Isoelectric Heterogeneity of Bovine Plasma Albumin*  

E. Martin Spencer† and Te Piao King  
From the Rockefeller University, New York, New York 10021

SUMMARY

Chromatography of bovine plasma albumin (BPA) on diethylaminoethyl cellulose resulted in a single asymmetric peak with considerable tailing. An identical result was obtained with half-cystinyl bovine plasma albumin (Cys-BPA), in which the single sulfhydryl group of BPA is in the form of a mixed disulfide with L-cysteine. This chromatographic behavior was related to the presence of albumins with different isoelectric points as revealed by isoelectric focusing of Cys-BPA on acrylamide gel containing 6 M urea. The two major components, about 76 and 20% of the sample, differed by 1 unit charge. This was shown by comparative isoelectric focusing studies of Cys-BPA and its mono\(^{14}C\)-acetylated derivative. The mono\(^{14}C\)-acetylated albumin and possibly the di- and triacetylated derivatives were prepared by the reaction of \(^{14}C\)-acetylsalicylic acid with Cys-BPA.

Investigation of chromatographic cuts, enriched with the different isoelectric components of Cys-BPA, indicated that these components were noninterconvertible on reversible denaturation or on treatment with thiol reagents, and that these components differed in their thermal and acid denaturation properties.

The isoelectric heterogeneity of Cys-BPA is not a result of heterozygosity of the structural gene since studies of Cys-BPA isolated from six separate calves showed the same result. Also studies with albumins isolated from eight humans and four mice showed similar findings.

Crystalline preparations of bovine plasma albumin are known to be essentially free of contaminating plasma proteins (1). But they usually contain bound fatty acids and possibly other materials. They also contain small amounts of diners and polymers of albumin. Furthermore BPA\(^1\) is known to be composed of two types of albumin, one devoid of sulfhydryl group and the other with a single sulfhydryl group (1). The albumin devoid of sulfhydryl group is a mixed disulfide compound of mercaptalbumin with L-cysteine and to a lesser extent with glutathione (2, 3). It is partly because of the above facts that crystalline BPA is heterogenous, as indicated by its chromatographic behavior on hydroxyapatite (4), diethylaminoethyl cellulose (5) and DEAE-Sephadex (6). Heterogeneity of albumin is also indicated by the physicochemical studies of its thermal denaturation (7), and its pH-dependent acid isomerization (8) which had been designated by its investigators as the N to F transition. However the aforementioned causes of heterogeneity, numerous as they are, were found to be still inadequate to explain the several different kinds of BPA monomers obtained on chromatography (5, 6). To account for these observations, it has been suggested that BPA may be mixtures arising from intramolecular sulfhydryl-disulfide exchange reactions (3, 6, 8).

In this paper we will report our findings on the nature of heterogeneity of albumin as revealed by a combination of different experimental approaches. Both crystalline BPA isolated from pools of animals as well as albumins from single calves, humans, and mice were studied. Unless stated otherwise, the albums used in this study were in the nonmercaptalbumin form prepared by the disulfide interchange reaction with L-cysteine, and they are referred to as half-cystinyl albumin.

EXPERIMENTAL PROCEDURE

Materials—The albumin used for most of the studies was crystallized bovine plasma albumin (Armour lot numbers A69702 and D71209). Studies were also made with albumins prepared from the plasma of individual calf, human, and mouse donors. Activated charcoal (Norit A from Fisher), used for defatting of albumin, was washed with water and air-dried. \(^{14}C\)-L-Cystine (uniformly labeled) with a specific activity of 264 mC/mM per mmole was from New England Nuclear. L-\(^{14}C\)-Acetic anhydride with a specific activity of 121 mC/mM per mmole was from Amersham-Searle. Both compounds were certified to be >99% radiochemically pure.

DEAE-cellulose was from Whatman of DE-32 grade. Hydroxylapatite (Bio-Gel HT) was from Bio-Rad, Richmond, California. Sephadex, G-100 grade, was from Pharmacia. Visking dialysis tubing, type 8/32, was used for ultrafiltration (9). Prior to use the tubing was soaked in 0.05 M disodium EDTA for several minutes then washed with water.

Ampholine was from LKB Instruments, Rockville, Maryland. All other chemicals used for electrophoresis in polyacrylamide gels were from Eastman Kodak, Rochester, New York.

METHODS

Isolation of Albumin from Plasma of Single Donors—The blood immediately after collection was diluted with 0.11 part of a...
solution of citric acid, sodium citrate, and dextrose (A.C.D. solution, Abbott). The cells were removed by centrifugation. To the plasma was added saturated (NH₄)₂SO₄ solution to give a final concentration of 55% in (NH₄)₂SO₄. The precipitate was removed by centrifugation, and the supernatant was brought to 85% saturation by the addition of solid (NH₄)₂SO₄.

Preparation of Defatted Cys-BPA Monomer—Cysteine was added to the plasma solution (3 to 25 mg) in 1 ml of 0.05 M Tris-0.04 M HCl. The solution was kept under a stream of nitrogen at 37°C for 24 hours. At the end of the experiment the cysteine concentration increased about 30% from the initial value of 1.6 mM, determined by the procedure of Ellman (11), as a result of evaporation. The solution was concentrated by ultrafiltration, dialyzed against 0.05 M Tris-0.04 M HCl and rechromatographed on DEAE-cellulose.

Acid Treatment of Cys-BPA—Cuts 1 (50 mg) and 2 (50 mg) were incubated with 14C-L-cystine in 0.10 M Tris-0.04 M HCl. The precipitate was removed by centrifugation, and the supernatant was brought to 85% saturation by the addition of solid (NH₄)₂SO₄. The precipitated albumin was processed as described below.

Preparation of Defatted Cys-BPA Monomer—BPA was converted to its half-cystinyl derivative by a disulfide exchange reaction with L-cystine according to the published method (10). The derivative contained less than 0.01 residue of sulfhydryl group as determined by the procedure of Ellman (11). Albumin concentration was determined by A₂₈₀ with the value of 0.64 for 1 mg per ml (1-cm cell). The molecular weight was determined by tritium equilibrated with the same buffer. The column was eluted with a buffer of 0.05 M Tris, 0.04 M HCl, and 0.40 M (NH₄)₂SO₄ (pH 7.96) at 30 ml per hour. The monomer peak was concentrated by ultrafiltration and stored as a frozen solution. Its yield was about 86%. The monomer was defatted according to the acid-charcoal method of Chen (12).

Chromatography of Cys-BPA on DEAE-cellulose—The sample (15 to 500 mg) in 2 to 4 ml of 0.05 M Tris-0.04 M HCl (pH 7.30) was applied to a column (2.5 x 400 cm) of Sephadex G-100. The column was eluted at room temperature by a linear gradient formed with 120 ml each of 0.05 M Tris-0.04 M HCl, and 0.05 M Tris-0.04 M HCl-0.30 M NaCl. The flow rate was 60 ml per hour and fractions of 3 ml were collected. The desired cuts were concentrated by ultrafiltration.

Chromatography of Cys-BPA on Hydroxylapatite (4)—The sample (3 to 25 mg) in 1 ml of 0.02 M sodium phosphate buffer (pH 6.8) was applied to a column (1 x 15 cm) of hydroxyapatite equilibrated with the same buffer. The column was eluted at room temperature by a linear gradient formed with 25 ml of the following phosphate buffers (pH 6.8): 0.04, 0.07, 0.11, and 0.40 M. The flow rate was 12 ml per hour and fractions of 3 ml were collected. Peaks 1, 2, and 3 were materials eluted by the 0.07, 0.11, and 0.40 M buffers, respectively.

Acid Treatment of Cys-BPA—Cuts 1 (50 mg) and 2 (50 mg) obtained from DEAE-cellulose chromatography of Cys-BPA were concentrated to 2 ml by ultrafiltration. The solutions were dialyzed at 25°C against four 400-ml changes of 0.05 M Tris-0.04 M HCl for a total of 24 hours. The pH of the dialyzed solutions was 2.82. Then they were dialyzed against several changes of 0.05 M Tris-0.04 M HCl for 24 hours before rechromatography on DEAE-cellulose.

Guandine Hydrochloride Treatment of Cys-BPA—To 3 ml of Cut 1 (45 mg) or Cut 2 (51 mg) in 0.05 M Tris-0.04 M HCl was added 4.94 g of guanidine hydrochloride (ultra pure grade, Mann) to give a final concentration of 6 M. After 4 hours at room temperature the solutions were dialyzed against several changes of 0.05 M Tris-0.04 M HCl before rechromatography.

Disulfide Exchange of Cys-BPA with ¹⁴C-L-Cystine—Cuts 1 and 2 were incubated with ¹⁴C-L-cystine in 0.10 M Tris-0.06 M HCl buffer (pH 7.96) at 22°C. The concentration of Cys-BPA was 63 μM and that of ¹⁴C-L-cystine was 384 μM. At 0, 2, 4, and 8 hours an aliquot (0.35 ml) was taken and passed through a Sephadex G-25 column (0.8 x 60 cm) equilibrated with 0.1 M NH₄HCO₃. The separation of protein and reagents was complete in 12 min. The radioactivity of the exchanged ¹⁴C-Cys-BPA was determined by counting a 0.50-ml aliquot in 5 ml of Bray’s solution (13); under these conditions the specific activity of ¹⁴C-L-cystine used was 2.44 x 10⁶ cpm per μmol.

Treatment of Cys-BPA with L-Cysteine—A solution of Cuts 1 or 2 (14 mg) in 3.2 ml of 0.05 M Tris-0.04 M HCl was diluted with 146 ml of 0.10 M Tris-0.06 M HCl (pH 7.96) which had been deaerated by bubbling nitrogen through it. L-Cysteine (30 mg) was added to give a final concentration of 1.67 mM. The solution was kept under a stream of nitrogen at 37°C for 24 hours. At the end of the experiment the cysteine concentration increased about 30% from the initial value of 1.6 mM, determined by the procedure of Ellman (11), as a result of evaporation. The solution was concentrated by ultrafiltration, dialyzed against 0.05 M Tris-0.04 M HCl and rechromatographed on DEAE-cellulose.

N to F Transitions of Cys-BPA—This was determined according to the method of Peterson and Foster (14).

Thermal Denaturation of Cys-BPA—This was followed spectrophotometrically in a Gilford model 2000 spectrophotometer. Solutions were deaerated by flowing a stream of helium over the surface while the samples were magnetically stirred then quickly transferred to cuvettes which were sealed with silicon rubber. The cuvettes were heated at a rate of approximately 15°C per hour. The absorbance changes at 278 and 300 nm were recorded at 2-min intervals.

Isoelectric Focusing—This was carried out in a thin layer of polycrylamide gel according to the method of Awdeh, Williamson, and Askones (15) with slight modifications. The following aqueous stock solutions were made and stored in ice box: Solution A, 28% acrylamide-0.5% N,N'-methylenebisacrylamide; Solution B, 0.8% N,N',N',N'-tetramethylethylenediamine; and Solution C, 0.002% ribofavin. The gel (15 x 23 cm) was formed by photopolymerizing for 1 hour 45 ml of the following mixture: 9 ml of Solution A, 5 ml each of Solutions B and C, 1.8 ml of 40% Ampholine, 12.2 ml of water, and 16.2 g of urea. For Figs. 7 and 8 (lower) pH 5 to 7 Ampholine was used. For Figs. 2 and 8 (upper) an equal volume mixture of pH 3 to 6 and pH 5 to 8 Ampholines were used.

Usually 10 samples (40 to 130 μg) in volumes of 5 to 50 μl of 0.05 M Tris-0.04 M HCl were applied to each gel plate by streaking across the length of the gel surface forming a band of about 5 mm in width. Electrofocusing was carried out at 400 volts for 14 hours at room temperature. For the determination of the pH gradient, a strip (0.5 cm in width) was cut off the gel and sliced into 1.3-cm segments. The segments were suspended in 1 ml of water and the pH was measured after 30 min. The remainder of the gel was stained overnight in a mixture of 200 ml of 5% trichloracetic acid-5% sulfosalicylic acid, 2 ml of 1% Coomassie Blue dye (16), and 40 ml of methanol. Before staining the distance between the two electrode marks on the gel surface was 20 cm; after staining the gel swelled and the distance became about 22.5 cm. For the determination of the isoelectric points of the stained bands, the pH gradient measured with the unstained gel was appropriately corrected for the swelling.

Synthesis of ¹⁴C-Acetylsalicylic Acid (17)—To 250 mg (1.81 mmoles) of salicylic acid (m.p. 160–161°C) was added 0.25 ml (2.65 mmoles) of L-¹⁴C-acetic anhydride (0.5 μCi) and two drops of pyridine. After 1 hour at room temperature, 10 ml of cold water was added. After 4 more hours, the crystalline product was collected by filtration and washed with water. The product after drying was recrystallized from a mixture of ethyl acetate (4 ml) and n-hexane (11 ml). The recrystallized product was...
Bella of this University. Obtained in a yield of 52\% with melting point of 138-139\°. Microanalysis of the product was carried out by Mr. Theodore Bella of this University.

\[ \text{C}_2\text{H}_4\text{O}_2 \]

Calculated: C 66.02, H 4.48

Found: C 69.89, H 4.00

**Reaction of \[^{14}C\]Acetylsalicylic Acid with BPA**—The reaction was carried out at 37\° in 1 to 2 ml of phosphate or Tris-HCl buffers. The concentration of BPA was 0.4 mM and that of \[^{14}C\]acetylsalicylic acid varied from 4.8 to 20 mM. At time intervals, 0.2-ml aliquots were removed and the reaction was terminated by adding an equal volume of cold 10 \% trichloroacetic acid. The precipitated BPA was washed three times with cold 5 \% trichloroacetic acid then it was dissolved in 2 ml of 0.10 M Tris-0.08 M HCl (pH 7.6). The concentration of the dissolved BPA was determined by A\(_{280}\) and the amount of \[^{14}C\]acetyl group incorporated was determined by counting a 50- to 100-\(\mu\)l aliquot in 10 ml of Bray’s solution. Under the counting conditions used, the specific activity of \[^{14}C\]acetylsalicylic acid was 127 cpm per mole.

The reaction products were examined by isoelectric focusing in 6 M urea gel. The protein contents in the stained bands were estimated by scanning the gel in a Photovolt 542 densitometer. Integration of the densitometer tracing was accomplished by cutting out and weighing the paper under each peak. Alternatively the protein content was estimated by measuring the \(A_{280}\) of the eluted dye. The dye was extracted from the band (about 3 \times 10 mm) by soaking in 1 ml of dimethyl sulfoxide at 25\° for 24 hours. The results from the two methods were in good agreement.

The radioactivity distribution of the stained bands was determined after digestion with 50 \% H\(_2\)O\(_2\) (0.1 ml) in a scintillation vial (18). After 5 hours at 50\°, 1.5 ml of Soluene-100 (Packard) was added followed with 10 ml of scintillator fluid (0.2 g of 1,4-bis[5-phenyloxazolyl]benzene and 5 g of 2,5-diphenyl-oxazole per liter of toluene).

**RESULTS**

**Chromatographic and Isoelectric Heterogeneity of BPA**—In the present work most of the studies were made with crystalline BPA samples which had been isolated from pooled animals. Some of the studies were made with albumin isolated from the plasma of a single calf by (NH\(_4\))\(_2\)SO\(_4\) precipitation. Prior to use the albumin samples were first converted to Cys-BPA, next freed of dimers and polymers by chromatography on Sephadex G-100 then defatted with the acid-charcoal method (19).

When defatted Cys-BPA from a single calf was chromatographed on DEAE-cellulose, an asymmetric peak with considerable tailing was observed (Fig. 1). The small broad peak eluted in front of the main peak is caused by other plasma proteins which were incompletely removed by the methods used for the albumin isolation. When defatted Cys-BPA from pooled animals was chromatographed, it gave a single peak with identical elution position and shape to that from a single calf.

The defatting process was found to influence the elution position, but not the profile of the albumin peak. Thus a nondefatted sample of Cys-BPA was eluted about 10 ml later than that shown for the defatted sample in Fig. 1. This is consistent with the fact that defatting removes bound fatty acid anions.

Monomeric BPA which was a mixture of mercaptalbumin (60\%) and Cys-BPA (40\%), showed a chromatographic behavior identical with that of Cys-BPA. Analysis of the sulfhydryl titer across the chromatogram of BPA indicated that there was a partial separation of bovine mercaptalbumin and Cys-BPA, as the sulfhydryl titer was 0.8 residue per mole at the height of the peak and it gradually decreased to about 0.3 residue at the trailing edge. This finding is in agreement with the results of Hartley, Peterson, and Sober (5) and Janatova, Fuller, and Hunter (6). However the partial separation of mercaptalbumin and Cys-BPA could not be the only explanation for the asymmetric

**Fig. 1.** DEAE-cellulose chromatography of defatted Cys-BPA from a single calf. A 50-\(mg\) sample was used. The column was eluted at 25\° with a linear NaCl gradient in Tris-HCl buffer (pH 7.3) at a flow rate of 60 ml per hour, and fractions of 3-ml volume were collected. The contents of the indicated tubes (1 to 7) were used for electrofocusing shown in Fig. 2. Cuts A to E were taken from an identical chromatogram of Cys-BPA from pooled animals, and these were used in the experiments of Figs. 4 and 5. For the experiment in Fig. 3, Cuts A + B and D + E were used and they are referred to as Cuts 1 and 2, respectively.

**Fig. 2.** Isoelectric focusing of Cys-BPA on polyacrylamide-6 \(\mu\) urea gel. Armour-defatted Cys-BPA was prepared from plasma of pooled animals. All other Cys-BPA samples were obtained from a single calf and they are described in Fig. 1. The following approximate amounts were used: Samples 1 to 4, 40 \(\mu\)g; Samples 5 to 7, 30 \(\mu\)g; and the remaining samples, 50 \(\mu\)g. The gel contained Ampholine of pH 3 to 8 range. Only a portion of the gel is shown. The Cys-BPA sample isolated from the plasma of a single calf by (NH\(_4\))\(_2\)SO\(_4\) precipitation was not homogeneous, as it contained faint bands other than those shown.
The two cuts were obtained from Cys-BPA of pooled animals as described in Fig. 1. Each cut (63 μg) was incubated with 14C-L-cystine (48 μm) in a buffer of 0.10 M Tris-0.06 M HCl (pH 7.96) at 22°C.

| Time (hrs) | Residue of half 14C-L-cystine incorporated per mole of Cys-BPA |
|-----------|---------------------------------------------------------------|
| 0.1       | 0.019                                                         |
| 2.0       | 0.172                                                         |
| 4.0       | 0.185                                                         |
| 8.0       | 0.212                                                         |

Table I

**Disulfide exchange of Cys-BPA with 14C-L-cystine**

The observed chromatographic and isoelectric heterogeneity of Cys-BPA was not a result of contamination with other plasma proteins. This was ascertained by studying two chromatographic cuts of Cys-BPA from pooled animals. These two cuts were obtained from Cys-BPA of pooled animals as described in Fig. 1. Approximately 62 mg of each cut were used.

**Chromatographic peak, since Cys-BPA also behaved in the same manner.**

Cys-BPA samples from six individual calves as well as those from pooled animals were examined by isoelectric focusing in polyacrylamide gels containing 6 M urea. All other conditions were the same as described in Fig. 1.

Fig. 3. DEAE-cellulose chromatographies of Cuts 1 and 2 before and after exposure to 6 M guanidine hydrochloride. The two cuts were obtained from Cys-BPA of pooled animals as described in Fig. 1. Approximately 62 mg of each cut were used.

Cuts 1 and 2 were subjected to reversible denaturing conditions, namely at pH 2.82 (21) and in 6 M guanidine hydrochloride (22). After removal from the denaturing environment, they were reexamined by chromatography on DEAE-cellulose. These treatments did not influence the elution positions or profiles of the two cuts. The results for the two cuts before and after guanidine hydrochloride treatment are shown in Fig. 3. These findings would suggest that the chromatographic heterogeneity of Cys-BPA is not caused by the presence of conformational isomers.

**TABLE I**

| Time (hrs) | Residue of half 14C-L-cystine incorporated per mole of Cys-BPA |
|-----------|---------------------------------------------------------------|
| 0.1       | 0.019                                                         |
| 2.0       | 0.172                                                         |
| 4.0       | 0.185                                                         |
| 8.0       | 0.212                                                         |

The observed chromatographic and isoelectric heterogeneity of Cys-BPA was not a result of contamination with other plasma proteins. This was ascertained by studying two chromatographic cuts of Cys-BPA from pooled animals. These two cuts were obtained from Cys-BPA of pooled animals as described in Fig. 1. Approximately 62 mg of each cut were used. All other conditions were the same as described in Fig. 1.

Cuts 1 and 2 corresponded to Cuts A + B and D + E of Fig. 1. The following experiments were performed. A, the two cuts have amino acid compositions identical with that of the starting material (10); B, cyanogen bromide cleavage of each of the two cuts gave two fragments N and C in yields identical with those from the unfractionated material (10); C, the two cuts showed identical rates of exchange of the half-cystinyl group with 14C-L-cystine (Table I); and D, these two cuts exhibited binding properties for octanoate ions identical with that of the starting material (10).

**Reversible Denaturation and Thiol Treatment of Cys-BPA**—The Cuts 1 and 2 were subjected to reversible denaturing conditions, namely at pH 2.82 (21) and in 6 M guanidine hydrochloride (22). After removal from the denaturing environment, they were reexamined by chromatography on DEAE-cellulose. These treatments did not influence the elution positions or profiles of the two cuts. The results for the two cuts before and after guanidine hydrochloride treatment are shown in Fig. 3. These findings would suggest that the chromatographic heterogeneity of Cys-BPA is not caused by the presence of conformational isomers.

Cuts 1 and 2 were exposed to reaction conditions known to promote disulfide bond isomerization, namely anaerobic incubations with cysteine or reduced glutathione at pH 7.9 and 37°C for 24 hours. The treatment was found not to change the elution behaviors of either cut on DEAE-cellulose chromatography toward that of the unfractionated Cys-BPA through the peak of Cut 2 trailed slightly more than that of the control. Since no interconversion of the two cuts occurred, this would suggest that the disulfide bond isomerization is not the cause of heterogeneity.

**N to F Transition and Thermal Denaturation of Cys-BPA**—The N to F transition was examined on the five chromatographic cuts of Cys-BPA with the pH solubility profile procedure of Peterson and Foster (14). The five cuts, A to E, were obtained...
The thermal denaturation of Cuts A, C, and E was investigated. The absorption changes on heating these samples in Tris-HCl buffer at pH 7.3 are reproduced in Fig. 5. In all samples there was an initial decrease in absorbance at 278 nm on heating and this decrease was not mirrored by a change in absorbance at 300 nm. Further heating increased the absorbance at 278 nm as well as that at 300 nm, and this represented aggregation of albumin. Cut A was the first to aggregate, followed by C and then E. Studies were also done at pH 6.3 in phosphate buffer and at pH 8.3 in Tris-HCl buffer. The aggregation temperature for each cut varied in the order: pH 8.3 > pH 7.3 > pH 6.3. The results indicate that the greater the negative charge on the molecule, the higher the temperature required for aggregation. The conclusion is in line with the fact that Cut A enriched with albumins of higher isoelectric points aggregated at a lower temperature than did Cut E enriched with albumins of lower isoelectric points.

Acetylation of Cys-BPA with Acetylsalicylic Acid—Hawkins et al. (23) have reported that the binding of acetylsalicylic acid by human plasma albumin was accompanied by transacetylation such that the ε-amino group of a single specific lysyl residue was modified. This reaction was investigated with BPA for the purpose of determining the charge differences of the BPA bands observed on isoelectric focusing.

The rates of acetylation of defatted Cys-BPA with 14C-acetylsalicylic acid in two different buffers are shown in Fig. 6. The reaction proceeded at a faster rate in phosphate buffer (pH 7.4) than in Tris-HCl buffer (pH 7.0). When nondefatted Cys-BPA was used, the reaction rate in phosphate buffer was one-fifth of that of the defatted sample. This result is not shown in the figure. The rate difference between these two samples may arise from the competitive or noncompetitive binding between the fatty acid and acetylsalicylate anions for Cys-BPA.

The products formed during the course of the reaction were examined by isoelectric focusing (Fig. 7). As acetylation proceeded, the intensity of Band 1, which is the strongest staining band in the control sample, gradually decreased while those of Bands 2, 3, and 4 increased (Samples 1 to 5 of Fig. 7A). Samples 1 to 5 were obtained with Tris-HCl as a buffer; Sample 5 contained 1.15 eq of 14C-acetyl group. The patterns of Samples 6, 6, and 7 are nearly identical indicating that the product distribution is independent of the buffer used for the acetylation and is dependent only on the degree of acetylation. Sample 1 of Fig. 7B was obtained with phosphate as a buffer and it contained 2 eq of 14C-acetyl group. Its pattern showed two addi-
FIG. 7 (upper). Isoelectric focusing of l*C-acetylated Cys-BPA on polyacrylamide-6 M urea gel. The gel contained Ampholine of pH 5 to 7 range. Only a portion of the gel is shown. A, Samples 1 to 6 are from Experiment S of Fig. 6 at 0, 2, 4, 8, and 24 hours, respectively; Sample 6 is from Experiment 2 at 6 hours; and Sample 7 is from Experiment 1 at 3 hours. The four components present in each sample were electrofocused to positions, with pH values of 6.02, 5.95, 5.89, and 5.83, respectively, from top to bottom. For each sample, 1 nmole of protein was applied to the gel. B, Sample 1 is from Experiment 2 at 22 hours and Sample 6 is a control. Two nanomoles of protein was used. The magnification of the pattern is twice that of A.

FIG. 8 (lower). Isoelectric focusing of half-cystinyl albumins from human, bovine, and mouse single donors. Upper, the gel contained Ampholine of pH 3 to 8 range. The samples from left to right are albumins from two mouse strains C3H/HeJ and C57B/6J, albumins from two humans, BPA from pooled animals, and BPA from a single calf. Approximately 50 µg of each sample were used. Lower, the gel contained Ampholine of pH 5 to 7 range. From left to right, two normal human sera (about 60 µg of albumin used) and a bisalbuminemias serum (about 120 and 200 µg of albumin used). The sera were reacted with L-cystine (10-mole excess of albumin) at pH 7.9 and 25° for 16 hours, then used directly. Only the portion of the gel containing albumins is shown.

### Table II

**Product distribution on isoelectric focusing of reaction mixtures of Cys-BPA and 1*C-acetylsalicylic acid**

| Sample no. | Reaction time | No. of 1*C-acetyl groups | Band nos. |
|------------|---------------|--------------------------|-----------|
|            | hrs           | %                        | 1 2 3 4   |
| 1          | 0             | 76                       | 20 4 0    |
| 2          | 2             | 61                       | 33 6 0    |
| 3          | 4             | 40                       | 39 16 5   |
| 4          | 8             | 32                       | 37 24 7   |
| 5          | 24            | 11                       | 40 32 17  |

### Table III

**Determination of 1*C-acetyl groups in bands from isoelectric focusing of (1*C-acetyl)-Cys-BPA**

Sample 5 of Fig. 7A was used. Radioactivity and protein determinations were made with separate but identical samples.

| Band | Radioactivity* | 1*C-Acetyl group* | Cys-BPA* | Amount of 1*C-acetyl group incorporated |
|------|----------------|-------------------|----------|--------------------------------------|
| 1    | 0.5            | 0                 | 0        | 0.07                                 |
| 2    | 25.3           | 0.23              | 0.24     | 0.96                                 |
| 3    | 28.3           | 0.26              | 0.20     | 1.30                                 |
| 4    | 14.8           | 0.14              | 0.10     | 1.40                                 |
| 5    | 3.1            | 0.03              | 0        | 0                                    |

* The blanks were taken from the region in front of Bands 1 and 2, and they averaged 17 ± 1 cpm. The areas between Bands 2 and 3 and Bands 3 and 4 contained a total of 4.5 cpm.

* Under these counting conditions 100 cpm represented the incorporation of 1 eq of 1*C-acetyl group.

* The amount of Cys-BPA was determined as described in Table II. The recovery from each band was assumed to be 61%, i.e., equal to the recovery of radioactivity.

The distribution of the products among the isoelectric bands was estimated by determining the amount of dye bound by each band. The results for Samples 1 to 6 are given in Table II. The amount of 1*C-acetyl group present in each of the bands for Sample 6 was determined. The results are given in Table III. The total recovery of radioactivity from the gel was 61%. All the bands except Band 1 contained radioactivity. The number of equivalents of 1*C-acetyl group present in Bands 2, 3, and 4 were calculated to be 0.96, 1.30, and 1.40, respectively, on the assumption that the protein and radioactivity recovery from the gel were of the same order. These results would indicate that Band 2 is the mono-1*C-acetyl derivative of Cys-BPA and that Bands 3 and 4 may be mixtures of mono- and di-1*C-acetyl derivatives. The results would also indicate that the two major components of Cys-BPA obtained on isoelectric focusing differ by one unit charge (Sample 2 of Fig. 7B).

### Isoelectric Focusing of Human and Mouse Cystinyl Albumins

Eight humans and four inbred strains of mice were examined. Some representative results together with those from two calves.
are shown in Fig. 8 (top). Within each group the pattern for all the individuals were alike. The half-cystinyl albumin from the inbred mouse strains appeared to be as heterogeneous as those from human and bovine sources. The pattern for a person with hisalubminemia (Fig. 8, bottom) suggests the presence of two sets of bands, one set corresponding to that from normal persons and another set at pH higher than 6.1.

**DISCUSSION**

The above results showed that BPA, isolated from pooled animals or single donors, contained several components which differ in their isoelectric points. These components were partially separable on DEAE-cellulose chromatography. Under the chromatographic conditions used they apparently do not behave ideally to give symmetric peaks without tailing. This is indicated by electrofocusing the albumins isolated from the different regions of the chromatogram (Figs. 1 and 2). Probably a combination of these two factors, the incomplete separation and the nonideal behavior of these components, give rise to the broad asymmetric peak of BPA on chromatography (Fig. 1).

The two major components of BPA with isoelectric points of 6.02 and 5.95 were found to differ by 1 unit charge. This was shown on electrofocusing the transacetylation products of Cys-BPA with 14C-acetylsalicylic acid. The reaction products showed a decrease of the component with isoelectric point of 6.02 and concomitant increases of the component with isoelectric point of 5.95 as well as those with lower isoelectric points. The component with isoelectric point of 5.95 was found to be a mono-14C-acetylated derivative of BPA while those with lower isoelectric points probably represent di- and triacetylated derivatives. At present we have no evidence whether or not the monoacetetyl group is introduced into a specific lysyl residue of BPA. Hawkins et al. (23) who originally discovered the transacetylation reaction of acetylsalicylic acid with human plasma albumin, have presented evidence that the reaction occurred at a specific lysyl residue.

The following possible origins may be considered for these BPA components. (a) they contain varying amounts of tightly bound charged ligands; (b) they are sequence variants; and (c) they are the results of modifications in vivo or in vitro. The presence of tightly bound ligands can not be readily excluded although it appears unlikely that the acid charcoal defatting procedure and reversible denaturation at low pH or in 6 m guanidine hydrochloride all failed to remove the ligands. Furthermore electrofocusing was carried out in gels containing 6 m urea, an environment which was shown to dissociate tightly bound fatty acids (Fig. 2). The second possibility of sequence variants arising from heterozygosity of the structural gene is also not likely as evidenced by the similarity of electrofocusing patterns from six different calves as well as those from eight human and four mouse single donors.

Of the third possibility, modification in vitro such as caused by the isolation procedures used appears unlikely as electrofocusing of whole plasma or serum (after conversion to half-cystinyl albumin) yielded identical results. Modifications in vivo could result from one of the following reactions: (a) isomers formed through intramolecular sulfhydryl-disulfide exchanges, (b) stable conformational isomers, (c) isomers formed by acetylation of the ε-amino group of lysyl residues, and (d) isomers formed by deamidation of asparaginyl or glutaminyl residues or by amidation of aspartyl or glutamyl residues.

Modification a is unlikely as Cuts 1 and 2, obtained as described in Fig. 1, were not altered by treatment with L-cysteine or glutathione. Also the two cuts showed identical rates of exchange of 14C-l-cysteine indicating that the half-cystinyl residue occupies the same environment. Furthermore CNBr cleavage of S-14C-carboxamidomethyl-BPA gave one 88-residue peptide containing all the label (10), and on subsequent trypptic hydrolysis of the 88-residue peptide, all the S-carboxamidomethyl cysteine residue was found to be localized in one unique tryptic peptide (24). Modification b is unlikely as reversible denaturation of Cuts 1 and 2 did not alter their chromatographic behavior. Only Modifications c and d remain to be considered, but at present we have no evidence to support or deny them.

It should not be surprising if one of the later possibilities is shown to be correct, as there are ample precedents in protein chemistry, e.g., desamido insulin (25) and ε-N-acetylhistone (26).

The isoelectric heterogeneity of plasma albumin appears to be widespread in mammals as suggested by the results with calves, humans, and mice. These albumins of different isoelectric points may have biological significance. One such possibility is suggested by the finding of Farr (27) that human plasma albumin on acetylation with acetylsalicylic acid showed enhanced binding for sodium aetrazoate. We were unable to detect any difference in the octaano-binding properties of Cuts 1 and 2 of Cys-BPA. However, these results are not conclusive as both cuts were still mixtures of the different isolectric components.

**Acknowledgments**—We would like to thank Doctors Morton Printz and Martin Jerry, respectively, for their help with the thermal denaturation studies and the electrofocusing experiments, and Dr. Bruce McEwen for the use of his Photovolt densitometer. Bovine plasmas, mouse sera, and human bisalbuminemia serum were generous gifts from Doctors Lewis Green, Anthony Cerami, and Leo Cawley, respectively.

**REFERENCES**

1. Hughes, W. L., in H. Neurath and K. Bailey (Editors), The proteins, Vol. IIIB, Academic Press, New York, 1965, p. 663.
2. King, T. P., J. Biol. Chem., 236, PC 5 (1961).
3. Anderson, L. O., Biochim. Biophys. Acta, 117, 115 (1966).
4. Temmink, A., Herren, S., and Levin, O., Arch. Biochem. Biophys., 65, 132 (1956).
5. Hartley, K. W., Jr., Peterson, E. A., and Sober, H. A., Biochemistry, 1, 60 (1962).
6. Janatova, J., Fuller, J. K., and Hunter, M. J., J. Biol. Chem., 243, 3612 (1968).
7. Stokovy, S., and Spolar, J., Collect. Czech. Chem. Commun., 25, 659 (1963).
8. Foreman, J. F., Sagami, M., Peterson, H. A., and Leonard, W. J., Jr., J. Biol. Chem., 240, 2495 (1965).
9. Berggard, I., Ark. Kemt, 18, 291 (1961).
10. King, T. P., and Spence, E. M., J. Biol. Chem., 245, 6134 (1970).
11. Edman, G., Arch. Biochem. Biophys., 82, 70 (1959).
12. Chern, K. F., J. Biol. Chem., 242, 173 (1967).
13. Bray, G. A., Anal. Biochem., 1, 279 (1960).
14. Felsen, H. A., and Foster, J. F., J. Biol. Chem., 240, 2569 (1965).
15. Ander, Z. L., Williamson, A. R., and Askonas, B. A., Nature, 219, 66 (1968).
16. Charles, A., Reif, R. A., Wyckoff, M., and Zagari, J., Anal. Biochem., 80, 150 (1967).
17. Piers, P. F., Experiments in organic chemistry, Heath, Boston, 1957, p. 265.
18. Tishler, P. V., and Epstein, E. J., Anal. Biochem., 22, 89 (1968).
19. Valmet, E., in H. Peeters (Editor), Proteins in Biological Fluids, Proceedings of 17th Colloquium, Pergamon Press, New York, 1969, p. 443.
20. Kaplan, L. J., and Foster, J. F., Fed. Proc., 29, 402 (1970).
21. Foster, J. F., in F. Putnam (Editor), The plasma proteins, Vol. 1, Academic Press, New York, 1960, p. 179.
22. Moore, W. E., and Foster, J. F., Biochemistry, 7, 3409 (1968).
23. Hawkins, D., Pinckard, R. N., Crawford, I. P., and Farr, R. S., J. Clin. Invest., 48, 536 (1969).
24. Spencer, E. M., Ph.D. thesis, The Rockefeller University, 1969.
25. Harfenist, E. J., and Craig, L. C., J. Amer. Chem. Soc., 75, 5598 (1953).
26. Gershey, E. L., Vidal, G., and Allfrey, V. G., J. Biol. Chem., 243, 5018 (1968).
27. Farr, R. S., J. Allergy, 45, 321 (1970).
