Patterson, M. S., and Greene, R. C. (1965), *Anal. Chem. 37*, 854.
Pollack, R. E., Green, H., and Todaro, G. J. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 126.
Robbins, P. W., Keller, J. M., Wright, A., and Bernstein, R. L. (1965), *J. Biol. Chem. 240*, 384.
Roseman, S. (1966), *in* Biochemistry of Glycoproteins and Related Substances, Rossi, E., and Stoll, E., Ed., Basal, S. Karger, 244.
Spiro, M. J., and Spiro, R. G. (1968), *J. Biol. Chem. 243*, 6520.
Spiro, R. G. (1969), *N. E. J. Med. 281*, 991, 1043.
Strauss, J. H., Burge, B. W., and Darnell, J. E. (1970), *J. Mol. Biol. 47*, 437.
Tsuiki, S., Hashimoto, Y., and Pigman, W. (1961), *J. Biol. Chem. 236*, 2172.
Warren, L. (1959), *J. Biol. Chem. 234*, 1971.
Warren, L., Blacklow, R. S. (1962), *J. Biol. Chem. 237*, 3527.
Warren, L., and Glick, M. C. (1966), *Methods Enzymol. 8*, 131.
Wu, H. C., Meezan, E., Black, P. H., and Robbins, P. W. (1969), *Biochemistry 8*, 2509.

Limited Digestion of Citraconylated Bovine Serum Albumin with α-Chymotrypsin*

Ana Jonas† and Gregorio Weber

ABSTRACT: Citraconyl bovine serum albumin was digested with α-chymotrypsin until eight to nine amide bonds had been broken per protein molecule. Under the conditions of the digestion (*i.e.*, pH 7.7, water solution, 23°) the citraconylated protein, which had nearly 70% of its ε-amino groups modified by citraconic anhydride, existed in an expanded conformation very similar to that of serum albumin in acid (Jonas, A., and Weber, G. (1970), *Biochemistry 9*, 4729). The digest was fractionated on ion-exchange and gel filtration columns, into a small peptide fraction and several macromolecular fragments. These chymotryptic fractions were subsequently studied as to their average molecular weights, rotational relaxation times, amino acid composition, fluorescence spectra, and anion binding properties. Molecular weights and rotational relaxation times for the five macromolecular fractions ranged from 35,000 to 8000 and from 33 to 6 nsec, respectively. The per cent amino acid compositions deviated significantly in polar residue content from that of bovine serum albumin; three of the larger fragments were negatively charged with respect to the intact protein. The small peptide fraction had an average molecular weight near 2000 and was basic relative to the parent protein, due mainly to a high content of arginine residues. At least one of the tryptophan residues was found in this fraction. The isolated fractions had low binding affinities for 1-anilinonaphthalene-8-sulfonate, but the total digest, especially in the presence of excess small peptide fraction, had an increased affinity for the ligand. The results suggest that 50–60% of the expanded bovine serum albumin is relatively compact at the carboxyl end, while the amino-terminal region is loosely organized and is easily broken down by proteases. The experiments also indicate that bovine serum albumin has regions of very uneven charge distribution. The "middle" of the protein appears to be more negative, and the adjacent zones appear to be more basic than the total bovine serum albumin. The anion binding experiments suggest that the small peptide fraction originates at or very near the regions of bovine serum albumin where strong anion binding takes place.

The reversible changes in the physicochemical properties of BSA,[1] in the pH region from 4 to 2, led Harrington *et al.* (1956) and Foster (1970) to propose three-dimensional models of the protein, which consisted of glubular regions joined by flexible polypeptide chains. Based on these models, limited protease digestion experiments were performed on expanded serum albumin, under the assumption that proteases would preferentially attack the extended polypeptide chains liberating a few compact macromolecular fragments.

After a short pepsin digestion of BSA at pH 3.0, Weber and Young (1964a,b) recovered two types of macromolecular fragments having average molecular weights of 32,000 and 12,500. A more prolonged digestion of BSA with pepsin, under similar conditions to those used by Weber and Young (1964a,b), gave several heterogeneous fractions. Purification of these fractions yielded two small, electrophoretically pure segments: the *Phe* fragment (mol wt 8500) from the carboxyl-terminal end, and the *Asp* fragment (mol wt 2800) from the amino-terminal end of BSA (Peters and Hawn, 1967).

Adkins and Foster (1965, 1966) and Pederson and Foster (1969) used subtilisin, in the presence of 100 moles of sodium

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[1] Abbreviations used are: BSA, bovine serum albumin; DNS, 1-dimethylaminonaphthalene-5-sulfonyl fluorescent label; BSA-DNS, BSA labeled with DNS; ANS, 1-anilinonaphthalene-8-sulfonate.

dodecyl sulfate/mole of BSA, at pH 9, to partially digest the protein expanded by the detergent. Two main fragments were recovered; each had approximately half the molecular weight of BSA, and together they accounted for most of the amino acids of the parent protein.

Even though macromolecular fragments are produced during the initial stages of the digestion of BSA by pepsin and subtilisin, the validity of the subunit-like models proposed for BSA cannot be established on the basis of such evidence alone—it is necessary to show that different proteases can, in effect, release essentially the same macromolecular fragments from similar expanded forms of BSA.

In this work, we partially digested citraconyl-BSA with α-chymotrypsin, in an attempt to recover macromolecular fragments comparable to those obtained by Weber and Young (1964a,b). Under the conditions of these experiments (70% modification of amino groups, pH 7.7, water solution) citraconyl-BSA had all the physical characteristics of the acid expanded form of BSA (Jonas and Weber, 1970). During the course of these studies we observed that some of the chymotryptic fractions of BSA had a markedly different charge from the net charge of BSA. We confirmed earlier observations on the decreased ligand binding affinity of digested BSA (Weber and Young, 1964a) and of BSA fragments (King and Spencer, 1968) as compared to the native protein. In addition, we found some correlation between the structural features of BSA, detected on the chymotryptic fractions, and the high affinity of this protein for hydrophobic anions such as ANS.

Materials and Methods

Citraconyl-BSA and citraconyl-BSA-DNS,[2] having from 60 to 80% of the amino groups modified, were prepared and characterized according to the procedures described in an earlier paper (Jonas and Weber, 1970).

Trypsin, α-chymotrypsin, and the soybean trypsin inhibitor, as well as ovalbumin, pepsin, and ribonuclease, were obtained from Worthington Biochemical Corp. Sephadex and Bio-Rad gels were purchased from Pharmacia Fine Chemicals and Bio-Rad Laboratories, respectively, while the DEAE-cellulose ion exchanger was obtained from Sigma Chemical Co.

The magnesium salt of ANS was prepared according to Weber and Young (1964a); *p*-dimethylaminobenzaldehyde was obtained from Eastman Organic Chemicals; hydrogen peroxide and formic acid were purchased from J. T. Baker Chemical Co. and Allied Chemical, respectively. All the common salts, acids, and bases were commercial, reagent grade chemicals. Sucrose, obtained from Allied Chemical was recrystallized once from ethanol. Deionized and glass-distilled water was used throughout these experiments.

All the instruments described in a previous paper (Jonas and Weber, 1970); except the cross-correlation phase fluorometer, were used in this work. A Metrohm automatic titrator and pH-Stat, Model Combi-Titrator 3D, equipped with a glass electrode and reaction vessels with thermostating jackets, was used during the proteolytic digestions. Column fractions were collected with a Research Specialties, Model 1205 D3, automatic fraction collector. Refractive indices were measured with a standard Abbe refractometer, and amino acids were determined on Beckman Model 120C and Beckman–Spinco automatic amino acid analyzers.

Fluorescence intensities, in the ANS binding experiments, were recorded with a Hitachi-Perkin Elmer MPF-2A fluorescence spectrophotometer. Ninety degree optics were used with solutions having absorbance values, at the exciting wavelength (345 mμ), lower than 0.5. A front face adapter with a 2 mm $\times$ 1 cm cuvet was required with solutions of higher absorbance.

Digestion Procedure. The protein solutions used in the digestions were from 0.5 to 1.0%, in water. Protein content was determined by the methods cited in an earlier paper (Jonas and Weber, 1970) and by measuring the refractive index increments of solutions of BSA and BSA fractions (Weber and Young, 1964b). α-Chymotrypsin solutions were prepared in water, just before use. A 1:400 enzyme to protein ratio by weight, which gave an initial reaction rate of approximately one bond hydrolyzed per minute, was used in the enzymatic reactions. All digestions were carried out under nitrogen, at 23°, and a constant pH of 7.7, with 5×10^{-3} to 5×10^{-2} M NaOH as the titrant.

The progress of the digestions was followed by two methods: base consumption, recorded with the pH-Stat, and changes in fluorescence polarization (p) measured on samples removed at regular time intervals from the reaction solution. It was determined by the procedure of Markus *et al.* (1967), using trypsin and soybean trypsin inhibitor, that at pH 7.7, 2.02 moles of amide bonds is broken in BSA per mole of base consumed.

Since a 70% citraconyl-BSA sample, which in water at pH 7.0 exists in an expanded form, can be refolded to the native configuration by increasing the ionic strength of the medium (Jonas and Weber, 1970), samples removed during the digestion, and diluted with 0.1 M sodium phosphate buffer (pH 7.0) containing 0.2 M LiCl, gave polarization values corresponding to the extent of fragmentation of citraconyl-BSA-DNS.

Deactivation of α-chymotrypsin and removal of citraconyl groups, after digestion, were accomplished simultaneously by stirring the digest solutions for 12 hr at pH 2–3, and at room temperature. The free citraconyl groups were removed by gel filtration through a Sephadex G-25 column equilibrated with water. The desalted BSA digests were lyophilized prior to the fractionation steps.

Fractionation of BSA Digests. A ratio of 20:1 (by weight) of DEAE-cellulose to protein was normally used in the ion-exchange procedure. Before packing the chromatography columns the ion exchanger was equilibrated for over 24 hr with 0.025 M sodium phosphate buffer (pH 6.0; with a few changes of buffer in order to remove an impurity which absorbs ultraviolet light at 280 mμ, and which is not displaced by repeated washing with water). The protein samples were eluted from the DEAE-cellulose columns with sodium phosphate buffer (pH 6.0), in a linear concentration gradient from 0.025 to 0.3 M. The total volume of eluent varied from column to column; 15-g DEAE-cellulose columns, used in the preparation of the BSA fractions, were eluted with 900 ml of buffer. Column fractions were collected automatically in equal volumes (3–5 ml); the absorbance at 280 mμ and the

[2] The physical properties of BSA and BSA-DNS are the same (Harrington *et al.*, 1956; Weber, 1952; Weber and Young, 1964a); therefore, the results obtained for the labeled protein also apply to the unlabeled species.

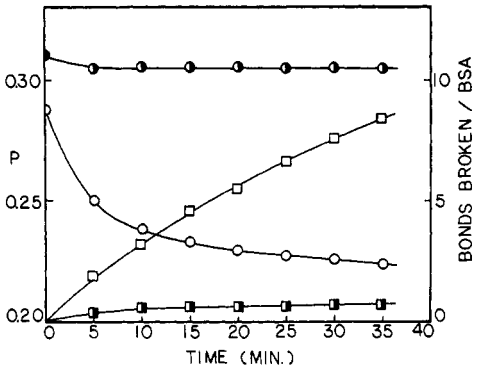


FIGURE 1: Progress of the digestion of citraconyl-BSA by α-chymotrypsin. (◑,◑) polarization (p) measurements on control BSA-DNS; (○,○) polarization measurements on citraconyl-BSA-DNS; (▮,▮) bonds broken per control protein molecule (bonds broken/BSA); (□,□) bonds broken per citraconylated protein molecule.

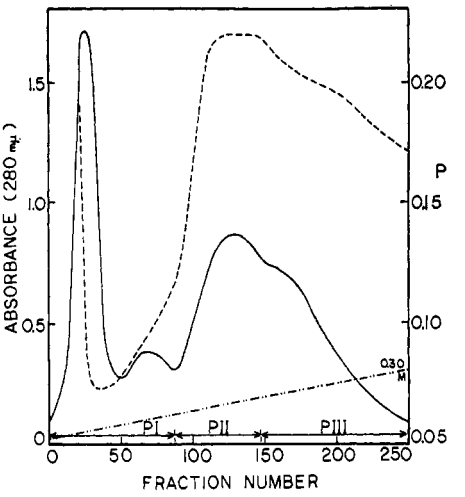


FIGURE 2: Fractionation of the chymotryptic BSA digest on a DEAE-cellulose column. Elution patterns: (———) absorbance at 280 mμ; (– – – –) fluorescence polarization (p); (–·–·–·–) sodium phosphate buffer (pH 6.0), concentration gradient from 0.025 to 0.3 M.

fluorescence polarization were measured every two and four fractions, respectively.

Further fractionation of the main DEAE-cellulose fractions was carried out on Bio-Gel, P-10 and P-30, columns. Following each of the fractionation steps, the pooled fractions were desalted either by passage through a Sephadex G-25 column, or—after preliminary experiments indicated that the protein fraction would not diffuse through dialysis tubing—by extensive dialysis against water. The desalted fractions were lyophilized and stored below −5°.

Characterization of BSA Fractions. Rotational relaxation times were determined for the main fractions of BSA following the same procedure and using the same conditions as in a previous paper (Jonas and Weber, 1970). Average molecular weights were estimated from their elution volumes on a Bio-Gel P-30 column (2.3 × 70 cm) equilibrated with 0.1 M NaCl in 0.05 M sodium phosphate buffer (pH 7.0). The standard plot of the logarithm of the molecular weights against the elution volumes was obtained by using four known proteins: ovalbumin (mol wt 44,000), pepsin (mol wt 35,500), trypsin (mol wt 24,000), and ribonuclease (mol wt 12,700). The molecular weight of the largest fragment (P II) was also determined with the ultracentrifuge, using the interference pattern of sedimentation equilibrium according to the technique by Nazarian (1968).

All the amino acids, except cysteine, cystine, and tryptophan, were determined by automatic amino acid analysis of BSA fraction samples which had been hydrolyzed at 105°, for 24 hr, in 5.7 N HCl and 0.05% mercaptoacetic acid. Cysteine and cystine residues were determined together as cysteic acid in samples which before acid hydrolysis had been oxidized with performic acid. Tryptophan was determined by the colorimetric method of Spies and Chambers (1949). Qualitatively, tryptophan was easily detected by its contribution to the intrinsic fluorescence spectra of BSA fractions excited at 275 and 295 mμ.

Binding of ANS. The binding of ANS by the fractions of BSA was represented graphically by the expression (Klotz, 1953; Weber, 1965)

$$\log \frac{n-r}{r} = -\log D + \log K_d$$

where, n is the total number of binding sites per mole of protein, r is moles of bound ligand per mole of protein, D is the molar concentration of free ligand, and K_d is the intrinsic dissociation constant.

Since the fluorescence yield of ANS bound to BSA and to the chymotryptic fractions of BSA was from 50 to 200 times higher than that of free ANS in the aqueous solvent, the fluorescence of BSA–ANS solutions could be attributed to bound ligand alone, even in the presence of considerable amounts of free ligand. The variables r and D were obtained from fluorescence intensity measurements using the expressions given by Weber and Young (1964a): $x = F/F_0$, $r = xD_0/P_0$, and $D = (1-x)D_0$, where x is the experimentally determined quantity, which represents the fraction of total ligand bound to the protein, F is the observed fluorescence intensity at any given protein to ligand ratio, F_0 is the fluorescence intensity at the same ligand concentration when it is totally bound to protein, and D_0 and P_0 are the total ligand and total protein concentrations, respectively.

The binding experiments were carried out in 0.05 M sodium phosphate buffer, at pH 7.0, by adding small amounts of 1–2% protein solution to a ligand solution. To avoid changes in total ANS concentration, the protein solutions contained the same concentration of ligand. After each protein addition, and careful mixing of the cuvet contents, the fluorescence intensity was measured at 470 mμ, using 345-mμ exciting light and 4-mμ band widths. Since the point of complete binding of ligand to the fractions of BSA could not be reached experimentally, F_0 was determined by extrapolating a plot of $1/F$ *vs.* $1/P_0$, to $1/P_0 = 0$.

Results

Digestion of Citraconyl-BSA by α-Chymotrypsin. Figure 1 shows the change in fluorescence polarization (p) and the number of amide bonds broken per protein molecule, against time of digestion, for a 70% modified citraconyl-BSA and an unmodified sample of BSA. The initial rate of reaction of

TABLE I: Percentage of Each Main Fraction in the Total Chymotryptic Digest of BSA.

Fraction	Percentage (by wt)
P Ia	13
P Ib	12
P Ic (small peptides)	28
P II	32
P IIIa	10
P IIIb	5

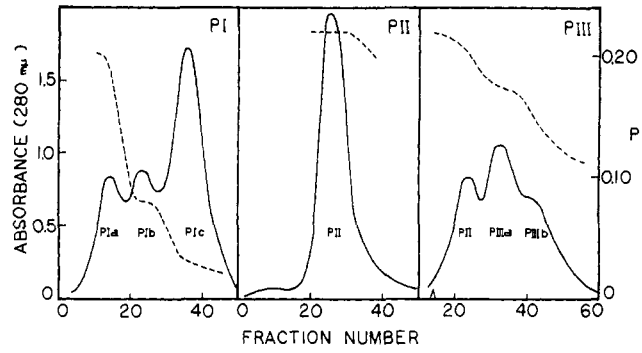


FIGURE 3: Fractionation of P I, P II, and P III by gel filtration on Bio-Gel columns. Elution patterns: (———) absorbance at 280 mμ, (- - - -) fluorescence polarization (p). P I, was fractionated on a P-10 column, equilibrated with water; P II and P III were fractionated on a P-30 column, equilibrated with 0.1 M NaCl in 0.05 M sodium phosphate buffer (pH 7.0).

the expanded citraconyl-BSA (in terms of bonds hydrolyzed) is nearly ten times higher than for the native protein. The change in fluorescence polarization for the control, after 35-min digestion is only 2%, while the polarization of the expanded sample decreases by 21%, in two stages. During the first 10–15-min digestion, the fluorescence polarization decreases rapidly from 0.285 to about 0.235, then it continues to drop slowly, approaching a value of 0.220 after 40 min. This polarization value is close to that of intact citraconyl-BSA (p = 0.210), measured in water at pH 7.0. Therefore, the fragments released after 30–40-min digestion have an average rotational freedom similar to that of expanded citraconyl-BSA.

For the preparation of the chymotryptic fractions, up to 1 g of citraconyl-BSA and citraconyl–BSA–DNS were digested until 8 to 9 amide bonds per BSA molecule had been broken and the fluorescence polarization in the DNS-labeled digest had decreased to about 0.220.

Fractionation of Digest on DEAE-cellulose and Bio-Gel Columns. The elution patterns of a chymotryptic digest of BSA from a DEAE-cellulose column, in terms of absorbance at 280 mμ and fluorescence polarization (p) of the fractions, are shown in Figure 2. Two main absorbance peaks and a trailing shoulder were eluted from the column; they were labeled P I, P II, and P III in the direction of increasing buffer concentration. The fluorescence polarization across P I and P III decreased from left to right from 0.20 to 0.08 and from 0.21 to 0.17, respectively, indicating the presence of more than one fragment in each of these fractions. P II had a constant polarization (p = 0.220) throughout the digestion (Jonas, 1970). In terms of protein content, P I accounted for 48% to 55% of the total digest and P II together with P III, for the remaining 45% to 52%.

Further fractionation and purification of the total fractions corresponding to P I, P II, and P III were carried out on Bio-Gel columns (Figure 3).

P I was passed through a Bio-Gel, P-10 column (2.5 × 33 cm), equilibrated with water. Absorbance at 280 mμ and polarization measurements revealed at least three components. The first two peaks corresponded to larger fragments having molecular weights of 20,000 and 8000 as estimated from their elution volumes. The last peak was eluted at the total bed volume of the column, corresponding to molecular weights of 2000 and lower. The three new fractions were labeled P Ia, P Ib, and P Ic in the order of their elution from the column, and they represented 26, 22, and 52%, respectively, of the total protein in P I.

The P II and P III fractions were passed through a Bio-Gel, P-30 column (2.3 × 70 cm), equilibrated with 0.1 M NaCl in 0.05 M sodium phosphate buffer (pH 7.0). P II appeared almost pure by gel filtration on the P-30 column; it had a single absorbance peak, with constant fluorescence polarization across it. P III, however, had two absorbance peaks and a shoulder at the trailing end; there also appeared to be three main components from the fluorescence polarization measurements. The first peak was identified with the overlapping P II fraction; the other two components were labeled P IIIa and P IIIb in the order of their elution from the column. The total protein content in the original P III fraction was represented by 49% of P II, 30% of P IIIa, and 21% of P IIIb.

The approximate percentage by weight, in the total chymotryptic digest, of each of the six main fractions recovered

TABLE II: Molecular Weights, Rotational Relaxation Times, and ρ_h/ρ_0 Ratios of the BSA Fractions.

Fraction	Av Mol Wt	ρ_h (nsec)	ρ_h/ρ_0
P II	35,000 (34,000 ultracentrifuge)	33	1.28
P IIIa	25,000	24	1.26
P IIIb	19,000	13	1.26
P Ia	20,000	23	1.20
P Ib	8,000	6	1.26
P Ic	2,000 or less[a]	—[b]	—[b]

[a] This molecular weight estimate may be somewhat low due to the retardation of basic proteins and peptides on Bio-Gel columns equilibrated with distilled water. [b] P Ic had an average fluorescence polarization of 0.030 ± 0.005, at 25.0° in 0.1 M sodium phosphate buffer. This polarization value, although too low for a meaningful determination of ρ_h, corresponds to small DNS peptides (molecular weight less than 3000). See Figure 3 for the average polarization values of BSA fractions.

TABLE III: Amino Acid Composition of the BSA Fractions per 100 Amino Acids Analyzed.

Amino Acid	BSA[a]	P II	P IIIa	P IIIb	P Ia	P Ib	P Ic
Asp	9.37	10.4	12.8	14.0	8.03	6.88	4.01
Thr	5.66	6.61	5.37	6.62	9.16	8.19	2.51
Ser	4.60	5.28	4.50	4.63	3.09	3.72	5.39
Glu	13.2	13.2	13.7	15.4	13.4	15.4	11.7
Pro	4.95	6.77	7.32	8.44	8.49	8.51	3.63
Gly	2.83	2.19	2.39	4.39	2.44	3.20	4.60
Ala	8.14	7.48	7.81	5.02	8.05	5.94	12.8
$^1/_2$-Cys	6.01	6.75	7.47	9.08	5.47	6.25	0.40
Val	6.18	6.14	3.99	2.39	8.70	8.90	5.56
Met	0.76	0.55	0.65	1.51	1.03	0.82	0.25
Ile	2.30	2.40	2.13	1.24	2.31	1.66	3.75
Leu	10.8	10.7	10.8	10.5	9.36	8.21	11.6
Tyr	3.33	2.65	3.01	1.19	1.22	1.31	8.30
Phe	4.60	3.63	3.40	4.14	4.20	3.40	5.32
His	3.01	2.48	2.75	1.52	2.10	2.83	4.07
Lys	9.90	9.36	9.49	8.35	10.4	10.8	6.69
Arg	3.89	3.34	2.45	1.43	2.52	3.82	9.30
Trp[b]	0.35	0.00	0.00	0.00	0.38	0.00	0.82

[a] Calculated from the data of Peters and Hawn (1967). [b] Determined by the Spies and Chambers (1949) method.

after DEAE-cellulose and Bio-Gel fractionation, is given in Table I.

For the characterization and the purity tests on the six final fractions, the middle tubes of the Bio-Gel column elution peaks were pooled.

Starch gel electrophoresis at pH 7.0 and polyacrylamide disc gel electrophoresis at pH 9.5 indicated that all the fractions, except P IIIb, were somewhat heterogeneous. Each fraction was represented by one main band or by a cluster of bands on polyacrylamide electrophoresis; most of the minor bands were due to contamination by adjacent fractions.

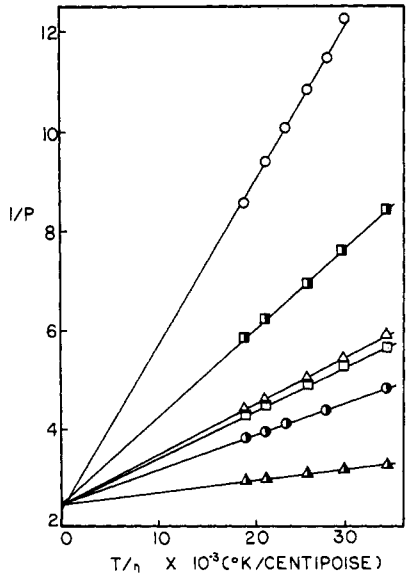


FIGURE 4: Rotational relaxation times of BSA fractions. Plots of $1/p$ *vs.* T/η, at 25.0°, and 0.1 M sodium phosphate buffer (pH 7.0) for: (Δ,Δ) BSA, (◑,◑) P II, (□,□) P IIIa, (△,△) P Ia, (■,■) PIIIb, and (○,○) P Ib.

Molecular Weights and Rotational Relaxation Times of BSA Fractions. The average molecular weights of the BSA fractions are given in Table II. The rotational ralaxation times (ρ_h), also listed in Table II, were calculated from the $1/p$ *vs.* T/η plots shown in Figure 4. All the fractions of BSA-DNS were assumed to have the same fluorescent lifetime (τ) of 12 nsec (Steiner and McAlister, 1957) because their DNS fluorescence emission was very similar to that of BSA–DNS. P Ic was an exception, with the fluorescence wavelength maximum (536 mμ) shifted to the red by about 30 mμ with respect to the other fractions (505 mμ).

In addition to molecular weights and rotational relaxation times, Table II includes the ρ_h/ρ_0 ratio, where ρ_0 is the rotational relaxation time calculated for a spherical particle of the same molecular weight as the BSA fraction. This ratio represents the deviation in shape and/or hydration of the BSA fragment from an unhydrated sphere of equivalent molecular weight. The ρ_h/ρ_0 values shown in Table II (1.28–1.20) correspond to unhydrated prolate ellipsoids of axial ratio 1:2 (Weber, 1953), or, assuming some hydration, to more nearly spherical particles.

Amino Acid Composition of BSA Fractions. Table III gives the per cent amino acid composition of the six BSA fractions. Tryptophan was determined by the Spies and Chambers (1949) method using native BSA, with its two tryptophan residues, as the standard.

A clear indication of the presence of tryptophan in P Ia and P Ic was obtained from the corrected fluorescence spectra of the BSA fractions excited at 275 mμ, where both tyrosine and tryptophan absorb, and at 295 mμ, where only tryptophan

TABLE IV: Acidic, Basic, and Nonpolar Amino Acid Content of BSA Fractions.

Fraction	$A - B$[a]	N[b]
BSA	5.77	32.5
P II	8.42	32.3
P IIIa	11.8	30.7
P IIIb	18.1	27.9
P Ia	6.41	34.7
P Ib	4.83	32.0
P Ic (small peptides)	−4.35	39.0
F1[c]	4.95	
F2	6.09	
F3	6.66	
Type II	11.2	
Type I	4.35	
Phe fragment	6.50	

[a] Per cent acidic residue content (aspartic and glutamic acids) minus per cent basic residue content (arginine, histidine, and lysine). [b] Sum of per cent content of: tryptophan, isoleucine, tyrosine, phenylalanine, proline, leucine, and valine. These seven amino acids are the most hydrophobic residues according to the hydrophobicity scale of Tanford (1962) and Nozaki and Tanford (1963). [c] The lower part of the table gives values calculated from literature data; the definition of the symbols and the references are given in the text.

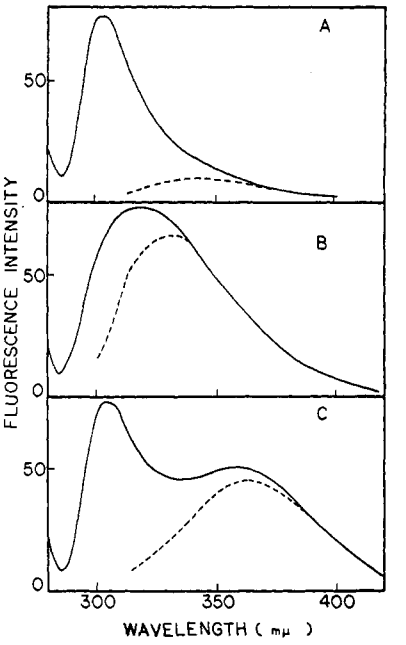


FIGURE 5: Intrinsic fluorescence spectra of BSA fractions excited at 275 mμ (———) and 295 mμ (– – – –). (A) Fluorescence spectra obtained for P II, P IIIa, P IIIb, and P Ib; (B) fluorescence spectra of P Ia; (C) fluorescence spectra corresponding to P Ic (small peptides). Fluorescence intensities are in arbitrary units.

is excited (Figure 5). P Ib, P II, P IIIa, and P IIIb had fluorescence emission spectra typical of tyrosine (wavelength maxima at 303–305 mμ). P Ia had a maximum emission wavelength at 318 mμ, with 70% of the total fluorescence, excited at 275 mμ, contributed by tryptophan. At 275-mμ excitation, P Ic had two emission peaks: 304 and 357 mμ, corresponding to tyrosine and tryptophan, respectively, each contributing nearly 50% to the total fluorescence of the fraction.

A closer examination of the per cent amino acid composition of the BSA fractions (Table III) reveals that the content of acidic and basic amino acid residues varies considerably from fraction to fraction; there are also some differences in the nonpolar amino acid content. The first entry in Table IV, $A - B$, represents the difference between the total per cent acidic residue content (glutamic and aspartic acids, disregarding the small proportion of glutamine and asparagine residues) and the total per cent basic residue content (lysine, histidine, and arginine) for each BSA fraction. The second entry, designated by N is the sum of the per cent content of tryptophan, isoleucine, tyrosine, phenylalanine, proline, leucine, and valine residues for the BSA fractions. Table IV also includes, under the $A - B$ heading, data calculated from the work of Weber and Young (1964a,b) (type I and type II fractions), Pederson and Foster (1969) (F2 and F3 fragments, and a breakdown product of F2, F1), and Peters and Hawn (1967) (*Phe*, carboxyl-terminal fragment).

Fractions P II, P IIIa, and P IIIb have progressively more acidic than basic residues when compared to native BSA. P Ia has a similar $A - B$ value, while P Ib and P Ic (small peptide fraction) are basic with respect to BSA. The small peptide fraction and P IIIb have the most marked differences in $A - B$ and N values from BSA. P Ic is basic and has a higher than average content of nonpolar amino acids, while P IIIb is acidic and has proportionately less nonpolar residues. The other fractions, even though they differ in acidic and basic residue content, are similar in N values, with each other, and with BSA.

pH Dependence of the Fluorescence Polarization of BSA Fractions. Figure 6 shows the polarization of the BSA fractions as a function of pH. Between pH 8 and 5 the polariza-

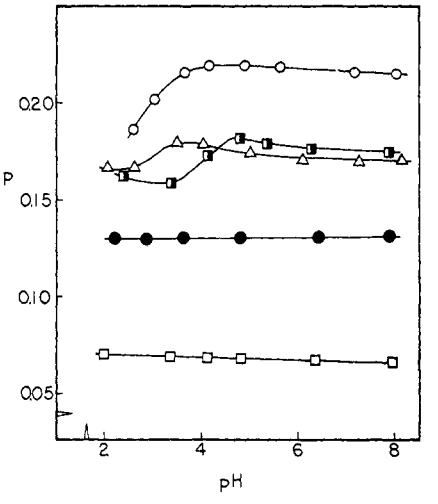


FIGURE 6: pH dependence of the fluorescence polarization of BSA fractions: (○,○) P II, (□,□) P IIIa, (△,△) P Ia, (●,●) P IIIb, and (□,□) P Ib.

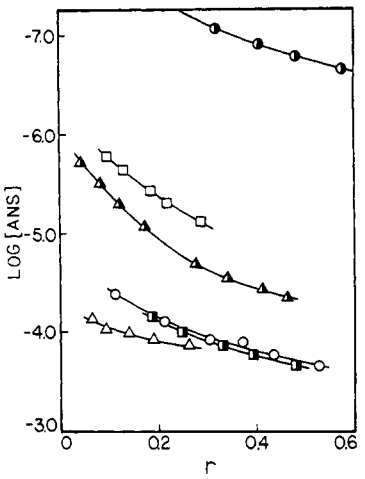


FIGURE 7: Binding curves of ANS by BSA fractions. Log [ANS] *vs.* *r* plots for: (◑,◑) BSA, (△,△) P I, (○,○) P II, (■,■) P III, (▲,▲) digest, and (□,□) digest plus P Ic, in 0.05 M sodium phosphate buffer (pH 7.0).

tion of the fractions is independent of pH. P Ib and P IIIb show no pH dependence down to pH 2. Fractions P II, P Ia, and P IIIa undergo a decrease in fluorescence polarization in the acid pH region, probably as a result of an electrostatic expansion analogous to that of intact BSA at low pH (Harrington *et al.*, 1956). Hence, some conformational flexibility and proteolytic fragmentation can be expected in P II, P Ia, and P IIIa in spite of their high disulfide bridge content.

Digestion of P II. P II was partially citraconylated and then digested with α-chymotrypsin (under very similar conditions to those used for BSA), in order to facilitate the identification of its breakdown products in the total digest of BSA. The digestion was carried out until 3 to 4 amide bonds were hydrolyzed per P II fragment, and the fluorescence polarization reached a constant value ($p = 0.200$). Polyacrylamide disc gel electrophoresis of the digest revealed bands corresponding to P IIIa, P IIIb, and one of the main bands of P Ia.

ANS Binding. Figure 7 shows the binding curves of ANS by the DEAE-cellulose fractions of BSA, at low values of *r*. The isolated fractions have binding affinities about three orders of magnitude lower than intact BSA at the same *r*. The total digest and the digest with excess P Ic fraction, however, show increased affinities, one and one and one-half orders of magnitude higher, respectively, than the individual fractions.

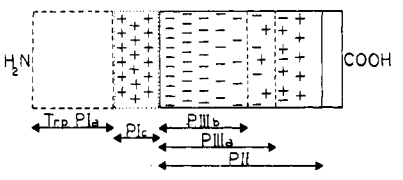
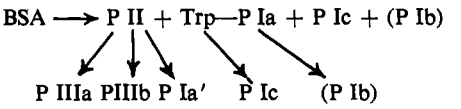


FIGURE 8: Schematic, linear representation of BSA, showing the origin of the main chymotryptic fragments and the regions of positive and negative charge with respect to the net charge of BSA. Heavy contours indicate compact regions, while dashes and dots include regions which are loosely organized in the expanded from of the protein.

Discussion

BSA Structure. The experiments on the molecular weights, amino acid composition of the chymotryptic fractions of BSA, and the fragmentation of P II, suggest that the primary breakdown of BSA by α-chymotrypsin yields P II, the tryptophan-containing component of P Ia (Trp-P Ia), and small peptides. Further degradation, which may occur simultaneously with the formation of the larger fragments, gives P IIIa, P IIIb, and part of P Ia (P Ia′) from the fragmentation of P II, and small peptides and perhaps P Ib from Trp- P Ia. The origin of P Ib is not clear.

To summarize



The relative positions of the chymotryptic BSA fragments in the linear amino acid sequence of the parent protein were determined on the basis of their amino acid composition, in particular the tryptophan and methionine content, and the acidic and basic residue content. Comparisons were made with the linear sequence of Pederson and Foster (1969), which gives the approximate location of the tryptophan and methionine residues in BSA, and with the $A - B$ values of the macromolecular fragments of Weber and Young (1964b), Pederson and Foster (1969), and Peters and Hawn (1967) (Table IV).

P II appears to originate close to the carboxyl end of BSA; however, it does not coincide completely with the F2 fragment of Pederson and Foster (1969). The type II fraction of Weber and Young (1964b), which by Pederson and Foster (1969) is located in the middle of the amino acid chain of BSA, such that one of the tryptophan residues is included, has a high $A - B$ value relative to BSA. P II, which has an $A - B$ intermediate between those of F2 and the type II fragment seems to include parts of both in its amino acid sequence.

The results suggest that P IIIa and P IIIb originate in the same region of P II, near the center of the linear amino acid chain of BSA, in the negative overlap region of P II and the type II fragment.

Trp-P Ia and P Ic originate in the amino-terminal region of BSA containing the two tryptophan residues and two of the four methionines. Trp-P Ia appears to originate near the amino-terminal end, while P Ic seems to arise from the region between Trp P Ia and P II, close to both tryptophans.

Figure 8 shows a schematic, linear model of BSA based on the results obtained with the chymotryptic fractions of BSA.

Almost 60% of the protein, near the carboxyl end, has a compact structure; the amino-terminal region, and in particular the region giving rise to P Ic is more loosely organized, and is consequently more easily digested than the P II region, in the expanded form of BSA. Zones of positive and negative charge, relative to the net charge of BSA, are also indicated in Figure 8.

The general region of BSA where P II, and F2 and the type II fragments are derived from, can be cleaved by proteases at several points, but it is more resistant to digestion than the rest of the protein, and it possibly resembles the globular regions of BSA proposed by Harrington *et al.* (1956) and Foster (1960), and the "core" structure of BSA

suggested by Luzzatti *et al.* (1961). Except for this overlap region of P II and the type II fraction, there are no other similarities between the chymotryptic and pepsin digestion products of BSA. Experiments on the digestion of BSA with trypsin and thermolysin, carried out under the same conditions and to the same final fluorescence polarization as the chymotryptic digestion, gave significantly different elution patterns, from DEAE-cellulose columns, for all three enzymes (Jonas, 1970). It appears, therefore, that the expanded form of BSA has extended regions where proteolytic attack can produce fragmentation that depends on the enzyme employed.

ANS Binding. The ANS binding experiments indicate that the fragments of BSA have lost the highest affinity binding sites of the intact protein. Some of the affinity, however, is regained in the digest, where all the fragments are present, including half of the small peptide fraction. The affinity for ANS is further enhanced by the addition of P Ic to the digest. It appears, that some of the structural characteristics of the strong binding sites of BSA are regenerated by the interaction of the BSA fragments with each other, and in particular with the small peptides.

The information available, at present, about some isolated properties of the strong anion binding sites of serum albumin, parallels in many aspects the findings made in this work on the properties of the small peptide fraction of BSA (P Ic).

It is known that some of the strong sites of serum albumin are located in the vicinity of the tryptophan residues (Herskovits and Laskowski, 1962; Polet and Steinhardt, 1968; Swaney and Klotz, 1970), in a hydrophobic environment (Klotz, 1953; Weber and Laurence, 1954); P Ic includes at least one of the tryptophans and has a high nonpolar residue content. In the specific case of ANS, it has been shown that the binding volume of the first two ligands is small compared to the total volume of BSA (Weber and Young, 1964a); P Ic comprises a small fraction of BSA, at least in terms of weight. Recently, the stronger set of binding sites of SCN^- on BSA has been related with the arginine residues (Pande and McMenamy, 1970). Even though SCN^- is not a hydrophobic ligand of the type of ANS, it is significant that the stronger interaction of the small anion occurs with the arginine rather than with the lysine residues. P Ic has a high arginine content when compared to the rest of the protein.

Thus, the evidence presented in this work suggests that the P Ic region of BSA, with a high local concentration of basic and nonpolar amino acids and without disulfide bridge constraints, is closely associated with the high-affinity anion binding sites of BSA.

References

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

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

Foster, J. F. (1970), *in* The Plasma Proteins, Vol. I, Putnam, F. W., Ed., New York, N. Y., Academic Press, p 179.

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

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

Jonas, A. (1970), Ph.D. Thesis, Univ. of Illinois, Urbana, Ill.

Jonas, A., and Weber, G. (1970), *Biochemistry 9*, 4729.

King, T. P., and Spencer, E. M. (1968), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 27*, 391.

Klotz, I. M. (1953), *Proteins 1*, 727.

Luzzatti, V., Witz, J., and Nicolaieff, A. (1961), *J. Mol. Biol. 3*, 379.

Markus, G., McKlintock, D. K., and Castellani, B. A. (1967), *J. Biol. Chem. 242*, 4395.

Nazarian, G. M. (1968), *Anal. Chem. 40*, 1766.

Nozaki, Y., and Tanford, C. (1963), *J. Biol. Chem. 238*, 4074.

Pande, C. S., and McMenamy, R. H. (1970), *Arch. Biochem. Biophys. 136*, 260.

Pederson, D. M., and Foster, J. F. (1969), *Biochemistry 8*, 2357.

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

Polet, H., and Steinhardt, J. (1968), *Biochemistry 7*, 1348.

Spies, J. R., and Chambers, D. C. (1949), *Anal. Chem. 21*, 1249.

Steiner, R. F., and McAlister, A. J. (1957), *J. Polym. Sci. 24*, 105.

Swaney, J. B., and Klotz, I. M. (1970), *Biochemistry 9*, 2570.

Tanford, C. (1962), *J. Amer. Chem. Soc. 84*, 4240.

Weber, G. (1952), *Biochem. J. 51*, 155.

Weber, G. (1953), *Advan. Protein Chem. 8*, 415.

Weber, G. (1965), *in* Molecular Biophysics, Pullman, B., and Weissbluth, M., Ed., New York, N. Y., Academic Press, p 369.

Weber, G., and Laurence, D. J. R. (1954), *Biochem. J. 56*, 31.

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

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