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Limited Hydrolysis of Bovine Plasma Albumin at Neutral and Alkaline pH Catalyzed by Associated Proteinases†

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ABSTRACT: Proteinase contaminants in some plasma albumin samples have previously been shown to produce cleavage of the albumin molecule at acid pH. The F conformer, existing at pH 3.8, is cleaved near residue number 400 to yield a large N-terminal fragment of approximately 46,000 daltons. No cleavage was found at pH above approximately 4.4. It is shown in this paper that the proteinase contaminants are active over a broad pH range from 2.5 to 11.4 provided conditions are such as to induce some breakdown of the native conformation of the albumin molecule. Addition of Tris–borate buffer (0.1 *M*) at pH 7.5–9.7 is sufficient to permit cleavage. At pH near 9 this occurs predominantly near residue 230 to yield two fragments of approximately 42,000 and 27,000 daltons. Near neutral pH substantial cleavage occurs in 4–8 *M* urea solution or in the presence of sodium dodecyl sulfate (AD_{110} complex). Under these conditions there are two large fragments (42,000 and 47,000 daltons) and essentially two small ones (20,000–27,000 daltons). Under conditions where there is no cleavage at 38–40°, substantial cleavage results at 50–65° but enzyme inactivation also occurs toward the top of this range. The alkaline activity is inhibited by soybean trypsin inhibitor but not by pepstatin; the reverse is true of the low pH activity. Cleavage at neutral or alkaline pH under the various conditions occurs primarily at X–Leu bonds while the low pH activity was already shown to occur at X–Phe. These facts suggest the presence of at least two enzymes. There is surprisingly little pH dependence over the range 7.5–9 in any of the media examined, even though albumin is known to undergo a significant conformational change in this range, the N → B transition. This transition is thought to be essentially a tertiary change with little loss of helix content. It is suggested that loss of native secondary structure, especially uncoiling of helical regions, is crucial to permit attack by these enzymes.

Wilson and Foster (1971) made the chance observation in disc acrylamide gel electrophoresis patterns that certain albumin preparations undergo limited proteolytic cleavage when exposed to pH below 4.3 for prolonged periods. They concluded that the enzyme responsible has no activity on the N form of the protein, but specifically cleaves the F form, existing at pH near 3.8, in one region. The initial cleavage yields two peptide chains of molecular weight approximately 46,000 and 24,000 which are disulfide linked and represent respectively the amino-terminal and carboxyl-terminal portions of the parent peptide chain. A subsequent cleavage removes approximately 30 additional amino acid residues from the smaller peptide. At lower pH, where molecular expansion of the albumin molecule is known to occur, more extensive degradation was observed.

The results thus indicated a remarkable dependence of the mode and degree of cleavage on the conformational state of the albumin substrate. From this point of view, it was somewhat surprising that no evidence of cleavage was seen above pH approximately 4.4 and up to pH 9 since the albumin molecule is known to undergo a conformational change in the neutral to alkaline pH range (Leonard et al., 1963; Harmsen et al., 1971; Zurawski and Foster, 1974) which has at least some superficial resemblance to the N–F transition.

Recently one of us (Aoki and Nagaoka, 1973) showed that Tris–borate–EDTA buffer systems provoke a small conformational change in the albumin molecule at pH 9. As the concentration of Tris buffer is increased from 0 to 0.1 *M* at substantially constant ionic strength there is an approximate 10% drop in $s_{20,w}$. In parallel, optical rotatory dispersion (ORD) studies indicated an approximate 5% decrease in the content of α helix. This prompted us to seek evidence of proteolytic degradation in Tris–borate buffer systems at pH 9. A relatively specific cleavage was found which, however, differs in position from that obtained with

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the F form. These results encouraged other experiments over the neutral and alkaline pH range which are reported now.

Experimental Section

Materials. Bovine plasma albumin preparations used were: Armour fraction V, lot G34805, Armour crystallized, lot D71104, Pentex fraction V, lot 121, and Pentex crystallized, lot BX6. Defatted albumin was prepared by the procedure of Chen (1967) as modified by Sogami and Foster (1968).

Acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, Naphthol Blue Black, and β-mercaptoethanol were purchased from Eastman Organic Chemicals. Coomassie Brilliant Blue R250 was purchased from Dickinson & Co. Sodium dodecyl sulfate from Matheson Coleman and Bell and iodoacetamide from Aldrich Chemical Co. were used after recrystallization. Urea, ultra pure grade purchased from Mann Research Co., was used without further purification.

The radioactive iodoacetamide ([1-^{14}C]iodoacetamide) was purchased from New England Nuclear Corp. and its specific activity was 65 μCi/mg. The scintillation grade 2,5-diphenyloxazole and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene were purchased from Packard Instrument Co. The NCS solubilizer was purchased from Amersham/Searle Corp.

Other chemicals were of highest purity commercially available.

Dialysis bags were prepared from cellophane tubing (Union Carbide Corp.) by boiling twice in distilled water and once in half-saturated $NaHCO_3$ solution. After rinsing well with distilled water, they were kept in the cold room. All water employed was passed through a mixed-bed ion-exchange column (Barnstead Bantam Model BD-1) and had a specific resistance greater than 10^6 ohms cm.

Buffer Solutions and Supporting Media. The principal buffer systems used were 0.1 *M* Tris plus 0.1 *M* boric acid at pH 9.1 and 0.1 ionic strength phosphate buffer at pH 7.4. Solutions containing KCl as supporting electrolyte were prepared by adding 0.01 *N* NaOH or HCl to the isoionic protein in 0.1 *M* KCl to give the desired pH value. Other buffers and supporting media were used in this study and will be mentioned in the text.

The pH values of the solutions were measured with a Radiometer Model 25 pH meter with a combination electrode GK2321C. Routine determinations of the protein concentration were made with a Perkin-Elmer spectrophotometer 124D assuming $E_{1\%}$(1 cm) 6.67 at 279 nm.

The urea solutions of bovine plasma albumin were prepared by adding urea to solutions of the sulfhydryl-blocked protein to give the desired concentrations (molal) of urea. Sulfhydryl-blocked albumin was employed to prevent serious aggregation which otherwise occurs in urea at alkaline pH (Aoki et al., 1974). The bovine plasma albumin–sodium dodecyl sulfate complexes AD_n and AD_{2n} (A is albumin and D is sodium dodecyl sulfate: $n = 55$) were prepared by mixing calculated amounts of albumin and the detergent.

Blocking of Sulfhydryl Group. Bovine plasma albumin and iodoacetamide (mol ratio 1:10) were dissolved in phosphate buffer at pH 6.8 and ionic strength 0.1. This solution was kept in the cold room overnight and then dialyzed extensively against Tris–boric acid buffer at pH 9.1 or against phosphate buffer at pH 7.4.

Incubation of Bovine Plasma Albumin. Glassware was dried in an oven, and syringes and rubber stoppers were sterilized by alcohol before use.

The bovine plasma albumin solution (0.1–0.2%) was filtered through a Millipore (0.45 μm) or Nucleopore (0.2 μm) filter and incubated for 70–100 hr at 37° unless stated otherwise. When the supporting electrolyte was NaOH–KCl, incubation was conducted in a sealed tube under nitrogen.

Dodecyl Sulfate β-Mercaptoethanol Electrophoresis. The procedure was similar to that described by Weber and Osborn (1969). The gels, concentration 10%, were 10 cm × 6 mm. Normally 75 μg of protein was applied, and the electrophoresis was continued for 7–9 hr at 10 mA/gel. After electrophoresis, the gel was stained by Coomassie Blue and destained in a mixture of methanol–acetic acid–water or, in later experiments, with a mixture of isopropyl alcohol and acetic acid (Fairbanks, Steck and Wallach, 1971) using a Bio-Rad diffusion destainer.

The gels were scanned with a Gilford Model 240 automatic gel scanner using a wavelength of 500 or 520 nm, and the resulting optical density tracings were resolved into components employing a Du Pont Model 310 curve resolver.

The molecular weight of protein fragments was estimated in the same way as Weber and Osborn (1969) using albumin monomer (66,000), ovalbumin (44,000), and β-lactoglobulin (17,500) as standards.

Dodecyl Sulfate β-Mercaptoethanol Electrophoresis Using Labeled Protein. The sulfhydryl group was blocked as described above using radioactive iodoacetamide. Incubation and electrophoresis were performed in the same way as described above. After the electrophoresis, the unstained gel was cut into slices of about 1 mm. These were placed in 1 ml of a mixture of NCS solubilizer and water (9:1) in a scintillation vial, and heated at 50° for 3 hr (Basch, 1968; Zaitlin and Hariharasubramanian, 1970). After the addition of 20 ml of scintillation cocktail, samples were counted in a Beckman Model LS-100 or a Packard Model 3320 liquid scintillation counter. The scintillation cocktail was prepared by dissolving 6 g of 2,5-diphenyloxazole and 0.075 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1.0 l. of toluene.

Gel Filtration of Incubated Albumin. A jacketed Sephadex G-150 column (3 x 90 cm) was equilibrated with 0.1*M* Tris–boric acid buffer at pH 9.1 and 5°. A solution of Armour fraction V albumin incubated at pH 9.1 in the Tris–borate system (200 ml of 0.3% solution) was concentrated to 4.5 ml by ultrafiltration in the cold room under nitrogen pressure of 40 psi using a Diaflo ultrafilter (Amicon Corp.) and membrane (UM-10). The concentrated solution was layered on top of the gel and the flow rate regulated at 20 ml/hr by a peristaltic pump. Fractions of 120 drops were collected and the absorbance of each fraction was read at 279 nm.

Determination of Amino-Terminal Residues. The incubated protein solution was dialyzed extensively against distilled water in the cold room and lyophilized. The protein was then dissolved in phosphate buffer at pH 8.2 in presence of 8 *M* urea. This was dansylated and hydrolyzed at 110° for 4 hr according to the procedure of Gros and Labouesse (1969) and was analyzed by thin-layer chromatography on a polyamide layer sheet (Chen Chin Trading Co., Ltd., Taiwan) and using solvents recommended by Woods and Wang (1967). Spots were visualized through their fluorescence when viewed under ultraviolet illumination. Select-

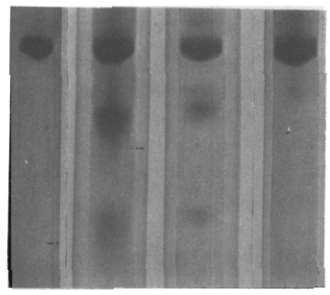

FIGURE 1: Sodium dodecyl sulfate β-mercaptoethanol gel electrophoresis patterns on various albumin preparations incubated 70 hr at 37° in 0.1 *M* Tris–borate buffer (pH 9.1). (a) Armour fraction V unincubated (control); (b) Armour fraction V; (c) Armour crystallized; (d) Pentex fraction V.

ed standard dansylated amino acids were applied to one side, and the sample solution was spotted at the same point on the reverse side.

Inhibition Experiments. The peptide inhibitor pepstatin (Umezawa et al., 1970) was tested at both pH 3.7 and 9.1. To 1 ml of a 0.15% solution of Armour fraction V albumin was added 0.2 ml of a solution containing 1 mg of pepstatin in 10 ml. This corresponds to a little more than a 1:1 equivalent of pepstatin to albumin, but of course the ratio to enzyme is entirely unknown. The resulting solution was kept in the cold room and then incubated in the usual manner.

Inhibition by whole plasma was tested by adding 0.1 ml of bovine plasma to 4 ml of 0.15% Armour fraction V in Tris–borate buffer at pH 9.1. Assuming the 0.1 ml of plasma to contain 3.5 mg of albumin it is calculated that there was a total of 9.5 mg of albumin in the incubation solution.

Similar inhibition studies were carried out using three main fractions of bovine plasma separated by gel filtration on Sephadex G-150. The three fractions presumably correspond roughly to (1) macroglobulin, (2) γ-globulin, and (3) albumin, respectively (Flodin and Killander, 1962).

Inhibition studies utilizing soybean trypsin inhibitor (Kunitz) (STI)[1] were carried out by adding various volumes of a 0.1% STI solution to 3 ml of a 0.1% Armour fraction V solution.

Results

Proteolytic Cleavage at pH 9.1. In Figure 1 are shown sodium dodecyl sulfate β-mercaptoethanol gel patterns obtained on albumin samples incubated for 70 hr at 37° in pH 9.1 Tris–borate buffer. Gel 1a is a control on unincubated Armour Fr.V. Gel 1b, resulting from incubation of this same protein, shows two additional bands of lower molecular weight than the albumin monomer. We estimate the molecular weights of these two fragments, based on a number of experiments, as 42,000 and 27,000. Gel 1d shows essentially no cleavage to occur with the corresponding Pentex protein, as was also observed under low pH conditions (Wilson and Foster, 1971). Gel 1c was conducted on crystallized bovine plasma albumin (Armour). While definite evidence of cleavage is seen in this case, it is far less than was obtained with the uncrystallized Armour protein.

Sulfhydryl-blocked Armour fraction V albumin exhibited the same cleavage pattern and to a similar degree as the unblocked protein under these incubation conditions. Similarly, defatting had little effect except that cleavage appeared to be slightly faster in the defatted protein. While no detailed kinetic study was made, it was observed that cleavage was significantly less at 18 hr but that 50- and 70-hr incubations yielded essentially the same degree of cleavage. In general, incubations were carried out for a fixed period of 70 hr.

[1] Abbreviation used is: STI, soybean trypsin inhibitor.

Table I: Effect of Supporting Electrolyte on the Degree of Cleavage, as Estimated from Densitometry on Sodium Dodecyl Sulfate β-Mercaptoethanol Gels.[a]

Electrolyte	Percent Cleavage of Monomer
0.1 *M* Tris–borate	60 ~ 65
0.1 *M* Tris–chloride	55
0.1 *M* Borate–NaOH	45
0.1 *M* $NaHCO_3$–Na_2CO_3	20
0.1 *M* NaOH–KCl	7

[a] All samples were incubated for 70 hr at 37° and pH 9.0 ± 0.1.

A comparison was made of the relative degree of cleavage in various buffers and supprting media at pH 9 ± 0.1. Table I gives the percent cleavage of monomer as estimated from densitiometry of the dodecyl sulfate β-mercaptoethanol electrophoresis gels. Tris–borate appears to be more conducive to the enzymatic cleavage than either Tris or borate alone. Significant cleavage occurs in $NaHCO_3$–Na_2CO_3 but cleavage in the 0.1 *M* chloride system is almost nil.

In an attempt to delineate more precisely the position of cleavage Armour fraction V albumin was sulfhydryl-blocked using [1-^{14}C]iodoacetamide, and incubation carried out in Tris–borate buffer at pH 9.1. Following dodecyl sulfate β-mercaptoethanol gel electrophoresis, the gels were sectioned and counted as described in the Experimental Section. The results, shown in Figure 2, demonstrate clearly that the small fragment is radioactive but that very little activity is associated with the large fragment. Since it is known that the single sulfhydryl residue of bovine plasma albumin occurs close to the N-terminal end of the chain (King and Spencer, 1972) and since the small fragment weighs approximately 27,000 daltons it can be concluded that the cleavage takes place at approximately residue number 230.

To ascertain whether cleavage occurs within a disulfide loop, dodecyl sulfate gel electrophoresis was conducted on incubated samples without addition of any reducing agent. The resultant pattern, shown in Figure 3a, is very similar to that with reducing agent (Figure 1b). Evidently cleavage takes place outside of any disulfide loops.

Close inspection of Figure 3a shows the existence of several bands in the region of the large fragment. It must be concluded that cleavage is not absolutely limited to a single peptide bond. Figure 4 shows an elution pattern of a sample incubated in Tris–borate at pH 9.1 obtained on Sephadex G-150, together with dodecyl sulfate β-mercaptoethanol gel electrophoresis patterns obtained in the regions of the large (tube 40) and small (tube 45) fragments. Clearly there is some heterogeneity of both the large and small fragments.

Cleavage in the Neutral pH Range. Having found cleavage due to associated proteinase in bovine plasma albumin preparations at both acid pH and at pH 9, it was of interest to look more closely at the behavior in the physiological range of pH. In agreement with the earlier findings (Wilson and Foster, 1971) no significant degree of cleavage was de-

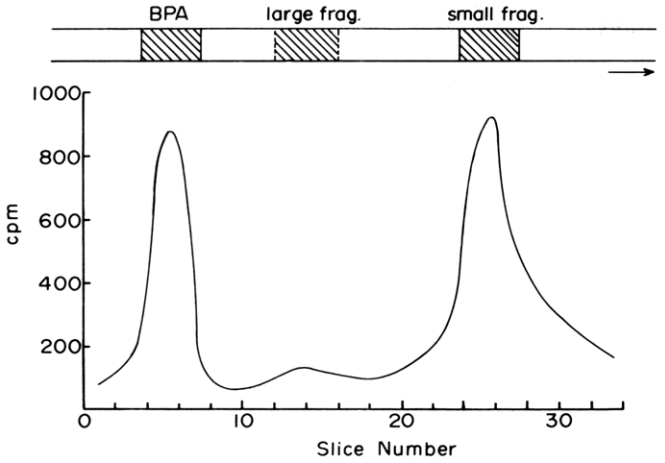

FIGURE 2: Separation by dodecyl sulfate β-mercaptoethanol gel electrophoresis of fragments resulting from pH 9.1 incubation of Armour fraction V albumin with the ^{14}C-label on Cys^{34}. Top, banding pattern; bottom, distribution of label as counts per minute.

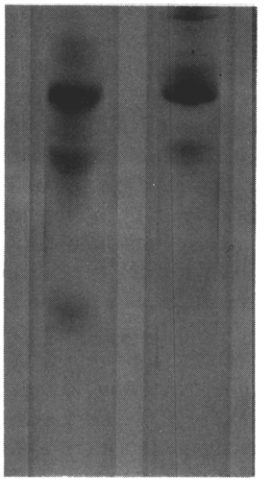

FIGURE 3: Dodecyl sulfate gel electrophoresis patterns without reducing agent following: (a) incubation at pH 9.1 in Tris–borate; (b) incubation at pH 7.4 in 4 *M* urea.

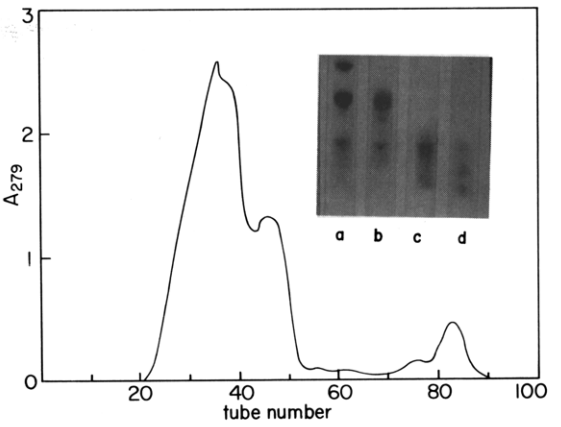

FIGURE 4: Elution pattern on Sephadex G-150 of Armour fraction V albumin following incubation in Tris–borate buffer at pH 9.1. The inset shows dodecyl sulfate β-mercaptoethanol gel patterns on (a) the incubated sample before separation; (b) fraction 40; (c) fraction 45; (d) fraction 50.

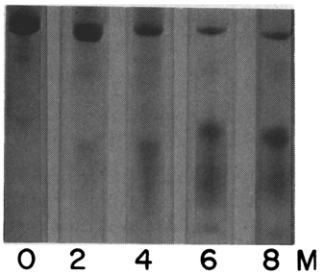

FIGURE 5: Dodecyl sulfate β-mercaptoethanol gel electrophoresis patterns following incubation of Armour fraction V albumin for 70 hr at pH 7.4 and 37° in various concentrations (molal) of urea, as shown.

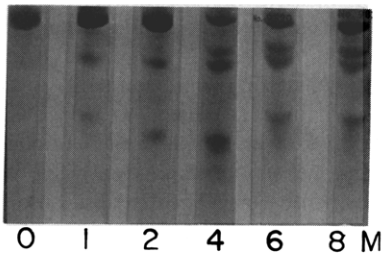

FIGURE 6: Dodecyl sulfate β-mercaptoethanol gel electrophoresis patterns on Armour fraction V albumin stored for 70 hr at pH 7.4 and 2° in various concentrations (molal) of urea, as shown.

tectable at neutral pH in the 0.1 *M* KCl system. Approximately 15% cleavage was seen in 70 hr at 37° in 0.1 ionic strength phosphate buffer at pH 7.4 but 50% cleavage was obtained in a Tris–borate system at pH 7.6. Comparing these results with those at pH 9 (Table I) it is seen that there is surprisingly little pH-dependence of the cleavage between pH 7.6 and 9.

In view of these results it was of interest to observe the effect of elevated temperature and of various protein denaturants in the physiological pH range. Figure 5 shows results of incubating Armour fraction V albumin for 70 hr at pH 7.4 and 37° in various molalities of urea. At 4 *M* and higher urea concentration proteolysis is extensive; in fact it is estimated that approximately 90% destruction of the albumin monomer has taken place in either 6 or 8 *M* urea. Interestingly, essentially no large fragments, as seen at pH 9 or 3.7, are formed, the fragments being approximately 24,000 or less in mol wt. Figure 6 shows results of a similar experiment conducted in the cold room (2°) instead of at 37°. Again substantial cleavage is seen at 4 *M* and higher urea concentrations but in this case two large fragments of approximately 46,000 and 42,000 daltons accumulate in good yield. Only the smaller of these is seen at 1 or 2 *M* urea, but they appear in approximately equal amounts at 4 *M* urea and above. The degree of destruction of albumin monomer appears to be almost independent of urea concentration over the range 4–8 *M* and amounts to approximately 60–70%. In the case of a sample stored at 2° for 12 days in 4 *M* urea, approximately 85% of the monomer was destroyed.

The molecular weights of the two large fragments produced in urea at pH 7.4 and 2° correspond closely to the 46,000-dalton fragment produced at pH 3.7 (Wilson and Foster, 1971) and the 42,000 fragment formed at pH 9. This suggests the possibility that both the acid and alkaline cleavages are proceeding in parallel at pH 7.4 in urea. To test this possibility, electrophoresis was conducted in dodecyl sulfate gels without reducing agent, since the acid cleavage is known to occur within a disulfide loop (Wilson and

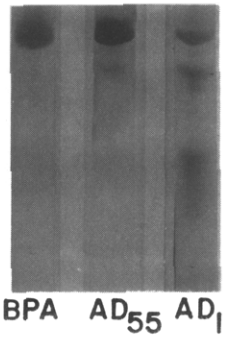

FIGURE 7: Dodecyl sulfate β-mercaptoethanol gel electrophoresis patterns following storage at 37° and pH 7.4 in phosphate buffer of Armour fraction V albumin (a) without detergent; (b) as AD_{55} complex; (c) as AD_{110} complex.

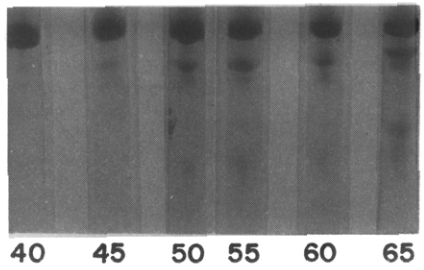

FIGURE 8: Dodecyl sulfate β-mercaptoethanol gel electrophoresis patterns following 2-hr incubation of Armour fraction V albumin at pH 6.8 in 0.1 ionic strength phosphate at various temperatures as indicated.

Foster, 1971). The resulting pattern, Figure 3b, is very similar to that obtained on incubation at pH 9 (Figures 1b and 3a). Evidently the cleavage giving rise to the 46,000-dalton fragment does occur within a disulfide loop. However, another experiment renders this simple interpretation of the two large fragments somewhat doubtful. A sample of Armour fraction V albumin labeled with [1-^{14}C]iodoacetamide was stored in 6 *M* urea at 2° and the dodecyl sulfate β-mercaptoethanol gel sectioned and analyzed for radioactivity as was described before. The distribution of radioactivity indicated that both the large fragments as well as the small fragments were radioactive.

Armour fraction V albumin was incubated at pH 7.4 and 37° in presence of a 55:1 molar ratio of sodium dodecyl sulfate (AD_{55}) or a 110:1 ratio (AD_{110}) corresponding to the two known stoichiometric complexes (Putnam and Neurath, 1945). The resulting electrophoretic patterns are shown in Figure 7. Again two large fragments of approximately 45,000 and 40,000 daltons result. Sometimes an extra zone was observed corresponding to approximately 34,000 daltons and two major small fragments of approximately 27,000 and 21,000 are also seen. The degree of cleavage is somewhat less than occurs in 4–8 *M* urea under similar conditions. Furthermore, wheras the two large fragments are produced in nearly the same amount in urea, the larger one clearly predominates in the case of the AD_{110} complex. Cleavage of AD_{55} is much less extensive (Figure 7) and only the larger of the two large fragments is seen.

To investigate the effect of temperature in the neutral pH range, samples of Armour fraction V albumin were incubated for 2 hr at pH 6.8 in 0.1 *M* phosphate buffer at elevated temperatures. Results are shown in Figure 8 over the temperature range 40–65°. Essentially no cleavage is seen at 40° but some is apparent at 45°. Over the temperature range 55–65° approximately 50% destruction of albumin monomer resulted. At higher temperatures the degree of hydrolysis decreased and there was none at 80°. Evidently at 80° the enzyme is instantly inactivated before it can produce significant degradation. To verify that this is the correct interpretation, a sample incubated 2 hr at 65° was dialyzed vs. the pH 9.1 Tris–borate buffer and incubated both without and with added substrate albumin (Pentex fraction V). No further cleavage was found in either case confirming that inactivation had been complete in 2 hr at 65°.

The cleavage pattern at elevated temperature in phosphate buffer clearly resembles that seen in urea and in the detergent complexes. Two large fragments of approximately 46,000 and 42,000 daltons appear, together with two or three small fragments. As in the case of the AD_{110} complex the larger of the large fragments predominates.

Additional Studies. Incubations were conducted in Tris–borate buffer at pH 9.1 in the presence of 4 *M* urea and on the AD_{110} complex. In each case, the cleavage pattern was very similar to that at pH 7.4 with the corresponding added denaturant. However, the degree of cleavage in 4 *M* urea decreased below pH 7.4 and was nil at pH 3.7. The low pH activity was restored by removal of the urea through dialysis, suggesting that the enzyme responsible for the acid cleavage is reversibly inactivated by 4 *M* urea. Studies over the pH range 7.6–9.7 in Tris–borate showed almost no pH dependence in the degree of cleavage. There was approximately 50–60% destruction of monomer in all cases (70 hr at 37°). Similar experiments in phosphate buffers of 0.1 ionic strength showed no pH dependence over the range 7.8–8.2 (20 ± 5% destruction of the monomer).

Harmsen et al. (1971) have shown low levels of calcium ion to increase the relative amount of the B isomer at physiological pH. Fraction V albumin was incubated for 21 hr at 45° in 0.154 *M* NaCl + 0.002 *M* $CaCl_2$. There was only a slight amount of cleavage, no more than in the absence of calcium under similar conditions.

A solution of Armour fraction V albumin in phosphate–NaOH buffer of pH 11.4 was stored at 2° for 70 hr. Extensive cleavage occurred with the formation of two large peptides of 46,000 and 43,000 daltons and a smaller one of 29,000. Again Pentex fraction V showed no cleavage indicating that the hydrolysis is enzymatically catalyzed even at this high pH.

Some additional incubations were conducted at acid pH and confirmed the earlier results of Wilson and Foster (1971). In particular, more than a dozen bands were found in the dodecyl sulfate gels when incubation was conducted at pH 2.5–3.3.

Inhibition Experiments. Tests for inhibition of the enzymatic cleavage at pH 9.1 by plasma inhibitors were carried out as described in the Experimental Section. Each of the first two fractions eluted on passing serum through Sephadex G-150 showed virtually total inhibition of the cleavage. This result is not surprising in view of the well-known α_2-macroglobulin inhibitor and α_1-antitrypsin which would presumably be concentrated, respectively, in these two fractions. On the other hand, somewhat surprisingly the sample to which whole serum was added showed evidence of a normal cleavage with little or no inhibition. We interpret this to mean that the amount of proteinase inhibitors in serum was not sufficient to inhibit fully under conditions where an excess of albumin (and proteinase) was present. The amount of (Armour fraction V) albumin added in this experiment was approximatley twice that present in the serum

itself. Pepstatin was found to inhibit the enzymatic cleavage in acid solution but not in alkaline solution. Conversely, soybean trypsin inhibitor showed inhibition in alkaline but not in acid solution. Guanidine hydrochloride at 6 *M* concentration deactivated the enzyme at pH 7.4.

Identification of the Amino-Terminal Residues Exposed. Amino-terminal residues were identified by dansylation and thin-layer chromatography as described in the Experimental Section. The results are given in Table II. Under all conditions tried in neutral and alkaline pH the predominant amino-terminal residue exposed is Leu. The Phe and Val seen in most cases in lesser amount, and also present in the fraction V albumin before incubation, may result from a minor amount of the low pH cleavage which could occur during the defatting procedure. As shown by Wilson and Foster (1971) the initial cleavage at pH 3.7 exposes amino-terminal Phe, and subsequent cleavage of the small fragment probably gives rise to the amino-terminal Val.

Table II: N-Terminal Amino Acid Residues as Determined by Dansylation.[a]

Protein	Incubation Conditions	Asp	Leu	Phe	Val	Ala
Crystallized	Control	+++	-	-	-	-
Armour fraction V	Control	+++	-	+	+	-
Armour fraction V	pH 9 (Tris–borate)	+++	+++	+	+	±
	pH 7.4, 45°	+++	+++	+	+	±
	pH 7.4, 4 *M* urea, 2°	+++	+++	+	+	+
	pH 7.4, AD_{110}, 37°	+++	+++	±	±	+
	pH 3.7[b]	+++	-	+++	+	±

[a] +++, intense; +, weak but definite; ±, questionable; -, absent. [b] From Wilson and Foster (1971).

Discussion

Clearly there are present in some albumin preparations proteinase contaminants which are catalytically active over the entire pH range from as low as 2.5 to 11.4. It seems probable that at least two different enzymes are responsible. Most suggestive in this regard are the findings that the low pH activity is inhibited by pepstatin but not by soybean trypsin inhibitor, while the alkaline activity shows the converse. These results suggest the presence of both an acid proteinase and a typical serine proteinase. The reversible urea inactivation of the low pH reaction together with the fact that hydrolysis in neutral and alkaline solution goes on in even 8 *M* urea supports the presence of two enzymes, as does also the limited specificity data. It seems very significant that under all conditions in neutral and alkaline solutions the cleavage occurs mainly at an X-Leu bond while at low pH an X-Phe bond is cleaved initially.

Both the acid and alkaline activities are most prevalent in the Armour fraction V albumin,[2] among the samples we have tested, but it is significant that even the crystallized protein has detectable activity. The absence of activity in the Pentex material suggests inactivation at some point in the preparative procedure. Both low and high pH activities are clearly destroyed by heating at relatively mild temperature. The low pH activity almost certainly exists in fresh plasma (Foster et al., 1973) and, though we have not yet demonstrated the fact, it seems probable that the alkaline activity does as well. Even the demonstration of these activities in fresh isolated plasma would not prove they are normal constituents of circulating plasma, however; the possibility certainly exists that they result from some chance cell lysis during the course of separation of the plasma from the formed elements.

The most remarkable aspect of these results is the extreme sensitivity of the enzymes to the conformational state of the substrate. The very small perturbation resulting in 0.1 *M* Tris–borate,[3] corresponding to about a 10% increase in hydrodynamic volume, permits cleavage in a localized region of the protein if not in a single peptide bond. Similarly, 2 *M* urea produces almost no detectable change in the optical rotation of bovine serum albumin and 4 *M* urea only about a 5–10% alteration (Kauzmann and Simpson, 1953); yet this is sufficient to permit some enzymatic cleavage (Figures 5 and 6). In the same way, cleavage in the KCl system is nil at 38° but substantial at 50°; yet there is not more than a 5% change in $-\alpha]_{233}$ on increasing the temperature through this interval (Wilson and Foster, 1972; Wallevik, 1973).

Given these results, the absence of any significant pH dependence of the susceptibility of the protein to enzymatic attack, over the pH range 7.5–9, is most unexpected. Over this pH range the protein undergoes a conformational transition (Leonard et al., 1963; Harmsen et al., 1971; Zurawski and Foster, 1974), the N → B transition. Moreover, no effect of Ca^{2+} ion was seen in spite of the fact that this ion strongly shifts the equilibrium toward the B form near neutral pH (Harmsen et al., 1971; Zurawski and Foster, 1974). Clearly the N → B transition does not enhance the sensitivity of the albumin molecule to this proteinase system, a result which is all the more surprising when contrasted with the pronounced pH dependence of the cleavage in acid solution. Wilson and Foster (1971) showed that the N form is totally resistant at pH 4.5 but the F form, existing near pH 3.8, is readily and specifically cleaved by the contaminating proteinase. Braam et al. (1971) demonstrated a similar difference in susceptibility of N and F forms to pepsin. There are substantial similarities in the changes in physical-chemical properties associated with the N → B and N → F transitions (Leonard et al., 1963; Harmsen et al., 1971; Zurawski and Foster, 1974). In both cases there are changes in optical rotatory properties, titration properties, and in the environment of the sulfhydryl group which probably result from similar changes in tertiary structure. There is one notable difference, however. While the N → F transition appears to be accompanied by an approximate 6% loss of helix content, as judged from optical rotatory parameters (Sogami and Foster, 1968), there is evidently almost no loss of helix associated with the N → B transformation (Leonard et al., 1963; Zurawski and Foster, 1974). This suggests that it is the unfolding of a helical segment which renders the peptide chain vulnerable to attack.

The region of the albumin molecule exposed to enzymatic attack at pH 9 is entirely different from that labilized in the N–F transition. The F form clearly is most susceptible to

[2] Recent samples of fraction V bovine plasma albumin obtained from the Armour Pharmaceutical Company have shown much less proteinase activity. Another grade of albumin provided by this same vendor, Leptalb 7, appears to be rich in such activity.

[3] It was stated by Aoki and Nagaoka (1973) that the unfolding occurs in 0.1 *M* Tris-EDTA-borate buffer but not in the presence of Tris-borate alone. That statement is now corrected since we have found a similar change in $-\alpha]_{233}$ with or without inclusion of EDTA.

enzymatic attack at a position near residue number 400. The protease contaminants under consideration here cleave the F conformer with great apparent specificity yielding a single N-terminal fragment of molecular weight approximately 46,000 (Wilson and Foster, 1971). Moreover, pepsin yields large fragments resulting from cleavage in the same general area (Braam et al., 1971). By contrast, the cleavage at pH 9 in presence of Tris buffer must occur near residue 230. It is of considerable interest that King and Spencer (1970) isolated a peptide of molecular weight approximately 40,000 from the C-terminal part of the albumin molecule following limited tryptic digestion at pH 8.8. That fragment also was separated without reduction and it seems clear the same region is preferentially attacked in both of these cases. It is interesting that King and Spencer used a Tris buffer, though only 0.025 *M*. In view of our results it seems conceivable this might have played a role in favoring formation of the large fragment they observed.

Experimentalists employing plasma albumin preparations should be alert to possible artifacts resulting from the proteinase contaminants. Albumin is certainly not unique in this regard since there are increasing examples of nominally pure proteins which are found to have at least trace amounts of proteinases associated with them (Pringle, 1970; Diezel et al., 1972; Ambesi-Impiombato and Pitt-Rivers, 1971). In our experience one cannot rely on any conventional purification procedure to remove all such contamination, since the proteinases appear to be rather tightly bound to the albumin. While the activity can be eliminated readily by heating, such a treatment is not always desirable. The danger of possible degradation is particularly real in any prolonged experiments at extremes of pH, in presence of denaturants, or at moderately elevated temperatures.

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