Conformation-Dependent Limited Proteolysis of Bovine Plasma Albumin by an Enzyme Present in Commercial Albumin Preparations*

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ABSTRACT: While conducting conformational studies on bovine plasma albumin (BPA) (Armour fraction V, charcoal-defatted, sulfhydryl-blocked, monomeric protein), it was discovered that a proteolytic enzyme, referred to as plasma protease, is present in protein preparations of this type. Crystallized BPA was also found to contain this enzyme, but in lower concentrations than fraction V. The enzyme was isolated from BPA on SE-Sephadex chromatography in the preparation of pure bovine mercaptalbumin. Mercaptalbumin prepared by this method appeared to be essentially free of plasma protease. Plasma protease does not hydrolyze the N form of BPA. It gives a specific cleavage of BPA in the F form, and it hydrolyzes several bonds in acid-expanded BPA. The hydrolysis of BPA in the F form produces a protein, BPA*, which does not give a conformational change in the N → F transition region, but does undergo the acid expansion typical of BPA.

BPA* seems to be in a conformational state quite similar to the F form of BPA. On reduction and carboxymethylation of BPA* a large fragment of molecular weight approximately 46,000 is obtained from the N-terminal region of BPA while a fragment of molecular weight approximately 24,000 is produced from the C terminal of the molecule. Plasma protease can hydrolyze another bond in BPA* to give BPA** which on reduction gives the same N-terminal fragment as BPA*, but a C-terminal fragment reduced in molecular weight by 3000. Alignment of the fragments with respect to the native BPA molecule was done using ^{14}C labeling of the sulfhydryl group of BPA, and by determination of the tryptophan content and N-terminal residues of the fragments. Commercial samples of fraction V BPA from a single supplier were found to be fairly uniform in their plasma protease content. Commercial preparations of fraction V albumins from other species were also found to contain proteolytic activity. The properties of plasma protease do not correspond to any of the better known proteolytic enzymes.

The plasma albumins have been useful as model proteins for fundamental studies of the solution behavior of proteins because of their commercial availability. These proteins have been shown to undergo several conformational transitions, the best known being the highly cooperative N ↔ F transition which takes place near pH 4. At somewhat lower pH there is a drastic expansion of the F form of the protein. The binding equilibria with detergent anions also are known to involve several cooperative conformational changes in the protein. The extensive literature on the plasma albumins with special emphasis on the conformational changes they undergo was reviewed a decade ago by one of us (Foster, 1960) and a model for the protein molecule was proposed which is capable of explaining many of the known transitions and the binding properties, at least in a superficial way. This model envisioned the single peptide chain, of molecular weight approximately 66,000, as being folded in such a way as to form four globular regions which, in the native protein at neutral pH, were assumed to be tightly packed to form a compact molecule. The various transitions were pictured as involving loosening of the structure and separation of the globular units which, however, remained connected through the peptide bonds. Since the protein contains 17 disulfide cross-linkages, it was assumed that these must be distributed in such a manner as to be within, but not interconnecting, the four globular regions. A somewhat similar model has been proposed by Bloomfield (1966).

One obvious approach for testing this model is to attempt to cleave, with proteolytic enzymes, the peptide backbone interconnecting the postulated globular regions. Successful attainment of this objective would be favored by carrying out the proteolytic attack under conditions where the protein is in one of the isomerized states with the globular units spatially separated and the interconnecting peptide bonds exposed. Numerous attempts to obtain such limited cleavage of the protein have been made and have met with some success. Large globular units were obtained from BPA[1] and characterized to a limited extent using pepsin (Weber and Young, 1964) and trypsin (King, 1970). Human albumins also give large globular units on hydrolysis by these enzymes (Franglen and Swaniker, 1968; Marcus *et al.*, 1967). In general these studies were conducted under conditions governed by the properties of the enzymes used, rather than by the conformational state of the albumin. In our laboratory the detergent-induced conformational changes of the albumin have been used to expand the molecule and expose the peptide links between subunits. The

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[1] Abbreviations used are: BPA, bovine plasma albumin; BMA, bovine mercaptalbumin; SDS, sodium dodecyl sulfate; βME, β-mercaptoethanol; RCM, reduced and carboxymethylated; Fr V, the fifth fraction from the Cohn procedure for albumin preparation; BPA*, BPA molecule with one peptide bond hydrolyzed; BPA**, BPA with two peptide bonds hydrolyzed; HAc, acetic acid.

enzyme subtilisin was employed because of evidence that it has a strong preference for unorganized peptide regions (Adkins and Foster, 1966). Two large fragments, each representing approximately half of the protein molecule, were formed in high yield from this detergent–albumin complex (Adkins and Foster, 1966; Pederson and Foster, 1969).

In studies of the behavior of bovine plasma albumin in the region of the N ↔ F transition we discovered by chance that some peptide-bond cleavage results when the protein is permitted to remain in solution for several days under conditions where it exists primarily in the F state. It was first thought that this reflected an inherent instability of some of the peptide bonds in the protein, possibly an autocatalytic type of cleavage. Further investigations, however, showed that this reaction is especially rapid when relatively crude BPA (commercial fraction V) is employed, slower but still measurable with crystallized BPA, and barely detectable when highly purified mercaptalbumin was employed. This suggested that the cleavage results from the presence of a proteolytic enzyme which is a contaminant in the fraction V protein and to a lesser extent in the crystallized BPA. This enzyme has now been isolated in moderately pure form and shown to have no detectable activity on native BPA. On the other hand, it attacks the F form of the protein readily and, apparently, quite specifically at only two points in the molecule. The acid-expanded form of BPA is more extensively hydrolyzed than the F form. This proteolytic enzyme thus appears to be uniquely suited for conformational studies on plasma albumin.

Experimental Section

Materials. Fraction V BPA was purchased from Armour Pharmaceutical Co. Several lots were used and the numbers are reported with the experiments. Fraction V was purchased from Pentex Inc. for comparison to the Armour fraction V and lot numbers are also given with the experiments.

SDS, lot 608814, was purchased from Matheson Coleman & Bell. Acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, and naphthol blue-black were purchased from Eastman. Coomassie brilliant blue R 250 was obtained from Colab Laboratories, Inc. 5,5'-Dithiobis-(2-nitrobenzoic acid) was purchased from Aldrich Chemical Co., Inc. Iodoacetamide from Aldrich was recrystallized twice from water before use. ^{14}C-labeled iodoacetamide from Amersham–Searle was also recrystallized from water. Its ^{14}C activity after dilution with unlabeled iodoacetamide was 1.08×10^{10} cpm/mmole.

For tryptophan analysis *p*-(dimethylamino)benzaldehyde was purchased from J. T. Baker Chemical Co., lot no. 1-2561. For scintillation counting 2,5-diphenyloxazole was purchased from Packard Instrument Co., Inc., lot no. 3866.

Other chemicals were of the highest purity commercially available.

BPA and BMA Preparation. For most of the experiments to be described fraction V BPA was used. This protein was charcoal defatted by the procedure of Chen (1967) as modified in this laboratory (Sogami and Foster, 1968), passed through a Sephadex G-150 column with 0.1 M NaCl to obtain monomeric protein, and sulfhydryl blocked with iodoacetamide at pH 7.0–7.1 using 6 moles of iodoacetamide to 1 mole of BPA or approximately 10 moles of iodoacetamide to 1 mole of free sulfhydryl group. (These conditions give essentially complete blocking when using the Armour fraction V protein.) After blocking, the pH of the solution was lowered to approximately 5.2, the solution was extensively dialyzed against water, the protein was obtained by lyophilization, and stored in stoppered vials at 2–3° in a desiccator over calcium sulfate.

Samples of BMA used in this research were a gift from Dr. Robert D. Hagenmaier and were prepared by SE-Sephadex chromatography using a method that he has recently developed (Hagenmaier and Foster, 1971).

Preparation of BPA.* A sample of 60–100 ml of a 1% solution of BPA in 0.1 M NaCl was adjusted to approximately pH 3.80. After approximately 10-days reaction at room temperature, the pH of the reaction solution was raised to approximately 5.3, the sample was dialyzed briefly to remove most of the salt, lyophilized, and dissolved in 10–12 ml of 0.1 M NaCl. It was then layered on top of a G-150 column (5.0 × 84 to 90 cm) and eluted with 0.1 M NaCl. Fractions of 120 drops were collected (approximately 6 ml/tube).

Reduction and Carboxymethylation. Reduction and carboxymethylation of BPA* was done by a procedure quite similar to that of Crestfield *et al.* (1963). If the large peptides produced from BPA* were applied directly to the column in the high pH urea solution used to do the RCM reaction, they precipitated and slowly redissolved in the 50% HAc solvent on elution. A dialysis step against 50% HAc was, therefore, inserted after the reaction to equilibrate the RCM peptides with 50% HAc. It was observed that the peptides quickly precipitated in the dialysis bag and redissolved over a period of several hours dialysis against 50% HAc. Mechanical shaking using a rotating shaker and screw-cap tubes of approximately 150-ml volumes was found to greatly shorten the time required for the peptides to redissolve. Several changes of 50% HAc were required. Due to the large size of the peptides, Sephadex G-150 was also substituted for the G-75 recommended by Crestfield *et al.*, and a 2.5 × 90 cm column was used rather than the recommended 4.0 × 40 cm column.

Liquid Scintillation Counting. Fragments from BPA labeled with radioactive iodoacetamide were counted by dissolving 0.400 ml of the appropriate sample in approximately 19 ml of the scintillation solution (6.0 g of 2,5-diphenyloxazole dissolved in 300 ml of ethylene glycol monomethyl ether diluted to 1 l. with toluene) and counting on a Packard Model 3320 liquid scintillation spectrometer using standard techniques. All samples were counted for at least 10 min. Samples were counted at least twice and the average of the countings taken and reduced to counts per minute for final data analysis.

Determination of Tryptophan Content in BPA and in BPA Fragments.* Tryptophan was determined using a slight modification of the procedure of Spies and Chambers (1948). The volumes recommended by Spies and Chambers were reduced by a factor of five so that less sample was required. The samples were dissolved directly in 1.8 ml of 14.6 N H_2SO_4 and 0.20 ml of the dimethylaminobenzaldehyde reagent was added in 2 N H_2SO_4 to give a final H_2SO_4 concentration of 13.2 N as recommended by Spies and Chambers (1948). After 4-hr reaction, 20 μl of 0.068% $NaNO_2$ was added to the reaction solution and the absorbance was read at 590 nm 1 hr later using a Cary 15 spectrophotometer. The standard graph of Spies and Chambers was used to determine the amount of tryptophan in the solution.

Determination of N-Terminal Amino Acid Residues. The RCM fragments of BPA* prepared as described above were dansylated and hydrolyzed by the procedure of Gros and Labouesse (1969). The chromatography was done using the solvents recommended by Gray (1967), on polyamide sheets (Woods and Wang, 1967). Selected standard dansylamino acids were applied to one side of the polyamide sheet and the

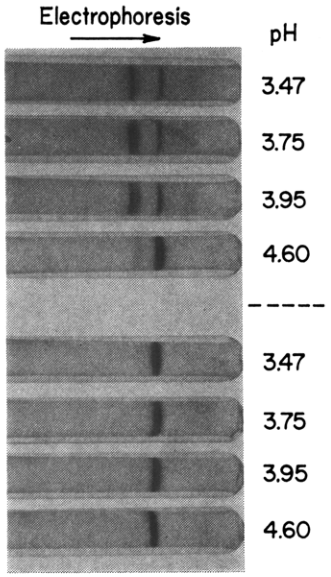

FIGURE 1: Gels from the disc gel electrophoresis of BMA samples stored at various pH values with and without plasma protease for 67 hr. Gels at the top had plasma protease added while those at the bottom did not. Hydrolysis pH values are indicated at the right of each gel. Each gel contained 20 μg of protein.

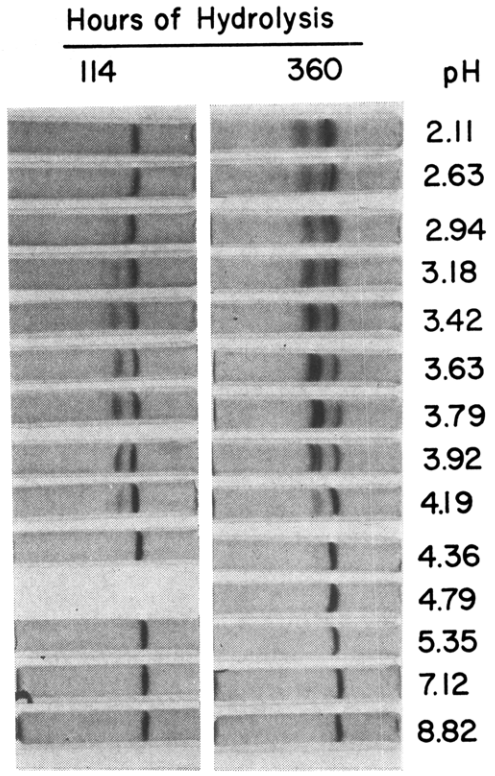

FIGURE 2: Gels from the disc gel electrophoresis of fraction V BPA samples hydrolyzed 114 and 360 hr at the pH values indicated at the right of the gel photographs. Each gel contained 20 μg of protein.

unknown to the other. The standards were identified using the data of Woods and Wang.

Following the Acid Transition of BPA Using the Optical Rotatory Dispersion Change.* A 0.100% solution of BPA* was prepared in 0.1 M NaCl. A 1.00-ml aliquot of this solution was pipetted into a 10-ml volumetric flask and diluted to approximately 8 ml with 0.1 M NaCl. The desired amount of 0.1 M HCl was added with a 100-μl Hamilton syringe and the solution was diluted to the mark with mixing. Optical rotatory dispersion curves were then recorded in a Cary 60 spectropolarimeter at 25.0° from 350 to 220 nm in a 2.0-cm jacketed cell which was kept clamped tightly in place during the experiment. A Beckman research pH meter, equipped with standard Beckman pH (41263) and calomel reference (39071) electrodes, was used to determine pH. Optical rotatory dispersion curves were taken 15–30 min after the sample was prepared and no time dependence was found in this period. To detect small base-line shifts, if they should occur, optical rotatory dispersion curves of BPA were typically recorded starting at 350 nm which is a wavelength that is particularly insensitive to changes in BPA conformation at the low concentrations used in these experiments (0.005–0.010%). The spectropolarimeter was programed to give a constant spectral bandwidth of 15 Å.

Disc Gel Electrophoresis. The Canalco formulation of 1965 for 7% polyacrylamide gels, which is based on the work of Ornstein (1964) and Davis (1964), was followed to prepare the gels except that double the amount of cross-linking agent was added. This resulted in greatly increased resolution of components formed by enzymatic hydrolysis of BPA. The gels used were 5.5 cm long and 5 mm in diameter. After the gel had polymerized, an approximately 1-cm layer of Sephadex G-150 equilibrated with the standard Tris–glycine buffer was placed in the tubes on top of the gel. The gel tube was then filled with buffer and the sample was applied into the Sephadex layer using a syringe of 10–50-μl capacity depending on the amount of sample. This procedure is similar to the one recommended by Broome (1963) and replaces the older stacking gel technique. Electrophoresis was conducted using a constant current of 5 mA/tube and required 35–40 min for the bromophenol blue tracking dye to reach the bottom of the gel.

The samples were stained with naphthol blue-black and destained electrophoretically. The resulting gels were frequently analyzed quantitatively at 470 nm on a Gilford 240 spectrophotometer equipped with a Heath recorder.

SDS–βME Gel Electrophoresis. The gel procedure was essentially that given by Weber and Osborn (1969). Longer gels (10 cm) were used and this resulted in longer electrophoresis times (usually around 10 hr) and increased resolution. Plexiglas tubes of 12 cm length and 6-mm i.d. were used.

Samples were prepared by mixing 1 ml of protein solution (typically around 0.1%) with 1 ml of a solution of 2% SDS, 2% βME, and 0.02 M sodium phosphate buffer (pH 7.0). The resulting solutions were heated at 37–38° for 2 hr in stoppered test tubes and then dialyzed for 12–15 hr against a solution of 0.1% SDS, 0.1% βME, and 0.01 M phosphate buffer (pH 7.0). The desired amount of this dialyzed sample was then applied to the gel.

Results

Isolation of an Enzyme from Fraction V BPA Preparations. We observed that samples of fraction V BPA stored at room temperature in the F form (pH 3.75) for several days always gave a very heavy band, corresponding to a peptide of molecular weight below that of BPA, on SDS–βME, gel electrophoresis. The protein did not give this extra band, even on prolonged storage, above pH 5.0. Storage of the protein in the F conformational state also produced new components which separated on disc gel electrophoresis and several different types of albumin samples were analyzed by this method.

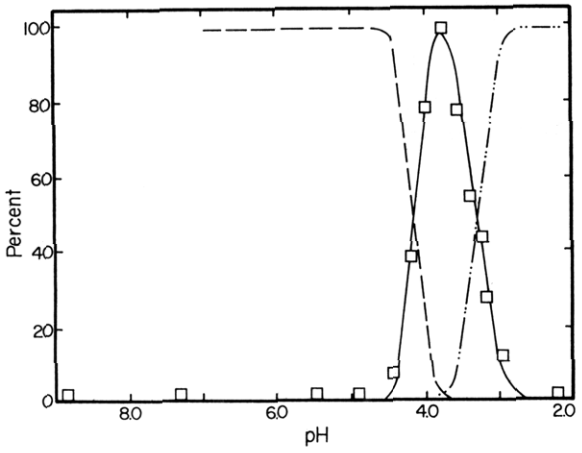

FIGURE 3: The normalized area under the BPA* peaks from disc gel electrophoresis gel scans of fraction V BPA samples hydrolyzed 114 hr at various pH values, □. Concentrations of N, ---; F, ——; and acid expanded BPA, ·-·-·, as a function of pH (data taken from Sogami and Foster (1968.) The area from the sample hydrolyzed at pH 4.79 is from the 360-hr hydrolysis (this makes no difference since the BPA* area for this pH is zero even after 360 hr).

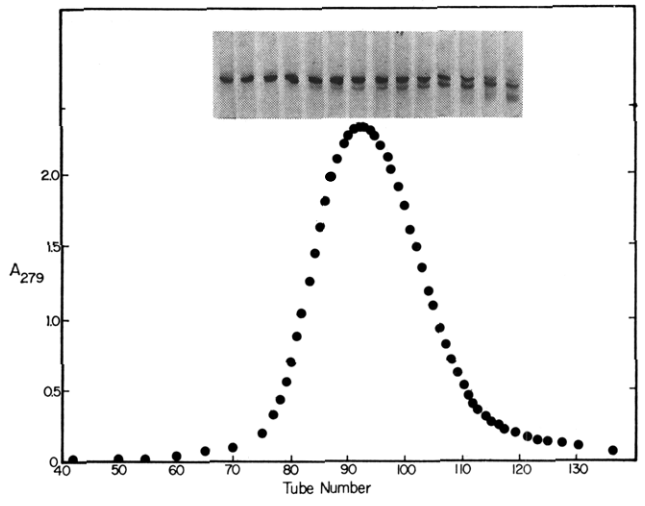

FIGURE 4: Preparation of BPA* by Sephadex G-150 chromatography: A_{279} as a function of tube number. Also shown are gels from the disc gel electrophoresis of samples from various tubes. From left to right the gels are from tubes 77, 80, 83, 86, 89, 91, 93, 95, 97, 100, 104, 108, 112, 116. Each gel contained 20 μg of protein.

Studies on BMA, prepared by chromatography on SE-Sephadex indicated that this preparation produced the extra bands at a very much slower rate. Blocking of the free sulfhydryl group had little effect on the rate or products. Charcoal defatting increased the rate, but had no detectable effect on the products formed. Crystallized BPA was found to react slower than fraction V, but much faster than BMA. All of these facts seemed to indicate that some proteolytic enzyme is present as a contaminant in fraction V BPA.

Small peaks containing proteins other than BMA (as indicated by disc gel electrophoresis) appear on SE-Sephadex chromatography of fraction V BPA ahead of the BMA peak (Hagenmaier and Foster, 1971). Careful examination of these peaks by disc gel electrophoresis indicated that the small peak preceding the mercaptalbumin peak (corresponding to approximately tubes 70–80 in Figure 4 of the paper by Hagenmaier and Foster (1971)) contained only one protein besides some BMA. The tubes from this peak were pooled and extensively dialyzed against 0.1 M NaCl. Sufficient BMA solution was added to each of two 25-ml volumetric flasks to give a final BMA concentration of 0.1%. One flask was then filled to the mark with the very dilute dialyzed protein solution and the other with the NaCl dialysate. Samples of each were then lowered to four pH values through the N ↔ F transition region and stored in plastic stoppered vials at room temperature (22–24°) for 67 hr. The results of disc gel electrophoresis on these samples are shown in Figure 1. The protein from the sulfoethyl column, which will be called plasma protease,[2] hydrolyzed BMA and the pattern of bands on the gels is the same as for fraction V samples stored in the same pH range.

Hydrolysis of BPA by Plasma Protease at Different pH Values. To determine whether albumin in other conformation states is hydrolyzed by plasma protease a 0.1% solution of fraction V BPA (Experimental Section) was prepared (0.1 M NaCl, pH 5.35) and 10-ml samples adjusted to pH values from 8.82 to 2.11. Each sample was placed in a stoppered vial (the vials were heated to approximately 250° before use and the stock protein solution was filtered through a 0.22 μ Millipore filter to help prevent bacterial growth) and the vials were stored in a parafilm-sealed beaker in a cabinet at room temperature (22–24°). It was unnecessary to add enzyme for these hydrolysis studies since the enzyme is already present in fraction V BPA. Figure 2 shows the disc electrophoresis gels of samples hydrolyzed 114 and 360 hr. Qualitative comparison of these gels to the pH dependence of the acid transitions of BPA (Sogami and Foster, 1968) indicates that no cleavage is obtained with the native, N, protein. In the region of the N ↔ F transition there is seen a specific pattern of two closely spaced bands, trailing the native BPA band. The two hydrolyzed proteins that give rise to this doublet on disc gel electrophoresis patterns from the hydrolysis of BPA in the F form will now be referred to collectively as BPA*. (A more detailed analysis of these forms will be presented later.) In the acid expansion region below pH 3.7 additional broad, weak bands are obtained which indicates that plasma protease can hydrolyze this conformational form of the protein more extensively than either the N or F forms. The characteristic BPA* doublet is totally absent at the lowest pH.

Gels for 114-hr hydrolysis from Figure 2 were scanned on the Gilford spectrophotometer and the areas under the BPA* peaks determined. The normalized areas were plotted *vs.* pH in Figure 3. The additional products obtained at very low pH values (acid-expansion region) make measurement of the area of the BPA* peaks more difficult in this region. The concentrations of the N, F, and acid-expanded conformational states of BPA at pH values in the acid region are also plotted in Figure 3. These curves were calculated from the pH dependence of the Cotton trough, $[\alpha]_{233}$, given by Sogami and Foster (1968). There are two transitions: N to F to acid-expanded protein. From Figure 3 it can be seen that the amount of BPA* produced increases and decreases essentially as the concentration of the F form increases and decreases.

*Preparation and Properties of BPA**. BPA* was prepared as described in the Methods section and an elution profile is

[2] The name plasma protease is adopted out of convenience in referring to the enzyme in this paper. This name was chosen since the enzyme is a protease and has been isolated from plasma. The enzyme *in vivo*, however, may have some other primary function than proteolytic degradation of proteins, and it may be a cellular enzyme that has simply leaked into the plasma in appreciable concentrations. It is hoped and assumed that a more descriptive name will be given to the enzyme when more is known about its properties and function.

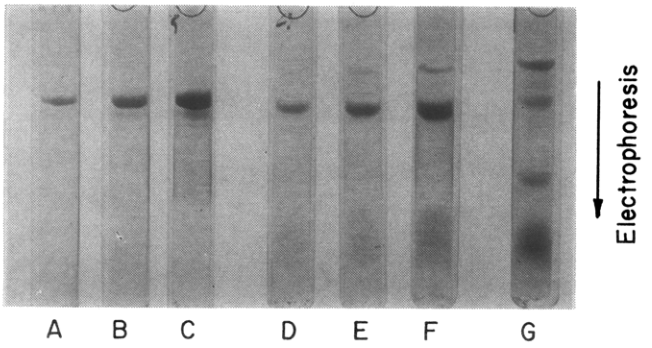

FIGURE 5: Gels from the SDS gel electrophoresis of unreduced (A-C) and reduced (D-F) BPA*. Gels A and D had 10 μg of protein applied; B and E had 20 μg; C and F had 40 μg. Gel G had 12.5 μg each of four standard proteins applied: (top to bottom) BPA, ovalbumin, chymotrypsinogen, β-lactoglobulin.

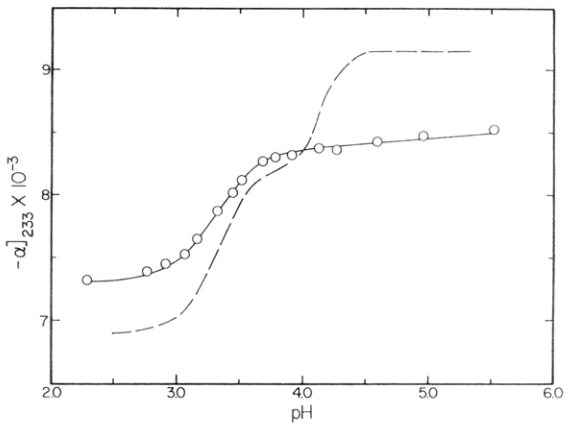

FIGURE 6: The acid transition of BPA*: $[\alpha]_{233}$ as a function of pH in presence of 0.10 M Cl^-. The results of Sogami and Foster (1968) on native BPA are shown, for comparison, by the dashed curve.

shown in Figure 4. Disc gel electrophoresis results on samples from various tubes from this column are also shown in Figure 4. Tubes 79, 80, and 81 were pooled to give essentially 100% BPA* and this sample will be called fraction I (FI). Tubes 82–90 were pooled to give 97–98% BPA* and will be called fraction II (FII). BPA* used in all experiments was from the FII sample unless otherwise indicated. As can be seen, there is some low molecular weight material trailing BPA* and BPA on the column. This material only accounts for a few percent of the original BPA. The fact that it is formed is not surprising since there is at least 1–2% of the acid-expanded form of BPA present at pH 3.8, as shown in Figure 3. The acid-expanded protein is more extensively hydrolyzed as was pointed out above.

One of the first facts to determine about any limited hydrolysis is whether the fragments are held together by disulfide bonds. This question was answered using a modification of the gel method of Weber (Weber and Osborn, 1969). Using BPA* prepared as described, one gel was run by the standard method using βME, and another gel of the same protein solution was run by the same gel method but omitting βME. The results are shown in Figure 5. With βME there is seen a trace of undegraded BPA, a fragment of molecular weight approximately 43,000 and low molecular weight fragments[3] of mol wt 20,000–25,000. The gels with no βME show only one main band indicating that the SDS-denatured BPA* has the same mobility as SDS-denatured BPA. This mobility is greater than that of BPA on the standard gels with βME, which is not surprising since the protein is unreduced and more compact without βME. The fact that only one band occurs in the absence of reducing agent, moving with a mobility which is quite probable for an unreduced protein of mol wt 66,000, is good evidence that the fragments are held together by disulfide bonds. The fact that the fragments do not dissociate on disc gel electrophoresis at high pH or on Sephadex G-150 chromatography at neutral pH is additional evidence that they are held together by disulfide bonds. Some slight amount of extra material can be seen on the SDS gels without βME. This probably represents the products of hydrolysis of BPA in the acid-expanded form which were not completely separated on Sephadex G-150 chromatography in the preparation of BPA*.

The behavior of BPA* was investigated in the acid region using optical rotation at 233 nm. Assuming $E^{1\%}_{1\,cm}$ for BPA* to be the same as for BPA, 6.67, $[\alpha]_{233}$ values were calculated and are plotted in Figure 6 *vs.* pH.

The stability of pure BPA* to plasma protease hydrolysis was examined using a sample of essentially 100% BPA* (FI) prepared as described above. The pH of approximately 5 ml of a 0.1% solution of BPA* in 0.1 M NaCl was adjusted to 3.74 with 0.1 M HCl, another 5-ml sample was kept at pH 5.60 as a control, and both samples were stored in plastic stoppered vials at room temperature. In Figure 7 photographs of gels from the disc gel electrophoresis of samples hydrolyzed for 18 days are shown. As can be seen, new bands are formed which have mobilities greater than that of BPA*. These new bands are formed fairly slowly, since they are present in only a few per cent concentration even after almost 3-weeks hydrolysis. BPA* seems actually to be more resistant to hydrolysis at pH 3.74 than at pH 5.60. The hydrolysis that occurs at pH 3.74 is probably due to enzymatic attack on acid-expanded BPA*.

The sample stored at pH 5.60 gives a quite sharp extra band on disc gel electrophoresis. This indicates that: (1) BPA* has a bond or area of the molecule that is labile to hydrolysis at this pH and this area is not present in BPA since BPA does not give any extra bands even after several weeks of storage with the enzyme at this pH; (2) since the enzyme is active even up to pH 5.60, the resistance to the enzyme of BPA in the native state must be due to the fact that BPA has no bonds available for hydrolysis, and not that the enzyme is inactive above pH 4.50.

*Separation and Analysis of the Fragments from Reduced and Carboxymethylated BPA**. BPA* was reduced, carboxymethylated, and the fragments separated on a 50% HAc column as described in the Experimental Section. An elution profile is shown in Figure 8. The first peak should be the large fragment and the second the small fragment. To determine whether this is true and also to assay the purity of the fragments, SDS gel electrophoresis was conducted on pooled tubes from the RCM column using the standard technique except that βME was omitted since the fragments are already reduced. The results are shown in Figure 9. As can be seen, the separation of the fragments by this procedure is quite good.

[3] These fragments are not easily seen on the gels, but this is not surprising since they contain only one-third of the BPA molecule and since later work showed that there are actually two fragments. Also low molecular weight peptides diffuse more on the gels and do not stain as well with coomassie blue.

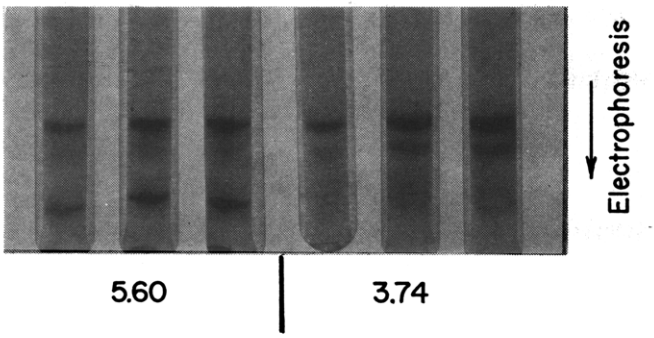

FIGURE 7: Gels from the disc gel electrophoresis of samples of BPA* hydrolysed by plasma protease at pH 5.60 and 3.74. In each series the gels contain 10, 20, and 30 μg of protein, from left to right. The pH of hydrolysis is indicated for each set of gels.

Some BPA can be seen on the gels from the early part of the large fragment peak since this sample (FII) is not 100% free of BPA. Another light band also appears in the large fragment peak (more concentrated on the trailing edge of the peak) and is probably a product of some nonspecific cleavage of BPA in the acid-expanded form that is not removed on Sephadex G-150 chromatography. These contaminants only account for a few per cent of the peak and can be largely removed by pooling only the center tubes of the peak. Two small fragments are obtained from the second peak of the RCM column and the reason for the two fragments will be discussed later.

Molecular weights for the large fragment and both small fragments were calculated from data obtained from several RCM column runs and SDS gel electrophoresis experiments of the type shown in Figures 8 and 9. The averaged results are 46,000 for the large fragment, and 24,000 and 21,000 for the two small fragments.

Blocked BPA was prepared in the standard manner except using [^{14}C]iodoacetamide. From this sample BPA* was prepared, the RCM reaction carried out, and the fragments separated as described. In Figure 8 both the absorbance and the ^{14}C counts per minute are plotted for samples from the RCM column. As can be seen, the radioactive label is in the large fragment, the small fraction being virtually devoid of radioactivity.

The tryptophan contents of the RCM fragments and a native BPA sample were determined and the results are shown

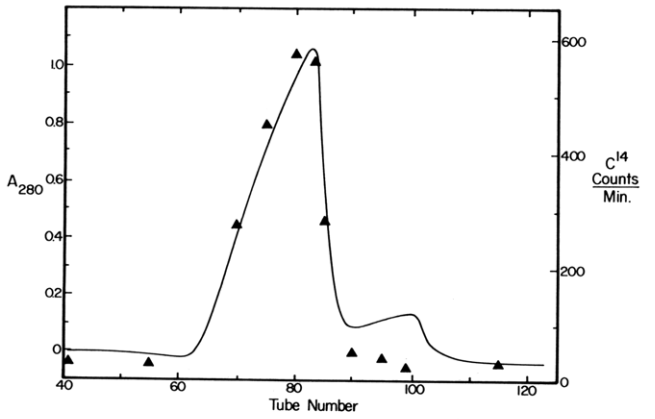

FIGURE 8: Chromatography of the RCM fragments from BPA* on a Sephadex G-150–50% acetic acid column: A_{280}, ——, and ^{14}C counts per minutes, Δ, as a function of tube number. A 50% acetic acid blank was used when measuring absorbances.

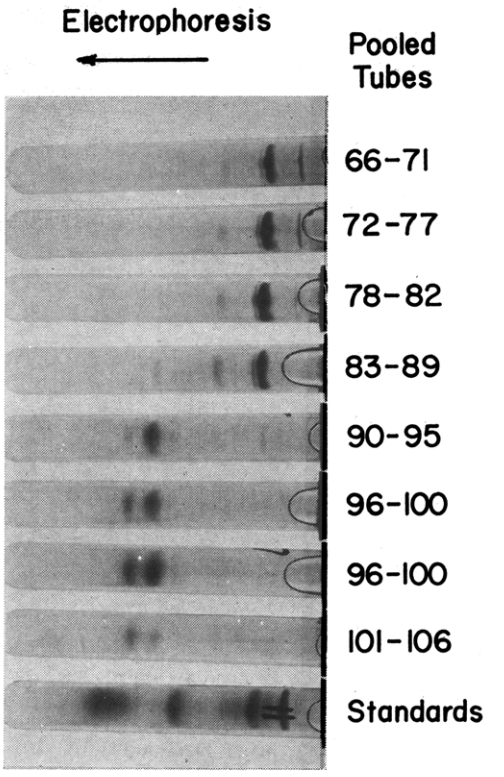

FIGURE 9: Gels from the SDS gel electrophoresis of samples from pooled tubes from the RCM chromatography described in Figure 8. The bottom gel contained 12.5 μg of each of the four standard proteins (see Figure 5). All other gels contained 20 μg of sample, except the lower gel from tubes 96–100 which contained 40 μg. The tubes pooled for each sample are indicated at the right of the photograph.

in Table I. Clearly, both of the tryptophan residues of BPA are located in the large fragment from the BPA* molecule. These results were obtained assuming the molecular weight of BPA to be 66,000, of the large fragment 45,600. An average molecular weight of 22,500 was used for the small fragments. The samples were weighed and added to the reaction solution as lyophilized powders which typically contain 5–10% water, and this perhaps explains the slightly low values obtained for BPA and the large fragment.

With both tryptophan residues and the sulfhydryl group positioned in the large fragment from BPA*, there can be no doubt that this fragment is from the N-terminal region of BPA (Pederson and Foster, 1969). The large fragment will now be referred to as the N fragment and the small fragments as the C fragments.

TABLE I: Tryptophan Content of BPA and the RCM Fragments from BPA*.

Sample	Moles of Tryptophan/ Mole of Sample	No. of Trp Residues
BPA	1.84	2
Large fragment	1.79	2
Small fragments	0.01	0

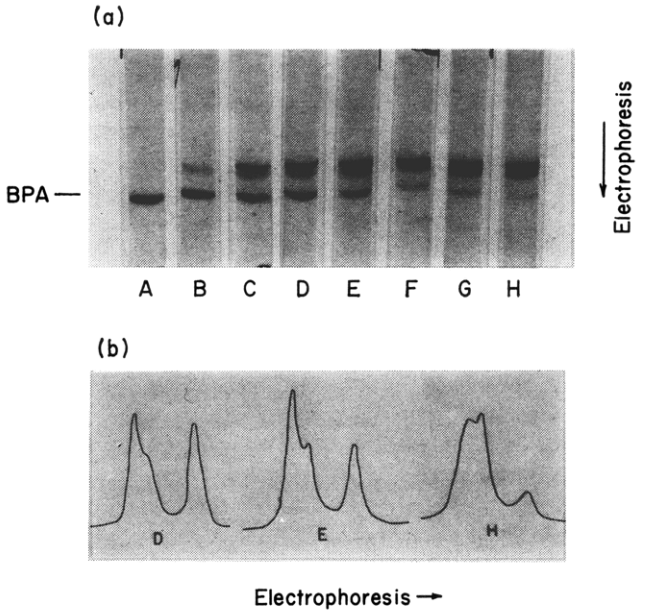

FIGURE 10: (a) Gels from the disc gel electrophoresis of fraction V BPA samples hydrolyzed for various times at pH 3.80 with plasma protease: (letter, hours of hydrolysis), A, 1.0: B, 24.0; C, 49.0; D, 97.0; E, 145; F, 192; G, 246; H, 296. (b) Reproductions of Gilford spectrophotometer traces of gels D, E, and H from 10 (a).

As an additional check on the alignment and purity of these fragments, their N-terminal residues have been determined. The N-terminal residue of the N fragment was indeed found to be the same as for BPA (aspartic acid) and the fragment gave only a single spot on thin-layer chromatography. The C fragment of mol wt 24,000 was obtained in fairly high purity from the RCM chromatography (gels on tubes 90–95 of Figure 9). This fragment, which results from the initial attack of plasma protease on BPA, gave essentially a single spot corresponding to an N-terminal phenylalanine residue. The other C fragment which results from plasma protease hydrolysis of BPA*, as will be discussed later, was not obtained in as high a purity as the other two fragments and it gave more than one spot on thin-layer chromatography. The results would indicate that valine is the N-terminal residue of this fragment, but more extensive purification of this fragment will be required before this can be determined with certainty.

Sequence of Events in the Plasma Protease Hydrolysis of BPA. To follow the formation of BPA* from BPA a 1% solution of BPA in 0.1 M NaCl was adjusted to pH 3.80. Aliquots were withdrawn at various times, analyzed by disc gel electrophoresis, and the results are shown in Figure 10a. These gels were scanned on the Gilford spectrophotometer and some of the results are reproduced in Figure 10b. As has been discussed previously, BPA* actually consists of two bands. The trailing BPA* band (which will now be referred to as BPA*) is formed first and, therefore, directly from BPA. The second band (now referred to as BPA**), which migrates between BPA and BPA* on disc gel electrophoresis, is formed later. The amount of BPA consistently decreases, while the amount of BPA* first increases, goes through a maximum and then begins to decrease. The amount of BPA** consistently increases after it is once formed. This behavior is characteristic of a consecutive reaction type of mechanism.

Plasma Protease in Various Fraction V Samples. Since plasma protease was found in commercial albumin samples, several different commercial samples were analyzed to see how common its presence might be. Three lots of Armour fraction V BPA and two lots of Pentex fraction V BPA (0.1% solution in 0.1 M NaCl) were stored at approximately pH 3.75, samples were withdrawn at various times, and analyzed by disc gel electrophoresis. The Armour samples all contained about the same amount of enzyme as indicated by hydrolysis rate. The Pentex samples, however, gave only barely detectable amounts of BPA* even after 1 week of standing. Samples of Pentex fraction V albumins from human, horse, and pig plasma were also analyzed for enzymatic activity at pH 3.75. All of these samples gave appreciable amounts of hydrolysis after several days, with the human sample being hydrolyzed at the fastest rate. Control samples of all proteins stored at near isoionic pH gave no detectable hydrolysis. It is interesting that only the Pentex bovine samples are relatively free of protease activity. Pentex literature indicates that the protein solution is raised to pH 7.0 before final preparation of bovine albumin. This pH is known to cause aging of BPA (Sogami *et al.*, 1969) and the possibility that it causes destruction of the enzyme also exists.

Some Properties of Plasma Protease. Plasma protease is eluted before BMA on SE-Sephadex chromatography which would indicate that it is somewhat more negative than BMA at pH 4.3. On disc gel electrophoresis the enzyme migrates as a single band slightly behind BPA. On SDS gels with βME, bands appear at positions corresponding to molecular weights of 46,700 and 14,300. Without βME the bands, at 46,700 and 14,300 remain and a band of higher molecular weight material appears. The 46,000 and 14,300 bands are evidently single chain peptide units since the addition of βME does not seem to affect them.[4]

Discussion

In the past most physical–chemical studies of BPA have been conducted with crystallized protein (Cohn *et al.*, 1947). Recent studies in our laboratory suggested that BPA monomer prepared from commercial samples of fraction V BPA (Cohn *et al.*, 1946) by charcoal defatting and chromatography on G-150 Sephadex is at least as pure as protein prepared in the same manner from crystallized BPA. In addition, due to the extraneous compounds (*e.g.*, decanol) added in crystallization, it seemed possible that fraction V might actually be a preferred starting material for the preparation of BPA. This is especially true since G-150 chromatography should largely remove the globulins remaining in fraction V along with the removal of dimer and higher polymeric forms of BPA. It is interesting to note that fraction V samples prepared in this manner show the presence of only one protein by SDS and disc gel electrophoresis and by exclusion chromatography, but have been shown beyond question by the results presented in the previous section to contain significant concentrations of a proteolytic enzyme. BPA* was also prepared in such a manner that it did not seem to contain other proteins (see gels from tubes 77–83 of Figure 4), but the results of Figure 7 clearly indicate that plasma protease is still present in this preparation.

The properties of plasma protease with respect to its action on BPA are quite striking. Although abundant evidence in the

[4] Preliminary equilibrium ultracentrifuge results, performed by Mr. Stephen Stroupe, are in substantial agreement with these findings. A main component of mol wt 46,000 (assuming $\bar{\nu} = 0.73$) was found, with appreciable amounts of components of both higher and lower molecular weight.

literature indicates that native BPA is readily hydrolyzed by most proteolytic enzymes, this enzyme does not hydrolyze the N form of the protein even after several weeks. In this same time period the acid-expanded form of BPA is nearly completely hydrolyzed to a heterogeneous mixture of peptides. The resistance of native BPA to hydrolysis by this enzyme is not due to the fact that the enzyme is inactive above pH 4.5 since BPA* is slowly hydrolyzed at pH 5.6 as indicated in Figure 7. The F form of BPA is hydrolyzed to only a limited extent by the enzyme, evidently only two bonds being hydrolyzed. Since the enzyme does extensively hydrolyze the acid-expanded form of BPA, this limited proteolysis of the F form is probably not accounted for by limited specificity of the enzyme. A more likely explanation of these results is that a small section of the BPA chain becomes exposed in going from the N-to-F conformational state and the enzyme can then attack this small section of the molecule.

The hydrolyzed proteins from the F form of BPA (which will be collectively referred to as BPA* in this paragraph for ease of discussion) seem to be quite similar to the F form in many properties. The behavior of BPA* as evidenced by changes of $[\alpha]_{233}$ in the acid region is quite different from that of BPA, as was shown in Figure 6. BPA* does not give a transition in the N–F region (pH 4.4–3.8) but does give a transition over the same pH region, and of nearly the same change in $[\alpha]_{233}$, as the acid expansion of BPA. This fact, together with the dependence of hydrolysis on concentration of the F form of BPA and the evidence from Sephadex G-150 chromatography and disc gel electrophoresis that BPA* is slightly more expanded than BPA, indicates that BPA* is in a state quite similar to the F form.

It should be mentioned that the separation of proteins on disc gel electrophoresis depends on size and charge. BPA and BPA* have quite similar mobilities on standard disc gel electrophoresis with the normal degree of cross-linking. The BPA* band travels only slightly behind the BPA band. On electrophoresis using gels with double the normal amount of cross-linking agent the mobility of BPA* is considerably more reduced than the mobility of BPA. These two results indicate that the proteins have quite similar charge at the pH of the electrophoresis, but differ slightly in size with the BPA* being somewhat more expanded than BPA. The fact that BPA* cannot undergo the N–F transition and is in an expanded state similar to the F form raises the intriguing possibility that many unanswered questions about BPA from over the years will now be open to experimental attack.

The question of where the enzymatic cleavage occurs in BPA is quite important in determining what occurs in the native BPA molecule on lowering pH to give the F-conformational state. The results have indicated that the cleavages give a single 46,000 molecular weight N fragment and two C fragments of mol wt 24,000 (C*) and 21,000 (C**). The cleavages are produced in a consecutive reaction mechanism as indicated in

$$\text{BPA} \xrightarrow[\text{protease}]{\text{plasma}} \text{BPA*} \xrightarrow[\text{protease}]{\text{plasma}} \text{BPA**} \qquad (1)$$

This explains the origin of the two RCM C fragments as shown in eq 2 and 3.

$$\text{BPA*} \xrightarrow{\text{RCM}} \text{N fragment} + \text{C* fragment} \qquad (2)$$

$$\text{BPA**} \xrightarrow{\text{RCM}} \text{N fragment} + \text{C** fragment} \qquad (3)$$

The molecular weights of the N fragment and C* fragment total 70,000 which is in good agreement with the molecular weight of BPA considering that this figure contains the errors in the molecular weights of both fragments. The difference in molecular weights between the small fragments is about 3000 which means that one or more peptides containing a total of approximately 30 residues must be lost in the second step of reaction 1 or in reaction 3 above. It should also be mentioned that only one large fragment is obtained on disc gel electrophoresis of the RCM fragments while two small fragments are obtained. This evidence taken with the SDS gel data and the N-terminal residue determinations seems convincing that the mechanism given in eq 1–3 is correct.

It seems likely that the 30-residue section of BPA that is produced by the action of plasma protease on BPA* to give BPA** comes from the internal region of the BPA molecule which serves as a link between the N and C fragments. That is, the N-terminal 30 amino acids are cleaved from the C* fragment to give the C** fragment. If the 30-residue peptide came from the C-terminal end of BPA, it would seem that it should be produced from both BPA and BPA* since there should be very little difference between the two proteins in this region. What seems most probable is that after cleavage of BPA to form BPA* a segment of the chain near this cleavage point becomes more exposed to the environment and available for plasma protease hydrolysis. A large fragment of molecular weight approximately 40,000 has recently been isolated from BPA by trypsin hydrolysis at high pH (King, 1970). This fragment came from the C-terminal end of the molecule, however, and not from the N terminal as with plasma protease. The SDS–BPA complex, on the other hand, is cleaved near the center of the molecule to give two fragments of similar size which largely resist subtilisin hydrolysis at both the plasma protease and trypsin point of attack (Pederson and Foster, 1969). It is possible that these three cleavages occur in the three peptide links between the four globular units postulated earlier (Foster, 1960), but one unit would have to be held to the parent molecule by a disulfide bond or bonds. This is true since the plasma protease fragments are held together by a disulfide bond or bonds.

From the few facts that are known about plasma protease at this time, it appears to be a most interesting proteolytic enzyme for use in protein conformational studies. The active site of this enzyme must be quite restricted since it is unable to hydrolyze the native form of BPA, which seems to be relatively easily attacked by most other proteolytic enzymes. Of the three components isolated from the plasma protease samples mentioned in the Results section, it would seem that the 46,000 molecular weight component must be the enzyme hydrolyzing BPA since it would remain with the BPA, as the enzyme does, through the standard Sephadex G-150 treatment to remove dimer.[5] The high and low molecular weight components should be removed by this technique. The 46,000 molecular weight protein is also present in significantly higher quantities than the other two components. The results presented are sufficient to indicate that plasma protease is not one of the commonly studied enzymes such as trypsin, chymotrypsin, pepsin, etc. The possibility exists that it may be a cellular enzyme which leaks into the plasma. Alternatively, due to its fairly high molecular weight, it seems possible that

[5] It is important to note that the purified enzyme used for these experiments was obtained by SE-Sephadex chromatography without use of a Sephadex G-150 step.

it may be a circulating protease. The fact that native BPA is completely resistant to this enzyme suggests the possibility that it might serve the role of a scavenger by degrading only denatured or otherwise damaged protein molecules. Alternatively, it is conceivable that it occurs as a zymogen and that the active form appears only as a result of activation in some stage of the commercial fractionation process. It might arise from one of the known zymogens of the blood clotting system. Studies in progress in our laboratories may lead to a clarification of the properties and possible physiological role of this interesting enzyme.

Acknowledgments

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Circular Dichroism and the Conformations of Membrane Proteins. Studies with Red Blood Cell Membranes*

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ABSTRACT: Previous studies of the circular dichroism spectra in the region of 190–230 nm of suspensions of red blood cell membranes and of other membrane preparations have shown some of the features characteristic of proteins in a partially α-helical conformation, with, certain anomalous features however, such as low values of $[\theta]$ near 190 nm and a "red shift" in the circular dichroism spectra above 220 nm. In this investigation, a careful comparison has been made of the circular dichroism and ultraviolet absorption spectra of suspensions of red blood cell membranes (1) before and after fragmentation in a French press; and (2) before and after treatment with preparations of phospholipase C. The magnitudes of the anomalies in the circular dichroism spectra were found to be correlated with increases in ultraviolet absorbance, probably due to light scattering from the suspensions, supporting the suggestions of Urry and coworkers that the circular dichroism anomalies may be explained as optical artifacts. With the aid of theoretical treatments of (1) the effects of light scattering on circular dichroism spectra from suspensions of large particles, and (2) the absorption flattening from suspensions of spherical shells, the circular dichroism spectra of suspensions of intact red blood cell membranes have been analyzed in detail. In particular it has been shown that the value of $[\theta]_{222}$ is not significantly influenced by optical artifacts, and that in the intact membranes, the protein is on the average about 40% in the right-handed α-helical conformation.

Considerable interest has been generated in recent years in the optical properties of the proteins of intact biological membranes. Optical rotatory dispersion and circular dichroism measurements in the peptide-bond absorption band around 200 nm have yielded the interesting information that a large fraction of the protein in a variety of membranes is in

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