Effect of Concentration of Substrate and of Chloride on the Optimum *p*H for the Proteolysis of Bovine Serum Albumin by Human Pepsin III*

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Human pepsin III digests bovine serum albumin (BSA) optimally between *p*H 2.6 and 2.9 in incubation mixtures composed of 4 ml 0.5% BSA in H_2O, 1 ml dilute HCl, and 1 ml of human pepsin III in 0.001 M sodium acetate buffer, *p*H 5.4. Substitution of the 0.5% BSA in the incubation mixtures by 1%, or 2.5% BSA in H_2O produces a decrease of proteolysis over the *p*H range 2.3–3.3. The *p*H adjustment of incubation mixtures containing higher BSA concentration (1% or 2.5%) requires more concentrated HCl solutions than incubation mixtures containing low BSA concentrations (0.5% BSA). This led us to test a hypothesis, namely that the higher chloride concentration of incubation mixtures was responsible for the observed inhibition of proteolysis of 2.5% and 1% BSA. Employment of 0.5% BSA substrate in 0.0025 M NaCl raises the final chloride concentration of incubation mixtures over the *p*H ranges 2.3–3.3 to values (0.0112–0.0045 M) approximating the final chloride concentration over this *p*H range in the incubation mixtures containing 1% BSA *without* added NaCl (0.0131–0.0044 M); use of 0.5% BSA in 0.0025 M NaCl results at the same time in an activity vs. *p*H curve grossly similar to the activity vs. *p*H curve of 1% BSA in H_2O. Employment of 0.5% BSA in 0.25 M NaCl or in 0.5 M NaCl results, over the *p*H range 2.3–3.3, in final chloride concentration (0.1760–0.1704 M and 0.3427–0.3370 M, respectively) exceeding those of 2.5% BSA in H_2O in this *p*H range (0.0248–0.0112 M); associated with addition of these amounts of chloride to 0.5% BSA, activity vs. *p*H curves are produced grossly similar to those seen with 2.5% BSA in H_2O. The grossly similar effects of increase in BSA concentration and increase of chloride content of incubation mixtures appears to substantiate our hypothesis. Reversible expansion of the BSA molecule in acid medium, which is decreased by addition of electrolyte, could provide an explanation for our observations, assuming that the expanded molecule is more accessible to peptic hydrolytic attack.

The optimum *p*H for the proteolysis of protein by pepsin may differ for different proteins (Northrop, 1923; Christensen, 1955a) and varies even for the same protein substrate, depending on the experimental conditions used (Christensen, 1955a; Tolckmitt, 1954). Observations on proteolysis by pepsin have recently been correlated with some hypotheses concerning the action of pepsin on proteins (Christensen, 1955a; Schlamowitz and Peterson, 1959).

This later work has been done almost exclusively with pepsins of animal origin. Although in several studies human gastric juice has been used, only two reports have described the optimum *p*H for the proteolysis of protein using a relatively pure human pepsin preparation (Seijffers *et al.*, 1963a; Tang *et al.*, 1959).

In a previous study we have described (Seijffers *et al.*, 1963a) *p*H activity curves for the proteolysis of bovine hemoglobin by human gastric juice and by two pepsin fractions isolated from human gastric juice. Following this we investigated the proteolysis of BSA[1] (bovine serum albumin) by human gastric juice. To ensure that enough substrate be present, proteolysis was initially followed at different *p*H values at two substrate concentrations. The higher BSA concentration was associated unexpectedly with an apparent decrease of proteolysis. We have tried to understand this in terms of change in ionic concentration (see Discussion).

We have recently fractionated human gastric mucosa and obtained three distinct human pepsinogen fractions designated as pepsinogens I, II, and III (Seijffers *et al.*, 1963b). Pepsin III (the pepsin corresponding to human pepsinogen III) has been employed in experiments similar to those done in a preliminary way with gastric juice. The use of a single pepsin such as pepsin III eliminates difficulties in interpretation incidental to the use of gastric juice with its mixture of pepsins.

This report describes the influence of substrate concentration on proteolysis of bovine serum albumin by chromatographically pure human pepsin III and seeks to interpret observations in terms of associated changes in chloride concentration.

METHODS

Preparation of Pepsin III.—The chromatographic separation of human pepsinogens and human pepsins from gastric mucosa has been described elsewhere in detail (Seijffers *et al.*, 1963b; Seijffers *et al.*, 1963c), but is outlined below. A stomach specimen was obtained immediately after excision at surgical operation on a patient with duodenal ulcer. The gastric mucosa was quickly dissected free from the underlying tissue and homogenized with 0.1 M potassium phosphate buffer (*p*H 7.3). The extract obtained after filtration of the homogenate and dialysis vs. 0.1 M acetate buffer, *p*H 5.3, was applied to a DEAE-cellulose column previously equilibrated with 0.1 M acetate buffer, *p*H 5.3. Elution, carried out with a continuously increasing sodium chloride gradient (0.11–0.16 M), yielded three distinct peaks of proteolytic activity. Eluate fractions corresponding to the third peak of proteolytic activity

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[1] Abbreviations used in this work: BSA, bovine serum albumin; DEAE-cellulose, diethylaminoethyl-cellulose.

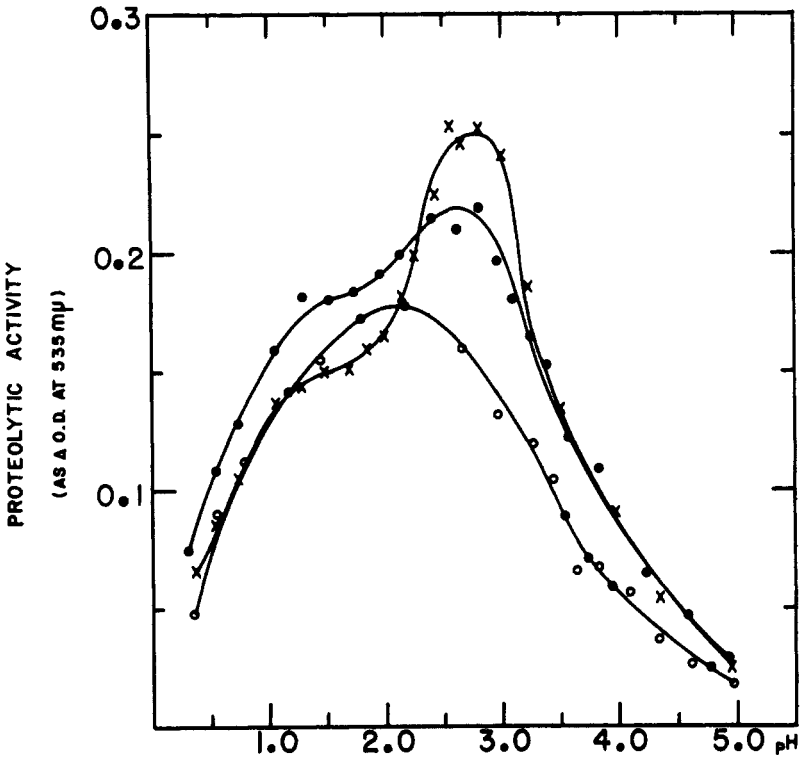

FIG. 1.—Effect of substrate concentration of BSA on protolysis. Proteolytic activity of human pepsin III as ΔOD at 535 mμ is plotted vs. *p*H of incubation mixture. O—O, 2.5% aqueous BSA as substrate; ●—●, 1% aqueous BSA as substrate; X—X, 0.5% aqueous BSA as substrate.

(human pepsinogen III) (Seijffers *et al.*, 1963b), were pooled. Human pepsin III was prepared from these pooled eluate fractions as follows: 2 N HCl was added until a *p*H of 2.0 was reached, and the solution was allowed to incubate for 5 minutes at 20°. The acidity was then adjusted with 2 M sodium acetate to *p*H 4.0 and the solution was left for 60 minutes at this *p*H (Seijffers *et al.*, 1964). After dialysis in the cold vs. 0.1 M acetate buffer, *p*H 5.3, the solution was applied by gravity to a DEAE-cellulose column previously conditioned with 0.1 M acetate buffer, *p*H 5.3. Elution, effected with NaCl gradient (0.1–0.27 M), resulted in two peaks of proteolytic activity, an early minor peak corresponding to human pepsin-pepsin inhibitor complex (Seijffers *et al.*, 1964) and a major peak corresponding to human pepsin III. Eluate fractions containing pepsin III were pooled and dialyzed in the cold vs. 0.001 M acetate buffer, *p*H 5.4. The resulting dialyzed solution was diluted with 0.001 M acetate buffer, *p*H 5.4, to obtain a pepsin III solution with proteolytic activity giving results in the linear range of the assay method. This proteolytic activity was of the same order as that of a crystalline porcine pepsin solution containing 2.5 μg pepsin/ml. The protein content of the purified pepsin III solution used in these studies was too low to measure either by absorption at 280 mμ or by available chemical micromethods. The same solution of enzyme was used for all experiments.

Substrate.—BSA (bovine serum albumin, fraction V, Armour and Co.) was dialyzed repeatedly against distilled H_2O at 4° and a 2.5% (w/v) aqueous stock solution made. The BSA substrate solutions were made by diluting this stock solution with water or sodium chloride solutions as required. The following BSA solutions were used as substrate:

Aqueous BSA: 0.5%, 1%, and 2.5% BSA in aqueous sodium chloride: 0.5% BSA in 0.0025 M NaCl; 0.5% BSA in 0.25 M NaCl; and 0.5% BSA in 0.5 M NaCl.

Determination of Proteolytic Activity at Various pH Values.—In a typical analysis 4 ml of BSA substrate solution and 1 ml from each of a series of dilute HCl solutions (ranging from 2.8 N to 0.0006 N) were mixed and allowed to equilibrate at 37°, and 1 ml enzyme solution was added. These mixtures (ranging in *p*H from 0.34 to 5.0) were incubated for 60 minutes at 37° and the reaction was terminated by the addition of 9 ml 0.3 M trichloroacetic acid. All determinations on test solutions, appropriate blanks, and on tyrosine standards were done in duplicate. The trichloroacetic acid supernatants were assayed colorimetrically for tyrosinelike substance with Folin's phenol reagent as follows: To 1 ml supernatant was added 3 ml 0.5 N NaOH followed by 1 ml phenol reagent; after color development optical densities were measured at 535 mμ with a Coleman Junior spectrophotometer. Whenever the term "proteolysis" is mentioned in this report, it is used with the understanding that it is measured in terms of trichloroacetic acid–soluble tyrosinelike substance only. All *p*H adjustments were made only with HCl, as a result of which the reaction mixtures contained variable amounts of chloride ion, but no added buffering ions except the very dilute acetate buffer in which the pepsin was dissolved. Determinations of *p*H at the beginning and end of the 60 minutes' incubation were done on parallel aliquots consisting of 4 ml BSA substrate, 1 ml enzyme solution, and 1 ml of appropriate HCl solution.

Time Course of Proteolysis of BSA.—The extent of proteolysis of BSA over a 60-minute incubation period was followed by withdrawing aliquots for assay at 1, 5, 10, 20, 30, 40, 50, and 60 minutes after the beginning of the incubation at 37°. Proteolysis at *p*H 1.5 and 2.7 of 0.5% aqueous BSA and 0.5% BSA in 0.25 M NaCl were studied in this fashion. The chloride present in these solutions from the HCl added for *p*H adjustment was 0.0374 M for *p*H 1.5 and 0.0054 M for *p*H 2.7. Accordingly the final chloride concentrations were at *p*H 1.5, 0.2035 M and 0.0374 M, and at

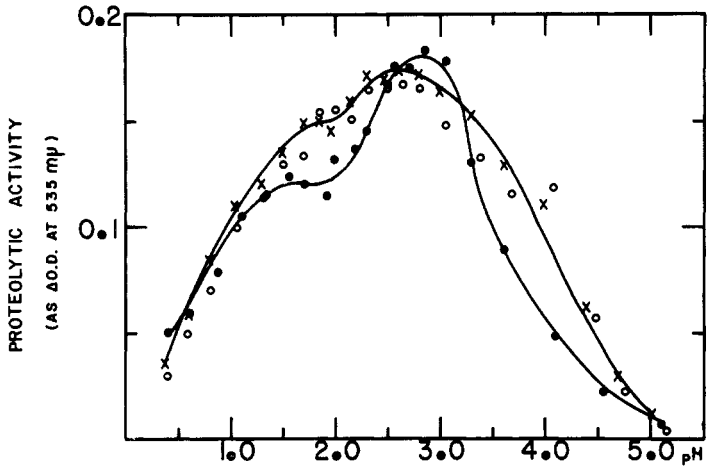

FIG. 2.—Effect of NaCl concentration on proteolysis of BSA. Proteolytic activity of Pepsin III as ΔOD at 535 mμ is plotted vs. *p*H of incubation mixtures. X—X, 0.5% aqueous BSA in 0.25 M NaCl; O—O, 0.5% aqueous BSA in 0.5 M NaCl; ●—●, 0.5% aqueous BSA in 0.0025 M NaCl. Experimental points for the proteolysis of 0.5% aqueous BSA in 0.5 M NaCl were not connected by a fluent line for the sake of clarity.

*p*H 2.7, 0.1729 M and 0.0054 M for BSA with and without added NaCl, respectively.

RESULTS

Effect of Substrate Concentration on Proteolysis of BSA.—The influence of substrate concentration on proteolysis of BSA by human pepsin III is depicted in Figure 1; proteolytic activity as ΔOD at 535 mμ is plotted against *p*H of incubation mixtures. The average *p*H obtained from measurements at the beginning and end of the 60-minute incubation period did not differ by more than 0.1 *p*H unit from either; this average was presumed to represent the *p*H of the incubation mixtures. With 0.5% aqueous BSA as substrate proteolysis is optimal between about *p*H 2.6 and 2.9, while a well-defined "shoulder" centers at *p*H 1.5.

In contrast, the activity vs. *p*H curve with 2.5% aqueous BSA as substrate shows a rather broad and symmetrical *p*H-activity curve with a maximum at about *p*H 2.0, and conspicuous absence of the higher activity noted at *p*H 2.3–3.3 with 0.5% aqueous BSA as substrate.

The use of 1% BSA as substrate results in an activity vs. *p*H curve having a maximum between *p*H 2.5 and 2.9 and a "shoulder" centering at *p*H 1.5. Comparison with the activity vs. *p*H curve of 0.5% BSA as substrate reveals decreased proteolysis in the *p*H range 2.3–3.3 and a variable increase in proteolysis below *p*H 2.3.

Effect of Sodium Chloride on Proteolysis of BSA.—The influence of the addition of sodium chloride to a 0.5% BSA substrate solution on proteolysis of BSA is shown in Figure 2.

An increased sodium chloride concentration results in activity vs. *p*H curves grossly similar to those obtained with increased BSA concentrations. The activity vs. *p*H curve with 0.5% BSA in 0.25 or 0.5 M NaCl as substrate is almost symmetrical, shows maximal activity between *p*H 2.4 and 2.8, and resembles the curve with aqueous 2.5% BSA as substrate (Fig. 1); the optimal *p*H has shifted to a slightly higher value compared to that seen with 2.5% BSA in H_2O. Increased proteolysis between *p*H 2.3 and 3.3 (relative to proteolysis at *p*H 1.5) such as was noted with 0.5% aqueous BSA as substrate is virtually absent. The activity vs. *p*H curve with 0.5% BSA in 0.0025 M NaCl has a well-defined "shoulder" at *p*H 1.5 and maximal activity between *p*H 2.6 and 3.0. Compared with the activity vs. *p*H curve of 0.5% aqueous BSA (Fig. 1) a distinct decrease in proteolysis is present over the *p*H range 2.3–3.3. This decrease in proteolysis results in a resemblance between the activity vs. *p*H curve with 0.5% BSA in 0.0025 M NaCl (Fig. 2) and of 1% aqueous BSA (Fig. 1) as substrate. Over this *p*H range of relatively decreased proteolysis, i.e., 2.3–3.3, 0.5% aqueous BSA in 0.0025 M NaCl and 1% aqueous BSA incubation mixtures have almost equal chloride concentration. Pertinent for later discussion are the chloride concentration ranges in incubation mixtures (4 ml substrate + 1 ml dilute HCl + 1 ml enzyme), calculated from the sodium chloride content of BSA (if present) and the HCl concentration used for *p*H adjustment over the *p*H range 2.3–3.3 (see Table I).

TABLE I
CHLORIDE CONCENTRATION RANGE OF INCUBATION MIXTURES[a]

Substrate	Chloride Concentration Range (moles/liter) *p*H 2.3		*p*H 3.3
0.5% BSA in H_2O	0.0091	—	0.0025
1% BSA in H_2O	0.0131	—	0.0044
2.5% BSA in H_2O	0.0248	—	0.0112
0.5% BSA in 0.0025 M NaCl	0.0112	—	0.0045
0.5% BSA in 0.25 M NaCl	0.1760	—	0.1704
0.5% BSA in 0.5 M NaCl	0.3427	—	0.3370

[a] The range of chloride concentrations in incubation mixtures, composed of 4 ml BSA substrate (indicated in first column), 1 ml of HCl of varying normality, and 1 ml of pepsin III in 0.001 M acetate buffer, *p*H 5.4, is indicated in the second column of the table. These final chloride concentrations of incubation mixtures ranging in *p*H from 2.3 to 3.3 were calculated from the normality of HCl used for *p*H adjustment, and NaCl content, if any, of BSA substrate.

It is unlikely that the above-mentioned effects of NaCl are due only to the particular technique employed, since addition of NaCl to trichloroacetic acid in the concentration range used had no effect on assay of tyrosinelike material.

Time Course of Proteolysis of 0.5% BSA at pH 1.5 and pH 2.7 with and without Addition of Sodium Chloride

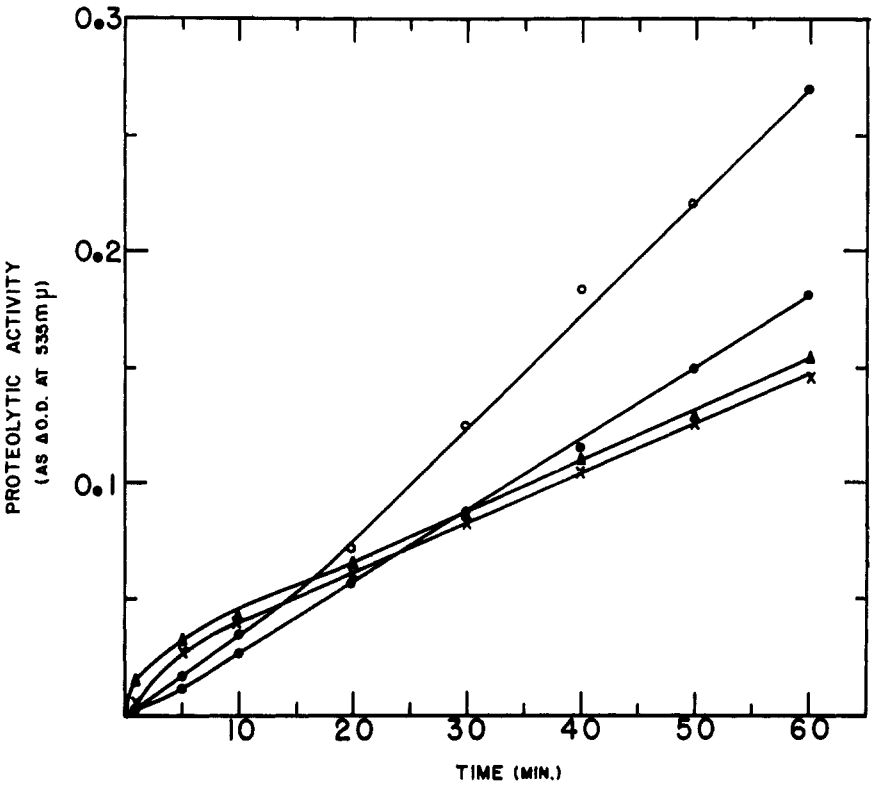

FIG. 3.—Time course of proteolysis of BSA. Cumulative proteolysis of BSA as ΔOD at 535 mμ is plotted vs. time of incubation. O—O, 0.5% aqueous BSA at *p*H 2.7; ●—●, 0.5% aqueous BSA in 0.25 M NaCl at *p*H 2.7; ▲—▲, 0.5% aqueous BSA at *p*H 1.5; X—X, 0.5% aqueous BSA in 0.25 M NaCl at *p*H 1.5.

to the Solution.—The proteolytic activity vs. time curves in Figure 3 corroborate the inhibitory effect of 0.25 M NaCl on proteolysis of 0.5% BSA. The effect is slight at *p*H 1.5 and more pronounced at *p*H 2.7, and accounts for the decreased proteolysis between *p*H 2.3 and 3.3 in the presence of NaCl depicted in Figure 2 as compared to 0.5% BSA curve of Figure 1.

These time studies show, furthermore, that even the lowest substrate concentration used (0.5% BSA) was sufficient to allow almost linear progression of proteolysis during 60 minutes' incubation.

DISCUSSION

The present study reveals a pronounced apparent effect of substrate concentration on proteolysis of BSA by human pepsin III as assayed by the liberation of trichloroacetic acid–soluble tyrosinelike material. Limitations of the use of a single method for determination of proteolysis have been emphasized (Christensen, 1955b). Ideally the progress of proteolysis should be followed by as many methods as possible in order to visualize the complexity of proteolysis (Sri Ram and Maurer, 1957). Unquestionably, the assay method used did not discern more subtle intermediate phases of proteolysis of BSA but afforded a comparison of the over-all results of proteolysis.

High substrate concentrations have been described in a few special cases to produce an inhibitory effect on their breakdown by enzymes (Dixon and Webb, 1958). We are not aware that such has been reported for protein substrates. However, such a possibility cannot be completely ruled out as an explanation for effects of substrate concentration shown in Figure 1. Conceivably a nondialyzable enzyme inhibitor could be contaminating the BSA, and at high substrate concentration have an obvious inhibitory effect. Our attempts to remove possible dialyzable inhibitors in BSA failed to alter the extent of proteolysis.

BSA substrate (0.5%) required less HCl to adjust the *p*H of incubation mixtures from *p*H 3.3 to *p*H 2.3 as compared with 1% and 2.5% BSA (see Table I). As a result, incubation mixtures containing various BSA concentrations in the same *p*H range differed apparently only in chloride concentration. This led to the hypothesis that the higher chloride concentration was responsible for the decrease of proteolysis of 1% and 2.5% BSA between *p*H 2.3 and 3.3. The hypothesis was tested by the addition of sodium chloride to BSA substrate and is supported by the results obtained as evidenced by a comparison of the 0.5% BSA curve of Figure 1 with curves of Figure 2, and comparison of chloride concentrations (Table I). From Figures 1 and 2 and the data on chloride concentration of Table I, one may surmise that the chloride effect is already maximal with 0.5% BSA in 0.25 M NaCl. Similar results have recently been obtained by addition of potassium nitrate to BSA solution (unpublished observations). The latter suggest that the ionic concentration effect is not specific.

Lack of reports from others that substrate concentration is a factor in proteolysis of BSA may be due to a number of factors. Adjustment of *p*H is rarely made with strong acid only. Buffers are normally employed and even different buffers may be used to cover one *p*H range. Conceivably use of a buffer could tend to suppress proteolysis over the *p*H range 2.3–3.3 in proportion to its ionic strength. Another factor may be that in the past almost exclusively animal pepsins have been used to study the proteolysis of BSA.

The presence of a "shoulder" at *p*H 1.5 and a *p*H optimum between *p*H 2.6 and 2.9 in the configuration of the proteolytic activity vs. *p*H curve with 0.5% aqueous BSA as substrate (Fig. 1) could be interpreted

to mean that we are dealing with two more or less independent proteolytic processes, one digesting BSA at *p*H 1.5, and the other between *p*H 2.6 and 2.9, with the latter process alone being inhibited by an increase in chloride concentration. Although such a possible dual proteolytic process could be caused by lack of homogeneity of BSA substrate or pepsin preparation used, it could equally well be related to a single enzyme acting on a single substrate over two *p*H ranges. Such a process would be in agreement with the findings of two different *p*H ranges for the hydrolysis of different synthetic substrates by pepsin (Baker, 1951; Fruton and Bergmann, 1939), and, among other factors, could be related to the presence of two active centers in the enzyme.

At present it is not possible to visualize exactly how substrate concentration and chloride concentration affect proteolysis of BSA by human pepsin III. In our hypothesis the effect of substrate concentration has been related to the chloride concentration of enzyme-BSA-incubation mixtures. Some reports concerning the mode of action of proteolytic enzymes on a protein substrate and the behavior of BSA in acid medium tend to support this interpretation of our experiments. Christensen has suggested that hydrogen ion concentration may be of importance to peptic hydrolysis of native protein sybstrates by effecting an unfolding of coiled peptide chains. This would make peptide bonds more readily accessible to enzymes (Christensen, 1955a). Of equal interest in this regard is a report of Yang and Foster (1954) who measured changes in intrinsic viscosity and specific rotation of BSA in acid medium. They found a parallel rise in both intrinsic viscosity and specific rotation (extrapolated to zero protein concentration) below *p*H 4.0, with a maximum between *p*H 2.2 and 2.7. The height of this maximum was markedly depressed by the addition of NaCl. As a basis for the observed changes, they suggested a reversible expansion of the BSA molecules in this *p*H range, decreased by addition of electrolyte.

In view of the above it is tempting to suggest that the increased proteolytic activity between *p*H 2.3 and 3.3 in our experiments with 0.5% aqueous BSA as substrate is related to an expansion of the BSA molecule, making peptide bonds more accessible to hydrolysis over this *p*H range. Correspondingly, the observed depression of the *p*H optimum by higher substrate concentration and/or increased chloride concentration would be referable to a reduction in this reversible expansion.

The activity vs. *p*H curve for the digestion of BSA by an animal pepsin reveals, under slightly different experimental conditions, at low substrate concentration and low ionic strength, a single *p*H optimum at about *p*H 1.5 (Schlamowitz and Peterson, 1961). The action of that animal pepsin on BSA may therefore be different from human pepsin III. It is nevertheless of interest that the proteolysis of BSA by animal pepsin is also significantly affected by addition of sodium chloride (Schlamowitz and Peterson, 1961).

One may speculate that by varying substrate concentration and chloride concentration either one or two *p*H optima could be demonstrated for proteolysis of BSA. This could be a factor in the existing controversy (Christensen, 1955b) regarding the presence of one or two *p*H optima for proteolysis of proteins by pepsin. It also renders hazardous some interpretations (Taylor, 1959) of *p*H optimum curves for elucidation of mechanisms of proteolysis. For comparison of proteolysis data from different experiments, pepsin source, ionic composition of incubation mixture, type of substrate and its concentration must all be taken into consideration.

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