Subtilisin-catalyzed Hydrolysis of Bovine Plasma Albumin: Implications with Respect to a Postulated Subunit Model*

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ABSTRACT: The limited subtilisin-catalyzed hydrolysis of bovine plasma albumin (BPA) and of BPA in the presence of 12 or 100 moles of sodium dodecylsulfate (SDS) has been investigated in the *p*H-stat and by sedimentation, electrophoretic, and chromatographic analyses of the hydrolysates. Subtilisin hydrolyzes BPA at *p*H 7.53 with the formation of relatively small (less than 0.6 S) fragments; at *p*H 9.30, 1.8 S, and 1.1 S, fragments are formed but in relatively low yields. Twelve moles of SDS per mole BPA retard hydrolysis of the protein-detergent complex, and only 0.7 S or smaller fragments are observed in the ultracentrifuge. In the presence of 100 moles SDS per mole BPA, subtilisin hydrolysis at *p*H 8.90 apparently proceeds in two well-delineated first-order kinetic steps. The initial reaction is 37-fold faster than the secondary reaction, involves the release of only 1–2 moles of hydrogen ion per mole of albumin, and results in cleavage of the albumin-detergent complex into hydrodynamically similar fragments of approximately one-half molecular weight. The secondary reaction corresponds to the slower hydrolysis of these fragments.

Removal of detergent after completion of the primary reaction yields a system whose state of aggregation is *p*H dependent: an aggregate with sedimentation properties resembling native BPA exists near neutral *p*H, while below *p*H 3 and at *p*H 9.3 the aggregate is dissociated into fragments having hydrodynamic properties consistent with a molecular weight of approximately 34,000.

Plasma albumin, according to a large body of evidence, consists of a single polypeptide chain of 65,000–69,000 mw. Foster (1960) has proposed a model for the albumin molecule, incorporating the earlier proposals of Harrington *et al.* (1956), which is designed to account for the N-F acid transformation (Aoki and Foster, 1956, 1957) and its relationship to the titration anomaly, the altered solubility behavior of the low *p*H form, and the cooperative binding of detergent ions to form AD_{12}, AD_n, and AD_{2n} complexes (Putnam and Neurath, 1945; Aoki, 1958) where n is approximately 50. In general, the model proposes four similar compact regions of the molecule connected by relatively short segments of the peptide chain. The research to be described attempts to partially test the model by means of preferential enzymatic cleavage of these connecting peptide chains. Subtilisin was chosen for the hydrolysis because its broad specificity (Harris and Roos, 1959) would enhance the probability of severing these peptide linkages.

Tryptic fragments from human plasma albumin with mw of 12,000–30,000, and which carry part of the antigenic structure, have been separated by column agar electrophoresis (Lapresle *et al.*, 1959). Porter (1957) isolated an immunologically active fragment of 12,000 mw from chymotryptic digests of BPA.[1] Richard and Kegeles (1959) obtained a fragment of about 19,000 mw which amounted to about 10% of the total chymotryptic hydrolysate of BPA. Press and Porter (1962) isolated from chymotryptic hydrolysates of human plasma albumin three fragments which contain some of the antigenic sites of the whole molecule. Their molecular weights were estimated to be 23,400 (Ia), 19,000 (Ib), and 7100 (II.2). The amino acid composition of fragment II.2 was found to contrast sharply with the composition of the whole molecule, and its antigenic site(s) is also a part of the antigenic structure of the two larger fragments. A fourth fragment (Ic), also antigenically active, was separated on Sephadex G-75 gel but was not characterized; from its elution volume it is presumably larger than Ia.

Limited peptic hydrolysis of equine albumin at *p*H 2.1 yielded two electrophoretic components at *p*H 8.0 (Holiday, 1939). At *p*H values from 1 to 4.5, pepsin cleaves the antigenic structure of human plasma albumin into three constituents (Kaminsky and Tanner, 1959). The recent research of Weber and Young (1964a,b) has shown that pepsin digestion at *p*H 3 results in the rapid cleavage of BPA into two or three Type I fragments of 12,500 mw and one Type II fragment of approximately 30,000 mw. These authors state that

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[1] Abbreviations used in this work: BPA, bovine plasma albumin; SDS, sodium dodecylsulfate.

their results are in complete agreement with the albumin model proposed by Foster (1960).

Experimental

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

Crystalline bacterial proteinase (referred to as "subtilisin" in the text) was obtained from Nagarse Co., Japan (batch CC G-2424). Ribonuclease (crystalline, salt free, lot R-567) was obtained from Worthington Biochemical Corp. Sodium dodecylsulfate (SDS) (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 meq/ml 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.

Concentration Determinations. BPA concentrations were determined with a Beckman Model DU spectrophotometer assuming $E^{1\%}_{1\,cm}$ to be 6.67 at 279 mμ (Sterman, 1955). In all calculations requiring a molecular weight for BPA, 69,000 was assumed.

Enzymatic hydrolysis of BPA with *p*H maintained constant to ±0.005 *p*H unit was performed (Ottesen, 1958) under nitrogen at 23.00 ± 0.05°, using a Radiometer automatic recording *p*H-stat consisting of a TTT-1b titrator and a type SBR2c/SBU1 titrigraph. The *p*H was continuously monitored with a Leeds and Northrup recorder during each experiment as a precautionary check on the constancy of *p*H. To a filtered solution of about 1% (g/dl) BPA in 0.10 M NaCl was added, when desired, sufficient SDS to obtain any desired molar-mixing ratio (m) which defines any given albumin-detergent system, AD_m, and determines the stoichiometry of the particular protein-detergent complex or complexes which will exist in the given system. Available literature on the SDS-BPA interaction (Strauss and Strauss, 1958; Foster and Aoki, 1958) justifies the assumption that 90% or more of the detergent in the AD_{100} system is bound. References in the text to specific albumin-detergent complexes should be interpreted in this light.

After adjustment to the desired *p*H and equilibration of the system, subtilisin (approximately 0.01 the weight of BPA and AD_{12} and 0.0002 in the case of AD_{100}) was added to initiate the hydrolysis. Hydrolysis was allowed to proceed to any desired extent, as indexed by the amount of base (B) per mole BPA which was required for the maintenance of constant *p*H, and was terminated by irreversible subtilisin deactivation (within about 5 minutes) at *p*H 2.1. Longer deactivation times were required at higher *p*H.

In the case of experiments with AD_{100}, about 99% of the hydrolyzed protein-detergent complex precipitates during acid deactivation of the subtilisin, allowing convenient concentration of the protein by centrifugation (11,000 rpm for 15 minutes). The protein-detergent precipitate dissolves completely at *p*H 6 or above.

Reconstitution of the protein-detergent digests by removal of detergent was accomplished by passage of the digest through a small column of AG1-X10 anion-exchange resin (chloride form, 300-fold excess). Barium ion has also been used for removal of detergent from albumin (Putnam and Neurath, 1943). However, the resin procedure is more convenient and probably affords more complete detergent removal since barium dodecylsulfate precipitate will dissolve in the presence of AG1-X10.

Sedimentation experiments were performed at 20.0° in a Spinco Model E ultracentrifuge equipped with a schlieren optical system including a Wolter phase plate and with the R.T.I.C. temperature control. Photographic plates were magnified by optical projection for analysis.

In sedimentation velocity experiments samples were centrifuged at 59,780 rpm in standard 4° sector-shaped Kel-F cells. Sedimentation coefficients were calculated from the position of the maximum ordinate of the sedimenting boundary. When boundaries were multiple, estimates of the sedimentation coefficients of ill-defined minor boundaries were made by arbitrarily forcing the log x versus time plot for minor components through the extrapolated intercept of the major boundary with the meniscus position. This procedure consistently yielded values that were plausible, whereas, if the slopes were determined solely from a few relatively imprecise points obtainable during a short period of the experiment, the calculated S values were often unreasonable or impossible. Sedimentation coefficients as recorded have not been corrected for solvent density or viscosity.

Relative areas under partially resolved schlieren patterns of paucidisperse systems have been estimated by simple visual inspection of the photographic plates. These quantities are subjective, but are sufficiently quantitative for the purpose intended, which is to give the reader an approximate idea of the percentage of material associated with a given sedimentation coefficient.

Prior to molecular weight determinations, samples were transferred to a solvent of known composition on Sephadex G-25 columns and were also checked at higher concentration for apparent homogeneity by sedimentation velocity experiments.

The partial specific volume $\bar{V}$ was assumed to be 0.734 ml/g (Dayhoff *et al.*, 1952) for native BPA and for reconstituted BPA digests. For the one case in which a molecular weight was determined on an unreconstituted digest, $\bar{V}$ for unhydrolyzed AD_{105} complex was used; it was estimated to be 0.773 ml/g by linear interpolation between $\bar{V}$ = 0.734 for BPA and $\bar{V}$ = 0.87 for

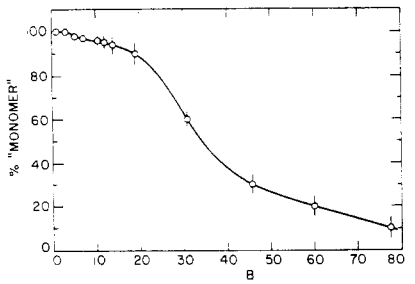

FIGURE 1: Relative concentration (%) of subtilisin digests (*p*H 7.53) of BPA sedimenting as "monomer" at *p*H 7.53 in 0.1 M KCl. The total protein concentrations are only approximately equal (*ca.* 0.8%).

SDS (Miller and Anderson, 1942), assuming complete binding of the dodecylsulfate anion (formula weight 265.4).

Molecular weights were determined by two approach-to-equilibrium methods: Ehrenberg's (1957) method was employed at 20,410 rpm. The method of Klainer and Kegeles (1956), which yields the weight-average molecular weight for nonideal solutions of polydisperse solutes (Kegeles *et al.*, 1957) as well as for chemically reacting systems (Kegeles and Rao, 1958), was used at 12,500 rpm to obtain molecular weights at the air-liquid meniscus. Photographs were chosen for analysis according to the criteria of Weston and Billmeyer (1963): identification of a linear region in the boundary pattern near the meniscus and absence of apparent evidence of convection. Linear extrapolation to the meniscus was also employed (Peterson and Mazo, 1961).

One molecular weight determination was performed by sedimentation equilibrium at 12,590 rpm employing a 3.92-mm solution column. Dow-Corning silicone oil 550 was used to form a solution-oil interface at the bottom of the cell. Average molecular weights were obtained at various r values from the slopes of tangents to plots of log $(1/r)(dc/dr)$ versus r^2 (Lamm, 1929; Ginsburg *et al.*, 1956):

$$\bar{M}_{zr} = (\text{slope})_r \times 2RT/(1 - \bar{V}\rho)\omega^2 \qquad (1)$$

For an ideal monodisperse solute these plots should be linear. For polydisperse systems, equation (1) yields the Z-average molecular weight at the point, r, of evaluation. In the determination of the best line, points within 0.3 mm of the ends of the column of solution were not used. In addition, the weight-average molecular weight for the entire sample was calculated from:

$$\bar{M}_w = 2RT(C_b - C_m)/C_o(1 - \bar{V}\rho)\omega^2(r_b^2 - r_m^2) \qquad (2)$$

Molecular weights were also determined at 59,780 rpm using a double-sector synthetic-boundary cell (filled Epon) by the Van Holde (1960) modification of Fujita's (1959) method for the calculation of diffusion coefficients from boundary spreading in the ultracentrifuge.

Fractionation of BPA on Sephadex G-200 gel was carried out by the method of Pedersen (1962) in order to obtain a sample of pure BPA monomer for sedimentation molecular weight determinations. The sample was checked for apparent heterogeneity by sedimentation velocity and ostensibly exhibited a perfectly symmetrical boundary. The apparent molecular weight was then determined by the Klainer and Kegeles (1956) method (*p*H 2.17, 0.10 M NaCl, 0.70% BPA).

Exclusion Chromatography. Molecular weights of reconstituted digests were estimated on Sephadex G-100 gel (Whitaker, 1963; Andrews, 1964) as a function of *p*H. Samples of approximately 1.0 ml of digest, normally containing approximately 1% protein, were passed through a 1.65 × 113.5-cm (bed volume, V_t, 243 ml) column previously equilibrated at the desired *p*H. Approximately 3-ml fractions were collected with a Gilson Medical Electronics constant-volume fraction collector. The absorbancies of effluent fractions were individually determined at 279 mμ, or the effluent optical density at 280 mμ was automatically monitored and recorded to locate the maximum absorbancy. When one monitoring technique was used to calibrate the column, the same method was also used for the molecular weight estimation. The column was calibrated at *p*H 2.17 and 6.48 with RNAase and BPA just prior to molecular weight estimations on the digested samples under the same conditions of *p*H and ionic strength. The relative elution volume, V_e/V_t, was assumed to be a linear function of the logarithm of molecular weight (Whitaker, 1963).

In order to determine the effect of concentration on the apparent molecular weights, one BPA digest was chromatographed using a starting protein concentration of 0.03%; effluent absorbance was determined at 220 mμ using a Beckman DU spectrophotometer.

The heterogeneity of one reconstituted subtilisin hydrolysate as a function of *p*H within the range 1.67–6.48 was also investigated by exclusion chromatography on Sephadex G-100 gel in 0.10 M NaCl.

Results

Hydrolysis of Native BPA. The results of several sedimentation velocity experiments (at *p*H 7.53 in 0.10 M KCl) on subtilisin hydrolysates of BPA are shown in Figure 1; the percentage of the hydrolysate sedimenting as "monomer" is shown as a function of the index of degradation, B. At the *p*H of degradation, 7.53, the number of peptide bonds actually hydrolyzed is probably significantly larger than B. At $B = 3$, the dimer originally present (about 7%) is not detectable in the ultracentrifuge and is apparently preferentially attacked by subtilisin. A relatively large number of peptide bonds must be hydrolyzed before an appreciable amount of "monomer" is fragmented; about 90% of "monomer" remains at $B = 20$. Not until approximately $B = 35$ is 60% of the "monomer" fragmented. About 10% "monomer" continues to persist in the digests at $B = 75$; the remainder of the digest consisted of 0.2–0.6 S fragments. Intermediates of larger size

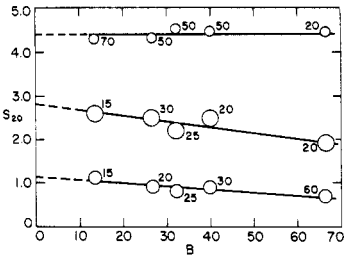

FIGURE 2: Sedimentation coefficients (Svedberg units) and corresponding relative component concentrations in subtilisin digests (*p*H 9.30) of BPA at *p*H 5.32 ± 0.05 in 0.1 M KCl. The total protein concentrations are only approximately equal (*ca.* 0.08%).

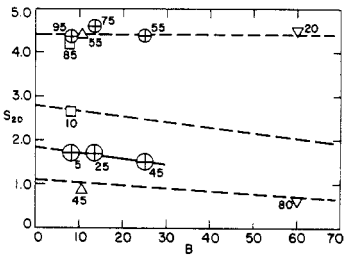

FIGURE 3: Sedimentation coefficients (Svedberg units) and corresponding relative component concentrations in solutions of BPA digested at various *p*H values. Sedimentation was at *p*H 5.31 ± 0.08 in 0.1 M KCl. The total protein concentrations are only approximately equal. Key: △, *p*H 6.58; ▽, *p*H 7.53; ⊕, *p*H 8.00; □, *p*H 10.0; - - - -, *p*H 9.30 (from Figure 2).

were detectable earlier in the degradation but not in high concentration at any degree of degradation.

One sedimentation velocity experiment was conducted to determine whether a significant fraction of the "monomer" component present after hydrolysis at *p*H 7.53 to $B = 31$ could be dissociated at *p*H 2.67. A sedimentation coefficient of 3.0 S was obtained, which corresponds well with the value of 3.11 S found for BPA at *p*H 2.94 in 0.10 M NaCl.

Estimates of the average molecular weight of BPA, digested to $B = 5$ at *p*H 9.3, by Sephadex G-100 exclusion chromatography at *p*H 2.1 and 9.3 (0.1 ionic strength) yielded elution volumes essentially identical to those of the undigested BPA monomer under the same conditions. The faster peaks normally associated with aggregates in undigested BPA solutions were not observed, whereas small amounts of material of lower molecular weight than BPA were detectable.

Shown in Figure 2 are the relative percentages of components produced by subtilisin digestion of BPA at *p*H 9.30 and their respective sedimentation coefficients as a function of *B*. The systems consist of three major components: one component sediments at about the same rate as BPA monomer and the other two have

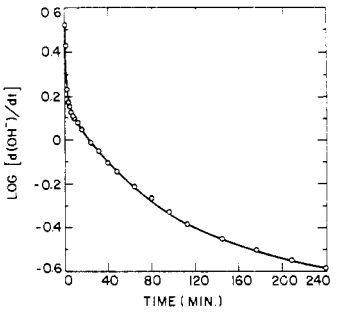

FIGURE 4: Logarithm of the rate of base uptake versus time for the subtilisin-catalyzed hydrolysis of BPA at *p*H 9.30.

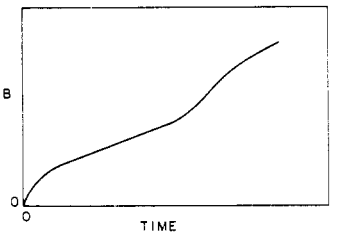

FIGURE 5: Schematic of *p*H-stat curves of subtilisin digestion of AD_{12} at various *p*H values over the range 7.45–9.30.

sedimentation coefficients (extrapolated to $B = 0$) of 2.8 and 1.1 S. All three components persist beyond $B = 67$. The relative concentration of monomer decreases montonically, while the 1.1 S fragment likewise increases. The concentration of the intermediate 2.8 S fragment, however, apparently passes through a maximum. Clearly, large intermediates are formed during the course of the degradation at *p*H 9.30.

The effects of variation of the *p*H of degradation on the size and relative abundance of the larger intermediates produced within the BPA-subtilisin system are shown as a function of *B* in Figure 3. Degradation at *p*H 8.00 results in the appearance of a 1.8–1.9 S boundary (at $B = 0$) which corresponds to a component intermediate in size to those formed at *p*H 9.30. Degradation at *p*H 7.53 and 6.58 resulted in the essentially exclusive formation of fragments with sedimentation coefficients less than 1 S.

Attempts by various methods to analyze quantitatively the titrimetrically observed kinetics of subtilisin hydrolysis of BPA at *p*H 9.30 failed. A plot of the logarithm of the rate of base uptake versus time for such an experiment is shown in Figure 4. The rates at various times were obtained from the slopes of tangents to the recorded *p*H-stat curve. As is readily observed, the reaction is kinetically complex.

Hydrolysis of BPA-Detergent Complexes. The general time course of the subtilisin degradation of the AD_{12} system at various *p*H-values is shown schematically in

TABLE I: Sedimentation Velocity and Approximate Relative Concentrations of Components in Subtilisin Digests of AD_{12} under Various Conditions.

Degradation *p*H	*B*	Sedimentation *p*H	Ionic Strength	Remarks	s_{20} (%)[a]
9.30	5.0	5.37	0.09		M_2 (+), 4.3 (98), ? (2)
9.30	4.1	2.19	0.08		3.1 (98), ? (2)
9.30	47	5.31	0.09		4.27 (65), 0.40 (35)
9.50	2.8	8.5	0.08	[b]	M_2 (0), 4.23 (90), ? (10)
9.50	2.8	8.5	0.1	[c]	1.12 (100)
	0	8.33	0.1	[d]	1.42 (30), 1.17 (70)
8.00	80	7	0.08	[e]	4.47 (40), 2.8 (60)
7.45	46	7	[f]	[e]	4.35 (55), 2.58 (45)
9.30	65	7.5	[f]	[e]	2.43 (98)

[a] M_2 (+), component sedimenting analogously to dimer. [b] In 0.029 M thioglycolic acid (385 moles thioglycolic acid/mole albumin). [c] In 0.029 M thioglycolic acid and 7.0 M urea. [d] Nondegraded control sample: 0.50 g/dl BPA, 0.035 M thioglycolic acid, 7.0 M urea. [e] Redissolved precipitates isolated from AD_{12} digests. [f] Ionic strength low but nonzero.

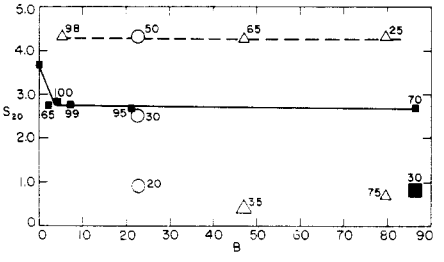

FIGURE 6: Sedimentation velocity and approximate relative concentration of components (%) in subtilisin digests (*p*H 8.90) sedimented at *p*H 5.2 in 0.10 M NaCl. Key: O, no detergent; △, AD_{12}; ■, AD_{100}.

Figure 5. The most striking features of subtilisin hydrolysis in these cases are: a rapid initial uptake of 1–2 moles of base per mole protein in a nonzero-order reaction, and a zero-order reaction segment involving 3–8 moles of base which is followed by a reaction segment in which the rate increases and then decreases. Relevant sedimentation data are collected in Table I.

A few of the sedimentation velocity experiments on the AD_{12} system are plotted in Figure 6. Comparison of these data with sedimentation velocity experiments on BPA hydrolysates produced at *p*H 8.00 and 9.30 (Figures 3 and 2, respectively) suggests that the AD_{12} complex suffers greater hydrolysis before collapse than does BPA. No intermediates with sedimentation coefficients larger than 0.7 S were observed in any of the experiments.

In recognition of the possibility that hydrolyzed BPA might not dissociate into large fragments even though the necessary bond or bonds had been cleaved, unsuccessful attempts were made to dissociate the hydrolyzed but apparently intact complex by various methods (Table I). The sedimentation coefficient obtained for the *p*H 9.30 digest of AD_{12} at *p*H 2.19 was 3.1 S, which is about the same as that of BPA under similar conditions. Thioglycolic acid (0.029 M; 385 moles thioglycolic acid/mole protein) also failed to cause a reduction in the sedimentation coefficient of the major component of an AD_{12} digest (*p*H = 9.5, *B* = 2.8) below that expected of unhydrolyzed AD_{12}, namely, about 4.3 S. Employment of the even more strenuous conditions of 0.029 M thioglycolic acid in 7.0 M urea also apparently failed to cause further fragmentation. The sedimentation coefficient of 1.12 S compares favorably with the sedimentation coefficient of the 1.17 S component of undigested AD_{12}. The 1.42 S component is presumably dimer. McKenzie *et al.* (1963) report a sedimentation coefficient of 1.01 S for BPA in 7 M urea and 0.02 M cysteine at *p*H 9.0 and 25°.

In the course of these experiments white precipitates were observed to form in AD_{12} hydrolysates which had been stored at about *p*H 5.3 and 0–5° for a few hours. In three cases these precipitates were isolated, redissolved by addition of dilute base to *p*H 7, and examined in the ultracentrifuge. The results are presented in Table I. In all three sedimentation velocity experiments relatively large quantities of 2.4–2.8 S material were observed. In two of these experiments relatively substantial amounts of 4.3–4.8 S material were observed.

Subtilisin degradation experiments were performed on the AD_{100} system at *p*H 8.90 to determine the effect of this increased amount of detergent on the kinetics and products of hydrolysis. The kinetics of *p*H 8.90 subtilisin hydrolysis of the AD_{100} system has been investigated by graphical analysis, similar to that of Leonis (1948), of the *p*H-stat curve and as presented in Figure 7. The rates of base uptake at various times were obtained from the slopes of tangents to the *p*H-

stat curve and their logarithms were plotted versus time.

The experimental curve so obtained can be factored reasonably well into a fast and slow reaction by assuming that two classes of peptide bonds, A and B, are undergoing independent, parallel, first-order hydrolysis at rates k_1 and k_2. The rate of base uptake for the overall reaction is then given by the sum of the hydrogen ion released by two reactions:

$$\begin{aligned} A &\xrightarrow{k_1} H^+ + \ldots \\ B &\xrightarrow{k_2} H^+ + \ldots \end{aligned} \qquad (3)$$

from which

$$\log(-d(A + B)/dt) = \log(k_1 A_0 e^{-k_1 t} + k_2 B_0 e^{-k_2 t}) \qquad (4)$$

If k_1 is sufficiently larger than k_2, A will have effectively disappeared after some time. The rate constant, k_2, and the constant B_0 were calculated from the slope and intercept of the linear extrapolation of the slower reaction. These parameters were used to calculate the rate of base uptake due to the fast reaction. A plot of the logarithm of this quantity versus time is also shown in Figure 7 and is seen to be essentially linear. The parameters k_1 and A_0 were calculated for the fast reaction as before.

The values obtained for the four kinetic parameters for the two reactions are: $k_1 = 68 \times 10^{-3}$ min^{-1}; $A_0 = 2.1$ moles of OH$^-$/mole BPA; $k_2 = 1.8 \times 10^{-3}$ min^{-1}; $B_0 = 23$ moles OH$^-$/mole BPA. These calculated parameters, when substituted into equation (4), lead to the calculated curve of Figure 7. As can be seen, the simple kinetic model assumed satisfies the experimental points quite well.

Sedimentation velocity experiments on subtilisin hydrolysates of the AD_{100} system are summarized in Figure 6. The unhydrolyzed AD_{100} complex yielded a sedimentation coefficient of 3.67 S at *p*H 5.24 in 0.10 M NaCl. It is apparent (Figure 6) that a sharp reduction of the sedimentation coefficient has been effected for approximately 65% of the complex at $B = 2.0$ and for the *entire* apparently homogeneous digest prior to $B = 4.6$. Thus the reduction of the sedimentation coefficient from 3.7 S to about 2.8 S correlates extremely well with the fast kinetic reaction which is estimated to be 99% complete at $B = 3.0$. The slower reaction must correspond primarily to the hydrolysis of the 2.8 S fragments.

The sedimentation velocity patterns of unreconstituted AD_{100} digests appeared sufficiently homogeneous (Figure 8a) to invite determination of the average molecular weight of the digest without further purification. The molecular weight of such a digest (*p*H 8.90, $B = 4.0$) was found at *p*H 6.7 to be 47,900 by the method of Van Holde (1960) (in presence of NaCl, ionic strength 0.10, $c = 0.45\%$). In the computation, the doubled area under the trailing half of the sedimentation pattern was used to minimize the effects of possible higher molecular weight components. Other computed values were: zero-time correction, $T(0) =$ 506 seconds; sedimentation coefficient, $s_{20} = 2.50$; concentration dependence parameter, $k = 0.030$ (dl/g); sedimentation coefficient corrected to zero concentration = 2.58, and the diffusion coefficient, $D = 5.81 \times 10^{-7}$ cm^2/sec. The approximate molecular weight of the AD_{105} complex would be about 97,000 if all of the dodecylsulfate anion is assumed bound.

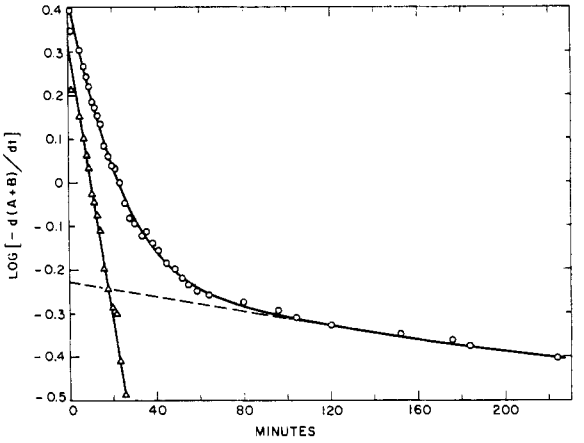

FIGURE 7: Logarithm of the rate of base uptake versus time for the subtilisin-catalyzed hydrolysis at *p*H 8.90 of AD_{100}. Key: O, experimental points; △, points calculated for fast reaction.

Sedimentation velocity experiments on resin-reconstituted AD_{100} digests at various *p*H values suggest a *p*H-dependent equilibrium to exist between the fragments. As can be seen from Table II the sedimentation coefficients and approximate relative concentrations (%) of the various species vary with sedimentation *p*H, *B*, and to some extent with ionic strength and concentration. From the data for $B = 4.0$ or greater, it is apparent that within the *p*H range 3.1–7.4 the observed boundaries are complex or multiple. The relative quantity of component sedimenting at a rate corresponding to BPA monomer becomes maximal within the *p*H range 6.4–7.1. The three experiments within this *p*H range imply that association is favored by low ionic strength. At *p*H values more alkaline than 8.1 and more acid than 3.0, the sedimentation boundaries are nearly symmetrical. The sedimentation patterns of three such typical digests are shown at seven *p*H values in Figure 8.

According to the previously presented sedimentation and kinetic analyses (Figures 6 and 7), the transformation of BPA into fragments is not complete until approximately $B = 3.0$. Within experimental error, then, the $B = 2.0$ values of Table II are consistent with their $B = 4.0$ to $B = 4.6$ counterparts.

Average molecular weights of reconstituted subtilisin digests determined by the methods of Klainer and Kegeles (1956), Ehrenberg (1957), Van Holde (1960), and by sedimentation equilibrium are presented in Table III. The graph of $\log (1/r)(dc/dr)$ versus r^2 from the sedimentation equilibrium experiment ex-

TABLE II: Sedimentation Coefficients and Relative Concentrations of Products in Resin-reconstituted Subtilisin Digest of AD_{105}.

B	Sedimentation pH	Approx. c (g/dl)	Approx. Ionic Strength	s_{20} (%)
2.0	6.89		0.003	4.0 (70), 3.0 (30)
2.0	4.51	0.64	0.1	4.10 (60), 3.16 (40)
2.0	3.00	0.6	0.1	3.20 (45), 2.50 (55)
4.0	11.7	0.9	0.1	1.65 (100)
4.0	10.1	0.9	0.1	2.57 (100)
4.0	8.08	0.9	0.1	3.05 (100)
4.0	7.39	0.9	0.1	3.49 (65), ? (35)
4.2	7.29	1.9	0.1	3.27 (80), ? (20)
4.0	7.08	1.2	0.005	? (5), 3.59 (95)
4.0	6.87	0.9	0.1	4 (65), 2.7 (35)
4.0	6.39	0.81	0.0035	3.5 (100)
4.0	6.3	0.76	0.1	4.11 (85), ? (15)
4.6	5.9	0.75	0.1	23.5 (5), 4.07 (55), 2.9 (40)
4.0	3.1	0.7	0.1	? (5), 2.23 (95)
4.6	3.0	0.78	0.05	2.11 (98)
4.0	2.90	0.8	0.0033	1.96 (99)
4.0	2.89	0.9	0.1	2.17 (100)
4.2	2.80	1.9	0.1	1.76 (100)

TABLE III: Apparent Average Molecular Weights of Reconstituted Subtilisin Digests (pH 8.90, B = 4.0) of AD_{100} at pH 2.17 and 0.8 g/dl.

NaCl, M	$\bar{M}_w$	$\bar{M}_z$	Method
0.10	31,400		Ehrenberg (1957)
0.10	29,600		Van Holde (1960)[a]
0.10	32,500		Klainer and Kegeles (1956)
0.10	34,700		Equilibrium (eq 2)
0.10		29,800	Equilibrium (eq 1)[b]
0.25	41,300		Klainer and Kegeles (1956)

[a] Presumed to yield $\bar{M}_w$. [b] At the meniscus.

hibited considerable curvature which suggests heterogeneity of the sample.

Velocity sedimentation of a 1% solution of the unhydrolyzed control sample of BPA monomer obtained by exclusion chromatography on Sephadex G-200 produced, as judged by visual inspection, an ideally symmetrical boundary pattern manifesting no evidence of the usual dimer content. The method of Klainer and Kegeles (1956) yielded an apparent molecular weight of 62,500 for this sample at a pH of 2.17 and 0.70 g/dl. This value implies the magnitude of the charge correction for BPA at pH 2.17 and 0.10 ionic strength to be of the order of 4.6–9.5%, depending upon assumed molecular weights of 65,600 and 69,000, respectively. (An attempt to apply a charge correction [Williams *et al.*, 1958] to this molecular weight data on BPA monomer yielded unreasonably high values for the molecular weight. Consequently, none of the molecular weight data presented in Table III are corrected for charge. The magnitude of the charge correction for BPA in the absence of salt is apparently not as large as had been thought on the basis of previous theories. Erlander and Senti [1964] conclude from sedimentation equilibrium experimentation with BPA that, in the absence of salt at low pH, the apparent molecular weight should be and is relatively close to the true molecular weight. In view of these considerations and the "control" experiment with BPA monomer the apparent molecular weights determined at pH values far removed from the isoelectric pH and presented in Table III acquire substantially more credibility than could previously have been attached.)

The experiments conducted at pH 2.17 and 0.10 ionic strength (Table III) yielded reasonably consistent average molecular weights for the entire AD_{100} digests; their mean value is 31,600, which is increased to 33,800 after a 7.0% empirical charge correction based on the mean of the assumed maximum and minimum BPA molecular weight and on that observed in the "control" experiment with unhydrolyzed BPA.

At pH 9.31 in 0.10 M NaCl the molecular weight of one reconstituted digest (pH 8.90, B = 4.0) of AD_{100} was estimated by the Van Holde (1960) method to be 34,100.

Polyacrylamide gel electrophoresis of a reconstituted subtilisin digest (pH 8.9, B = 2.0) of the AD_{105} system was performed at pH 2.8 in 0.02 M NaCl as described

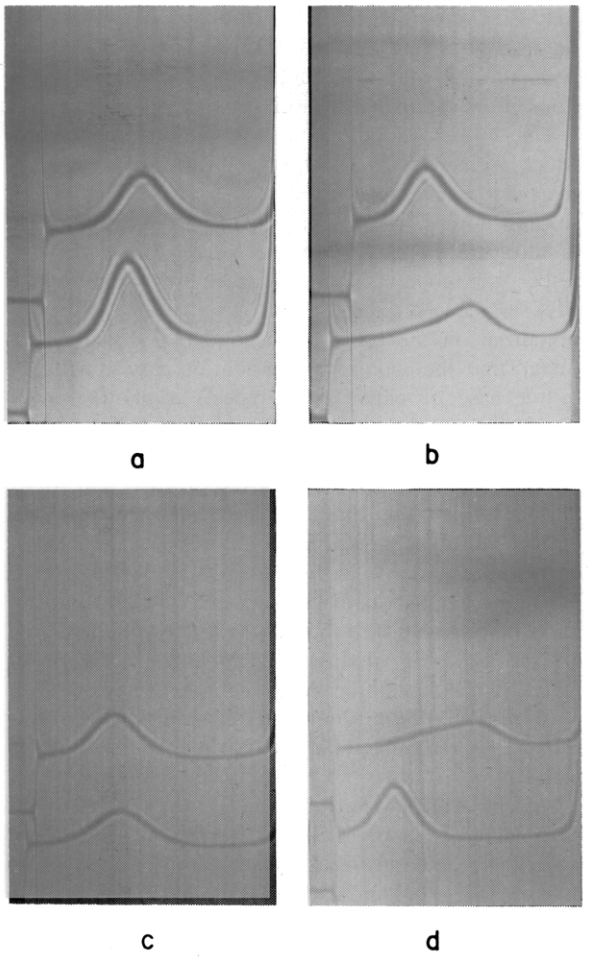

FIGURE 8: Ultracentrifuge patterns (59,780 rpm) as a function of *p*H of nonreconstituted and reconstituted subtilisin digests (*p*H 8.90, $B = 4$) of AD_{105}: (a) Upper pattern, reconstituted, *p*H 2.95, $c = 0.8\%$, 0.05 M NaCl, 144 minutes, 2.11 S; lower pattern, nonreconstituted, *p*H 6.6, 0.02 M NaCl, 144 minutes, 2.25 S. (b) Upper pattern, reconstituted, *p*H 3.1, $c = 0.7\%$, 0.1 M NaCl, 112 minutes, 2.23 S; lower pattern, reconstituted, *p*H 6.3, $c = 0.8\%$, 0.1 M NaCl, 112 minutes, 4.11 S. (c) Upper pattern, reconstituted, *p*H 10.1, $c = 0.9\%$, 0.1 M NaCl, 64 minutes, 2.56 S; lower pattern, reconstituted, *p*H 8.08, $c = 0.9\%$, 0.1 M NaCl, 64 minutes, 3.05 S. (d) Upper pattern, reconstituted, *p*H 6.87, $c = 0.9\%$, 0.1 M NaCl, 112 minutes, 4 S; lower pattern, reconstituted, *p*H 11.7, $c = 0.9\%$, 0.1 M NaCl, 112 minutes, 1.65 S.

by Sogami and Foster (1962). Representative microdensitometer tracings are shown in Figure 9. Essentially only "monomer" (M_1') and fragments were observed in the digest (solid line), which was also run with added unhydrolyzed BPA (M_1', M_1, dashed line).

Estimates of the molecular weights and degree of heterogeneity, as a function of *p*H, of reconstituted subtilisin digests of BPA were obtained using Sephadex

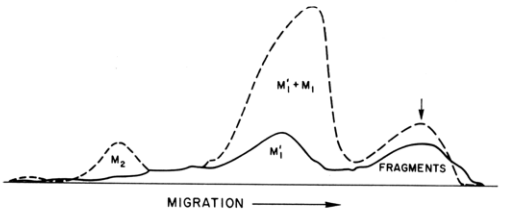

FIGURE 9: Microdensitometer tracings from zone electrophoresis (*p*H 2.8 in 0.02 M NaCl) on polyacrylamide gel of a reconstituted subtilisin digest (*p*H 8.90, $B = 2.0$) of AD_{105} (solid line) and of the same digest plus added nonhydrolyzed BPA (dashed line). The curves are normalized at the arrow.

G-100 gel. Chromatograms of reconstituted subtilisin digests (*p*H 8.90, $B = 4.0$) of AD_{105} at *p*H 2.17 and 6.48 in 0.10 M NaCl are reproduced in Figure 10 as a function of relative elution volume, V_e/V_t. The patterns have been normalized to the relative elution volume of the BPA monomer. In addition to the obvious *p*H dependence of the elution patterns, it is striking that almost none of the fragments have molecular weights lower than about 25,000.

At *p*H 2.17 small amounts of approximately 70,000 and 27,000 mw fragments are observed on either side of the major 39,000 mw material. No further significant change in the *p*H 2.17 chromatogram is produced by lowering the *p*H to 1.67 nor by 33-fold reduction of the initial sample concentration (to 0.03%), which implies further dissociation of these fragments to be improbable.

At *p*H 6.48 a decrease in the amount of 38,000–39,000 mw material and a corresponding increase in the amount of material of apparent molecular weight (59,000) approaching that of BPA are observed. The distinct resolution at *p*H 2.17 of the smaller 27,000 mw peak is lost at *p*H 6.48.

The apparent molecular weight of the subtilisin sample (Nagarse, CC G-2424) used throughout these experiments was estimated by exclusion chromatography at *p*H 6.48 to be 17,500. Its elution pattern suggests the preparation to be essentially homogeneous in molecular weight.

Several attempts to fractionate reconstituted subtilisin digests of AD_{100} into well-defined components by means of anion- and cation-exchange cellulose chromatography were generally unsuccessful; the fractionations were incomplete and the yields were low.

Discussion

The sedimentation data of Figures 1, 2, and 3 must be interpreted within the following restrictions relative to their accuracy. The apparent relative concentrations are merely visual estimates. The sedimentation coefficients are uncorrected and in most cases concentrations are only approximately known. Any given value of the extent of degradation, B, probably represents the lower

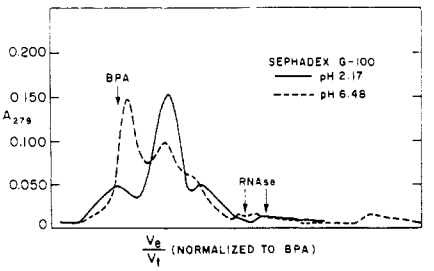

FIGURE 10: Sephadex G-100 chromatography of a reconstituted digest (*p*H 8.90, $B = 4.0$) of AD_{105}.

limit of the number of peptide bonds hydrolyzed. Richards (1955) determined the average pK for the liberated α-amino groups of subtilisin-hydrolyzed ribonuclease to be 7.4; the amount of acid liberated per BPA peptide bond opened would analogously be: *p*H 9.3, 0.99 H^+; *p*H 8.0, 0.80 H^+; *p*H 7.53, 0.57 H^+; and *p*H 6.58, 0.13 H^+.

The sedimentation experiments (Figure 3) on digests of BPA in absence of detergent are somewhat difficult to interpret because of the fact that they were conducted near *p*H 5.3. Later studies show clearly that there is a powerful tendency of large intermediates to aggregate under these conditions. Nevertheless, the limited results suggest a gradual alteration of the pattern of fragmentation by subtilisin as the *p*H of digestion is increased from 7.5 to 9.3. In the *p*H range 6.5–7.5, no evidence of intermediates of $S > 1.0$ is seen, while at *p*H 8.0–10 such intermediates, though in poor yield, do appear. It is suggested that this result can be rationalized in terms of the alkaline transition observed earlier (Leonard *et al.*, 1963) if the exposure of some susceptible interior peptide bonds accompanies the transition.

The initial fast kinetic step (Figure 4) for BPA hydrolysis at *p*H 9.30 is not easily dismissed as an artifact. The experiment was carefully begun with only 15 μg of subtilisin which had been previously titrated to *p*H 9.30. Approximately 3 H^+/BPA are released during this initial step, which probably corresponds to the early fragmentation of BPA aggregates observed by sedimentation and exclusion chromatography. The extreme susceptibility of BPA aggregates to enzymatic attack suggests that their constituent monomers exist in a form having critical peptide segments exposed. The possibility that one or more small peptides are also liberated in the initial kinetic step cannot be ruled out. That the initial reaction is not associated with any substantial fragmentation of BPA is suggested from the results of molecular weight estimations by exclusion chromatography at *p*H 2.1 and 9.3.

The results presented on hydrolysis of AD_{12} complexes are generally consistent with observations by other workers (Epstein and Possick, 1961; Kondo, 1962) that strongly bound compounds retard the rate of enzymatic hydrolysis of plasma albumin. Detergent might change the nature of subtilisin attack in the following ways: steric hindrance of susceptible peptide bonds, steric hindrance of enzyme-substrate binding sites, alterations in the structure of albumin, or alterations in the specificity of subtilisin.

The 2.4–2.8 S fragments found in the precipitates which formed in the AD_{12} digests on standing must represent a very small proportion of the total material, since in no case were fragments of $S > 1.0$ observable in ultracentrifuge runs on the whole digests. In this respect the mode of hydrolysis resembles that of native BPA in the *p*H range 6.7–7.5. This result can be rationalized on the basis of the above-mentioned postulate, that the change in degradation pattern with *p*H in the case of native BPA reflects a *p*H-dependent isomerization of the substrate molecule, together with available evidence as to the effect of detergent on the possibly similar N–F equilibrium. Foster and Aoki (1958) pointed out that low levels of detergent ion, up to 12 ions per molecule, should stabilize the N form of the protein relative to the F form. At higher detergent levels, the opposite is to be expected, detergent shifting the equilibrium toward the F form. Following this same line of reasoning, it might be anticipated that enzymatic hydrolysis of the AD_{100} complex, in which the BPA molecule is presumed to exist in the F form, should lead to a relatively good yield of large intermediates.

For the AD_{100} system the kinetic and sedimentation data strongly support the view that the AD_{100} complex is smoothly converted by the faster reaction into hydrodynamically similar half-molecules. The apparent homogeneity of the digest in gel electrophoresis and sedimentation velocity experiments makes it difficult to assume that substantial quantities of higher or lower molecular weight fragments are present. It is possible to argue, however, that different sized fragments exist in dynamic, rapid association-dissociation equilibrium which produces, as an artifact, a single, symmetrical sedimentation boundary. In the presence of detergent this situation would seem improbable, however. It is more likely that the albumin-detergent complex has been divided symmetrically into halves, thirds, or quarters, and so forth. Of these possibilities the choice of division into halves is forced if the error in determination of molecular weight by the Van Holde (1960) method is less than ± 8000 or 16.5%, as it is believed to be from test experiments with RNAase and β-lactoglobulin, in which the maximum error was 11%. The mw of 33,800, obtained by applying an empirical charge correction to averaged molecular weights of reconstituted AD_{105} digests, lends further strong support for cleavage into half-molecules. Coupled with the mw of 34,100 obtained at *p*H 9.31, the entire range of *p*H-dependent behavior observed (Table II) in sedimentation velocity experiments on reconstituted digests appears interpretable in terms of varying degrees of association, which apparently does not exceed the molecular weight of BPA, of two hydrodynamically very similar halves of the albumin molecule. The dissociation constant of the aggregate increases significantly with increasing ionic strength at neutral

*p*H and markedly increases at either higher or lower *p*H values; between *p*H 2 and 3 and at *p*H 9.3 the aggregate is essentially completely dissociated into hemimeric fragments.[2]

The increase in apparent molecular weight at *p*H 2.17 with an increase in ionic strength to 0.25 (Table III) is counter to the effect apparent in sedimentation velocity data near *p*H 7 (Table II). This suggests ion-pair formation to be a significant force for aggregation at neutral *p*H while electrostatic repulsion is dominant at low *p*H.

The two densitograms (Figure 9) from polyacrylamide gel electrophoresis of a reconstituted ($B = 2$) digest probably allow the following conclusions: the fragments have very similar charge and molecular weight; BPA aggregates are apparently preferentially hydrolyzed by subtilisin, as previously observed from sedimentation velocity experiments; and about half of the albumin remains apparently unfragmented at $B = 2$, which is also consistent with previous sedimentation velocity experiments (Table II).

The approximate molecular weight data obtained by exclusion chromatography are in qualitative agreement with that from sedimentation methods and with the observed *p*H dependence of the sedimentation coefficient of reconstituted digests. The mw of 38,000–39,000 observed for the preponderant amount of the reconstituted digests is somewhat higher than that of 33,800 estimated for the major component from sedimentation data. However, the difference is probably within experimental error.

The nature of the two minor peaks at *p*H 2.17 (Figure 10) is difficult to assess. The minor component with an apparent mw of about 70,000 seems unlikely to be due to incompleteness of the primary kinetic reaction. Possibly it is a product of hydrolysis of disulfide-linked dimer, i.e., consists of two hemimers linked by a disulfide bond involved in dimer formation. Alternatively this fraction may derive from a small percentage of the more slowly degradable AD_{12} complex which might be in equilibrium with the AD_{100} complex at *p*H 8.9 (Aoki, 1958). The fragments of about 27,000 mw observed by exclusion chromatography are present in sufficiently low quantity (by weight) to be sensibly accounted for in terms of degraded hemimer.

From the results presented, the conclusion appears warranted that the AD_{100} complex contains a relatively short vulnerable segment near the center of the BPA peptide chain, of which segment subtilisin catalyzes the hydrolysis of 1–3 peptide bonds at a rate approximately 37-fold faster than other peptide bonds in the complex. The rapid hydrolysis results in formation of two very similar fragments, each of molecular weight approximately one-half that of the parent BPA molecule. While other explanations are possible, these results can be rationalized on the basis of the previously proposed subunit model for the albumin molecule (Foster, 1960). It was proposed that the N-F transformation consists of a separation of the postulated subunits and that the same structural alteration occurs on formation of the AD_{100} complex. It is conceivable that such a separation could render available to enzymatic attack one or more of the interconnecting peptide segments. In the case of the AD_{100} complex and in terms of this model it would appear that only the central bond is exposed, or, which seems less likely, the other bonds do not contain peptide linkages susceptible to subtilisin.

In this connection, the work of Weber and Young (1964a,b) is most significant. Those workers cleaved BPA with pepsin at low *p*H and isolated, in fairly good yield, three fragments. Their Type I fragments, of mw 12,500, comprised approximately 55% of the total recovered digest. The other two, classified as Types IIa and b, had similar mw (about 30,000) as well as similar absorption and fluorescence spectra and amino acid compositions. Together they were considered to account for the remaining 45% of the recovered digest. They concluded that the original albumin molecule consists of one fragment of Type II and either two or three of Type I. While both their and our results can be taken as consistent with the proposed subunit model, it is clear that the fragmentation pattern is somewhat different in the two cases. It remains to be seen whether this difference is owing to differences in specificity of the two enzymes employed or, as seems equally possible, to differences in the degree of exposure of connecting peptide linkages under the two sets of experimental conditions.

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[2] It seems justifiable to employ the adjective "hemimeric" and the noun "hemimer" in referring to these fragments in view of their similarity in size and hydrodynamic behavior, and in the interest of brevity. It is not contended that the two hemimers of BPA are identical.

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