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TITRATION AND ELECTROPHORESIS STUDIES OF BOVINE ALBUMIN AND TWO AMIDINATED DERIVATIVES

by

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By John R. Van Atta

Abstract

Previous workers have shown that bovine serum albumin undergoes two conformational changes as the pH is lowered below 5. The first conformational change, the N-F transformation, is rather specific and is believed to consist of a slight opening-up of the molecule which permits the titration of previously-unavailable ionized protein carboxyl groups. The second, lower pH, conformational change consists of a general expansion of the molecule.

The purpose of this research was the investigation of this N-F transformation with regard to the structure of the protein and the nature of the forces involved in the stabilization of the abnormal carboxyl groups.

By the conversion of the protein ε-amino groups to similarly-charged substituted amidino groups (by reaction with appropriate imidoesters), small aliphatic groups were introduced into the protein. Essentially completely modified acetamidino- and butyramidino-albumin derivatives were prepared without any measureable changes in the general properties of the protein at neutral pH values.

Moving boundary electrophoresis was performed on the native protein and the derivatives at acid pH values. The electrophoretic mobility of the F form of the protein is greater than that of

the N form at the same pH value. The extent of the N-F transition at a given pH can thus be determined by analysis of the heterogeneous electrophoretic patterns which are obtained in the pH region.

Hydrogen ion titration curves of deionized, monomer preparations of the native protein and of the amidino-albumins were performed in the presence of 0.1M KCl. All carboxyl group titration curves were abnormal, indicating that the 100 protein carboxyl groups could not be considered as one set of 100 equivalent groups. The acid titration curves were essentially superimposeable except in the pH region 4.8 to 3.0. In this region more amidino-albumin carboxyl groups had been titrated at any given pH.

Both the titration and the electrophoretic data demonstrated that the stability of the N form of the protein was not as great in the case of the amidino-derivatives as it was in the case of the native protein.

The use of Abramson's rule (1) permitted comparison of the electrophoretic and titration data. The two methods gave excellent agreement above pH 4. At lower pH values the increased expansion of the derivatives caused divergence of the electrophoretic and titration data. Abramson's rule does not apply under these conditions.

Analysis of the electrophoretic data showed that about 35 of the 100 protein carboxyl groups appeared to be stabilized in the anionic form in the N form of albumin.

Foster's (2) hypothesis of a crevice which contains approximately 40 abnormally-titrating carboxyl groups is the most reasonable explanation of the titration behavior of these groups. From the differences in the titration and electrophoretic behavior of the derivatives and the native protein, it is apparent that some of the lysyl residues of the protein are present in this crevice and are involved in the stability of this crevice-containing or N form of the protein.

Since this crevice-structure appears less stable in the case of the derivatives with their added hydrophobic groups, the data would suggest that electrostatic interactions between the abnormal, anionic carboxyl groups and the cationic ε-amino or amidino groups play a significant role in maintaining the stability of the crevice-containing or N form of albumin.

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To my wife, Pam

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INTRODUCTION

The study of protein derivatives can give information on the structure of the unmodified protein provided that the derivatives can be prepared under nondenaturing conditions. Interpretation of the data obtained with the protein derivatives is greatly facilitated if the reagent is group-specific and if homogeneous, completely modified derivatives can be obtained. One such group of reagents are the imidoesters which react specifically with the amino groups of proteins under mild conditions converting the amino group to a similarly-charged substituted amidino group.

Human and bovine serum albumin undergo a reversible conformational change below pH 4.5. This conformational change is related to a certain amount of opening up of the molecule and more carboxyl groups become available for titration as the pH is lowered. As a result of various studies on the nature of this conformational change it has been postulated that, at neutral pH, many of the charged carboxyl groups of the protein are involved in intrachain interactions in crevices of the molecule. The exact nature of the structural changes that occur on lowering the pH is not known. It was thought, however, that some evidence as to the validity of the crevice hypothesis might be obtained by an investigation of certain amidino-albumin derivatives which contained added aliphatic residues. Such residues should have little if any effect on the titration properties of free carboxyl groups on the surface of the molecule. By steric and other effects, however, they could alter the strength of carboxyl intrachain

interactions in crevices of the molecule and hence alter the titration properties of these carboxyl groups.

The present investigation is thus concerned with a comparison of certain physical properties of native albumin and some amidino-albumin derivatives which contain additional aliphatic residues. Most of the studies described in this thesis involve the hydrogen ion titration and electrophoretic behavior of these albumin preparations.

Physical Properties of Serum Albumin

The physical and chemical properties of serum albumin have been extensively studied. The scope of this investigation on albumin is, in part, a reflection of the availability of the protein in reasonable amounts and of its favorable stability and solubility characteristics. However, much of the interest in this protein stems from its rather complicated behavior: it undergoes reversible conformational changes at acid and alkaline pH, it binds negative ions and dye molecules with a relatively high affinity and it is heterogeneous with respect to sulfhydryl content, dimer content and the ease with which the conformational change occurs at acid pH values.

Serum albumin is a globular protein with a molecular weight within the range of 65,000 - 67,000 (1-3). It has been shown to be a single polypeptide chain (4,5) containing 16 to 17 disulfide bonds. Two-thirds of the albumin contains one free sulfhydryl group (mercaptalbumin) and the remaining one-third has no free sulfhydryl. Dielectric studies (6,7) describe albumin as a

prolate ellipsoid with an axial ratio of 3 to 4 having a semi-major axis of approximately 70 Å and a semi-minor axis of approximately 20 Å. Amino acid analysis (8) and titration studies (9) demonstrate that albumin has approximately 200 ionizable groups of which 100 are carboxyl groups and approximately 60 are ε-amino groups. A typical preparation of albumin contains 5-10% dimer with a fatty acid content of 1-2 moles fatty acid per mole of protein.

Molecular Expansion

Bovine serum albumin (BSA) has been shown to have abnormal titration behavior by Tanford, et al. (9). An analysis of the titration results have shown an abnormally low intrinsic pK for the carboxyl and ε-amino groups and an abnormally high intrinsic pK for the phenolic groups when compared to other proteins. The method of analysis employed was the application of the Linderstrøm-Lang equation to the titration data (10)

$$pH - \log \frac{r}{n-r} = pK_{int.} - 0.868\ \omega Z \qquad (1)$$

where n represents the total number of intrinsically identical groups (e.g. carboxyl groups) of which a number r have dissociated at any pH, $pK_{int.}$ is the pK_a for these n equivalent groups, and $0.868\ \omega Z$ represents the electrostatic interaction between Z, the net charge on the protein, and the dissociating hydrogen ion. The electrostatic factor, ω, derived from Debye-Hückel theory (11,12) for an impenetrable sphere with charges distributed evenly on the surface is

$$\omega = \frac{e^2}{2DkT}\left(\frac{1}{b} - \frac{K}{1+Ka}\right) \qquad (2)$$

where b is the radius of the spherical molecule, a is the exclusion radius for this molecule (usually taken as b + 2.0 Å), K is the Debye-Hückel constant proportional to the square root of the ionic strength, e is the electronic charge, D is the dielectric constant of water, k is Boltzmann's constant and T is the absolute temperature. An empirical value for ω can be obtained by plotting pH - log $\frac{r}{n-r}$ versus Z and equating the slope to -0.868ω. A number of proteins exhibit a constant ω over a wide range of pH. The empirical value of ω for BSA obtained by Tanford (9) closely agrees with the calculated value of ω from equation (2) in the pH range 4.3 to 10.5. However, outside this range ω decreases drastically. Because ω is inversely related to the molecular radius, the rapid decrease in ω has been attributed to an expansion of the molecule. Reversible titration curves have not demonstrated any hysteresis effect, indicating that the expansion is reversible and not a denaturing process. Also the process appears to be rapid (less than 5 minutes) since no time dependence has been observed during the titration experiments. The postulate of expansion was in agreement with the findings of Scatchard (13) for titration curves of human serum albumin (HSA).

Yang and Foster (14) have found that both the intrinsic viscosity and specific optical rotation of BSA increases rapidly below pH 4. These authors have interpreted the data in terms of an "all-or-none" expansion. Both the viscosity and specific rotation parameters were found to be extremely sensitive to ionic strength. The ionic strength dependence was of a nature to lead

the authors to suggest that the expansion was essentially electrostatic in origin.

A re-examination of the viscosity of BSA at low pH by Tanford, et al. (15) has described the expansion as being more complex than that previously assumed. They have observed a slight increase in the intrinsic viscosity from pH 4.3 to pH 4.0 and no further change until pH 3.5, where a rapid increase in the viscosity begins. The existence of a plateau from pH 4.0 to 3.5 has led to the conclusion that expansion takes place through an intermediate which they have termed the "expandable" form. Below pH 3.5 there appeared to be a rapid expansion of this "expandable" form. In the presence of 2M urea, Sherman and Foster (16) have found that the low pH expansion process was shifted to higher pH and they have described this effect as urea disruption of internal bonding in the protein. Kronman and Foster (17) have studied the optical rotation and sedimentation coefficient of BSA at low pH. In the presence of various strongly bound anions the measured parameters indicated that the expansion was a function of the net charge on the protein. This result agrees with the proposed electrostatic nature of the expansion.

The N-F Transformation

The electrophoretic pattern of a highly purified HSA sample at pH 4 was first shown to be heterogeneous by Luetscher (18). In light of the postulate that albumin exists as an "expandable" form in the neighborhood of pH 4, Aoki and Foster (19,20) have examined the low pH behavior of BSA using moving boundary electro-

phoresis. Through the use of low protein concentration and low ionic strengths they were able to obtain enantiographic patterns in the ascending and descending channels. In the pH range 3.5 to 4.5, two components were resolved whereas outside this region homogeneous patterns were observed. The components were classified as the normal or slow (N) form and the fast (F) form. As the pH was lowered from 4.5, the N form decreased in concentration. At pH 3.5 the pattern was homogeneous in the F form. The point at which the two forms were in a 1:1 ratio was pH 4.0 in chloride, pH 4.1 in acetate, and pH 3.8 in thiocyanate. The interconversion from the N form to the F form was named the N-F transformation (isomerization). Based on the percentages of the two components in the transformation range, the value of m for the equilibrium, $N + mH^+ \rightleftarrows F$, was placed at 3. This transformation appeared to be relatively insensitive to temperature and to ionic strength and was shown to be completely reversible. Protein charge and electrophoretic mobility may be related by Henry's equation (21)

$$Q = 6\pi\eta b \left(\frac{1+Ka}{f(Ka)(1+K(a-b))}\right)\cdot\mu \qquad (3)$$

where Q is the charge on the protein, a and b are as defined in equation (2), a-b is the average radius of the electrolyte ion, η is the viscosity coefficient of the solvent, K is the Debye-Hückel constant, μ is the electrophoretic mobility, and f(Ka) is a slowly varying function of Ka given by Henry. Aoki and Foster have calculated the effective radius of BSA at various low pH from equation (3). In media of ionic strength 0.02 and 0.1 the radius of the protein was found to be 33 Å down to pH 3.5. Below

pH 3.5 the radius began to increase and became sensitive to ionic strength. At pH 2.1 the radius was 50 Å at 0.02 ionic strength and 43 Å at 0.1 ionic strength. The fact that the expansion was suppressed at higher ionic strengths was in accord with the results obtained from optical rotation and viscosity measurements by Yang and Foster (14).

The appearance of two electrophoretically distinct components at low pH led Aoki and Foster (20) to suggest that the anomalous titration of BSA between pH 3.5 and 4.5 be explained in terms of the N-F transformation instead of molecular expansion. From their electrophoretic results they felt that the gross molecular expansion began at pH values lower than those at which, from titration data, ω would appear to decrease. In the titration studies, the calculation of ω was based on the assumption that $pK_{int.}$ and n remained constant over the pH range of the carboxyl groups. In the pH region of the N-F transformation, Aoki and Foster have contended that, on the assumption of a constant molecular radius (and hence a constant ω), the carboxyl $pK_{int.}$ would increase gradually from an abnormally low value of 3.7 above pH 4.5 to a normal value of 4.4 at pH 3.5. The carboxyl groups were presumed to be stabilized as anions in the N form but free to titrate normally in the F form.

In an attempt to explain the N-F transformation with respect to a specific model, Foster and Aoki (22) and Foster (23,24) have proposed a step-wise mechanism for conformational changes of a four sub-unit model of BSA. To explain the appearance of two

components at low pH, it was necessary to assume that each subunit contained approximately 25 carboxyl groups and each had to undergo a transition that was third order in hydrogen ion.

Microheterogeneity of Serum Albumin

Microheterogeneity, i.e. slightly varying molecules within a total population of a specific protein, was suggested by Colvin, et al. (25) in 1954. In recent years a number of investigators have obtained distinguishable subfractions by the chromatography of serum albumin on columns of DEAE-cellulose. Subfractions have been shown to differ in dimer content and in the content of titratable sulfhydryl groups by Hartley, et al. (26). Keller and Block (27) have shown differences in cystine and cysteine content between subfractions. Sponar, et al. (28) have shown differences between subfractions in their resistance to heat denaturation and in their optical rotation.

Microheterogeneity as an Explanation for Electrophoretically Resolvable N and F Components at Low pH

The evidence that an albumin population might be microheterogeneous prompted Foster to reject his hypothesis of a step-wise mechanism for the N-F transformation (29). Sogami and Foster (29) have explained the relatively wide pH range of the N-F transformation on the basis of a population built up of a large number of individual subfractions, each subfraction undergoing the transformation at a slightly different pH value. The subfraction least resistant to the transformation would undergo a rapid conversion to the F form around pH 4.5 and the progres-

sively more resistant subfractions would undergo the transformation at progressively lower pH values. The extent of proton uptake for $N + mH^+ \rightleftarrows F$ for each subfraction was placed at m = 12 to 15; each subfraction was considered to be completely converted from the $N \rightarrow F$ form in 0.1 pH units. On the basis of the above hypothesis, at any specific pH within the transformation range, there would exist molecules stable in the F form, molecules stable in the N form, and a small percentage of the population undergoing the N to F conversion.

In a series of papers (30-32) Foster, et al. have studied the N-F transformation with respect to the postulate of the microheterogeneity of BSA. A necessary prerequisite to their N-F transition hypothesis was the fact that the N and F forms were stable components within the transformation pH range. On a square poly-acrylamide gel plate, electrophoresis was carried out along one edge of the gel. When the N and F forms had separated into resolvable components, the current was turned off for as long as 72 hours. Electrophoresis was again carried out for the same length of time at 90° to the initial migration. If re-equilibration of the N and F forms had taken place during the 72 hour waiting period, zones of protein would be observed at off-diagonal positions. However, zones were only present on the diagonal showing that the second migration was identical to the first and that the N and F forms were stable components.

A question brought up in one of these papers (30) was the possibility that microheterogeneity might have arisen from the

use of albumin obtained from pooled sources. A curve relating percent N form versus pH showed that an albumin sample from one individual was as heterogeneous, if not more heterogeneous, than the pooled albumin sample.

Albumin subfractions have been obtained by Foster, et al. (31) by making use of the difference in solubility between the N and F forms of the protein. Isoionic BSA was dissolved in 3M KCl and the pH lowered into the upper portion of the transformation range. The proportion of the sample in the F form at this pH readily precipitated out while the molecules in the N form remained in solution. The precipitate was removed and the pH again lowered to obtain a second precipitate. This procedure yielded a number of subfractions on which physical-chemical studies were performed.

The optical rotatory behavior between subfractions was studied. A plot of the specific rotation at 313 mμ versus pH showed that the earliest precipitating subfraction underwent specific rotation changes at a pH higher than later precipitating subfractions. The result was consistent with the microheterogeneity, N-F transition postulate.

Titration curves for three subfractions in 0.1M NaCl at 22°C showed significant differences between subfractions. The curves showed the greatest difference in the pH range 3.5 to 6, and above pH 10. The earliest precipitating subfraction showed the least resistance to protonation whilst the later precipitating subfractions were progressively more resistant. The titration curves were identical throughout the pH range 6.8 to 9.7 sug-

gesting that the N forms of the protein were identical at least as far as the imidazole and α-amino groups were concerned. At pH 3.5 the titration curves came together but below this pH they again diverged. The authors have speculated that the expansion process below pH 3.5 may also be slightly different for each subfraction.

Sources of Heterogeneity in Serum Albumin

Heterogeneity in serum albumin can be attributed to several factors; the presence of dimer, varying sulfhydryl and fatty acid content. Whether these factors contribute to the differing resistance of subfractions to undergo the N-F transformation has not been ascertained. Another aspect which, as yet, has not been demonstrated is the possible existence of slight variations in primary structure or possible differences in S-S pairing between subfractions.

Dimer. The extent to which dimer exists in a particular sample of albumin appears to be associated with the preparative procedure. Andersson (33) has reported that aggregates were not present in fresh serum and noted that they appear to be artifacts of the preparation. One type of dimerization of BSA has been shown by Williams and Foster (34) to be a function of pH and ionic strength. In 0.1M NaCl, this type of dimerization was maximal at pH 3.3 but decreased as the pH was lowered below 3.0 or raised above 3.5. In 0.02M NaCl the dimerization at low pH was insignificant. The extent of dimerization was also shown to be greatly enhanced by removal of lipid impurities. By

applying the gel filtration method of Pedersen (35) using Sephadex G-150 or G-200, all traces of dimer can be separated from the monomer.

Hartley, et al. (26) and Janatova, et al. (36) have shown that fractionation of serum albumin on DEAE-Sephadex produces subfractions of unequal dimer content. The dimer fraction has been shown to consist of both a disulfide and non-disulfide type (36). Petersen and Foster (31) have shown this type of dimer (as distinguished from that produced at pH 3.3) to be concentrated in the early subfractions in their solubility studies.

Sulfhydryl Content. Two-thirds of an albumin sample contains one free sulfhydryl group (mercaptalbumin). Hughes and Dintzis (1) discuss the reaction of mercaptalbumin with mercuric chloride to produce a less soluble mercury dimer of albumin. This dimer can be crystallized under conditions in which the monomer remains soluble. The mercury can then be removed most satisfactorily by use of ion exchange resins. Although the resulting mercaptalbumin sample contains one sulfhydryl group per molecule of protein, the sulfhydryl content diminishes with storage and can result in a sample that contains roughly 0.7 sulfhydryl per molecule of protein (36).

Andersson (33) has expanded on the work of King (37) and concluded that most albumin samples contain four components. One component is the mercaptalbumin and two components are of the non-mercaptalbumin type containing mixed disulfides between a modified form of mercaptalbumin and glutathione or cysteine.

The fourth component is a mixed disulfide between mercaptalbumin and cysteine formed by a disulfide exchange reaction with the cystine in the plasma.

Fatty Acid Content. The extent of bound fatty acid in a particular albumin sample depends on the preparative procedure. Several techniques have been employed to remove lipid impurities. Williams and Foster (38) have removed lipid material by lowering the pH of an albumin solution to 3 and allowing it to stand for 2 to 3 days at 5°C. The insoluble material was then filtered off and the remaining fat-free protein passed through a mixed-bed ion exchange resin. The authors concede, however, that this procedure is not 100% efficient. McMenamy and Lee (39) have discussed the use of a modified Goodman defatting process (40). This procedure uses an acetic acid-heptane mixture for removing lipid from albumin. Chen (41) has recently reported a low pH charcoal method for the removal of 99% of the bound fatty acid. This method is extremely rapid and gentle and the resulting fat-free albumin has been judged native by a number of criteria.

The presence of bound lipid impurities has been suggested by McMenamy and Lee (39) to be responsible for the observation of microheterogeneity seen in solubility studies. Petersen and Foster (31) have shown that when subfractions, obtained from solubility experiments, were re-treated with 3M KCl and decreasing pH, the solubility profiles between subfractions were separated by as much as 0.5 pH units. The albumin used in the preparation of the subfractions had been defatted using the Williams and Foster

method (38). McMenamy and Lee, using protein defatted by the modified Goodman process, noticed no difference in the solubility profiles between subfractions taken at early and late stages of an initial precipitation experiment. They have suggested that solubility microheterogeneity is an artifact caused by "contaminants (in a large part fatty acid) which can be removed by a proper defatting process". Sogami and Foster (42) using the method of Chen (40) for defatting albumin, have found differences in the solubility profiles between subfractions. These differences, however, were greatly reduced from those observed when albumin was defatted by the Williams and Foster method (38).

Structural Studies on Serum Albumin

In 1960, Foster (24) suggested that the single polypeptide chain of albumin is folded into four globular sub-units. At neutral pH he envisioned the four sub-units to be in close association having a single hydrophilic interface and two hydrophobic interfaces per albumin molecule. He postulated that the hydrophilic interface consist of anionic (carboxyl) groups and cationic groups paired through electrostatic interaction. In the pH range of the N-F transformation this interaction is progressively weakened as the pH is lowered, thus permitting titration of previously stabilized groups. At even lower pH values the sub-units would then halve again exposing hydrophobic regions to the environment.

Evidence for Sub-units in Serum Albumin. Weber (43), on the basis of fluorescence polarization measurements on dye-

complexed BSA, had concluded that the molecule dissociates at low pH. An increase in depolarization of fluorescence at low pH has also been observed by Harrington, et al. (44). However, on the basis of light scattering and sedimentation measurements, they have shown that the increase of depolarization is not due to dissociation. Their explanation of the fluorescence data is that, at low pH, there exists two connected segments of the molecule, each free to rotate independently. At neutral pH these segments are not free to rotate independently.

Adkins and Foster (45,46) have subjected a BSA-sodium dodecyl sulfate complex to limited subtilisin digestion. The four major products of enzymatic cleavage have been designated M, H_1, H_2, and T. M appears to be the native protein, H_1 and H_2 have molecular weights of 38,000 and 31,000 respectively, and T has a molecular weight of 21,000. The sum of the amino acid analyses of H_1 and H_2 correspond to that of the original protein. Also a mixture of H_1 and H_2 at neutral pH displays a single peak in the ultracentrifuge having a sedimentation coefficient identical to that of the native molecule. The tendency towards aggregation into the native molecule is much greater for a mixture of H_1 and H_2 than self-aggregation of either fragment. The fraction T is explained as a further breakdown product of either H_1 or H_2 or possibly both.

Weber and Young (47,48) have studied the fragmentation of BSA using short digestion times with pepsin. The rate of breakdown into fragments and the separated fragments themselves have

been studied using fluorescence polarization, ultracentrifugation and binding affinity for a polycyclic anion. The attack by pepsin appears to take a biphasic time course. The first phase appears to be a rapid breakdown into a small number of high molecular weight fragments. The second slow phase suggests a breakdown of the fragments themselves. A reversible, concentration-dependent association exists between first phase fragments when the pH of the solution containing the fragments is raised to neutral pH values. The interaction between fragments exhibits bonding of an electrostatic nature. Although the number of fragments obtained from pepsin digestion differs from the number obtained using subtilisin (45,46), Weber and Young feel that their data is in accord with Foster's model.

Markus, et al. (49) have obtained 51,000 and 18,500 molecular weight fragments of HSA using trypsin as the proteolytic enzyme. There also appears to be a second breakdown mechanism which yields 13,500 molecular weight fragments as well as smaller fragments. The enzymatic cleavage of HSA complexed with anionic dyes has been found to be diminished from that of native HSA by Markus, et al. (50). These authors have concluded that specific conformations of HSA are stabilized by the presence of these dyes, that the dye-stabilized conformations are different, and that the conformations are characteristic for the dye used. This conclusion is in agreement with the idea of the "configurational adaptability" of albumin suggested by Karusch (51) in 1950.

Luzzati, et al. (52) have suggested the possibility of a sub-unit model of BSA based on low-angle x-ray scattering experiments. Bloomfield (53) has examined the low-angle x-ray scattering, diffusion coefficient, and intrinsic viscosity of BSA at pH 3.6 and has correlated experimental results with calculated data for several different models. A molecule consisting of three linearly arranged spherical sub-units gave the best agreement with experimental data.

Evidence for Crevice(s) in Serum Albumin. Solvent perturbation is a technique which can be used to study the availability of specific protein chromophores to the solvent. The influence of solvent on tyrosyl residues causes a small change in the extinction coefficient and a slight shift in the wavelength of maximal absorption. Information concerning buried tyrosyl residues or tyrosyls residing in crevices can be obtained by comparing data for the urea denatured protein with data obtained with the protein dissolved in various other solvents. If the availability of tyrosyl residues is dependent on the molecular size of the solvent molecules, then it is possible that the chromophore resides in a crevice (54).

Herskovitz and Laskowski (55) using the method of solvent perturbation, have shown that the tyrosyls of BSA are unaffected by the small perturbant glycerol from pH 2 to pH 8. However, in the presence of a large perturbant such as polyethylene glycol, 3-5 tyrosyls appear to be unavailable to solvent above pH 4. Below pH 4 the availability of tyrosyls to both small and large

perturbants is the same. It has been suggested that 3-5 tyrosyls exist in crevice(s) in the N form which are penetrable to glycerol but not to polyethylene glycol. The transition from the N form to the F form would destroy the crevice(s) making the tyrosyls available to both large and small perturbants. On the basis of volume of exclusion calculations for different perturbants, Shormuller and Laskowski (56) have shown that the size of the crevice(s) of BSA in the N form would be approximately 5 Å. Wishnia and Pinder (57) have studied the hydrophobic interaction of BSA with butane and pentane. The binding behavior has been explained in terms of the existence of relatively large hydrophobic regions within the N form of BSA which are able to interact with the hydrocarbons. The F form of the protein can bind only one-fourth as much butane and one-fifth as much pentane as the N form, suggesting disruption of the integrity of the hydrophobic regions upon conversion to the F form.

Evidence for Stabilized Carboxyl Groups in Serum Albumin. The extent of carboxyl group stabilization in human mercaptalbumin (HMA) has been studied by Foster, et al. (58,59). They suggest that, because of uncertainties in the assignment of values to the protein net charge, Z, the evaluation of titration data using calculated Z values is tenuous. The Linderstrøm-Lang equation (eqn. 1), written in terms of the potential, ψ, at the surface of a charged sphere, becomes

$$pH - \log \frac{r}{n-r} = pK_{int.} - 0.434 \frac{\varepsilon\psi}{kT} \qquad (4)$$

Hartley and Roe (60) have shown that the electrophoretic mobility, μ, and Henry's equation (eqn. 3) can be used to calculate ψ. Although Henry's equation yields a " ζ potential", i.e. that potential at an imiginary surface of shear somewhat displaced from the surface of the molecule, it is assumed that at low ionic strength of 1:1 electrolytes the difference between ζ and ψ is small. This method has been discussed and used by Beychok and Warner (61) for an analysis of the behavior of lysozyme at low pH. The potential is therefore given as

$$\psi \doteq \zeta = \frac{6\pi\eta}{Df(Ka)} \cdot \mu \qquad (5)$$

By setting $\nu = n-r$ (the number of H^+ equivalents associated with protein at a specific pH), taking anti-logarithms, and rearranging equation (4), an equation of the form

$$\frac{\nu}{[H^+]} e^{\varepsilon\Psi/kT} = \frac{1}{K_{int.}} (n-\nu) \qquad (6)$$

is obtained for equivalent, non-interacting sites. Foster and Clark (59) have used the graphical procedure of Scatchard, et al. (62) to relate the parameters given in equation (6). With the data obtained for HMA, a linear plot of $\frac{\nu}{[H^+]} e^{\varepsilon\Psi/kT}$ versus ν has a negative slope equalling $\frac{-1}{K_{int.}}$ and an intercept on the abscissa equal to n. In the pH range above the N-F transformation the plot is linear and gives a $pK_{int.}$ of 4.3. Extrapolation of this line to the abscissa gives a best estimate of n = 55 equivalent sites. Below the N-F transformation the plot again becomes linear and gives $pK_{int.}$ = 4.4 and n = 106, approximately

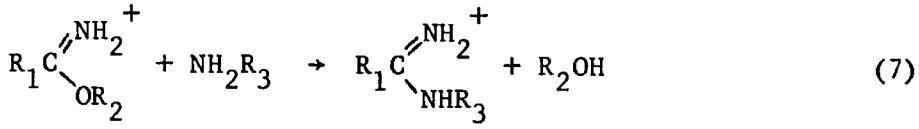
the total number of carboxyl groups in HMA. In the region of the N-F transformation the plot is not linear implying non-equivalent sites. The interpretation of these findings is that the molecule contains approximately 55 normally titrating carboxyl groups on its surface. The remaining carboxyl groups have a pK < 4 above the N-F transformation range. These stabilized groups become normal when the protein is converted to the F form and titrate with a normal pK below the transition. The authors postulate that in order to have such a large number of charged carboxyl groups stabilized in the N form there must be pairing between these carboxyl groups and cationic groups, possibly of the ε-amino groups of lysine.

Vijai and Foster (63) have studied BSA using electrophoresis and titration. In order to shift the range of the N-F transformation to lower pH, they have also studied BSA complexed with twelve dodecyl sulfate ions. Using the analytical procedure outlined above, the data would suggest that 60 normal carboxyl groups are present with a $pK_{int.} = 4.3$ and 40 carboxyl groups are stabilized in the N form. Below the transformation BSA appears to have 100 equivalent sites titrating with $pK_{int.} = 4.1$.

The Chemical Modification of Serum Albumin

The amidination of proteins and related small molecules using imidoesters has been thoroughly investigated by Hunter and Ludwig (64,65) and Hand and Jencks(66). The requirements of reaction specificity, mild reaction conditions, and lack of alteration in the net charge of the protein are satisfied by

this reaction procedure. The imidoester-amine reaction to form amidines is



(7)

where, in the present investigation,

R_1 = a) CH_3
b) $CH_3(CH_2)_2$
c) $CH_3(CH_2)_8$

R_2 = CH_3CH_2

R_3 = Bovine Mercaptalbumin (BMA)

The names and abbreviations for the specific derivatives are a) Acetamidino - BMA, (A-BMA), b) Butyramidino - BMA (B-BMA), and c) Decylamidino - BMA (D-BMA).

The reaction to form amidines has been shown (64) to be strongly pH-dependent over the pH range 7-11. The pH for maximum reactivity is a function of the type of imidoester used and the nature of the reacting amine. The specificity of the imidoester for amines has been established by the absence of reaction with model compounds containing imidazole groups, phenolic groups, guanidino groups, sulfhydryl groups or peptide bonds. Amidines are stronger bases than amines but not quite as strong bases as guanidines; the amidines have a pK near 12.

Several studies have looked into the possibility of three-dimensional structural changes in amidinated BSA. Wofsy and Singer (67), in a study on amidinated antigens and antibodies, have found no significant changes in $[\alpha]_D$, E_{280}, or $s_{w,20}$ in an

extensively (80-90%) modified acetamidino derivative of BSA. Exhaustive amidination (over 95%) resulted in a protein which showed slight differences in the above parameters. Habeeb (68,69), in papers discussing conformational differences in native and chemically modified derivatives of BSA, has shown that any structural differences between amidinated and native BSA are slight.

MATERIALS AND METHODS

The bovine mercaptalbumin (Thompson I and II) (BMA) used in these studies had been prepared by Dr. T. E. Thompson by the method of Hughes and Dintzis (1). It had been stored as an isoionic, salt-free powder at -10°C for over ten years. The preparation contained 0.72 sulfhydryl groups per molecule and 8-9% dimer. The molecular weight of albumin was taken as 65,000.

Acetonitrile, reagent grade, was obtained from the J. T. Baker Chemical Co. N-butyronitrile and n-nonyl cyanide, reagent grade, were obtained from the Aldrich Chemical Co., Inc. These nitriles were used directly without further drying. Formaldehyde solution (36-38%), reagent grade, was obtained from Merck and Co. 1,3-diphenyl guanidine, reagent grade, was obtained from Eastman Organic Chemicals and sodium carbonate (anhydrous), reagent grade, was obtained from Allied Chemicals. Standard 2M hydrochloric acid, standard 2M potassium hydroxide, and prediluted buffers (pH 1.68, pH 4.01, pH 6.86, and pH 9.18) were obtained from Hartman-Leddon Co. (Harleco).

Standard buffers, potassium tetroxalate (pH 1.680) standard sample (s.s.) 189, potassium hydrogen phthalate (pH 4.008) s.s. 185c, potassium hydrogen phosphate and disodium hydrogen phosphate (pH 6.864), s.s. 186-I-b and s.s. 186-II-b respectively, and borax (pH 9.180), s.s. 187a, were obtained in powder form from the National Bureau of Standards, Washington, D.C. Sephadex G-150 and G-200 were obtained from Pharmacia Fine Chemicals Inc. Dialysis tubing was obtained from Union Carbide Corporation and

washed in distilled water prior to use. A Millipore filter apparatus using Millipore filters, RAWP 01300, 1.2μ, was obtained from the Millipore Filter Corporation. "Aged" nitroprusside stain was prepared according to Block (70).

Conductivity measurements were performed on a Conductivity Bridge, Type 3216B, with an LKB 3225J-2 cell, cell constant = 4.60 cm^{-1} (LKB Instruments, Inc.). The cell constant was determined by measuring the resistance of 0.01 D and 0.1D KCl standard solutions. The specific conductivities of these solutions had been determined by Jones and Bradshaw (71).

Partial specific volume measurements and dry weight analyses were performed by the method of Hunter (72). The partial specific volumes of A-BMA and B-BMA were measured by Mr. D. Turk and Dr. M. J. Hunter.

Absorbancy measurements were made on a Beckman Model DU Spectrophotometer and a Zeiss PMQII Spectrophotometer using Beckman quartz cells.

Sedimentation velocity analyses were performed on a Spinco Model E Ultracentrifuge using the schlieren optical system. Ultracentrifuge and electrophoresis plates were read on a modified Nikon Shadow Graph, Model 3A (Nippon Kogaku K. K., Japan), equipped with a Mann two-dimensional Comparator, Type 829 (David W. Mann, Inc., Lincoln, Massachusetts).

Gel filtration was carried out in a Sephadex K 50/100 column (I.D., 5 cm.; length, 100 cm.) (Pharmacia Fine Chemicals, Inc.) and column flow rates were controlled by a Beckman

Solution Metering Pump, Model 746. Column fractions were collected by a G.M.E. Linear Fractionator (Gilson Medical Electronics) and the column eluates were monitored at 280 mμ by a Vanguard 1056 Automatic Ultraviolet Analyzer (Vanguard Instrument Co.).

Fatty acid determinations were done by Dr. W. Scheider using a modification of the method of Dole (73).

Titration Studies

pH measurements were performed on a Radiometer pH Meter 4, type PHM 4c, using a Radiometer combination glass electrode, type GK 2021 B. A Heto constant temperature circulating bath, model 623, (Heto, Birkerod, Denmark) circulated water (25°C±0.1°) through two identical pyrex jacketed vessels (I.D., 2 cm; length, 8 cm.). A 5 ml pyrex burette, graduated to 0.01 ml and equipped with a platinum tip, was used to deliver acid (base). Drawn polyethylene tubing was attached to the platinum tip when it was necessary to titrate directly into the solution. Samples were stirred using teflon coated magnets driven by a Mag-Mix magnetic stirrer. A Radiometer titrator, type TTT 1c, was attached to a Radiometer combination glass electrode in a 2°C cold room for recording low temperature pH values. The titrator served as a pH stat when connected to a magnetic valve.

N.B.S. standard buffers were prepared by the procedure of Bates (74). Standard buffers prepared by this method should not vary by more than 0.005 pH units from the given pH at 25°C. All buffers were prepared in CO_2-free glass distilled water (specific conductivity $< 10^{-6}$ mhos/cm) and stored in polyethylene

bottles under nitrogen. The buffers were delivered from the bottles by nitrogen pressure and stored at 2°C when not in use.

Standard 0.1M, 0.2M, and 0.5M HCl and KOH solutions were prepared by quantitative dilution of 2M HCl and 2M KOH standard solutions obtained from Harleco. Two standard acid (base) solutions were prepared for each of the above concentrations: one with a final KCl concentration of 0.1M and the other with a final KCl concentration of 0.02M. All solutions were prepared with CO_2-free glass distilled water. Concentrations of the HCl solutions were obtained by standardization with standard solutions of 1,3-diphenyl guanidine (in 95% ethanol) and sodium carbonate. Concentrations of the KOH solutions were obtained by standardization with a standard solution of potassium hydrogen phthalate. A final standardization of HCl against KOH, in which each solution was of the same molarity and KCl concentration, was made to assure accurate concentration values. The resulting HCl and KOH concentrations were reproducible to 5 parts in 1000. The HCl solutions were stored in glass bottles with ground glass stoppers and the KOH solutions were stored in Nalgene bottles under nitrogen.

Moving Boundary Electrophoresis Studies

Moving boundary electrophoresis was performed on a Spinco Model H Electrophoresis and Diffusion Apparatus using a Rayleigh interference optics system. The optics were modified by Mr. R. H. Matthews in the course of his work for a Ph.D. thesis, utilizing a multiple slit source and raster to obtain uniform,

intense fringes. A standard 11 ml interference Beckman quartz cell was used for all electrophoretic runs. Kodak metallographic plates (4 x 5 in.) were used for recording fringe patterns. Kodak D19 developer, indicator stop bath, fixer, hypo clearing agent, and photo-flo 200 were used in the development procedure of the plates.

EXPERIMENTAL

Preparation of Imidoesters

The procedure for the synthesis of imidoesters is basically that of Pinner (75);

$$RCN + R'OH \xrightarrow[HCl]{dry} R\text{-}\underset{\underset{NH\cdot HCl}{\|}}{C}\text{-}OR' \qquad (8)$$

where, for this study,

R = a) CH_3 R' = CH_3CH_2
b) $CH_3(CH_2)_2$
c) $CH_3(CH_2)_8$

In this thesis the class of compounds will be referred to as imidoesters; specific compounds will be called imidates, viz., ethyl acetimidate. The method reported by Hunter and Ludwig (64) was used for the preparation of ethyl acetimidate·HCl (R = a) and the method of McElvain and Nelson (76) for the preparation of imidoesters utilizing nitriles with larger R-groups (R = b, c).

Ethyl Acetimidate·HCl Preparation. 6.4 ml anhydrous ethanol (0.10 mole) was cooled in a dry ice-ethanol bath. Dry HCl gas was bubbled into the ethanol until 4.0-5.0 gm (0.11-0.14 mole) had been added. After the final weighing of the ethanol:HCl mixtures the reaction mixture was recooled and 5.3 ml acetonitrile (0.10 mole) was rapidly added to the reaction vessel. The reaction vessel was left in the dry ice-ethanol bath until the acetonitrile had frozen out of the reaction mixture. The reaction vessel was then transfered to an ice-water bath and placed in a 2°C cold room. After 48 hours an equal volume of dry ether was added to the mixture causing the immediate formation of

crystals. The crystals were washed in a filtering flask with ethanol:ether (1:2) and then with ether and stored at room temperature in a desiccator over desiccant. The yield was about 7 gm (>70%). The crystals had a m.p. of 109°-112°C. Bubbling and gas evolution at the m.p. was indicative of imido-esters.

Ethyl Butyrimidate·HCl and Ethyl Decylimidate·HCl Preparation. 6.4 ml anhydrous ethanol (0.10 mole) and either 8.7 ml butyronitrile (0.10 mole) or 18.5 ml decanenitrile (0.10 mole) were added to a reaction vessel and cooled in an ice-water bath. After an initial weighing of the reaction vessel, 4.0-5.0 gm (0.11-0.14 mole) dry HCl gas was bubbled slowly into the nitrile:alcohol mixture. The reaction vessel was then placed in a 2°C cold room. After 48 hours crystals appeared in the decanenitrile:ethanol:HCl mixture to which an equal volume of anhydrous ether was added. Crystals were formed in the butyronitrile:ethanol:HCl mixture after addition of 60 ml (4 times the mixture volume) anhydrous ether. The crystals were placed in a filtering flask and washed with an equal volume of ether. The crystals were then stored at room temperature in a desiccator over desiccant. A yield of about 8 gm (>70%) was obtained for ethyl butyrimidate·HCl with a m.p. of 78-80°C. A yield of about 13 gm (>60%) was obtained for ethyl decylimidate·HCl with a m.p. of 68-70°C. There was bubbling and gas evolution at the melting points of these crystals.

Preparation of Amidinated Derivatives of Albumin

The procedure for the amidination of BMA (see formula 7) is a modification of the method of Wofsy and Singer (67). Complete modification of the ε-amino groups of BMA was desired in the preparation of A-BMA and B-BMA. The procedure that was found most suitable for this objective was repeated additions of imidoester reagent to the protein solution with the reagent aliquots in a ten-fold excess of the ε-amino groups of the protein. The reactions were done in a 2°C cold room.

Prior to protein modification a test was done on the likelihood of reaction of ε-amino groups with the prepared imidoesters. "Aged" nitroprusside stain gives an orange color with unsubstituted amidines whereas substituted amidines give a pink color. The imidoesters were reacted with ε-aminocaproic acid and chromatographed with an unsubstituted amidine, acetamidine hydrochloride, on a circular paper chromatograph. Upon staining, pink spots were obtained for the products of the acetimidoester, butyroimidoester, and decylimidoester reactions with ε-aminocaproic acid and an orange spot observed for acetamidine hydrochloride. Crystals obtained from imidoester synthesis using hexanenitrile and octanenitrile proved to be unreactive with ε-aminocaproic acid. These crystals were probably something other than imidoesters due to the use of improperly dried nitriles.

Preparation of A-BMA. A 5% solution of BMA (2.2 gm in 40 ml) was adjusted to pH 9.2 and placed in the 2°C cold room.

Ethyl acetimidate·HCl (2.1 gm) was dissolved in 1.0M NaOH (8 ml) and the pH adjusted to 9.2 using 10.0M NaOH. After addition of the imidoester to the cold protein solution the pH was readjusted to 9.2. The pH was maintained at this value by the addition of 1.0M HCl using the titrator as a pH stat. After approximately four hours a further 10 fold excess of imidoester was added to the reaction vessel and the reaction allowed to continue overnight. In the morning the pH of the solution was lowered to 7. The solution was then dialyzed for 5 hours in 0.01M phosphate buffer at pH 7 (2 liters). The dialyzed solution was then passed through a millipore filter (1.2μ pore size), dialyzed for two days in three changes of distilled water (2 liters each), freeze-dried, and stored at 2°C. The yield of product was over 85%.

Preparation of B-BMA. The preparation of B-BMA was the same as that for A-BMA except that the reaction was done in the presence of 10% methanol. Both the 5% protein solution and the ethyl butyrimidate·HCl solution contained 10% methanol before mixing. Three additions of ethyl butyrimidate·HCl were necessary for complete reaction of the ε-amino groups of BMA. In addition, the initial dialysis in phosphate, after the reaction was complete, was done in the presence of 10% methanol. The yield of product was over 80% of the starting material.

In an earlier attempt to prepare B-BMA, the addition of a 20-fold excess of butyrimidoester to a 5% solution of BMA in 20% dimethylformamide at 25°C and pH 9.2 resulted in considerable precipitation of grossly denatured albumin during the course of the reaction.

Preparation of D-BMA. Exhaustive modification of BMA with ethyl decylimidate·HCl produced an insoluble precipitate. For this reason amidination using decylimidoester was restricted to incomplete modification of the ε-amino groups of BMA. The reaction time for one addition of decylimidoester (ten-fold excess) to a 5% BMA solution was limited to four hours. The reaction was carried out in the presence of 15% methanol. Upon lowering the pH to 7 the solution remained clear. The reaction mixture was dialyzed at pH 7 in 0.01M phosphate buffer (2 liters) in the presence of 15% methanol for several hours. Amidination was then repeated with another ten-fold excess addition of decylimidoester with a reaction time of four hours. The mixture appeared to be a two phase system at the end of this time. The mixture was centrifuged and the clear bottom phase was removed, dialyzed as before, and filtered through a millipore filter (1.2μ pore size). After two days of dialysis in three changes of distilled water (2 liters each) the protein was freeze-dried and stored at 2°C. The yield was over 80% of the starting material.

Analysis for the Extent of Amidination of Albumin

The ε-amino groups in a protein can be estimated by titration in the presence of formaldehyde (77). Model studies with glycine, ε-aminocaproic acid and acetamidinoacetic acid showed that, in the presence of 10% formaldehyde, glycine and ε-aminocaproic acid were completely titrated by pH 9. Acetamidinoacetic acid, however, did not begin to titrate until this pH was reached (64). Below pH 9, therefore, protein amidino groups will not be titrated

even in the presence of formaldehyde. It is thus possible to determine by titration the ε-amino groups remaining after protein modification.

A 0.8% protein solution in 0.1M KCl (6 ml) was titrated to pH 9.00 with 0.0201M KOH. The protein solution was then titrated back to pH 9.00 after the addition of 4 ml of 37% formaldehyde solution (previously adjusted to pH 7). In a control experiment, 0.1M KCl (6 ml) was brought to pH 9.00, 4 ml of formaldehyde added and the solution titrated back to pH 9.00. Several formaldehyde titrations on native BMA resulted in values of 56.5-57.5 for the total number of lysyl residues per mole protein. These values agree well with the value of 57 lysyl residues reported for acid-base titration studies of BSA (9) and a composite value of 57 reported for amino acid analysis of BSA (8, 78-80).

Preparation of Deionized, Monomer Albumin

Preparation of Albumin Monomers. Native and amidinoalbumin preparations were separated into monomer, dimer, and higher polymers by the method of Pedersen (35). Approximately 75 gm of Sephadex G-150 or G-200 was suspended in 3 liters of 0.1M KCl and the resin allowed to swell for at least 48 hours at 2°C. A Sephadex column was filled with the swollen Sephadex under gravity and 0.1M KCl passed through the resin column overnight. The pressure head on the column was always maintained at less than 20 cm in height.

Albumin (1.5-1.75 gm) was dissolved in 20-25 ml 0.1M KCl and the solution applied to the Sephadex column. KCl (0.1M)

was then passed through the column. Flow rates of approximately 25 ml per hour were maintained with the metering pump. The absorbancy of the eluate was monitored with the Vanguard analyzer and the 11 ml fractions were collected by the GME fraction collector. All operations were conducted at 2°C. After inspection of the absorbancy profile the eluate tubes were pooled to give monomer and dimer fractions of albumin. The fractions were than dialyzed against distilled water, freeze-dried, and stored at 2°C. The protein monomers were dissolved in 0.05 $\Gamma/2$ acetate buffer (pH 4.74) and sedimented at 59,780 r.p.m. and 20°C in the Spinco Model E ultracentrifuge to determine homogeneity.

Preparation of Deionized Albumin Monomers. The mixed-bed resin method of Dintzis (7) was used for deionizing the native and amidino-albumin monomer preparations. The column used had a volume of approximately 23 ml. A 5% albumin solution was applied to the column and the deionized protein was eluted from the column by passage of deionized water down the column; the specific conductivity of the water was less than 10^{-6} mhos per cm. The flow rate of the column was approximately 15 ml per hour. All operations were conducted at 2°C.

The following precautions were taken to assure fresh, CO_2-free protein solutions. 125 ml polyethylene bottles were cleaned for one hour in dichromate cleaning solution and soaked for one week in many changes of distilled water. Air tight caps with entrance and exit tubes were made for the bottles. The bottles were purged with nitrogen just prior to deionizing the protein

solutions and the exit tubes were attached to CO_2 traps when the entrance tubes were connected to the foot of the column. The albumin solutions were deionized just prior to the titration studies in order to assure that the CO_2 content of the solutions was minimal. Enough deionized protein was removed from the stock solution for dry weight and extinction coefficient determinations and for titration studies and stored, under nitrogen, in polyethylene bottles. The remainder of the stock solution was shell frozen in its polyethylene bottle and stored at -10°C.

The deionized B-BMA became flocculent at room temperature necessitating special handling procedure. After deionizing the B-BMA solution, the protein was dialyzed with stirring, against 0.04M KCl. In the presence of 0.04M KCl, B-BMA remained in solution at room temperature. Since B-BMA had been dialyzed against 0.04M KCl, a portion of the 0.04M KCl dialysate was saved for the dry weight determinations of B-BMA.

A 3% D-BMA solution precipitated out on the mixed-bed resin column. The application of 1% D-BMA solution did not cause precipitation; however, the eluted deionized protein became flocculent at 2°C. In the presence of 0.1M KCl D-BMA remained in solution at 2°C and at room temperature. Because of the solubility problems associated with deionized D-BMA, studies on this protein were limited to sedimentation velocity analysis and moving boundary electrophoresis.

Acid-Base Titration Studies

Acid-base titration studies were performed on BMA, A-BMA, and B-BMA deionized monomer preparations. Initial titration studies were performed at 25°C on 0.5% protein solutions (4 ml) in 0.1M KCl. The protein solutions were titrated with 0.1M, 0.2M, and 0.5M standard HCl solutions and 0.1M and 0.2M standard KOH solutions. The standard acid (base) solutions contained 0.1M KCl. When titrating in the alkaline pH range, nitrogen was passed over the protein solution. The above titration procedure was also carried out in the presence of 0.02M KCl. The resulting corrected acid titration curves for BMA, for the various standard acid solutions in 0.1M KCl, differed from one another by less than one mole H^+ per mole protein from the isoionic point to pH 3.0. This result demonstrated that the dilution of the protein solution, introduced by the use of the acid solutions of low molarity (0.1M and 0.2M), was not a source of error in the titration procedure. The base titration curves of the protein were conducted in this manner. However, a lack of correspondence among the corrected acid titration curves in the presence of 0.02M KCl prompted some refinements of the experimental technique for the acid titration experiments.

The Titration Method. The two basic methods for titration are (1) a continuous titration whereby increments of titrant are added consecutively to a protein solution and (2) the addition of titrant to a series of separate protein solutions, the pH of each solution being measured separately (81). Method (2) is

capable of greater accuracy but necessitates the use of larger amounts of protein than method (1). Although method (1) places severe demands on the pH measuring equipment because of lengthy (several hours) experimental times, it was chosen for these titration studies because of the limited supply of protein. It was hoped that by controlling the different time-dependent factors, e.g., stability of the equipment, correction for dilution by titrant, KCl leak from the glass electrode, that titration results approaching the accuracy of method (2) could be obtained.

Control titration experiments were performed to establish whether pH values should be taken on stirring or stationary solutions. Although the results of these control experiments showed little difference in pH, there appeared to be greater stability when pH values were taken on stationary solutions. Therefore, in the course of a titration experiment, the protein solution was stirred for eight minutes following the addition of titrant. The magnetic stirrer was then turned off for two minutes at which time the pH of the stationary solution was recorded. These time limits were adequate for equilibration as noted by the lack of pH drift at the end of each time interval.

Control of KCl Diffusion From Glass Electrode. A single Radiometer combination glass electrode was used in all titration experiments. This particular electrode was found to be exceptionally stable. Because of the time involved for a single titration experiment, the rate of saturated KCl diffusion through the porous plug of the electrode was examined. The glass

electrode was immersed in 5 ml of a stirred 0.1M KCl solution in a titration vessel. At 30 minute intervals, samples of the KCl solution were removed for conductivity measurements. The rate of increase of KCl in the solution varied considerably from electrode to electrode. The electrode with which the protein titration studies were conducted showed a change in KCl concentration of 0.006M per hour or a 30% increase in the KCl concentration over a five hour time span. This increase in the KCl concentration was too great to permit the titration curves to be conducted at approximately the same ionic strength.

An attempt was made to decrease the KCl leak by lowering the hydrostatic head of the saturated KCl in the side-arm of the glass electrode. An air-tight cap, made to fit this arm, was connected to 1 mm I.D. tubing of a length such that the end of the tubing was just above the height of the solution in the titration vessel. The cap and the tubing were then filled with saturated KCl. Repetition of the above conductivity measurements on a 0.1M KCl solution showed that the rate of change in KCl concentration had been reduced to 0.002M per hour or only a 10% increase in the KCl concentration over a five hour time span. This increase was considered to be within practical limits. The stability of the glass electrode was not affected by the addition of the cap and tubing to the electrode arm. Any further decrease in the rate of KCl diffusion from the electrode decreased the stability markedly.

Standardization of the pH Meter. Two National Bureau of Standards buffers, pH 6.864 and pH 4.008, were used in the standardization of the Radiometer pH meter. 5 ml pH 6.864 buffer was added to one titration vessel and 5 ml pH 4.008 buffer was added to a second titration vessel. The buffer adjustment dial and the temperature control dial of the pH meter were adjusted until both buffers gave the correct pH on the pH meter. By making these "slope factor" adjustments of the pH meter the need for a pH correction to the titration data between pH 6.864 and pH 4.008 was eliminated (82). 5 ml N.B.S. standard buffer pH 1.680 was then added to a titration vessel and its pH value recorded. This pH was usually below 1.680 by 0.02 pH units. Below pH 4.01, therefore, a slight pH correction was made to the titration data.

Protein Concentrations. Dry weight determinations, reproducible to 2 parts in 1,000, were obtained for the salt-free protein preparations. The concentration of B-BMA in 0.04M KCl was found by obtaining a dry weight on the 0.04M KCl dialysate and subtracting this weight from the dry weight determination of the B-BMA:KCl solution. The salt-free protein preparations were then diluted, by weight, to final 1% protein solutions (~25 ml) in 0.1M KCl. A 5 ml volumetric pipette (Pyrex, Class A, 5 ml ± 0.01) was used to introduce the 1% protein solution into the titration vessel. The calculation of the number of moles protein in the 5 ml sample involved the protein's partial specific volume ($\bar{v}$), the dry weight of the salt-free protein

preparation, the weights obtained for dilution, and the protein's molecular weight. The molecular weights of the amidino-albumins were based on the molecular weight of BMA (65,000) and the additional weight due to modification. The calculated molecular weights of A-BMA and B-BMA were 67,430 and 68,850, respectively.

Correction Curves. The concentration of unbound or free hydrogen ion in the titration of a protein-containing solution can be obtained by the titration of a protein free solution providing the two solutions have the same ionic strength at any given pH value (81). 5 ml 0.1M KCl was used in place of the protein solution. At any given pH, enough 0.1M KCl was added to the blank solution such that the protein-free and protein-containing solutions had the same volume and ionic strength. The pH value of the blank solution was then recorded. The concentration of hydrogen ion bound to the protein was calculated by subtracting the correction titration curve from the protein titration curve.

Reproducibility of Titration Curves. To check for time dependence the protein titration experiments were conducted in two ways. One type of titration was performed by adding small amounts of acid to the protein solution throughout the acid pH range. The second type of titration was performed by initially adding relatively large amounts of acid to the protein solution and then titrating with acid volumes equal to those in the first type of titration. The criteria for acceptance of each type of titration was that, at the end of the experiment, the standard

pH 4.008 buffer read to within 0.01 pH units of its stated pH value. Titrations performed in these two ways agreed to ±0.01 pH units. Also, separate titrations performed in either of the two ways were reproducible to ±0.01 pH units. The titration curves, therefore, were experimentally reproducible and showed no time dependence.

In the studies conducted in 0.02M KCl, however, a time-dependent variation in pH was still observed in the pH region of the N → F transition although the pH values obtained above and below this region were reproducible and showed no time dependence.

Moving Boundary Electrophoresis. Moving boundary electrophoresis was performed on BMA, A-BMA, and B-BMA monomer, deionized preparations and on a D-BMA deionized preparation. Standard 2% protein solutions were diluted with 0.1M KCl to a final volume of 15 ml and a concentration of 0.2%. The 0.2% protein solution in 0.1M KCl and a 0.1M KCl solution (2 liters) were adjusted to a specific acid pH and the protein solution was dialyzed overnight, at 2°C, against the 0.1M KCl solution.

The electrophoresis cell and electrode vessels were assembled at 2°C. After a 30 minute equilibration of the electrophoresis assembly in the constant temperature bath (0.9°C ± 0.1°C) of the Spinco Model H electrophoresis and diffusion apparatus, the protein-solvent boundaries were formed and a baseline picture taken for both ascending and descending channels. Compensation was carried out for approximately 35 minutes using a current of 4 mamp. after which time initial boundary pictures

were taken. Electrophoresis of the protein was carried out using a current of 20 mamp. and a potential gradient of 5.3 volts per cm. Pictures of the boundaries were taken every hour over a five hour time span.

The rate of heat production by the current in the cell is expressed by

$$H = \frac{i^2}{q^2(S.C.)} \tag{9}$$

where i is the current, q is the cross-sectional area of the cell, and S.C. is the specific conductivity of the solvent. At 1°C the maximum power which might be dissipated in 0.1M buffers in the electrophoresis cell without causing convection is about 0.15 watts per cm^3 (83). In this investigation H = 0.073 watts per cm^3 which is well below the maximum power dissipation limit.

Plate exposure times of 5.7 sec. were used when recording the electrophoretic Rayleigh fringe patterns. The development procedure for the metallographic plates was as follows: D-19 developer, 2 min.; stop bath, 1 min.; water rinse, 30 sec.; fixer, 12 min.; water rinse, 30 sec.; hypo clearing agent, 2 min.; water rinse, 15 min.; photo flo, 1 min.

Conductivity measurements were performed on the 0.1M KCl solutions with the conductivity cell immersed in the electrophoresis constant temperature bath. At the completion of an electrophoretic experiment, the protein in the reservoir of the electrophoresis cell was recovered. The protein from the reservoir and the Harleco buffers (pH 6.86, pH 4.01, and pH 1.68)

were added to test tubes and placed in an ice-water bath. The pH meter was standardized using the pH 4.01 buffer (pH 4.01 at 0°C). The pH of the pH 6.86 (pH 6.98 at 0°C) buffer and the pH 1.68 (pH 1.67 at 0°C) buffer were recorded and a linear pH correction was calculated. The pH of the reservoir protein solution was then measured and the linear pH correction applied to obtain the true pH of the protein solution.

The electrophoresis plates were read on the Nikon-Mann microcomparator (reproducibility, ± 5 microns). Fringe pattern analyses were carried out on the IBM 7090 computer and the IBM 763/780 Cal-comp plotter (see appendix). Mobility calculations were based on the distance the weight-mean of the boundary had travelled in a one hour time interval. The mobilities obtained for each of these intervals over a five hour electrophoresis run were averaged to give the mobility at a specific pH value. The resulting mobilities for the ascending and descending channels differed by less than 0.1×10^{-5} cm^2 per volt sec. at pH values near the isoelectric point and by less than 0.2×10^{-5} cm^2 per volt sec. at pH values at which the protein had a relatively great mobility. An arithmetic average of the weight-mean of the ascending and descending mobilities was used as the mean mobility at any given pH.

RESULTS

Comparative Studies on Native and Amidinated Albumins Above the pH Region of the N-F Transformation

Chloride Binding at the Isoionic Point. Chloride binding studies at the isoionic point of BMA, A-BMA, and B-BMA were performed by the method discussed by Lowey (84). Differing extents of chloride binding between the derivatives and the native protein would complicate comparison of electrophoretic mobilities and titration curves of these preparations.

The extent of chloride binding can be determined by making pH measurements on the isoionic proteins in the presence of varying concentrations of sodium chloride. Standard 0.002M, 0.02M, 0.2M, and 2.0M sodium chloride solutions were prepared using CO_2-free glass distilled water and stored under nitrogen. 2 ml isoionic protein (1%) and 2 ml of the various standard sodium chloride solutions were mixed and the resulting pH recorded. The binding of chloride to the protein was calculated using the equation given by Scatchard (85):

$$\Delta \bar{\nu} = \left(\frac{2.3}{2\omega}\right) \Delta pH \qquad (10)$$

where $\Delta \bar{\nu}$ is the number of chloride ions bound per mole protein, ΔpH is the difference between the pH at a given ionic strength and that of the salt-free protein, and ω is the electrostatic factor. ω was considered to be the same for the three protein preparations. The values of $\left(\frac{2.3}{2\omega}\right)$ given by Scatchard for HSA (85) were used in the calculation of chloride binding. The values

of $\Delta \bar{\nu}$ for the native and amidino-albumins are presented in Table I.

TABLE I

Chloride Binding at the Isoionic Point

protein	$\Delta\bar{\nu}$ $\Gamma/2 = 0.001$	$\Gamma/2 = 0.01$	$\Gamma/2 = 0.1$	$\Gamma/2 = 1.0$
BMA	1.0	2.6	7.1	20
A-BMA	0.6	2.2	7.2	22
B-BMA	0.3	2.0	6.5	19
HSA (85)	1.3	4.1	8.5	24

Partial Specific Volumes. Three density measurements were performed on the A-BMA preparation of known protein concentration. The calculated temperature coefficient $(\frac{d\bar{v}}{dT})$ of 0.00037 agrees with that obtained by Hunter (72) for BMA ($0.00037_5 \pm 0.00003$). One density measurement was made on B-BMA. Partial specific volumes for A-BMA and B-BMA were calculated for various temperatures using the slope $\frac{d\bar{v}}{dT}$. Using a temperature coefficient of 0.00037_5, the partial specific volume of the derivatives at 25°C were found to be: A-BMA, 0.742; B-BMA, 0.751. The partial specific volume of the native protein at this temperature is 0.735. The radius, b, for a sphere representing the albumin molecules can be calculated from the partial specific volume data using the equation

$$b = \left(\frac{M \cdot \bar{v}}{\frac{4}{3}\pi N}\right)^{(1/3)} \qquad (11)$$

where M equals the molecular weight of the hydrated protein,

$\bar{v}$ is the partial specific volume of the hydrated protein molecule, and N is Avrogadro's number. On the assumption that albumin binds about 0.2 gm water per gm protein and that the specific volume of water is 1.00, the calculated radii for BMA, A-BMA, and B-BMA were found to be 30.7Å, 31.1Å, and 31.5Å, respectively.

Extinction Coefficients. The extinction coefficients of the BMA, A-BMA, and B-BMA preparations were measured at 279 mμ. Protein concentrations were determined by dry weight measurements; these determinations were reproducible to 2 parts in 1000. Samples were removed from the stock solutions of known concentration and diluted, by weight, with distilled water. The B-BMA solution was diluted by weight with 0.04M KCl. Each protein solution was prepared in triplicate for optical density measurements in the Zeiss spectrophotometer. The extinction coefficients ($E^{1\%}_{1\ cm}$) at 279 mμ were found to be: BMA, 7.05; A-BMA, 6.84; B-BMA, 6.64. The molar extinction coefficients ($\varepsilon_{279m\mu}$) based on the molecular weight of BMA and the calculated molecular weights of the derivatives were found to be: BMA, 4.58; A-BMA, 4.61; B-BMA, 4.57.

Sedimentation Velocity Analyses for Homogeneity and Sedimentation Coefficients. Sedimentation velocity experiments were performed on native and amidino-albumins. 1.2% protein solutions were dialyzed overnight in pH 4.74 acetate buffer ($\Gamma/2 = 0.05$) and sedimented at 59,780 r.p.m. and 20°C in a double sector cell. The sedimentation coefficients ($s_{20°}$) in acetate buffer for 1.2% monomer solutions of BMA, A-BMA, and B-BMA were

4.2_9S, 4.2_7S, and 4.2_5S, respectively, and 4.5_2S for a 1.2% solution of D-BMA. Ultracentrifuge runs were done on protein solutions before and after the isolation of the monomers. Sedimentation patterns for BMA, A-BMA, and B-BMA, prior to the removal of dimer and higher polymers, revealed less than 10% dimer for each solution. The D-BMA solution contained 18% dimer. Sedimentation patterns for the monomer fractions displayed symmetrical boundaries and a homogeneous peak (see Fig. 1).

Fatty Acid Determinations. Fatty acid determinations were performed on BMA and A-BMA. The fatty acid content of both proteins was 1.8 moles fatty acid bound per mole protein. This result demonstrated that amidination did not change the fatty acid content of albumin.

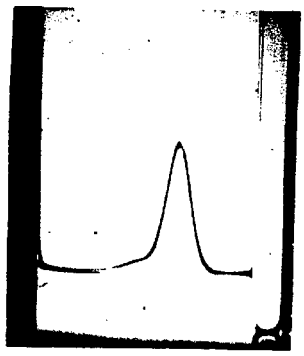
Extent of Amidination of the Amidino-Albumin. The results of the formaldehyde titrations for the extent of amidination of the amidino-albumins are presented in Table II.

TABLE II

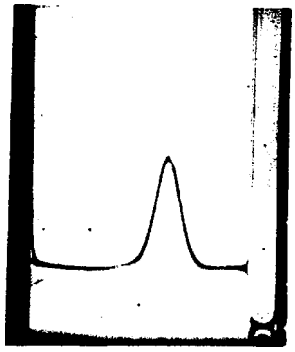
Extent of Amidination

protein	ε-amino groups remaining	% completion of reaction
A-BMA	1	98%
B-BMA	2	96%
D-BMA	36	37%

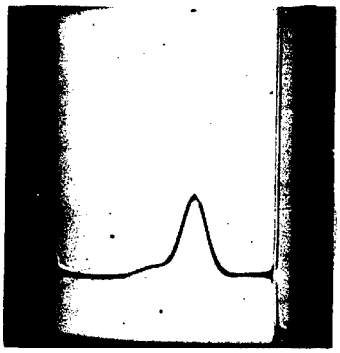
Electrophoresis of Albumins in Phosphate Buffer at pH 7. Control electrophoresis experiments were performed on BMA, A-BMA, and B-BMA in pH 7.0 phosphate buffer ($\Gamma/2 = 0.05$) at 1°C. The resulting protein-solvent boundaries of BMA and A-BMA



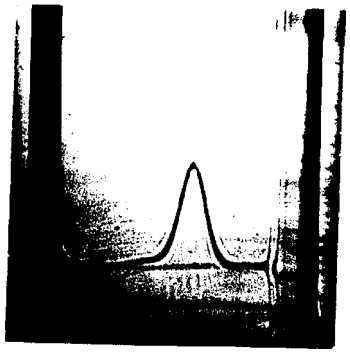
BMA



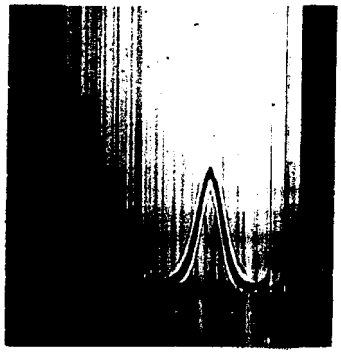
Monomer BMA



A-BMA



Monomer A-BMA



Monomer B-BMA

Figure 1. Ultracentrifuge Sedimentation Patterns in pH 4.74 Acetate Buffer ($\Gamma/2 = 0.05$), 20°C.

were homogeneous with mobilities of 5.7 and 5.5 x 10^{-5} $\frac{cm^2}{volt\ sec}$, respectively. The B-BMA pattern displayed some boundary spreading with a leading shoulder that was approximately 10% of the protein concentration. The mobility of the main portion of the B-BMA boundary was 5.2 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1}. Radius calculations using equation (3) revealed that a change of 1 Å in the effective radius of the protein would result in a difference in mobility of approximately 0.4 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1} under the conditions of the control experiment. This observation is consistent with the differences in radii and mobilities between the native and derivative albumins.

The results of the comparative studies on the native and amidino-albumins are summarized in Table III.

Titration Studies

Titrations with Base. The base titration curves for deionized monomer preparations of BMA, A-BMA, and B-BMA in 0.1M KCl at 25°C, shown in Figure 2, have been corrected for unbound hydrogen ions. These curves are plotted as hydrogen ions dissociated per molecule protein versus pH. On this hydrogen ion scale, the pH at which zero hydrogen ions have been dissociated is the isoionic pH. In the pH range of the titration of imidazole groups (pH 5.4 - 8.5), the native and amidino-albumin curves are superimposable. The divergence of the curves above pH 8.5 is a reflection of the increase in pK on the conversion of the ε-amino groups to amidino groups. Table IV presents a comparison of the base titration data reported by Tanford (9)

TABLE III

Physical Properties of Native and Amidinated Albumins Above the pH Range of the N-F Transformation

	Protein		
	BMA	A-BMA	B-BMA
% Amidination	-	98%	96%
Molecular Weight	65,000	67,430[a]	68,850[a]
$E^{1\%}_{1\ cm}$ (279 mμ)	7.0_5	6.8_4	6.6_4[b]
$\epsilon_{279\ m\mu} \times 10^{-4}$	4.5_8	4.6_1	4.5_7[b]
Electrophoretic Mobility x 10^5 cm^2 $volt^{-1}$ sec^{-1} [c]	5.7	5.5	5.2
$s_{20°} \times 10^{13}$ [d]	4.2_9	4.2_7	4.2_5
Isoionic pH in 0.1M KCl	5.39_0	5.36_0	5.42_0
Chloride Binding ($\Gamma/2$=0.1)	$7._1$	$7._2$	$6._5$
Partial Specific Volume (T=25°)	0.735[e]	0.742	0.751
Fatty Acid Content[f]	1.8	1.8	-

[a] Based on the percent amidination and the molecular weight of the modifier plus the molecular weight of native BMA (65,000).

[b] In 0.04M KCl.

[c] In 0.05 $\Gamma/2$ phosphate buffer, pH 7.0 at 0°C.

[d] In 0.05 $\Gamma/2$ acetate buffer, pH 4.74; 59,780 r.p.m. and 1.2% protein.

[e] Taken from Hunter (72).

[f] moles fatty acid/mole protein.

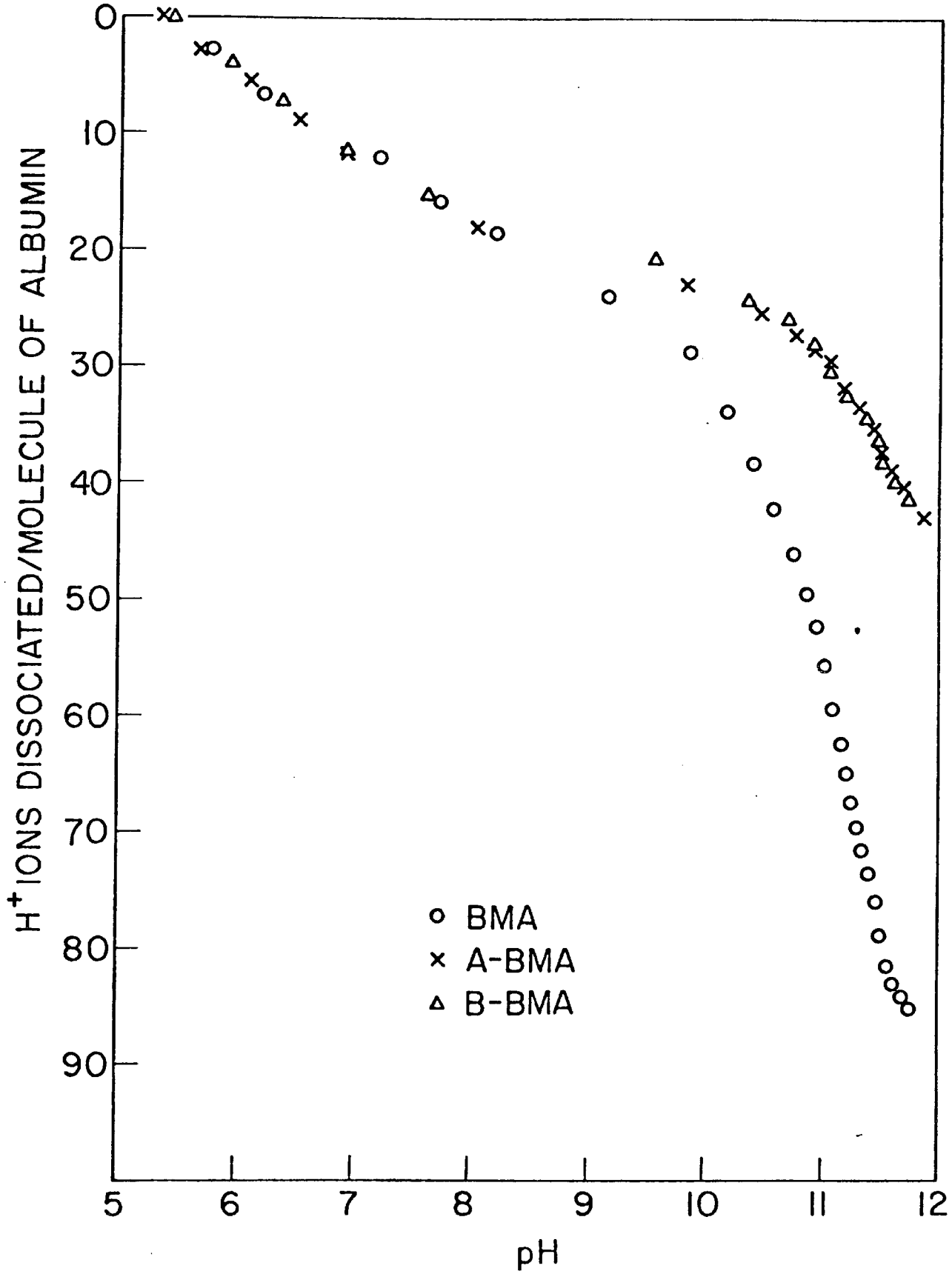


Figure 2. Base Titration Curves (0.1M KCl, 25°C).

for BSA at 0.15 ionic strength with the data from Figure 2 for BMA at 0.10 ionic strength.

TABLE IV

Comparison of Base Titration Data

	Hydrogen ions dissociated/molecule protein	
pH	Tanford (9) - BSA	Figure 2 - BMA
6	5	5
7	10	11
8	18	17
9	24	23
10	32	31
11	54	55
12	89	87

Between pH 5.4 and pH 8.5 it can be assumed that approximately 16 imidazole groups (pK_{int} = 6.9), 5 carboxyl groups, and 1 α-amino group per albumin molecule have lost hydrogen ions. The value of hydrogen ions dissociated per albumin molecule at pH 8.5 from Fig. 2 is in agreement with the sum of 22 for the above groups. Tanford (9) has reported a pK_{int} = 9.8 for the 57 ϵ-amino groups and a pK_{int} = 10.4 for the 19 phenolic groups in BSA. Decker and Foster (86), on the other hand, have reported a normal pK_{int} = 10.7 for the ϵ-amino groups and a pK_{int} = 11.0 for the phenolic groups in BSA as obtained from comparison of electrophoresis and titration studies. Subtraction of the value of 22 groups (i.e., the total number of

carboxyl, imidazole and α-amino groups above the isoionic pH) from the amidino-albumin titration curves at pH 11.5 results in a value of 16 groups. The value of 16 groups is a reasonable value for the phenolic groups dissociated at pH 11.5 assuming the amidino and guanidino groups begin to titrate above this pH. There is no evidence for ε-amino group titration in the amidino-albumins below pH 11.5.

Titrations with Acid. The acid titration curves for deionized monomer preparations of BMA, A-BMA, and B-BMA in 0.1M KCl at 25°C, represented by circles in Figure 3, have been corrected for unbound hydrogen ions. These curves are plotted as H versus pH where H represents the number of hydrogen ions associated per molecule protein. H is zero at the isoionic pH.

Three acid titration curves for any one of the three albumin types (the pH values of the triplicate titrations agreed to within 0.01 pH units) were averaged to give the final titration curve as presented in Figure 3. The reproducibility of the protein titrations in 0.1M KCl was not impaired by the experimental procedure (see Experimental section) of varying the volume of acid addition in separate titration experiments. All titration experiments were performed with the same glass electrode and the same standard acid solution. The pH values in Figure 3 have a maximum of error of ±0.01 pH units from the isoionic pH to pH 3.0. In the region of steepest ascent of the titration curves, this error would be equivalent to an error of ±0.5 in the determination of the number of hydrogen ions dissociated at a given pH.

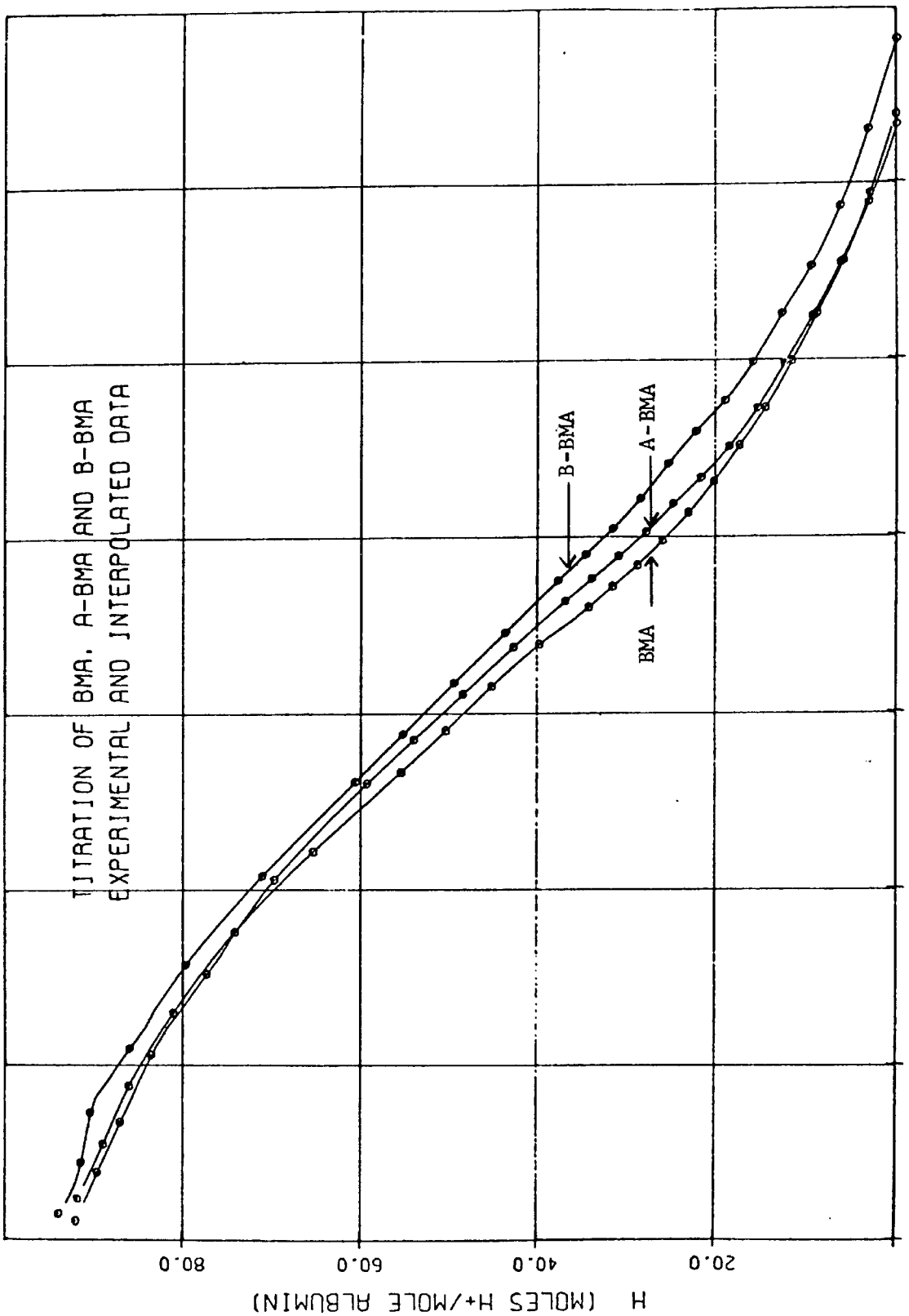

TITRATION OF BMA, A-BMA AND B-BMA
EXPERIMENTAL AND INTERPOLATED DATA
B-BMA
A-BMA
BMA
H (MOLES H+/MOLE ALBUMIN)
80.0
60.0
40.0
20.0
2.7
3.2
3.7
4.2
4.7
5.2
PH

Figure 3

In order to compare the acid titration data for the three different albumins at any given pH, a successive four point linear interpolation (see Appendix) was performed at 0.05 pH intervals on the corrected experimental titration data reported in Table V. The interpolation was carried out on the IBM 7090 computer and plotted by the IBM Cal-comp 763/780 plotter (Fig. 3). The interpolated titration points were connected by line segments and are represented in Figure 3 by the solid curves.

Upon analysis of the acid titration curves the isoionic pH of B-BMA was questioned. In chloride binding studies the affinity of B-BMA for chloride ions at 0.1 ionic strength had been found to be no greater than that of BMA. Therefore, the difference in isoionic pH between BMA and B-BMA was not due to differences in chloride binding. As previously mentioned, the deionized B-BMA monomer preparation was dialyzed against 0.04M KCl at 2°C prior to room temperature dry weight determinations and to acid titration studies. To test if the pH of the B-BMA solution had changed during the lengthy dialysis procedure, a portion of the B-BMA monomer preparation was deionized again and diluted, at 2°C, to a final KCl concentration of 0.1M in the manner in which the BMA and A-BMA had been treated. The isoionic pH of a 5 ml sample of this undialyzed B-BMA preparation was 5.420, 0.175 pH units lower than the value obtained after dialysis (Table V). Comparison of the isoionic pH of this B-BMA preparation with that of BMA and A-BMA, where each preparation had been treated in an identical manner prior to pH

TABLE V

Corrected Acid Titration Data for
BMA, A-BMA and B-BMA in 0.1M KCl at 25°C

BMA		A-BMA		B-BMA	
pH	H	pH	H	pH	H
5.390	0.0	5.360	0.0	5.605	0.0
5.166	2.9	5.138	3.1	5.347	3.2
4.976	5.8	4.970	6.2	5.129	6.3
4.829	8.7	4.819	9.3	4.959	9.5
4.692	11.5	4.684	12.4	4.825	12.7
4.560	14.4	4.558	15.4	4.688	15.9
4.454	17.3	4.449	18.5	4.579	19.0
4.352	20.1	4.361	21.6	4.492	22.2
4.264	22.9	4.287	24.7	4.401	25.3
4.186	25.8	4.208	27.7	4.302	28.4
4.116	28.6	4.139	30.8	4.217	31.5
4.056	31.4	4.076	33.8	4.144	34.6
3.997	34.1	4.013	36.8	4.070	37.7
3.893	39.7	3.883	42.7	3.923	43.7
3.774	45.1	3.750	48.4	3.781	49.5
3.649	50.3	3.621	54.0	3.636	55.3
3.531	55.4	3.497	59.3	3.501	60.7
3.306	65.3	3.224	69.7	3.234	71.1
3.077	74.1	2.955	77.3	2.981	79.7
2.847	81.0	2.725	83.5	2.742	86.0
2.639	86.0	2.534	87.0	2.559	90.4
2.472	88.9	2.390	89.6	2.416	91.5
2.317	91.8	2.251	92.0	2.270	94.0

H = moles H^+/mole protein

measurements, showed differences of only ±0.03 pH units. The acid titration curve of B-BMA was therefore shifted vertically to pass through pH 5.420; this shift was equivalent to 2 moles H^+ per mole B-BMA.

In order to represent the acid titration data solely on the basis of hydrogen ions bound to carboxyl groups, a small correction was made for the titration of imidazole groups below the isoionic pH. Computer reference tables for the dissociation of mono-functional acids described by the Henderson-Hassalbach equation (program 07B, written by Dr. G. Nordby) were used for the correction. In conjunction with the reference tables the value of 17 imidazole groups per albumin molecule (pK_{int} = 6.9) was used to obtain values for the hydrogen ion required to titrate any remaining uncharged imidazole groups. The maximum correction at the isoionic point amounted to less than 1 imidazole group per protein molecule.

Tanford, from titration studies, has reported that BSA contains 100 carboxyl groups, 96 of which exist in the anionic form at an isoionic point of pH 5.5 (9). Tanford has also shown that a plot of pH-log (r/n-r) versus Z was linear between the isoelectric point (Z = 0) and the isoionic point (see eqn. 1). A similar plot for the acid titration data of BMA, A-BMA, and B-BMA proved to be quite sensitive to the number of carboxyl groups (h) assumed to be available for titration at the isoionic pH. Linear plots were obtained for values of h equalling 94.3 ± 0.05, 94.0 ± 0.05, and 94.6 ± 0.05 for BMA, A-BMA, and

B-BMA, respectively. The carboxyl titration for the native and derivative albumins are presented in Figure 4. These curves are plotted as the number of carboxyl groups dissociated per protein molecule versus pH. The data for Figure 4 is presented in Table VI at 0.1 pH intervals.

A plot of pH-log (r/n-r) versus Z, with n = 100, is shown in Figure 5. As can be seen in equation (1), the slope of the curve is equal to -0.868ω. The protein net charge, Z, equals $(h-r-\nu_{cl})$ where h is the number of dissociated carboxyl groups at the isoionic point, r equals the number of dissociated carboxyl groups at any given pH and ν_{cl} is the number of chloride ions bound to the protein at any given pH. In these calculations (91), chloride binding was assumed to increase linearly from $\nu_{cl} = 10$ at pH 5.4 to $\nu_{cl} = 14$ at pH 4.0 and to be constant below pH 4. ω was calculated by equation (1) using the value of pH-log $(r/n-r) = pK_{int}$ at the isoelectric point (Z = 0). A plot of ω versus pH is also presented in Figure 5.

Difference curves obtained by subtracting the modified albumin titration data from that of the unmodified albumin (Table VI) are shown in Figure 6. The resulting number of BMA carboxyl groups dissociated minus the number of A-BMA or B-BMA carboxyl groups dissociated at various pH values were plotted against pH.

Titration of BMA with Acid in 0.02M KCl. The acid titration data for BMA in 0.02M KCl were not reproducible to within 0.01 pH units in the region of the N-F transformation (pH 4.5-3.5) when

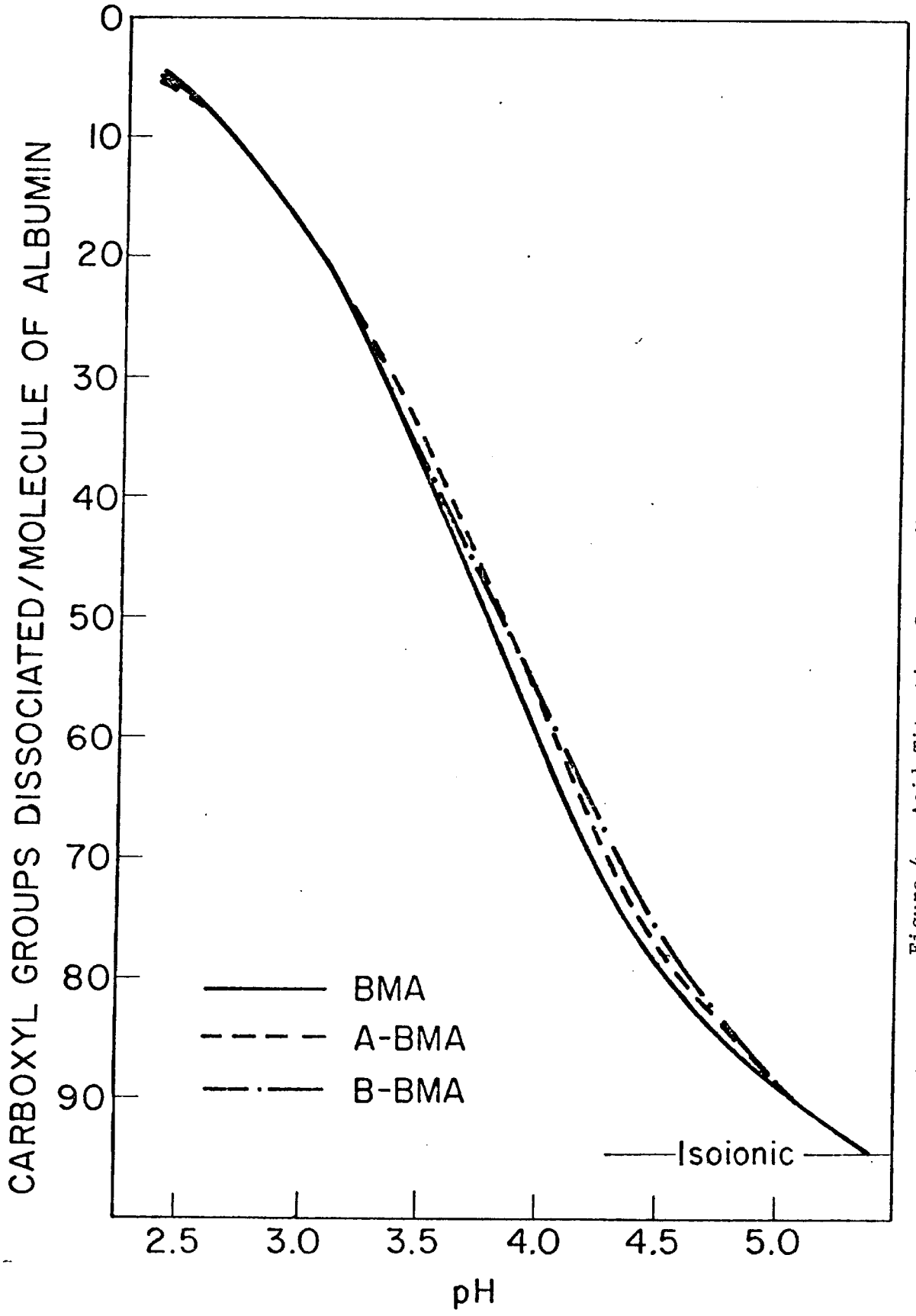


Figure 4. Acid Titration Curves (0.1M KCl, 25°C).

TABLE VI

Dissociation of BMA, A-BMA and B-BMA Carboxyl Groups in 0.1M KCl, 25°C at Various pH Values

	Carboxyl Groups Dissociated		
pH	BMA	A-BMA	B-BMA
5.3	93.2	93.4	93.2
5.2	92.0	92.1	91.9
5.1	90.7	90.5	90.4
5.0	89.1	88.6	88.6
4.9	87.4	86.6	86.4
4.8	85.4	84.5	84.1
4.7	83.4	82.2	81.7
4.6	81.3	80.0	79.1
4.5	78.8	77.4	75.5
4.4	76.0	74.3	72.1
4.3	73.1	70.3	68.9
4.2	69.5	66.5	65.2
4.1	65.5	61.8	60.9
4.0	60.8	57.1	56.8
3.9	55.5	52.5	52.8
3.8	50.8	48.2	48.7
3.7	46.6	43.9	44.6
3.6	42.4	39.6	40.7
3.5	38.0	35.3	36.7
3.4	33.6	31.2	32.7
3.3	29.2	27.5	28.8
3.2	25.2	24.0	25.0
3.1	21.5	21.1	21.5
3.0	18.2	18.5	18.3
2.9	15.2	15.6	15.4
2.8	12.6	12.7	13.1
2.7	10.1	10.5	10.2
2.6	8.1	8.7	7.6
2.5	6.4	6.9	6.6
2.4	4.6	5.1	5.8

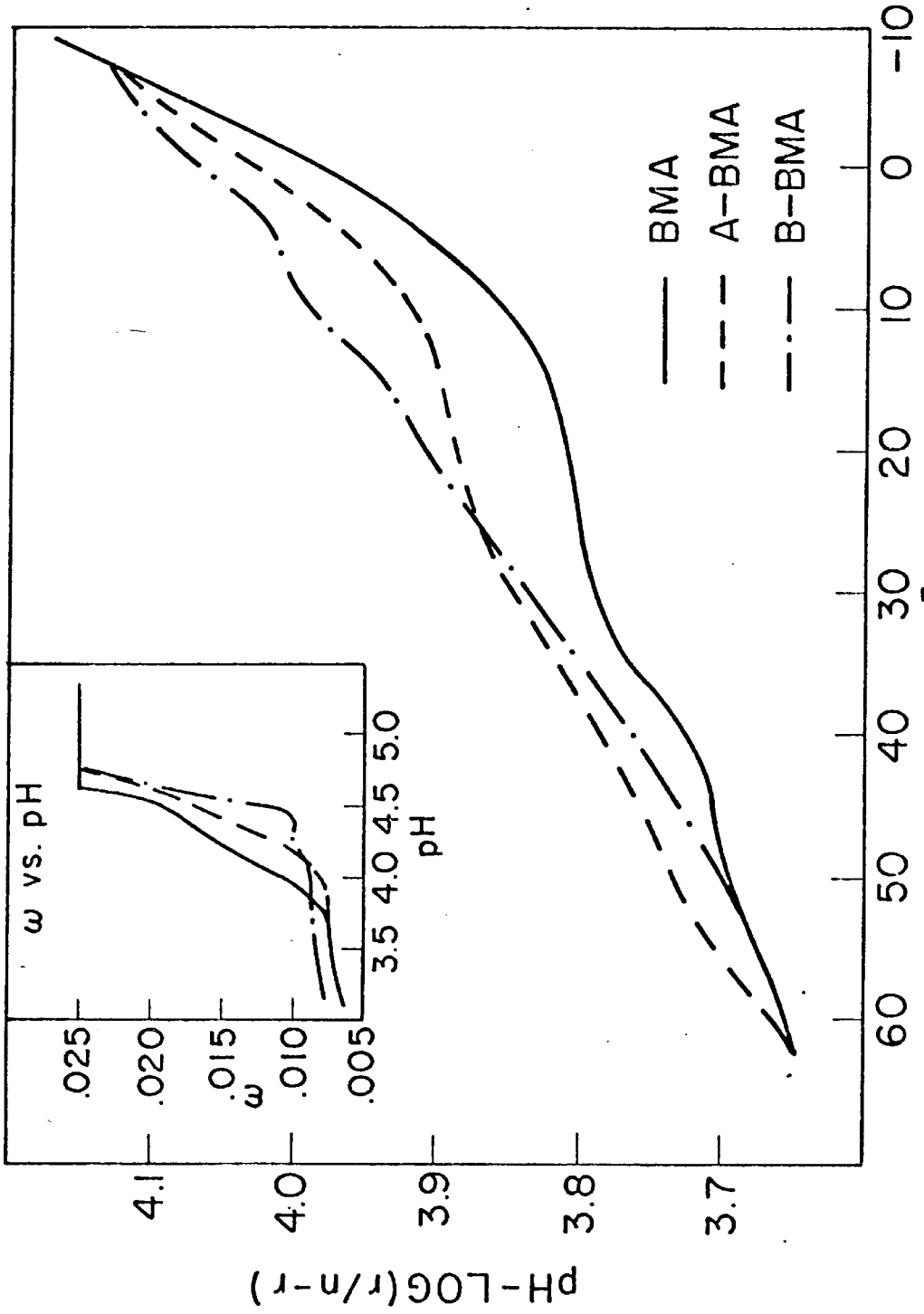


Figure 5. pH-log (r/n-r) vs. Protein Net Charge, z.

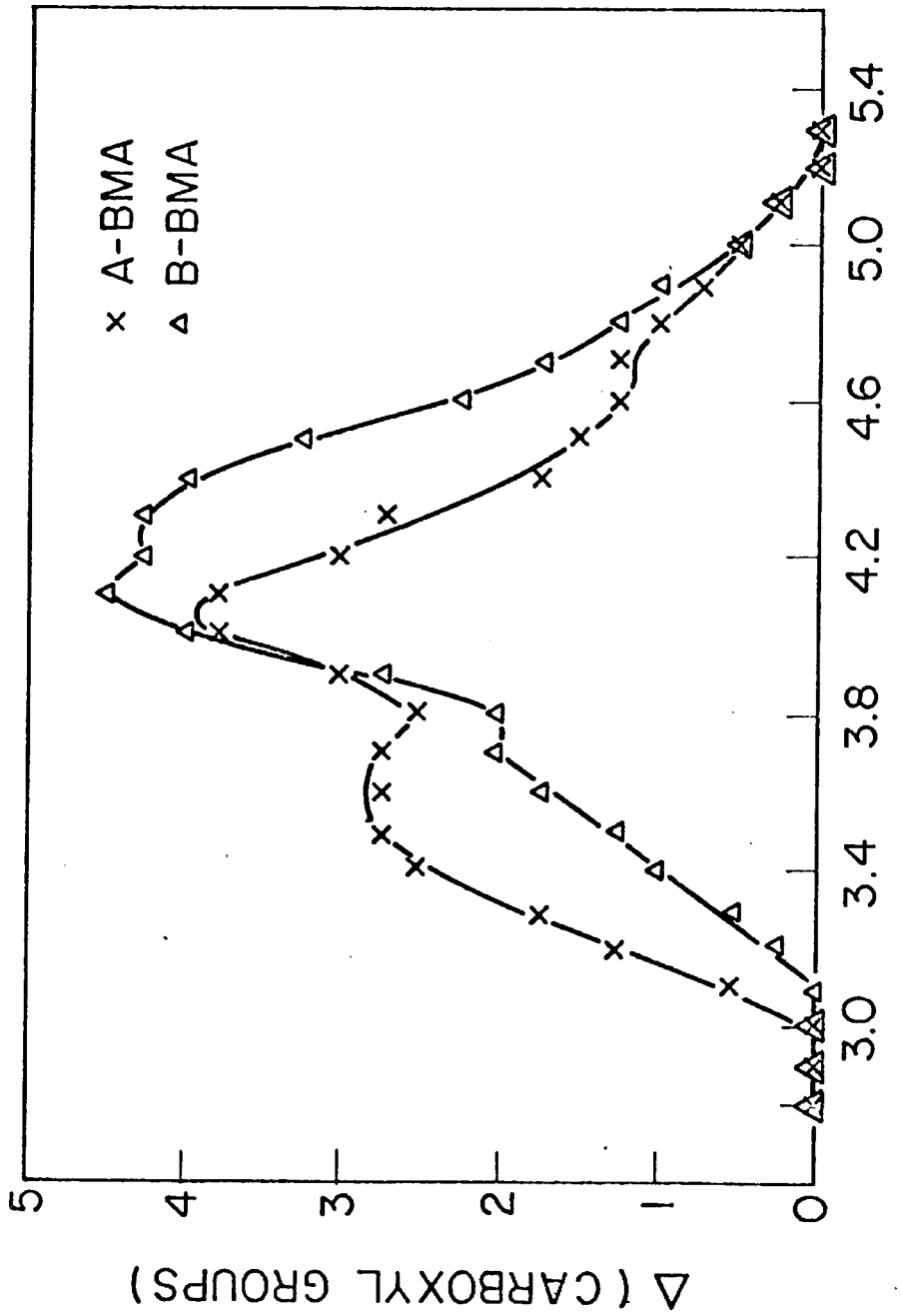


Figure 6. Titration Difference Curves of Modified versus Unmodified Albumin

the rate of addition of HCl to the albumin solution was varied. Uncorrected pH data obtained with four replicate samples of the same albumin solution are shown in Table VII. Each pH reading was taken 10 minutes (stirred, 8 minutes; unstirred, 2 minutes) after the addition of a certain volume of acid. This type of behavior had not been observed in 0.1M KCl and it was thought that the irreproducibility at the lower ionic strength might be due to an inherent instability of the electrode and pH meter. Control experiments were therefore carried out to check the stability of the equipment under these conditions.

5 ml samples of 0.02M KCl were titrated with HCl. Between pH 6 and 3, the pH of the KCl solutions was reproducible to 0.01 pH and the value obtained appeared to be independent of time. The pH of one 0.02M KCl solution was brought to pH 4 by the addition of HCl and the pH measured every 10 minutes for one hour. No significant variation in pH was observed. The time of stirring required to achieve equilibration of the solution was also investigated. The pH of an albumin solution in 0.02M KCl was adjusted to 6.9 by the addition of 0.2M KOH. A relatively large volume of the HCl solution (0.05 ml) was added to the protein solution. The pH of the solution was still well above the pH region of the N-F transformation. After the solution had been stirred for 5 minutes no further change in pH was observed. The 8 minute stirring time which had been employed in the determinations reported in Table VII should therefore have been more than adequate to achieve complete equilibration of the protein sample.

TABLE VII

Acid Titration of Four Replicate Samples of BMA in 0.02M KCl

total ml HCl added	1	2	3	4
0.0	5.322	5.322	5.321	5.322
0.01	5.098			
0.02	4.900		4.897	
0.03	4.750			
0.04	4.599		4.596	4.590
0.05	4.464	4.440	4.464	
0.06	4.353		4.353	
0.07	4.252			
0.08	4.168		4.161	4.143
0.09	4.080			
0.10	4.012	3.983	4.014	
0.12		3.867	3.898	3.879
0.14		3.755	3.792	3.768
0.16		3.667	3.696	3.670
0.18		3.568	3.593	3.568
0.20		3.467	3.494	3.469
0.24		3.288		3.290
0.32		2.952		2.954
0.40		2.623		2.629

pH values taken at 10 min. intervals

1% protein solution (5 ml) in 0.02M KCl

0.195_3M HCl in 0.02M KCl

From these control studies it appeared that the lack of reproducibility observed with the BMA samples in the pH range 4.5-3.5 was not due to instrument instability or to insufficient equilibration of the protein solutions prior to pH measurement.

Various experiments were conducted in which the albumin solution was titrated to pH values within the range of the N-F transformation and the pH of the solution measured as a function of time. One such set of data is given in Table VIII. The pH measurements were taken at 10 minute intervals. By comparison of the data in Table VII and Table VIII it can be seen that, above the pH region of the N-F transformation (>4.5), the pH of the albumin solution was independent of time. When 0.08 ml HCl were added rapidly to the albumin solution (Table VII, run 4; Table VIII) the pH of the solution was 4.143 or 4.144. If this volume of acid were added slowly to the albumin solution (Table VII, runs 1 and 3) or the solution was allowed to stand for some time after the addition of the acid (Table VIII) the pH of the solution was 4.168-4.161. Similar comparisons can be made of the data obtained after the addition of 0.20 ml of acid to the protein solution. The results of all these titration studies may be summarized as follows: Above the pH of the N-F transformation no measurable alteration of pH was observed. Within the pH region of the transformation relatively large increases in pH with time occurred. The magnitude of the drift was related to the magnitude of the volume of acid which had been added. If sufficient acid had been added to the BMA solution

TABLE VIII

Acid Titration of BMA in 0.02M KCl

total ml HCl added	pH	ΔpH
0.0	5.318	0.002
	5.320	0.0
	5.320	0.0
	5.320	
0.08	4.144	0.007
	4.151	0.004
	4.155	0.003
	4.158	0.002
	4.160	0.002
	4.162	
0.20	3.480	0.009
	3.489	0.005
	3.494	0.003
	3.497	0.002
	3.499	0.002
	3.501	
0.32	2.977	0.002
	2.979	0.002
	2.981	

1% BMA solution in 0.02M KCl

0.195_3M HCl in 0.02M KCl

to just enter the pH region of the transformation, relatively small changes in pH with time were observed. If a large volume of acid had been added to the isoionic albumin solution at one time so that the pH of the protein solution was on the acid side of the pH region of the transformation a large increase in pH with time was observed. The rate of change of pH with time decreased to a value of about 0.002 pH units per 10 minutes as the time of equilibration of the sample increased. Below the pH region of the transition a small increase in pH with time was observed, but whether this was due to instrument instability or some slow process involving the protein has not been determined.

From the data obtained in these preliminary studies it was obvious that any investigation of the acid titration behavior of albumin and its derivatives in the presence of 0.02M KCl would be a major undertaking in itself, and hardly pertinent to the scope of the present investigation. Titration studies in 0.02M KCl were therefore not extended beyond this point.

Moving Boundary Electrophoresis Studies

The Rayleigh interference fringe patterns in the protein-solvent boundary regions of the electrophoresis cell were analyzed using a program written for the IBM 7090 computer (see Appendix). The resulting derivative data (i.e., the derivative of the relative protein concentration with respect to distance) were plotted versus distance by the IBM Cal-comp 763/780 plotter. Representative plots for the electrophoresis of BMA at three pH values are shown in Figures 7-10. The sharpest peak in the ascending plots repre-

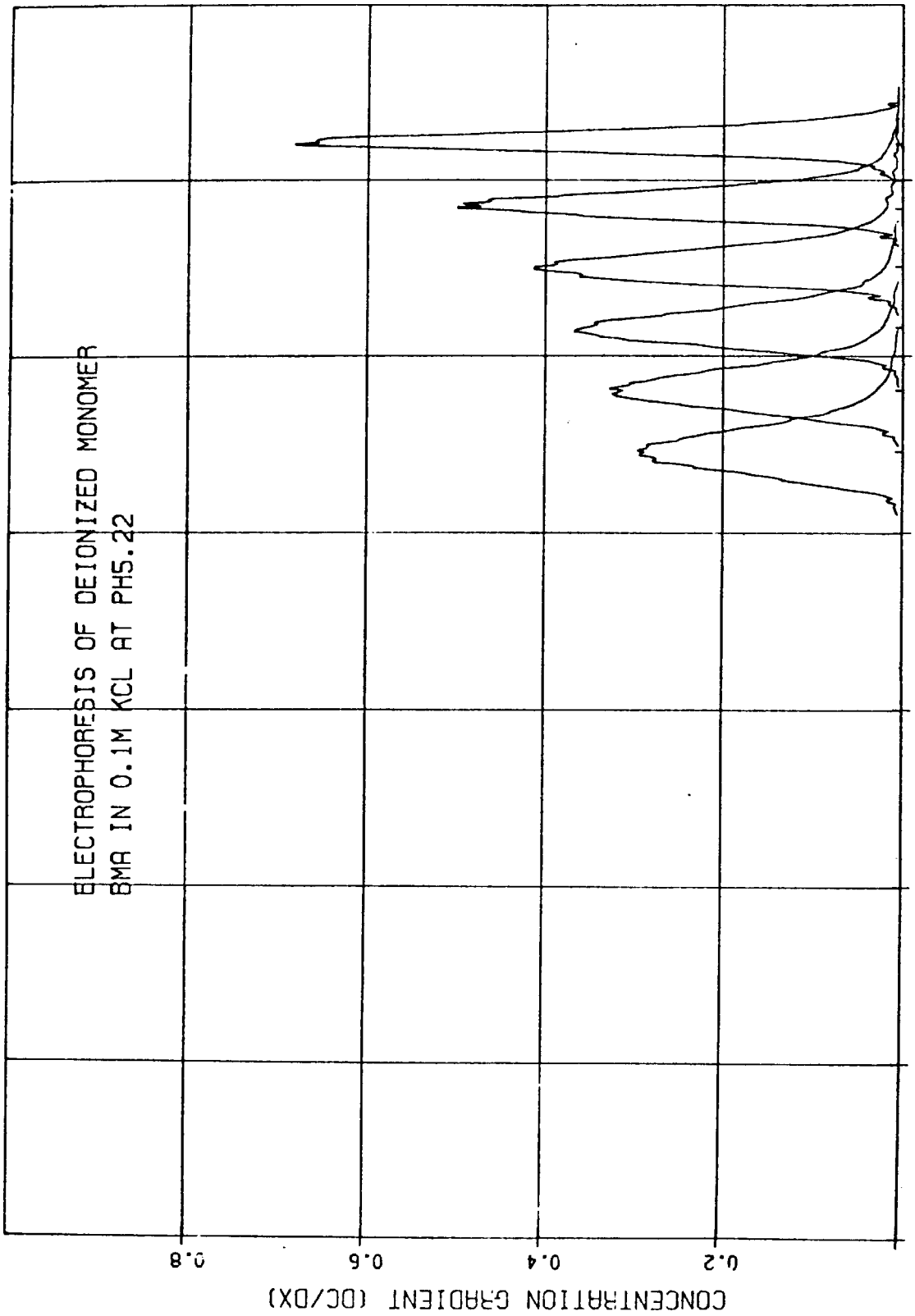

ELECTROPHORESIS OF DEIONIZED MONOMER
BMA IN 0.1M KCL AT PH5.22
CONCENTRATION GRADIENT (DC/DX)
0.2
0.4
0.6
0.8
15.0
25.0
35.0
45.0
55.0
65.0
DISTANCE (X) IN MM. ASCENDING

Figure 7.

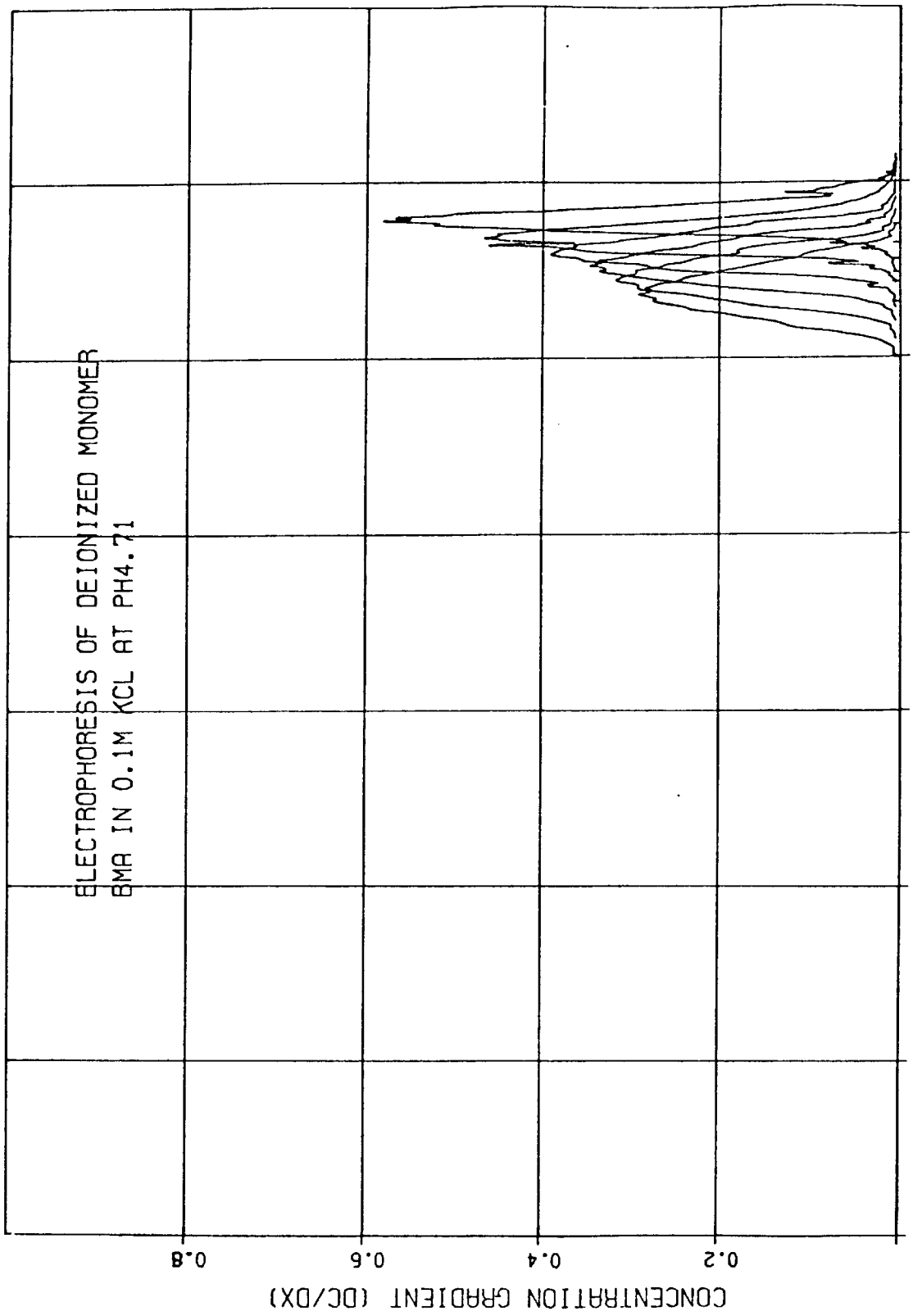

ELECTROPHORESIS OF DEIONIZED MONOMER
BMA IN 0.1M KCL AT PH4.71
CONCENTRATION GRADIENT (DC/DX)
0.8
0.6
0.4
0.2
15.0
25.0
35.0
45.0
55.0
65.0
DISTANCE (X) IN MM. ASCENDING

Figure 8.

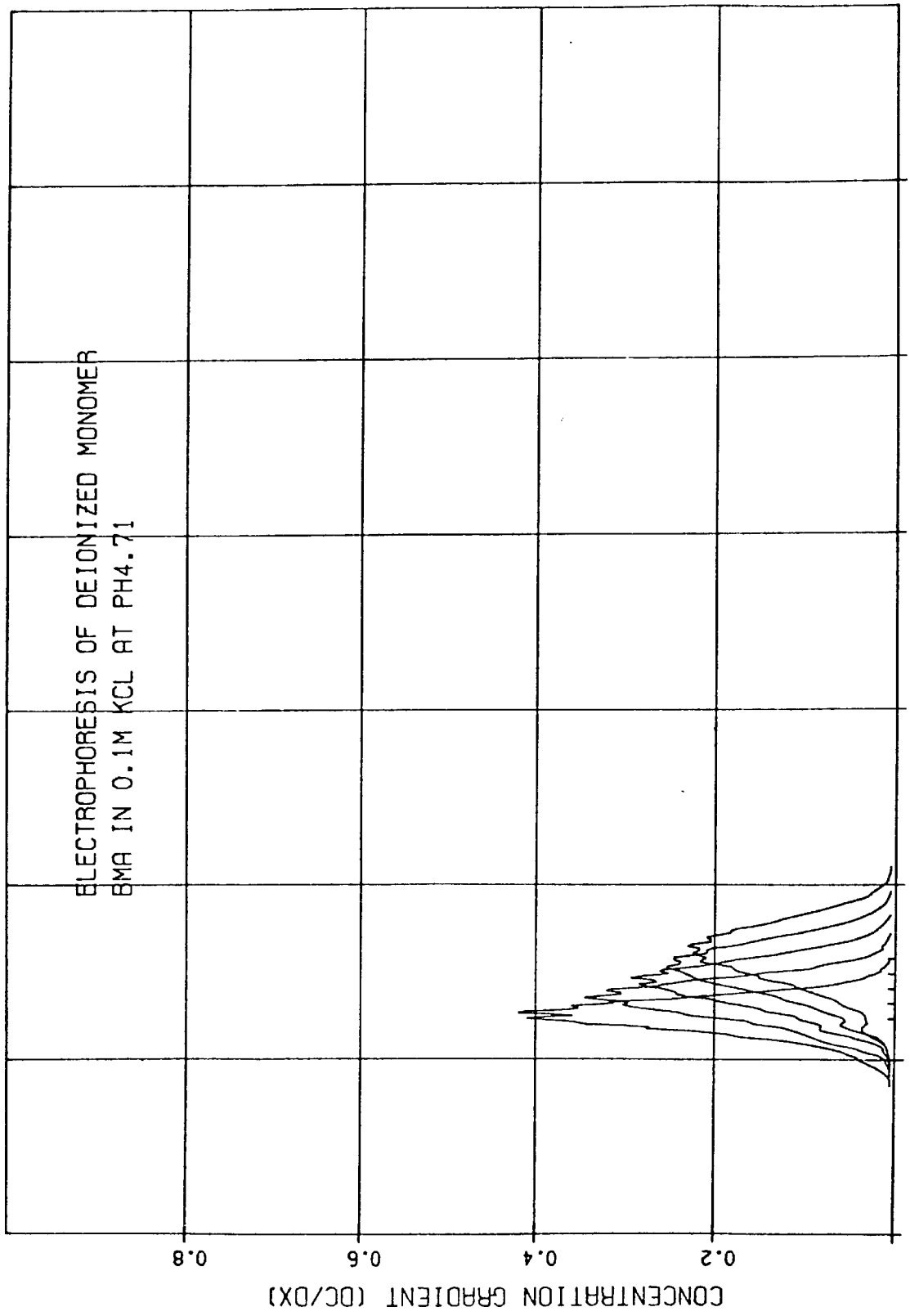

ELECTROPHORESIS OF DEIONIZED MONOMER
BMA IN 0.1M KCL AT PH4.71
CONCENTRATION GRADIENT (DC/DX)
0.2
0.4
0.6
0.8
15.0
25.0
35.0
45.0
55.0
65.0
DISTANCE (X) IN MM. DESCENDING

Figure 9.

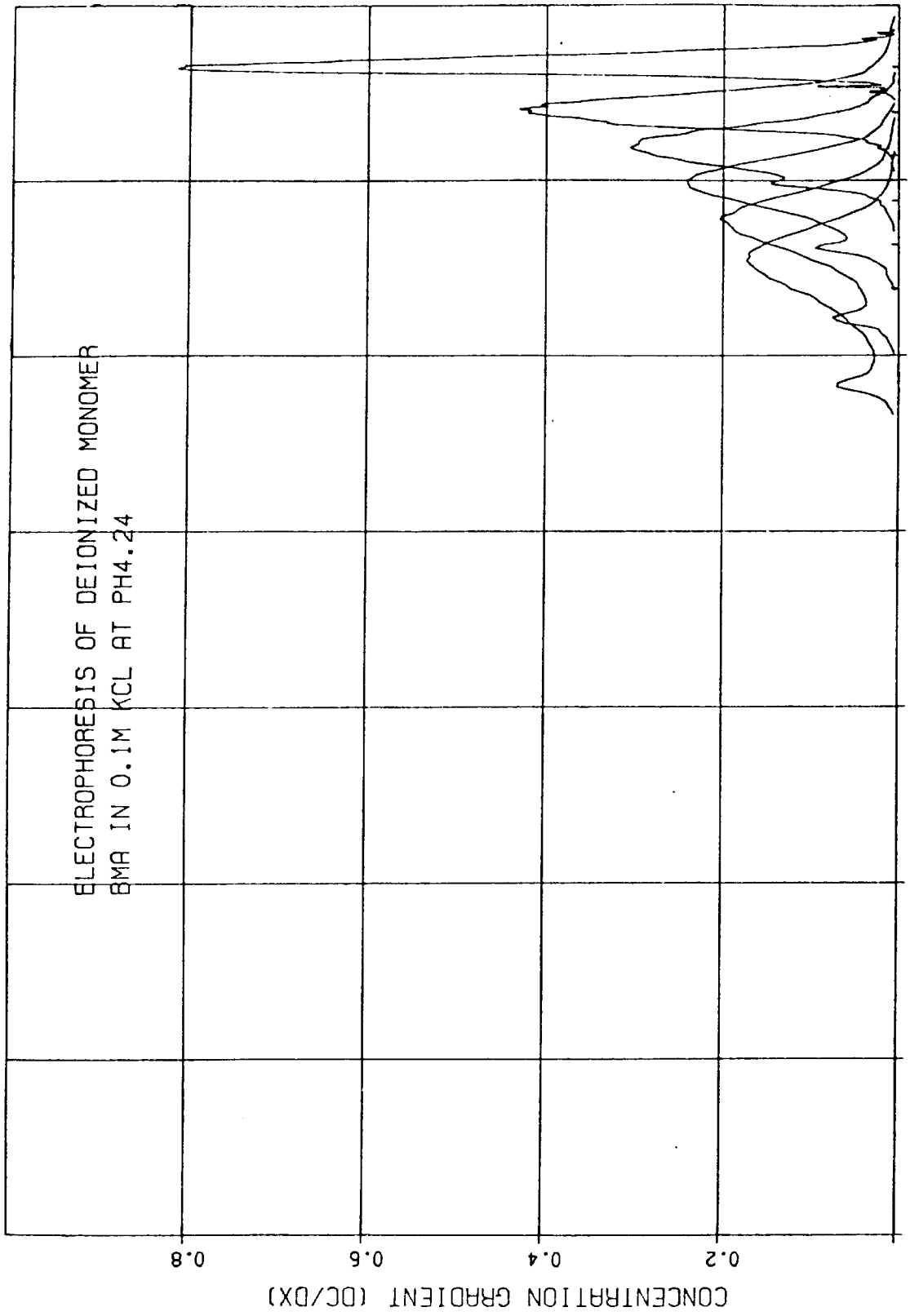

ELECTROPHORESIS OF DEIONIZED MONOMER
8MA IN 0.1M KCL AT PH4.24
CONCENTRATION GRADIENT (DC/DX)
0.2
0.4
0.6
0.8
15.0
25.0
35.0
45.0
55.0
65.0
DISTANCE (X) IN MM. ASCENDING

Figure 10.

sents the initial boundary after compensation, prior to turning on the current. The other patterns were obtained at one hour time intervals after the start of electrophoresis. The initial boundary for the descending channel was always too sharp to read. The patterns obtained from the descending channel, therefore, represent hourly intervals of electrophoresis. The short vertical lines on the abscissa represent the weight-mean positions of the boundary at the specified times.

The electrophoretic patterns of BMA, A-BMA, and B-BMA (ascending channel) at certain pH values are presented in Figure 11. The electrophoretic mobilities of the proteins versus pH are presented in Figure 12. The mobilities were calculated using the equation

$$\mu = [x/t]/[I/A(S.C.)] \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1} \qquad (12)$$

where x is the distance in cm the weight-mean position of the boundary has travelled in t = 3600 seconds, I is the current in amps, A is the cross-sectional area of the electrophoresis cell channel in cm^2 and S.C. is the specific conductivity of the solvent in mhos per cm. The rate of travel per hour of the weight-mean position of the boundary for a specific pH was obtained by comparison of patterns at adjacent time intervals and the five mobilities thus obtained were averaged to give a mean value. The mean mobility values obtained for both the ascending and descending channels were averaged to give a final mean electrophoretic mobility for any given pH value. The mean mobility found for the ascending channel agreed to within 5% of that obtained for the descending channel.

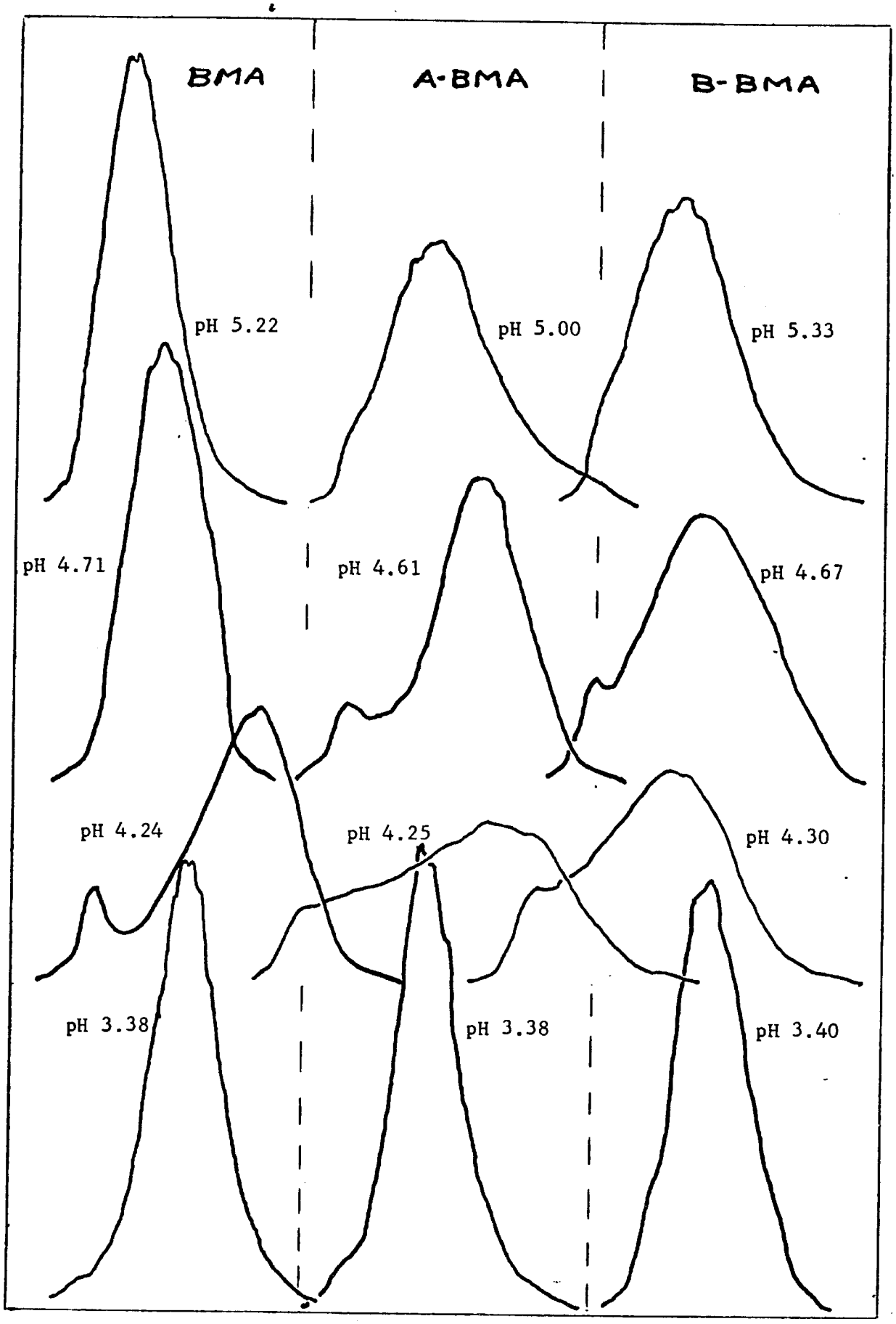


Figure 11. Representative Electrophoretic Patterns of BMA, A-BMA, and B-BMA at Equivalent Times.

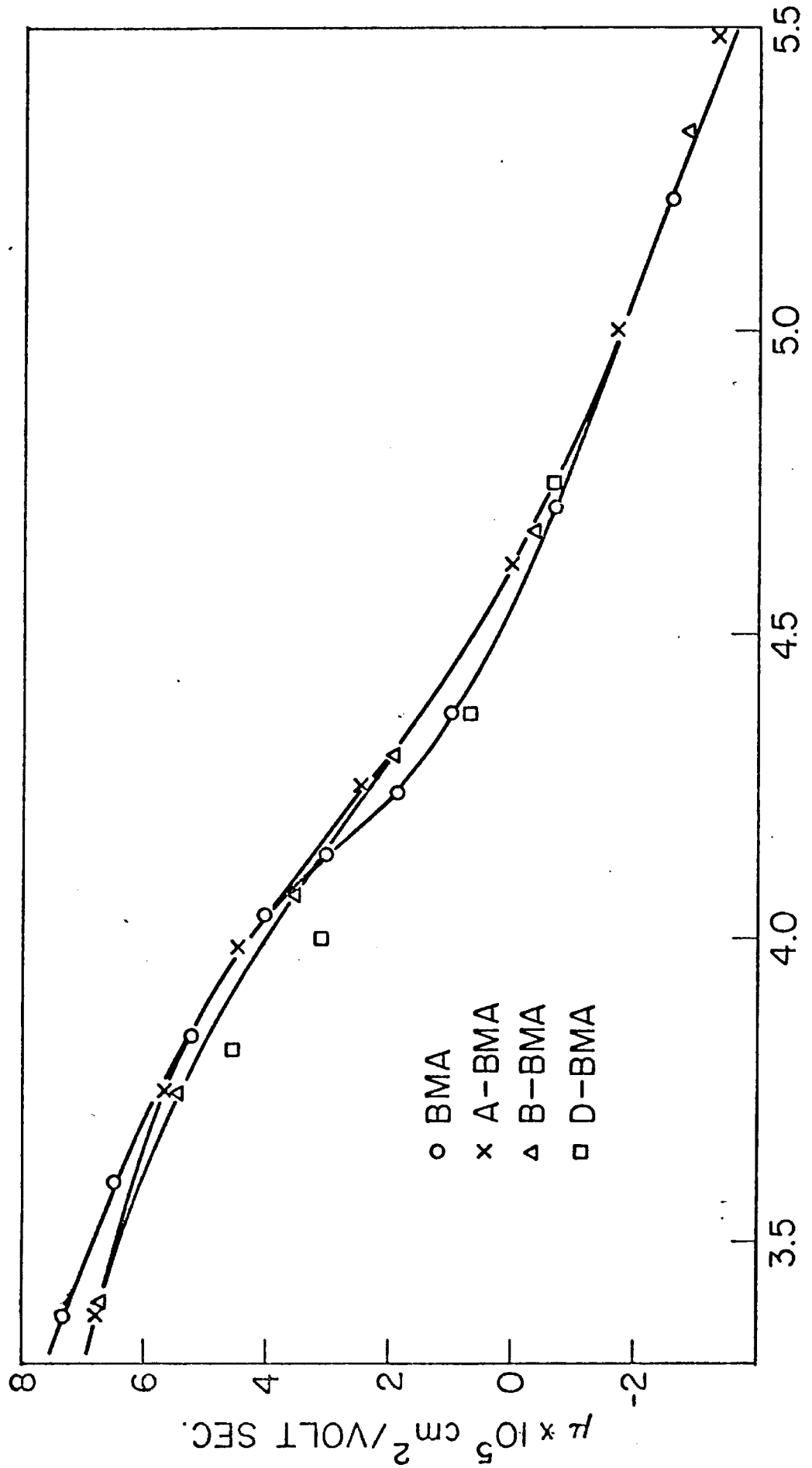


Figure 12. Electrophoretic Mobility versus pH (0.1M KCl, 0°C).

Comparison of Electrophoretic Mobilities and Titration Data

Abramson's rule states that "in solutions of the same ionic strength, the electric mobility of the same protein at different hydrogen ion activities should be directly proportional to the number of hydrogen ions bound" (87). A comparison between electrophoretic mobilities and titration data at 25°C is presented in Figure 13. The mobility data was converted to 25°C by use of the equation

$$\mu_{0°}\ \eta_{0°} = \mu_{25°}\ \eta_{25°} \qquad (13)$$

where μ and η are the protein mobility and solvent viscosity, respectively, at the two temperatures (88). Aoki and Foster (19) have reported that, at a specific pH, the ratio of the mobilities of BSA at 0° and 25° was equal to the reciprocal ratio of the solvent viscosities at the two temperatures.

In order to use Abramson's relationship, a proportionality factor between the mobility data and titration results must be found. This proportionality factor was determined by first plotting the mobility data versus pH. The titration data was then plotted, in relative units, on the mobility-pH graph and vertically adjusted until the two curves crossed at the isoelectric point ($\mu = 0$). A proportionality factor was then obtained by dividing the mobility at a specific pH by the titration value at the same pH. The entire titration curve was then multiplied by this proportionality factor (BMA and A-BMA, 2.10; B-BMA, 1.95) and the titration curve replotted to obtain Figure 13. If the assumptions for Abramson's rule hold, the mobility and titration curves should be superimposeable within experimental limits.

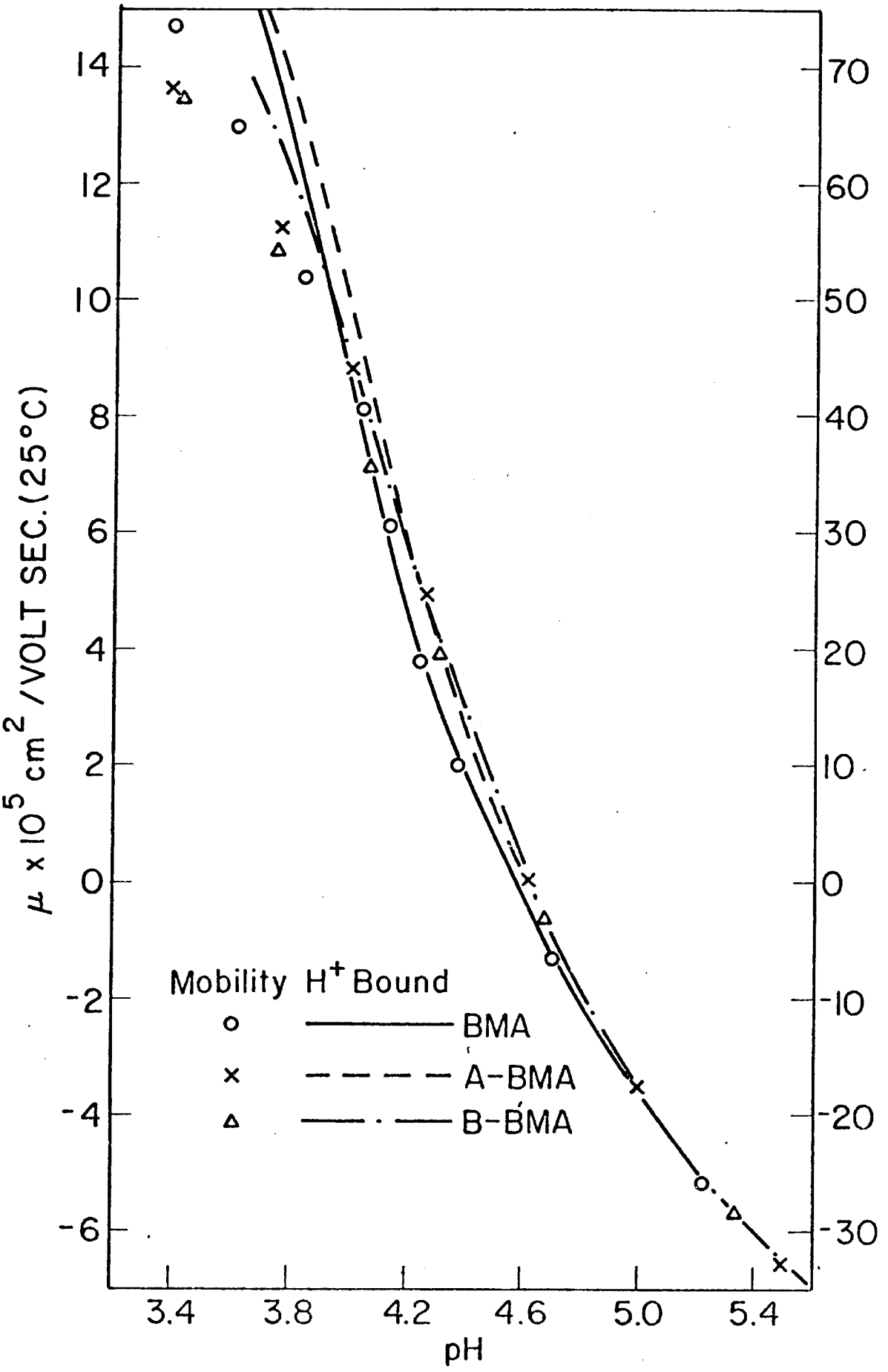


Figure 13. Comparison of Mobilities and Titration Data with pH.

Calculation of Equivalent Carboxyl Sites and Related pK_{int} Values

The electrostatic interaction factor for the Linderstrøm-Lang equation (eqn. 4) may be calculated through the use of electrophoretic mobilities. The term $\epsilon\psi$ /kT is related to the mobility, μ, by the conversion factor $6\pi\eta\epsilon/DkTf(Ka)$ which, under the conditions of this investigation, equals 6.93×10^3 volt sec per cm^2. For the calculation of this conversion factor albumin was considered to be a sphere of 30 Å resulting in a value of f(Ka) equal to 1.10 (89). The calculated values of $\epsilon\psi/kT$ were introduced into equation (6)

$$\frac{\nu}{[H^+]} e^{\epsilon\psi/kT} = \frac{1}{K_{int}} (n - \nu) \qquad (6)$$

where ν equals the total number of carboxyl groups (100) minus the dissociated carboxyl groups as presented in Table VI at the pH values at which the $\epsilon\psi/kT$ values were calculated. ν is thus also the number of hydrogen ions bound to carboxyl groups at any given pH.

As can be seen in equation (6), an extrapolation of a plot of ν vs. $\frac{\nu}{[H^+]} e^{\epsilon\psi/kT}$ will lead to a value of ν which represents the maximum number of carboxyl groups, n, with a pK_{int} calculated from the slope $(\frac{-1}{K_{int}})$ of the extrapolated line. If the graph is not totally linear, then an extrapolation of linear portions of the curve to a zero ordinate value will lead to values of ν which represent subsets of equivalent, non-interacting carboxyl sites. When the data obtained for albumin and the derivatives

(Table IX) were plotted in this manner (Figure 14) the linear portions of the curves at low pH extrapolated to a ν_{max} of about n = 100 with a pK_{int} = 4.2. At pH values above the isoelectric point, the linear region of the BMA curve extrapolated to approximately n = 65 with pK_{int} = 4.2.

Radius Calculations for the Albumins

Radius calculations of BMA, A-BMA, and B-BMA were carried out in the manner of Aoki and Foster (19) from mobility and titration data at low pH values. Henry's equation (eqn. 3) relates Z and μ to the radius of the protein molecule. However, albumin behaves more like an ellipsoid of revolution with an axial ratio of 4/1 than like a sphere (7). Gorin (90) has shown that a radius calculated by equation (3), using the mobility obtained for a molecule with an axial ratio of 4/1, should be multiplied by 0.67 to give the radius of an equivalent sphere. At pH values above the isoelectric point, the radii of equivalent spheres for BMA, A-BMA, and B-BMA were calculated as 30 Å ± 2. In the region of the N-F transformation the radii for the three albumins were calculated to be 32 Å ± 2. Within the pH range of the transformation the radii represent the mean values of the slow and fast components. The radii of the modified albumins began a rapid increase approximately 0.2 pH units higher than the unmodified in the lower portion of the N-F transformation range. The increase in radius for both the native and derivative albumins in this pH region was approximately 2 Å per 0.1 pH unit down to pH 3.4 where a value of 40 Å was obtained for BMA and 44 Å for A-BMA and B-BMA.

TABLE IX

Parameters for the Linderstrøm-Lang Equation

protein	pH	μ	$\epsilon\Psi/kT$	ν	$\frac{\nu e^{\epsilon\Psi/kT}}{[H^+]} \times 10^{-4}$
BMA	5.22	-5.2	-0.36	7.7	89.5
	4.71	-1.3	-0.09	16.4	76.5
	4.37	+2.0	+0.14	24.8	66.9
	4.24	3.8	0.26	29.0	65.6
	4.14	6.1	0.42	32.8	68.8
	4.04	8.1	0.56	37.4	71.7
	3.84	10.4	0.72	47.4	67.0
	3.60	13.0	0.90	57.6	56.2
	3.38	14.7	1.02	67.4	44.8
A-BMA	5.49	-6.6	-0.46	4.8	93.3
	5.00	-3.6	-0.25	11.3	88.1
	4.61	0	0	19.8	80.5
	4.25	+4.9	+0.34	31.6	78.6
	3.99	8.8	0.61	43.4	77.5
	3.75	11.2	0.78	53.9	66.0
	3.38	13.6	0.94	69.6	42.7
B-BMA	5.33	-5.7	-0.40	6.4	91.6
	4.67	-0.7	-0.05	19.0	84.3
	4.30	+3.9	+0.27	31.1	81.2
	4.07	7.1	0.49	40.3	77.1
	3.74	10.8	0.75	53.8	62.7
	3.40	13.5	0.94	67.3	43.1

$T = 25°$; $\Gamma/2 = 0.1$; $\mu = \mu \times 10^{+5}$ cm^2 volt^{-1} sec^{-1}

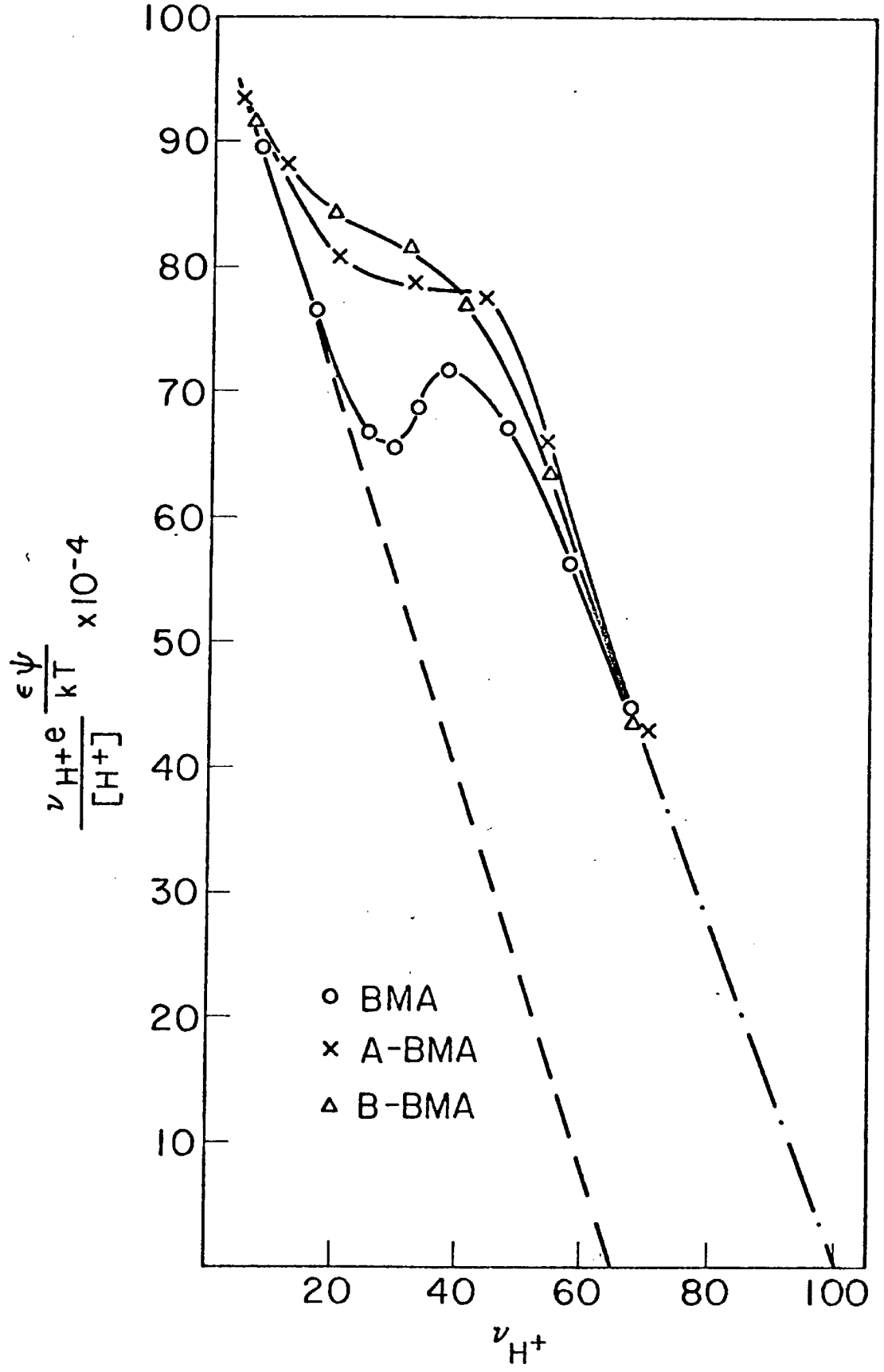


Figure 14. Evaluation of Equivalent Carboxyl Groups.

DISCUSSION

The Modified and Unmodified Albumins in the N-Form

A necessary prerequisite for this investigation was the preparation of completely-modified amidino-albumins which had not been denatured by the modification procedure. Several comparative studies on the modified and unmodified albumins were conducted to examine the physical properties of the proteins near the isoionic pH. Any gross differences observed in these comparative studies would complicate comparison of the proteins at low pH values. Formaldehyde titrations revealed that over 95% of the BMA ε-amino groups had been converted to amidino groups upon amidination; therefore, little heterogeneity was introduced due to incomplete modification of the proteins. The molar extinction coefficients of the albumin and derivatives differed by less than 1%. This finding showed that the modified albumins had not undergone structural changes which had altered the ultraviolet absorbancy of certain chromophores. Wofsy and Singer (67) found that exhaustive amidination (over 95%) increased the extinction coefficient of A-BSA over that of BSA. The modification procedure adopted for this investigation was milder than the amidination procedure used by Wofsy and Singer. The A-BMA preparation used in this investigation displayed a homogeneous electrophoretic pattern in pH 7.0 phosphate buffer ($\Gamma/2 = 0.05$).

The affinity of the amidino-albumins for chloride ions at isoionic pH values was the same as that of the native albumin.

This observation led to the assumption that the chloride binding behavior of the three albumin types was the same at low pH values. This assumption was reasonable in light of the finding that the major contributing sites for the binding of chloride ions were the arginyl residues of BSA (91). The importance of deionizing the derivative proteins was emphasized in the chloride binding studies. The pH of the non-deionized modified albumins were slightly above 6 after thorough dialysis against distilled water. This initial pH value was essentially unchanged by the addition of solutions of varying chloride ion concentration to the derivative protein solutions. It appeared likely that the sites of anion binding in the non-deionized amidino-albumins were occupied with by-products of the modification reaction. After deionization, the derivative albumins behaved in the same manner as the BMA preparation with respect to chloride binding and had isoionic pH values which differed from that of BMA by only ±0.03 pH units. On the basis of the results of the comparative studies (Table III) it appeared that essentially complete amidination of the ε-amino groups of BMA had not altered the gross physical properties of the N form of the molecule.

Possible Effects of Amidination on the Titration Behavior of Albumin

The titration of the carboxyl groups of albumin is abnormal and much, at least, of this abnormality does not appear to be due to a gross change in the size or shape of the molecule. From analysis of the titration data it would appear that many of the albumin carboxyl groups are present under conditions in

which the ionized form of the carboxyl group is stabilized (the N form of albumin). It has been suggested that these "abnormal" carboxyl groups are present in a crevice (or crevices) of the molecule (24) and are stabilized in the ionized form by interaction with the other amino acid residues which are present in the crevice. The stability of this crevice-containing form of the molecule could be due to hydrophobic (Van der Waals), electrostatic, or hydrogen-bond interactions or to all three types of interactions.

By the addition of small hydrophobic groups to the ε-amino groups of the lysyl residues of albumin it was hoped that it might be possible to obtain information on which type of interaction predominated in the maintenance of the N form of the molecule. If the ε-amino groups were in no way involved with the situation which obtained in the crevice, then the behavior of the albumin derivatives should not differ from that of the native protein. If the interaction was mainly hydrophobic and ε-amino groups were present in the crevice, then the presence of additional hydrophobic groups in the crevice in the case of the derivatives might lead to greater stabilization of the N form of the molecule, although there is the possibility that steric requirements might overwhelm any extra interaction energy which would result from the presence of the added aliphatic residues. If charge-charge interactions involving the ε-amino groups of the lysyl residues were of major importance in maintaining the N form of albumin then the added aliphatic residues might interfere with the charge-

charge interactions and lower the stability of the N form of albumin.

Since the stabilized form of the carboxyl groups is the ionized one it is tempting to conclude that the type of interaction which predominates in the crevice is hydrophilic in nature and that electrostatic interactions are mainly responsible for the stabilization of the N form of albumin.

The following discussion will attempt to interpret the experimental data in terms of the type of interaction which predominates in the crevice of the N form of the albumin molecule.

Titrations with Acid

Analysis of the Titration Data using the Linderstrøm-Lang Equation. The customary analysis of titration data is based on the application of the Linderstrøm-Lang equation (eqn. 1). Albumin is the classic example of a protein which does not lend itself to a Linderstrøm-Lang interpretation at low pH values. The titration behavior of BSA was extensively studied by Tanford (9) at several ionic strengths. An interpolation of Tanford's data to 0.10 ionic strength from BSA carboxyl group titration data at 0.08 and 0.15 ionic strength gave empirical values of pK_{int} = 3.98 and ω = 0.023 in comparison with a calculated value of ω = 0.025 (eqn. 2) for a spherical molecule with a radius equal to 33 Å. The calculated value of the electrostatic factor for a protein of 30 Å radius at 0.10 ionic strength was ω = 0.033. In order to use the Linderstrøm-Lang equation it was necessary to know the albumin low pH chloride binding behavior for the

calculation of the net charge, Z, of BMA and the derivative albumins. Chloride binding studies (62, 91, 92) have shown that approximately 10 chloride ions per albumin molecule were bound when the isoionic salt-free protein was dissolved in 0.1M KCl. Other chloride binding data (91) have suggested that the chloride binding increased to a maximum value of about 14 chloride ions bound at pH 4.0 and remained constant at this value as the pH was further lowered. In light of more precise information it has been assumed that the number of chloride ions bound varied linearly with pH in the pH range 5.4 - 4.0. Using the above assumptions as to chloride binding in the calculation of Z, the titration behavior of the 100 carboxyl groups of BMA resulted in values of pK_{int} = 3.98 and ω = 0.025 at 0.10 ionic strength when data from the linear portion of the graph (Fig. 5) above pH 4.6 was used to calculate pK_{int} and ω. These values were in good agreement with those obtained from the BSA titration data of Tanford (9) (see previous paragraph). If chloride binding was assumed to be constant and have a value of 10 chloride ions bound to the protein at all low pH values then a pK_{int} of 4.01 and a ω of 0.018 was obtained for the BMA preparation. This $pK_{int} \doteq 4.0$ for the carboxyl groups of albumin is abnormally low when compared to the pK_{int} of carboxyl groups of other proteins (pK_{int} = 4.3-4.7).

The major assumptions that were made in the calculation of the electrostatic factor, ω (eqn. 1), were: a) that n equalled 100 equivalent, non-interacting carboxyl groups on the surface of an impenetrable, spherical protein, and b) that the intrinsic

pK of these carboxyl groups was constant throughout the acid pH range. Foster (24) has suggested that a relatively large number of these 100 carboxyl groups are stabilized against protonation above the pH region of the N-F transformation. Therefore, in the linear region of the BMA curve for negative values of Z (Fig. 5), Foster suggested that the value of n should be considerably less than 100. The use of lower values of n, ω remaining constant, will increase the value of pK_{int}. Also, in the pH range within which albumin undergoes conformational changes, the assumption that pK_{int} should remain constant did not appear to be valid. With these objections in mind, the electrostatic factor was calculated (assuming pK_{int} and n to be constant) as a function of pH for the three albumin preparations (Fig. 5). Above the isoelectric point (Z=0), ω was constant for BMA. The ω versus pH curves revealed that changes in the electrostatic factor of the amidinated proteins began at higher pH values than for BMA and that changes in the electrostatic factor of BMA began at approximately the beginning of the N-F transformation range. While qualitatively these data could be interpreted as being a reflection of a facilitated formation of the F form of the protein in the case of the amidinated albumins, rigorous interpretation of the data was impossible since resolution of the differences in terms of n, ω, and pK_{int} (all of which could be varying to a different extent) was not possible.

Titration Difference Curves. The carboxyl titration difference curves (Fig. 6) between the titration data of the modified albumins and the unmodified albumin appeared bimodal in shape

The maximum differences occurred in the pH range of the N-F transformation with the B-BMA curve shifted to slightly higher pH values than the A-BMA curve. The smaller peak in the A-BMA difference curve around pH 3.5 was in the pH range of the rapid expansion of albumin (see later).

Electrophoresis at Acid pH

In order to interpret the difference curves and the data obtained in Figure 6 in a more meaningful manner, moving boundary electrophoresis experiments were performed on the three albumin preparations in the pH range in which differences in the carboxyl group titration behavior of these albumin preparations had been observed. Electrophoresis experiments on the native and derivative albumins were conducted at low protein concentrations (0.2%) with the protein-solvent boundaries represented by fringes through the use of Rayleigh interference optics. Low protein concentrations were employed in order to minimize conductivity changes and pH gradients across the boundary. A Rayleigh interference optical system was chosen over a schlieren optical system even though the analysis of fringe patterns was far more tedious and complicated than that of schlieren patterns. Accuracy is increased by the use of interference optics because a minimum in optical density between fringes can be located more reproducibly than the edge of the shadow produced in schlieren optics. The fringe patterns also lend themselves to computer calculations of the weight-mean position of the protein-solvent boundaries. One indication of the reproducibility of the electrophoresis procedure used in this investigation was the fact that the electrophoretic

patterns of BMA at equivalent times were superimposeable at pH values above and below the range of the N-F transformation. The patterns observed for A-BMA and B-BMA at pH 3.4 were superimposeable on the BMA patterns at equivalent time intervals. At pH values removed from the isoelectric point, the ascending and descending anomalies at the position of the initial boundaries were not included in the calculations for the weight-mean positions of the protein-solvent boundaries. Near the isoelectric point where the protein-solvent boundary was not substantially removed from the origin, the anomaly had to be included in the calculations. A representation of the anomaly is shown in Figure 9 for BMA at pH 4.71 in the descending channel of the cell. It is evident from this figure that the anomaly (the small peak on the left in each hourly pattern) had not moved during the course of the five hour experiment.

Electrophoresis in the Region of the N-F Transformation. On the basis of the curves presented in Figure 11 and the graph of mobility versus pH presented in Figure 12 it was obvious that the modified albumins had a larger portion of their molecules in the F form at a specific pH within the N-F transformation region than did the native albumin. The derivative albumins also appeared to enter into the N-F transformation at a higher pH value than did the BMA preparation. Comparison of these electrophoresis findings with the carboxyl group titration difference curves (Fig. 6) demonstrated that the regions of maximal carboxyl group titration differences ($\sim$ pH 4.2) were related to the differences

in pH at which the modified and native albumins underwent the N-F conversion.

Expansion of the Albumins at Low pH. The fact that below pH 4 the mean electrophoretic mobilities of the derivatives were less than those of the native albumin was hard to reconcile with the titration data since the net charge of the derivatives was greater than or equal to that of BMA at these pH values. A comparison of the titration data of each albumin preparation with the electrophoretic mobility of the albumin preparations at various acid pH values (Fig. 13) demonstrated that Abramson's rule (87) did hold above approximately pH 4. This coincidence provided assurance that: a) the calculated weight-mean mobilities of the homogeneous and heterogeneous electrophoretic patterns accurately reflected the extent of titration of the protein carboxyl groups at a specific pH value and b) that in this pH range no gross change in size or shape had occurred. One of the assumptions on which Abramson's rule is based is that the radius of the molecule does not change in the pH region under consideration. Divergence of the mobility data from the titration data (Fig. 13) did occur at approximately pH 3.9 for BMA and approximately pH 4.1 for A-BMA and B-BMA. In order to test whether this divergence could be due to expansion of the molecule, the radii of the proteins were calculated from the low pH electrophoretic mobility data using Henry's equation (eqn. 3). On the assumption that the number of chloride ions bound to the derivative and native albumins was constant and had a value of 14 below approximately

pH 4.2 (91), the net charge of the albumins was calculated from titration data at the pH values at which the mobilities had been obtained. These calculations showed that the mobility data was compatible with a rapid increase in the radius of BMA below approximately pH 3.9, whereas the radii of A-BMA and B-BMA appeared to increase with a rate paralleling that of BMA below approximately pH 4.1-4.2. These radius calculations were consistent with viscosity and optical rotation data (14) and with other electrophoresis investigations (19). On the basis of these results the smaller peak at lower pH in the titration difference curves (Fig. 6) could be interpreted as a reflection of the increased expansion of the amidino-albumins in this pH region. The fact that titration differences were observed in the region of molecular expansion could be attributed to either or both of the following explanations. Firstly, an increase in the radius of the molecules would decrease the electrostatic interaction factor, ω, and hence increase the propensity of the remaining anionic carboxyl groups to undergo titration. At a specific pH value in the region of expansion, the derivative albumins have larger radii than does the native albumin, thus increasing the probability of increased carboxyl group titration. Secondly, upon changes in the radius of the albumin molecules, a small number of previously stabilized ionized carboxyl groups could become available for titration. The increased expansion of the derivatives relative to the native protein would permit titration of these groups at higher pH values in the case of the amidino-albumins.

Electrophoresis of D-BMA. The electrophoretic mobilities of D-BMA in the region of the N-F transformation were considerably slower than those of the other three albumin preparations (Fig. 12). This, in part, could be due to the larger size of this protein derivative. Heterogeneity was not observed in the electrophoretic patterns of D-BMA even at the lowest pH value (pH 3.84) and, unlike BMA and the other two derivatives the mobility of D-BMA varied linearly with pH. It appeared as if the N form of the protein had been stabilized by the addition of long hydrocarbon chains to the albumin molecule.

It has been shown (93) that the N-F transformation of BSA complexed with 12 sodium dodecyl sulfate (SDS) ions was shifted by about 0.3 pH units to lower pH. Markus et al. (94) have suggested that SDS anions were binding with the positively charged ε-amino groups of BSA and that the hydrophobic tails of the SDS anions were interacting with hydrophobic regions on the protein, thus inhibiting conformational changes of the molecule. This cross-linking mechanism could be an explanation of the behavior of D-BMA at low pH values. It is interesting to note that the solubility of the D-BMA molecule decreased greatly after about 21 of the 57 ε-amino groups had been modified. Goldfarb (95) found that only about 19 ε-amino groups of HSA reacted with trinitrobenzenesulfonic acid. It has been speculated (63) that the unreactive ε-amino groups (~ 37) of albumin are involved in electrostatic interaction with an equal number of carboxyl groups in crevices of the protein. The data on D-BMA,

such as it is, would support this hypothesis. The ε-amino groups on the surface of the molecule could react freely with the reagent; the reaction of the ε-amino groups with these long-chain imidoesters would only occur with difficulty and would cause a considerable change in the gross structure of the protein. This change in structure would be paralleled by a loss of solubility.

Analysis of Equivalent Carboxyl Sites in Albumin. Electrophoretic mobilities were used to calculate the electrostatic interaction parameter in the Linderstrøm-Lang equation (eqn. 4) written in terms of the surface potential of the molecule. Although not enough data points were available for a definitive analysis of this nature (Fig. 14), the qualitative results were in close agreement with values obtained in other investigations (59, 96). The analysis of the native albumin data revealed approximately 65 equivalent, non-interacting carboxyl groups with $pK_{int} = 4.2$ above the region of the N-F transformation. Below the N-F transformation pH region the data from the three albumin preparations fell on or near a line which extrapolated to about 100 carboxyl groups, the total number present in albumin with $pK_{int} = 4.2$. The explanation given by Foster and Clark (59) for these findings was that below the N-F transformation range all 100 carboxyl groups titrate as equivalent, non-interacting sites. Above the pH range of the transformation the protein titrates as a molecule containing about 65 equivalent carboxyl groups, supposedly on the surface of the molecule, with approximately 35 carboxyl groups stabilized against protonation presumably in

crevices of the molecule. In the region of the N-F transformation, Foster and Clark (59) maintain that the data points have no physical significance because the value of n changes throughout this region. Use of the electrophoretic data to determine the electrostatic parameter enables this type of analysis to be made even though the size and shape of the molecule may be varying in the pH region under consideration.

The Relationship of the Electrophoretic and Titration Data to the Structure of the Albumin Molecule

The data obtained on the albumin derivatives above pH 5 would indicate that gross physical changes in the structure of the molecule had not occurred on modification and that the N form of the derivatives had much the same 3-dimensional structure as the native protein.

The difference in the electrophoretic and titration behavior of albumin and its derivatives in the pH region of the N-F transformation are small but real and the electrophoretic and titration data are mutually compatible without recourse to tenuous extrapolation of electrolyte theory.

The differences in behavior between the native protein and the derivatives at acid pH values are great enough that it would be difficult to explain the data were all the ε-amino groups of albumin completely uninvolved in the N-F transformation of the protein. Any explanation of the data must take into account the following facts.

The titration of the carboxyl groups of albumin and the amidino-albumins is abnormal. Above pH 4.0, at least, this abnormality is not caused by expansion of the molecule. From the characteristics of the titration curves a considerable portion of the albumin carboxyl groups appear to be stabilized in the anionic form in the N form of albumin. The stability of this form of the molecule is not as great in the case of the derivatives as it is in the case of the native protein. Below the pH region of the N-F transformation all protein carboxyl groups are equivalent. Considerable expansion of the molecule occurs in this pH range and this expansion occurs more readily in the albumin derivatives.

Since many (approximately 35) of the 100 albumin carboxyl groups appear to be abnormal and stabilized in the anionic form, a considerable portion of the molecule must be involved in a structure which permits this type of abnormal behavior to occur. The simplest explanation would be that of a crevice (or crevices) within the molecule with Van der Waals or electrostatic interactions occurring between the amino acid residues on the sides of the crevice. Since modification of the ε-amino groups of the protein lysyl residues affects the ease with which the N or crevice-containing form of the molecule can be converted to the F or opened-up form of the molecule some of the 57 lysyl residues must be present in this crevice as well as about 35 of the protein carboxyl groups. The finding that about 38 of the ε-amino groups do not readily react with the relatively large reagent trinitrobenzenesulfonic acid (95) is also indicative of some masking of part of the lysyl groups of the protein.

If the interactions between the residues in the crevice were mainly hydrophobic in nature it is energetically unfavorable for the carboxyl groups to be stabilized in the charged form in this type of environment. The only manner in which these groups could be stabilized in the charged form would be by some mechanism like hydrogen-bond formation with a residue like tyrosine. Furthermore, the added hydrophobic residues in the case of the derivatives should enhance the stability of the N form of the molecule rather than render it less stable unless, of course, some very special steric requirements were disrupted by the addition of these groups. Since most of the hydrophobic residues have considerable freedom of rotation, it is unlikely however that these largely undirected hydrophobic interactions would be greatly disrupted by the addition of the small aliphatic residues.

The most straight-forward explanation of the data can be made in terms of Foster's hypothesis of a crevice with a hydrophilic environment. The addition of small hydrophobic groups to the lysyl residues in such a crevice could interfere somewhat with the electrostatic interactions between the ionized carboxyl groups and the charged lysyl residues and thus decrease the stability of the N form of the protein. If some such charge-charge interaction does occur, stabilization of the carboxyl groups in the charged form would result. It would also be expected that some parallel abnormalities would be observed in the pH region of the titration of ϵ-amino groups and such abnormalities have been reported (9, 86). If the differences in the ease of

the N-F transition for the derivatives and the native albumin are related to differences in electrostatic interaction energies, titration studies at different ionic strength would show predictably differing results. Unfortunately the drift in the pH values in the region of the N-F transition at low ionic strength (0.02) made any correlation of the titration data with ionic strength impossible.

The data that has been obtained in the presence of 0.1M KCl, however, can be satisfactorily explained in terms of a hydrophilic crevice in the albumin molecule; electrostatic interactions between the charged carboxyl groups and the charged ε-amino groups must play a significant role in the maintenance of the crevice structure of the N form of albumin.

CONCLUSIONS

The ε-amino groups of the lysyl residues of albumin can be essentially completely converted to substituted amidino groups by reaction with short chain aliphatic imidoesters without measureable alteration of the structure of the protein at neutral pH values. The electrophoretic and titration behavior of these amidino-albumin derivatives in the pH region of the N-F transformation has shown that this transformation occurs at somewhat higher pH values than has been observed with the native protein. It has been possible to interpret the data obtained with the albumin derivatives in terms of a hypothesis on the nature of this transformation.

Foster (24) has suggested that the abnormal titration curves of albumin in the regions of titration of the carboxyl groups are due to the stabilization of the anionic form of these groups in a hydrophilic environment in a crevice of the molecule.

It would appear from the differences between the titration and electrophoretic behavior of the derivatives and the native molecule that some of the lysyl residues must be present in the crevice. The experimental data can be best explained by the thesis that attractive electrostatic interactions between the charged carboxyl groups and the charged amino groups play a significant role in the maintenance of the crevice structure of the N form of the albumin molecule.

The study of suitable protein derivatives has thus made it possible to obtain experimental evidence to the type of forces

which are involved in the maintenance of a specific protein structure.

APPENDIX

A computer program was written in Fastran language for the IBM 7090 computer in order to accurately calculate the concentration gradients of the electrophoretic protein-solvent boundaries and the weight-mean positions of these boundaries for subsequent mobility calculations. A plot routine, utilizing the Cal-comp 763/780 plotter, was included in the source program to graph the resulting data as shown in Figures 7-10. The source program was written to maximize the efficiency of plate reading; plate reading was not limited to reading fringes either at constant x values or constant integral fringe values. Constant x intervals were obtained for use in the equation for the weight-mean position of the boundary by interpolating the data at unequal x intervals to a minimum constant x interval through the use of Aitken's successive four-point linear interpolation method. This interpolation routine was also used in the calculation of titration data at equal pH values. A flow diagram for the source program is presented in Figure 15 as well as several of the essential equations used in the program. The source program itself is presented at the end of the Appendix.

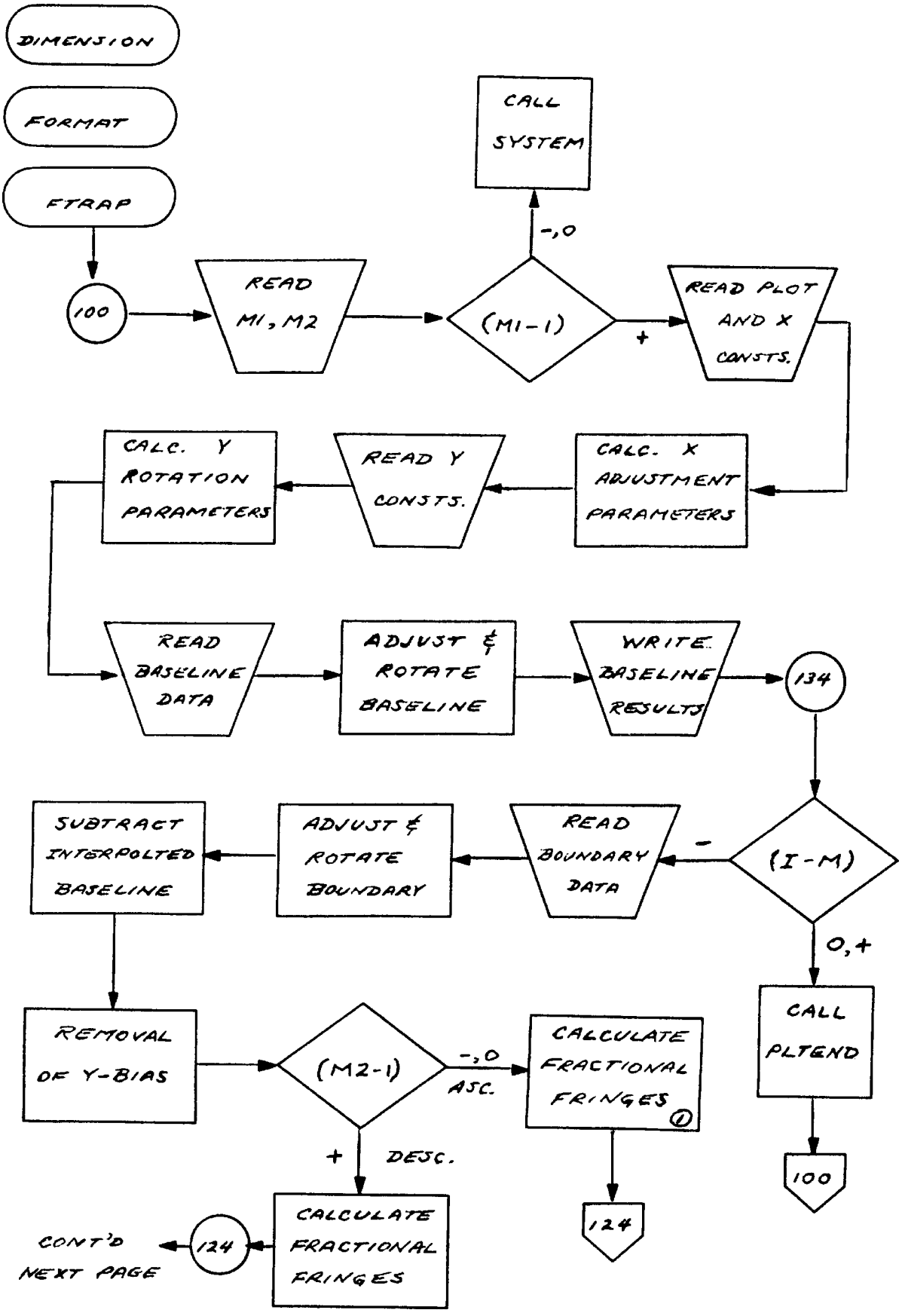
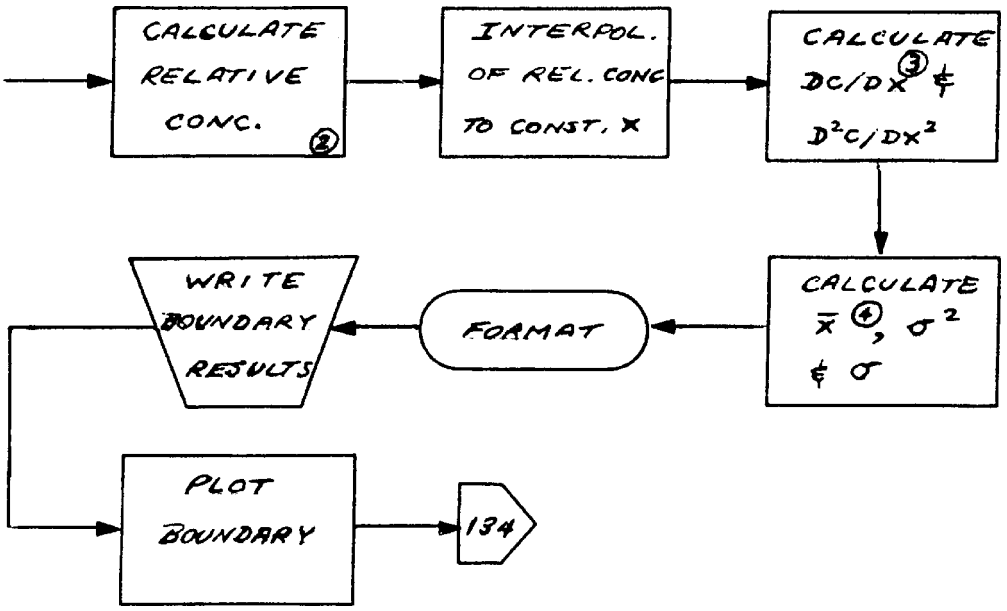


Figure 15. Flow Diagram for the Fringe Analysis Program.



ESSENTIAL EQUATIONS FOR PROGRAM

①

$$A^{\dagger} = \text{FRACTIONAL FRINGES} = \text{INTEGRAL FRINGE NO.} + \frac{Y \text{ DEFLECTION}}{\text{FRINGE WIDTH}}$$

②

$$B = \text{RELATIVE CONCENTRATION} = \frac{A}{\text{TOTAL NO. OF FRINGES}}$$

③

$$C = DC/DX = \frac{B(J-2) - 8B(J-1) + 8B(J+1) - B(J+2)}{(12.0)(\Delta X)}$$

④

$$\bar{X} = \sum_i X_i C_i \Delta X \Big/ \sum_i C_i \Delta X$$

$\bar{X}$ WEIGHT-MEAN POSITION OF THE PROTEIN-SOLVENT BOUNDARY

†WHERE $0.0 \leq A \leq$ TOTAL NO. OF FRINGES

```
C       ANALYSIS OF FRINGE DATA FROM MOVING BOUNDARY ELECTROPHORESIS
        DIMENSION XMARK1(7),XMARK2(7),XMARKM(7),XREF1(7),XREF2(7)
        DIMENSION YREF1(7),YREF2(7),YREFC1(7),YREFC2(7),DELYR(7)
        DIMENSION X(200),Y(200),FRNGNO(200),XB(200),YB(200),VARI(7)
        DIMENSION XI(400),CRELI(400),DELX(7),CREL(200),XMEAN(7),DCDX(400)
        DIMENSION D2CDX2(400),DER(400),SIGCSQ(7),SIGCPL(7)
        DIMENSION A(4,6),CONC(7)
D       DIMENSION RX(200),RY(200)
D       DIMENSION XLBL(6),YLBL(6),TTL(12)                                  PLT
  010 FORMAT (2I5)                                                         PLT
  011 FORMAT (2E12.6,I2)
  012 FORMAT (6E12.0)
  030 FORMAT (12A6)
  031 FORMAT (4E12.5)
      FLOATING ROUND
      CALL FTRAP
  100 CONTINUE
      RIT 7,10,M1,M2
      IF (M1-1) 101,101,102
  101 CONTINUE
      CALL SYSTEM
  102 CONTINUE
      RIT 7,11,FACMAG,FRNGWD,M
      RIT 7,12,(DELX(I),I=2,M)
      RIT 7,30,(XLBL(I1),I1=1,6)
      RIT 7,30,(YLBL(I1),I1=1,6)
      RIT 7,30,(TTL(I1),I1=1,12)
      RIT 7,30,GX2,GX3,GX4,GX5,GX6,GX7
      RIT 7,30,GY2,GY3,GY4,GY5
      RIT 7,31,XMIN,DX,YMIN,DY
      RIT 7,12,(XMARK1(I),XMARK2(I),I=1,M)
```

```
      RIT 7,12,(XREF1(I),XREF2(I),I=1,M)
C     LOOP FOR CALCULATING AND SUBTRACTING XMARKM AND DIVIDING
C     SUBSEQUENT VALUES BY FACMAG
      DO 103 I=1,M
      XMARKM(I)=(XMARK1(I)+XMARK2(I))/2.0
      XMARKM(I)=XMARKM(I)-80000.
      XMARK1(I)=XMARK1(I)-XMARKM(I)
      XMARK2(I)=XMARK2(I)-XMARKM(I)
      XREF1(I)=XREF1(I)-XMARKM(I)
      XREF2(I)=XREF2(I)-XMARKM(I)
      XMARKM(I)=XMARKM(I)/FACMAG
      XREF1(I)=XREF1(I)/FACMAG
      XREF2(I)=XREF2(I)/FACMAG
  103 CONTINUE
C     LOOP FOR COMPARISON OF REFERENCE FRINGE DISPLACEMENTS
      RIT 7,12,(YREF1(I),YREF2(I),I=1,M)
      AUXVR1=0.0
      DO 104 I=1,M
      DELYR(I)=YREF2(I)-YREF1(I)
      AUXVR1=AUXVR1+DELYR(I)
  104 CONTINUE
      AUXVR2=M
      DELYRM=AUXVR1/AUXVR2
      DO 105 I=1,M
      YREFC1(I)=YREF1(I)
      YREFC2(I)=YREFC1(I)+DELYRM
  105 CONTINUE
C     READING IN BASELINE DATA
      RIT 7,12,XA,XN
```

```
      XNB=((XN-XA)/500.0)+1.0
      NB=XNB
      RIT 7,12,(YB(J),J=1,NB)
      XINC=-1.0
      DO 106 J=1,NB
      XINC=XINC+1.0
      XB(J)=XA+XINC*500.0
  106 CONTINUE
      I=1
C     LOOP FOR ADJUSTING XB(J) AND ROTATING YB(J)
      DO 107 J=1,NB
      XB(J)=XB(J)/FACMAG
      XB(J)=XB(J)-XMARKM(I)
      YB(J)=YB(J)+(YREFC2(I)-YREF2(I))*(XB(J)-XREF1(I))/(XREF2(I)-XREF1(
     1I))
  107 CONTINUE
C     WRITING OUT BASELINE RESULTS
      WOT 6,13
      WOT 6,14
      WOT 6,15,I,XMARKM(I),XMARK1(I),XMARK2(I),XREF1(I),XREF2(I)
      WOT 6,16
      WOT 6,17
      WOT 6,15,I,DELYR(I),DELYRM,YREFC2(I),YREF1(I),YREF2(I)
      WOT 6,25,I
      WOT 6,40
  040 FORMAT (/,37H    J                    XB                    YB    )
      WOT 6,41,(J,XB(J),YB(J),J=1,NB)
  041 FORMAT (I5,1X,F15.2,1X,F15.2)
  134 CONTINUE
      IF (I-M) 136,135,135
  135 CALL PLTEND
```

```
      GO TO 100
  136 DO 137 J=1,200
      X(J)=0.0
      CREL(J)=0.0
  137 CONTINUE
C     LOOP FOR READING IN ELECTROPHORESIS DATA
  109 NP=3
  110 NO=NP+1
      KIT 7,12,(X(J),Y(J),FRNGNO(J),J=NP,NO)
      IF (X(NP)-X(NO)) 111,112,113
  111 NP=NP+2
      GO TO 110
  112 NP=NP-1
  113 CONTINUE
      I=I+1
C     LOOP FOR ADJUSTING X(J) AND ROTATING Y(J)
      DO 114 J=3,NP
      X(J)=X(J)/FACMAG
      X(J)=X(J)-XMARKM(I)
      Y(J)=Y(J)+(YREFC2(I)-YREF2(I))*(X(J)-XREF1(I))/(XREF2(I)-XREF1(I))
  114 CONTINUE
C     LOOP FOR SUBTRACTION OF INTERPOLATED BASELINE FROM Y(J)
      J1=3
      J=0
  115 J=J+1
  116 IF ((X(J1)-XB(J))-500.0) 117,117,115
  117 YBI=(((XB(J+1)-X(J1))*(YB(J)))-((XB(J)-X(J1))*(YB(J+1))))/500.0
      Y(J1)=Y(J1)-YBI
      J1=J1+1
```

```
      IF (J1-NP) 116,116,118
  118 CONTINUE
C     LOOP FOR REMOVAL OF YBIAS
      VAR=Y(3)
      DO 119 J=3,NP
      Y(J)=Y(J)-VAR
  119 CONTINUE
C     LOOP FOR COMPUTING CREL FOR ASCENDING OR DESCENDING DATA
      IF (M2-1) 120,120,122
  120 DO 121 J=3,NP
      ERNGND(J)=ERNGND(J)+Y(J)/ERNGWD
  121 CONTINUE
      GO TO 124
  122 DO 123 J=3,NP
      ERNGND(J)=ERNGND(J)-Y(J)/ERNGWD
  123 CONTINUE
  124 DO 125 J=3,NP
      CREL(J)=ERNGND(J)/ERNGND(NP)
  125 CONTINUE
C     LOOP FOR INTERPOLATING CREL TO CONSTANT X INCREMENTS
C     AITKENS METHOD USING FOUR POINTS
      DEL=-1.0
      J=2
      J2=0
  200 J=J+1
  201 DEL=DEL+1.0
      J2=J2+1
      XI(J2)=X(3)+DEL*DELX(I)
      IF (ABSF(X(J+2)-XI(J2))-ABSF(X(J-1)-XI(J2))) 202,205,204
  202 IF (ABSF(X(J+3)-XI(J2))-ABSF(X(J-1)-XI(J2))) 203,203,205
  203 J3=J
```

```
      GO TO 207
  204 IF (ABSF(X(J+2)-XI(J2))-ABSF(X(J-2)-XI(J2))) 205,205,206
  205 J3=J-1
      GO TO 207
  206 J3=J-2
  207 N1=0
      J4=J3+3
      DO 208 M3=J3,J4
      N1=N1+1
      A(N1,1)=X(M3)
      A(N1,2)=X(M3)-XI(J2)
      A(N1,3)=CREL(M3)
  208 CONTINUE
      K=0
      DO 210 N2=3,5
      K=K+1
      DO 209 N1=K,3
      A(N1+1,N2+1)=((A(K,N2)*A(N1+1,2))-(A(N1+1,N2)*A(K,2)))/(A(N1+1,1)-
     1A(K,1))
  209 CONTINUE
  210 CONTINUE
      CRELI(J2)=A(4,6)
      IF ((X(NP)-XI(J2))-DELX(I)) 212,211,211
  211 IF ((X(J+1)-XI(J2))-DELX(I)) 200,201,201
  212 CONTINUE
C     LOOP FOR COMPUTING DC/DX AND D2C/DX2
      L=J2-2
      SIGCSQ(I)=0.0
      XMEAN(I)=0.0
```

```
      CONC(I)=0.0
      DO 130 J=3,L
      DER(J)=(CRELI(J-2)-8.0*CRELI(J-1)+8.0*CRELI(J+1)-CRELI(J+2))/12.0
  130 CONTINUE
      DO 131 J=3,L
      DCDX(J)=DER(J)/DELX(I)
      D2CDX2(J)=(CRELI(J-1)-2.0*CRELI(J)+CRELI(J+1))/DELX(I)**2.0
  131 CONTINUE
C     CALCULATING XMEAN(I) AND SIGCSQ(I) FOR VALUES OF DCDX(J)
C     GREATER THAN 0.000005
      J=2
  300 J=J+1
      IF (DCDX(J)-0.000005) 302,301,301
  301 XMEAN(I)=XMEAN(I)+XI(J)*DER(J)
      CONC(I)=CONC(I)+DER(J)
  302 IF (J-L) 300,303,303
  303 CONTINUE
      XMEAN(I)=XMEAN(I)/CONC(I)
      J=2
  304 J=J+1
      IF (DCDX(J)-0.000005) 306,305,305
  305 SIGCSQ(I)=SIGCSQ(I)+(XI(J)-XMEAN(I))**2.0*DER(J)
  306 IF (J-L) 304,307,307
  307 CONTINUE
      VARI(I)=ABSF(SIGCSQ(I))
      SIGCRL(I)=SQRT(VARI(I))
  132 CONTINUE
C     WRITING OUT RESULTS
      WOT 6,13
  013 FORMAT (70H1 FINAL VALUES OF XMARKM(I) XMARK1(I) XMARK2(I) XREF1(I
     1) AND XREF2(I) )
```

```
      WOT 6,14
014 FORMAT (66H   I XMARKM(I)      XMARK1(I)      XMARK2(I)       XREF1(I)
     1   XREF2(I) )
      WOT 6,15,I,XMARKM(I),XMARK1(I),XMARK2(I),XREF1(I),XREF2(I)
015 FORMAT (I3,1F10.2,4F13.2)
      WOT 6,16
016 FORMAT (//66H  FINAL VALUES OF DELYR(I) DELYRM YREFC2(I) YREF1(I)
     1AND YREF2(I) )
      WOT 6,17
017 FORMAT (66H   I   DELYR(I)        DELYRM     YREFC2(I)      YREF1(I)
     1   YREF2(I) )
      WOT 6,15,I,DELYR(I),DELYRM,YREFC2(I),YREF1(I),YREF2(I)
      WOT 6,25,I
025 FORMAT (//,18H  DATA SET NUMBER ,I2)
      WOT 6,26
026 FORMAT (/,61H    J            X(J)       FRNGMD          Y(J)          REL.
     1 CONC.   ,/)
      WOT 6,27,(J,X(J),FRNGMD(J),Y(J),CREL(J),J=3,NP)
027 FORMAT (I5,1X,F15.2,1X,F8.2,F15.8,2X,F15.8)
      WOT 6,18,I
018 FORMAT (18H1 DATA SET NUMBER ,I2)
      WOT 6,19
019 FORMAT (/,66H    J           XI(J)          CRELI(J)                    DCDX
     1    D2C/DX2    ,//)
      WOT 6,20,(J,XI(J),CRELI(J),DCDX(J),D2CDX2(J),J=3,L)
020 FORMAT (I5,F15.2,F15.8,2X,E15.8,1X,E12.6)
      WOT 6,21,(XMEAN(I),SIGCSQ(I),SIGCRL(I))
021 FORMAT (//, 7H XMEAN=,F10.2,12H      SIGCSQ=,E15.8,12H      SIGCRL=,
     1 E15.8)
```

```
133 CONTINUE
    IF (I-2) 500,500,600
500 CONTINUE
    DX=DX/1.9685
    DY=DY/1.9685
    CALL PLTREC                                                              PLT
    CALL PLTPAP (3H400)                                                      PLT
    CALL PLTDES (XMIN,DX,YMIN,DY,2.0,1.0)                                    PLT
    CALL PAXIS (2.0,1.0,XLBL(1),-0,-13.7795,00.0,XMIN,DX,1.9685)             PLT
    CALL PAXIS (2.0,1.0,YLBL(1),+0,-09.8425,90.0,YMIN,DY,1.9685)             PLT
    CL=PSYMLN (0.20,36)/2.0                                                  PLT
    CALL PSYMB (1.40,5.92125-CL,0.20,YLBL(1),90.0,36,0)                      PLT
    CALL PSYMB (8.88975-CL,0.20,0.20,XLBL(1),00.0,36,0)                      PLT
    CALL PSYMB (8.88975-CL,9.8740,0.20,TTL(1),00.0,36,0)                     PLT
    CALL PSYMB (8.88975-CL,9.4740,0.20,TTL(7),00.0,36,0)                     PLT
    CALL PGRID (2.0,1.0,1.9685,1.9685,7,5)                                   PLT
    CL=PSYMLN (0.15,6)/2.0                                                   PLT
    CALL PSYMB (03.9685-CL,0.6,0.15,GX2,00.0,6,0)                            PLT
    CALL PSYMB (05.9370-CL,0.6,0.15,GX3,00.0,6,0)                            PLT
    CALL PSYMB (07.9055-CL,0.6,0.15,GX4,00.0,6,0)                            PLT
    CALL PSYMB (09.8740-CL,0.6,0.15,GX5,00.0,6,0)                            PLT
    CALL PSYMB (11.8425-CL,0.6,0.15,GX6,00.0,6,0)                            PLT
    CALL PSYMB (13.8110-CL,0.6,0.15,GX7,00.0,6,0)                            PLT
    CALL PSYMB (1.8,02.9685-CL,0.15,GY2,90.0,6,0)                            PLT
    CALL PSYMB (1.8,04.9370-CL,0.15,GY3,90.0,6,0)                            PLT
    CALL PSYMB (1.8,06.9055-CL,0.15,GY4,90.0,6,0)                            PLT
    CALL PSYMB (1.8,08.8740-CL,0.15,GY5,90.0,6,0)                            PLT
600 DO 700 K=1,200
    RX(K)=0.0
    RY(K)=0.0
700 CONTINUE
```

```
      K=0
      J=3
  701 J=J+1
      IF (DCDX(J)-0.000005) 703,702,702
  702 K=K+1
      RX(K)=XI(J)
      RY(K)=DCDX(J)
  703 IF (1-(K/200)-(J/L)) 704,704,701
  704 CONTINUE
      CALL PLINE (RX(1),RY(1),K,1,00.0,1)                    PLT
      PX=XMEAN(I)
      CALL PLINE (PX,YMIN,1,1,-1,16,1)
      GO TO 134
      END
```

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