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Isoelectric Focusing Behavior of Bovine Plasma Albumin, Mercaptalbumin, and β-Lactoglobulins A and B*

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ABSTRACT: The basic causes for the microheterogeneity of plasma albumin have remained obscure, and, in fact, there is not even an estimation of the number of species involved. This study was undertaken in the hope that isoelectric focusing might help answer these questions. To this end, crystallized bovine plasma albumin and mercaptalbumin were studied extensively by isoelectric focusing in a sucrose-stabilized column and in polyacrylamide gels. β-Lactoglobulin provided a standard of comparison because it focused well under all conditions employed, yielding two sharp, fairly symmetrical peaks corresponding to isoelectric pH values of 5.21 for the A and 5.34 for the B genetic variants. Difficulties were encountered with albumin samples which are attributed to the propensity of this protein to bind various trace impurities in the ampholyte mixture and in polyacrylamide gels. Focusing on washed gels yielded essentially the same results as were obtained on the column using sucrose density gradients. Monomeric, charcoal-defatted albumin yielded a main peak at pH 5.28 with shoulders on both high and low pH sides plus a broad peak at pH 4.8. Purified mercaptalbumin gave the main peak, essentially devoid of the shoulders, but also gave rise to the peak at 4.8.

Evidence is presented that the low pH component resulted from an interaction of the protein with a minor constituent of the ampholyte mixture. The results indicate that the purified mercaptalbumin is as homogeneous, by the electrofocusing criterion, as either of the β-lactoglobulin variants. The nonmercaptalbumin components clearly make a major contribution to the microheterogeneity of plasma albumin preparations.

Many investigators have demonstrated that plasma albumin preparations are not homogeneous. One aspect of the heterogeneity is associated with the nonintegral sulfhydryl content which has been shown to be due to the presence of two types of albumin: mercaptalbumin and nonmercaptalbumin. The nonmercaptalbumin has been shown to consist of mixed disulfides of cysteine and glutathione (King, 1961; Andersson, 1966). Another type of heterogeneity involves the state of aggregation of the albumin molecules. Plasma albumin can exist as monomer, dimer, trimer, and higher polymers. These forms can be readily separated by Sephadex exclusion chromatography as was first shown by Pederson (1962). Microheterogeneity has been demonstrated by electrophoresis at low pH (Sogami and Foster, 1963), by pH-solubility studies (Foster *et al.*, 1965; Petersen and Foster, 1965), and by salting out studies (Wong and Foster, 1969a, 1969b). Evidence has been presented that the presence of bound impurities (McMenamy and Lee, 1967; Sogami and Foster, 1968) and fluctuations in disulfide pairing (Sogami *et al.*, 1969) contribute to the observed microheterogeneity.

Isoelectric focusing (electrofocusing) in natural pH gradients, as described by Vesterberg and Svensson (1966), has proven valuable in studying the heterogeneity of myoglobin (Vesterberg and Svensson, 1966), cytochrome *c* (Flatmark and Vesterberg, 1966), and a number of other proteins (Vesterberg, 1968). Electrofocusing experiments have been performed in either sucrose-stabilized density gradients (Vesterberg and Svensson, 1966) or in polyacrylamide gels (Dale and Latner, 1968; Wrigley, 1968) where the pH gradient is established by a system of synthetic low molecular weight ampholytes called "carrier ampholytes." These experiments yield information on the isoelectric spectrum as well as the isoelectric pH of the sample.

Isoelectric focusing was used in this investigation in an attempt to examine the heterogeneities or microheterogeneities of charcoal-defatted monomeric bovine plasma albumin and of purified bovine mercaptalbumin. The β-lactoglobulin genetic variants A and B provided a convenient system by which to calibrate the electrofocusing technique. The reported isoionic points of the β-lactoglobulins are similar to that of plasma albumin, so that focusing in the same pH range was possible.

Experimental Section

Materials. Crystallized bovine plasma albumin, lot D71209, was obtained from Armour Pharmaceutical Co. This lot con-

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tained approximately 5% dimer, determined by densitometric measurement of disc electrophoresis gels. The sulfhydryl titer was 0.65 as determined by the Ellman (1959) procedure. Armour crystallized bovine plasma albumin, lot F71601, and Armour fraction V bovine plasma albumin, lot F32101, were used to prepare mercaptalbumin.

Crystallized bovine β-lactoglobulin was obtained from Pentex, Inc. Lots 36 and 38, which contained both β-A[1] and β-B, were employed to prepare samples of the pure genetic variants.

Ampholine carrier ampholytes, LKB 8152, were obtained from LKB Produkter AB as 40% solutions. Batch numbers 9, 10, 11, and 12, covering the pH range 4–6 were employed.

Mann Ultra Pure sucrose was employed in the density gradients, while Mallinckrodt analytical reagent grade sucrose was used in the electrode solutions and in all other cases. Acrylamide and other gel reagents were from Eastman Organic Chemicals. The acrylamide was recrystallized from hot chloroform before use. All other chemicals were the purest analytical reagent grade available.

Darko KB-activated charcoal from Atlas Chemical Industries, Inc., was used after extensive washing with deionized water. Sephadex G-150 and the 2.5 × 100 cm Sephadex column, equipped with upward-flow adaptors, were from Pharmacia Fine Chemicals, Inc. Visking cellophane casing, obtained from Union Carbide Corp., was used as dialysis tubing after repeated boiling in half-saturated sodium bicarbonate and extensive washing with deionized water. Deionized water with a specific conductivity of less than 10^{-6} mho was obtained by passing distilled water through a Bantam demineralizing column (Model BD-1, Barnstead Still and Sterilizer Co.).

Preparations. Commercial samples of crystallized bovine plasma albumin were defatted by the charcoal adsorption method of Chen (1967) as modified by Sogami and Foster (1968). Monomeric albumin was prepared by Sephadex G-150 exclusion chromatography with 0.1 N KCl as eluent. The protein was stored in a 0.1 N KCl solution in the cold room for immediate use or was lyophilized and stored as a powder for later use.

Mercaptalbumin samples were prepared from either crystallized charcoal-treated bovine plasma albumin, or from charcoal-treated fraction V employing a novel procedure consisting of a separation on SE-Sephadex with carefully controlled ionic strength and temperature. Details of this procedure have been described recently (Hagenmaier and Foster, 1970).

The β-lactoglobulin genetic variants A and B were obtained from the commercial sample by a modification[2] of the procedure by Piez *et al.* (1961).

Isoelectric Focusing in Column. Isoelectric focusing in the LKB 8102 column was performed essentially as described by Vesterberg and Svensson (1966) and as outlined in the preliminary instruction sheet from LKB. A few modifications that were employed and the essential details of the electrofocusing will be presented.

The preparation of the sucrose (Mann Ultra Pure) density gradient was done manually. The appropriate volume of protein sample (previously dialyzed against 0.5% ampholyte solution) containing 30–35 mg of protein was introduced in place of the corresponding volume of less dense solution. The anode solution of sulfuric acid was placed at the bottom of the column. The fractions were introduced sequentially into the column and the ethylenediamine cathode solution was applied at the top.

During electrofocusing, the column was maintained at 4° with a Lauda-Brinkman K-2/R circulator. An initial potential of 300 V was maintained for 3–5 hr. The voltage was then increased stepwise to obtain a limiting value of 800 V in 10–15 hr. The total electrofocusing time was approximately 48 hr. The time needed to achieve the steady state was experimentally determined by plotting mA *vs.* time at the limiting voltage. The current decreased rapidly at first and then approached a limiting value of 4.0–4.5 mA in 30–36 hr.

The contents of the column were removed at a constant flow rate of 2 ml/min which was maintained with a peristaltic pump. To avoid disturbances caused by the pulsing of the pump, it was not attached to the outlet of the column. Instead, the space remaining above the upper electrode solution was filled with water and water was pumped from a reservoir into the upper gas outlet forcing the contents out of the column outlet. The effluent was monitored with a Gilford 2000 spectrophotometer at 279 mμ. Fractions (30 drop) were collected and analyzed individually for pH and absorbance at 279 mμ. Some of the fractions were pooled to give samples suitable for further analysis. The pooled samples were exhaustively dialyzed to remove the ampholytes and sucrose and were lyophilized to concentrate the protein.

Isoelectric Focusing in Polyacrylamide Gels. Isoelectric focusing in polyacrylamide gels has been reported by a number of workers (Dale and Latner, 1968; Wrigley, 1968; Awdeh *et al.*, 1968). The procedure of Dale and Latner (1968) in which the protein is initially distributed throughout the gel was first employed but later abandoned due to an apparent interaction of the protein and some component of the gel. A modification of Wrigley's (1968) procedure was employed with good results. The gels were prepared in Plexiglas tubes (12 × 0.6 cm, i.d.) from 2 ml of 28% acrylamide, 0.73% *N,N'*-methylene bisacrylamide, 1 ml of 0.23% *N,N,N',N'*-tetramethylethylenediamine (TMED), 4 ml of 0.14% ammonium persulfate, and 1 ml of water. The gels were then removed from the tubes, dialyzed profusely with deionized water, equilibrated with an excess ampholyte solution (25 ml of 1% for two gels), and reinserted into the Plexiglas tubes. The actual electrofocusing was conducted as described by Wrigley, that is, the sample was layered under a protective ampholyte–sucrose layer and focused for 4–5 hr with a limiting potential of 350 V.

After electrofocusing the gels were removed from the tubes, washed with 10% trichloroacetic acid to remove the ampholytes, stained with 0.5% Amido Schwartz in 7% acetic acid, and destained transversely with 7% acetic acid. An alternative procedure for analyzing the gels after electrofocusing was also employed. After soaking briefly in trichloroacetic acid, they were scanned at 279 mμ in the Gilford 240 spectrophotometer equipped with a linear transport. This gave a direct quantitative measure of the components present and eliminated the time-consuming trichloroacetic acid wash, staining, and destaining.

Other Techniques. Optical rotatory dispersion was performed at 27° with the Cary 60 recording spectropolarimeter using a 0.1-cm Cary quartz cell. The specific rotation was calculated from the observed rotation at the 233-mμ trough by use of the equation, $[\alpha] = 100\alpha/dc$, where $[\alpha]$ is the specific rotation, α is the observed rotation in degrees at a given wavelength, d is the path length, in decimeters, and c is the concentration of solute in g/100 m of solution.

[1] Abbreviations used are: β-A, β-lactoglobulin A; β-B, β-lactoglobulin B.

[2] S. D. Stroupe and J. F. Foster, to be published.

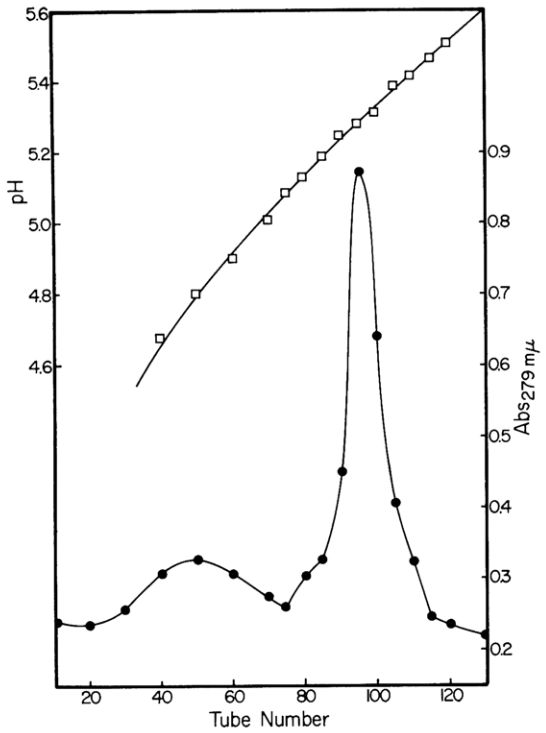

FIGURE 1: Isoelectric focusing of charcoal-treated, monomeric albumin. Experimental conditions were: 33 mg of bovine plasma albumin, 4–6 pH range, 1% ampholyte, 48 hr, 800 V, 4.2 mA; squares, pH gradient; filled circles, absorbance 279 mμ.

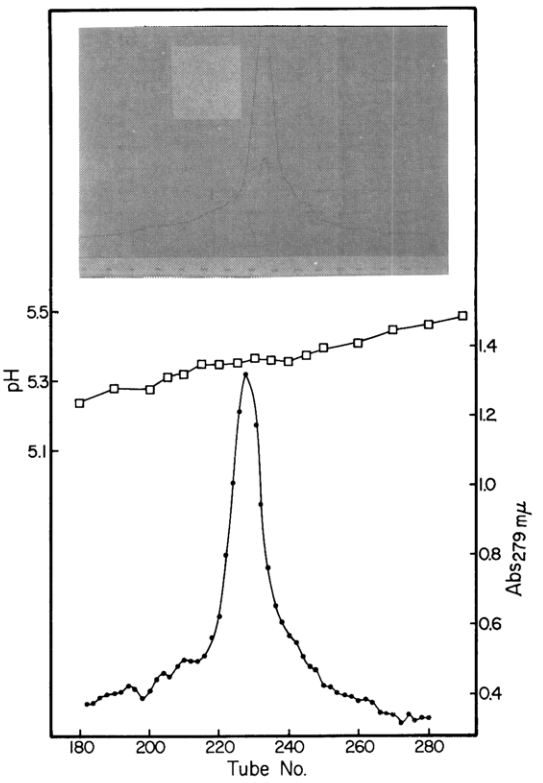

FIGURE 2: Isoelectric focusing of charcoal-treated, monomeric albumin in one pH unit gradient. Experimental conditions were: 57 mg of bovine plasma albumin, 4.7–5.7 pH gradient, final ampholyte concentration, 1%, 64 hr, 1000 V, 3.0 mA; squares, pH gradient; points, absorbance 279 mμ; inset at top, elution diagram from Gilford 2000 spectrophotometer.

Determinations of pH were made with a Radiometer Type 25 pH meter equipped with a scale expander and a Radiometer GK 2321C combined electrode. Measurements were made at room temperature and Sargent standard buffers, pH 4.01 and 7.00, were used to standardize the pH meter.

The concentration of bovine plasma albumin and β-lactoglobulin solutions was routinely determined with the Hitachi Perkin–Elmer 139 UV-VIS spectrophotometer assuming $\epsilon^{1\%}_{1\text{ cm}}$ 6.67 at 279 mμ for bovine plasma albumin and 9.6 at 278 mμ for β-lactoglobulin.

Disc gel electrophoresis was conducted using a modification of Broome's (1963) modification of the original procedure of Ornstein (1964) and Davis (1964). Sephadex G-150 previously equilibrated with Tris-glycine buffer was layered on top of the gel. Then 15–20 μg of the protein sample was applied directly into the Sephadex.

Results

Electrofocusing of Plasma Albumin. The initial isoelectric focusing experiments were designed to obtain isoelectric spectra of charcoal-treated, monomeric bovine plasma albumin. Figure 1 shows a spectrum, typical of these experiments, characterized by a main peak at pH 5.28 ± 0.03 with acidic and basic shoulders and an additional broad peak at pH around 4.8. Due to the known background absorbance of the sucrose and the ampholytes, it was difficult to ascertain precisely which peaks and shoulders were due to background and which to protein. The problem was eventually solved by employing disc gel electrophoresis as an assay for the protein. The protein migrated normally on the disc gel while the ampholytes migrated with the tracking dye and were stained at the bottom of the gel. (This procedure was also used to assay for free ampholytes.) Disc gel electrophoretic analysis confirmed the existence of a protein peak at pH 4.7–4.8. In fact, if the disc gels used to analyze various electrofocusing fractions were stained and scanned in a densitometer, a plot of the area *vs.* tube number was in very good agreement with the electrofocusing plots of absorbance *vs.* tube number. A major peak at 5.28 with acidic and basic shoulders and an additional peak at low pH were obtained by disc electrophoresis analysis of the electrofocused bovine plasma albumin. This low pH protein was thought to be an experimental artifact and an extensive investigation was undertaken to characterize it. These results will be presented later.

Further electrofocusing of bovine plasma albumin in a narrower pH gradient (4.7–5.7) was performed to increase the resolution of the components present. In Figure 2, the isoelectric spectrum shows more pronounced shoulders than previously obtained. The main peak corresponded to a pH of 5.35.[3] The inset at the top of Figure 2 shows the ultraviolet monitor trace of the column effluent. The shoulders can be seen more readily here than with the absorbance determined on individual fractions.

Since the column electrofocusing experiments were time consuming a system for rapidly analyzing multiple samples was developed utilizing isoelectric focusing in polyacrylamide gels. The initial experiments, conducted in both riboflavin–

[3] This relatively high value for the main peak position was found only in this single experiment with the narrow pH gradient. Several experiments with the pH 4–6 gradient yielded reproducible values of 5.28 ± 0.03. No explanation for the discrepancy can be offered.

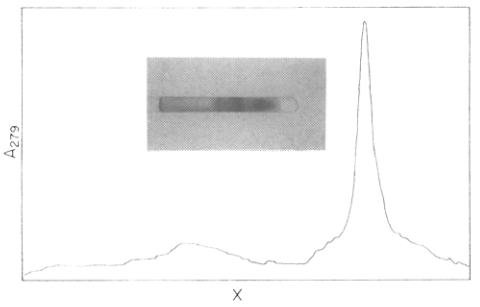

FIGURE 3: Gel electrofocusing of charcoal-treated, monomeric albumin. The inset is a photograph of the stained gel. The ultraviolet scan of the gel before staining is shown as A_{279} *vs.* coordinate along the gel (×) for the region of the bands only. The pH gradient is from left to right.

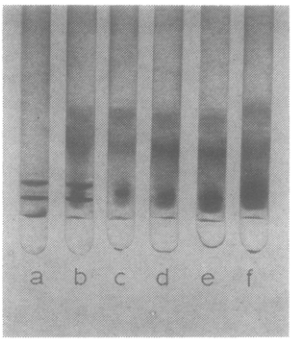

FIGURE 4: Isoelectric focusing in washed gels of β-lactoglobulins A and B and charcoal-treated, monomeric albumin: (a) mixture of 30 μg of β-A and 30 μg of β-B; (b) mixture of 30 μg of β-A, 30 μg of β-B and 90 μg of bovine plasma albumin; (c) 90 μg of bovine plasma albumin; (d) 120 μg of bovine plasma albumin; (e) 170 μg of bovine plasma albumin; (f) 215 μg of bovine plasma albumin. Anode at the top.

light polymerized gels and in ammonium persulfate polymerized gels, did not produce good results with bovine plasma albumin. Bovine plasma albumin focused into a broad, poorly defined band considerably acidic to the well-focused β-lactoglobulins. Since an interaction of impurities in the polyacrylamide with the albumin was suspected, further gel electrofocusing was performed according to the procedure of Wrigley (1968) and a modification of that procedure.

Isoelectric focusing on prefocused gels had the advantages of partially establishing the pH gradient and removing charged impurities before applying the sample. While β-lactoglobulin focused very well with this technique, bovine plasma albumin still focused rather poorly although the bovine plasma albumin pattern was greatly improved with two distinct bands and a number of weak, poorly defined bands between them. The major band focused between the β-lactoglobulins while an intense secondary band focused in a more acidic position.

Since prefocused gels resulted in some improvement, washed gels were employed with the hope that all of the impurities could be removed with extensive washing. Electrofocusing on washed gels is shown in Figure 3. Bovine plasma albumin focused quite well and yielded a spectrum that was essentially the same as the results obtained in the column electrofocusing. The main band focused between the β-lactoglobulin genetic variants (Figure 4b) with additional smeared bands at lower pH.

An experiment on washed gels with varying amounts of albumin was performed to test the possibility that the ampholytes, or impurities in the ampholytes, were interacting with the albumin. As Figure 4c shows, the low pH band formed predominantly when small quantities of albumin were used. As larger amounts of albumin were focused (at a fixed ampholyte concentration), the main band increased in intensity, while the low pH band reached a constant intensity (Figure 4d,e,f). This result indicated that something was still present, in a limited amount, which interacted with the protein.

Since essentially the same results were obtained with the column and gel electrofocusing, and since the ampholyte solution was the only common component besides albumin, the ampholytes or impurities in the ampholyte solution were considered to be the cause of the "ampholyte-modified protein."

Electrofocusing of β-Lactoglobulin. A second series of column isoelectric focusing experiments employed β-lactoglobulins A and B in an attempt to demonstrate the resolving power of the technique. The results of focusing approximately equal quantities of β-A and β-B (mixed together prior to application to the column) are shown in Figure 5. The charge difference between the β-lactoglobulins is caused by an amino acid replacement which gives β-A an additional carboxylic acid. Since the pK of this group is estimated to be 4.66 (Basch and Timasheff, 1967), the actual charge difference between β-A and β-B at the isoelectric point is between one and two charge units per 36,000 molecular weight. The separation achieved was very good. The peaks were fairly symmetrical and corresponded to isoelectric pH values of 5.21 ± 0.02 for β-A and 5.34 ± 0.02 for β-B.

Focusing of β-lactoglobulin on washed gels is shown in Figure 4a. The bands obtained were quite sharp and well separated. The gel in Figure 4b compares the β-lactoglobulin and bovine plasma albumin focusing. That the bovine plasma albumin band shown in Figure 3 is quite broad becomes obvious from this comparison.

Electrofocusing of Mercaptalbumin. In an attempt to identify the shoulders on the main bovine plasma albumin peak,

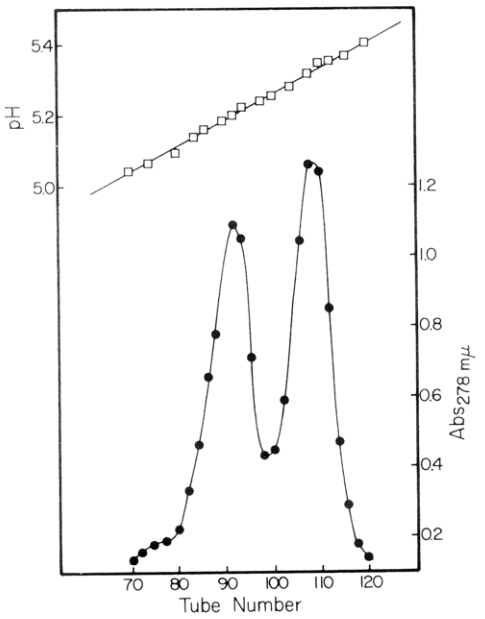

FIGURE 5: Isoelectric focusing spectrum of β-lactoglobulins A and B. Experimental conditions were: 26 mg of β-A, 28 mg of β-B, 4–6 pH gradient, 1% ampholyte, 45 hr, 800 V, 4.5 mA; squares, pH gradient; filled circles, absorbance 279 mμ.

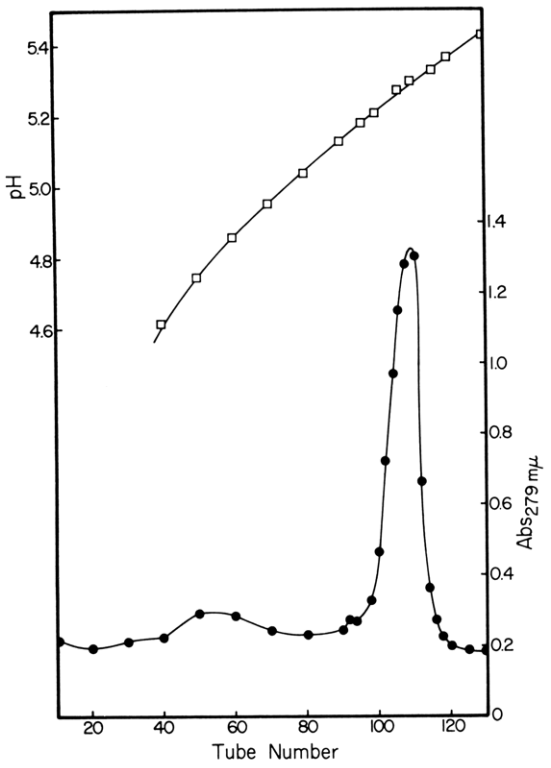

FIGURE 6: Isoelectric focusing of charcoal-treated, monomeric mercaptalbumin prepared from fraction V. Experimental conditions were: 36 mg of bovine mercaptalbumin, 4–6 pH gradient, 1% ampholyte, 43 hr, 800 V, 4.2 mA.

the isoelectric focusing of bovine mercaptalbumin was investigated (Figure 6) and compared to the electrofocusing behavior of the charcoal-treated monomeric bovine plasma albumin samples which contained both mercaptalbumin and nonmercaptalbumin. Similar quantities of each were focused in a 4–6 pH gradient as shown in Figures 1 and 6. The shoulders present in the bovine plasma albumin spectrum were absent in the bovine mercaptalbumin spectrum and the bovine mercaptalbumin had focused into a more concentrated peak with p*I* of 5.30. The low pH (4.7–4.8) peak was, however, still present as in the case of bovine plasma albumin.

Mercaptalbumin was also studied by gel electrofocusing (Figure 7). Very good agreement between the column and gels

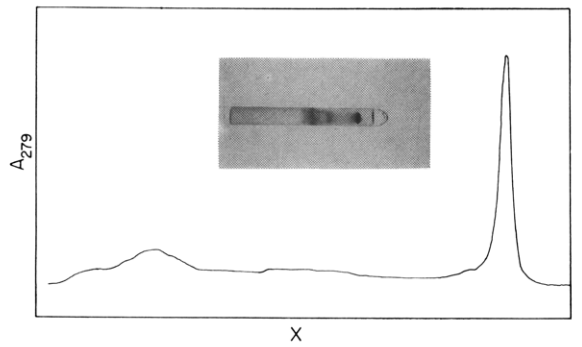

FIGURE 7: Gel electrofocusing of charcoal-treated, monomeric mercaptalbumin. The inset is a photograph of the stained gel. The ultraviolet scan of the gel before staining is shown as A_{279} *vs.* coordinate along the gel (×) for the region of the bands only. The pH gradient is from left to right.

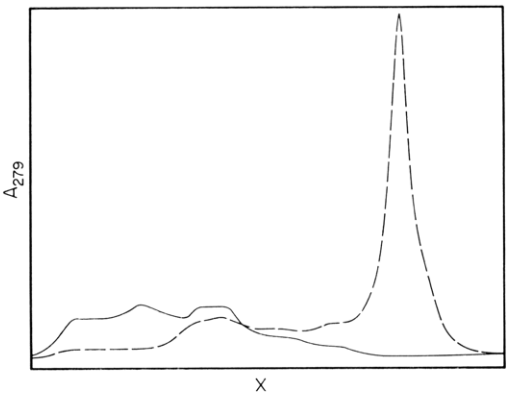

FIGURE 8: Refocusing of ampholyte-modified albumin and albumin from the main peak. Solid line (ampholyte modified albumin) and dashed line (main peak albumin) are ultraviolet scans of gel before staining given as A_{279} *vs.* position along the gel (×) for region of the bands only. The pH gradient is from left to right.

was obtained. The protein band was quite sharp, lacking the minor bands present with bovine plasma albumin. The low pH bands were, however, still present.

Characteristics of Ampholyte-Modified and of Main Peak Albumin. Since the low pH material was present with both albumin preparations, a further study was undertaken to characterize the protein in this region. Two protein samples were obtained by pooling the fractions from the main peak and low pH region, respectively. Disc gel electrophoresis on these samples after extensive dialysis and lyophilization indicated the absence of free ampholytes. Figure 8 shows a scan of the gels on which these samples were refocused. The dashed line, the spectrum obtained upon refocusing protein from the main peak, shows the normal pattern of main peak and low pH peak. With low pH material, refocusing yields only low pH protein. No main peak protein is regenerated.

To further characterize the ampholyte-modified protein, optical rotatory dispersion was employed. While the dispersion curves were qualitatively the same, the ampholyte-modified protein had a greatly reduced trough at 233 mμ. The specific rotation of this protein was approximately −6600, while the protein from the main peak had a specific rotation of −9300, the value for native bovine plasma albumin. The ampholyte-modified protein, which arose from the mercaptalbumin was also investigated to determine its refocusing behavior on gels and its optical rotatory dispersion properties. The results were essentially the same as those obtained above. The main peak protein yielded a normal pattern while the ampholyte-modified protein gave only a broad low pH band. The specific rotation at 233 mμ for the ampholyte-modified protein was −6600, while −9200 was obtained for the main peak protein.

Discussion

While much evidence for microheterogentity of plasma albumin has been brought forward, the basic causes remain obscure. In particular, it is by no means clear how many contributing species are involved, even as to the order of magnitude. This study was undertaken in the hope that the highly sensitive electrofocusing method might shed some light on this question. If the resolving power of isoelectric focusing were sufficient to distinguish between the species, discrete peaks would be expected. If electrofocusing were unable to resolve

the components, a (presumably broad) continuum would be obtained. To this end, charcoal-treated, monomeric plasma albumin and charcoal-treated, monomeric mercaptalbumin were extensively studied by isoelectric focusing in natural pH gradients in sucrose-stabilized density gradients and in polyacrylamide gels. The β-lactoglobulins provided an internal standard with which to calibrate the electrofocusing technique.

The isoelectric spectrum of bovine plasma albumin showed a main peak at pH 5.28 with a number of acidic and basic shoulders and a broad peak at pH 4.7–4.8. In the column experiments, the background absorbance of the ampholytes and sucrose caused a serious problem in detecting minor protein components. Disc gel electrophoresis proved useful to detect these minor components. In fact, analysis of the fractions from the electrofocusing column by this method indicated that small but significant quantities of albumin were present from pH 4.6 to 5.6. An analogous spectrum was obtained by Reis and Wetter (1969) who focused human serum albumin and obtained a number of peaks between 4.7 and 5.7. Since Vesterberg and Svensson (1966) have shown that the isoelectric pH determined in electrofocusing is also the isoionic point, comparison of these results is made to reported isoionic points. The value obtained in this investigation for the main peak, 5.28 ± 0.03, is in good agreement with previously reported isoionic pH values which range from 5.1 to 5.4 (Dintzis, 1952; Dandliker, 1954). As is to be expected, since bovine plasma albumin strongly binds anions, isoelectric points determined by moving-boundary electrophoresis are lower than that determined by electrofocusing.

In contrast to bovine plasma albumin, β-lactoglobulins A and B produced well-resolved, fairly symmetrical peaks in the column. The isoelectric points of 5.21 for β-A and 5.34 for β-B were reproducibly obtained. These values agree quite well with the values previously reported by Treece *et al.* (1964) of 5.23 for β-A and 5.30 for β-B. They also agree well with the values reported by Tanford and Nozaki (1959) and Basch and Timasheff (1967).

In order to avoid the time-consuming column electrofocusing and to focus multiple samples simultaneously, electrofocusing on polyacrylamide gels was adopted. Unfortunately, an interaction of bovine plasma albumin with some component of the gels necessitated the use of washed gels. Both bovine plasma albumin and β-lactoglobulin focused well on the washed gels yielding isoelectric spectra very similar to those obtained in the column experiments. β-Lactoglobulin focused into sharp well-defined bands, while bovine plasma albumin focused into a main band with shoulders on both acidic and basic sides and a broad low pH band.

An investigation of mercaptalbumin was undertaken in an attempt to identify some of the shoulders and minor bands obtained with bovine plasma albumin. The isoelectric spectrum of bovine mercaptalbumin consisted of a main peak at pH 5.30 devoid of shoulders and a broad peak centered about 4.8. Essentially the same results were obtained on washed polyacrylamide gels. A direct comparison of the column and gel results for bovine plasma albumin can be made from Figures 1 and 3. For bovine mercaptalbumin, a similar comparison can be made from Figures 6 and 7.

A number of results established that the low pH component is an artifact resulting from the interaction of the protein with some minor constituent of the ampholyte mixture. For example, this component is generated when main peak protein from a column-focusing experiment is refocused on fresh gels. The component migrates with native bovine plasma albumin on disc gel electrophoresis and exhibits an ultraviolet absorption spectrum characteristic of proteins. Additional evidence comes from focusing increasing amounts of protein at a fixed ampholyte concentration (Figure 4c–f). The ampholyte-modified component reaches a limiting value while the main peak continues to increase.

A study was undertaken to characterize the ampholyte-modified albumin. The samples pooled from the column electrofocusing were extensively dialyzed to remove the ampholytes and sucrose and then lyophilized. Optical rotatory dispersion studies indicated that the ampholyte-modified albumin had considerably less-ordered structure ($[\alpha]_{233}$ −6600°) than native albumin ($[\alpha]_{233}$ −9300°). Refocusing the ampholyte-modified protein on washed gels indicated that no native albumin was generated. These results indicated a profound, irreversible alteration of the albumin molecules probably resulting from a tenacious interaction with a minor constituent of the ampholyte mixture.

A recent paper by Vesterberg (1969) reporting the complete separation of ampholytes from human serum albumin with G-50 Sephadex might appear to contradict the conclusions presented here. However, Vesterberg's experimental conditions did not resemble the isoelectric focusing conditions. For example, small quantities of radioactively labeled pH 3–10 ampholyte were mixed with the albumin and then applied to the Sephadex column which was equilibrated with a pH 7 phosphate buffer and 0.5 M NaCl. While it is felt that the experiments reported by Vesterberg demonstrate a convenient, rapid method of separating ampholytes and protein, they do not invalidate our conclusion that some minor component of the ampholyte mixture interacts strongly with, and irreversibly modifies, the protein.

While Reis and Wetter (1969) observed an isoelectric spectrum with human serum albumin similar to that obtained in this investigation with bovine plasma albumin, they failed to recognize that some of the components might be artifacts of the experimental procedure. In general, however, their data provide considerable support for the conclusions presented here, since the relative amounts of the two main peaks they observed at pH 5.5 and 4.7 were dependent upon the ampholyte to protein ratio. In addition, since they identified the albumin by its specific immunoelectrophoretic behavior, independent evidence is provided to substantiate the claim that the low pH component contains albumin.

In spite of the presence of the ampholyte-modified albumin, an important conclusion can be drawn by comparing the isoelectric spectra of bovine plasma albumin and bovine mercaptalbumin. It becomes obvious that the nonmercaptalbumin components make an important contribution to the microheterogeneity of plasma albumin. The importance of this conclusion can be appreciated when one considers that, as recently as the work by Wong and Foster (1969a,b), microheterogeneity was thought to be independent of the sulfhydryl heterogeneity. A direct comparison of the isoelectric spectra of bovine mercaptalbumin and β-lactoglobulins A and B is given in Figure 9 in the form of plots of absorbance *vs.* pH. The bovine mercaptalbumin spectrum is essentially gaussian and at least as sharp as that of either of the β-lactoglobulin variants. In view of these results it would be easy to reach the conclusion that the microheterogeneity of bovine plasma albumin resides only in the nonmercaptalbumin component and that bovine mercaptalbumin is a homogeneous protein. These results appear to support the conclusion drawn in a recent paper by Andersson (1969) based on solubility studies in the acid pH range, that mercaptalbumin is a homogeneous protein. The

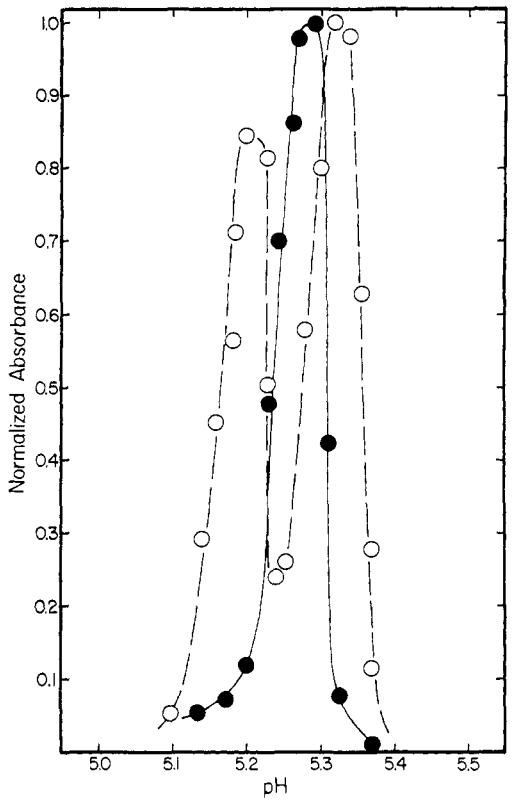

FIGURE 9: Isoelectric spectra of mercaptalbumin and β-lactoglobulins A and B plotted as normalized absorbance *vs.* pH: open circles, β-A (left) and β-B (right); filled circles, bovine mercaptalbumin. Normalization was achieved by dividing the observed absorbance by the maximum observed absorbance in each experiment.

present results indicate that if bovine mercaptalbumin is a microheterogeneous protein, the molecular forms must be so closely similar that they are not resolvable by the isoelectric focusing method, at least at the current stage of its development. Additional studies on the microheterogeneity of mercaptalbumin have recently been reported by Hagenmaier and Foster (1971) and are in accord with conclusions drawn here.

Added in Proof

Frater (1970) has recently presented evidence for the formation of protein–ampholyte complexes in a study of the isoelectric focusing behavior of an acidic wool protein.

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