BPA; *p*H 3.91 ± 0.02; 0.02 *M* Cl^- at 0°.

equilibrium clearly shifts toward the N form with increasing detergent concentration. Above 0.5 mg. SDS per 20 mg. protein only N form exists in measurable concentration at this *p*H.

In Fig. 3 are shown mobilities of the two components as a function of the amount of SDS added. Somewhat surprisingly the mobilities of both forms are found to be constant regardless of SDS concentration so long as both forms exist in measurable concentration. In the region where the N form alone exists the mobility appears to decrease significantly with increasing detergent concentration. However, it should be pointed out that mobilities were somewhat less reproducible at the higher SDS concentrations.

Figure 4 shows the effect of *p*H on the N–F equilibrium in the presence of a constant concentration of SDS, 0.1 mg. per 20 mg. protein. At this small concentration the SDS is seen to have an appreciable effect on the position of the equilibrium. To this figure are added new data ob-

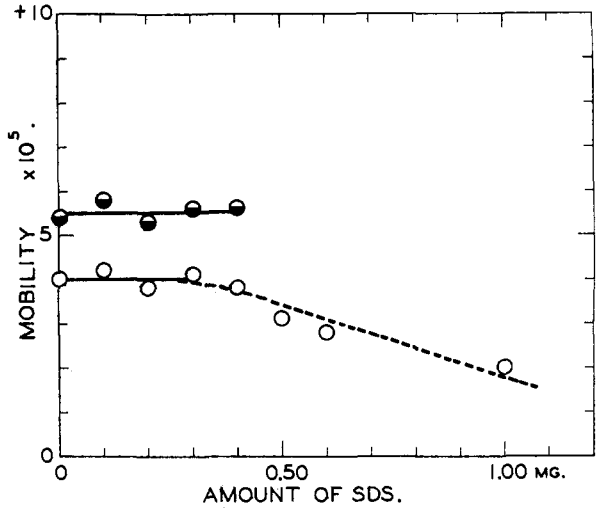

Fig. 3.—Mobility values *vs.* amount of SDS added to 20 mg. of BPA; *p*H 3.91 ± 0.02; 0.02 *M* Cl^- at 0°.

tained without SDS which are seen to be in agreement with the data found previously.[8]

A few measurements were made in the presence of acetate (0.02 *M*) at *p*H 4.08 with variation of SDS concentration. The results were in essential agreement with those in chloride and also indicated the mobilities of the two forms to be independent of SDS concentration.

The cationic detergents DTAB and DTB were found to be without appreciable effect on the distribution of N and F forms up to concentrations of even 1 mg./20 mg. protein. In one experiment with 2 mg. DTAB quantitative interpretation was precluded by slight convective disturbances.

A few sedimentation velocity measurements were made to check on the possible alteration of the frictional properties of the protein by detergent and by high *p*H. At *p*H 4.0 in presence of 1 mg. of SDS to 20 mg. of protein a single sedimenting boundary was obtained with normal sedimentation coefficient ($S_{20,w}$ 4.5 svedbergs). At *p*H 10.5 and 10.8 values of 3.2 and 2.2, respectively, were noted.

Discussion

The distance migrated by both N and F boundaries, both in the ascending and descending limbs of the electrophoresis cell, was in all cases strictly proportional to the elapsed time of electrophoresis. This together with the reasonably high degree of enantiography obtained again indicates the absence of re-equilibration effects which might invalidate interpretation of the boundaries, as we recently have pointed out.[12]

The shift toward more acid values of the midpoint of the N–F equilibrium in presence of the strongly bound anion SDS is in accord with the previous finding that this shift parallels in all cases the relative binding affinity of anions.

Previous studies of the interaction of SDS with proteins have been confined almost entirely to the alkaline side of the protein isoelectric point. Putnam and Neurath[13] observed that acid to the

(12) J. F. Foster and K. Aoki, THIS JOURNAL, 80, 1117 (1958).
(13) F. W. Putnam and H. Neurath, *ibid.*, 66, 692 (1944).

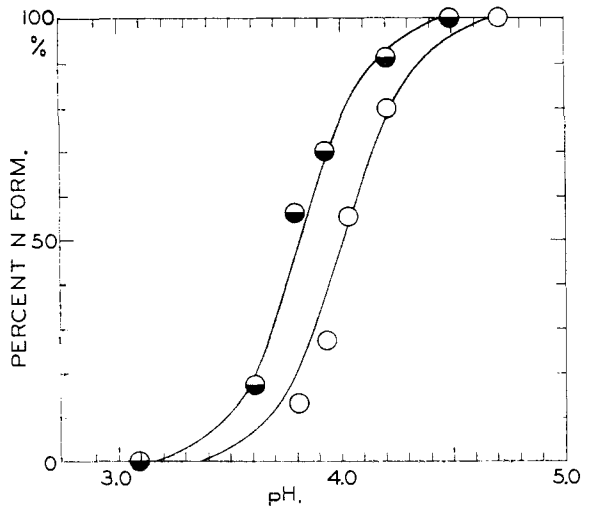

Fig. 4.—Per cent. composition *vs.* *p*H, 0.02 *M* Cl^- at 0°: ◒, % N form when 0.1 mg. SDS was added to 20 mg. BPA; O, % N form without SDS, new data. The curve drawn is from previously published data.[3]

isoelectric point a precipitate is generally obtained when SDS and horse serum albumin are mixed. It was observed, however, that when the protein is in relatively large excess no precipitate results. Similar experiments were performed by one of the authors.[8] When the weight mixing ratio horse serum albumin/SDS was between 100/0 and approximately 95/5 there was no precipitate. All of the present studies with SDS were within this range and no precipitation took place. It should further be emphasized that the free detergent concentration was of the order 10^{-6} molar as compared to the critical micelle concentration (c.m.c.) value of about 0.0035 at 0.02 ionic strength.[8,10] It is clear that the possible participation of detergent micelles in the reaction need not be considered.

The coöperative nature of the binding of detergent ions by globular proteins was first clearly shown by Lundgren.[4] Putnam and Neurath[5] showed that in the interaction of horse serum albumin with SDS two discrete protein complexes are formed, PD_n and PD_{2n}, where n is approximately 55. Yang and Foster,[6a] using dodecylbenzenesulfonate and BPA, showed that there is first a statistical reaction, detergent ions being bound one by one up to a binding limit of approximate 12. As this binding limit is approached there is a complete change in the nature of the reaction and approximately 35–40 additional detergent ions are taken up coöperatively to yield the complex PD_n. Essentially the same conclusion was reached by Pallansch and Briggs[7] using BPA and SDS. They concluded that 10 sites are available for the initial statistical reaction and 80 for the second. Especially clear evidence for the three discrete complexes, PD_{12}, PD_n and PD_{2n} has been noted recently.[14]

These results indicate rather clearly that relatively few, perhaps 10 or 12, strong binding sites exist on the native albumin molecule and that additional binding sites are exposed through some coöperative structural change which takes place as saturation of the original sites is approached. At the time of initiation of the present studies it was postulated that this coöperative reaction might be identical with the N–F transformation. The fact that coöperative interaction with cationic detergents does not take place at low *p*H, as has been shown,[6b] thus would be explainable readily since under such conditions the F form would pre-exist in absence of detergent.

It might be postulated that the conversion of N to F takes place at high *p*H as well as low. Tanford, *et al.*,[15] have shown expansion of the BPA molecule to take place above *p*H 10. If it is true, as we have suggested,[3] that conversion of N form to F is a necessary prerequisite to expansion this should indeed be true. A few preliminary electrophoresis experiments have been carried out above *p*H 9 which indicate isomerization to take place. Using 0.2% protein in carbonate buffers of ionic strength 0.02 a single boundary was obtained at *p*H 9.8. At *p*H 10.2 a small percentage of a slower moving boundary appeared[16] and with increase in *p*H the proportion of this form increased.[17] A pattern at *p*H 10.8 is reproduced in Fig. 5. Enantiography is clearly not as good as in the low *p*H studies.

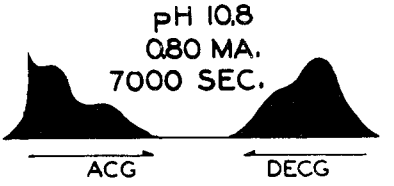

Fig. 5.—Electrophoretic pattern obtained on BPA at the *p*H and ionic strength indicated, carbonate buffer.

A detailed analysis of the effect of SDS on the N–F equilibrium is rendered difficult by our lack of detergent binding data under the conditions used. Obtaining such data of sufficient precision at the very low detergent concentrations employed appears to be out of the question by conventional techniques and would probably best be accomplished by means of radioactive SDS. However, some enlightening calculations are possible without such data.

The fact that N combines more strongly with SDS than does F seems clear from the direction of the shift in the equilibrium. The free detergent concentration (D) is given by

$$(D) = (D_0) - r_N(N) - r_F(F) \cong (D_0) = r_N(N)$$

since $r_F << r_N$ and (F) << (N). Here (D_0) is total detergent concentration, (N) and (F) the concentrations of N and F forms of the protein and r_N and r_F the average number of anions bound per N and F molecule, respectively. But r_N can be related to the intrinsic dissociation constant K_N and the number of binding sites n through[18]

(14) K. Aoki, to be published.

(15) C. Tanford and J. Buzzell, *J. Phys. Chem.*, **60**, 225 (1956).

(16) It is of interest that the new component has in this case a slower mobility than the N form.

(17) Unfortunately, in this case the reaction does not seem to be entirely reversible. A sample held at *p*H 10.8, then dialyzed back to *p*H 9.35 showed some remaining slow component.

(18) This equation of course neglects the usual correction for electrostatic interactions. This approximation is justified by the inde-

$$\frac{n - r_N}{r_N} = \frac{K_N}{(D)} \text{ or } r_N = \frac{n[D]}{K_N + [D]} \quad (1)$$

Hence

$$(D) \cong (D)_0 - \frac{n(D)(N)}{K_N + (D)} \quad (2)$$

At constant pH of the effect of (D) on the equilibrium may be expressed through an expression for the fraction of N form, f_N[19]

$$f_N = \frac{\sum_i ND_i}{\sum_i ND_i + \sum_i FD_i} = \frac{1}{1 + \sum_i FD_i / \sum_i ND_i}$$

But[20]

$$\sum_i FD_i = (F_0)(1 + (D)/K_F)^m \text{ and}$$

$$\sum_i ND_i = (N_0)(1 + (D)/K_N)^n$$

where (F_0) and (N_0) refer to concentrations of the species with no combined SDS, K_F is the intrinsic dissociation constant appropriate to the F form and m the number of sites in this form. Hence

$$1/f_N = 1 + \frac{\sum_i FD_i}{\sum_i ND_i} = 1 + K\frac{(1 + (D)/K_F)^m}{(1 + (D)/K_N)^n} \quad (3)$$

where $K = (F_0)/(N_0)$ is the equilibrium constant in the absence of SDS appropriate to the pH in question. This equation can be conveniently written in the logarithmic form

$$\log K' - \log K = \log (1/f_F - 1) - \log K = m \log (1 + (D)/K_F) - n \log (1 + (D)/K_N) \quad (4)$$

Hence at any experimental point the value of (D) can be estimated with sufficient precision for any chosen value of n and K_N using equation 2. This value may then be substituted in (4), together with an assumed value of K_F and m, to obtain a value of $\log (1/(f_N - 1))$ or f_N for comparison with experiment. Pallansch and Briggs[4] concluded that $n = 10$ and $K_N = 5 \times 10^{-5}$ from experiments with BPA and SDS at pH 6.8 ionic strength 0.20. Using these values and assuming $K_F = \infty$ (*i.e.*, no binding by F form) reasonably satisfactory agreement with experiment is obtained. The result is shown in Fig. 6. This form of plot tends to accentuate strongly the lack of agreement at higher detergent levels as compared, for example, with a plot of the type of Fig. 2. Actually it might be expected that K_N under our conditions would be considerably smaller than at pH 6.8 due to difference in electrostatic interaction. The correction should be given by

$$K_N = K_N'^{2z'w'}/e^{2zw}$$

where the primed quantities refer to the conditions of Pallansch and Briggs and the unprimed quantities to our own. We estimate $Z' = -15$, $\omega' = 0.025$, $Z = +12$ and $\omega = 0.050$ so that

$$K_N = (5 \times 10^{-5})(e^{-0.75}/e^{1.20}) = 7.5 \times 10^{-6}$$

Using this K_N value gives somewhat poorer fit of the data[21] (Fig. 6).

Incorporation of a finite K_F value does not improve the fit of the data appreciably if the value of m is chosen equal to n, *i.e.*, 10. However, Pallansch and Briggs concluded[7] that the new form arising at r approximately 10 at pH 6.8 has *lost* the original 10 strong sites and *gained* a large number of weaker sites. They concluded that $m = 80$ and $K = 2 \times 10^{-4}$ although these values are subject to considerable uncertainty owing to the extreme scatter in their experimental points. If m is taken as 100, a value which seems somewhat preferable to 80 in view of the considerable evidence for the PD_{2n} complex ($2n$ being approximately 100) and K taken as 2.7×10^{-4} together with $K_N = 7.5 \times 10^{-6}$ excellent fit of the data is obtained (Fig. 6).[22]

This evidence suggests strongly that the N–F transformation we have observed at low pH is structurally the same as the one taking place upon detergent combination in alkaline solution. Under our conditions, where predominantly F form was present originally, addition of SDS at the low levels employed converts F to N because of the fact that at these low detergent levels N is much the stronger binder. At higher levels, however, N would become saturated and further addition of detergent would result in an opposite shift in the equilibrium, *i.e.*, of N to F. This is clear from eq. 4 and is illustrated in Fig. 7 where theoretical values of $\log K' - \log K$ have been plotted *versus* $\log$ [D] employing the binding constants deduced above. Unfortunately it does not appear possible to test for this reversal of the effect of detergent in the acid pH range because of the precipitation which results at higher detergent levels.

The deduction that the N–F equilibrium may be shifted in either direction by anions, depending upon their activity and binding affinity, might have important implications with regard to the properties of plasma albumin. As an example, it has long been known that organic anions of intermediate size (butyrate and caprylate for example) stabilize plasma albumins toward heat and urea denaturation.[23,24] Detergent anions, on the other hand, are powerful denaturing agents. It seems entirely probable that this results purely from a difference in degree, rather than kind, of interaction, *i.e.*, from a difference in binding affinity. Thus under the conditions of their use as stabilizing

pendence of electrophoretic mobility on SDS binding (Fig. 3) which indicates constancy of net charge. Previously it has been observed that detergent binding to BPA in the first binding range can be treated as purely statistical without electrostatic correction.[6,7]

(19) This expression of course yields the mole fraction. It might be expected that the area distribution in the Schlieren patterns would correspond more closely to the weight fraction composition but the difference is trivial at the low degree of binding considered.

(20) See for example I. Klotz, in Neurath and Bailey, "The Proteins," Vol. 1B, 1953, p. 768.

(21) Again, this result is perhaps not surprising. See footnote 18.

(22) Note that the value of K_N used is the one obtained from Pallansch and Briggs' data after electrostatic correction. If their K_F value were similarly corrected it would be approximately 3×10^{-5}. However, this does not seem to seriously invalidate the general conclusion reached.

(23) P. D. Boyer, F. Lum, G. Ballou and J. M. Luck, *J. Biol. Chem.*, **162**, 181 (1946); P. D. Boyer, G. Ballou and J. M. Luck, *ibid.*, **162**, 199 (1946).

(24) Evidence that this stabilization is only apparent and due to repression of aggregation has been presented recently by Loseva and Tsyperovich.[25] It seems likely, however, that the effect is real and not limited to repression of aggregation.

(25) A. L. Loseva and S. Tsyperovich, *Colloid J.* **19**, 233 (1957).

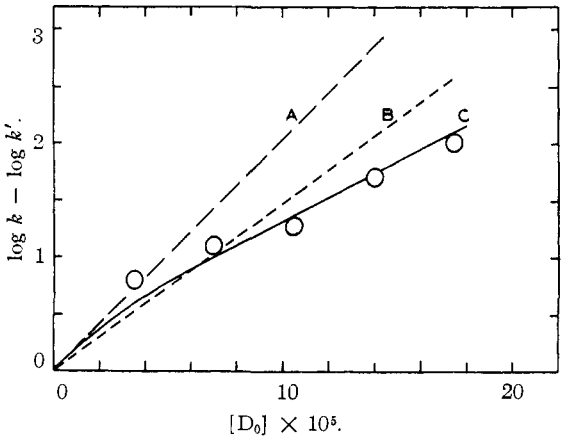

Fig. 6.—Plots of log K − log K' *versus* total detergent concentration. Circles are experimental points. Curves drawn are theoretical. Curve A, $K_N = 7.5 \times 10^{-6}$; curve B, $K_N = 5 \times 10^{-5}$; curve C, $K_N = 7.5 \times 10^{-6}$, $K_F = 2.7 \times 10^{-4}$, $m = 100$. In all cases $n = 10$.

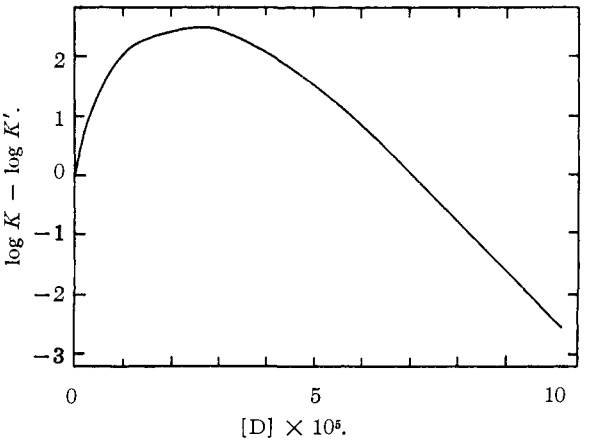

Fig. 7.—Theoretical plot of log K − log K' *versus* free detergent activity showing reversal in the direction of the shift of the equilibrium at high detergent concentration.

agents it seems probable that the effect of anions is to shift the N–F equilibrium toward the native or N form. At similar concentrations with detergent ions the N form would already be saturated with anion and the effect would be strongly in the reverse direction.

Interpretation of the electrophoretic mobilities raises some puzzling problems. It was previously calculated[3] using Henry's equations with appropriate corrections for molecular asymmetry that for BPA at 0.02 ionic strength and 0° the ratio net charge to electrophoretic mobility would be 2.7×10^5. The mobility increment $\Delta\mu/\Delta Z$ corresponding to this value would be 0.37×10^{-5}. This relation appeared to be highly satisfactory in interpreting our previous data in absence of detergent.[26]

In their electrophoretic study of the system BPA–SDS at *p*H 6.8 Pallansch and Briggs[7] found a linear relationship between mobility and r in the first statistical binding range ($r < 10$). The slope corresponded to $\Delta\mu/\Delta r = 0.27 \times 10^{-5}$ at 0.2 ionic strength and 20°. The calculated value of $\Delta\mu/\Delta Z$ under these conditions is 0.31×10^{-5} indicating an increase of one net negative charge per SDS anion bound. On the other hand using dodecylbenzenesulfonate and BPA Yang and Foster[6] found a relatively small increase in mobility in the first binding region indicating the charge to increase much less than proportionately with the increased anion uptake. In studies of dye binding it also has been found that the increase in mobility with binding corresponds to only 0.33 to 0.4 that expected.[27,28] These last mentioned results indicate that in many cases detergent or dye binding is competitive with the binding of other anions. Klotz, *et al.*,[29] have shown competitive binding of detergent and methyl orange to plasma albumin.

The present studies are striking in that experimentally no change in mobility results from SDS binding over most of the range of detergent concentrations studied. This indicates an extreme competition, one chloride ion being ejected for each SDS anion bound.[30] Why a decrease in mobility of the N form should set in after the disappearance of the F form is not clear. The implication is that not all of the SDS binding sites are occupied by chloride ions and those so occupied are first occupied by detergent. This clearly suggests that not all of the assumed 10 or 12 sites are equivalent.

In conclusion it is a pleasure to acknowledge the interest and helpful suggestions of Dr. M. Laskowski, Jr.

LAFAYETTE, INDIANA

(26) The corresponding calculated values of $\Delta\mu/\Delta Z$ at 0.10 and 0.20 ionic strength and at 0° are 0.22 and 0.175×10^{-5}, respectively. These values appear to be in good accord with other results in the literature, for example those of Longsworth and Jacobsen.[27]

(27) L. G. Longsworth and C. F. Jacobsen, *J. Phys. Colloid Chem.*, **53**, 126 (1949).

(28) R. Smith and D. R. Briggs, *ibid.*, **54**, 33 (1950).

(29) I. Klotz, H. Triwush and F. Walker, THIS JOURNAL, **70**, 2935 (1948).

(30) Alternatively, of course, it could be postulated that SDS binding is accompanied by uptake of protons. This seems much less likely. It is worthy of note that the difference in mobilities between N and F corresponds almost exactly to three net charge units over the entire range in which they coexist in measurable quantity. The hydrogen ion dependence of the equilibrium also indicates a difference in binding of three protons. A difference in mobility corresponding to three charge units was found also in our earlier studies.[3]

Competitive binding must be assumed for both forms since binding to F is not negligible. It is calculated, using the binding constants deduced above, that $r_F = 4$ and $r_N = 5.7$ at the highest SDS concentration where F is observed, *i.e.*, at 0.4 mg. SDS/20 mg. protein.