Subtilisin Cleavage of Bovine Plasma Albumin. Reversible Association of the Two Primary Fragments and Their Relation to the Structure of the Parent Protein*

Dennis M. Pederson† and Joseph F. Foster

ABSTRACT: Bovine plasma albumin was hydrolyzed by subtilisin at pH 8.9 in the presence of approximately 100 moles of sodium dodecyl sulfate/mole of bovine plasma albumin. Modification of the free sulfhydryl group in bovine plasma albumin with iodoacetamide or cysteine accelerated the hydrolysis and led to a decrease in the number of fragments formed. Chromatography on DEAE-Sephadex at pH 9.5 of digests of sulfhydryl-blocked bovine plasma albumin gave two major fragments (F_2 and F_3) of approximately equal molecular weight (30,000 and 35,000, respectively), but differing in amino acid composition. Several smaller fragments, resulting from further hydrolysis of F_2, appeared under one chromatographic peak (F_1). The reaction path was confirmed by amino-terminal analysis and chromatographic studies at various stages of the reaction. The N-terminal residue of F_3 was found to be aspartic acid, the same as in the intact molecule. On the other hand, F_2 had N-terminal phenylalanine and F_1 had both phenylalanine and alanine, but no N-terminal aspartic acid. Amino acid analysis of fragments produced from iodoacetamide-blocked bovine plasma albumin placed the originally free sulfhydryl group in F_3. Difference spectra between F_2 and F_3 showed both tryptophan residues of the parent molecule to be in F_3 also. On the basis of these results together with other published information on the degradation of bovine plasma albumin, a schematic model of the covalent structure is developed.

A significant feature of this structure is the presence of four domains which are not interconnected by disulfide bonds. Molecular weight analysis of mixtures of F_2 and F_3 showed a reversible pH-dependent association to take place. The apparent association constant was 10^6 l./mole at pH 6 and dissociation was virtually complete at pH 4. This equilibrium is in qualitative agreement with the structural transition (N–F) known to occur in bovine plasma albumin. Association at pH 6 between F_2 and F_3 gave a tyrosine difference spectrum whose magnitude corresponded to approximately one-third that observed in the formation of N from F. By various arguments it is shown that the fragments F_2 and F_3 correspond to the fragments H_2 and H_1, respectively, of Adkins and Foster (Adkins, B. J., and Foster, J. F. (1966), *Biochemistry 5*, 2579).

The plasma albumins in general and bovine plasma albumin in particular have been the subject of extensive physical–chemical investigation for many years. The model most consistent with all the experimental evidence is one in which the polypeptide chain is folded into two (Harrington *et al.*, 1956) or perhaps four (Foster, 1960) globular regions. Although these globular domains are connected by peptide linkages, the molecule can in a sense be regarded as consisting of subunits.

This representation is useful in considering ligand-induced structural changes known to occur in bovine plasma albumin. The most widely studied of these induced changes, the N–F transition, occurs when the pH is lowered to approximately 4 (Aoki and Foster, 1956, 1957). All studies of this transition are consistent with a structural change resembling an association–dissociation equilibrium between "subunits," *i.e.*, domains of the type visualized. The transition has been pictured as involving more than one step (Leonard and Foster, 1961; Sogami and Foster, 1968) and a meaningful description of the molecular changes involved in the various stages is difficult to obtain. One approach, both to probe the three-dimensional structure and to help delineate the steps of the N–F transition, involves limited preferential proteolysis of the peptide chains connecting the various globular domains, isolation and characterization of the fragments, and a study of their association properties.

Adkins and Foster (1965, 1966) have shown that subtilisin-catalyzed hydrolysis of bovine plasma albumin in the presence of approximately 100 moles of sodium dodecyl sulfate/mole of bovine plasma albumin results in the preferential scission of one or more vulnerable peptide bonds near the center of the single polypeptide chain, liberating large fragments of approximately half the molecular weight of the substrate. Owing to the similarity in size of these fragments together with their strong tendency to associate in neutral aqueous solution, attempts at separation and purification met with only limited success. The separation method (recycling chromatography on Sephadex G-100 at pH 2.3) employed by Adkins and Foster was extremely time consuming and yielded only small amounts of moderately pure materials.

In the present work, final selection of the separation conditions for these fragments was based on the observation of Adkins and Foster (1966) that the fragments resolve well in polyacrylamide gel disc electrophoresis at pH 9.5 in a Tris–glycine buffer system. Salt-gradient elution chromatography on

* From the Department of Chemistry, Purdue University, Lafayette, Indiana 47907. *Received January 21, 1969.* This research was supported by Grants CA-02248 and T01 GM-1195 of the U. S. Public Health Service. Presented in part at the Federation of American Societies for Experimental Biology at Atlantic City, N. J., April, 1968.

† National Science Foundation Cooperative Fellow, 1965–1966. Present address: Department of Biochemistry, School of Medicine, University of North Carolina, Chapel Hill, N. C. 27514.

DEAE-Sephadex using similar solvent conditions resulted in separation of fragments which appear to be pure on the basis of various criteria. The isolated fragments were characterized with respect to their disc electrophoresis homogeneity, amino acid composition, N-terminal and other specific residues, sedimentation equilibrium molecular weight, association–dissociation properties, and alignment within the parent molecule. These data have been used to correlate results of other limited proteolytic studies on bovine plasma albumin (Weber and Young, 1964a,b; Peters and Hawn, 1967; King and Spencer, 1968) and a model consistent with this information has been proposed.

Experimental Section

Materials. Bovine plasma albumin (Armour Pharmaceutical Co., lot B70411) was treated with charcoal (Darko KB) according to the method of Chen (1967) as modified by Sogami and Foster (1968) to remove lipid contaminants.

Subtilopeptidase A (Nargase and Co., Ltd., Osaka, Japan, Batch CC G-2424), prepared from cultures of *Bacillus subtilis* N′, is the same preparation used by Adkins and Foster (1965, 1966). It is referred to simply as "subtilisin" in the text.

Sodium dodecyl sulfate (Matheson Coleman and Bell, 95%) was recrystallized twice from methanol. Iodoacetamide (Sigma Chemical Co.) and L-cysteine (Eastman Kodak Co.) were used without further purification. Tris of primary standard grade was obtained from Fisher Scientific Co. Urea and guanidine hydrochloride were Ultra-Pure grade purchased from Mann Research Laboratories. Sephadex G-150 and DEAE-Sephadex (A-50) were products of Pharmacia, Uppsala, Sweden.

All other chemicals used were reagent grade materials. Deionized water, prepared by passing distilled water through a mixed-bed-deionizing column (No. 0808, Barnstead Still and Sterilizer Co.), was used in the preparation of all solutions. For preparation of the Tris–glycine buffers deionized water was boiled before use.

Concentration Determinations. Bovine plasma albumin concentrations were determined with an Hitachi Perkin-Elmer spectrophotometer, assuming $E^{1\%}_{1\ cm}$ to be 6.67 at 279 mμ (Sterman, 1956). The concentrations of solutions of F_2 and F_3 were based on $E^{1\%}_{1\ cm}$ values of 5.51 and 7.55, respectively, at 278 mμ. These values were determined from samples dried to constant weight at 110° in a vacuum oven.

Enzymatic Hydrolysis. Enzymatic digestion of bovine plasma albumin–detergent complexes and removal of detergent followed the methods described by Adkins and Foster (1965). For studies of the digest as a function of *B*, the moles of base consumed per mole of bovine plasma albumin, 10-ml aliquots were removed from the reaction vessel (initial volume, 100 ml) at the desired intervals. The amount of base required per *B* unit was corrected for the new volume after each withdrawal.

DEAE-Sephadex Chromatography. Resolution of the reconstituted digests was effected on DEAE-Sephadex (2.5 × 45 or 2.5 × 100 cm columns obtained from Pharmacia) at 0–5° using a Tris–glycine buffer system (pH 9.5 at room temperature) and linear gradient in NaCl. Constant flow was maintained at 5–10 ml/hr by a peristaltic pump (Holter Co., Model R134). The gradient components were: starting buffer, 0.025 M Tris–0.025 M glycine–0.12 M NaCl, adjusted to pH 9.5 using 0.05 M NaOH in 0.12 M NaCl; and limiting buffer, starting buffer adjusted to 0.30 M in NaCl. For short-column runs 400 ml of each buffer was used, and 1000 ml was used in the long-column runs. Fractions were collected with a Gilson Medical Electronics (GME) constant-volume fraction collector, and the concentration of protein was determined by measurement of the absorbance using either the Hitachi Perkin-Elmer or Cary Model 14 (Applied Physics Corp.) spectrophotometer. In some cases where the protein concentration was too low for accurate detection at 279 mμ, the absorption was measured at 230 mμ. Homogeneity of the fractions was determined by disc electrophoresis (see below), and the appropriate tubes were pooled, dialyzed, and concentrated in an ultrafiltration cell (Diaflo Model 50, Amicon Corp.) and lyophilized.

Exclusion Chromatography. Additional purification of F_3 (when needed) was performed on a 2.5 × 100 cm column filled with Sephadex G-150. For the purification, Tris–glycine starting buffer was used as the eluent. Fractions were collected using the GME fraction collector fitted with either the constant-volume or drop-counter attachment. Protein content was monitored as described above.

Sulfhydryl Modification. Blocking of the sulfhydryl group with iodoacetamide was performed according to the procedure of Brush *et al.* (1963). The pH was kept at 7.0 to minimize the changes observed in charcoal-defatted bovine plasma albumin (Sogami and Foster, 1968) which occur faster at higher pH (Sogami *et al.*, 1969). Blocking by cysteine followed essentially the procedure of Andersson (1966). Modifications were addition of the cysteine before raising the pH, and addition of solid Tris and 1 M HCl in small increments so as to maintain the pH hear 8. Analysis for free sulfhydryl content using the 5,5′-dithiobis(2-nitrobenzoic acid) method of Ellman (1959) showed 80–90% blocking by iodoacetamide and 100% by cysteine. Excess reagents were removed by dialysis and the samples were lyophilized.

Disc Electrophoresis. This was performed as described by Adkins and Foster (1966). Best results were found with 0.1% protein solutions and 20–30-μg sample loads. The stained gels were scanned in a microdensitometer (Chromoscan, Joyce-Loebl and Co., Ltd.).

Amino Acid Analysis. Analyses were made using the Beckman-Spinco Model 120B amino acid analyzer and the procedure of Spackman *et al.* (1958) as modified by Benson and Patterson (1965). Dry protein samples (3–5 mg) or 1.0-ml aliquots of 0.3–0.5% solutions were placed in Pyrex ignition tubes (18 × 150 mm) which had been constricted at the top, and either 2 ml of 6 M HCl (in the case of the solid samples) or 1 ml of 12 M HCl (in the case of the solutions) was added. The solutions were frozen in a Dry-Ice–acetone bath, evacuated, sealed, and placed in an oven at 110 ± 1° for the desired length of time. Hydrolysates were dried by rotary evaporation, deionized water was added, and the process was repeated three times to assure removal of HCl. The residue was dissolved in the desired amount of pH 2.2 citrate buffer and an appropriate aliquot was analyzed.

In work with the purified fragments F_2 and F_3, hydrolyses were carried out for 24, 48, and 72 hr and the serine, threonine, and tyrosine contents were determined by extrapolation to zero time of hydrolysis. For the slowly released amino acids, isoleucine and valine, the 72-hr values were used. Values for all other amino acids were the average of all runs. Tryptophan values were estimated from difference spectral results. In the case of the fraction containing a mixture of fragments, F_1,

only one analysis was performed and approximate destruction corrections, based on the values of F_2, were applied for serine (24.4%), threonine (21.8%), and tyrosine (18.1%).

Amino acid residue values were estimated using the small methionine content of bovine plasma albumin (four residues per molecule) (Spahr and Edsall, 1964) and the sedimentation equilibrium molecular weights to unambiguously assign a number of these residues to each fragment. Requiring the methionine content to be equal to or less than the assigned value, all other residues were estimated so as to minimize deviations from integral values.

N-Terminal Analysis. Qualitative results were obtained from the 1-fluoro-2,4-dinitrobenzene method of Sanger (1945, 1949) as described by Fraenkel-Conrat *et al.* (1954). Quantitative values were determined according to the cyanate method of Stark and Smyth (1963). Both urea and guanidine hydrochloride were found to be equally effective denaturants when measured by the extent of carbamylation (80–90%). Carbamylation reaction times of longer than 12 hr had no effect on the yield of the hydantoin. Values for the N-terminal residues were based on: (1) the dry weight of the carbamyl derivative as determined by amino acids analysis, (2) the approximate recovery corrections of Stark and Smyth (1963), and (3) the following molecular weights: bovine plasma albumin, 66,000; digests, 66,000; F_1, 30,000 (for comparison with F_2); F_2, 30,000; and F_3, 35,000.

Velocity Sedimentation. Sedimentation velocity experiments were performed at 20.0° in a Spinco Model E ultracentrifuge equipped with a Wolter phase-plate schlieren optical system and R.T.I.C. temperature control. Samples were centrifuged at 59,780 rpm in standard 4° Kel-F cells. Sedimentation coefficients were calculated from the position of the maximum ordinate of the sedimentating boundary using a Nikon Shadowgraph Model 6C optical microcomparator.

Equilibrium Sedimentation. Using the Spinco Model E ultracentrifuge, equipped with Raleigh interference optics, high-speed equilibrium runs were performed at 35,600 rpm according to the method of Yphantis (1964). All samples were dialyzed against 0.1 M KCl (final protein concentration 0.01–0.09%) and loaded into a three-channel Yphantis-type Epon cell (12 mm). Using 0.5-ml syringes fitted with polyethylene delivery tubes, solvent (0.12 ml) was first loaded into the three reference channels. Solution channels were loaded in order of decreasing concentration and increasing distance from the center of the rotor. Protein solutions were placed in their proper channel, left there for 5 min, removed, and the channel was filled and emptied two additional times. This was done to minimize protein adsorption on the cell walls. For the final filling, 0.10 ml of sample was layered over 0.01 ml of FC 43 fluorocarbon oil (Minnesota Mining and Manufacturing Co.). Attainment of equilibrium required 18–24 hr. Kodak spectroscopic plates type I-D or II-G were analyzed on the Nikon microcomparator, equipped with a 50× lens. The z-average molecular weights were calculated as described by Yphantis (1964) by evaluation of the weight-average molecular weights at the base of the cell, obtained from the slope of plots of the natural logarithm of the blank-corrected fringe displacements *vs.* the comparator x-coordinate. These plots were linear for displacements greater than 10 μ. Partial specific volumes for the isolated fragments were estimated from the amino acid composition and the partial specific volume for each amino acid (Cohn and Edsall, 1943).

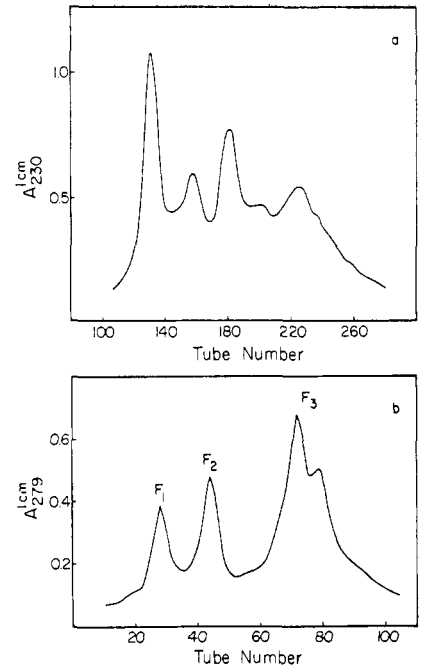

FIGURE 1: DEAE-Sephadex chromatography of subtilisin digests of bovine plasma albumin. Samples were applied to a 2.5 × 38 cm column and eluted with a linear salt gradient composed of 400 ml each of starting and limiting buffer. In part a the gradient ranged from an ionic strength of 0.13 at tube 60 to 0.21 at tube 260. In part b the gradient ranged from an ionic strength of 0.13 at tube 15 to 0.21 at tube 95. Fraction volumes were 2.0 ml for the B = 4.0 digest of unblocked bovine plasma albumin (a), and *ca.* 5 ml for the B = 3.8 digest of iodoacetamide-blocked bovine plasma albumin (b).

For low-speed equilibrium runs (12,590 rpm), ultraviolet absorption optics, a photoelectric scanner (Beckman-Spinco), and the Yphantis-type cell (Yphantis, 1964) were employed. Preparation of solutions of F_2 and F_3 was based on the dry weight extinction coefficients and the high-speed equilibrium molecular weights. The pH of each solution was adjusted to the desired value with small amounts of 0.1 M HCl or 0.1 M KOH. The apparent optical density weight-average molecular weight ($\bar{M}_{ODW} = \Sigma\epsilon_i M_i/\Sigma\epsilon_i$) for each channel was calculated using the conservation of mass equation

$$\bar{M}_{ODW} = \frac{(c_b - c_m)}{c_0(r_b^2 - r_m^2)} \frac{2RT}{\omega^2(1 - \bar{v}\rho)}$$

with c_b and c_m being replaced by extrapolated optical densities at the base and meniscus, and c_0 being the initial optical density of the solution in the ultracentrifuge cell.

Difference Spectroscopy. Difference spectra were determined by scanning from 350 to 250 mμ using a Cary Model 14 or Cary Model 15 recording spectrophotometer (Applied Physics Corp.) and tandem cells as described by Herskovits and Laskowski (1962). Equal volumes of F_3 were pipetted into one compartment of each cell and the same volume of 0.1 M KCl into the other compartment using the same pipet. After setting a base line, equal increments (50–100 μl) of a concentrated F_2 solution (1%) were added to the solution compartment in the sample cell and the solvent compartment in the reference cell and mixed using a small magnetic stirring bar placed in

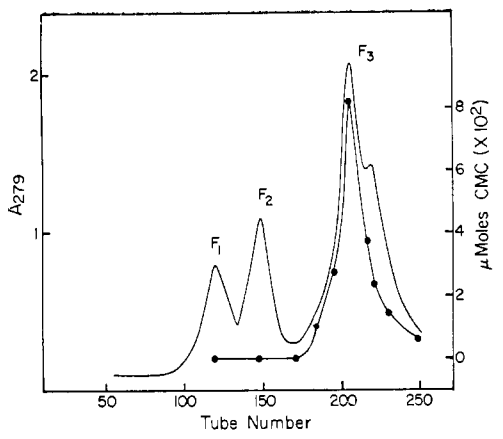

FIGURE 2: Analysis of the CM-cysteine content as a function of the fraction number for a $B = 3.8$ subtilisin digest of iodoacetamide-blocked bovine plasma albumin. (——) Absorbance measured on a GME ultraviolet monitor (fractions 50–170) and an Hitachi Perkin-Elmer spectrophotometer (fractions 170–250). (●) Micromoles of CM-cysteine obtained from amino acid analysis.

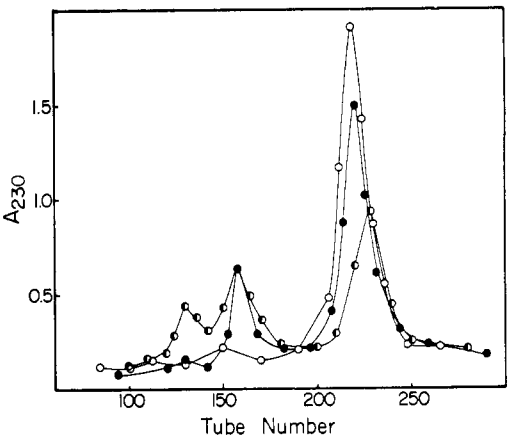

FIGURE 4: DEAE-Sephadex chromatography at pH 9.5 of subtilisin digests of cysteine-blocked bovine plasma albumin at various values of B. Equal sample loads, as measured by the product (A_{279} × volume), were applied to a 2.5 × 38 cm column and eluted with a linear salt gradient composed of 400 ml each of starting and limiting buffer. Fraction volumes were 2.0 ml and the gradient ranged from an ionic strength of 0.13 at tube 60 to 0.21 at tube 260. (○) $B = 0$, (●) $B = 1.9$, and (◐) $B = 3.8$.

each compartment. To achieve spectral resolution, the dynode setting and sensitivity were selected so as to keep the maximum slit width less than 0.7 mm.

Results

Resolution of Fragments by DEAE-Sephadex Chromatography and the Effect of Blocking the Sulfhydryl Group. After exposure of nonblocked charcoal-defatted bovine plasma albumin to a pH greater than 7, marked changes have been observed in the acid pH–solubility curves in 3 M KCl (Sogami *et al.*, 1969). This effect was attributed by them to sulfhydryl-catalyzed disulfide interchange and was shown to be markedly repressed by blocking of the SH group. Since both the enzymic digestion and DEAE-Sephadex chromatography were performed at alkaline pH, an effect due to the free sulfhydryl group might be expected here also. Indeed, hydrolysis of the unblocked sample was found to proceed at a much slower rate than that for bovine plasma albumin in which the SH group had been blocked with iodoacetamide or cysteine, taking nearly three times as long to reach a similar stage of digestion ($B = 3.8$). In addition, DEAE-Sephadex chromatography of a nonblocked reconstituted $B = 3.8$ digest yielded a multiplicity of peaks (Figure 1a). Decrease in this multiplicity was obtained with digests of iodoacetamide-treated bovine plasma albumin (Figure 1b). In this chromatogram the components have been labeled F_1, F_2, and F_3 in order of their elution.

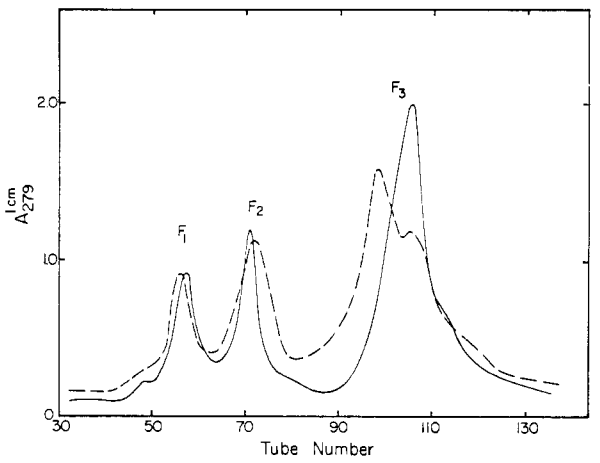

FIGURE 3: DEAE-Sephadex chromatography at pH 9.5 of subtilisin digests of cysteine-blocked bovine plasma albumin (——) and iodoacetamide-blocked bovine plasma albumin (– – –). Samples applied to a 2.5 × 36 cm column and eluted with a linear salt gradient composed of 400 ml each of starting and limiting buffer. Fraction volumes were *ca.* 4.5 ml and the gradient ranged from an ionic strength of 0.13 at tube 30 to 0.21 at tube 120.

The F_3 region of the chromatographic elution pattern of a digest of iodoacetamide-blocked bovine plasma albumin (Figure 1b) still shows evidence for at least two components. A plausible explanation for this heterogeneity can be offered on the basis of the known fact that bovine plasma albumin consists of two major components, mercaptalbumin and nonmercaptalbumin. In nonmercaptalbumin the SH group is blocked, in the main, by mixed-disulfide formation with cysteine (Andersson, 1966). Hartley *et al.* (1962) demonstrated by frontal analysis that the nonmercaptalbumin fraction of bovine plasma albumin is more strongly bound to DEAE-cellulose than is the mercaptalbumin fraction. They suggested that this difference in binding affinity is due to the fact that the group covalently bonded to the SH group in nonmercaptalbumin carries a dipolar ionic group. At the pH of their experiments, 7.0, the sulfhydryl groups would be essentially un-ionized. Since blocking by iodoacetamide would contribute no ionic groups and in fact would prevent ionization of the SH group (which otherwise would occur at the pH of our chromatographic separation), it might be anticipated that a difference in binding affinity would exist between the iodoacetamide-blocked mercaptalbumin component of bovine plasma albumin and the nonmercaptalbumin species. Amino acid analysis confirmed this hypothesis by locating the carboxymethyl derivative of cysteine only in the main subcomponent of F_3 (Figure 2). In view of this result, it would be expected that

TABLE I: Amino Acid Compositions of F_2, F_3, and Bovine Plasma Albumin.

	No. of Residues Estimated				
Amino Acid	F_2	F_3	$F_2 + F_3$	Bovine Plasma Albumin	Bovine Plasma Albumin[a]
Aspartic acid	23.3	32.2	55.5	54.9	54.1
Threonine	19.4	13.8	33.2	32.1	34.2
Serine	13.2	15.4	28.6	26.6	26.0
Glutamic acid	39.1	45.0	84.1	84.8	77.6
Proline	12.9	14.1	27.0	28.6	29.9
Glycine	6.1	10.3	16.4	16.1	15.9
Alanine	18.1	25.7	43.8	48.6	46.1
Half-cystine	15.5	18.9	34.4	33.5	36.1
Valine	22.1	13.5	35.6	36.5	36.5
Methionine	1.8	2.0	3.8	3.9	3.8
Isoleucine	4.8	8.3	13.1	14.1	14.0
Leucine	26.0	33.2	59.2	63.7	61.7
Tyrosine	11.0	10.6	21.6	21.7	20.2
Phenylalanine	11.9	15.0	26.9	26.6	26.7
Lysine	29.0	33.0	62.0	57.2	61.7
Histidine	6.1	11.0	17.1	15.9	16.8
Arginine	10.7	12.2	23.0	23.5	22.4
Tryptophan		(2)	(2)	(2)	2.0
Total	271.0	316.3	587.3	590.3	585.9
Calculated molecular weight	31,000	36,300	67,300	67,400	66,000

[a] Spahr and Edsall (1964).

blocking with cysteine, instead of iodoacetamide should eliminate the heterogeneity in the F_3 region. This is in fact so, as is seen in results from a digest of cysteine-blocked bovine plasma albumin shown in Figure 3. In this case there is only one peak in the F_3 region and its position corresponds to the shoulder observed in the case of iodoacetamide-blocked bovine plasma albumin. All further characterization studies were therefore performed on digests of cysteine-blocked bovine plasma albumin.

Chromatographic studies at various stages of the digestion (Figure 4) showed F_2 and F_3 to be the primary fragments produced, with F_1 appearing later in the course of proteolytic events. For these analyses all conditions were maintained as identical as possible and the same amount of protein was applied as measured by the product $A_{279} \times$ volume.

Disc Electrophoresis. Tracings of densitograms of disc electrophoresis gels of components F_1, F_2, F_3, and of cysteine-blocked bovine plasma albumin are given in Figure 5. F_1 was found to contain two major and some minor components (Figure 5a); F_2 was consistently homogeneous (Figure 5b); and F_3 at times showed minor amounts of a slower moving component (Figure 5c). This minor component could be removed by chromatography on Sephadex G-150 and was concluded to be undigested albumin. The identical elution position of cysteine-blocked bovine plasma albumin and F_3 on DEAE-Sephadex (Figure 4) supports this conclusion.

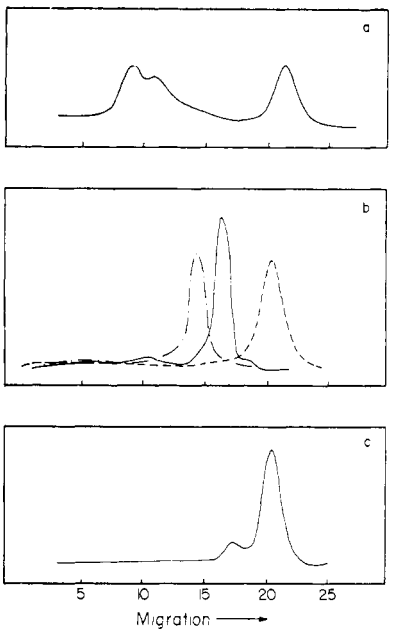

FIGURE 5: Densitograms of polyacrylamide gel disc electrophoresis separations at pH 9.5 of the fractions from B = 3.8 digests of cysteine-blocked bovine plasma albumin. (a) Fraction F_1, (b) fraction F_2 (-·-), cysteine-blocked bovine plasma albumin (——), fraction F_3 (- - -), and (c) fraction F_3.

Velocity Sedimentation. Sedimentation velocity runs gave symmetric patterns for F_1, F_2, and F_3 with the order of broadness being $F_3 < F_2 < F_1$. Sedimentation coefficients at pH 6.5 of 2.89 for F_3, 3.12 for F_2, and 2.52 for F_1 indicated F_2 and F_3 to be approximately half the size of bovine plasma albumin and F_1 to be smaller.

Amino Acid Compositions. Results of amino acid analyses for F_2, F_3, and bovine plasma albumin are presented in Table I as number of residues per mole. These values revealed a strong asymmetry in composition between F_2 and F_3 with the latter containing a larger amount of all of the amino acids except threonine and valine. The fact that the content of the latter two amino acids is higher for F_2 than for F_3, demonstrated unequivocally that the smaller fragment (F_2) could not have originated from further proteolysis of F_3. The sum of the individual residues ($F_2 + F_3$) gave excellent agreement with both our experimentally determined values and published data (Spahr and Edsall, 1964) for bovine plasma albumin.

The amino acid composition of the mixture of smaller fragments (F_1) has been summarized in Table II together with that of F_2 and F_3. All data are given here in the form of g/100 g of sample so as to allow a meaningful comparison of the clearly heterogeneous F_1 fraction with F_2 and F_3. The homology between F_1 and F_2, especially in those amino acids strongly asymmetric between F_2 and F_3, *i.e.*, histidine, threonine, glycine, and valine, indicated F_1 to have originated from the same part of the albumin molecule as did F_2.

An ultraviolet difference spectrum of F_3 *vs.* F_2, with the solutions matched at 278 mμ, gave a peak at 292 mμ and a shoulder at 284 mμ demonstrating a pronounced difference in tryptophan content. Spahr and Edsall (1964) report only two tryptophan residues in bovine plasma albumin. Therefore, as-

TABLE II: Amino Acid Compositions of DEAE-Sephadex Chromatographic Fractions from a B = 3.8 Digest of Sulfhydryl-Modified Bovine Plasma Albumin.[a]

Amino Acid	F_1	F_2	F_3
Aspartic acid	8.56	8.66	10.21
Threonine	6.16[b]	6.33	3.86
Serine	3.12[b]	3.70	3.71
Glutamic acid	15.17	16.28	16.04
Proline	3.99	4.05	3.77
Glycine	1.14	1.12	1.62
Alanine	4.45	4.15	5.04
Half-cystine	4.94	5.18	5.39
Valine	6.97	7.07	3.70
Methionine	0.64	0.76	0.71
Isoleucine	1.44	1.76	2.59
Leucine	9.08	9.47	10.38
Tyrosine	4.50[b]	5.80	4.78
Phenylalanine	5.78	5.65	6.10
Lysine	11.89	11.99	11.67
Histidine	2.52	2.69	4.15
Arginine	4.62	5.37	5.29

[a] Expressed as g/100 g of protein. [b] Uncorrected for hydrolytic destruction.

suming that the proteolytic split is unique, as argued below, and that there was no loss of tryptophan, both residues must be located in F_3. Further evidence for this distribution of tryptophan residues may be found in the experimentally determined extinction coefficients ($E_{278}^{1\%,1\,cm}$) of 5.51 and 7.55 for F_2 and F_3, respectively. These values are in good agreement with those expected for two protein molecules having similar molecular weights and tyrosine contents, but with one (F_2) having no tryptophan and the other (F_3) having two tryptophan residues. Using the amino acid molar extinction coefficients reported by Wetlaufer (1962), the determined amino acid compositions,

TABLE III: N-Terminal Residues of F_1, F_2, F_3, Bovine Plasma Albumin, and a B = 3.8 Digest.[a]

N-Terminal Residue	F_1 (moles/ 3 × 10^4)	F_2 (moles/ 3 × 10^4)	F_3 (moles/ 3.5 × 10^4)	Bovine Plasma Albumin (moles/ 6.6 × 10^4)	Digest (moles/ 6.6 × 10^4)
Aspartic acid	0.12	0.14	1.10	0.86	1.16
Threonine	0.43			0.13	0.30
Glycine			0.12		0.14
Alanine	0.88				0.38
Leucine			0.18	0.11	0.16
Phenylalanine	0.66	0.64			0.66

[a] All amounts in excess of 0.10 mole are reported.

TABLE IV: Equilibrium Sedimentation Molecular Weights of F_2 and F_3.

Sample	C (%)	pH	$\bar{M}_z^0$
	High Speed (36,500 rpm)		
F_2	0.017	6.00	30,400
	0.034	5.95	30,600
	0.035	6.29	31,500
	0.053	6.48	30,300
	0.068	6.16	28,400
	0.070	6.32	29,100
F_3	0.022	6.02	36,100
	0.044	5.99	35,300
	0.088	6.00	34,000
			$\bar{M}_w^0$
	Low Speed (12,590 rpm)		
F_2	0.053	6.48	30,500
	0.070	6.32	29,500
	0.033	2.98	29,200
F_3	0.030	2.98	36,400

and the molecular weights from equilibrium sedimentation, values of $E_{278}^{1\%,1\,cm}$ of 4.8 for F_2 and 7.2 for F_3 may be estimated.

N-Terminal Analysis. 1-Fluoro-2,4-dinitrobenzene analysis yielded aspartic acid as the major N-terminal residue in F_3 and phenylalanine in F_2. Quantitative data obtained by the cyanate method of Stark and Smyth (1963) for F_1, F_2, F_3, cysteine-blocked bovine plasma albumin, and a B = 3.8 digest are presented in Table III. The data for the digest show the formation of little or no new N-terminal aspartic acid to occur during the reaction. Thus the one such residue found in F_3 must be the same as that found in intact albumin. In addition, since the predominant new N-terminal residue produced during the reaction was phenylalanine and since F_2 contained this terminus almost exclusively, the occurrence of a specific proteolytic split yielding F_2 and F_3 was indicated. Finally, the determination of only N-terminal alanine and phenylalanine[1] in F_1 indicated that a second moderately specific proteolytic split in F_2 (and not F_3) gave rise to the mixture F_1. N-Terminal analysis as a function of B value (Figure 6) confirmed the formation of little new N-terminal aspartic acid, the early production of new N-terminal phenylalanine, and the delayed formation of N-terminal alanine.

Association–Dissociation Equilibrium. Molecular weights of F_2 and F_3 at pH 6 were determined by equilibrium sedimentation using the high-speed meniscus depletion technique (Yphantis, 1964). In addition some low-speed runs were performed using ultraviolet absorption optics. Molecular weight values are summarized in Table IV. Linearity of the Yphantis plots in all cases and agreement between the two molecular weight averages for F_2 and F_3 from the high- and low-speed experiments demonstrated a high degree of homogeneity for these

[1] N-Terminal threonine was also detected, but because of the large destruction correction which had been applied (Stark and Smyth, 1963) to obtain the final values, the presence of this N-terminal residue is open to question.

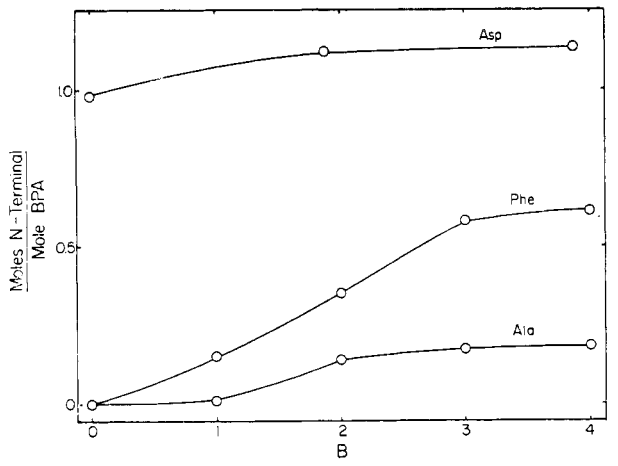

FIGURE 6: Amounts of N-terminal residues present per mole of cysteine-blocked bovine plasma albumin as a function of the extent of subtilisin digestion. Values for Phe and Ala corrected for background by subtracting the $B = 0$ values.

fragments. Lack of concentration dependence and no change in the molecular weights when the pH was lowered to 3 indicated negligible self-association of the fragments.

F_2 and F_3 revealed a strong tendency to associate with each other in the pH range near neutrality. In an attempt to determine the molar ratio of F_2 to F_3 which gave the highest degree of association, molecular weight studies were performed at pH 6.8 as a function of the molar mixing ratio of F_2 and F_3, with the total concentration of protein held constant at 1×10^{-5} M. The results, shown in Figure 7, revealed a molecular weight maximum of 53,500 at a molar ratio of 1:1. In addition, the

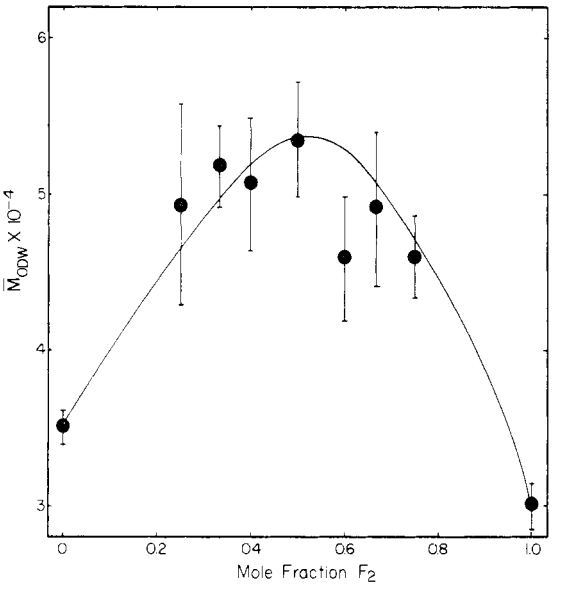

FIGURE 7: Dependence of the optical density weight-average molecular weight on the mole fraction of F_2 in various mixtures of F_2 and F_3 at pH 6.8. Total protein concentration maintained at 1×10^{-5} M. Filled circles are an average of the experimental values with error bars drawn so as to include the entire range of results. In general six measurements were performed at each mixing ratio. The solid line corresponds to a completely interacting F_2–F_3 system with an association constant of 10^6 l./mole.

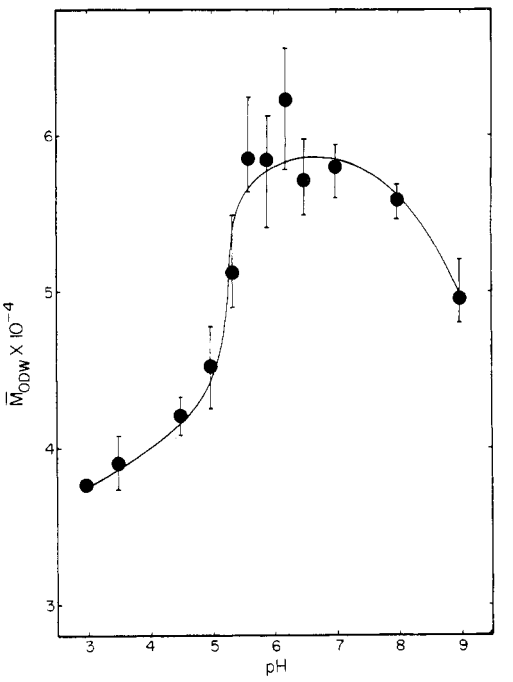

FIGURE 8: The pH dependence of the optical density weight-average molecular weight of an equimolar mixture of F_2 and F_3. Error bars drawn include the entire range of results. In general three measurements were performed at each pH. Protein concentration *ca.* 0.03 g/100 ml.

data could be fitted reasonably well assuming formation of a 1:1 complex and an association constant of 10^6 l./mole. This would indicate that the complex formed contained one molecule each of F_2 and F_3 and that all F_2 and F_3 molecules present participated in the formation of this complex.

Results of low-speed molecular weight analyses of equimolar mixtures of F_2 and F_3 at various pH values are shown in Figure 8. The maximum molecular weight for the mixture, 55,000–60,000, was attained in the pH range 6–7 and corresponds to approximately 75% association. The results also

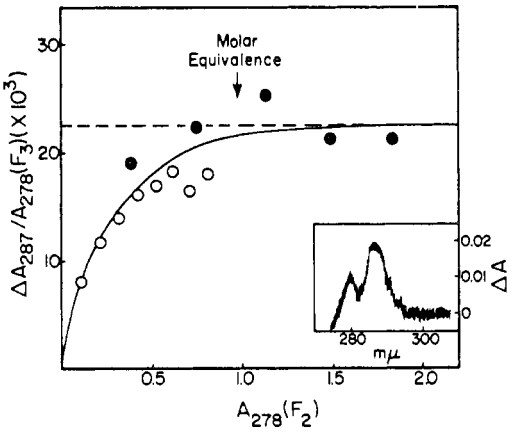

FIGURE 9: Difference spectral titration of F_2 with F_3 at pH 6. The fractions were from a $B = 3.8$ subtilisin digest of cysteine-blocked bovine plasma albumin. (○) 5.029 ml of 0.092% F_3 titrated with 50-μl aliquots of 0.972% F_2; (●) 5.00 ml of 0.052% F_3 titrated with 100-μl aliquots of 1.005% F_2. The inset shows a typical difference spectrum obtained at approximately molar equivalence.

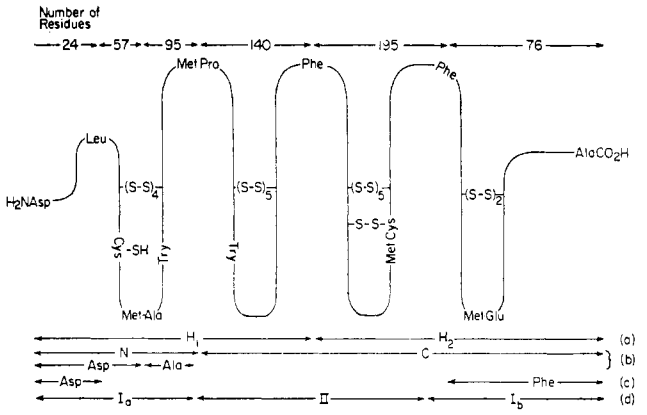

FIGURE 10: Schematic model of the covalent structure of the bovine plasma albumin molecule. (a) Fragments obtained in the present study; (b) fragments obtained by cleavage at the methionine residues with cyanogen bromide (King and Spencer, 1968; T. P. King, 1968, personal communication); (c) small fragments isolated from a peptic digest (Peters and Hawn, 1967); (d) large fragments isolated from a peptic digest (Weber and Young, 1964a,b; Pesce *et al.*, 1967)

show that almost complete dissociation had occurred by pH 3 with the optical density weight-average molecular weight decreasing to nearly the value expected for an equimolar non-reacting mixture (*ca.* 34,000).

Mixtures of F_2 and F_3 when compared with the separate components gave difference spectra characteristic of a perturbation of tyrosyl residues (Figure 9, inset). Plotting $\Delta A_{287}/A_{278F_3}$ *vs.* A_{278F_2} (Figure 9) gave the extent of the reaction as a function of the amount (in absorbance units) of F_2 added. The values of A_{278F_3} and A_{278F_2} were obtained for each point by making appropriate volume corrections to the direct spectra of F_3 and F_2, which were determined from the solutions in the reference compartments at the completion of the titration.

The method of plotting employed in Figure 9 is analogous to that used by Lebowitz and Laskowski (1962) for the analysis of data on the association of trypsin and soybean trypsin inhibitor. They published theoretical curves for various assumed association constants. The curvature seen is consistent with expectation for an association constant of the order 10^6 l./mole.

Discussion

Comparison with the Results of Adkins. Adkins and Foster (1966), using recycling chromatography at pH 2.3 on Sephadex G-100, were able to resolve reconstituted $B = 3.8$ digests of acid-defatted unblocked bovine plasma albumin into three major components which they designated T, H_1, and H_2. Although disc electrophoresis showed their fractions to contain small amounts of impurities, a definite correlation can be shown between them and the more homogeneous fragments obtained from SH-blocked charcoal-defatted bovine plasma albumin in the present work.

Adkins and Foster found the mobilities in disc electrophoresis of their fractions to be in the order H_2 < bovine plasma albumin < H_1, and we find F_2 < bovine plasma albumin < F_3 (Figure 5b). In addition, velocity sedimentation and amino acid analysis (Table V) demonstrate a positive correlation between F_1 and T, F_2 and H_2, and F_3 and H_1, indicating these to have fundamentally the same origin with respect to the bovine plasma albumin molecule. Because of this correlation and in order to maintain a constant notation, the symbols T, H_1, and H_2 will be used in the remaining discussion in place of F_1, F_3, and F_2, respectively.

TABLE V: Amino Acid Composition of Bovine Plasma Albumin Fragments Obtained by Adkins and DEAE-Sephadex Chromatographic Fractions of a $B = 3.8$ Bovine Plasma Albumin Digest.

Amino Acid	F_1	T	F_2	H_2	F_3	H_1
Aspartic acid	15.3	16.0	23.3	25.5	32.2	34.9
Threonine	15.8	12.3	19.4	16.5	13.8	14.5
Serine	9.8	10.2	13.2	11.2	15.4	15.0
Glutamic acid	24.2	26.1	39.1	38.4	45.0	48.7
Proline	8.5	10.4	12.9	13.0	14.1	14.9
Glycine	4.1	5.1	6.1	7.1	10.3	11.1
Alanine	12.9	11.0	18.1	20.5	25.7	27.9
Half-cystine	9.8	10.0	15.5	14.1	18.9	16.9
Valine	14.5	14.4	22.1	19.8	13.5	15.3
Methionine	1	1	1.8	2	2.0	2
Isoleucine	2.6	3.1	4.8	5.5	8.3	9.8
Leucine	16.5	19.4	26.0	28.8	33.2	37.6
Tyrosine	6.9	7.7	11.0	9.8	10.6	9.2
Phenylalanine	8.1	7.3	11.9	12.8	15.0	15.0
Lysine	19.1	16.9	29.0	30.3	33.0	37.1
Histidine	3.8	4.2	6.1	7.2	11.0	11.8
Arginine	6.1	10.0	10.7	11.2	12.2	13.6
Tryptophan	0	N.d.	0	N.d.	(2)	N.d.
Total		185.1	271.0	273.3	316.3	335.2
Calculated molecular weight		21,000	31,000	31,000	36,300	38,000

Sequence of Proteolytic Events. From the chromatographic studies and N-terminal analyses as a function of B value, we were able to trace the reaction pathway. Chromatographic runs at various stages of digestion (Figure 4) revealed that T (F_1) appeared very late in the reaction. N-Terminal results on these digests at different B values (Figure 6) also showed a lag time in the appearance of N-terminal alanine, *i.e.*, T indicating the following sequence of proteolytic events

$$\text{cysteine-blocked bovine plasma albumin} \longrightarrow H_1 + H_2$$

$$H_2 \longrightarrow T$$

Relationship of Fragments to the Parent Molecule. Based on the N-terminal analyses and the additivity of amino acid composition, the arrangement of the fragments in the intact bovine plasma albumin molecule must be H_2N–H_1–H_2–CO_2H. The possibility of loss of a small peptide fragment between H_2 and H_1 or from the C-terminal end of H_2 has not been completely excluded, but from the summed residue values (Table I), it can be concluded that any such peptide would have very few residues.

On the basis of the present findings, together with degradation results of other workers (Weber and Young, 1964a,b;

Peters and Hawn, 1967; King and Spencer, 1968; T. P. King, 1968, personal communication), a model is proposed for the covalent structure of bovine plasma albumin (Figure 10). In agreement with our assignment of the single cysteine residue to H_1, King (T. P. King, 1968, personal communication) has shown this residue to be located within the first 81 amino acids from the N terminus. Sequence work of Shearer *et al.* (1967) further positions this amino acid between residues 24 and 81. The position of the two tryptophan residues could also be further pinpointed by combination of our results, which indicated both to be in H_1, and the work of King (T. P. King, 1968, personal communication), who found one tryptophan residue in each of the fragments N and C (see Figure 10).

A most interesting characteristic of this model is its subunit-like structure. At least four regions of the molecule are found to be intralinked by disulfide bonds, but connected to each other only through the peptide backbone. This covalent structure is entirely compatible with the schematic model proposed earlier by one of us (Foster, 1960).

Relationship of the Association–Dissociation Equilibrium to the N–F Transition. Both the pH dependence of the dissociation of the complex and the difference spectral titration are in substantial accord with the N–F transition. The equilibrium constant for the formation of N from F at pH 6 is not known but must be at least of the order 10^3 since F is never observed at this pH. It must be emphasized that this is a concentration-independent constant which is not directly comparable with the constant observed for mixtures of H_2 and H_1 (approximately 10^6 l./mole). In qualitative terms, the fact that association of H_2 and H_1 is not complete at pH 6 and the fact that dissociation occurs at a higher pH than the N–F transition can be explained, at least in part, by an additional entropy of mixing term which tends to favor dissociation for the case where the peptide bond has been cleaved. The magnitude of $\Delta A_{287}^{max}/A_{278}^{total}$ for the association at pH 6 was 1.4×10^{-2}. This corresponds to about one-third that observed by Sogami and Foster (1968) for the N–F transition (4.0×10^{-2}), and may indicate that the association of H_2 and H_1 corresponds to only one of the two steps pictured as occurring in the total transition (Sogami and Foster, 1968; Leonard and Foster, 1961).

Acknowledgment

We are indebted to Miss Lydia Heim for carrying out the disc electrophoresis experiments, to Professor A. Light for his advice with respect to amino acid analysis and determination of N-terminal residues, and to Professor David Filmer for assistance with some of the low-speed equilibrium ultracentrifuge runs.

References

Adkins, B. J., and Foster, J. F. (1965), *Biochemistry 4*, 634.

Adkins, B. J., and Foster, J. F. (1966), *Biochemistry 5*, 2579.

Andersson, L. O. (1966), *Biochim. Biophys. Acta 117*, 115.

Aoki, K., and Foster, J. F. (1956), *J. Am. Chem. Soc. 78*, 3538.

Aoki, K., and Foster, J. F. (1957), *J. Am. Chem. Soc. 79*, 3385.

Benson, J. V., Jr., and Patterson, J. A. (1965), *Anal. Chem. 37*, 1108.

Brush, J. S., Jensen, E. V., and Jacobsen, H. I. (1963), *Biochim. Biophys. Acta 74*, 688.

Chen, R. F. (1967), *J. Biol. Chem. 242*, 173.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides as Ions and Dipolar Ions, New York, N. Y., Reinhold.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Foster, J. F. (1960), *in* The Plasma Proteins, Putnam, F. W., Ed., New York, N. Y., Academic, Chapter 6.

Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L. (1954), *Methods Biochem. Anal. 2*, 359.

Harrington, W. F., Johnson, P., and Ottewill, R. H. (1956), *Biochem. J. 62*, 569.

Hartley, R. W., Jr., Peterson, E. A., and Sober, H. (1962), *Biochemistry 1*, 60.

Herskovits, T. T., and Laskowski, M., Jr. (1962), *J. Biol. Chem. 237*, 2481.

King, T. P., and Spencer, E. M. (1968), *Federation Proc. 27*, 391.

Lebowitz, J., and Laskowski, M., Jr. (1962), *Biochemistry 1*, 1044.

Leonard, W. J., Jr., and Foster, J. F. (1961), *J. Biol. Chem. 236*, 2662.

Pesce, A. J., Pasby, T. L., and Weber, G. (1967), *Federation Proc. 26*, 602.

Peters, T., Jr., and Hawn, C. (1967), *J. Biol. Chem. 242*, 1566.

Sanger, F. (1945), *Biochem. J. 39*, 507.

Sanger, F. (1949), *Biochem. J. 45*, 563.

Shearer, W. T., Bradshaw, R. A., Gurd, F. R. N., and Peters, T., Jr. (1967), *J. Biol. Chem. 242*, 5451.

Sogami, M., and Foster, J. F. (1968), *Biochemistry 7*, 2172.

Sogami, M., Peterson, H. A., and Foster, J. F. (1969), *Biochemistry 8*, 49.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Spahr, P. F., and Edsall, J. T. (1964), *J. Biol. Chem. 239*, 850.

Stark, G. R., and Smyth, D. G. (1963), *J. Biol. Chem. 238*, 214.

Sterman, M. D. (1956), Ph.D. Dissertation, Purdue University, Lafayette, Ind.

Weber, G., and Young, L. B. (1964a), *J. Biol. Chem. 239*, 1415.

Weber, G., and Young, L. B. (1964b), *J. Biol. Chem. 239*, 1424.

Wetlaufer, D. B. (1962), *Advan. Protein Chem. 17*, 303.

Yphantis, D. A. (1964), *Biochemistry 3*, 297.