Structural Studies and Organic Ligand-binding Properties of Bovine Plasma Albumin*

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SUMMARY

Cyanogen bromide cleavage of bovine plasma albumin in 75% formic acid gave two fragments, N and C, in yields of 80%. After reduction of the cross-linking disulfide bonds of Fragment N, two peptides containing 88 and 98 amino acid residues were isolated. The single sulfhydryl group of albumin was found to be located in the 88 amino acid residue peptide which occupies the amino-terminal position of the molecule. After reduction of Fragment C, three peptides containing 211, 148, and 34 amino acid residues were isolated. These five peptides together account for the albumin molecule.

Limited tryptic hydrolysis of defatted bovine plasma albumin at pH 8.8 and 0° gave a fragment of molecular weight of about 40,000 in a yield of 21%. The fragment is derived from the carboxyl-terminal two-thirds of the albumin molecule as shown by a comparison of its three cyanogen bromide peptides with those from albumin. These data were used for the alignment of the five cyanogen bromide peptides of albumin.

Binding studies showed that the tryptic fragment and defatted albumin both had one primary site for octanoate and L-tryptophan. D-Tryptophan competitively displaced either of these two ligands from the fragment and from albumin, thus suggesting that all three ligands bind at the same site. The binding constants of the fragment for the three ligands were all about one-third of those for albumin. The ratio of the binding constants of L- and D-tryptophan for the fragment was nearly the same as that for albumin, and this was also the case for octanoate and L-tryptophan. These similarities strongly suggest that the binding site in the fragment is the one present in albumin.

One of the interesting properties of bovine or human plasma albumin is its propensity to bind a wide variety of organic and inorganic ligands. Because of this property, albumin is believed to function as a transport protein, and possibly serves as a mechanism in regulating the blood level of certain drugs and metabolites (2). The binding affinities of albumin for different ligands are often as high as those of the specific interactions. The present studies were made with the intention to understand better what molecular parameters of albumin are required for this binding property.

Our approach has consisted of stepwise degradation of albumin into fragments and an examination of the binding properties of such fragments. This has the advantage of providing structural information about this most abundant plasma protein for which only fragmentary data are presently available (3, 4). Both bovine and human plasma albumins are readily available and studies carried out with either one will be equally useful for the understanding of the other because of probable similarities in structure.

Bovine albumin was chosen since our first degradation procedure was cyanogen bromide cleavage at methionyl bonds, and bovine albumin has one less methionine than does the human albumin (4). Our second degradation procedure follows the findings of other workers that limited tryptic hydrolysis of bovine albumin gave intermediates of large molecular sizes (5, 6).

EXPERIMENTAL PROCEDURE

Materials—Crystalline bovine plasma albumin samples (lot B-70411 and D-71209) were from Armour. Cyanogen bromide was from Aldrich. Formic acid, 97 to 100% from Matheson Coleman and Bell (Division of the Matheson Company, East Rutherford, New Jersey) was purified by distillation before use. Dithiothreitol was from Calbiochem. Urea and guanidine hydrochloride were of ultrapure quality from Mann. Carboxypeptidase A was from Sigma (lot C-33B80). Trypsin, crystallized twice was from Worthington (TRL 6254).

14C-Iodoacetamide with specific activity of 3.87 mCi per mmole from New England Nuclear was diluted with cold carrier to a specific activity of 0.43 mCi per mmole before use. On reaction with excess cysteine followed by acid hydrolysis, 99% of the radioactivity was found in the carboxymethylcysteine peak separated on the amino acid analyzer.

3-14C-n-Cystine with a specific activity of 17 mCi per mmole was from Schwarz BioResearch (lot 6803-P). For preparation of 14C-half-cystinyl-BPA1, it was diluted with cold L-cystine to yield 7 × 104 cpm per μmole. The radiochemical purity of the

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diluted cystine was established by radioactivity determination of the cystine peak separated on an amino acid analyzer. 

L- and D-Tryptophan obtained from Calbiochem were purified by recrystallization from water; they had identical ultraviolet spectra, but they had opposite optical rotations, \([\alpha]_D \approx -30.8^\circ \text{ and } +30.8^\circ \text{ (C, 1, in water)}, \) respectively. 1-\[^{14}\text{C}\]-L-Tryptophan with a specific activity of 9.6 mCi per mmole was also obtained from Calbiochem (lot 860011). For binding studies, it was diluted with cold L-tryptophan to give \(1.5 \times 10^6 \text{ cpm per pmol.}\) The radiochemical purity was established by chromatography on Sephadex G-25; tryptophan was eluted after the total column volume, and there was coincidence of the radioactivity distribution with the tryptophan peak. The stereochemical purity of \(^{14}\text{C}-\text{L-tryptophan was not checked. The manufacturer stated that it was prepared by enzymatic resolution and that it was free of }\text{D isomer as tested by cocrystallization in the presence of excess D isomer.}\n
Sodium \(^{1-14}\text{C-octanoate with a specific activity of 17 mCi per mmole was from Amersham-Searle Corporation, Arlington Heights, Illinois (lot CPA 90). For binding studies, it was diluted with cold sodium octanoate (acid purified by vacuum distillation) to give }1.5 \times 10^6 \text{ cpm per pmole.}\) To check its radiochemical purity, binding studies were also carried out after further dilution with carrier to give 0.5 \(\times 10^6 \text{ cpm per pmole.}\)

**Methods**—Amino acid analyses were made on a Beckman-Spinco model 120-B analyzer after hydrolysis of the samples in \(6 \times \text{HCl} \) at 110°C for 22 and 72 hours (7). For good separation of the homoserine lactone and ammonia peaks, a short column (15 cm) was used; for resolution of homoserine and glutamic acid peaks, analyses on the long column (55 cm length) were started at 48°C, and the temperature was changed to the normal operating value of 55°C at the position of threonine peak. For 22-hour hydrolyses, the following decomposition factors were used for carboxymethylcysteine, threonine, cystine, and serine: 3%, 5%, 7%, and 10%, respectively (7). Tryptophan was determined on the basis of the molar extinction coefficients of the peptides at 280 nm.

For quantitative NH\(_2\)-terminal end group analysis, the cyanate procedure was used, and only the hydantoin fractions A and B of the isolation scheme of Stark (8) were examined. For qualitative analyses, the Edman procedure described by Fambrough and Bonner (9) and the dansylation (10) procedure were used. The phenylthiohydantoins were identified by chromatography on Eastman Kodak silica sheets with fluorescent indicator using two solvent systems; heptane-1,4-dichloroethane-formic acid (99%/3% or 3:6:1 only the upper phase used). The spots were visualized under ultraviolet light, and also after spraying first with starch solution, followed by heating with 3.6 ml of acetic acid per liter of water; the electrophoretic conditions were about 20 volts per cm for 3 hours.

Radioactivity determinations of samples (50- to 200-\(\mu l\) aliquots) were made after dilution with 4 ml of ethanol and 15 ml of scintillant fluid. The scintillant fluid was prepared by diluting 53 ml of Liquifluor (New England Nuclear) with 1 liter of toluene. Alternatively, the radioactivity determinations of samples (0.2-ml aliquots) were made after dilution with 5 ml of Bray's solution (17). The scintillation counter which was used had an efficiency of 60% for the \(^{14}\text{C}-\text{isotope.}\)

For calculation of the molar extinction coefficients of peptides at 280 nm, the extinction coefficients of tryptophan, tyrosine, and cystine were taken to be 5060, 1280, and 120, respectively (18). The molar extinction coefficient at 280 nm for BPA in Tris-HCl buffer (pH 7.96) was found to be \(4.2 \times 10^4 \text{ (±5%),}\) employing amino acid analysis and the composition given in Table I in order to establish the concentration of the BPA solution.

**Preparation of Half-cystinyl Bovine Plasma Albumin**—To a solution of BPA (31.2 g, 44 mmole) in 100 ml of 0.10 M Tris and 0.06 M HCl (pH 7.96) was added a solution of L-cystine (67.4 mg, 280 mmole) in 142 ml of the same buffer. Because of the limited solubility of L-cystine, it was dissolved first in 1.5 ml of 1.08 M NaOH, then immediately diluted with 140 ml of buffer. After 17 hours at 25°C, the sulphhydril titer had decreased from the initial value of about 0.65 mmole per mole of BPA to less than 0.01 mmole, as determined by the Ellman procedure (19). The BPA solution was concentrated to about 25 ml by ultrafiltration through Visking tubing (20) and then stored frozen until use. For limited tryptic hydrolysis studies, the half-cystinyl-BPA samples used for binding studies were freed of dimers and polymers by chromatography on a column of Sephadex G-100 (200 \(\times 0.9\) cm). The column was equilibrated with a buffer containing 0.05 M Tris, 0.03 M HCl, and 1 M NaCl (pH 7.95).

**Preparation of \(^{14}\text{C-Carboxamidomethylated Bovine Plasma Albumin**—To a solution of BPA (520 mg, 7.3 mmole) in 5.2 ml of 0.5 M Tris and 0.15 M HCl was added 3.75 ml of \(^{14}\text{C-iodoacetic acid (3.63 \times 10^{-3} \text{ m in water). After 20 min at 25°C, the pH of the solution was 8.38, and the solution was freed of excess reagents by passage through a Sephadex G-25 column (2.2 \times 60 \text{ cm}) which was equilibrated with a buffer of 0.174 M formic acid and 0.026 M ammonium formate (pH 2.85). The front peak was concentrated by ultrafiltration. Radioactivity determination showed 0.78 eq of carboxamidomethyl group had been incorporated, whereas amino acid analysis indicated the presence of 0.64 residue of carboxamidomethyl cysteine.**

**Cyanogen Bromide Cleavage of Half-cystinyl Bovine Albumin or \(^{14}\text{C-Carboxamidomethylated Bovine Plasma Albumin in 70\% Formic Acid—To a 12-ml solution containing 13.3 mmole of dialyzed salt-free BPA was added a solution of cyanogen bromide (245 mg, 2.31 mmole) in 36 ml of 99% formic acid. After 16 hours at 25°C, the excess reagents were removed by passage through a Sephadex G-25 column (4 \times 62 \text{ cm}). The column was**
eluted with a buffer of 0.174 M formic acid and 0.026 M ammonium formate (pH 2.86) at a flow rate of 240 ml per hour. The front peak was concentrated by ultrafiltration to a volume of about 10 ml, and the slightly hazy concentrate was clarified by centrifugation. It was next applied to a Sephadex G-75 column (2.8 x 105 cm) and eluted with the pH 2.86 ammonium formate buffer at a flow rate of 24 ml per hour. Fractions of 12-ml volume were collected. Cuts containing Fragments N and C (Fig. 1) were concentrated by ultrafiltration, then rechromatographed on the same column. Lyophilization of the fragments was used. The reaction time required for cyanogen bromide cleavage in formic acid could be decreased to 3 hours by increasing the concentration of cyanogen bromide 12-fold. This gave the other 25 min, the mixture was desalted on a Sephadex G-25 column. Lyophilization of the fragments to be described below, lyophilized fragments were used. The reaction time required for cyanogen bromide cleavage in formic acid could be decreased to 3 hours by increasing the concentration of cyanogen bromide 12-fold. This gave the fragments in yields and purities identical with those obtained after the longer reaction time. Cleavage in 0.1 M formic acid could be decreased to 3 hours by increasing the concentration of cyanogen bromide 12-fold. This gave the another 25 min, the mixture was desalted on a Sephadex G-25 column. Lyophilization of the fragments to be described below, lyophilized fragments were used.

Reduction and Carboxymethylation of Fragment N—To Fragment N (1 μmole) in 1.75 ml of 0.10 M Tris and 0.06 M HCl (pH 7.96) was added urea (1.52 g) to a final concentration of 8 M. After 3 hours at 25° under N2 and in the presence of diithiothreitol (28 mg, 180 μmole), a solution (0.75 ml) of iodoacetate (85 mg, 450 μmole) in 1.2 M Tris was added. After another 30 min, the mixture was freed of reagents by dialysis in Visking tubing against 0.1 M ammonium bicarbonate and used directly for the separation of reduced peptides on Sephadex G-100 (Fig. 2).

Reduction and Carboxamidomethylation or Carboxymethylation of Fragment C—To a suspension of Fragment C (5 μmole) in 2 ml of 2 M Tris and 0.5 M HCl (pH 8.7) was added guanidine hydrochloride (3.3 g) to a final concentration of 8 M. As reduction proceeded under N2 in the presence of diithiothreitol (47.4 mg, 307 μmole), all the suspension dissolved. After 3 hours at 25°, solid iodoacetamide (300 mg, 1600 μmole) was added. After another 35 min, the mixture was desalted on a Sephadex G-25 column (2 x 66 cm) equilibrated with 2 N acetic acid. The front peak was collected and lyophilized. For separation of the reduced peptides on Sephadex G-100 (Fig. 3), loads larger than 1 μmole of Fragment C led to poor resolution.

For carboxamidomethylation, 0.8 μmole of Fragment C was reduced in 1 ml of 2 M Tris and 0.5 M HCl containing guanidine hydrochloride (1.65 g) and diithiothreitol (31 mg) for 3 hours, then a solution of iodoacetate (130 mg) in 1 ml of 2 M Tris was added. After 30 min the mixture was freed of excess reagents by dialysis against 0.1 M ammonium bicarbonate and used directly for separation on a Sephadex G-100 column (Fig. 4). Lyophilization of the reduction mixture was avoided, since one of the peptides (the arginyl peptide) redissolved with difficulty.

Limited Tryptic Hydrolysis of Half-cystinyl Bovine Plasma Albumin—A solution of half-cystinyl-BPA (150 mg) in 3.0 ml of 0.025 M Tris and 0.015 M HCl was cooled to 0°, and its pH was adjusted to 8.8 by the addition of 0.21 ml of 0.1 N NaOH. A 150-μl aliquot of the trypsin solution (10 mg per ml in 1 x 10^{-4} M HCl) was added. The pH of the digestion mixture was monitored with a pH meter and was kept at pH 8.8 ± 0.05 by periodic addition of 20-μl aliquots of 0.1 N NaOH. After reaching the desired extent of hydrolysis, the reaction mixture was acidified to pH 3.1 with 25 μl of 6 N HCl, and 0.3 ml of a buffer consisting of 1.74 M formic acid and 0.20 M ammonium formate (pH 2.86) was added.

The mixture was next separated on a column of Sephadex G-150 (200 × 0.9 cm). The column was eluted at 25° with a buffer of 0.174 M formic acid and 0.026 M ammonium formate at a flow rate of 12 ml per hour, and fractions of 3-ml volume were collected. The desired Fraction T-2 (Fig. 6) was concentrated by ultrafiltration and rechromatographed on the same column.

The concentration of rechromatographed Fraction T-2 was neutralized to pH 7.4 by addition of 2 M Tris and was dialyzed against 0.10 M Tris and 0.08 M HCl (pH 7.61). It was next separated on a column (0.9 x 22 cm) of DEAE-cellulose (Whatman DE-32 grade). The column was eluted at 25° with a linear gradient formed with 200 ml each of 0.10 M Tris and 0.08 M HCl, and 0.10 M Tris, 0.08 M NaCl, and 0.10 M NaCl at a flow rate of 36 ml per hour, and 3-ml fractions were collected. The desired fractions, T-22 and T-23 (Fig. 7), were concentrated and dialyzed against the starting buffer. They were each rechromatographed to reduce their cross contamination with a recovery yield of 50 to 60%. For cyanogen bromide cleavage of Fragment T-23, the concentrate was dialyzed against water and then lyophilized. The procedures used for cyanogen bromide cleavage and for reduction and carboxamidomethylation were the same as those used for half-cystinyl-BPA.

Equilibrium Dialysis—The binding of organic ligands by half-cystinyl-BPA and its fragments were determined by equilibrium dialysis in a simple apparatus containing 14 cells. The two cylindrical compartments (width, 0.004 inch and diameter, 0.7 inch) of each cell were separated by a sheet of Visking dialysis tubing. The total volume of each compartment was 0.5 ml.

Binding experiments were carried out at 24 ± 1° in a buffer of 0.05 M Tris, 0.03 M HCl, and 0.10 M NaCl (pH 7.95). Into one compartment of each cell were introduced 300 μl of a protein solution (about 0.6 x 10^{-4} M), and into the other compartment were introduced 300 μl of a ligand solution. The protein solution was thoroughly dialyzed against the desired buffer before use. Duplicate analyses at several different ligand concentrations ranging from 0.3 x 10^{-4} M to 70 x 10^{-4} M were made. After equilibrating for about 16 hours, a 200-μl aliquot was withdrawn from each compartment for determination of the concentration of free and bound ligands by radioactivity. The concentration of bound ligand was calculated by taking the difference of the concentrations in the two compartments, and the concentration of free ligand was that in the compartment without protein. The average number of moles of ligand bound per mole of protein, denoted as N, was calculated by dividing the concentration of bound ligand by the protein concentration. The protein concentration was taken to be equal to that initially introduced into the compartment. At the end of an experiment, about 95% of the volume of solution initially introduced into each compartment was recoverable, and the total recovery of ligand from each cell ranged from 85 to 98%. The low recovery was probably due to absorption to the cell, since this occurred when the free ligand concentration was less than 0.1 x 10^{-4} M.

Binding of L-tryptophan was also determined spectrophotometrically. After equilibration, a 200-μl aliquot from each compartment was diluted with 0.8 ml of 10% trichloracetic acid. The solution from the protein-containing compartment was centrifuged to remove the precipitates before measuring its absorbance.

The binding data given in Figs. 11 and 12 are in the forms of Scatchard plots (22). The curves in the figures were calculated.
as described below. The binding of ligands by a protein with \(i\) classes of \(n\) equivalent and independent sites is described by the following equation:

\[
\frac{p}{A} = \frac{n_iK_i}{1 + K_i(A)}
\]

Equation 1

where \(p\) represents the average number of moles of ligand bound per mole of protein, \(K_i\) is the apparent binding constant for the site of class \(i\), and \(A\) is the free ligand concentration. In this study, the data were treated on the assumption that there are only two classes of binding sites, since our main interest is concerned with the tight binding primary class.

In the case where another ligand \((B)\) competitively binds only to one of the two classes of ligand \(A\) binding sites, Equation 1 becomes:

\[
\frac{p}{A} = \frac{n_iK_i}{1 + K_i(A)} + \frac{n_sK_s}{1 + K_s(A)}
\]

Equation 2

In this work, the free ligand concentration of \(B\) was not determined but was approximated to be equal to the total concentration used. The error from this approximation is small since the molar ratio of ligand \(B\) to protein was greater than 100.

**RESULTS**

Most albumin preparations are mixtures in which about two-thirds of the molecules have a free cysteinyl residue (23), but the remaining one-third has this cysteine in the form of a mixed disulfide with \(l\)-cysteine or glutathione (24). To avoid oxidation of the cysteine residue during cyanogen bromide cleavage, the albumin preparations used in this work were converted to the mixed disulfide form by reaction with \(l\)-cysteine or to the \(S\)-carboxamidomethylated derivative with iodoacetamide. These two derivatives will be referred to as half-cystinyl-BPA and \(S\)-carboxamidomethylated-BPA, respectively.

**Isolation of Cyanogen Bromide Fragments of Bovine Plasma Albumin**—Five peptides should result on cyanogen bromide cleavage of BPA, since albumin contains 4 methioninyl residues. However, the presence of cross-linking disulfide bonds between the peptides decreases the number of products to two. The two products designated as Fragments N and C were isolated from the cleavage mixture by chromatography on Sephadex G-75. The upper graph of Fig. 1 was obtained with half-cystinyl-BPA after cleavage with cyanoformic acid in 0.1 \(\times\) HCl (25), and the lower graph was obtained with \(^{14}C\)-\(S\)-carboxamidomethylated-BPA after similar cleavage in 75\% aqueous formic acid. The elution positions of Fragments N and C indicated their molecular weights to be about 20,000 and 40,000, respectively. There was significantly more of the materials eluted in front of Fragment C when the cleavage was done in HCl rather than in formic acid. This difference is due to the different solvents used for cleavage and is not related to the nature of the protecting group. When half-cystinyl-BPA was cleaved in formic acid, the result was identical with that obtained with \(S\)-carboxamidomethylated-BPA. The radioactivity distribution of the \(^{14}C\)-labeled protecting group showed that the single cysteine residue of albumin is present in Fragment N.

The yields of the two fragments after rechromatography were both 80\% from cleavage of half-cystinyl-BPA in formic acid. Their homogeneity was studied by disc electrophoreses in Tris-urea gel and in acetate-urea gel. In both buffer systems the fragments migrated as a single band together with a very faint faster moving band. The results obtained with Tris-urea gel are given on the left of Fig. 1. The amino acid compositions of the fragments are reported in Table I. The sum of their compositions agrees well with the composition of BPA. The weight recoveries of Fragments N and C as amino acid residues were 98 and 94\%, respectively. The low weight recovery for Fragment C was probably due to bound buffer ions, since its nitrogen recovery was nearly quantitative. The sample of Fragment C analyzed was prepared by lyophilization from 0.1 \(\times\) ammonium bicarbonate. The molar extinction coefficients of Fragments N and C at 280 nm in ammonium formate buffer of pH 2.86 were found to be 17,100 \pm 1,000 and 20,500 \pm 1,200, respectively, and the values calculated from their amino acid compositions are 16,600 and 19,600.

Fragment N was found to contain 2 NH\(_2\)-terminal residues, aspartic and glutamic acid and alanine (Table II). It contained 2 COOH-terminal residues of homoserine as indicated by its composition, although carboxypeptidase A digestion released only 0.45 residue of it together with traces of other amino acids (less than 0.05 residue each). These data indicate that Fragment N contains two cyanogen bromide peptides. Fragment C was found to contain 3 NH\(_2\)-terminal residues, glutamic acid and proline, by the cyanate procedure (Table II) and by Edman degradation.
since no such end groups were found. This is believed to be an artifact, addition to the residues reported, about 0.5 mole of carboxy-

| Amino acid | Residues per 21,600 g | Nearest integer | Residues per 46,000 g | Nearest integer | Residues per 65,000 g | Nearest integer |
|------------|----------------------|----------------|-----------------------|----------------|-----------------------|-----------------|
| Lysine     | 17.7 ± 0.4           | 18             | 40.0 ± 0.7            | 40             | 57.6 ± 1.4            | 58              |
| Histidine  | 7.8 ± 0.1            | 8              | 0.1 ± 0.2             | 9              | 16.6 ± 0.5            | 17              |
| Arginine   | 5.2 ± 0.1            | 5              | 17.8 ± 0.4            | 18             | 23.0 ± 0.5            | 23              |
| Aspartic acid | 18.3 ± 0.1       | 19             | 34.2 ± 0.4            | 34             | 53.0 ± 1.4            | 34              |
| Threonine  | 7.0 ± 0.2            | 7              | 25.7 ± 0.5            | 26             | 22.2 ± 0.3            | 32              |
| Serine     | 7.1 ± 0.3            | 7              | 20.0 ± 0.8            | 20             | 26.2 ± 0.3            | 26              |
| Glutamic acid | 27.6 ± 0.4        | 28             | 52.2 ± 0.7            | 52             | 80.0 ± 1.4            | 80              |
| Proline    | 8.6 ± 0.3            | 9              | 18.4 ± 0.4            | 18             | 27.8 ± 1.4            | 27              |
| Glycine    | 7.6 ± 0.2            | 8              | 7.8 ± 0.1             | 8              | 14.9 ± 0.3            | 15              |
| Alanine    | 6.0 ± 0.2            | 6              | 27.0 ± 0.5            | 27             | 34.9 ± 0.2            | 34              |
| Methionine | <0.1                 |                |                       |                | 3.7 ± 0.1             | 4               |
| Isoleucine | 3.9 ± 0.1            | 4              | 8.8 ± 0.2             | 9              | 13.8 ± 0.1            | 13              |
| Leucine    | 18.1 ± 0.1           | 18             | 38.9 ± 0.4            | 39             | 57.7 ± 1.4            | 57              |
| Tyrosine   | 8.1 ± 0.3            | 8              | 10.0 ± 0.4            | 10             | 19.4 ± 0.5            | 19              |
| Phenylalanine | 10.7 ± 0.3     | 11             | 15.2 ± 0.1            | 15             | 25.5 ± 0.3            | 25              |
| Tryptophan | 1.0                  | 1              | 1.0                   | 1              | 2.0                   | 2               |
| Homoserine | 1.6 ± 0.2            | 2              | 1.6 ± 0.2             | 2              | 2                     | 2               |

| Total      | 184                  |                | 383                   | 43,421         | 571                   | 65,243          |
| Molecular weight | 21,219             |                | 43,421                |                | 65,243                | 64,640          |
| Nitrogen recovery | N.D.            |                | 98.0%                 |                | 100.0%                |                |
| Weight recovery | 98.3%            |                | 94.3%                 |                | 96.7%                 |                |

* Averages of five preparations ± standard deviations.

* Averages of six preparations ± standard deviations.

* Averages of three preparations ± standard deviations.

* The sample of half-cystinyl-BPA analyzed was purified by successive chromatographies on Sephadex G-100 and DEAE-cellulose. The composition found is in close agreement with those in the literature (26, 27).

* Values in parentheses were not included in the totals.

* Molecular weights were calculated from the composition determined.

* N.D., not determined.

and arginine by the procedure of dansylation. The yield of dansyl arginine was qualitatively estimated to be about 30%, but affirmative evidence for its presence is given by the work of Brown et al. (28), who isolated a tryptic peptide of BPA containing the sequence of Met–Arg. Fragment C contains 2 homoserine residues, and carboxypeptidase A digestion released 0.60 residue of homoserine together with 1.39 residues of alanine, 0.56 residue of leucine, 0.60 residue of threonine, and 0.68 residue of valine. These data together would indicate that Fragment C contains three cyanogen bromide peptides. As aspartic acid and alanine are, respectively, the known NH₂- and COOH-terminal residues of Fragments N and C, it follows then that Fragments N and C contain, respectively, the NH₂-terminal and the COOH-terminal portions of BPA.

The end group results of Table II indicated that the NH₂-terminal residues of Fragments N or C were not found in equimolar quantities when they were obtained from cleavage in HCl, but that they were nearly equimolar when the cleavage was done in formic acid. This finding suggested incompletely cleaved methionyl bonds in the fragments, a finding useful for establishing the relative positions of the two peptides of Fragment N to be described later.

During the course of isolation, Fragment N was found to have the interesting property of aggregation in the pH range of 4.1 to 5.3, as detected by chromatography on a Sephadex G-100 column with acetate buffers. At pH 4.6, about 50% of it was aggregated in the form of a polymer, and 30% of it was monomeric. The polymeric Fragment N could not be dissociated...
Fig. 2. Separation of reduced and carboxymethylated peptides of Fragment N on a Sephadex G-100 column (400 × 0.9 cm). The column was eluted with 0.1 M ammonium bicarbonate at a flow rate of 12 ml per hour, and fractions of 4-ml volume were collected. The fractions were analyzed by ninhydrin color after alkaline NH₂-terminal aspartyl peptide hydrolysis of 100–1200 µg aliquots (29). The upper graph was obtained with 0.6 µmole of chromatography. Their yields were about 65% when Fragment N was the starting material. The amino acid composition of the purified peptides

| Amino acid       | Aspartyl peptide Residues per 103 Residues per 15,000 g Nearest integer | Alanyl peptide Residues per 103 Residues per 15,000 g Nearest integer | Sum of peptides | Fragment N |
|------------------|---------------------------------------------------------------|---------------------------------------------------------------|----------------|-------------|
| Lysine           | 7.18 ± 0.17                                                   | 10.70 ± 0.20                                                  | 18             | 18          |
| Histidine        | 5.63 ± 0.16                                                   | 2.28 ± 0.06                                                   | 8              | 8           |
| Arginine         | 4.20                                                         | 5.70                                                         | (12)           |             |
| Aspartic acid    | 2.14 ± 0.02                                                   | 3.03 ± 0                                                      | 5              | 5           |
| Threonine        | 7.29 ± 0.04                                                   | 11.80 ± 0.30                                                  | 19             | 19          |
| Serine           | 4.86 ± 0.11                                                   | 2.37 ± 0.13                                                   | 7              | 7           |
| Glutamic acid    | 5.17 ± 0.12                                                   | 2.30 ± 0.31                                                   | 7              | 7           |
| Proline          | 13.30 ± 0.40                                                  | 14.70 ± 0.30                                                  | 28             | 28          |
| Glycine          | 1.93 ± 0.10                                                   | 7.64 ± 0.44                                                   | 9              | 9           |
| Alanine          | 4.75 ± 0.21                                                   | 3.19 ± 0.15                                                   | 8              | 8           |
| Half cystine     | 6.10 ± 0.13                                                   | 7.08 ± 0.25                                                   | 13             | 13          |
| Valine           | 4.94 ± 0.23                                                   | 1.15 ± 0.06                                                   | 6              | 6           |
| Isoleucine       | 1.98 ± 0.09                                                   | 2.01 ± 0.09                                                   | 4              | 4           |
| Leucine          | 9.75 ± 0.30                                                   | 9.00 ± 0.31                                                   | 4              | 4           |
| Tyrosine         | 2.20 ± 0.07                                                   | 6.92 ± 0.14                                                   | 8              | 8           |
| Phenylalanine    | 5.79 ± 0.20                                                   | 4.91 ± 0.25                                                   | 11             | 11          |
| Tryptophan       | 0.74 ± 0.02                                                   | 0.74 ± 0.14                                                   | 2              | 2           |
| Homoserine       | 3.84 ± 0.15                                                   | 6.60 ± 0.07                                                   | 11             | 11          |
| Total            | 88                                                          | 98                                                           | 18             | 18          |
| Molecular weight | 10,192                                                       | 11,872                                                       | 21,813         | 21,219      |
| Nitrogen recovery| N.D.                                                         | N.D.                                                         |                 |             |
| Weight recovery  | 99%                                                          | 100%                                                         |                 |             |

- Averages of five preparations ± standard deviations.
- Averages of four preparations ± standard deviations.
- One of twelve half-cystine residues of Fragment N is not linked by a peptide bond to the molecule, and it was removed on reduction and carboxymethylation to yield the two peptides.
- Molecular weights were calculated from the compositions determined.

into the monomeric form when rechromatographed in pH 2.8 buffer. Fragment C did not aggregate under these conditions.

Aspartyl and Alanyl Peptides of Fragment N—These two peptides were so designated according to their amino-terminal residues, and they were isolated as the carboxymethylated derivatives after reduction of the cross-linking disulfide bonds of Fragment N. The chromatogram in the upper graph of Fig. 2 illustrates the separation of the peptides on a Sephadex G-100 column using 0.1 M ammonium bicarbonate as a solvent. The Fragment N used was from cyanogen bromide cleavage of half-cystinyl-BPA in 0.1 n HCl. Cuts 2 and 3 contained the alanyl and the aspartyl peptides, respectively, and their amino-terminal residues were determined qualitatively on Edman degradation. Cut 1 represented incompletely cleaved Fragment N, since its amino acid composition was identical with that of Fragment N. The only difference was that it contained 1 residue each of homoserine and methionine. Its yield, about 20%, is in line with the finding in Table II that the yield of the amino-terminal alanine was 0.3 mole less than that of the aspartyl residue. This finding establishes that, in intact albumin, the alanyl peptide occupies a position penultimate to that of the NH₂-terminal aspartyl peptide.

The aspartyl and the alanyl peptides were purified by rechromatography. Their yields were about 65% when Fragment N, obtained from cleavage in formic acid, was used as the starting material. The amino acid composition of the purified peptides.
are given in Table III. The molecular weights of the aspartyl and the alanyl peptides were taken to be 10,300 and 12,100, respectively, for the calculation of their amino acid compositions, since only with these molecular weight values was the sum of the compositions of the peptides in close agreement with that of Fragment N. The molar extinction coefficients at 280 nm of the aspartyl and the alanyl peptides in 0.1 M ammonium bicarbonate were found to be 3,500 ± 500 and 12,800 ± 1,500, respectively. The corresponding values calculated from their amino acid compositions were 2,560 and 13,570.

To determine the location of the cysteinyl residue of BPA, the [14C]-carboxamidomethylated Fragment N was next reduced and carboxymethylated, and the two resulting peptides were separated as shown in the lower graph of Fig. 2. About 80% of the radioactivity in Fragment N was present in the aspartyl peptide faction, and about 6% was in the alanyl peptide fraction. On rechromatography of the alanyl peptide fraction, all of its radioactivity was removed, thus indicating that the single cysteinyl residue of albumin is located in the aspartyl peptide region.

**Arginyl, Prolyl, Glutamyl Peptides of Fragment C**—These three peptides designated according to their NH2-terminal residues, were isolated in the form of the carboxamidomethylated derivatives, as well as in the form of the carboxymethylated derivatives after reduction of the disulfide bonds of Fragment C. In the experiments to be described below, only Fragment C, isolated from cleavage of albumin in formic acid, was used.

The separation of the carboxamidomethylated peptides on Sephadex G-100 in 2 N acetic acid is given in Fig. 3. Cuts 2, 3, and 4 contained the desired peptides. The NH-terminal residue of Cut 2 was shown qualitatively to be arginine using the nearest integer method.

**Table IV**

| Amino acid     | Arginyl peptidea | Prolyl peptideb | Glutamyl peptidec | Sum of peptides |
|----------------|------------------|-----------------|-------------------|----------------|
|                | Residues per 26,500 g | Nearest integer | Residues per 15,000 g | Nearest integer | Residues per mole | Nearest integer | |
| Lysine         | 21.0 ± 0.3        | 21              | 16.7 ± 0.1        | 17             | 3.0 ± 0.1        | 3               | 41 |
| Histidine      | 4.7 ± 0.1         | 5               | 4.0 ± 0.2         | 4              | 2              | 9               | 9  |
| Arginine       | 11.6 ± 0.3        | 12              | 4.8 ± 0.1         | 5              | 3.8 ± 0.1        | 4               | 17 |
| Aspartic acid  | 20.3 ± 0.3        | 20              | 10.8 ± 0.1        | 11             | 2.2 ± 0.2        | 2               | 35 |
| Threonine      | 10.6 ± 0.1        | 11              | 15.9 ± 0.4        | 16             | 6.3 ± 0.2        | 6               | 20 |
| Serine         | 12.3 ± 0.4        | 12              | 6.3 ± 0.3         | 6              | 4.0 ± 0.1        | 4               | 53 |
| Glutamic acid  | 28.8 ± 0.2        | 20              | 19.8 ± 0.3        | 20             | 1.1 ± 0.1        | 1               | 20 |
| Proline        | 9.0 ± 0.1         | 9               | 6.0 ± 0.3         | 10             | 1.1 ± 0.1        | 1               | 8  |
| Glycine        | 5.6 ± 0.2         | 6               | 8.0 ± 0.1         | 8              | 6.4 ± 0.3        | 6               | 31 |
| Alanine        | 18.8 ± 0.2        | 17              | 8.0 ± 0.1         | 8              | 6.4 ± 0.3        | 6               | 31 |
| Half-cystine   | 14.3 ± 0.2        | 14              | 10.3 ± 0.2        | 10             | 4.9 ± 0.2        | 5               | 29 |
| Valine         | 5.1 ± 0.1         | 5               | 4.0 ± 0.1         | 4              | 2.2 ± 0.2        | 2               | 40 |
| Isoleucine     | 21.2 ± 0.3        | 21              | 17.4 ± 0.2        | 17             | 3.0 ± 0.1        | 3               | 10 |
| Leucine        | 5.0 ± 0.2         | 7               | 5.0 ± 0.3         | 6              | 2.6 ± 0.1        | 3               | 15 |
| Tyrosine       | 7.0 ± 0.1         | 7               | 6.8 ± 0.2         | 6              | 2.3 ± 0.1        | 2               | 24 |
| Phenylyalanine | 1.0 ± 0.1         | 1               | 1.0 ± 0.1         | 1              | 2               | 2               | 2  |
| Tryptophan     | 0.8 ± 0.1         | 1               | 0.8 ± 0.1         | 1              | 4               | 4               | 4 |
| Homoserine     | 12.7 ± 0.4        | 13              | 8.6 ± 0.2         | 9              | 2               | 2               | 2  |
| Carboxymethylleucine | 121.0 ± 0.3 | 121             | 148              | 148            | 34             | 393             | 380 |
| Molecular weight | 24,785           | 24,785          | 17,504           | 17,504         | 3,719          | 3,719           | 43,421 |
| Nitrogen recovery | 98.3%            | 99.6%           | N.D.             | N.D.           | 85             | 95              | 90  |
| Weight recovery | 93.7%            | 94.8%           | N.D.             | N.D.           | 85             | 95              | 90  |

**Notes:**

a Averages of four preparations ± standard deviations.
b Averages of seven preparations ± standard deviations.
c Averages of five preparations ± standard deviations.
d Molecular weights were calculated from the compositions determined.
e N.D., not determined.
the dansyl technique, and those of Cuts 3 and 4 were found to be
proline and glutamic acid in yields of about 70% using the
cyanate method. The yields of the arginyl, the prolyl, and
the glutamyl peptides were 75, 65, and 75%, respectively.
After rechromatography the yields of the arginyl and the prolyl
peptides were 52 and 42%. Their homogeneity was checked
on disc electrophoresis in acetic-urea gel, each showing one
heavy band together with a series of faint slower moving bands
suggestive of aggregates of increasing sizes. On paper electrophoresis at pH 6 of the glutamyl peptide, only a single ninhydrin-
positive spot migrating toward the anode was observed. The
amino acid compositions of these peptides are given in Table IV,
and the molecular weights of the arginyl, the prolyl, and the
glutamyl peptides were taken to be 26,500, 18,300, and 4,130,
respectively, on the basis of their elution positions shown in
Fig. 3. The sum of the compositions of the peptides accounts
satisfactorily for the composition of Fragment C. The weight
recovery of amino acid residues was about 94% for the arginyl
and the prolyl peptides, but their nitrogen recovery was nearly
quantitative. The low weight recovery of these two peptides
might be due to tightly bound acetic acid, since these samples
were obtained on lyophilization from 2 N acetic acid. The
molar extinction coefficients at 280 nm in 2 N acetic acid for the
arginyl and the prolyl peptides were found to be 13,800 ± 1,400
and 4,660 ± 500, respectively. The calculated values from their
amino acid compositions are 14,650 and 3,940.

The separation of these three peptides as carboxymethylated
derivatives was carried out on a column of Sephadex G-150 in
0.1 M ammonium bicarbonate (top graph, Fig. 4). The arginyl
peptide was isolated in 80% yield from Cut 1. Its amino acid
composition is identical with that shown in Table IV. It was
also present in Cut 2 as indicated by the similar patterns of these
two cuts on disc electrophoresis (top of Fig. 4). The arginyl
peptide in Cut 1 was aggregated because, when it was rechroma-
tographed on the same column using 0.1 M ammonium bicar-
bonate containing 0.04% sodium dodecyl sulfate as a solvent,
it elution position was significantly retarded. It was eluted
just in front of the proline peptide (the lower graph of Fig. 4)
and in accord with their molecular weight differences. Cut 3 of Fig. 4
has not been characterized. It was electrophoretically different
from Cuts 2 and 4 (top of Fig. 4). Cut 4 contained the prolyl pep-
tide in 50% yield. Its amino acid composition was the same
as that given in Table IV, with the exception that its glycine
content was consistently found to be only about 0.4 residue.
Disc electrophoresis of Cut 4 in Tris-urea gel showed the presence

![Fig. 4. Separation of reduced and carboxymethylated peptides of Fragment C on Sephadex G-150 column (200 × 0.9 cm). The upper graph was obtained with 0.9 amole of Fragment C, and the column was eluted with 0.1 M ammonium bicarbonate at a flow rate of 12 ml per hour. The lower graph shows the rechromatographies of Cuts 1 (●) and 4 (○) from the upper graph, and the column was eluted with 0.1 M ammonium bicarbonate containing 0.04% sodium dodecyl sulfate. The photograph at top shows the disc electrophoretic patterns of the cuts in Tris-urea gel; from left to right, Cuts 1 to 4 and an admixture of Cuts 3 and 4.](http://www.jbc.org/)

![Fig. 5. Rate of tryptic hydrolysis of half-cystinyl-BPA at pH 8.8. The concentrations of half-cystinyl-BPA and trypsin were 48 and 0.48 mg per ml, respectively.](http://www.jbc.org/)
FIG. 6. Separation of tryptic digests of half-cystinyl-BPA on a Sephadex G-150 column (200 × 0.9 cm). The column was eluted with a buffer of 0.174 M formic acid and 0.026 M ammonium formate (pH 2.86) at a flow rate of 10 ml per hour. The upper graph was obtained with a 51-min digest of nondefatted half-cystinyl-BPA (135 mg). The lower graph was obtained with a 29-min digest of defatted 14C-half-cystinyl-BPA (147 mg).

FIG. 7. Separation of Fraction T-2 on a DEAE-cellulose column (22 × 0.9 cm). The column was eluted with a linear NaCl gradient (0 to 0.10 M) in a buffer of 0.10 M Tris and 0.08 M HCl (pH 7.61) at a flow rate of 36 ml per hour. The upper graph was obtained with T-2 (23 absorbance units at 280 nm) from the 29-min digest of defatted half-cystinyl-BPA. The lower graph shows the rechromatography of Fraction T-23 (15 absorbance units at 280 nm).

Table V

| Amino Acid | Fragment T-22 | Fragment T-23 |
|------------|---------------|---------------|
|            | Residues per 43,100 g | Nearest Integer | Residues per Mole | Nearest Integer |
| Lysine     | 36.6 ± 1.0 | 37 | 36.0 ± 0.9 | 36 |
| Histidine  | 9.3 ± 0.5 | 9 | 8.7 ± 0.2 | 9 |
| Ammonia    | 13.6 ± 0.3 | 14 | 14.0 | 14 |
| Arginine   | 13.9 ± 1.7 | 35 | 34.8 ± 0.6 | 35 |
| Aspartic acid | 24.2 ± 1.0 | 24 | 25.0 ± 0.4 | 25 |
| Threonine  | 18.3 ± 0.6 | 18 | 18.7 ± 1.0 | 19 |
| Serine     | 18.3 ± 0.6 | 18 | 18.7 ± 1.0 | 19 |
| Glutamic acid | 47.5 ± 2.3 | 48 | 48.0 ± 0.6 | 48 |
| Proline    | 10.2 ± 1.2 | 10 | 10.9 ± 0.2 | 10 |
| Glycine    | 7.5 ± 0.6 | 8 | 7.8 ± 0.3 | 8 |
| Alanine    | 28.0 ± 1.1 | 28 | 27.4 ± 0.1 | 27 |
| Half-cystine | 23.4 ± 0.9 | 24 | 22.8 ± 1.0 | 23 |
| Valine     | 22.6 ± 1.4 | 23 | 23.4 ± 0.5 | 23 |
| Methionine | 1.7 ± 0.1 | 2 | 1.6 ± 0.1 | 2 |
| Isoleucine | 8.1 ± 0.5 | 8 | 8.1 ± 0.7 | 8 |
| Leucine    | 34.1 ± 1.2 | 34 | 33.3 ± 0.1 | 33 |
| Tyrosine   | 10.1 ± 0.6 | 10 | 11.0 | 11 |
| Phenylalanine | 12.1 ± 0.4 | 12 | 14.1 | 14 |
| Tryptophan | 1.1 | 1 | 1.0 | 1 |
| Total      | 354 | 356 |
| Weight recovery | 92.8% | 43,421 |

* Averages of five preparations ± standard deviations.
+ Averages of two preparations ± standard deviations.
* The 2 methionine residues were present as homoserine.

Isolation of Tryptic Fragments of Bovine Plasma Albumin—Both defatted and nondefatted half-cystinyl-BPA were tested as starting materials. This is because most albumin preparations contain bound fatty acids (21), and albumin with bound organic ligands is known to be stabilized against denaturation (20) and proteolytic digestion (31). The rate of tryptic hydrolysis of defatted and nondefatted half-cystinyl-BPA at pH 8.8 is shown in Fig. 5. At 25° the nondefatted half-cystinyl-BPA was hydrolyzed too rapidly for convenient isolation of the fragments. Therefore, the temperature was lowered to decrease the rate of hydrolysis. At 0° the defatted and the nondefatted samples both showed initial rapid cleavages followed by slow cleavages. But the nondefatted sample was hydrolyzed more slowly than the defatted one, probably as a result of ligand-stabilized conformation or conformations.

The digests after acidification were separated on a column...
Table VI

| Amino acid | T-1 | T-22 | T-23 | CNBr-T-23 | RCAM-CNBr-T-23* |
|------------|-----|------|------|-----------|----------------|
| Aspartic acid | 1.30 | 0.11 | 0.10 | 0.12 | 0.10 |
| Glutamic acid | 0.38 | 0.32 | 0.07 | 1.11 | 1.2 |
| Proline      | 0.09 |      |      | 0.94 |      |
| Valine       | 0.05 | 0.12 | 0.75 | 0.73 | 0.56 |
| Leucine      | 0.09 | 0.78 | 0.16 | 0.14 |      |
| Tyrosine     | 0.62 | 0.13 |      |      |      |

* CNBr-T-23, Fragment T-23 after cyanogen bromide cleavage.
* RCAM-CNBr-T-23, Fragment T-23 after cyanogen bromide cleavage followed with reduction and carboxamidomethylation. The two cuts were obtained as shown in Fig. 9. In addition to the amino acids reported, about 4.5 residues of carboxymethylcysteine were found, and these are believed to be an artifact.

Fig. 8. Separation of cyanogen bromide cleaved T-23 (18 mg) on a Sephadex G-150 column (200 × 0.9 cm). The chromatographic conditions are the same as Fig. 6. Lyophilization of the indicated cut yielded 14 mg of product.

Fig. 9. Separation of reduced and carboxamidomethylated peptides of cyanogen bromide cleaved T-23 on a Sephadex G-75 column (200 × 0.9 cm). The column was eluted with 2 N acetic acid at a flow rate of 11 ml per hour. Ninhydrin analyses were made with 25-μl aliquots after alkaline hydrolysis. The upper graph was obtained with 0.31 μmole of cleaved T-23. ○, A at 230 nm; □, A at 570 nm. Indicated cuts were recovered by lyophilization. Rechromatographies of Cuts 1 and 2 are shown in the lower graph.

cule. The materials in Cut T-8 have molecular weights of about 20,000 since trypsin was eluted in this region.

The two fragments in Cut T-2 were separated by chromatography on DEAE-cellulose, designated as T-22 and T-23 in the upper graph of Fig. 7. Both fragments were rechromatographed, but only the rechromatography of T-23 is shown in the lower graph of Fig. 7. After rechromatography, the two fragments were still slightly cross contaminated with each other as shown by disc electrophoresis at pH 8.9. The disc electrophoretic mobilities of Fragments T-22 and T-23 were, respectively, slightly slower and faster than that of half-cystinyl-BPA. The amino acid compositions of the two fragments were indistinguishable from that of Fragment C obtained from cyanogen bromide cleavage of BPA. The two tryptic fragments differed in their NH₂-terminal end groups (Table VI); T-23 has valine as its end group, and T-22 has two end groups, leucine and tyrosine.

The molecular weight of Fragment T-23 was established to be 40,024 on the basis of its amino acid composition and its elution position from Sephadex G-150. If the methionine content of T-23 were taken to be any integral number other than 2, its molecular weight would not be consistent with its elution position. Using the molecular weight of 40,024, the molar extinction coefficient of Fragment T-23 at 280 nm was determined to
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Amino acid compositions of reduced and carboxamidomethylated peptides from cyanogen bromide cleaved Fragment T-23

Values given are the averages of duplicate analyses from a single preparation with the exception of those of the glutamyl peptide which were from a single 22-hour analysis. The number of residues per mole of peptide was calculated by taking the glutamic acid and the alanine contents to be, respectively, 26 and 14 residues for the valyl peptide, 20 and 8 for the prolyl peptide, and 4 and 6 for the glutamyl peptide.

Table VII

| Amino acid          | Valyl peptide | Prolyl peptide | Glutamyl peptide | Sum of peptides |
|---------------------|---------------|----------------|-------------------|-----------------|
|                     | Residues found| Nearest integer| Residues found    | Nearest integer| Residues found| Nearest integer| Fragment T-23 |
| Lysine              | 17.6 ± 0.3    | 18 (21)        | 17.4 ± 0.2        | 17 (17)        | 3.0           | 3 (3)           | 38              |
| Histidine           | 4.5 ± 0.3     | 5 (5)          | 4.4               | 4 (4)          | 0.1           | 0 (1)           | 9               |
| Arginine            | 8.5 ± 0.3     | 9 (12)         | 5.3 ± 0.1         | 5 (9)          | 0.1           | 0 (1)           | 14              |
| Aspartic acid       | 19.9 ± 0.8    | 20 (20)        | 10.6 ± 0.1        | 11 (11)        | 4.0           | 4 (4)           | 35              |
| Threonine           | 9.0 ± 0.1     | 9 (11)         | 10.4 ± 0.4        | 16 (16)        | 2.0           | 2 (2)           | 27              |
| Serine              | 10.2 ± 0.1    | 10 (12)        | 5.9 ± 0.3         | 6 (6)          | 1.1           | 1 (1)           | 17              |
| Glutamic acid       | 26.5 ± 0.1    | 27 (29)        | 20.1 ± 0.1        | 20 (20)        | 4.0           | 4 (4)           | 51              |
| Proline             | 10.2 ± 0.1    | 10 (9)         | 10.2 ± 0.2        | 10 (10)        | 1.3           | 1 (1)           | 21              |
| Glycine             | 5.4 ± 0.2     | 5 (6)          | 0.7 ± 0.1         | 1 (1)          | 1.0           | 1 (1)           | 7               |
| Alanine             | 13.8 ± 0.2    | 14 (17)        | 7.8 ± 0.2         | 8 (8)          | 6.1           | 6 (6)           | 28              |
| Half-cystine        |               |               |                   |                |               |                 |                 |
| Valine              | 9.8 ± 0.2     | 10 (14)        | 9.6               | 10 (10)        | 3.7           | 4 (5)           | 24              |
| Methionine          |               |               |                   |                |               |                 |                 |
| Isoleucine          | 3.7 ± 0.3     | 4 (5)          | 3.7 ± 0.2         | 4 (4)          | 8             | 8               | 8               |
| Leucine             | 17.7 ± 0.5    | 18 (21)        | 16.5 ± 0.4        | 17 (17)        | 2.0           | 2 (2)           | 37              |
| Tyrosine            | 7.2 ± 0.1     | 7 (7)          | 9.8 ± 0.2         | 5 (6)          | 2.5           | 3 (3)           | 12              |
| Phenylalanine       | 5.3 ± 0.1     | 5 (7)          | 4.6 ± 0.2         | 5 (6)          |               |                 |                 |
| Tryptophan          | 1.0           | 1 (1)          |                   | 1 (1)          |               |                 | 1               |
| Homoserine          | 1.0           | 1 (1)          | 1.0               | 1 (1)          |               |                 | 2               |
| Carboxymethylcysteine | 12.0 ± 0.4   | 12 (13)        | 8.6 ± 0.1         | 9 (9)          | 2.3           | 2 (2)           | 23              |
| Total               | 185 (211)     |               |                    | 147 (148)      | 33 (34)       | 364 (364)       | 354             |

* Values in parentheses are for the corresponding peptides obtained from Fragment C.

Fig. 10. A schematic structure of bovine plasma albumin. The molecule contains a total of 579 residues. The positions of the 4 methionine residues are, respectively, residue 88, 156, 397, and 545. The indicated positions of peprisin and subtilisin cleavages are taken from References 3 and 37, respectively.

be 19,100 ± 1,000, while the value calculated from its tyrosine, tryptophan, and cystine contents is 19,900.

The yields of Fragments T-22 and T-23 were 15 and 13%, respectively, from the 51-min digest of nondefatted half-cystinyl-BPA (the upper graph of Fig. 6), and were 11 and 18% from the 29-min digest of defatted half-cystinyl-BPA (the lower graph of Fig. 6). The yields of Fragments T-22 and T-23 could be increased to 13 and 21% by terminating the digestion of defatted half-cystinyl-BPA at 20 min, and at this stage there was still 16% of undigested albumin. In practice, it was found more convenient to terminate the digestion at 29 min, since this obviates the need of a careful separation of the undigested material. By carrying the digestion of the defatted half-cystinyl-BPA to 50 min, the yield of Fragment T-23 decreased to 17%, while that of Fragment T-22 was about 12%. Thus, at all stages of digestion, the yield of Fragment T-23 was higher than that of Fragment T-22, while the yield of Fragment T-22 remained approximately constant. This dependence of the yields of the two fragments on the length of digestion suggests that they both are unstable intermediates, and that Fragment T-22 is probably formed from Fragment T-23.

Cyanogen Bromide Cleavage of Fragment T-23—Cleavage of the 2 methionyl residues of Fragment T-23 yielded a single product in 80% yield (Fig. 8). The product had an amino acid composition identical with that of the intact fragment with the difference of replacement of 2 methionine residues by 2 homoserine residues. The product had two new NHz-terminal end groups of proline and glutamic acid in addition to the expected end group of valine (Table VI). These results indicated that the product was a complex of three peptides cross-linked by disulfide bonds. This finding paralleled that of Fragment C.


FIG. 11. Octanoate binding by Fragment T-23 (upper graph) and by half-cystinyl-BPA (lower graph). Experiments were carried out in a buffer of 0.05 M Tris, 0.03 M HCl, and 0.10 M NaCl (pH 7.95) at 24 ± 1°C. The protein concentration was about 0.6 × 10⁻⁸ M, and the initial ligand concentration ranged from 0.3 to 30 × 10⁻⁴ M. ●, the data for octanoate binding; X, the data for competitive binding of octanoate in the presence of 50 × 10⁻⁴ M of n-tryptophan. The curves are calculated with the constants given in Table VIII. Competitive binding of octanoate by half-cystinyl-BPA was also determined in the presence of 90 × 10⁻⁴ M of n-tryptophan, and the results were in agreement with the calculated curve.

which is also a complex of three peptides cross-linked by disulfide bonds with NH₂-terminal end groups of arginine, proline, and glutamic acid.

The three peptides of the cleaved Fragment T-23 were separated after reduction and carboxamidomethylation. This is depicted in the upper chromatogram of Fig. 9. Cuts 1, 2, and 3 contained the desired peptides. Cuts 1 and 2 were rechromatographed to reduce their cross contamination as shown in the lower graph of Fig. 9. The yield of Cut 3 was 75%, while the yields of the rechromatographed Cuts 1 and 2 were 31 and 48%, respectively. The NH₂-terminal end groups of Cuts 1 and 2 were found to be valine and proline, respectively (Table VI). Cut 3 must have glutamic acid as its NH₂-terminal end group as deduced from the end group results of the cyanogen bromide cleaved Fragment T-23 and Cuts 1 and 2. The amino acid compositions of the three cuts are given in Table VII. Cuts 2 and 3 had compositions identical with those of the prolyl and the glutamyl peptides isolated from Fragment C, while Cut 1 had a composition very similar to that of the arginyl peptide of Fragment C. These findings, therefore, provide strong support that

Fig. 12. L-Tryptophan binding by Fragment T-23 (upper graph) and by half-cystinyl-BPA (lower graph). 

The alignment of the three cyanogen bromide peptides in the intact Fragment T-23 must be in the order of valyl peptide, prolyl peptide, and glutamyl peptide, since this is required by the NH₂-terminal residue of the valyl peptide and by the absence of a homoserine residue in the glutamyl peptide. This information has also permitted the alignment of the three peptides of Fragment C in the intact BPA as shown in Fig. 10.

Organic Ligand-binding Properties of Fragment T-23—The tryptic Fragment T-23 was found to bind octanoate anion, L-, and n-tryptophan, as does intact BPA (32-34). The binding of octanoate and of L-tryptophan was determined by equilibrium dialysis, while the binding of n-tryptophan was determined indirectly by competitive binding with L-tryptophan and with octanoate. The data for the fragment, as well as those for defatted half-cystinyl-BPA are shown in Figs. 11 and 12. The data were analyzed according to the equations given under “Experimental Procedure,” with the assumption that there is a maximum of two classes of binding sites present in the protein. The number of sites in each class and their apparent binding constants which would best fit the data are listed in Table VIII. These values were used to calculate the curves shown in the figures.

Binding studies were also carried out with Fragment C. The
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Summary of organic ligand-binding constants of Fragment T-23 and half-cystinyl bovine plasma albumin

| Ligand         | Fragment T-23                  | Half-cystinyl-BPA          |
|----------------|--------------------------------|----------------------------|
| Octanoate      | \( n_1 = 1, K_1 = 5.0 \times 10^4 \) | \( n_1 = 1, K_1 = 16 \times 10^4 \) |
| \( n_2 = 3, K_2 = 0.2 \times 10^9 \) | \( n_2 = 10, K_4 = 1 \times 10^9 \) |
| \( n_1 = 1, K_1 = 0.84 \times 10^4 \) | \( n_1 = 1, K_1 = 2.1 \times 10^4 \) |
| \( n_2 = 2, K_2 = 0.5 \times 10^5 \) | \( n_2 = 2, K_2 = 0.5 \times 10^5 \) |
| \( n_1 = 1, K_1 = 0.32 \times 10^8 \) | \( n_1 = 1, K_1 = 1.2 \times 10^9 \) |
| \( (K_4 = 0.24 \times 10^9) \) | \( (K_4 = 0.7 \times 10^9) \) |

* The first value was obtained from competitive binding with L-tryptophan, and the second value in parentheses was from competitive binding with octanoate.

The data suggested that Fragment C had one weak binding site for octanoate with a binding constant of about \( 10^3 \) or less. No detectable binding of L-tryptophan was observed. Under the experimental conditions used, binding with a constant of less than 500 would have escaped detection.

**DISCUSSION**

On cyanogen bromide cleavage of BPA, two fragments, N and C, were formed. After reduction of the crosslinking disulfide bonds of the fragments, five peptides were isolated in over-all yields of 45 to 60%. These peptides with NH2-terminal residues of aspartic acid, alanine, arginine, proline, and glutamic acid contain 88, 89, 211, 148, and 34 amino acid residues, respectively. The five peptides together account for the molecule of BPA with 580 amino acid residues. The molecular weight calculated from this composition is 65,500. This value is slightly less than the value of about 67,000 determined by physical techniques (4), but the difference is well within the experimental error of the methods used.

It was possible to position three of the five peptides in the BPA molecule from the results of end-group analyses and by the isolation of an incompletely cleaved Fragment N. The positions of these three peptides, the aspartyl, the alanyl, and the glutamyl peptides, are shown in Fig. 10. The alignment of the remaining two peptides was made possible from studies of a tryptic fragment of BPA. This trypic fragment, T-23, containing 2 methionyl residues, was derived from the COOH-terminal two-thirds of BPA, just as Fragment C was. The data on the relative positions of the three cyanogen bromide peptides of Fragment T-23 provided the necessary information to align the two remaining arginy1 and prolyl peptides in BPA (Fig. 10). This unique arrangement of the five cyanogen bromide peptides of BPA is consistent only with the interpretation that BPA is a molecule of a single polypeptide chain.

BPA has a single cysteine residue and 34 half-cystine residues forming 17 disulfide bonds. The present work has located the single cysteine residue in the NH2-terminal region of the molecule, Residues 1 to 85. Almost half of the sequence in this region is already known. The structure of a nonapeptide containing the cysteine residue of BPA was determined by Witter and Tuppy (35), and an identical structure was found for that from human albumin. The sequences of Residues 1 to 24 of bovine, rat, and human albumins were elucidated by Bradshaw and Peters (36), and extensive similarities were observed.

Of the 17 disulfide bonds of BPA, 5 are located in the Fragment N region and 12 in the Fragment C region. On the basis of the findings of other workers, the disulfide bonds in the Fragment C region may be divided further into three separate sets as given in Fig. 10. The presence of two disulfide bonds in the COOH-terminal one-fifth of BPA molecule follows from the work of Peters and Hawn (3) who isolated a 77-residue peptic fragment corresponding to this region of the molecule, and from our own observation that the glutamyl peptide is released from Fragment C only after reduction. The presence of two sets of five disulfide bonds in the central region of BPA can be deduced from the work of Pederson and Foster (37). These workers found that subtilisin cleaved a detergent-complexed BPA into two half-molecules of 270 and 320 residues having eight and nine disulfide bonds, respectively.

This distribution of the disulfide bonds in four separate regions of BPA is in accord with its physical property of conformational flexibility in acid and in alkaline solutions (38, 39). It is also in accord with proposed models of BPA consisting of compact regions linked by flexible parts as indicated by the low angle x-ray diffraction studies (40), as well as by the isolation of large fragments on limited proteolysis of albumin with pepsin (41), trypsin (5, 6), and chymotrypsin (42, 43).

The homogeneity of BPA is still an unresolved issue, since a number of workers have reported the heterogeneity of BPA by chromatographies on hydroxylapatite (44), DEAE-cellulose (45), and DEAE-Sephadex (46) as well as by solubility studies (47, 48). The data on the compositions of the cyanogen bromide peptides of BPA do not help to settle the issue of whether or not heterogeneity of albumin has a structural origin because of the large sizes of two of the five peptides. However, the present work does offer an orderly approach to the study of this problem.

A comparison of the organic ligand-binding properties of the tryptic Fragment T-23 and defatted half-cystinyl BPA showed that they have similar properties. The fragment and half-cystinyl-BPA both had one primary binding site for the three ligands tested. The primary binding constant of the fragment for each ligand was about one-third of that of half-cystinyl-BPA. The primary binding constants in Table VIII are estimated to have an error of about 20% with the exception of the value for octanoate binding by half-cystinyl-BPA. This value may have an error of as much as 50% due to the scattering of data in the region of \( 1 \) (lower graph of Fig. 11). \( \nu \)-Tryptophan competitively displaced L-tryptophan and octanoate from the primary site of the fragment, as well as from that of half-cystinyl-BPA. This suggests that all three ligands bind at the same site. The ratio of the primary binding constants of L- and...
d-tryptophan is 30 for the fragment and 22 for half-cystinyl-BPA, and the ratio of the primary binding constants of octanoate and L-tryptophan is 6 for the fragment and 7.6 for half-cystinyl-BPA. These ratios for the fragment and for half-cystinyl-BPA are nearly identical within the experimental error. Therefore, these similarities suggest strongly that the primary binding sites of the fragment and half-cystinyl-BPA share common steric and structural features, thus implying that the primary binding site of the fragment is the same as one present in half-cystinyl-BPA.

A notable difference between the fragment and half cystinyl-BPA is the contribution of the secondary sites to the over-all binding process. The secondary sites of the fragment made only a small contribution to the over-all binding of octanoate, and were apparently absent for the binding of L-tryptophan. This is not the case for half-cystinyl-BPA, since the secondary sites contributed significantly to the over-all binding of octanoate or L-tryptophan. The number of secondary sites and their sites contributed significantly to the over-all binding of octanoate and were apparently absent for the binding of n-tryptophan. The secondary sites of the fragment made BPA is the contribution of the secondary sites to the over-all binding of octanoate. The value of the primary binding constant of half-cystinyl-BPA for L-tryptophan is about 50% higher than that originally determined by McMenamy and Oncley (33) in 0.05 M Tris-HCl containing 0.10 M NaCl (pH 7.75). However, they carried out the binding studies with undefatted BPA, and the single sulfhydryl group of BPA was not blocked, as is the case in the present work. To eliminate these variables, binding studies were carried out with undefatted BPA which had been freed of polymers by chromatography on Sephadex G-100. No difference was observed between the binding properties of undefatted BPA and defatted half-cystinyl-BPA. Fairclough and Fruton (34) have also studied the binding of L- and d-tryptophan by BPA. Since their studies were carried out at a different pH from that used in the present work, it is not feasible to compare their values directly with the present ones. However, it is possible to compare the ratio of their binding constants, 21, with the present value which is 22.

Teresi and Luck (32) have studied the octanoate binding by BPA in phosphate buffer at pH 7.6. They interpreted their binding data to indicate that BPA contained 4.2 primary sites with binding constants of 0.65 \times 10^4 and 27 secondary sites with binding constants of 0.01 \times 10^4. These values differ significantly from the present ones. The discrepancy is not related to the fact that defatted half-cystinyl-BPA was used in the present work, since experiments with nondefatted BPA yielded the same result. The discrepancy may be a result of the different buffer anions used in the two studies.

The decrease in the primary binding constant of the tryptic fragment may be a result of one factor or a combination of several factors. (a) The fragment has an altered conformation as compared to its state in half-cystinyl-BPA so that it binds the ligand less efficiently. (b) The fragment has several conformational states, of which one is favored for binding. (c) The fragment has lost a portion of the primary binding site of half-cystinyl-BPA. At present, there is no evidence to support the first two factors, but there is suggestive evidence for the third.

For simplification, the primary binding site of half-cystinyl-BPA may be considered to consist of two parts, one interacting with the hydrophobic portion of the ligand, and the other interacting with the anionic portion of the ligand. This consideration is indicated by the observations that BPA binds L-tryptophan methyl ester less tightly than it binds L-tryptophan (33), and that acetyl L-tryptophan amide is bound less tightly than acetyl-L-tryptophan (34). Since the fragment has the same steric and structural features for its binding site as half-cystinyl-BPA does, this will suggest that the two hydrophobic binding sites are the same. If that is the case, the decrease in the primary binding constant of the fragment may be due to the loss of the anionic binding site. The resistance of nondefatted half-cystinyl-BPA toward tryptic digestion may be interpreted to support the present hypothesis, that the stabilization is due to the ligand bridging the different segments of half-cystinyl-BPA molecule. It should be possible to test this hypothesis by comparing the binding properties of the fragment for L-tryptophan and its methyl ester.

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