Limited Subtilisin-Catalyzed Hydrolysis of Bovine Plasma Albumin. Isolation and Partial Characterization of Products*

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ABSTRACT: Subtilisin digestion of bovine plasma albumin at pH 8.9 in the presence of 100 moles of sodium dodecyl sulfate/mole of protein results in rapid cleavage with the liberation of one or more intermediates of molecular weight approximately one-half that of the native substrate. In the present studies the major products of hydrolysis, designated M, H_1, H_2, and T, have been isolated by recycling chromatography on Sephadex G-100 and partially characterized by exclusion chromatography, velocity, and equilibrium sedimentation, disc electrophoresis, and amino acid analysis. Fraction M, constituting about 12% of the initial digest, resembles native albumin, both in terms of physical properties and amino acid composition. Yields of H_1 and H_2 relative to total fragmented substrate are estimated at 40 and 48%, respectively. Molecular weights are estimated on the basis of sedimentation equilibrium and amino acid composition as approximately 38,000 for H_1 and 31,000 for H_2, which sum to approximately the molecular weight of the substrate. Their amino acid compositions are distinctly different, but together they account for virtually all of the amino acid content of the substrate. Fraction T, estimated yield approximately 12%, has a molecular weight of approximately 21,000 and appears to arise from further hydrolysis of either H_1 or H_2. Mixtures of H_1 and H_2 aggregate, in neutral solution, to give a globular unit having the sedimentation velocity of albumin. The results appear to be consistent with a model for the plasma albumin molecule proposed earlier.

Subtilisin-catalyzed hydrolysis of bovine plasma albumin[1] in the presence of 100 moles of SDS/mole of BPA results in the preferential scission of one or more vulnerable peptide bonds near the center of the single polypeptide chain with consequent liberation of hydrodynamically similar fragments of roughly one-half the molecular weight of the substrate (Adkins and Foster, 1965). This initial reaction is approximately 37-fold faster than subsequent hydrolytic reactions. After acid deactivation of the enzyme, SDS was removed by ion exchange. The digest reconstituted in this way exhibits reversible pH-dependent association–dissociation equilibria. The present report will describe the resolution of these fragments in relatively good yield and state of purity by means of recycling chromatography. The isolated fractions have been partially characterized by exclusion chromatography, sedimentation, disc electrophoresis, and amino acid analysis.

Experimental Section

Materials. Bovine plasma albumin (Armour and Co., Lots W69102 and W69204) was maintained at pH 2.4–2.7 and 0–5° for 48 hr and then centrifuged to remove lipid contaminants (Williams and Foster, 1960). The solution was then deionized (Dintzis, 1952) and lyophilized. Ultracentrifugation showed this preparation (preparation I) to contain the usual 5–10% of 6S (probably dimeric) material.

For use in one degradation experiment BPA was further purified by column fractionation in 0.10 M NaCl on Sephadex G-100 gels (Pederson, 1962); the monomer fraction was concentrated 1.9-fold with Sephadex G-25 and used without freeze drying for digestion experiments. BPA prepared in this way (referred to as preparation II) contained no dimer or polymer observable on exclusion chromatography; after freeze drying small but significant amounts are readily detected.

Crystalline bacterial proteinase (BPN′), "Nagarse" (Batch CC G-2424), was prepared by Nagarse and Co., Ltd., Osaka, Japan, from cultures of *Bacillus subtilis* N′ (Okunuki *et al.*, 1956) and is the same preparation used in the previous paper (Adkins and Foster, 1965). It is referred to simply as "subtilisin" in the text. Sodium dodecyl sulfate (Matheson Coleman and Bell, 95%) was recrystallized twice from methanol. Sephadex gels G-25, G-100, and G-200 were obtained from Pharmacia, Uppsala, Sweden. Strongly basic anion-exchange resin, AG 1-X10 (chloride form, 50–100 mesh, 3.0 mequiv/ml of wet resin), was obtained from Bio-Rad Laboratories.

All salts used were reagent grade chemicals. Deionized water (Barnstead Still and Sterilizer Co., mixed-bed deionizing column no. 0808) was used in the preparation of all solutions.

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[1] Abbreviations used: BPA, bovine plasma albumin; SDS, sodium dodecyl sulfate; D, dodecyl sulfate.

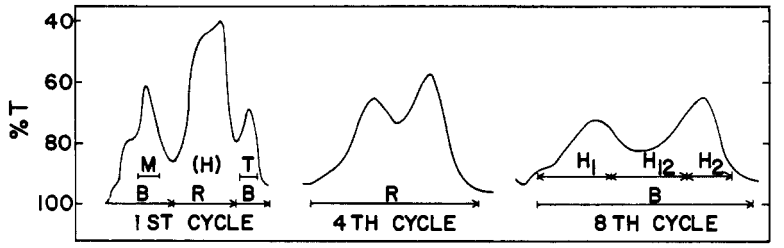


FIGURE 1: Sephadex G-100 recycling chromatography at pH 2.3 of reconstituted pH 8.9 subtilisin digest. Effluent was continuously monitored at approximately 280 mμ. B and R signify, respectively, fractions which were bled from the column or recycled.

Concentration Determinations. BPA concentrations were determined with a Beckman Model DU spectrophotometer at 279 mμ assuming $E^{1\%}_{1cm}$ 6.82. This value is based on the data of Sterman (1955) and on a recent value of 16.43% nitrogen for bovine mercaptalbumin (Spahr and Edsall, 1964). Sterman's (1955) value, $E^{1\%}_{1cm}$ 6.67, is based on Tristam's (1953) value of 16.07% N for BPA.

Enzymatic Hydrolysis. Enzymatic digestion and reconstitution of BPA detergent digests followed the procedures previously stated (Adkins and Foster, 1965). Two digestions (one using BPA preparation I and the other preparation II) were carried out at pH 8.9 in the presence of 0.1 M Cl^- and 105 moles of SDS/mole of BPA and were terminated at the same relative degree of hydrolysis, B = 4.0 (moles of base consumed in the pH-Stat per mole of BPA).

Recycling Chromatography. Recycling chromatography on Sephadex G-100 (Porath and Bennich, 1962) of reconstituted digests was carried out at room temperature using descending flow (~50 ml/hr) on a 4.50 × 117 cm column (bed volume, V_t = 1.86 l.). Each cycle required about 22 hr. Bacterial growth was assumed sufficiently well controlled by the conditions of the experiment: 0.1 M NaCl adjusted with 0.1 M HCl to pH 2.3. pH 2.3 was chosen on the basis of experiments at pH 2.93, 2.17, and 1.67 with a small analytical column. The per cent transmittance of the effluent at 280 mμ was automatically recorded on a Gilson Medical Electronics (GME) ultraviolet monitor. After completion of the first cycle, undesired fractions were removed from the system during each succeeding cycle (*cf.* Figure 1). At the completion of eight cycles it was necessary to bleed the entire column to prevent mixing. Fractions were collected as indicated in Figure 1 with a GME constant-volume fraction collector (~16 ml/tube). Pooled fractions were neutralized and then dialyzed four times with a 20-fold excess of deionized water prior to freeze drying.

Equilibrium Sedimentation. Molecular weights were estimated using the high-speed equilibrium sedimentation technique of Yphantis (1964) and a Spinco Model E ultracentrifuge equipped with Rayleigh interference optics and RTIC temperature control. The Spinco An-D rotor was used at 24,630 rpm and 20.0 ± 0.1°. Sufficient protein solution to form a column of 3.0 ± 0.1 mm was layered over Dow-Corning 550 silicone oil in a three-channel Yphantis-type Epon cell (12 mm) with sapphire windows. Attainment of equilibrium required about 18 hr. Kodak spectrographic plates Type I-D were measured with the Nikon Shadowgraph Model 6C optical microcomparator. Weight-average molecular weights were calculated using the slope of plots of the natural logarithms of the blank-corrected fringe displacements *vs.* the comparator x-coordinate. These plots were linear for fringe displacements greater than approximately one fringe (298 μ). Molecular weights were evaluated at the radial distance corresponding to $(C_m + C_b)/2$, where C_m and C_b are the concentrations in fringe displacements at the meniscus and base of the cell as estimated by extrapolation. Even relatively large errors in C_b do not affect molecular weights significantly for present purposes. Partial specific volumes of isolated albumin fragments were assumed equal to that of BPA, *viz.*, $\bar{V}$ = 0.734 ml/g (Dayhoff *et al.*, 1952).

Sedimentation Velocity. Sedimentation velocities were determined as described in the earlier paper (Adkins and Foster, 1965).

Exclusion Chromatography. The procedures employed for estimation of molecular weights of isolated albumin fragments using Sephadex G-100 gels have been previously stated (Adkins and Foster, 1965).

Disc Electrophoresis. This was performed according to Broome's (1963) modification (20% sucrose and Sephadex G-200 combined with the sample) of the original procedure of Ornstein and Davis (Ornstein, 1964; Davis, 1964). Experiments were performed at room temperature on 7.5% cross-linked polyacrylamide gels as prepared according to instructions supplied by Canal Industrial Corp. A constant current of 5 ma/tube was simultaneously applied to four tubes (3 in. × 5.0 mm i.d.) for 30 or 34 min. The Tris–glycine buffer employed is assumed to maintain the pH of the gels at 9.5. Gels were stained with amido black and densitograms were obtained using the Joyce Loebl and Co., Ltd., Chromoscan. No visible bands were observed in control "blank" runs. Component mobilities, in arbitrary units, were computed from the densitograms as the difference between the absorption maximum and the top of the running gel.

Amino Acid Compositions. Analyses were made

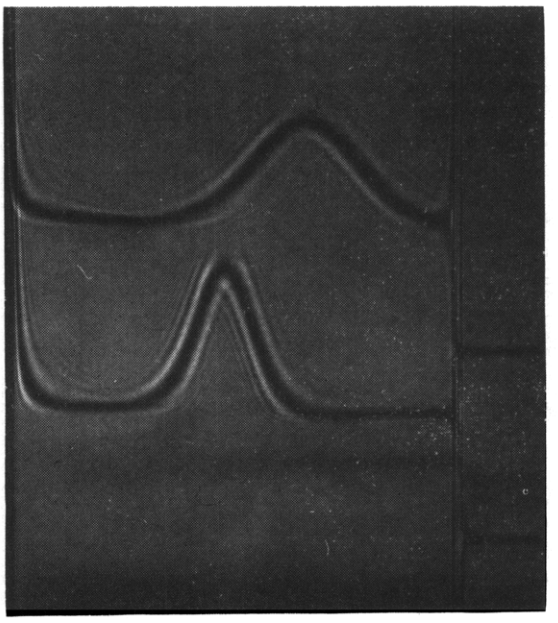
using the Beckman–Spinco Model 120B amino acid analyzer and the procedure of Spackman *et al.* (1958) as modified by Benson and Patterson (1965). Vacuum-dried protein samples (4–7 mg) were placed in Pyrex tubes, dissolved in 3 ml of 6 N HCl, evacuated, frozen in a Dry Ice–acetone bath, sealed, and placed in an oven at 110 ± 1° for 40 hr. The hydrolysate was vacuum dried over solid NaOH at room temperature. The residue was dissolved in 5.00 ml of pH 2.2 citrate buffer. Any insoluble residue was removed by sedimentation and decantation.

Approximate corrections were applied for the hydrolytic destruction of tyrosine (+4%), threonine (+6%), and serine (+13%) using the data on subtilisin BPN′ of Matsubara *et al.* (1965) and linear interpolation between their 20- and 70-hr hydrolysis data. Tryptophan was not determined nor were the two tryptophan residues of BPA (Spahr and Edsall, 1964) included in any computations described herein; the resulting systematic errors should not exceed 0.6%.

Amino acid compositions were estimated by assuming four residues of methionine for BPA (Spahr and Edsall, 1964) and from one to four residues for any of its given hydrolysis products. The number of methionine residues assumed in every case was determined without apparent ambiguity from molecular weights estimated by exclusion chromatography and equilibrium sedimentation. The corrections for hydrolytic destruction described above were then applied to the relevant amino acids. Molecular weights were estimated by summing the products of the nearest integral number of residues and the corresponding residue weight.

Results

Recycling Chromatography. Reconstituted albumin digests were resolved by Sephadex G-100 recycling chromatography into fractions M, H, H_1, H_2, and T as defined in Figure 1.[2] Yields could not be estimated definitively due to loss of material during recycling chromatography. However, the following indicative statements can be made based on approximate correction for effluent loss and on spectrophotometric estimation of concentrations in which the extinction coefficients of all fragments were assumed equal to that of BPA, $E^{1\%}_{1cm}$ 6.82. Over-all approximate recoveries at various stages of the preparative procedure were accordingly as follows: after enzymatic hydrolysis and concentration of the digest by acidic precipitation, 99%; after resin reconstitution, 87%; and after recycling chromatography, 69%. Over-all approximate recoveries of individual fractions resolved by recycling chromatography were: M, 12%; T, 9%; H_1, 15%; H_{12}, 15%; H_2, 18% (total 69%). Relative product recoveries based on the recycling chromatography



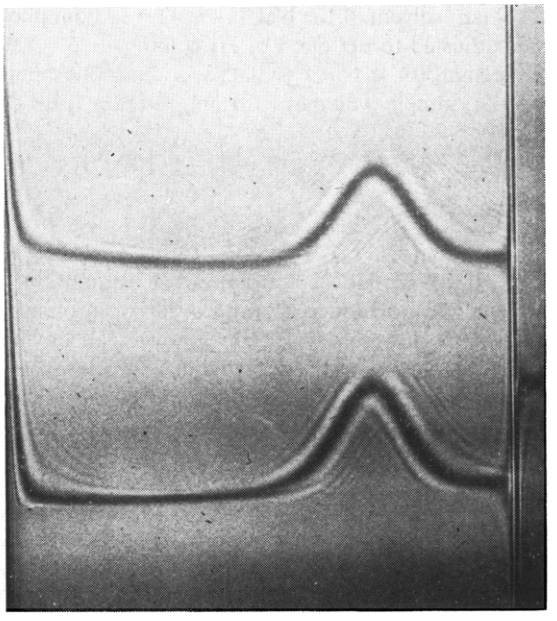


FIGURE 2: Ultracentrifuge schlieren patterns of fractions isolated by recycling chromatography. All patterns were obtained at 59,780 rpm in the presence of 0.10 M NaCl at protein concentrations of 0.5–1.0 g/dl. The direction of sedimentation is from right to left. Upper frame: sedimentation time 96 min; upper pattern fraction T at pH 6.2, 2.34 S; lower pattern, fraction M, pH 6.5, 3.93 S. Lower frame: sedimentation time 121 min; upper pattern, fraction H_2 at pH 2.2, 2.25 S; lower pattern, fraction H_1, pH 2.2, 2.19 S.

[2] The symbol H was derived from the earlier inference (Adkins and Foster, 1965) that these fragments constituted halves or "hemimers" of the protein. This symbolism is perhaps misleading in view of the evidence to be presented that H_1 and H_2 are distinctly different peptides.

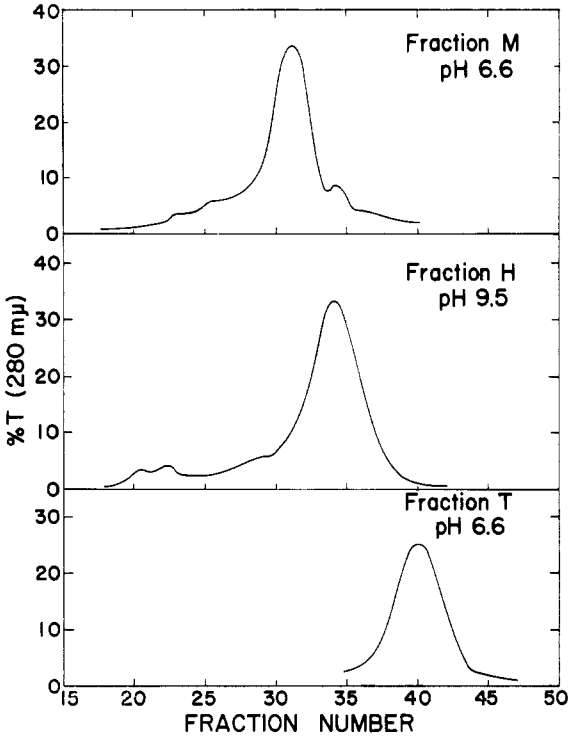


FIGURE 3: Sephadex G-100 exclusion chromatography of fractions M, H, and T. For M and T: column, 1.65 × 114 cm; solvent, 0.1 M NaCl + 0.02 M sodium phosphate adjusted to pH 6.6. For H: column, 1.75 × 116 cm; solvent, 0.1 M NaCl + 0.05 M sodium phosphate, pH 9.5; sample volumes, 1.0 ml, elution rate 15 ml/hr.

TABLE I: Sedimentation Coefficients of Isolated Fractions.[a]

Fraction	pH	C (%)	S_{20} (Svedberg Units)
H_1	6.6	0.87	2.87[b]
H_1	6.5	0.62	2.85
H_1	6.3	0.62	2.82
H_1	2.2	0.6	2.12
H_{12}	2.2	0.6	2.17
H_2	6.6	0.85	3.17[b,c]
H_2	6.2	0.62	3.24[c]
H_2	6.6	0.62	3.15[c]
H_2	2.2	0.6	2.25
$H_1 + H_2$	6.4	0.8	3.98[d]
M	6.5	1.0	3.93
M	6.6	1.0	4.07
M	9.5	1.0	4.01
M	2.2	0.6	3.10
H	6.8	1.0	4.12[d]
T	6.2	1.0	2.34
T	2.2	0.6	1.79

[a] As measured at 20.0° at the single concentration indicated, uncorrected for solvent density, viscosity, or nonideality. The solvent was 0.10 M NaCl unless otherwise indicated. [b] In 0.10 M NaCl plus 0.006 M sodium phosphate. [c] Boundary slightly skewed on high molecular weight side. [d] Boundary slightly skewed on low molecular weight side.

sample load (0.45 g) were: M, 14%; T, 11%; H_1, 17%; H_{12}, 17%; H_2, 22% (total 80%). Resolution of fractions was not noticeably improved by chromatography at the lower pH of 1.67; however, an obvious loss of resolution between H and T fractions was observed at pH 2.93. Use of either BPA preparation I or II appeared to make no difference in the pattern of BPA fragmentation or in relative product yields.

Velocity Sedimentation. Data for fractions isolated by recycling chromatography at pH 2.3 are presented in Table I. Fractions M, H, and T, which correspond to the three major chromatographic peaks of the first cycle, yielded essentially single schlieren peaks though H skewed slightly on the trailing side. The s_{20} values of fractions M and H between pH 6 and 7 are essentially the same as that found for BPA under similar solvent conditions, *i.e.*, 4.11 S. Representative sedimentation patterns for T and M are shown in Figure 2.

An equimolar mixture of H_1 and H_2 sedimented at pH 6.4 yielded results very similar to those obtained with whole fraction H, *i.e.*, a main peak with s approximately 4.0 and having a trailing shoulder. On the other hand, fractions H_1 and H_2 run separately sedimented as essentially single boundaries at pH 6.6, though their sedimentation coefficients (2.87 S for H_1 and 3.17 S for H_2) are different and the H_2 peak is more broad and skewed than the H_1 peak. At pH 2.2 their sedimentation coefficients (2.12 for H_1, 2.17 for H_{12}, and 2.25 S for H_2) are closely similar, and their schlieren patterns are barely distinguishable (Figure 2). In particular the H_2 pattern is considerably more symmetrical than at pH 6.6. At pH 2.2 the sedimentation rate of M was 3.10 S, practically identical with that found for BPA at this pH.

Exclusion Chromatography. Fractions obtained from recycling chromatography were analyzed on Sephadex G-100 columns as a function of pH and concentration to provide estimates of their heterogeneity and apparent molecular weight. Chromatograms of fractions isolated after one cycle of recycling chromatography are presented in Figure 3. It is apparent that fractions M and H contain some aggregated material and that M is contaminated by a significant amount of probably lower molecular weight material. Fraction T appears relatively homogeneous. The apparent molecular weights inferred from the elution volumes are: M, 69,000 at pH 6.6; H, 43,000 at pH 9.5; and T, 31,000 mol wt at pH 6.6.

The effect of sample concentration on the elution behavior of fraction H at pH 6.5 is illustrated in

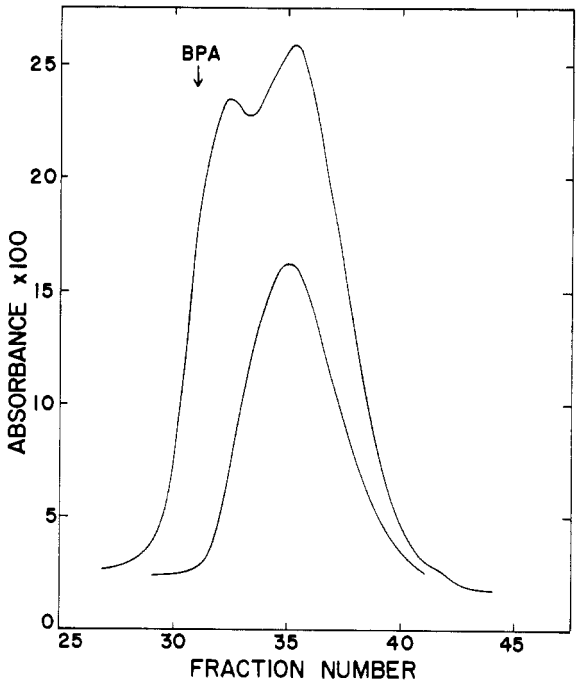


FIGURE 4: Sephadex G-100 chromatography at pH 6.5 of fraction H at two initial sample concentrations. Initial 1.0-ml samples were applied to a 1.75 × 115 cm column, V_t = 276 ml, and eluted (15 ml/hr) with a buffer consisting of 0.1 M NaCl and 0.01 M sodium phosphate. Fraction volumes were 3.55 ml and the elution volume of BPA monomer is indicated by a vertical arrow. Upper curve: C_0 = 1.0%, absorbance read at 279 mμ. Lower curve: C_0 = 0.04%, absorbance read at 220 mμ.

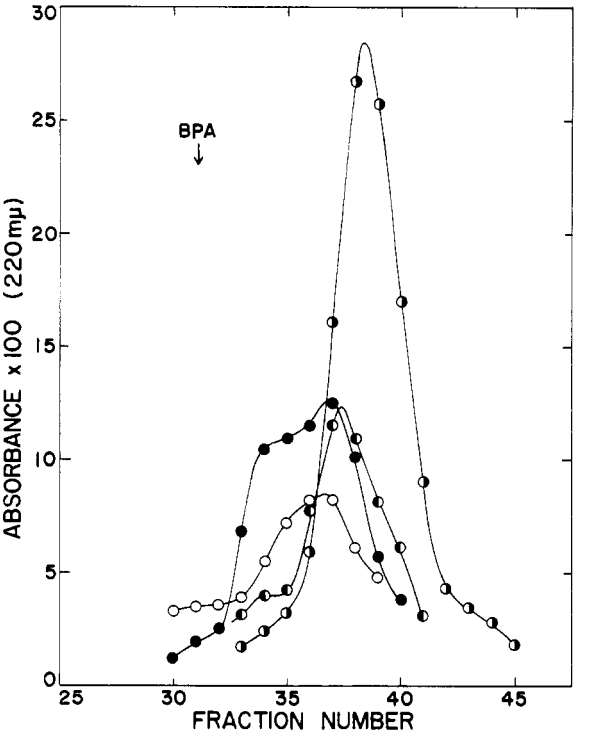


FIGURE 5: Sephadex G-100 chromatography at pH 6.5 of samples H_1, H_{12}, H_2, and an approximate 1:1 mixture of H_1 and H_2. Separations were made on a 1.75 × 115 cm column, V_t = 276 ml, using a buffer consisting of 0.1 M NaCl and 0.01 M sodium phosphate; elution rate 15 ml/hr; volume/fraction = 3.55 ml. Key: ◐ = H_1; ◑ = H_{12}; ○ = H_2; ● = mixture of H_1 + H_2.

Figure 4. With an initial sample concentration of 1.0%, two distinct peaks were observed. When the initial sample concentration was reduced to 0.04% and the effluent absorbance measured at 220 mμ a single, nearly symmetrical peak was observed corresponding to the slower of the two peaks seen at higher concentration.

Shown in Figure 5 are chromatograms determined at pH 6.5 on fractions H_1, H_{12}, H_2, and an approximate 1:1 mixture of H_1 and H_2 at pH 6.5. These samples were removed from the tubes after recycling chromatography (Figure 1). Since the samples were not concentrated (C_0 = 0.01–0.04%), absorbance was determined at 220 mμ. Estimated apparent molecular weights from these data were as follows: H_1, 41,000; H_{12}, 44,000; and H_2, 38,000. The (H_1 + H_2) mixture produced one peak corresponding to 43,000 mol wt and a distinct "shoulder" corresponding roughly to 54,000 mol wt. Essentially symmetrical peaks were observed for H_1 and H_2; the H_{12} peak exhibited only a slightly skewed leading edge instead of the "shoulder" of (H_1 + H_2), though the peak maximum eluted at virtually the same volume. The larger absorbance of H_2 (Figure 5) is the result of a larger sample load.

It is interesting to note that at higher initial sample concentration (1%) the order of elution of H_1 and H_2 was reversed from that of Figure 5. In this case H_2 eluted before H_1 with corresponding apparent molecular weights of 42,000 and 38,000 implying that H_2 has a greater tendency toward aggregation than H_1.

TABLE II: Apparent Equilibrium Sedimentation Molecular Weights for Fractions H_1 and H_2.[a]

Fraction	pH	Concn (g/dl)	$\bar{M}_w$
H_1	2.3	0.05	39,900
		0.03	39,600
		0.01	42,800
H_2	2.1	0.05	33,100
		0.03	32,300
		0.01	35,700

[a] Runs conducted at 24,630 rpm and 20.0 ± 0.1°. The solvent was 0.10 M NaCl adjusted to the indicated pH with 0.10 M HCl. The fractions were from the subtilisin digest of BPA preparation II.

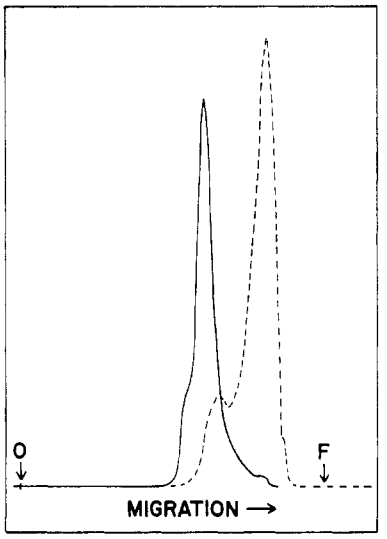


FIGURE 6: Densitograms of polyacrylamide gel disc electrophoresis separations at pH 9.5 of fractions H_1 (dashed curve) and H_2 (solid curve) run separately. Patterns were normalized to the position of the dye front, indicated by F. Conditions of electrophoresis are described in the text under Experimental Section.

Equilibrium Sedimentation. Molecular weights as determined for H_1 and H_2 at approximately pH 2 for three solute concentrations are collected in Table II. No obvious curvature was noted in any of the Yphantis-type plots for fringe displacements exceeding one fringe. The average values of the three molecular weights for H_1 and H_2 given in Table II are 40,800 ± 1400 and 33,700 ± 1300 mol wt, respectively.

Disc Electrophoresis. Tracings of densitograms of disc electrophoretic analyses of fractions H_1 (dotted line) and H_2 (solid line) are given in Figure 6. These results suggest that H_2 is a relatively homogeneous fraction while H_1 contains approximately 15% of a minor, slower component (probably H_2). The mobilities of H_1 and H_2 are distinctly different. A 1:1 mixture by weight of H_1 and H_2 resolved into two peaks whose mobilities corresponded almost exactly to those of H_1 and H_2 (Figure 6). These results indicate interaction between H_1 and H_2 to be minor at pH 9.5 in the buffer employed for electrophoresis. In general the electrophoretic mobilities of the major components under the conditions employed were found to be related qualitatively as follows: $H_1 > M \sim BPA > H_2 >> T$.

Amino Acid Compositions. The results on BPA and BPA fractions T, H_1, H_2, and M are presented in Table III. It is apparent from the compositions of fractions H_1 and H_2 that they constitute distinctly different peptides. The sum of individual residues (H_1 + H_2) in most cases is equivalent to the number determined for BPA. As calculated from the data of Table III the minimal molecular weights of H_1 (38,000) and H_2 (31,000) account for 69,000 mol wt units, in excellent agreement with the value for BPA. The composition of fraction M is clearly very similar to that of BPA. The amino acid distribution in fraction T is substantially different from all other samples analyzed and yielded a minimum molecular weight of 21,000.

The amino acid analyses reported are thought to be internally consistent and sufficiently precise to justify the limited conclusions drawn from them. They are not to be considered highly accurate in an absolute sense, particularly because only a single hydrolysis time was employed and the corrections applied were rather arbitrary. Comparison of the BPA control data with the much more reliable and extensive data of Spahr and Edsall (1964) shows reasonably good agreement for most of the amino acids but substantial discrepancies for a few, for example, glutamic acid.

Discussion

The apparent molecular weights for H_1 and H_2 obtained by sedimentation equilibrium (Table II) are not highly reliable because of the necessity for making the measurements at low pH to repress aggregation. Under such conditions there is doubtless a charge effect which would tend to decrease the apparent values. On the other hand, some aggregation may persist under such conditions so that the apparent values might actually be high. The latter uncertainty applies also to the molecular weight estimates from exclusion chromatography. All things considered the molecular weights estimated from amino acid composition, namely 38,000 for H_1 and 31,000 for H_2, are probably the most reliable. In any event, it seems safe to conclude that the molecular weight of H_1 is somewhat greater than that of H_2 and that the two together sum to very nearly the value for the original BPA.

The apparent molecular weight of M from exclusion chromatography (69,000), its sedimentation velocity (*ca.* 4.0 S at pH 6.5 and 1% concentration), and its amino acid composition (Table III) all point to this fraction being essentially unfragmented substrate. On the other hand, fraction T is of distinctly smaller molecular weight than either H_1 or H_2 as judged by both exclusion chromatography and amino acid composition.

The results show clearly that the tendency toward aggregation, in neutral solution, of mixtures of H_1 and H_2, greatly exceeds the self-aggregation of either H_1 or H_2. The sedimentation velocity of whole fraction H at pH 6.8 is virtually indistinguishable from that of BPA though evidence of some dissociation is seen in the skewed trailing edge of the sedimentation boundary. Exclusion chromatography, while also showing some dissociation, again indicates the presence of a large amount of material of av mol wt *ca.* 64,000 (Figure 4). It can be concluded that the initial cleavage by subtilisin is such that the two large resulting fragments H_1 and H_2 retain a marked tendency to coalesce at neutral pH, presumably in a globular form resembling the unaltered substrate.

Yields of the various fractions are difficult to establish with confidence because of mechanical losses incurred in the recycling chromatography. Assuming

TABLE III: Amino Acid Compositions of BPA and Isolated BPA Fractions.

Amino Acid	Number of Residues Estimated					
	T	H_1	H_2	$H_1 + H_2$	M	BPA
Aspartic acid	16.0	34.9	25.5	60.4	62.2	60.5
Threonine[a]	12.3	14.5	16.5	31.0	32.6	37.5
Serine[a]	10.2	15.0	11.2	26.2	26.3	26.3
Glutamic acid	26.1	48.7	38.4	87.1	89.6	89.7
Proline	10.4	14.9	13.0	27.9	30.5	29.9
Glycine	5.1	11.1	7.1	18.2	18.0	17.6
Alanine	11.0	27.9	20.5	48.4	49.4	52.8
Half-cystine	10.0	16.9	14.1	31.0	31.1	35.5
Valine	14.4	15.3	19.8	35.1	37.1	40.2
Methionine	1	2	2	4	4	4
Isoleucine	3.1	9.8	5.5	15.3	16.2	15.3
Leucine	19.4	37.6	28.8	66.4	68.6	68.1
Tyrosine[a]	7.7	9.2	9.8	19.0	18.0	19.7
Phenylalanine	7.3	15.0	12.8	27.8	27.2	28.6
Lysine	16.9	37.1	30.3	67.4	68.4	66.4
Histidine	4.2	11.8	7.2	19.0	19.2	18.0
Arginine	10.0	13.6	11.2	24.8	25.4	24.7
Total[b]	185.1	335.2	273.7	608.9	623.8	634.8
Calcd mol wt	21,000	38,000	31,000	69,000	71,000	72,000

[a] Empirically corrected for hydrolytic destruction or formation. [b] Excluding tryptophan and ammonia.

that M is composed of unfragmented BPA, that fraction H_{12} consists of equal weights of H_1 and H_2, and that all fractions have the same ultraviolet extinction coefficients at 279 mμ, the yields expressed as a percentage of total fragmented material recovered on recycling chromatography become: H_1, 39%, H_2, 45%, and T, 16%, expressed on the basis of total fragmented material. If it is assumed that the actual extinction coefficients of the various fractions are proportional to their tyrosine contents (Table III), the figures are altered slightly to: H_1, 47%, H_2, 41%, and T, 12%. Assuming the molecular weights of the three fragments to be those deduced from amino acid composition the latter correspond to relative molar yields of 1.0, 1.1, and 0.5 for H_1, H_2, and T, respectively. If it is assumed that the impurity found in H_1 by disc electrophoresis corresponds to 15% contamination by H_2, as suggested above, the weight yields become 40% for H_1 and 48% for H_2 and the relative molar yields for H_1, H_2, and T are modified to 1.0, 1.5, and 0.5.

It seems probable that fraction T results from further hydrolytic degradation of fraction H_1 or H_2 or perhaps both. (The possibility clearly cannot be ruled out that T is a mixture of two or more peptide fragments.) The data on relative molar yields presented in the previous paragraph clearly suggest H_1 as the probable precursor. On the other hand, careful comparison of the amino acid compositions (Table III) of T with, respectively, H_1 and H_2 shows a much closer homology with H_2. The generation of T from either H_1 or H_2 should clearly result in the production of other small fragments. Evidence that the products of the reaction include small quantities of peptides in the molecular weight range 12,000 and lower has been observed by exclusion chromatography (Figure 10 of Adkins and Foster, 1965). It should also be pointed out that small peptide fragments could have been lost in at least three points: (1) in the concentration step which is also the acid-deactivation step, (2) during ion-exchange removal of detergent; and (3) during recycling-chromatography.

The investigation of the enzymatic degradation of BPA described in this and the preceding paper (Adkins and Foster, 1965) was undertaken as a means of testing a model of the albumin molecule derived from physical-chemical investigations. This model (Foster, 1960) pictures the single peptide chain to be folded into two globular units with essentially hydrophobic interior and hydrophilic exterior, the two units being closely associated in neutral solution. The results obtained by subtilisin degradation, together with the closely related results of Weber and Young (1964a,b), appear to be in excellent general accord with and lend credence to this model.

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Primary Structure of the Cytochrome *c* from the Snapping Turtle, *Chelydra Serpentina**

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ABSTRACT: The primary structure of the cytochrome *c* from hearts of the snapping turtle, *Chelydra serpentina*, has been determined from the complete amino acid sequences of peptides isolated from a chymotryptic digest. The positions of these peptides in the over-all sequence were assigned by homology to the structures of the other 15 cytochromes *c* of known structure.

The *C. serpentina* protein bears the characteristics of all "mammalian-type" cytochromes *c* including the clustered distribution of hydrophobic and basic residues, an acetylglycine NH_2-terminal residue, a single polypeptide chain 104 residues long, a heme prosthetic group bound to cysteinyl residues in positions 14 and 17, and the typical invariant sequence Asn-Pro-Lys-Lys-Tyr-Ile-Pro-Gly-Thr-Met at residues 70–80. Position 33 is occupied by an asparaginyl residue, as contrasted to the histidyl residue commonly occurring at this location in other cytochromes *c*. The importance of this substitution is discussed in connection with recent findings concerning the nature of the hemochrome-forming groups in cytochrome *c*.

Following the determination of the complete amino acid sequence of horse heart cytochrome *c* (Margoliash and Smith, 1961; Kreil and Tuppy, 1961; Margoliash *et al.*, 1961, 1962; Margoliash and Smith, 1962; Margoliash, 1962a; Tuppy and Kreil, 1962), the primary structures of the cytochromes *c* from a number of different species have been established through the efforts of several groups of investigators. To date, in addition to the horse heart protein, the cytochromes *c* of known structure include those from man (Matsubara and Smith, 1963), pig (Stewart and Margoliash, 1965), cow (Yasunobu *et al.*, 1963), chicken (Chan and Margoliash, 1966a), tuna (Kreil, 1963, 1965), a moth, *Samia cynthia* (Chan and Margoliash, 1966b), baker's yeast (Narita *et al.*, 1963), a rhesus monkey (Rothfus and Smith, 1965), the dog (McDowall and Smith, 1965), the rattlesnake (Bahl and Smith, 1965), the mold *Neurospora crassa* (Heller and Smith, 1965), the rabbit (Needleman and Margoliash, 1966), the great grey kangaroo, *Macropus canguru* (Nolan

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