Studies on the Optimum pH for the Action of Pepsin on "Native" and Denatured Bovine Serum Albumin and Bovine Hemoglobin*

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Several studies (2-6) of the hydrolysis of proteins by proteolytic enzymes have led to the suggestion that only denatured forms of the substrate are acted on; and that where an equilibrium between native (N) and denatured (D) forms, N \rightleftharpoons D, is not spontaneous, the initial action of a protease may be a non-hydrolytic denaturation of native substrate.

Green and Neurath (7) have suggested that native as well as denatured proteins can serve as protease substrates, a view also held by Chernikov (8). A simplified version of the scheme proposed by Green and Neurath to depict these ideas is shown below,

\[
\begin{align*}
D & \rightarrow \text{Products} \\
N & \rightarrow N' \rightleftharpoons D' \rightarrow \text{Products}
\end{align*}
\]

where \( N \) and \( D \) refer to native and denatured protein. \( N' \) designates the product of limited proteolysis of \( N \); and \( D' \), its denatured form, is considered like \( D \), to be susceptible to extensive proteolytic action. The \( N \rightleftharpoons D \) and \( N' \rightleftharpoons D' \) conversions may be either spontaneous or induced by environmental conditions.

The significance of this scheme is that all the postulated pathways are multistage processes, any one of which may be rate limiting. Insofar as pH may affect the various steps unequally, it would appear that the optimum pH for the action of a protease may be in essence an apparent one.

It is conceivable that for substrates where the \( N \rightleftharpoons D \) step is rate limiting, the use of denatured substrates would result in a shift in the optimum pH of proteolysis. This is particularly relevant to the proteolytic action of pepsin, generally accepted to be optimal in the range of pH 1.5 to 2.2 (7, 9, 10), a pH where most proteins may be presumed to be in a denatured state. For this reason, a re-evaluation of the apparent optimum pH for the proteolytic action of pepsin on native and denatured substrates was undertaken.

Christensen (6) has shown that the optimum pH for the action of pepsin on native ovalbumin was pH 1.0, for the heat-denatured substrate it was pH 1.5, and for the urea-denatured substrate, pH 1.8. Optimal activity against hemoglobin was found at about pH 1.5 to 2.0, but the effects of denaturation conditions other than those achieved by the pH values used to estimate optimal pH were not investigated. With casein too, where a broad maximum was observed in the pH-activity curve, other denaturation conditions were not studied. Sri Ram and Maurer (11) reported changes in hydrolysis rates but no significant change in pH optimum (pH 2.0) for the action of pepsin on heat-treated, acetylated, guanidinated, and deaminated bovine serum albumin.

In the present study bovine serum albumin and hemoglobin were used. These proteins are known to undergo configurational alterations readily in the presence of hydrogen ions, hydroxyl ions, and hydrogen bond-breaking agents like urea (12-18). It was felt that these properties would make them useful as model substances to establish a dependence of the apparent pH optimum of the action of pepsin upon the secondary and tertiary structural properties of proteins.

Pepsin activity on these proteins after their denaturation with urea (5.4 M), HCl (pH 0.8, 1.0), NaOH (pH 12.5), or combinations of these treatments, was found to be optimal at about pH 3.5. In some cases considerable activity was manifest even at pH 5. Pepsin activity on "native" substrates was optimal at pH 1.7 to 2.0.

The relation of these observations to the structural changes which these proteins are known to undergo and to the action of pepsin on synthetic substrates is discussed.

EXPERIMENTAL

Materials and Methods

Pepsin—Pepsin, three times crystallized, prepared by ethanol fractionation according to Northrop (19), was obtained from Pentex Company, Inc. (Lot No. D 3700, C 3709).

Stock solutions containing 400 \( \mu g \) per ml in 0.005 M acetate buffer, pH 4.0, were found to be stable for at least 4 days when stored in the cold (about 5°). The pH range for optimal stability of pepsin is reported to be between pH 4 to 5 (7, 20, 21). Dilutions of the stock solution with water (usually 1:10) were prepared just before use.

Bovine Serum Albumin—Crystalline bovine serum albumin was obtained from Pentex Company, Inc. (Lot No. 12019P).

* A preliminary report of some of these studies has been presented (1).

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1 The term "native" is used simply to designate proteins, which before their use in pH-activity studies, were not subjected to treatments specifically designed to denature them. It is recognized that at low pH values even the "native" protein is no longer truly native.
Inasmuch as the conditions and extent of denaturation of the protein may influence its behavior as a substrate for pepsin, the preparation of each type of BSA substrate is described.

"Native"-BSA—Solutions of BSA (1%) in water were prepared at room temperature (pH 5.1) and stored in the cold.

HCl-BSA—BSA (1.1%) in water was adjusted with 10 N HCl to pH 0.8. After 10 minutes portions of this solution were adjusted to pH values as required in the pH-activity studies, diluted with water to give a 1% solution of BSA, and used immediately.

NaOH-BSA—This substrate was prepared in an analogous manner to HCl-BSA. However, the pH was adjusted to about pH 12 with 10 N NaOH, and after 15 minutes at this pH, the solutions were adjusted to the desired pH values and used immediately.

Urea-BSA—Solutions of BSA (1%) in urea (5.4 M) were prepared at room temperature, pH 6.1, and stored in the cold.

HCl Urea-BSA—Solutions of BSA (2.2%) in water were first adjusted to pH 0.8 with HCl. After 10 minutes, urea was added, the pH adjusted to 6.3, and the solution diluted with water to give a 1% BSA solution in 5.4 M urea. It was stored in the cold before use.

NaOH Urea-BSA—This substrate was prepared as described for HCl urea-BSA except that the primary adjustment was made with NaOH to about pH 12.5. The pH of the final solution was pH 6.6.

Except for HCl-BSA and NaOH-BSA, all BSA substrates were stored in the cold for 24 hours before use.

Hemoglobin—All hemoglobin substrates were prepared from a stock solution (approximately 5% Hb, dialyzed 16 to 24 hours, and centrifuged) made from bovine hemoglobin substrate powder (Pentex Company, Inc., Lot No. 1700).

"Native"-Hb—A solution of Hb (2.2%) in water, pH 6.9, was prepared by dilution of the stock solution.

HCl-Hb—A 2.2% solution of Hb in water was adjusted with HCl to pH 1.0 and allowed to stand at room temperature for 1 to 2 hours. Portions of the solution were then adjusted to the desired pH values and used immediately.

Urea-Hb—A solution of 2.2% Hb in 5.4 M urea, pH 7.2, was prepared by the appropriate addition of urea and water to the stock solution.

HCl Urea-Hb—A stock solution was adjusted with HCl to pH 1.0. It was allowed to stand for 10 minutes, urea was added, and it stood for another 30 minutes at room temperature, after which it was adjusted with NaOH to pH 5.9 and diluted. The final concentrations were 2.2% Hb and 5.4 M urea.

NaOH Urea-Hb—Except for initial adjustment of the pH of the stock solution to about pH 12.5 instead of pH 1.0, this substrate was prepared in the same way as HCl urea-Hb.

Like the BSA substrates, all Hb substrates except HCl-Hb were stored in the cold for 24 hours before use.

**Determination of Proteolytic Activity**—Enzyme-substrate digestion mixtures were deproteinized with trichloroacetic acid. The digestion products in the trichloroacetic acid filtrates were analyzed by a modification of the colorimetric procedure with Folin's phenol reagent (22). Optical densitometry was measured in the Klett photoelectric colorimeter (660 micrometer filter) against controls in which the enzyme was omitted from the reaction mixture. In some experiments, the optical density of the trichloroacetic acid filtrates at 278 micrometer (Beckman model DU spectrophotometer) was used to measure the extent of proteolysis. Results obtained by the two methods paralleled each other.

In a typical analysis, 2 ml of substrate solution (1% BSA, or 2.2% Hb) were adjusted to the desired pH with HCl or NaOH. Buffer (0.50 ml of 0.05 M glycine buffer for pH 1.0 to 3.5, or 0.50 ml of 0.05 M acetate buffer for pH 4.0 to 5.5) and water were added and finally, after equilibration at 37°, the enzyme was added. These reaction mixtures (3.0 ml) were incubated at 37° for 10 minutes (except when time studies were done). Five milliliters of 5% trichloroacetic acid were added to stop the reaction and to deproteinize the solutions. For all cases where "native" and urea-treated substrates were compared, deproteinization of the "native"-protein mixtures was done with trichloroacetic acid solutions which contained urea (2.18 M) so that the final concentration of urea in the deproteinization mixtures from both "native" and urea-treated substrates was the same (1.35 M). After standing for 10 minutes at 37° the mixtures were filtered through Whatman No. 3 paper (once for Hb, three times for BSA). Portions of 2 ml of the clear filtrates were then analyzed by the aforementioned methods.

**RESULTS**

*Action of Pepsin on "Native"-BSA and "Native"-Hb*—The optimum pH for the action of pepsin on "Native"-BSA was found to be about pH 1.7 (Fig. 1), whether deproteinization of the reaction mixture was carried out with aqueous trichloroacetic acid or with trichloroacetic acid-containing urea. However, in the presence of urea, the amount of cleaved products which appears in the protein-free filtrate is approximately 2.5 times as great as in the filtrates prepared in its absence. This is true at all the pH values, so that neither the shape of the curve nor the pH for optimal activity is appreciably affected. This relationship is significant for it establishes that the shifts in optimal pH, to be described later for urea-treated substrates, cannot be ascribed to any change in the selection of cleaved products precipitated by trichloroacetic acid when urea is present.

The possibility that the increased amounts of material in the urea-containing trichloroacetic acid filtrate could be the result of a failure of trichloroacetic acid to precipitate the products of digestion was investigated in a series of control experiments. Reaction mixtures of BSA where pepsin was added immediately after the addition of trichloroacetic acid, and of partial digests of BSA where trichloroacetic acid was added after a 10-minute incubation with pepsin, were allowed to stand at 37° and were analyzed at varying times up to half an hour after the addition of the trichloroacetic acid. In no case was there evidence of continued peptic activity. The explanation for the increased amounts of material in urea-containing trichloroacetic acid filtrates of the proteolytic digest appears then to be due to an increase in the solubility of the products of digestion in this medium.

Fig. 1 shows further that at pH 3.5 "Native"-BSA is hydrolyzed only about one-fourth as much as it is at the optimal pH (pH 1.7). At pH 4.0, this value falls to less than one-tenth and at still higher pH values, the activity is even less. These rela-
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NATIVE-BSA, H2O-TCA.
I I, UREA-TCA.

FIG. 1. Activity-pH curves for the action of pepsin on "Native"-bovine serum albumin. Lower curve (O——O), deproteinized with aqueous trichloroacetic acid (TCA); upper curve (X——X), deproteinized with trichloroacetic acid in the presence of 1.35 m urea.

FIG. 2. Activity-pH curves for the action of pepsin on bovine serum albumin. "Native"-BSA (X——X); acid-denatured BSA (O——O); alkali-denatured BSA (●——●).

FIG. 3. Activity-pH curves for the action of pepsin on bovine serum albumin. "Native"-BSA (X——X); BSA denatured with urea (O——O); BSA denatured with acid and urea (●——●); BSA denatured with alkali and urea (△——△).

relationships are important for comparison with the curves for the denatured substrates, to be described later.

Experiments analogous to the one just described for BSA were were carried out with "Native"-Hb. They established that hydrolysis by pepsin is optimal at pH 2.0, that at pH 3.5 the activity is only about 40% of maximal, and that at pH values of 4.0 or higher this figure is 5% or less. These results are in fair agreement with those reported by Christensen (6). As in the case of BSA, it was found that although the amount of cleaved products which appear in the protein-free filtrate is enhanced (1.67-fold) by the presence of urea in the trichloroacetic acid mixture, neither the shape nor the optimum pH value of the pH-activity curve is appreciably effected, and as in the experiments with BSA, the possibility that pepsin activity on Hb continued in trichloroacetic acid in the presence of urea was ruled out.

Action of Pepsin on NaOH-BSA and HCl-BSA—The pH-activity curves obtained with these two substrates are compared in Fig. 2 with the curve obtained for "Native"-BSA, under like conditions of deproteinization (aqueous trichloroacetic acid). It is seen that although pretreatment of BSA with NaOH enhances the susceptibility of the protein to the action of pepsin, the position of optimal pH and the general character of the pH-activity is largely unaffected. This is not so in the case of HCl-BSA, where in contrast with its action on "Native"-BSA, the action of pepsin is characterized by a very broad range of activity, with a maximum at about pH 3. At pH 4.5, where the "Native" substrate is scarcely degraded under the experimental conditions used, the action on HCl-BSA is still approximately 65% of its peak value.

Action of Pepsin on Urea-BSA, NaOH Urea-BSA, and HCl Urea-BSA—Fig. 3 shows the pH-activity curves obtained with...
on the above substrates and on "Native"-Hb are compared insofar as digestion with pepsin was concerned.

By analysis of trichloroacetic acid filtrates in the spectrophotometer at 278 nm.

About pH 2.0. The curve for urea-Hb, on the other hand, shows the shift in apparent optimal pH and the shoulder on the curves, as found by the use of the colorimetric method, were confirmed by analysis of trichloroacetic acid filtrates prepared with acid and urea (A-A); Hb denatured with alkali and urea (D-D).

These urea-denatured substrates compared with the curve for "Native"-BSA. In these experiments, all deproteinizations with trichloroacetic acid were carried out in the presence of urea (1.35 M final concentration). In contrast with the "Native" substrate the maximal activity against all the denatured ones was at about pH 3.5 and was, in addition, almost as great or greater than that of the "Native" substrate at its optimal pH (pH 1.7). At pH 4.0, where "Native"-BSA was almost unaffected, pepsin showed 50 to 80% of maximal activity against the denatured substrates.

The possible significance of the diminished activity against the denatured substrates at around pH 1.5, and of the presence of a shoulder on the acid side of the curves is discussed later. Both the shift in apparent optimal pH and the shoulder on the curves, as found by the use of the colorimetric method, were confirmed by analysis of trichloroacetic acid filtrates in the spectrophotometer at 278 nm.

Urea-BSA from which the urea had been dialyzed out was indistinguishable from "Native"-BSA at both pH 1.5 and 3.5, as far as digestion with pepsin was concerned.

**Action of Pepsin on HCI-Hb, Urea-Hb, NaOH Urea-Hb, and HCI Urea-Hb**—The pH-activity curves for the action of pepsin on the above substrates and on "Native"-Hb are compared in Fig. 4. Deproteinization of the reaction mixtures of urea-containing substrates was carried out with aqueous trichloroacetic acid. For the "Native" and HCI-Hb substrates trichloroacetic acid solutions containing urea were used, so that the final trichloroacetic acid and urea concentrations achieved (3.1% and 1.35 M, respectively) were the same for all cases.

The curve for HCI-Hb does not differ greatly from that of "Native"-Hb, and the optimal pH with both substrates is at about pH 2.0. The curve for urea-Hb, on the other hand, shows activity over a broader pH range, with considerable activity at pH 3.5, and a dip at about pH 2.5. This dip was reproducible and showed up when 2 or 3 μg of pepsin were used, but was obscured when 6 μg were used.

The greatest changes in the nature of the curves appear when NaOH urea-Hb and HCl urea-Hb are used as substrates. With these, a broad range of high activity from pH 1.5 to 4.5 is obtained, with an apparent optimum pH at about pH 3.5. About 50% of maximal activity is shown even at pH 5.5 where the action of pepsin on "Native"-Hb is almost undetectable.

**Effect of Pepsin Concentration and Time of Reaction on Hydrolysis of "Native" and Denatured BSA and Hb**—The purpose of these experiments was to determine whether the shift in optimal pH shown in the pH-activity curves for denatured substrates was unique for the particular combination of enzyme concentration and incubation period (10 minutes) used, or whether they applied over broader ranges of concentration and time (i.e. extent of protein breakdown). Toward this end the activity-pepsin concentration and activity time of incubation relationships were established for each of the BSA substrates and for most of the Hb substrates.

Representative examples of such studies are the results obtained with "Native"-BSA and urea-BSA, shown in Figs. 5 and 6. Over the ranges tested, these concentration and time studies show the same relationships observed in the pH-activity curves. Thus, the relative susceptibility of these substrates to peptic action is: urea-BSA, pH 3.5 > "Native"-BSA, pH 1.5 > urea-Hb, pH 1.5 > "Native"-BSA, pH 3.5. Furthermore these studies show that the conditions selected for the pH-activity curves (8 μg of pepsin, 10 minutes incubation time for BSA substrates) are on the linear or near linear portions of the concentration and time curves. Analogous studies with the other BSA and Hb substrates showed that for these too the pH-activity curves (Figs. 2 to 4) had been obtained under conditions which were on the linear or near linear portions of their respective enzyme concentration and time curves.

It appears then that the relationships of activity at pH 1.5 and pH 3.5 seen in the pH-activity curves for the various "Native" and denatured substrates are not confined to a single enzyme concentration or incubation time.

**Effect of Trichloroacetic Acid Concentration on Apparent Activity of Pepsin on BSA and Hb**—Although size and composition appear to be among them, the factors which govern the precipitation of proteins and protein cleaved-products by trichloroacetic acid (23, 24), are not all known. A study was therefore made to determine whether the activity-pH relationships already established from analysis of trichloroacetic acid filtrates prepared by deproteinization with trichloroacetic acid at a final concentration of 3.1% would apply as well to filtrates obtained with other concentrations of trichloroacetic acid.

"Native" and urea-treated BSA and Hb, acted on for 10 minutes by pepsin at pH 1.5 and pH 3.5, were precipitated by trichloroacetic acid (5 ml) of such concentrations that the final levels achieved were 2.0, 3.1, 6.2, and 10%. Trichloroacetic acid solutions used for deproteinization of the "Native" substrates contained urea to make them comparable with the urea-substrate systems. The results of such a study (Fig. 7) show that the relationships of "Native"- and urea-BSA at pH 1.5 and 3.5, seen in the pH-activity curves (Figs. 3, 5 and 6), apply over a wide range of trichloroacetic acid concentrations. At the 2% level of trichloroacetic acid, where the slope of the curves is very
Fig. 5. The effect of enzyme concentration on the action of pepsin on bovine serum albumin. "Native"-BSA, pH 1.5 (O-O); "Native"-BSA, pH 3.5 (■■■■); BSA denatured with urea, pH 1.5 (△-△); BSA denatured with urea, pH 3.5 (▲-▲).

The shape of these trichloroacetic acid precipitation curves suggests a distribution in the size of products formed during peptic action. This, coupled with the observation of a lag in the early phases of the concentration and time curves (Figs. 5, 6), and the marked difference in appearance of the precipitates of control BSA and BSA after only brief exposure to pepsin, all suggest that intermediates occur and perhaps accumulate during the peptic digestion of BSA. This aspect of the mechanism of peptic action will be the subject of a separate communication.

A comparable study with "Native"-Hb and urea-Hb (not shown) indicated that for Hb, too, the relationships observed in the pH-activity curves (Fig. 4) are valid over a wide range (2 to 10%) of trichloroacetic acid concentrations used for deproteinization.

Influence of Urea and pH on Stability of Pepsin—Pepsin solutions were exposed for at least 10 minutes in media simulating actual experimental conditions (3.6 M urea, pH 1.5 and 3.5, 37°C) but in the absence of substrate. The activity of these pepsin solutions when tested against both "native" and urea-denatured substrates at pH 1.5 and 3.5 was found to be the same as that given by untreated pepsin solutions. This stability is also indicated by the activity-time response of the enzyme against BSA substrates (Fig. 6), where it is seen that activity continued in a linear fashion for at least 30 minutes. These results also indicate that the differences in the action of pepsin against "native" and denatured substrates (Figs. 2 to 6) is not attributable to an
irreversible alteration in the pH activity characteristics of the enzyme.

These results are in accord with results of studies on other properties of pepsin, e.g., crystallization from solutions of 6 M acetic acid (25), the constancy of its sedimentation constant in water or in strong urea solution (20), the constancy of its optical rotation over the range pH 1 to 6.5 (27), and the slow denaturing action of 3.6 M urea on pepsin at 37°, calculated from the work of Perlman (28).

**Action of Pepsin on “Native”-BSA and urea-BSA as Determined by Analysis of Trichloroacetic Acid Precipitates**—The dangers inherent in basing an interpretation of proteolysis on results obtained by use of a single analytical procedure are recognized. For example, it is conceivable that the distribution of tyrosine and tryptophan residues along the peptide chain of BSA and Hb might be grossly nonuniform, and that the native and denatured substrates might be cleaved by pepsin at different points along the chain. Under these circumstances, granting even that the particles screened by trichloroacetic acid are of uniform size, the tyrosine and tryptophan contents of trichloroacetic acid filtrates from partial peptic hydrolyses of the various substrates might differ widely, even if the number of bonds cleaved was the same. It is clear that results arising from such a set of circumstances could erroneously be interpreted as increased rates of peptic action and shifts in the optimal pH for peptic action.

If the possibility considered above is true it should be revealed by a study of the insoluble residues from the trichloroacetic acid filtrates. Firstly, these residues, when analyzed for total protein and polypeptide material should show little or no differences. Secondly, the tyrosine and tryptophan content per unit protein and polypeptide substance derived from the various substrates should be different and should bear an inverse relation to the content of tyrosine and tryptophan content of the corresponding filtrates.

“Native” and urea-BSA were digested at pH 1.5 and 3.5 (8 µg of pepsin for 20 minutes), deproteinized (final concentration of trichloroacetic acid, 3.1%; urea, 1.35 M), and the deproteinization mixtures centrifuged. The supernatant solutions were analyzed for their tyrosine and tryptophan content in the Beckman spectrophotometer at 278 µ. Precipitates prepared in a similar manner were dissolved in 35% urea (30) and analyzed for their tyrosine and tryptophan content in the Beckman spectrophotometer at 278 µ. Precipitates of the undigested substrates were also analyzed by these procedures.

From these experiments it was found that at pH 3.5 the digestion of “Native”-BSA was 23% that of urea-BSA when calculated from the biuret analysis of the trichloroacetic acid residues, and 18% when calculated from analysis of the respective trichloroacetic acid supernatant solutions by the phenol method. These values are in relatively good agreement with each other and with the value, 19% estimated from the pH-activity data for these substrates (Fig. 3). Furthermore, the compositions of the trichloroacetic acid residues, expressed in empirical units as the ratio of ultraviolet-absorbing material (tyrosine and tryptophan) to biuret-reactive material (protein and polypeptide), were similar to each other and to the composition of the trichloroacetic acid precipitate from undigested protein (48, 50, and 43 units, respectively, for the precipitates of digests of “Native”-BSA, urea-BSA, and undigested BSA).

In a like manner, analysis of residues and supernatant solutions obtained after the action of pepsin on “Native” and urea-BSA at pH 1.5 were in good agreement and reflected the relation shown in Fig. 3 (digestion of urea-BSA is about 40% that of “Native”-BSA).

The results of these experiments rule out the operation to any great extent of factors of the type considered above. Instead they lend support to the interpretations already set forth, namely that denaturation of the protein substrates is accompanied by changes in the rates and optimal pH for peptic action in the early stages of digestion.

**Action of Pepsin on “Native”-BSA and Urea-BSA as Determined by Acidimetric Titration at Constant pH**—Verification of the results of the analysis of trichloroacetic acid filtrates and residues for tyrosine and tryptophan and for total protein and polypeptide was further sought by methods which might reflect a little more directly the cleaving of peptide bonds. The titration of groups, both newly created and unmasked during the course of peptic action on “Native”-BSA and urea-BSA at pH 3.5 and 30° was therefore carried out.

pH measurements were made with a Beckmann pH meter, model G, equipped with extension electrodes. The reaction vessels were contained in a constant temperature bath at 30°, and rapid and continuous stirring was accomplished by rotation of the vessels through a pulley system connected to a stirring motor. The substrate solutions (8 ml of 0.67% BSA in water or 0.67% BSA in 3.6 M urea) were adjusted to pH 3.45 at 30°. The pH was checked for a 15-minute period to be sure that it was constant. Pepsin (24 µg) was then added and the solutions were titrated from a microburet with 0.05 N HCl at brief intervals so that the pH did not rise by more than about 0.02 pH unit from the starting pH.

Results of the average from 2 runs with “Native”-BSA and from 3 runs with urea-BSA are shown in Fig. 8. As a result of the stirring buffer action of the protein itself at this pH, the precision and sensitivity of the measurements leaves much to be desired. This is especially true as more and more buffer ions (carboxyl groups) are produced by the hydrolysis. Even the

![Figure 8](http://www.jbc.org/)
residual buffer capacity of urea, small as it is at this pH, becomes a significant factor in view of its high concentration and the small titrations. Despite this the results of these titrations are in substantial agreement with those obtained from analysis of trichloroacetic acid filtrates and precipitates. Thus, from the titration experiment, the action of pepsin on urea-BSA in the early stages of digestion is about 4.5 times that of its action on "Native"-BSA. From Figs. 3, 5 and 6 and from the analysis of trichloroacetic acid residues described in the previous experiment, values of about 4.0 to 4.7 were found for the ratios of the action of pepsin on the two substrates.

Unfortunately it is not feasible to analyze reliably the titration data in terms of the number of peptide bonds cleaved. The pK values of the newly formed carboxyl groups in the cleaved products are not known, nor is the contribution to the titration due to changes in the pK values of the carboxyl groups of aspartic and glutamic acids, as these groups are unmasked during hydrolysis. The latter factor would be particularly relevant to an analysis of data of studies where the substrate was "Native"-BSA.

Comparison of Action of Pepsin and Gastricsin on BSA and Hb—The presence of other proteases besides pepsin in noncrystalline preparations of pepsin as well as in gastric mucosa and gastric juice has been indicated by the work of Pope et al. (31), Buchs (32), Merten (33), and Richmond et al. (34). Whether such proteases are unique entities or simply active derivatives from pepsinogen or from pepsin itself remains to be established, but the optimal pH for their action is claimed to be in the region of pH 3.0 to 3.5. It was therefore important to rule out any heavy contamination by them in the crystalline pepsin used in the present studies, even though the electrophoretic data of Merten has already shown that contamination of similar crystalline pepsins by such proteases is of the order of 5% or less. The following tests were carried out: the action of a twice crystallized pepsin (Worthington Biochemicals Corporation, Lot No. PM 534) and of the three times crystallized pepsin (Pentex Company, Inc.) on both "Native" and urea-BSA were compared at pH 1.5 and 3.5. No differences were found between these two enzyme preparations. Further, the activity of a sample of the three times crystallized pepsin, fractionated with phosphate buffer to remove other proteases, was the same as the unfraccionated enzyme action on bovine Hb or on equine hemoglobin at these pH values. Finally, a sample of chromatographically homogeneous gastricsin, estimated to be 80% pure, was studied for its activity at pH 1.5 and 3.5 on "Native" and urea-BSA and on "Native" and NaOH urea-Hb. The results were compared with the action of pepsin under similar conditions (i.e. on linear portions of their respective concentration curves) and are shown in Fig. 9. The two enzyme preparations differ in their activity against each substrate and also in response to the influence of urea, especially at pH 3.5, the region in question. Hence, the strong activity of pepsin at pH 3.5 against the denatured BSA and Hb cannot be attributed to contamination with gastricsin or a gastricsin-like enzyme. However, this study does not exclude the possibility that crystalline pepsin is contaminated with gastric proteases of a type different from gastricsin.

The foregoing experiments demonstrate that whereas the apparent optimum pH for the action of pepsin on "native" bovine serum albumin and hemoglobin is at pH 1.7 to 2.0, it is shifted to about pH 3.5 when the substrates have been suitably denatured. In several instances considerable activity is manifest at pH values well above pH 3.5, in the region where the enzyme is essentially without action on the "native" proteins. The differences in the pH-activity curves for the different denatured substrates indicate that the denatured forms produced by the various denaturation procedures are not identical.

In terms of the schemes for proteolysis (cf. "Introduction"), these findings suggest that the rate-limiting steps in the over-all action of pepsin on the "native" proteins are N = D or N' = D', or both N = D and N' = D', and that the role of the denaturants used has been to reduce or eliminate the limitations imposed by these steps. The low optimum pH for the action of pepsin against "native" proteins seems then to be only an apparent one, resulting from the elimination of these restrictive steps by the denaturing action of the acid conditions. Many of the facts concerning BSA and Hb and alterations in their structure under conditions related to the ones used in the present study are consistent with these views. Thus, for BSA, isomerization of the native protein to an "expandable" form is known to occur between pH 4.5 and 3.5. Upon further acidification this "expandable" form expands reaching its maximum (about 20-fold calculated for zero protein concentration and ionic strength) at about pH 2.0 to 2.5 (12, 15, 38, 39). This pH is seen to be in the region of optimal activity for pepsin on "native" proteins. Studies of BSA in urea solutions have led to similar conclusions (12, 13, 16, 40-43). However, in urea the isomerization and expansion are shifted up by roughly 1 pH unit. Interestingly, in the present experiments, it will be recalled that the apparent

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Fig. 9. A comparison of the activities of pepsin and gastricsin on bovine serum albumin and hemoglobin (native and denatured, tested at both pH 1.5 and 3.5).

DISCUSSION

We wish to thank Dr. R. E. Trucco for providing us with details of the unpublished fractionation procedure.

The authors are indebted to Dr. R. E. Trucco for the sample of gastricsin used in this study.

Molecular association or dissociation have been ruled out (12, 35-37).
optimum pH against the various urea-denatured substrates was shifted up to about pH 3.5. Hence in both cases a correlation is seen between the pH for optimal activity of pepsin and the pH where the proteins have become greatly expanded or unfolded.

For bovine hemoglobin, the picture is complicated by the numerous changes which this protein undergoes. Apart from the dissociation of Hb (bovine, horse, human) into half-molecules, which takes place at pH 5 to 6 (44, 45), there is an unmasking of titratable groups and a change in shape or size between pH 4.5 and pH 3.1 (17, 18). At still lower pH values more drastic structural alterations of the protein occur and it may even be cleaved into its heme and globin moieties (45, 46). In the present study the most profound effects on apparent optimum pH of pepsic action were achieved with Hb exposed to conditions of high or low pH plus urea, where such multiple changes may have occurred. This substrate is obviously a more denatured hemochromogen than the altered form, described by Steinhardt et al. (17), which exists at values around pH 3 to 4.

In the preceding discussion the postulated restrictions, whose removal is accomplished by hydrogen bond-breaking agents (urea) and by conditions which favor intramolecular repulsions by like charges (low or high pH), are considered to be largely steric in nature. It must be recognized, however, that inseparable from the stearic changes will be a redistribution of existing charges and the unmasking of new ones. The role that the charge on the substrate may play in the action of pepsin on proteins has been considered by Northrop (49) and by Sri Ram and Maurer (11).

It may be pointed out that restrictions on extensive proteolysis imposed by the $N \rightarrow D$ and $N' \rightarrow IV$ steps, shown in these studies, might be turned to advantage. They open up possibilities of a far greater application of pepsin, at pH values removed from its acid optimum, for studies of controlled hydrolysis (N $\rightarrow$ N') of proteins. The fragmentation of antibodies by pepsin at around pH 4 to yield active fragments (90, 51) may be cited as an example of this application.

The present work also suggests that various groups of peptide bonds in the protein molecule are optimally split at different pHs. Thus, the shoulder on the pH-activity curves for urea-BSA, HCl urea-BSA and NaOH urea-BSA substrates, the dip at about pH 2.5 in the curve for the Urea-Hb substrate, and the great breadth of the curves for the HCl-BSA, HCl urea-Hb, and NaOH urea-Hb substrates are all consistent with this idea.

* The lower activity observed for the urea-denatured BSA substrates at the low pH values (pH 1.0 to 1.5), compared with "Native"-BSA, may be due to the high ionic strengths of their solutions. The effect of high ionic strength at these pH values is to reduce any expansion of the protein (12). The contribution to the ionic strength factor to the total effect was not separably quantitated.

In experiments where the effects of denaturation by prior exposure to low or high pH without or in conjunction with urea were studied at pH 3.5, the contribution of the ionic strength factor to the total effect was not separably quantitated.

A comparative study of the action of pepsin on "native" and denatured bovine serum albumin and hemoglobin is presented.

1. The apparent optimum pH for the action of pepsin on the "native" substrates was pH 1.7 for bovine serum albumin and pH 2.0 for bovine hemoglobin.

2. A shift in the apparent optimum pH to about pH 3.5 could be produced by suitable denaturation of both substrates with HCl, urea, or combinations of acid and urea or alkali and urea. In many instances, considerable activity could be demonstrated at pH values of 4.5 or higher, where the "native" substrates are refractory to the action of pepsin.

3. As an explanation for these changes, artifacts arising from the use of selected conditions of enzyme concentration, incubation period, or trichloroacetic acid deproteinization, have been ruled out. Such factors as instability of pepsin, errors arising from the analytical procedure, and contamination with gastrin have also been eliminated.

4. The results are discussed in terms of rate-limiting steps in peptic proteolysis and in terms of the peptide bond specificity of pepsin.

REFERENCES


Studies on the Optimum pH for the Action of Pepsin on "Native" and Denatured Bovine Serum Albumin and Bovine Hemoglobin
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