eq. at 25° to that at 0° is taken (suffix means the temperature), it becomes

$$\frac{Z_{25}}{Z_0} = \frac{r_{25}g(r_{25})}{r_0 g(r_0)}$$

where $g(r)$ is a new function of r. The hydrogen ion binding of proteins in the carboxyl titration region is known to be nearly temperature independent.[53,54] Evidence also exists that chloride ion binding to human serum albumin has a low temperature coefficient (enthalpy).[39] The change of the isoelectric point with temperature is not great, in this study less than 0.1 pH unit between 0 and 25°. These results suggest Z to be nearly independent of temperature. If we assume that Z is constant at the two temperatures, the radius of BPA is also constant.

(53) C. Tanford, THIS JOURNAL, **72**, 441 (1950).
(54) J. Wyman, *J. Biol. Chem.*, **127**, 1 (1939).

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, PURDUE UNIVERSITY]

Electrophoretic and Hydrogen Ion Binding Behavior of Bovine Plasma Albumin in the Presence of 0.02 *M* Thiocyanate Ion[1,2]

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Electrophoretic studies have been conducted on bovine plasma albumin over the pH range 2.0–4.5 in 0.02 *M* thiocyanate. Below pH 3.0 and above 4.3 single boundaries were obtained. Between 3.2 and 4.3 two boundaries were found, the composition varying continuously with pH in accord with the same N–F equilibrium previously reported in presence of chloride. The mid-point of the equilibrium occurs at pH 3.75 in comparison to 4.0 in chloride. Hydrogen ion titration studies were conducted in the region acid to the isoionic point in presence of 0.02 *M* thiocyanate. Molecular radii estimated from electrophoretic mobilities through Henry's equation are in agreement with values in chloride and indicate no appreciable molecular expansion to take place over the pH range of the molecular transition. Assuming a constant value for the electrostatic interaction parameter w, and constant number of binding sites, the apparent intrinsic pK of the carboxyl groups is shown to parallel very closely the electrophoretic composition in both chloride and thiocyanate. These results strongly suggest that the anomaly in the titration behavior of this protein is due essentially to the N–F transition, the carboxyls in the N form having a pK of approximately 3.7 due to stabilization by the native protein structure, while in the F form these stabilizing interactions are largely removed (pK = 4.4).

Introduction

It has been shown in chloride media that bovine plasma albumin (BPA) undergoes an isomerization reaction (N–F equilibrium)[3] in the pH range 4.5 to 3.5, and that the molecule expands below pH 3.5.[3,4] It has been suggested that the anomalous character of the titration curve of this protein above pH 3.5 can be explained on the basis of this equilibrium if it is assumed that the carboxylate groups are stabilized in the N form.[5] To test this idea further, electrophoretic studies have been conducted in the presence of 0.02 *M* thiocyanate ion, which is known to be much more strongly bound to this protein than is chloride. In addition hydrogen ion titration curves were determined in the same medium.

Experimental

Materials.—Pentex bovine plasma albumin, Lot No. A1201, was used. In the electrophoretic study this was used without further purification. In the titration study the protein solution was deionized by passing through the mixed ion-exchange column of Dintzis.[6] Potassium thiocyanate was of C.P. grade. Thiocyanic acid was obtained by passing potassium thiocyanate solution through cationic ion exchanger in the hydrogen form. In the titration study only ion-exchanged water was used.

Procedure.—Electrophoresis was carried out at 0° in a Tiselius type 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] The total ionic strength of the media was 0.02 and the concentration of protein was 0.2%.

In the titration study the same apparatus as used by Foster and Sterman[7] was used. It consisted of a Beckman Model G pH meter and an external electrode cell assembly. The cell assembly consisted of a Beckman No. 1190-80 General Purpose glass electrode and a Beckman No. 1070-71 sleeve type calomel reference electrode. All temperature sensitive parts of the electrode cell assembly were maintained at a constant temperature, 25 or 0°. The procedure for determining the titration curve at 25° was also the same as was followed by the above workers.[7] In the studies at 0° the electrode system was standardized at each measurement against a standard phthalate buffer of pH 4.00, and the pH reading of the solution, which was prepared in a cold room, was taken after attaining thermal equilibrium (about 15 minutes).[8] In the calculations the molecular weight of BPA was assumed to be 70,000.

Results

Electrophoresis of BPA in the thiocyanate media of 0.02 ionic strength and at 0° was carried out in the pH range of 2.0 to 4.5. Below pH 3.0 there was a single boundary, and between this pH and 4.3 there were two boundaries. The percentage of the area of each form changed continuously with pH in the same way as in chloride media.[9] Here

(1) Supported in part by the National Cancer Institute, National Institutes of Health, Grant C-2248.
(2) Presented in part at the 41st Meeting of the Federation of American Societies for Experimental Biology, Chicago, 1957.
(3) K. Aoki and J. F. Foster, THIS JOURNAL, **78**, 3538 (1956); **79**, 3385 (1957).
(4) J. T. Yang and J. F. Foster, *ibid.*, **76**, 1588 (1954); C. Tanford, *et al.*, *ibid.*, **77**, 6421 (1955); W. F. Harrington, P. Johnson and R. H. Ottewill, *Biochem. J.*, **62**, 569 (1956).
(5) J. F. Foster and K. Aoki, Abstracts 130th ACS Meeting, September 1956.
(6) H. M. Dintzis, Ph.D. thesis, Harvard University, 1952.
(7) J. F. Foster and M. D. Sterman, THIS JOURNAL, **78**, 3656 (1956).
(8) In measurements at 0°, the temperature dial of the pH meter was adjusted to 25° and the pH reading multiplied by 1.09 (= 298/273).
(9) The areas were measured on the ascending pattern. Patterns were reasonably enantiographic in all cases. Resolution of the two components was not quite as good as in either chloride or acetate probably because of the decreased mobility in presence of thiocyanate. Mobilities at zero concentration were estimated using equation 1 in the earlier paper.[3]

again we call the faster moving component F form and the slower one N form. The *p*H–mobility curve[9] is shown in Fig. 1. The percentages of the areas are plotted against *p*H in Fig. 2.

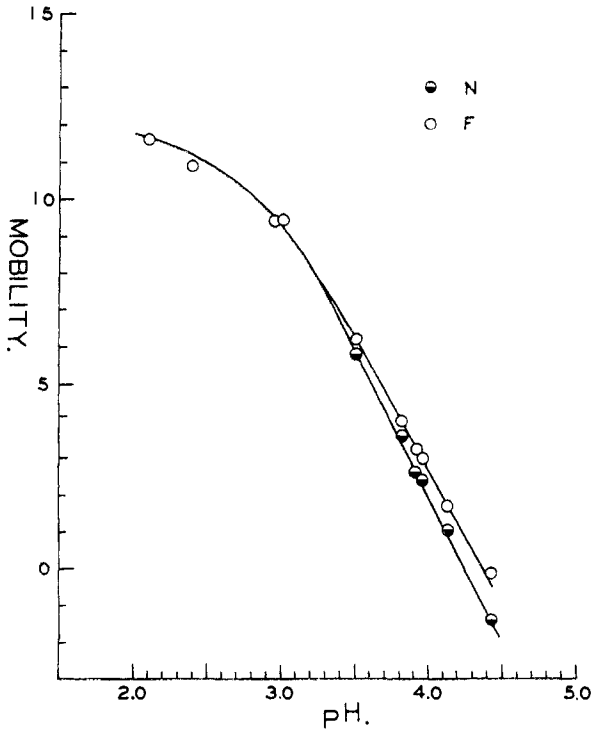

Fig. 1.—*p*H–mobility curve of BPA in 0.02 ionic strength SCN^- and at 0°. The mobility is given in the units cm.²/volt sec. × 10^5.

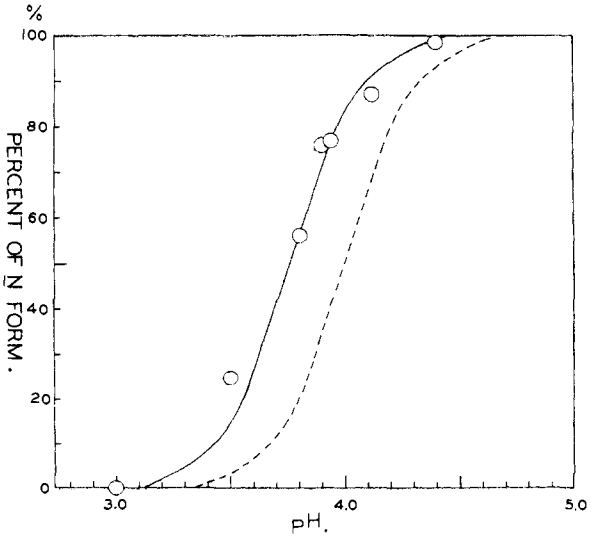

Fig. 2.—Dependence of electrophoretic composition on *p*H at 0°: ——, 0.02 SCN^-; - - - -, 0.02 Cl^- (ref. 2).

Titration curves of BPA in 0.02 thiocyanate media were determined at two temperatures, 25 and 0°. These are shown in Fig. 3, to which the titration curve in 0.02 chloride media at 25° by Foster and Sterman[7] is added.

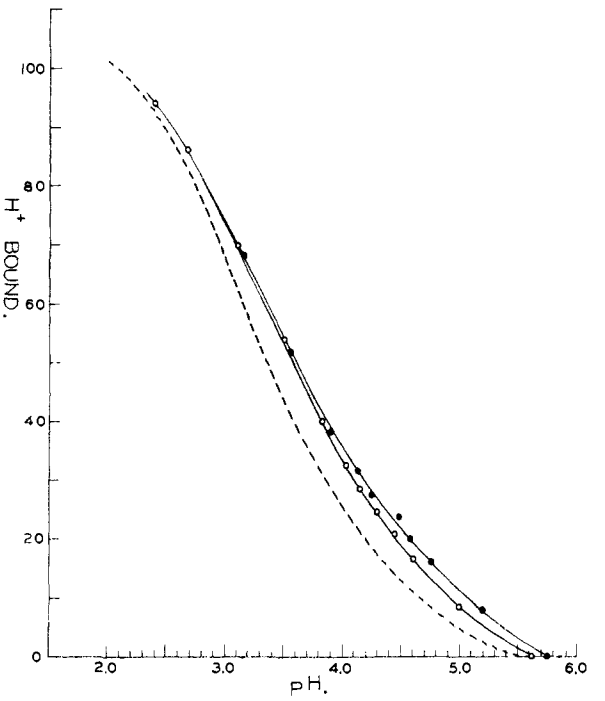

Fig. 3.—Titration curves of BPA in 0.02 ionic strength SCN^- at 25° (o) and at 0° (●). Equivalents H^+ bound per mole of BPA are shown against *p*H; - - - -, 0.02 Cl^- (ref. 6).

Discussion

In all experiments between *p*H 3.2 and 4.3, two boundaries were observed in the electrophoretic patterns. Further, the percentage composition changed continuously with *p*H in close accord with the equilibrium

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

The result is in qualitative agreement with the finding of Longsworth and Jacobsen[10] that BPA gives a single boundary, in presence of thiocyanate, at *p*H 4.31 and 4.61 but two boundaries at *p*H 4.06. The results supplement and extend our earlier finding[2] that such an equilibrium exists in both chloride and acetate. (In the case of acetate the equilibrium corresponded more closely to a two-hydrogen-ion equilibrium.) The "mid-point" *p*H values in the three systems are, respectively, 3.75, 4.0 and 4.1 in 0.02 *M* thiocyanate, chloride and acetate. It should be emphasized that a third component such as was observed over a narrow *p*H range in chloride (3.80–3.95) was not observed in the present studies.

The net charge Z was calculated at various *p*H values using the hydrogen-ion titration curve at 25° and the thiocyanate binding equation of Coleman.[11] The latter may be expressed as

$$\nu_{x^-} = \sum \frac{k_i n_i C_{x^-} \exp(2Zw)}{1 + k_i C_{x^-} \exp(2Zw)}$$

where ν_{x^-} represents the number of anions (x^-) bound to one BPA molecule. C_{x^-} is the molar concentration of the anion and n_i the number of

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

(11) J. S. Coleman, Ph.D. thesis, Massachusets Institute of Technology, 1953.

binding sites with intrinsic constant k_i. In the case of thiocyanate, $n_1 = 1$, $n_2 = 8$, $n_3 = 18$, $k_1 = 45{,}000$, $k_2 = 1925$, and $k_3 = 63$. The parameter w is a function which mainly depends upon the molecular size and the ionic strength. The method of calculation of Z is exactly the same as previously used on the data in chloride media. The Z–pH curve thus calculated predicted the isoelectric point to be pH 4.50. The value found experimentally was 4.25. Therefore the thiocyanate binding curve was corrected so that the Z–pH curve yielded the isoelectric point experimentally found. This was carried out by raising the thiocyanate binding curve by four units over the entire range. Using the Z–pH curve thus corrected a plot of mobility (μ) *versus* net charge (Z) was drawn (Fig. 4).[12] Employing Henry's equation, the effective

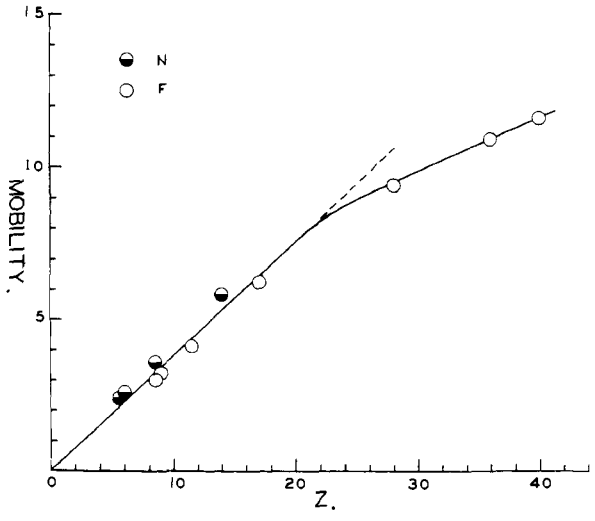

Fig. 4.—Relation between electrophoretic mobility (u) and net charge (Z).

radius of the BPA molecule was calculated from Z/u, in the same manner as previously, at the various pH values. Results are shown in Fig. 5. The radius is constant, 32 ± 2 Å., for both F and N forms above pH 3.2, and is the same as found in chloride media.[3] Slight expansion of BPA takes place below pH 3.2, considerably less than was observed in chloride media. This is in agreement with the finding of Kronman and Foster[14] that the sedimentation constant of BPA at pH 2 and 0.02 ionic strength is larger in thiocyanate media than in chloride. They have estimated effective radii at pH 2.0 of 57.0 and 40.5 Å. in chloride and thiocyanate media, respectively.

It is seen in Fig. 3 that hydrogen ion binding is enhanced in thiocyanate over that in chloride media at similar pH. This is in agreement with

(12) The calculation of the net charge on N and F involved the assumption that $Z_F = Z_N + 3$. The procedure and assumptions were identical with those used earlier.[3] The Z–pH curve was also drawn by use of the thiocyanate binding data of Carr.[13] This Z–pH curve predicts the isoelectric point to be around 4.8. However, there is agreement at pH 3.0 between the Z value calculated by this method and that calculated by our curve.

(13) C. W. Carr, *Arch. Biochem. Biophys.*, **40**, 291 (1953).

(14) M. J. Kronman and J. F. Foster, *ibid.*, in press.

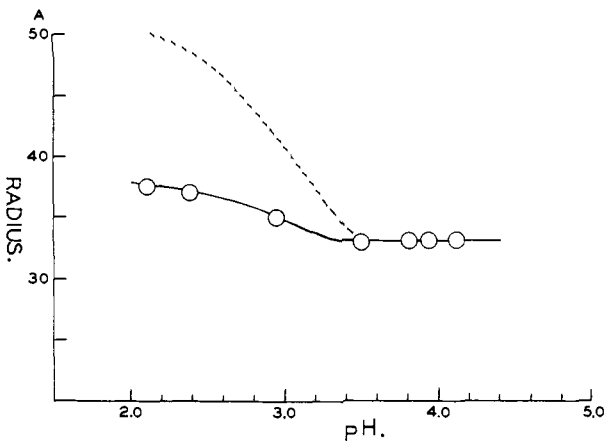

Fig. 5.—Radius of BPA *versus* pH as calculated by Henry's equation: ——, 0.02 SCN^-; - - - -, 0.02 Cl^- (ref. 2).

results described by Scatchard and Black[15] and is also to be expected on the basis of the enhanced binding of thiocyanate ion. This is seen easily from the following form of the titration equation

$$pH = pK_0 + \log \frac{n - r}{r} - 0.868Zw \qquad (I)$$

where n is the total number of basic sites of a particular species in the protein molecule, r the number of hydrogen ions bound to such sites, pK_0 the negative logarithm of the intrinsic dissociation constant for the particular species considered, Z the net charge on the protein and w the Debye–Hückel parameter.[16] Clearly, enhanced anion binding will diminish Z, which is in every case positive on the acid side of the isoelectric point, hence increasing the number of bound hydrogen ions (r).

Tanford has pointed out the inadequacy of eq. I in accounting for the titration behavior of plasma albumin acid to the isoelectric point.[17] In particular, he has shown the titration curve to be too steep in the carboxyl titration region and has suggested that this results from expansion of the protein molecule with a resultant decrease in the interaction parameter w. While the now well-known low pH expansion of this protein appears at first glance to be in good accord with this idea, it has been pointed out that gross molecular expansion takes place only at pH values below that range in which most of the apparent decrease in w occurs.[5,7,18] It has been suggested alternatively that the titration anomaly in the pH region above 3.5 is to be associated with the N–F transition.[5] The present data provide a further test for this idea.

(15) G. Scatchard and E. S. Black, *J. Phys. Colloid Chem.*, **53**, 88 (1949).

(16) See, for example, C. Tanford, in T. Shedlovsky, ed., "Electrochemistry in Biology and Medicine," John Wiley and Sons, Inc., New York, N. Y., 1955.

(17) C. Tanford, THIS JOURNAL, **72**, 441 (1950); *Proc. Iowa Acad. Sci.*, **59**, 206 (1952).

(18) The alternative assumption, n variable (unmasking of binding sites) is considered less likely for reasons that will not be discussed here. This problem, together with a more detailed consideration of the mechanism implied in the present discussion, will be presented in a future paper.[19]

(19) J. F. Foster and K. Aoki, *J. Phys. Chem.*, in press.

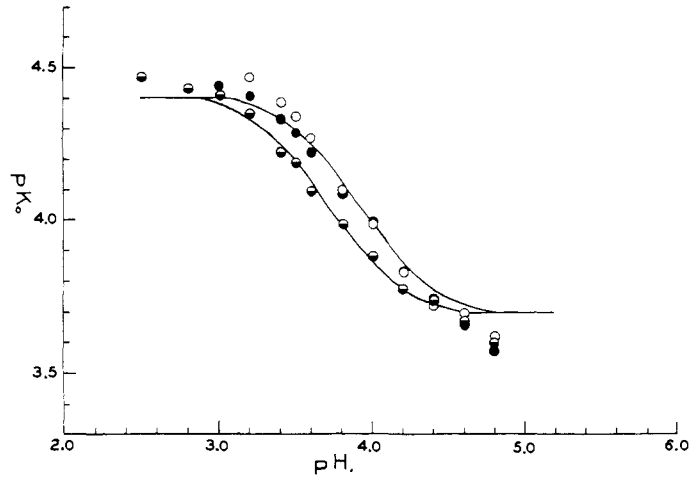

Fig. 6.—Relation between pK_0 and pH: ◒, 0.02 SCN^-; ○, 0.02 Cl^-; and ●, 0.10 Cl^-.

Tanford's calculations of w clearly pre-suppose the constancy of pK_0 and of n, the number of binding sites. We adopt instead the approximation that w is constant over the pH range in which no significant increase in molecular radius occurs. In case of chloride, the radius is substantially constant down to pH 3.5; in thiocyanate down to pH 3.2. We further adopt tentatively the position that n is constant and that all of the deviation from ideal behavior is to be attributed to changes in the intrinsic constant, pK_0.[18] Figure 6 shows results of calculations utilizing data in chloride at two ionic strengths, 0.02 and 0.10, and in thiocyanate at 0.02 ionic strength. The radius of the protein was assumed to be 33 Å. in all cases, corresponding to a w value of 0.046 at 0.02 ionic strength, and 0.029 at 0.10 ionic strength. It is seen that in all cases sigmoid curves are obtained which coincide remarkably well with the electrophoretic composition curves. In particular, the curves in chloride are seen to be independent of ionic strength, as was essentially true also of the electrophoretic composition. The mid-point of the pK_0–pH curve is shifted toward the acid side, when chloride is replaced by thiocyanate, by 0.2 pH unit in comparison with a shift of 0.25 unit in the electrophoretic composition curve. A minor discrepancy is the fact that the pK_0–pH curves in both thiocyanate and chloride appear to fit a two hydrogen ion equilibrium somewhat better than three.[20] The fact that calculated pK_0 values deviate from the drawn curves near pH 5 as well as at the lower end of the curves is not at all surprising. Near pH 5, imidazolium groups must almost certainly begin to titrate, a fact which is not taken into account in the present calculations. At the lower end of the transition, all evidence indicates that molecular expansion is beginning to take place, which will result in a decrease in w and an increase in the apparent pK_0.

To a reasonably close approximation it appears that the anomalous character of the titration curve can be accounted for through the N–F transition. It further appears that the mean pK_0 value for the carboxyl groups is about 3.7 for N form and 4.4 for F form, and that in each case these intrinsic pK_0's are substantially independent of such environmental factors as ionic strength and salt type. It is natural to conclude that in the native N form the carboxyl groups are stabilized in the basic (anionic) form by hydrogen bonding and/or localized electrostatic interactions[21] yielding the abnormally low value of pK_0. The nearly normal value of 4.4 found for the F form suggests that these stabilizing interactions are essentially completely lost in the "isomerized" protein.

Some evidence that electrostatic interactions may be of more importance than carboxyl hydrogen bonds in this stabilization may also be found in the present results. The mid-point of the N–F transition in both 0.02 M chloride and thiocyanate is found to occur at precisely the same net protein charge, namely, plus 11. In 0.1 M chloride the mid-point occurs at net charge plus 8. It thus appears that electrostatic factors are of key importance in governing the position of the N–F equilibrium. *Were carboxyl hydrogen bonds of primary importance it would be expected that thiocyanate binding would actually shift the equilibrium to higher pH rather than lower.* This inference is based on the patent fact that competition of hydrogen ions for hydrogen-bonded carboxylate anions would be greater, at given pH, in the presence of thiocyanate, due to the enhanced hydrogen ion binding. Tanford, *et al.*,[23] have similarly concluded that the forces stabilizing this protein against molecular expansion may be predominantly electrostatic.

LAFAYETTE, INDIANA

(20) A possible explanation for this fact can be given on the basis of a mechanism to be presented in a future paper.[19]

(21) The importance of such localized electrostatic effects has been emphasized recently by Hill.[22]

(22) T. L. Hill, THIS JOURNAL, **78**, 3527 (1956).

(23) C. Tanford, J. G. Buzzell, D. G. Rands and S. A. Swanson, *ibid.*, **77**, 6421 (1955).