ORGANIC AND BIOLOGICAL CHEMISTRY

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, PURDUE UNIVERSITY]

The Isomerization Equilibrium of Bovine Plasma Albumin in the Presence of Urea[1]

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The electrophoretic behavior of bovine plasma albumin in 2.0 *M* urea has been explored extensively over the *p*H range 3.9 to 7.3 in 0.02 ionic strength chloride. Below *p*H 4 and above 7 a single boundary was obtained; between 4 and 7 there were two boundaries the proportion of which varied with *p*H. The two forms are considered identical with the N and F forms previously demonstrated in absence of urea. As was predicted, the *p*H range over which transition from N to F takes place is shifted upwards by urea and the *p*H dependence is less pronounced. Between *p*H 4 and 5 the equilibrium is approximately second order in hydrogen ion, between 5 and 6.7 is independent of hydrogen ion concentration and between 6.7 and 7.0 again approximately second order. More limited studies have been conducted in presence of acetate at 0.02 ionic strength, in chloride at various ionic strengths from 0.01 to 0.1 and in 0.02 chloride at 1, 3 and 4 molar urea. The equilibrium is approximately third order in urea and is dependent on chloride concentration only at the lower chloride concentrations. The results indicate that in addition to its effect on the N–F equilibrium, urea diminishes the affinity of F, but not N, for chloride ions. Limited sedimentation experiments indicate N and F to have similar frictional properties in 2 *M* urea. The results are considered to be in substantial agreement with previous suggestions of the authors as to the nature of the N–F transition and its relationship to the amphoteric behavior of this protein.

Introduction

The authors recently have demonstrated electrophoretically the existence of two "isomeric" forms of bovine plasma albumin (BPA) which exist in equilibrium over a narrow *p*H range just acid to the isoelectric point.[3-5] The equilibrium is strongly *p*H dependent, obeying closely the equation

$$N + 3H^+ \rightleftarrows F$$

under most conditions. Here N represents the normal or native form existing at neutral *p*H while F is the isomeric "fast" form which migrates with a mobility corresponding to a charge three units more positive than N. Independently, Phelps and Cann[6] have presented evidence for the isomerization of this as well as several other globular proteins but have not examined the *p*H dependence in any detail.

In our work primary consideration has been given to the hydrogen ion dependence of the equilibrium because of its probable relationship to (a) the expansion of the molecule which takes place at somewhat lower *p*H than isomerization[7] and (b) the anomaly in the low *p*H titration behavior of this protein.[8] With regard to point (a) we are inclined to believe that the isomerization is a necessary preliminary to expansion, the F form probably being liberated from certain intramolecular constraints which exist in N. Form F is thus analogous to the "expandable" form first postulated by Tanford, *et al.*[7b] As to the relationship to the titration anomaly, we have suggested that this can be accounted for quantitatively down to *p*H 3.5 if it is assumed that all carboxyl groups have an anomalous intrinsic pK (pK^0) in the N form due to stabilization of the anionic COO–groups by about 1 kcal. free energy each and that all are essentially normal in the F form. Evidence for this interpretation is based on the parallel change with *p*H in pK^0 and per cent. F form, which exists in the presence of both chloride and thiocyanate ions.[5]

The effect of 2 *M* urea on the titration curve of BPA has been investigated recently.[9] It was shown that hydrogen ion binding is markedly enhanced, at given *p*H, by urea and that the effect is probably due in the main to an alteration in the pK^0 of the carboxyl groups. With the development of our present picture of the N–F equilibrium and its relationship to amphoteric behavior, it seemed probable that the effect of urea might essentially reside in a shift of the equilibrium toward the F form at a given *p*H. In other words, the transition should take place at higher *p*H in presence of 2 *M* urea than in absence thereof. Electrophoretic studies presented herein amply confirm this prediction and lead to other important deductions as to the effect of urea on the ion binding behavior of BPA.

Experimental

Materials.—Pentex bovine plasma albumin, Lot No. A1201, was used without further purification. Sodium chloride and hydrochloric acid was of C.P. grade. Urea was B & A purified and used without further recrystallization. Fresh urea solution was employed in all experiments.

Procedure.—Electrophoresis was carried out at 0° in a Tiselius electrophoresis apparatus, Model 35 of the Perkin–Elmer Corporation, equipped with the schlieren scanning system. All the procedures in electrophoresis and the method of calculation were exactly the same as used in the previous work.[3-5] The total ionic strength of the media was 0.02 in most cases and the concentration of the protein was 0.2%. Dialysis of samples was carried out in a cold room at 1–2° for 14–21 hr. with continuous mechanical agitation. As Moore[10] stated, urea solutions have a tendency toward convection during electrophoresis. The voltage gradient was less than 4.0 volt cm.$^{-1}$ in this study. In one experiment conducted at 4.0 volt cm.$^{-1}$ and 0.1 ionic strength evidence of convection was observed.

Sedimentation measurements were conducted at room temperature (24.5–26.5°) at 59,780 r.p.m. using a Spinco Model E ultracentrifuge. Samples were prepared in the

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(3) K. Aoki and J. F. Foster, THIS JOURNAL, **78**, 3538 (1956).

(4) K. Aoki and J. F. Foster, *ibid.*, **79**, 3385 (1957).

(5) K. Aoki and J. F. Foster, *ibid.*, **79**, 3393 (1957).

(6) R. A. Phelps and J. R. Cann, *ibid.*, **78**, 3539 (1956).

(7) (a) J. T. Yang and J. F. Foster, *ibid.*, **76**, 1588 (1954). (b) C. Tanford, J. G. Buzzell, D. G. Rands and S. A. Swanson, *ibid.*, **77**, 6421 (1955).

(8) C. Tanford, S. A. Swanson and W. S. Shore, *ibid.*, **77**, 6414 (1955).

(9) (a) M. D. Sterman and J. F. Foster, *ibid.*, **78**, 3652 (1956); (b) J. F. Foster and M. D. Sterman, *ibid.*, **78**, 3656 (1956).

(10) D. H. Moore, *ibid.*, **64**, 1090 (1942).

same way as for electrophoresis, to permit comparison with the electrophoretic results. Solutions were dialyzed 14–16 hr. in a cold room and permitted to stand 30 minutes to equilibrate with the room temperature just prior to sedimentation.

Results

Electrophoresis of BPA was conducted mainly at 0.02 ionic strength chloride plus 2 *M* urea in the *p*H region between 3.9 and 7.3. When the *p*H was lower than 4.0 and higher than 7.0, there was a single boundary. Between these two *p*H values there were two boundaries, and the percentage of each boundary changed with *p*H. Some typical electrophoretic patterns are shown in Fig. 1 and per cent. composition *versus* *p*H in Fig. 2. Between *p*H

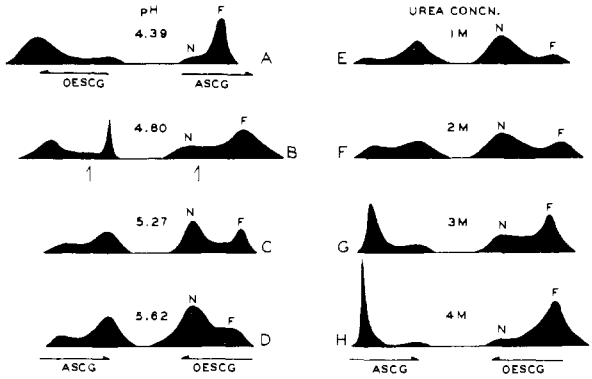

Fig. 1.—Electrophoretic patterns: A, 0.02 Cl^- + 2 *M* urea, 10,000 sec., 3.9 volt cm.$^{-1}$; B, 0.02 Cl^- + 2 *M* urea, 8,000 sec., 3.9 volt cm.$^{-1}$; C, 0.02 Cl^- + 2 *M* urea, 6,400 sec., 3.9 volt cm.$^{-1}$; D, 0.02 Ac^- + 2 *M* urea, 8,000 sec., 3.95 volt cm.$^{-1}$; E, 0.02 Cl^- + 1 *M* urea, 11,000 sec., 3.75 volt cm.$^{-1}$, *p*H 5.80; F, 0.02 Cl^- + 2 *M* urea, 10,800 sec., 3.6 volt cm.$^{-1}$, *p*H 5.84; G, 0.02 Cl^- + 3 *M* urea, 6,000 sec., 3.7 volt cm.$^{-1}$, *p*H 5.98 H, 0.02 Cl^- + 4 *M* urea, 5,800 sec., 3.8 volt cm.$^{-1}$, *p*H 6.31. In pattern A, both N and F are cationic. In pattern B, N is anionic and F is cationic. The vertical arrows represent the position of the initial boundaries. In patterns C to H both N and F are anionic. In all patterns the ascending direction is toward the right, descending toward the left.

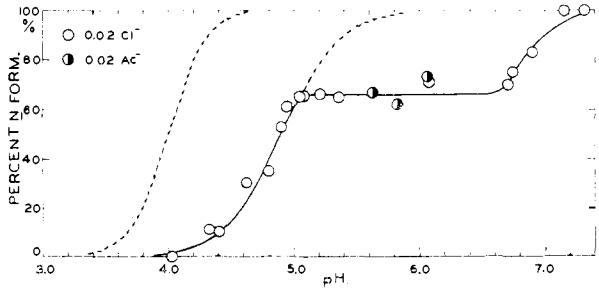

Fig. 2.—Per cent. composition *vs.* *p*H in 2 *M* urea. Dotted line on the left indicates the N–F equilibrium in 0.02 Cl^- in absence of urea (ref. 4).

4.0 and 5.0 the change of the area followed closely a curve corresponding to a two hydrogen ion equilibrium. Between *p*H 5.0 and 6.7 the percentage composition was almost constant, the percentage of the slower moving (F) boundary being 30–35%. The *p*H–mobility curves are shown in Fig. 3. The mobility values decreased continuously up to *p*H 6.0 and then were constant between *p*H 6.0 and 6.8. To Fig. 3 are added the *p*H–mobility curves of BPA at 0°, 0.02 Cl^- without urea.[4]

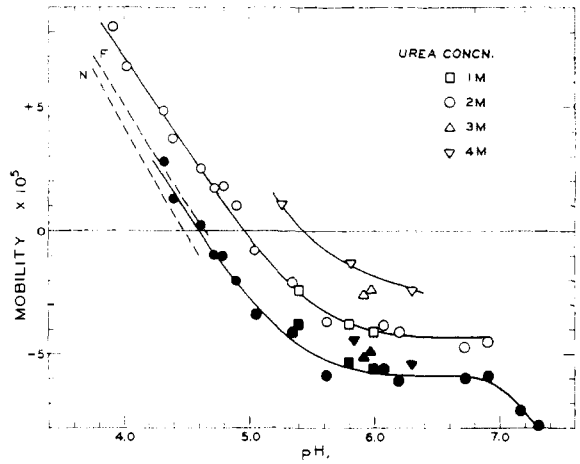

Fig. 3.—*p*H–mobility curves in 0.02 Cl^- plus urea. Shaded marks are for N form and open marks for F form. Dotted lines indicate results in absence of urea (ref. 4). Mobility is given in units of cm.^{2}/volt sec.

Some experiments were carried out at 0.02 ionic strength acetate plus 2 *M* urea in the *p*H region between 5.4 and 6.1 (Fig. 2). The per cent. composition of the boundary was almost the same as that in 0.02 Cl^-, although the mobilities were lower in this medium (due undoubtedly to the lesser binding of acetate as compared to chloride).

The effect of urea concentration upon the per cent. composition was studied around *p*H 6. The concentration of urea was changed from 1 to 4 *M*, keeping the ionic strength of chloride constant at 0.02. The per cent. composition changed markedly with urea concentration, results being shown in Fig. 4. The effect of ionic strength upon the per cent.

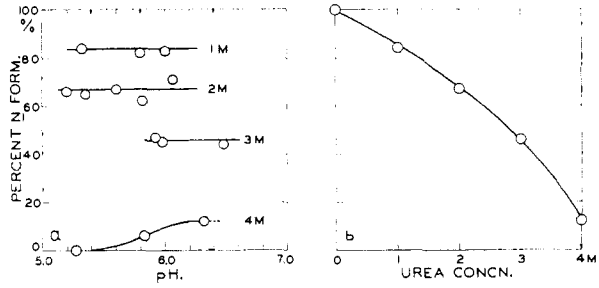

Fig. 4.—(a) Relation between *p*H and per cent. composition at various urea concentrations; (b) per cent. composition as a function of urea concentration around *p*H 6.

composition also was studied around *p*H 6. The ionic strength was changed from 0.01 to 0.10 at 2 *M* urea, but an appreciable effect was observed only in the lower part of this range. Results are shown in Fig. 5.

Ultracentrifuge runs were conducted at 0.02 ionic strength chloride near *p*H 6 changing the urea concentration from 1 to 4 *M*. All the patterns exhibited only a single boundary. The sedimentation coefficients, $s_{20,w}$, corrected to water at 20°, are shown in Fig. 6. It should be emphasized that

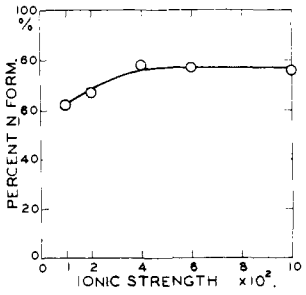

Fig. 5.—Effect of chloride concentration on the per cent. composition around *p*H 6 in 2 *M* urea.

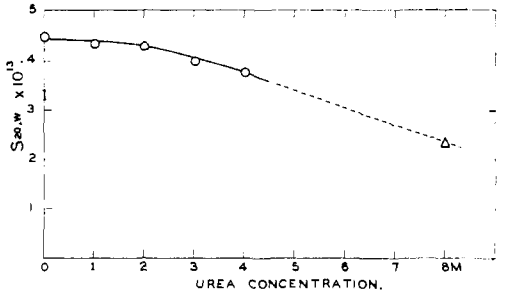

Fig. 6.—Sedimentation coefficients of BPA as a function of urea concentration: *p*H 6, 0.2% protein concentration, 0.02 ionic strength chloride and room temperature. Δ was taken from Charlwood's data[11] at *p*H 9.9, 8 *M* urea, 0.245% protein concentration at 960 min. after protein was exposed to urea.

these sedimentation coefficients are not extrapolated but refer to 0.2% protein. The coefficient decreases gradually above 2 *M* urea. Density and viscosity values used for correction were found in the literature.[11,12] The partial specific volume ($\bar{v}$) used was 0.734.[13] Recently Charlwood[14] published data on the partial specific volumes of BPA in aqueous solution and in 8 *M* urea solution. It was shown that $\bar{v}$ in 8 *M* urea solution is 0.746, which is not greatly different from the normal value.

Discussion

The enantiography achieved in these experiments is in general quite satisfactory (Fig. 1). Perhaps the most serious deviation is seen in pattern B which was carried out very close to the mean isoelectric point, *p*H 4.80. Here the N form is migrating as an anion, the F form as a cation. Considering the strong interaction effects possible under such conditions the result is quite satisfying. It might be pointed out that resolution at the isoelectric point is much more readily studied in presence of 2 *M* urea because of the roughly equal proportions of N and F which exist under these conditions. It will be recalled that in absence of urea the proportion of F form existing at the mean isoelectric point was small, of the order 5–10%.

The resolution obtained in these studies is actually somewhat better than achieved previously, in spite of the necessity of working at lower current density. This improved resolution results from the enhanced mobility difference existing between the two forms, a point to be discussed further below.

The expected shift of the transition region to higher *p*H clearly occurs upon adding 2 *M* urea. In terms of the mid-point, the *p*H where N and F forms exist in equal proportions, the shift corresponds to 0.9 *p*H units (*p*H 4.9 as compared to 4.0 in absence of urea). For comparison the shift in mean isoelectric point is 0.3 *p*H unit (4.80 as compared to 4.52). The transition up to *p*H 5 is distinctly flatter than in absence of urea following closely a second-order hydrogen ion dependence rather than third order. This result was also anticipated on the basis of the mechanism we have proposed.[15] Briefly the transition is envisioned as a coöperative structural change such that the intrinsic dissociation constant of all carboxyl groups, pK^0, is simultaneously altered from about 3.7 in N to 4.4 in F. The hydrogen ion dependence of the equilibrium under a given set of conditions would be a consequence of the difference in mean number of protons bound per mole to the two forms. Over the *p*H range 3.5 to 4.5 this is fairly constant and equal to about 12. To reconcile the third-order dependence observed with the expected 12th order dependence we have proposed the existence of four sub-units per molecule which can undergo coöperative transition sub-unit by sub-unit. On the basis of this picture the order in hydrogen ion would not be constant and in fact should diminish above *p*H 4.5, becoming zero near *p*H 6.

The fact that the order in hydrogen ion suddenly becomes zero at *p*H 5 and remains so up to *p*H 6.7 was entirely unexpected and necessitates some revision in our proposed mechanism. The conclusion that any groups titrating in this range must be independent, so far as acidity is concerned, of the state of the protein molecule, whether N or F, seems unavoidable. Unfortunately, *p*H titration curves have not been extended into this range in presence of urea and would be difficult to interpret because of ignorance as to the effect of urea on the titration constants of the various groups which might be expected to titrate here. However, the mobility data (Fig. 3) indicate that groups are titrated between *p*H 5.0 and 6.0, probably about 10 hydrogen ions being released from each form over this range. On the basis of the proposed pK^0 values 3.7 and 4.4 and taking into consideration the relative proportion of the two forms at *p*H 5, it can be estimated that on the average about 10 carboxyl groups should remain untitrated at *p*H 5.0. It thus appears that a few, possibly ten or so, carboxyl groups are so arranged in the protein molecule that their titration behavior is uninfluenced by the N–F equilibrium.

The mobility data do show a plateau from about *p*H 6.0 to 6.8 suggesting that there are no groups titrating in this range.[16] It seems possible that

(11) H. M. Chadwell and B. Asnes, THIS JOURNAL, **52**, 3507 (1930).

(12) Using values determined at 25.07°, density and viscosity values at 1,2, and 3 *M* urea were interpolated. Those at 4 *M* urea were extrapolated.

(13) M. O. Dayhoff, G. E. Perlmann and D. A. MacInnes, *ibid.*, **74**, 2515 (1952).

(14) P. A. Charlwood, *ibid.*, **79**, 776 (1957).

(15) J. F. Foster and K. Aoki, *J. Phys. Chem.*, **61**, 1369 (1957).

(16) These deductions as to hydrogen ion binding from mobility are clearly provisionary being based on the tacit assumption that chloride binding is independent of *p*H. It is well known that this is not strictly true. An extension of the *p*H titration curve in 2 *M* urea appears clearly to be desirable.

the imidazole titration region has been shifted above *p*H 7 by the presence of urea. If so, the implication is that the completion of the transition to N above *p*H 6.7 is associated with the imidazole titration which would imply a dependence of the acidity of imidazolium groups on the N–F transition. Another, though possibly less likely, interpretation is that the imidazolium groups titrate in the *p*H 5 to 6 region and that some of the carboxyl groups have been shifted to much higher pK^0.

Closer consideration of the *p*H–mobility curves (Fig. 3) is most instructive. In the first place, the mobility difference between N and F is much larger than in absence of urea. The difference must be due primarily, if not entirely, to difference in charge and not to difference in molecular size or shape. This conclusion is based primarily on the fact that the isoelectric points of the two forms are so widely separated, namely, by almost 0.4 *p*H unit as compared to only about 0.2 *p*H unit in absence of urea. Further, the mobility difference between the two forms is quite constant over most of the *p*H range. This would not be the case if the difference arose primarily from difference in frictional properties, especially in the neighborhood of the mean isoelectric point where the relative charge is changing very sharply with *p*H. Further evidence for the conclusion that frictional properties of the two forms are very nearly the same is seen in the sedimentation results (Fig. 6). Single, symmetrical boundaries were obtained and further the mean sedimentation coefficient at 2 *M* urea is not significantly different from that in absence of urea.

Assuming the radii of the two forms to be the same an estimate of the charge difference ΔZ can be made utilizing Henry's equation. Henry's equation[17] is given as

$$\frac{Z}{u} = \frac{300}{4.80 \times 10^{-10}} \times \frac{6\pi\eta r(1 + \kappa r + \kappa r_i)}{f(\kappa r)(1 + \kappa r_i)}$$

where κ is the Debye–Huckel function

$$\kappa = \sqrt{\frac{8\pi e^2 N}{1000\ DkT}}\sqrt{\mu}$$

Here, Z is the net charge, u the electrophoretic mobility, η the viscosity of the solvent, r the radius of the protein and r_i that of the electrolyte ion constituent i, e the electronic charge, D the dielectric constant of the medium, k Boltzmann's constant, N Avogadro's number, T the absolute temperature and μ the ionic strength. The function $f(\kappa r)$ has been given by Henry.[16]

In the previous work it was shown that Z/u is equal to 2.7×10^5 at 0.02 ionic strength in either chloride or thiocyanate.[4] At constant ionic strength but varying urea concentration the ratio Z/u will be a function of η, r and D. Viscosity data on aqueous urea solutions[12] as well as dielectric constants[18] have been reported. Therefore a relation between Z/u and r can be calculated at various urea concentrations at 0°. The sedimentation data (Fig. 6) indicate substantially no change in molecular radius up to 2 *M* urea, and perhaps 10 and 15% increases, respectively, at 3 and 4 *M*. In this way we estimate the following ratios of Z/u at 1, 2, 3 and 4 *M* urea, respectively: 2.7, 3.0, 3.4 and 4.0×10^5. On the basis of these values, estimates have been made of the net charge Z on both N and F form under various conditions, as well as the difference in net charge, ΔZ. This difference is of the order six charge units in 2 *M* urea.

The difference of six charge units in 2 *M* urea raises an obvious problem since the equilibrium composition data (Fig. 2) indicate a difference of only two in hydrogen ion binding up to *p*H 5 and no difference between 5 and 6.7. The result is quite different from that in absence of urea where the mobility difference corresponded closely to the value expected on the basis of difference in hydrogen ion binding. One is forced to conclude that in 2 *M* urea F binds fewer chloride ions than N, the difference being perhaps 6 ions per molecule. Further, the fact that the *p*H–mobility curve for N is not greatly different from that of either N or F in absence of urea (dotted curves in Fig. 3) suggests that *urea has little effect on N in regard to either proton or chloride binding ability.* The difference in chloride binding must thus result from a decreased tendency for F to bind anions in presence of 2 *M* urea.

The binding data of Scatchard, *et al.*,[19] indicate that approximately 4 chloride ions are bound by BPA near *p*H 6 and ionic strength 0.02. We have suggested that this binding value must be low by about 3 units at this ionic strength.[4] If our deduction of the preceding paragraph is correct, N could bind only about 7 chloride ions under the conditions of present interest, and therefore in 2 *M* urea F must bind essentially no chloride ions. An obvious problem is encountered when the urea molarity is increased because the apparent difference in charge now exceeds the number 7. It seems probable that our inference of an equality of radii for the two forms may in fact be in error, and at 3 *M* urea and above a part of the mobility difference arises from expansion of the F form.[20]

The conclusion that chloride ions are released on conversion of N to F in urea clearly implies that the N:F ratio should be dependent on chloride activity. In particular, increase in chloride concentration would be expected to shift the equilibrium toward the N form. Our earlier studies in absence of urea indicated the equilibrium to be remarkably insensitive to chloride concentration. The results in presence of urea (Fig. 5) show the expected effect near 0.02 ionic strength though the data do not permit quantitative calculation of the order in chloride ion. Surprisingly, above 0.04 ionic strength the chloride ion dependence disappears.

The dependence of the N–F equilibrium on urea concentration is striking (Fig. 4). For the study of urea dependence experiments were restricted to the

(17) D. C. Henry, *Proc. Roy. Soc.* (*London*), **A133**, 106 (1931).

(18) "Physikalisch-chemische Tabellen," Dritter Ergänzungsband, p. 1962.

(19) G. Scatchard, J. S. Coleman and A. L. Shen, THIS JOURNAL, **79**, 12 (1957).

(20) A related problem is encountered in absence of urea at low *p*H. Thus, Kronman and Foster[21] have concluded on the basis of sedimentation that some expansion takes place even above *p*H 3.5 where both N and F forms exist, yet no resolution of the sedimenting boundary is observed. If F is to be regarded as an expandable form, as suggested by Tanford, *et al.*,[7b] and discussed further below, the reduction in sedimentation coefficient should be due solely to F and a difference in radii between N and F should exist.

(21) M. J. Kronman and J. F. Foster, *Arch. Biochem. Biophys.*, **72**, 205 (1957).

region of the *p*H plateau to prevent confusion which could arise easily from the additional *p*H variable. Progressively increasing difficulty was encountered in electrophoretic experiments with increasing molarity of urea. The data fit rather closely a third-order dependence in urea over the entire range studied.

The results in urea provide additional evidence for the intimate relationship between the N–F equilibrium and the molecular expansion. The latter process, which results in a substantial increase in intrinsic viscosity, decrease in sedimentation coefficient and increase in levorotation, has been found to begin under conditions where the conversion of N to F is substantially complete. In comparison with chloride, thiocyanate ion shifts both the *p*H for incipient expansion and the *p*H range for the N–F transition downward,[5,20] in both cases by approximately 0.3 *p*H unit at 0.02 ionic strength. While 2 *M* urea shifts the mid-point of the N–F transition upwards by 0.9 *p*H unit, it shifts the onset of expansion by about 0.6 unit.[9a] The same general result appears to hold in regard to the expansion provoked by urea near neutral *p*H. The sedimentation results reported here suggest expansion to begin near 3 *M* urea at *p*H 6. The increase in specific rotation[22] and intrinsic viscosity[23] takes place at nearly this same molarity. Our electrophoretic results show that this corresponds to the condition in which the conversion of N to F form is approximately 50–75% complete. The concept that isomerization is a necessary prelude to molecular expansion,[24] whether by low *p*H or by urea, is a compelling one.

In the foregoing discussion we have interpreted rather literally the area distributions and mobilities obtained in our electrophoretic experiments. Such interpretations may be legitimately questioned on the grounds that in electrophoresis of an equilibrium system re-equilibration may seriously affect the migration behavior. We have discussed this problem briefly in our previous papers and have presented some evidence to justify our interpretations. It seems desirable at this time to discuss this matter in somewhat more detail and to present further experimental results which in our opinion demonstrate rather clearly the fact that both our area and mobility data may be interpreted in a straight forward manner.

The problem of electrophoretic resolution of equilibrium systems was first stated clearly, though qualitatively, by Longsworth and MacInnes.[25] They rightly concluded that the result obtained would differ depending upon the relative rates of the two opposing reactions and the rate of electrophoretic separation. The seriousness of the problem was shown by their patterns on mixtures of ovalbumin and yeast nucleic acid. The most characteristic feature of these patterns was their extreme deviation from enantiography. Similar results have been obtained by others for the interaction of BPA with thymus nucleic acid,[26] conalbumin with lysozyme[27] and BPA with anionic dyes.[28] In all such cases the number of boundaries differed in the opposite limbs or the relative areas under such boundaries differed greatly. In only three general cases, so far as we are aware, have equilibrium systems been found to give satisfactory enantiography. One of these is the case of protein detergent interaction.[29] The second example is that of the antigen–antibody reaction studied by Singer and Campbell[30] and the third, our own studies on the isomerization of BPA. In none of these cases is there any good data on the rate of the equilibration reaction, but in each case there is some evidence that it is reasonably fast. It seems probable, however, that in all of these cases the rate is slower than in the first group of reactions mentioned which are probably essentially instantaneous ionic interactions.

Gilbert and Jenkins[31] have given a quantitative derivation which predicts that meaningful resolution might be attained in spite of the existence of a rapid equilibration reaction if the equilibrium involved is bimolecular. It seems unlikely that this is the case in the albumin isomerization as we have previously pointed out.[4]

Recently Kirkwood, Cann and Brown[32] have given a treatment of the problem for the case of a simple equilibrium between two species, assuming, however, ideal electrophoretic conditions.[33] They conclude on the basis of resulting mobilities and areas would be complicated by re-equilibration unless the time of electrophoresis does not exceed the order of magnitude of the half-time for equilibration. An important result which arises from their calculations is that under conditions where re-equilibration is serious the mobilities would be affected much more seriously than relative areas of

(22) W. Kauzmann and R. B. Simpson, THIS JOURNAL, **75**, 5154 (1953).

(23) H. K. Frensdorff, M. T. Watson and W. Kauzmann, *ibid.*, **75**, 5167 (1953).

(24) It might be pointed out that the addition of urea offers the possibility of studying the hydrodynamic behavior of the F or expandable form in absence of electrostatic repulsion effects, hence possibly in the unexpanded state. Thus, in 4 *M* urea the isoelectric point is approximately 5.4–5.5 (Fig. 3). At this *p*H in 4 *M* urea the protein exists essentially 100% in the F form (Fig. 4a). Some preliminary sedimentation experiments have been conducted under these conditions. Surprisingly, two sedimenting boundaries invariably were observed. This result is in contrast to that obtained at *p*H 6 and discussed above. The faster of the two components sedimented slightly, but perhaps significantly, faster than native BPA and the percentage present increased with decreasing *p*H. The slower form had a sedimentation velocity similar to that of the single component at *p*H 6 (see Fig. 6). The situation is thus somewhat complex and merits further study.

(25) L. G. Longsworth and D. A. MacInnes, *J. Gen. Physiol.*, **25**, 507 (1942).

(26) E. Goldwasser and F. W. Putnam, *J. Phys. Colloid Chem.*, **54**, 79 (1950).

(27) S. Ehrenpreis and R. C. Warner, *Arch. Biochem. Biophys.*, **61**, 38 (1956).

(28) R. F. Smith and D. R. Briggs, *J. Phys. Colloid Chem.*, **54**, 33 (1950); R. A. Alberty and H. H. Marvin, Jr., *ibid.*, **54**, 47 (1950); J. R. Colvin and D. R. Briggs, *ibid.*, **56**, 717 (1952).

(29) H. P. Lundgren, D. W. Elam and R. A. O'Connell, *J. Biol. Chem.*, **149**, 183 (1943); F. W. Putnam and H. Neurath, *ibid.*, **159**, 195 (1945); J. T. Yang and J. F. Foster, THIS JOURNAL, **75**, 5560 (1953); M. J. Pallansch and D. R. Briggs, *ibid.*, **76**, 1396 (1954); K. Aoki and J. Hori, *Bull. Chem. Soc. Japan*, **29**, 104 (1956).

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(32) J. G. Kirkwood, J. R. Cann and R. A. Brown, Abstr. 131st Meeting American Chemical Society, April 1957. We are indebted to Dr. Cann for supplying the manuscript of this paper and for helpful discussions.

(33) That is, absence of, for example, ionic or density gradients.

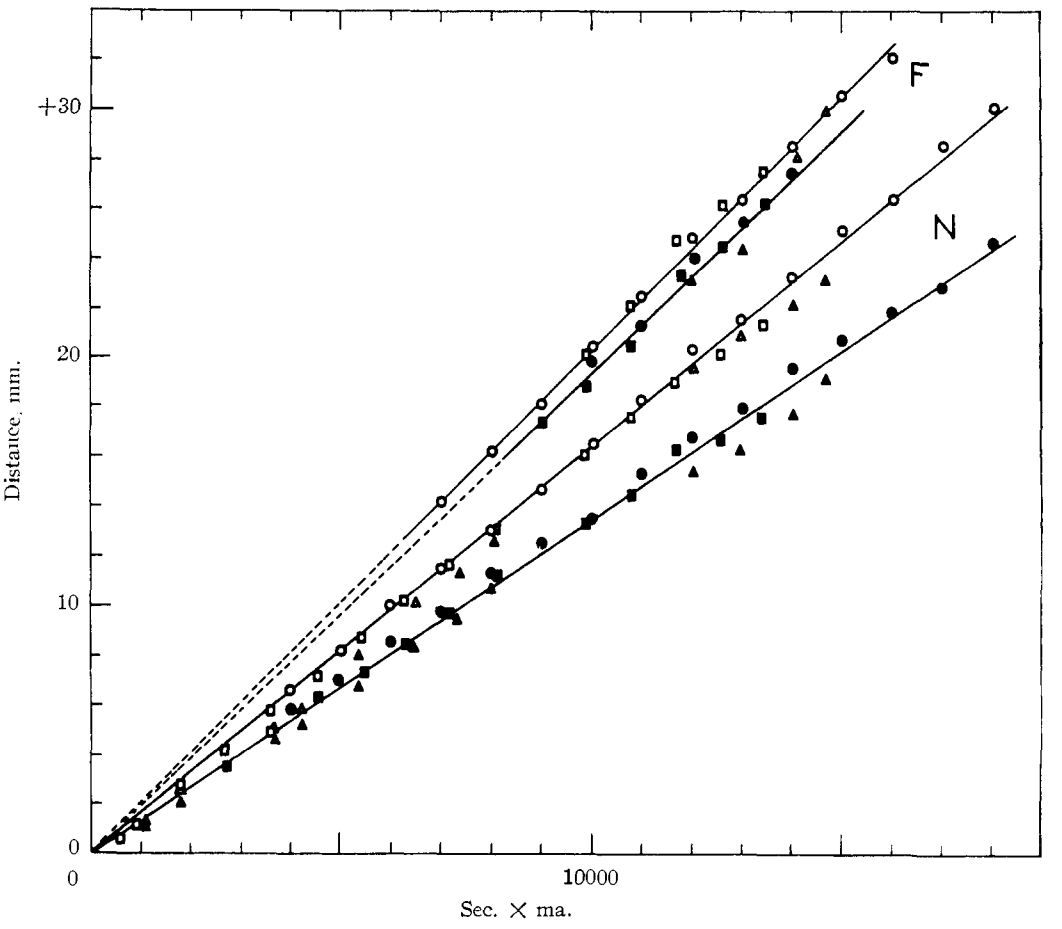

Fig. 7.—Distance migrated by N and F boundaries as a function of the product of current density and time of electrophoresis. All experiments were conducted at *p*H 4.12 in 0.02 ionic strength acetate at 0°. Open symbols are for ascending and filled symbols for descending boundaries: ○, 4.0 v./cm.; □, 1.2 v./cm.; △, 0.7 v./cm.

the gradient patterns.[34] It is thus quite possible that meaningful composition, and hence equilibrium, data could be obtained even under conditions of fairly serious re-equilibration. It further follows that analysis of mobility results is a more critical test for the absence of such effects than analysis of areas.

The most obvious experimental approach to assessing the importance of re-equilibration is that of varying the potential gradient and hence the rate of separation of components. We have previously reported results of such experiments[4] showing that both the mobilities and composition are independent of potential gradient over the range 1.05–5.80 volts/cm. We report now results of additional and more critical experiments of this nature. A single stock solution of BPA at *p*H 4.12 in 0.02 ionic strength acetate was subjected to electrophoresis at 1.0, 0.30 and 0.175 milliampere corresponding to 4.0, 1.2 and 0.7 volts/cm., respectively. Frequent exposures were taken over the course of electrophoresis which extended, in the last case, to 24 hr. In Fig. 7 are given distance migrated as a function of the product of time and current density for both N and F boundaries in both limbs of the cell. Clearly, all points for a given boundary fall on a common straight line, passing through the origin, to well within experimental error. Similarly, in Fig. 8 the composition is seen to be independent of time and current density and to be the same in both limbs of the cell.[35]

A further, possibly even more critical, test arises from the Kirkwood–Cann–Brown theory. Thus, it may be concluded that in the event of serious re-equilibration the mobilities of the two moving boundaries will not only differ from the true values of the corresponding components but will change with time. In other words the position of a given boundary will not be a linear function of the time of electrophoresis (nor of the current density). Figure 7 shows quite clearly that for the components of the albumin isomerization reaction the boundary positions are in fact linear functions of both time and current density in both limbs of the cell. In the case of F form meaningful boundary positions could be obtained only relatively late in the electrophoresis under these conditions. However, the best straight lines through the points clearly pass through the origin indicating that no deviation from linearity occurred earlier in the experiment. In another experiment at *p*H 4.08 and 0.02 *M* ace-

(34) We are indebted to Dr. Robert L. Baldwin for first calling our attention to this fact.

(35) Only a few patterns selected at random were included in Fig. 8 because of the magnitude of the labor involved in enlarging and planimetering patterns.

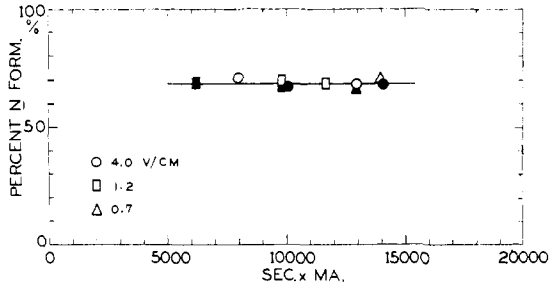

Fig. 8.—Percentage composition as a function of the product of current density and time of electrophoresis.[35] Conditions were the same as in Fig. 7. Open symbols are for ascending and filled symbols for descending boundaries.

tate the same result was obtained at 4.0 and 8.0 v./cm. Therefore it can be stated that mobilities and areas are independent of time and of current density over the range 0.7 to 8.0 v./cm.

Figure 9 gives another example which is more pertinent to the present work since it involves a study in urea. This experiment is the more striking because of the fact that N and F forms here have opposite sign of charge. Again the boundary positions are seen to be strict linear functions of the time of electrophoresis. We have now re-examined a great many of our earlier runs for linearity of boundary positions *versus* time and find linearity to hold in the vast majority of the cases. In systems containing only chloride ion near the mid-point of the *p*H transition region, *i.e.*, near *p*H 4, slight curvature was occasionally seen in the early stages of electrophoresis only. That this curvature is to be attributed to *p*H gradients seems amply substantiated by the fact that systems containing acetate, hence weakly buffered, give linear plots.

In our opinion these results amply substantiate our thesis that both boundary areas and boundary mobilities may be meaningfully interpreted in terms of the equilibrium constituents. As to why such a result should be obtained in spite of the relatively fast rate of the isomerization process, we have nothing further to add beyond the points raised previously.[4,36]

(36) This paradox has been discussed further by J. R. Cann, Abstr. 132nd Meeting American Chemical Society, September, 1957. His results indicate the half-time of equilibration to be of the order 5 hr. in the electrophoresis cell but less than 0.5 hr. otherwise, indicating some stabilizing force to arise in electrophoresis.

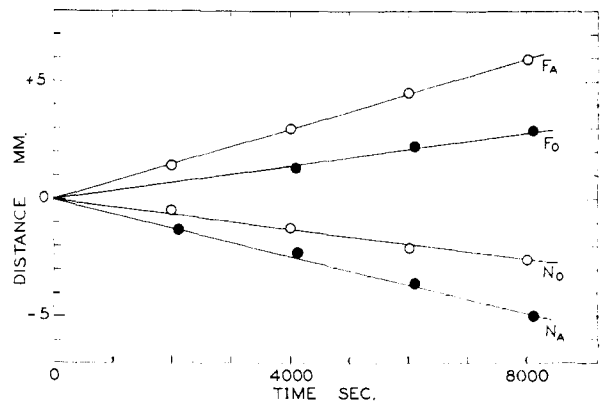

Fig. 9.—Distance migrated as a function of time, BPA at *p*H 4.92 in 0.02 ionic strength chloride plus 2 *M* urea, 0°. Under these conditions N and F forms, which are present in approximately equal proportions, move in opposite directions. Open circles refer to the rising limb of the cell (*i.e.* ascending F and descending N boundaries), filled circles to the descending limb.

By way of summary, the present results are taken as strong substantiating evidence for our general thesis, presented in previous communications, that two electrophoretically resolvable forms of BPA exist in acid solution and that the equilibrium between these forms is intimately related to the anomaly which exists in the *p*H titration curve of this protein. In presence of urea the equilibrium situation may be summarized by the equation

$$N + m\text{Urea} + n\text{H}^+ \rightleftharpoons F + y\text{Cl}^-$$

Here m appears to be approximately equal to three; n is two below *p*H 5, zero from *p*H 5 to 6.7, and again approximately two above *p*H 6.7. The coefficient in chloride, y, appears to be approximately six in the region where n is zero at 0.02 ionic strength but appears to approach zero at higher ionic strength. The situation is obviously complex but its solution would appear to be essential to an ultimate understanding of the unusual properties of this protein.

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Formation of Keto-pyruvate in the Dehydrogenation Catalyzed by Yeast Lactic Oxidase[1]

BY ABRAHAM MARCUS[2] AND BIRGIT VENNESLAND

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With the help of deuterium, it is shown that when lactate is oxidized to pyruvate in the presence of yeast lactic oxidase there is no labilization of the hydrogen atoms of the methyl group.

There are two well-defined and different types of enzymatic reactions whereby lactate may be oxidized to pyruvate. One type involves oxidation by a pyridine nucleotide in the presence of lactic dehydrogenase. The other type of reaction is mediated by a flavoprotein. Previous studies of the diphosphopyridine-nucleotide (DPN)-linked reaction catalyzed by lactic dehydrogenase have shown that the enzyme acts on the keto form of py-

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(2) Postdoctoral Fellow, United States Public Health Service (1956–1958).