Electrophoretic Mobility-Ionic Strength Studies of Proteins

IV. THE EFFECT OF LIPIDS ON THE ELECTROPHORETIC PATTERNS OF HUMAN SERUM ALBUMIN AT ACID pH*

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In previous publications from this laboratory, we have investigated the anomalous behavior of human serum albumin (1), various animal albumins (2) and human γ globulin (3) in the Tiselius electrophoresis apparatus at acid pH. Because of increasing interest in the behavior of proteins in the acid region below their isoelectric point, this phenomenon has been receiving increasing attention from several groups of investigators as discussed in a recent review article by Brown and Timasheff (4).

The main characteristics of the electrophoretic patterns of proteins in the isoelectric point region are the complex boundary patterns obtained and the lack of symmetry between the ascending and descending boundaries (5-7). Longsworth and Jacobsen (8) suggest that the nonenantiographic patterns obtained for bovine serum albumin under such conditions indicate continuously readjusted equilibria across the moving boundaries. Charlwood (9) studied the variations in the electrophoretic pattern of human serum albumin (2%) in 10 different organic buffer anion systems at pH 4.0 and 0.02 ionic strength. He concluded that the complex ascending patterns obtained must involve specific interactions between buffer anions and protein although they may be partly dependent on buffer capacity. Aoki and Foster (10-12) investigated the electrophoretic behavior of bovine serum albumin in the pH region between 3.0 and 4.5 with 0.2% protein solution at 0.02 ionic strength of chloride, thiocyanate, and acetate. Two main boundaries were observed whose percentage composition varied with pH although the patterns obtained were fairly enantiographic at the low protein concentration employed. These authors explained the anomalous electrophoretic behavior of bovine serum albumin at pH 4.0 as due to an isomerization reaction.

Cann and Phelps (13-15) have also performed studies of the electrophoretic behavior of bovine serum albumin and ovalbumin in low and high ionic strength acetate buffers at pH 4.0 and at protein concentrations of approximately 1.3%. These investigators explain the complex nonenantioptic patterns at these higher protein concentrations as being due to false boundaries, i.e., those not due to single components, which result partly from the conductance and pH gradients in the electrophoresis cell but are also associated with the complex forming of the protein with the undissociated buffer acid (16). Recently, Woods (17) investigated the electrophoretic behavior of a number of proteins including bovine serum albumin and ovalbumin in various amino acid buffers between pH 1.5 and 3.0. This author concluded from his experimental data that the boundaries obtained are not false boundaries of the Svensson type (18) and that the patterns can be interpreted qualitatively in terms of the moving boundary theory of weak electrolytes.

Brown and Timasheff (4) conclude their review of this subject by stating, "It seems probable, however, that the observed electrophoretic behavior in acid pH's is the result of the cooperative influence of true heterogeneity, isomerization, and the binding of ions and small molecules. Thus, a great deal of work remains to be done before this question is fully elucidated."

The original observation of Luetscher (19) that highly purified serum albumins could be separated into two or more components electrophoretically at pH 4.0 has been repeatedly confirmed by other investigators (1, 2, 7, 9, 11). The same electrophoretic behavior at acid pH is also obtained with purified albumin fractionated with the aid of mercury salts (20), as well as with crystalline albumin preparations (21). Such experimental findings have led Hughes (22) to conclude in a review article that the electrophoretic patterns obtained at pH 4.0 are particularly sensitive to the ionic environment.

It has been found by both Kendall (23) and Cohn et al. (21) that purified human serum albumin, obtained either by salt or alcohol fractionation, contains appreciable amounts of bound fatty acids and that these compounds are still present in significant amounts after repeated recrystallizations. That such binding of fatty acids to proteins has a pronounced effect on the electrophoretic mobility of human serum albumin at alkaline pH is known from the work of Ballou et al. (24). Studies of the interaction of human serum albumin with long-chain fatty acid anions with partition analysis have recently been published by Goodman (25, 26) who has reviewed much of the earlier literature in this field. From the experimental work in this paper (26) and that of other investigators (21, 23), it is evident that practically all previous work dealing with the electrophoresis of purified serum albumins at acid pH has been carried out with preparations which contain appreciable amounts of bound lipids or other anions. It has been suggested by Hughes (22) and Charlwood (9) that such bound anions, rather than true protein heterogeneity, might be the real cause of the anomalous electrophoretic behavior of this protein at acid pH.

The present paper deals with the effect on the electrophoretic patterns obtained with human serum albumin in the acid pH...
region after lipid removal with various techniques and the subsequent effect on the nature of the electrophoretic boundaries obtained, of the addition of fatty acids and related substances which bind to albumin.

**EXPERIMENTAL**

**Materials and Reagents**

1. **Albumin, Human Serum**—Squibb Fraction V. Lyophilized.
2. **Albumin, Human Serum—25% solution of Squibb Fraction V containing 0.02 M sodium caprylate and 0.02 M acetyl tryptophanate as stabilizers.** Squibb and Company, New Brunswick, New Jersey.
3. **Oleic Acid, Purified—Lot No. 17. Hormel Foundation, Austin, Minnesota.**
6. **Dodecyl Alcohol—Eastman Kodak No. 873.**
7. **Sodium Acetate-Sodium Chloride Buffers (27)—Buffers of 0.04 ionic strength were prepared containing 90% sodium chloride and 10% sodium acetate. The pH was then adjusted to the desired level with 3.5 M acetic acid before diluting to volume.**
8. **Acetate Buffers—Buffers of the desired ionic strength were prepared by weighing out reagent grade sodium acetate and adjusting to the proper pH with 3.5 M acetic acid before diluting to volume.**
9. **Sodium Chloride-Hydrochloric Acid Buffers—Sodium chloride, 0.1 M, was adjusted to the desired pH with 1 N hydrochloric acid.** A Beckman model G pH meter with glass electrodes was employed for all the pH measurements.

**Methods**

**Total Protein**—The determinations were performed by the biuret method of Gornall et al. (28) with a standard serum protein solution which was checked with the micro-Kjeldahl procedure. **Moving Boundary Electrophoresis**—These analyses were performed with the portable Amino-Stern electrophoresis apparatus with albumin samples whose concentrations were adjusted to 2.0 ± 0.2% with the appropriate buffer solution. Dialysis of the protein was then performed for 48 hours at 4°C with two or more changes of buffer, followed by 4 hours of mechanical dialysis (29) just before performing the electrophoretic run at 2°C with the technique described in a previous publication (30). **Ether Treatment of Albumin Solutions**—Procedure employed was essentially that of MacFarlane (31). The pH of approximately 5% albumin solutions was adjusted to about pH 4 with 1 N HCl. A Dry-Ice-acetone bath was used to freeze the 4 to 5 ml aliquots and the slurry was extracted at least four times with 2 ml of ethyl ether for each extraction. The other was then removed by dialysis against deionized water at 4°C. **Isooctane Treatment of Albumin Solution**—Two-gram portions of albumin were treated at 0°C in an ice bath with isooctane containing 5% of glacial acetic acid according to the method of Goodman (32). The treatment was carried out twice with 100-ml aliquots of acidified dehydrated isooctane followed by dialysis in the cold against deionized water. A small amount of white low density material was removed by centrifugation. After dialysis the pH of the lipid-free solution was found to be 4.95.

**Deionization of Albumin Solutions**—Solutions of albumin, 5%, were deionized by passage through a column containing the mixed-bed ion exchange resins of Dintzis (33). In some experiments, the albumin solution was passed through a series of two columns to test whether further material was removed.

**Effect of pH Variation in Acid Region**

The electrophoretic patterns obtained for human serum albumin (Squibb Fraction V with stabilizers) in the pH region below the isoelectric point are shown in Fig. 1. The asymmetry between the ascending and descending boundary, and the formation of multiple ascending boundaries in the pH 4.0 to pH 4.2 region noted by Miller et al. (7) at higher ionic strengths (0.20) with this buffer becomes even more pronounced at lower ionic strength (0.04). The results obtained in these experiments are generally similar to those reported by Cann (16) and Phelps and Cann (15) for bovine serum albumin in acetate-chloride buffer at acid pH. Miller et al. (7) reported that the resolution of human serum albumin into 3 or 4 "components" was most clearly revealed between pH 4.0 and 4.4 for both the isolated albumin fraction and with serum.

**Effect of Variation of Protein Concentration**

The nature of the protein concentration effect in acidic buffer of low ionic strength is shown in Fig. 2. The protein solutions used to obtain these data were prepared from the 25% solution of Squibb Fraction V which contains caprylate and tryptophanate as stabilizers. In general it would be expected that the area under each peak, expressed as grams per 100 ml of protein, would increase proportionately with the increase in the total protein content of the sample. Although this is true for the first and third peaks (Fig. 1C), the nonideal behavior of Peak 2 is clearly evident. The amount of protein represented by this peak increases but slightly with increasing protein concentration, indicating that some other...
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Effect of Preliminary Treatment

It had been assumed that any stabilizers or other ions bound to albumin would be removed by the prolonged and intensive dialysis against the buffer solution employed in our electrophoretic runs. In view of the results shown in Fig. 2, such a conclusion appeared questionable and the possibility was then considered that the continued presence of such bound anions might be a factor in effecting the electrophoretic patterns obtained in the acid pH region. The Squibb Fraction V albumin preparation was then treated in several ways in order to remove such bound anion without denaturing the protein. Such preliminary treatment included passage of the albumin through a Dintzis (33) column, the isooctane-acetic acid treatment of Goodman (32) and the low temperature ether treatment of MacFarlane (31).

Fig. 3A shows the electrophoretic patterns obtained with barbital buffer, pH 8.6, 0.1 ionic strength for the Squibb Fraction V albumin and Fig. 3B the patterns for the same sample in acetate-NaCl (90%) buffer at pH 4.2, 0.04 ionic strength. Fig. 3C demonstrates that passage of the albumin fraction through the Dintzis column resulted in a considerable decrease in the area under the second peak but not in its complete disappearance. However, this was very nearly achieved with either the ether treatment (Fig. 3D) or the isooctane-acetic acid procedure (Fig. 3E). Since the ether treatment was more conveniently applicable to proteins in solution and because it removed any denatured protein as an insoluble product, it was employed in all subsequent experiments for the removal of lipid substances from albumin samples.

Effect of Addition of Substances with High Binding Affinities

In view of the known high affinity of many small molecules including fatty acids, higher alcohols, detergents, and others, for binding to serum albumins, it was of interest to ascertain whether the addition of such materials would increase the area under the second peak (Fig. 1C) beyond the limiting value of Fig. 2.

Three such materials were added to samples of Fraction V from which the stabilizers and other anions had been removed by the deionization procedure previously described. In the case of the caprylic and oleic acids the protein solution was first saturated with the fatty acid and the bag containing the preparation was then dialyzed against buffer. For the experiments shown in Figs. 4A and 4B an aliquot of the albumin solution saturated with the fatty acid was diluted with an equal volume of deionized albumin and then dialyzed against the buffer. In the case of dodecanol the albumin solution in the bag was dialyzed against repeated changes of dodecanol saturated buffer and then an aliquot of this solution was dialyzed against pure buffer so as to remove part of the bound dodecanol.

The results obtained in these electrophoretic runs are shown in Fig. 4. The data demonstrate that caprylic acid, oleic acid, and dodecanol all had the effect of increasing the area under the second peak of the ascending pattern and that the extent of this effect is dependent both upon the amount and nature of the materials present at the time of electrophoresis.

Effect of Variation of Ionic Strength

The sodium acetate-sodium chloride buffer used in these experiments was low both in ionic strength and buffer capacity. It was therefore considered important to determine whether any factors in the ionic environment of the protein influenced the areas of any of the peaks at a constant protein-lipid ratio. For these experiments, deionized Fraction V (Fig. 3C) was utilized since it contained a small but significant amount of bound lipid so that changes due to ionic strength variation could be readily detected regardless of their direction. Some typical results ob-
tained with the electrophoresis of deionized and ether treated albumin (Fraction V) samples at different ionic strengths in acetate buffer systems (without sodium chloride) and in an unbuffered HCl-NaCl system at the same pH and ionic strength are shown in Fig. 5.

For the deionized 1 to 2% albumin samples, as the ionic strength increased from 0.02 to 0.10, the relative area under the second peak also increased. The small amount of the third peak present at 0.02 ionic strength (Fig. 5Al) tended to diminish, or to coalesce with the delta boundary, upon increasing the ionic strength and disappeared completely at 0.10 ionic strength or higher. The pattern obtained with sodium chloride at 0.10 ionic strength, pH 3.9, (Fig. 5Cf) was very similar to that for acetate at the same ionic strength (Fig. 5Bi). The results obtained by the 1 to 2% protein concentration in general agreement with those of 5% albumin (Fraction V) previously reported from this laboratory (2). At a given pH, both the protein-lipid ratio and ionic strength influence the area under the 2nd peak. That is, at constant lipid-protein ratio, the area under the second peak increases with increasing ionic strength, and at constant ionic strength, the area increases with increasing amounts of lipids.

It should also be noted that preliminary treatment of the albumin samples with ether to remove bound lipids results in ascending patterns (Figs. 5A2, 5B2, and 5C2) in which the second peak is absent or very nearly so, at about a pH of 4 regardless of the ionic strength employed. These results are in marked contrast to the 2 or more ascending boundaries usually obtained with the untreated albumin (Fig. 6A) in this pH region (7, 9, 11). In addition, as seen in Figs. 5A1 and 5A2, the removal of lipid by ether treatment has little or no effect on the third peak.

Effect of Cysteine Treatment

Keltz and Mehl (34) had isolated from rat plasma and Schmid (35) from human plasma, albumin fractions which gave a single electrophoretic boundary in pH 4.0 acetate buffer, 0.10 ionic strength. The latter author found that after cysteine treatment two peaks were obtained. It was therefore of great interest to us whether our preparation would behave in a similar manner after such treatment.

The human albumin sample (Fig. 6A) was deionized and then carried through the ether treatment. The electrophoretic pattern of the treated preparation at acid pH is shown in Fig. 6B and that in barbital buffer at pH 8.6 in Fig. 6D. Cysteine treatment of this sample followed by deionization according to Schmid's technique (35) also resulted in two boundaries after electrophoresis in acetate buffer as is demonstrated in Fig. 6C. However, the phenomenon has not been investigated sufficiently to ascertain whether the additional boundary resulted from the reappearance of Peak 2, or the splitting of Peak 1.

Effect of Oleic Acid Binding

A more detailed study of the effect on the electrophoretic pattern at acid pH of lipid binding to the ether-treated albumin was now undertaken. A weighed amount of oleic acid was neutralized with sufficient sodium hydroxide to form the soap and diluted with water to give a final volume such that 0.10 ml gave an amount of oleate equimolar to 9 ml of a 2% human serum albumin solution (assumed molecular weight = 65,000). The oleate was then added to samples of ether-treated Fraction V albumin which had been equilibrated with 0.10 ionic strength pH 4.0 acetate by dialysis. The oleate and albumin were allowed to reach equilibrium by gentle rocking overnight at 4°. The addition of oleate brought about no detectable change in pH up to the addition of 0.7 ml of oleate per 9 ml of protein in buffer.

The electrophoretic patterns obtained in pH 4 acetate buffer, after the addition of increasing increments of pure sodium oleate, are shown in Fig. 7 and reveal some interesting relationships. The first peak shows a gradual diminution in area with increasing amounts of oleic acid up to 4 moles per mole of albumin.

![Fig. 4. Effect of addition of (A) caprylic acid, (B) oleic acid, and (C) dodecanol on the ascending electrophoretic patterns of human serum albumin (deionized Squibb Fraction V) in acetate-NaCl buffer, pH 4.2, 0.04 ionic strength. The protein concentration of each sample was 2.0 ± 0.2% and all runs were performed at 8.6 volts per cm for 4500 seconds.](http://www.jbc.org/)

![Fig. 5. Effect of variation in ionic strength and of different buffer systems on the electrophoretic pattern of human serum albumin (deionized Squibb Fraction V) at pH 4.0 ± 0.1. A, protein concentration = 1.9 g per 100 ml, \( E = 25.6 \) volts per cm, \( t = 1500 \) seconds; B, Deionized and 2, ether treated. Acetate buffer, 0.02 ionic strength; B, protein concentration = 1.9 g per 100 ml, \( E = 7.2 \) volts per cm, \( t = 5400 \) seconds; C, Deionized and 2, ether treated. Acetate buffer, 0.10 ionic strength; C, protein concentration = 1.3 g per 100 ml, \( E = 6.8 \) volts per cm, \( t = 5400 \) seconds; D, Deionized and 2, ether treated. HCl-NaCl, 0.10 ionic strength.](http://www.jbc.org/)
were constant at 2.0 ± 0.2 g per 100 ml for all these experiments.

The variations in both peaks show a sharp break in their rate of change when between two and three moles of oleic acid per mole of albumin. There is some indication from these patterns that the rate of decrease is greater for small amounts of oleic acid than for larger quantities. The second peak shows a continual increase in area with increasing quantities of oleic acid per mole of albumin. This increase is directly related to the decrease in the first peak. The variations in both peaks show a sharp break in their rate of change when between two and three moles of oleic acid per mole of albumin have been added.

The electrophoretic patterns obtained appear to show a single peak both at very low concentrations of oleic acid per mole of albumin (Fig. 7A) and at very high concentrations (Fig. 7E). It was not possible to extend our observations on oleic acid binding beyond 7 moles of added oleic acid per mole of albumin because of the turbidity of the solutions. Addition of larger amounts of oleic acid did not bring about resolution into two boundaries even when the acid was present in amounts in excess of that which would bind.

RESULTS

The marked variation of the electrophoretic pattern of human serum albumin (Fraction V) with pH is illustrated in Fig. 1. The virtual disappearance of the multiple ascending boundaries at less than pH 3.7 (below the isoelectric point) and at pH values greater than 4.0 (above the isoelectric point) confirms the findings of Miller et al. (7), for human serum albumin and of Aoki and Foster (11) for bovine serum albumin although our experimental conditions were somewhat different from theirs. These differences may account for the experimental fact that our ascending boundaries were most clearly defined at pH 4.2 when acetate-NaCl (90%) buffer was used as is shown in Fig. 1C. Aoki and Foster (11) have attributed the changes in the electrophoretic pattern in 0.02 ionic strength NaCl-HCl buffer as being due to an isomerization reaction between a normal (N) form and a fast (F) form. The percentage composition of the two forms changed continuously with pH according to the equilibrium reaction, N + 3H⁺ ⇔ F. Since these authors employed a bovine plasma albumin prepared by the Cohn ethanol-low temperature fractionation procedure (36) without further purification, it is equivalent in lipid content to our human albumin samples and probably contains at least two moles of bound lipid per mole of protein (26,32). The data in Fig. 3 show that although the pattern in barbital buffer, pH 8.6, gives a single boundary (Fig. 3A), multiple boundaries are obtained at pH 4.2 in acetate-NaCl (Fig. 3B). However, as is seen in Figs. 3D and 3E, prior treatment with ether or isooctane to remove lipid material resulted in elimination of the second boundary and a slight diminution of the area of the third boundary in the same buffer system.

The results in Figs. 5A1, 5B1, and 5C1 show that even when a relatively small amount of bound lipid is present, variation in the ionic strength influences the area under the second peak as has been found by Aoki and Foster (11,12) for such small anions as chloride, acetate, and thiocyanate as well as by anionic detergents such as dodecyl sulfate (37). The ether-treated samples are not effected by changes in the ionic strength at pH 4.0 over a wide range of values, i.e. 0.02 to 0.10, and in different buffers systems as is illustrated in Figs. 5A2, 5B2, and 5C2. These acid pH ascending patterns are in fact, similar in appearance with that obtained in barbital buffer at pH 8.6 (Fig. 3A). The albumin electrophoretic patterns at acid pH were nonenantiotropic when compared to those obtained in pH 8.6 barbital buffer.

The data presented in Fig. 2 for the effect of protein concentration on the electrophoretic pattern confirms our previously reported results (2). A decrease in protein concentration does not change the number of boundaries formed, but does cause an increase in the relative percent area under Peak 2 in comparison with Peak 1. This change then is in the same direction as that produced by an increase in the bound lipid per mole of protein as is illustrated in Figs. 4 and 7. It was for this reason that in most of these experiments the protein concentration was main-
tained at 2.0 ± 0.2% as in the case of the oleate binding data in Fig. 7.

The data in Fig. 4 illustrate that not only lipids but other substances as well, e.g., dodecanol, which bind to albumin form a slower migrating boundary. From the degree of change produced in the relative areas of the first peak to that of the second peak in employing a sample fully-saturated as compared to one half-saturated, the binding affinities of the three materials could be listed in decreasing order as: oleate > dodecanol > caprylate. The quantitative aspects of the use of this technique are clearly apparent from the data in Fig. 7 and confirm the findings of other investigators that normal human serum albumin (Cohn Fraction V) contains about 1 to 2 moles of bound lipid per mole of protein (21, 26).

Before the present investigation, work was presented from this laboratory suggesting that the electrophoretic patterns of serum albumin at acid pH were influenced by the ionic composition of the buffer system although the pH, ionic strength, and protein concentration were kept constant (38). This was attributed to the different binding affinities of the specific ions and their influence on the ionization of the carboxyl groups of the protein molecule. Charlwood (9) studied the variations in the electrophoretic patterns of human serum albumin produced by various organic buffer anions at constant pH (4.0 ± 0.05), ionic strength (0.02), and protein concentration (2%). This investigator concludes that the effects observed, whether or not partly dependent on buffer capacity, must involve specific interaction between buffer anions and protein and that his experimental evidence supports the equilibrium hypothesis of Phelps and Cann (4, 14–16), i.e., \( P \text{(slow)} + HAc \rightleftharpoons PHAc \text{(fast)} \).

From the data presented in Fig. 5, it is evident that when the albumin contains bound lipid, increasing the ionic strength increases the area under the second peak in relation to the first peak but decreases that under the third peak. Therefore lowering the ionic strength in acetate buffer from 0.10 to 0.02 enhances the area under the third peak but it is still not clearly resolved. Addition of NaCl to an extent of 90% at the same pH and at even higher ionic strength (0.04) reduces the amount of undissociated acid present and results in a clearly defined third boundary as is illustrated in Fig. 3C. Removal of the second peak by prior ether treatment serves only to reduce slightly the area under the third peak which still remains clearly resolved from the stationary boundary. These results confirm those of Phelps and Cann (4, 14–10) and Charlwood (9) and are explainable on the basis that this third peak constitutes a boundary which results from the binding of undissociated acetic acid (4, 16), although the extent of the binding may be influenced by the kind of buffer anions present in solution (9, 15).

Schmid (35) published the preparation of a “new” form of albumin from human serum (Fraction VI) which was shown to be electrophoretically homogeneous at pH 4.0 acetate buffer, 0.1 ionic strength. This homogeneity is lost after treatment with cysteine followed by deionization of the sample with ion exchange resins. As is illustrated in Fig. 6, our ether-treated sample of Fraction V albumin behaved in a similar manner. Since Schmid carried his albumin through a series of precipitations with heavy metal salts, the possibility exists that some bound fatty acid was removed during the various preparative states. Pending the preparation of an albumin sample with Schmid's method, no further comparative tests were performed with the ether treated sample.

In a subsequent paper Schmid (39) performed a systematic study of the effect of organic acids and alcohols upon the electrophoretic behavior of human serum albumin (Cohn Fraction V) at pH 4.0 and 0.1 ionic strength. He concluded that the interaction between albumin and the undissociated or dissociated form of organic acids seems to be of primary importance in modifying the electrophoretic behavior of albumin at pH 4.0. Although our results, as illustrated in Figs. 4 and 6 are in general agreement with his, the albumin sample used by Schmid contained both bound lipids as well as tightly bound stabilizers.

The data in Fig. 7 depicting the patterns obtained with increasing molar concentration of oleate per mole of human serum albumin are quite similar to the results obtained by Aoki and Foster (11) for bovine serum albumin with respect to pH variation. An increase in the lipid to protein ratio resulted in an increased proportion of the slower migrating peak but the mobilities of the two boundaries remained nearly constant. When the moles of oleate bound per mole of albumin reached a value of about 5, only a single electrophoretic protein species of lower mobility was present in the system.

Goodman (32) had reported that low temperature isocanolic-acetic acid extraction of albumin caused no significant change in the physical or immunological properties of albumin. However, it should be emphasized that a small amount of lipid, i.e., about 0.5 mole per mole of albumin, still remains firmly bound and according to Kendall (23) cannot be completely removed without denaturing the protein. Goodman (32) found a value of 0.10 mole of fatty acid per mole of protein after isocanolic-acetic acid extraction but our patterns would be influenced by the binding of lipids other than fatty acids as is shown in Fig. 4.

Gordon (40) found that the addition of oleate to serum at alkaline pH caused a linear increase in the mobility of albumin with increasing mole ratio up to a value of 3 or 4 to one. If one assumes that serum albumin already contains 2 to 3 moles of bound lipid per mole of albumin, then this value of the total bound lipid at which the mobility becomes constant is between 5 to 7 moles per mole of albumin as compared to a value of approximately 6 moles with our data. It is of interest to note that Goodman (25, 26, 32) concluded from his studies of the interaction of long-chain fatty acids with albumin, by means of partition analysis, that oleate binds to three classes of sites, \( n_1 = 2 \), \( n_2 = 5 \pm 1 \), and \( n_3 = 20 \) (with a large error). This would imply that mainly the first two classes of sites are involved in the binding of oleate to albumin and in producing changes in the electrophoretic patterns observed in the acid pH region in acetate buffer.

**DISCUSSION**

Luetscher (19) was the first to report that crystalline human and horse serum albumins showed two electrophoretic boundaries in acetate buffer of pH 4.0 and ionic strength 0.02. When he applied this technique to purified albumins derived from the sera of such pathological conditions as nephrosis and cirrhosis of the liver, Luetscher (41) found a reversal of the normal ratio of the areas under the two peaks formed under these conditions.

On the basis of the experimental findings reported in this paper it is now possible to offer a rational explanation for Luetscher's results for albumins derived from both normal and abnormal sera based on their different lipid-albumin ratios. If one assumes that normal serum albumin contains 1 to 2 moles of bound fatty acid, or other lipids, as has been reported by several investigators (21, 23, 26) then an electrophoretic pattern would be
obtained at pH 4.0, 0.10 ionic strength acetate buffer, in which the area under the first peak is considerably greater than that under the second peak as is shown in Fig. 7C. Any increase in the ratio of the lipid to protein, as would be expected to occur where there is a large decrease in the albumin to globulin ratio in the disease state, would result in an electrophoretic pattern in which the area under the second peak is greater than that under the first peak as can be seen in Fig. 7D. The application of these findings to the study of albumins isolated from the sera of patients with various chronic diseases is presently under investigation.

The pronounced variability of the second peak obtained in the low ionic strength electrophoresis of purified albumin samples in the acid pH region (Fig. 3B) has been observed over a 5-year period. Differences in the area under this peak were not only obtained with various human albumin samples but also with albumin from different mammalian species (2). The data in Figs. 3C, D, and E show that this boundary is partially removed by deionization and is almost completely removed by lipid extraction at low temperature with ether or isooctane. From these results one would expect an increase in the area under the second boundary to occur whenever additional substances which bind are added to an albumin preparation or when the albumin is isolated from serum with a high lipid to protein ratio. The addition of stabilizers such as sodium caprylate or sodium acetyltryptophanate to albumin solutions, which are not readily removed by dialysis, would result in an increased second boundary as compared to the same sample run without added stabilizers. The results obtained in our previous publication (2) showing marked differences in the area under the second boundary for different animal albumins is explainable on the basis that human serum has the highest lipid to protein ratio of the species tested. In partial confirmation of this finding, Burstein and Samaille (42) have reported that the β-lipoprotein level of human serum is much higher than in most animals.

The experiments in this paper demonstrate a profound effect of bound fatty acids and higher alcohols on the electrophoretic behavior of human serum albumin in acetate buffers and in NaCl-HCl at pH 4.0. As in the case of bovine serum albumin, the electrophoretic patterns of human serum albumin (Fig. 3B) in 0.04 ionic strength acetate buffer (90% NaCl) reflect two types of interaction of the protein with acidic media: (a) isomerization and (b) interaction with undissociated buffer acid. In contrast, only the isomerization reaction is observed in 0.1 M sodium acetate buffer and in 0.1 M NaCl-HCl (Figs. 5B and C). It can therefore be concluded that whereas bound fatty acids and higher alcohols exert a pronounced effect upon the isomerization reaction, they seem to have a relatively small effect on the interaction of the protein with undissociated buffer acid.

The data in Fig. 7 depicting the patterns obtained with increasing molar concentration of oleate per mole of human serum albumin are quite similar to those described by Aoki and Foster (11) for bovine serum albumin with respect to pH variation. One possible explanation of these data is that the protein solution is an equilibrium mixture of the type $P \rightleftharpoons P'$, the equilibrium position depending upon the amount of oleic acid bound. In that event, the slow peak might represent $P$ and the fast peak, $P'$. At low oleic acid to albumin ratio, the protein is largely in the form $P'$ (Fig. 7A); at high ratios, the protein existing largely in the form $P$ (Fig. 7E). It is conceivable that at any given oleic acid to albumin ratio, both isomeric forms contain the same mean amount of bound oleic acid. The theory of isomerization equilibrium and electrophoresis of Cann et al. (43) predicts that for a simple, unimolecular, isomerization reaction, two moving boundaries will be observed for times of electrophoresis less than, or of the order of, the half-time of the reaction. For longer times of electrophoresis a single moving boundary of intermediate mobility should be observed. However, it has been shown by Gilbert and Jenkins (44) that separation into two boundaries may occur even if the reaction is very rapid. Cann (4) has recently offered an explanation for the nonenantiorgraphic nature of the electrophoretic patterns based upon the formation of a complex of higher mobility between the protein and undissociated buffer acid, e.g. PHAc, and the differences in the readjustment of the equilibrium mixture when it moves into the protein solution in the descending limb as compared to its movement into buffer in the ascending limb.

The separation of bovine or human serum albumin into two or more discrete fractions by means of column chromatography has been reported by several investigators (45–47). The role which the bound lipids and buffer acids may play in the production of such discrete fractions is presently under investigation.

**Summary**

1. Human serum albumin (Column Fraction V) prepared by low temperature ethanol fractionation gives a single electrophoretic boundary in barbital buffer, pH 8.6, 0.10 ionic strength ($\mu$) but not in the acid pH region. Two ascending boundaries are obtained in pH 4.0 acetate buffer, 0.10 $\mu$ and three boundaries in acetic acid-NaCl (90%) buffer at pH 4.2, 0.04 $\mu$ (exclusive of the stationary or delta boundaries). There is marked asymmetry between the ascending and descending boundaries.

2. Removal of lipid from the albumin preparation by means of ether or isooctane-acetic acid extraction at low temperature yields samples which give a single ascending boundary in both 0.10 $\mu$ acetate (pH 4.0) and barbital (pH 8.6) buffer systems. Two boundaries are still obtained with 0.04 $\mu$ acetate-NaCl (90%) buffer but the slowest moving boundary results from the influence of the binding of buffer acids to the protein.

3. In acetate-NaCl buffer, pH 4.2, 0.04 $\mu$, an increase in albumin (Fraction V) concentration causes a corresponding increase in the relative areas under the first and third peaks but that under the second peak remains constant. The first two peaks in these patterns appear to arise as the result of an isomerization reaction (P = $P'$) which is sensitive to bound lipids.

4. When 0.10 $\mu$ acetate buffer is used to eliminate the third boundary, then the addition of dodecane, caprylic, or oleic acids to other-treated albumin (Fraction V) causes the second boundary to reappear. The increase in the area under the slower boundary ($P$) and the decrease under the faster first boundary ($P'$) is directly proportional to the moles of lipid (oleate) bound per mole of albumin. At a molar ratio of about 5.0, all the albumin is present as the isomeric form $P$ which migrates as a single electrophoretic boundary.

5. Deionization of the albumin (Fraction V) preparation by passage through an ion exchange resin column (Diolac) serves to eliminate most but not all of the second peak ($P$) as a result of a shift in equilibrium upon removal of lipids. At a constant lipid-protein ratio, the area under the second peak increases with increasing ionic strength in acetate buffer, pH 4.0. When an
ether-treated albumin sample is used, a single ascending boundary is obtained regardless of the variation in the ionic strength, i.e. from 0.02 to 0.10.

6. The ether-treated albumin (Fraction V) preparation resembles the "new" kind of albumin prepared by Schmid (35) from Cohn Fraction VI by precipitation with heavy metal salts in that 16. of interaction of the protein with acidic media; (a) isomerization and (b) interaction of undissociated buffer acid. In contrast, have a relatively small effect on the interaction of the protein 0.04 ionic strength acetate buffer (90% NaCl) reflect two types ionization, two peaks are obtained.

8. In 0.1

7. The electrophoretic patterns of human serum albumin in 0.04 ionic strength acetate buffer (90% NaCl) reflect two types of interaction of the protein with acidic media; (a) isomerization and (b) interaction of undissociated buffer acid. In contrast, only the isomerization reaction is observed in 0.1 M acetate buffer with undissociated buffer acid. It can be concluded from this study that, whereas bound fatty acids and higher alcohols exert a pronounced effect upon the isomerization reaction, they seem to have a relatively small effect on the interaction of the protein with undissociated buffer acid.

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