The Effect of Guanidine Hydrochloride on Crystalline Pepsin*†

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In a previous communication, it was shown that on prolonged exposure to urea at temperatures above 20° pepsin is irreversibly inactivated (1). The loss of activity is most marked in the pH range of 4.6 to 5.6 and is always accompanied by the formation of nonprotein material that originates from autodigestion of the protein.

Since urea is known to affect the secondary structure of proteins by rupturing hydrogen bonds, our studies have been extended to an investigation of the effect on pepsin of another reagent with similar properties, namely guanidine hydrochloride. Although high concentrations of this reagent cause an irreversible loss of the proteolytic activity, its effects on the protein differ somewhat from those of urea and will, therefore, be presented in this report.

EXPERIMENTAL

Materials and Methods—In most of the experiments, samples of Worthington crystalline pepsin, Lot No. 629, with a nitrogen content of 14.9% and a molar extinction coefficient, $\epsilon = 51.7 \times 10^3$ at $\lambda 278$ m\(\mu\), per molecular weight of 35,000, were dissolved in guanidine hydrochloride solutions of the desired pH to give a final protein concentration of 1%. The concentration of guanidine hydrochloride, twice recrystallized from absolute methanol, was varied from 2.0 M to 6.0 M. In the pH range of 3.6 to 5.6, the salt was dissolved in 0.1 N acetate buffers; at more alkaline values, 0.066 M phosphate buffers served as solvents, whereas hydrochloric acid was used in the acid pH range. pH measurements were made with a MacInnes type glass electrode (2) and no attempt was made to correct the observed pH values for the effects of guanidine hydrochloride.

Experimental Procedure—The pepsin solutions were placed in a water bath regulated at 37, 30, or 25°, and portions were withdrawn at the desired time interval. One aliquot was assayed for enzyme activity with the aid of the hemoglobin method (3) and the activity was compared with that of pepsin samples in guanidine hydrochloride-free solutions or with samples in which the guanidine salt had been replaced by sodium chloride. In all of the experiments, the specific activity was expressed as optical density per minute/protein nitrogen and the factor of 6.70 was used for conversion of dry weight to nitrogen. The first order reaction constants of inactivation, $k$, are then derived from the slope of the line obtained by plotting the logarithm of the residual specific activity against time.

A second aliquot of each mixture was deproteinized by the addition of an equal volume of 20% trichloroacetic acid. After immersion in a boiling water bath for 6 minutes, the protein precipitate was removed by filtration, and the amount of nonprotein material was estimated from the optical density, at 276 m\(\mu\), of the filtrate.

Viscosity Specific viscosities were measured at 25° ± 0.01° in an Oswald type viscometer with a flow time for water of approximately 87 seconds. Viscosities of each pepsin solution were obtained at four different protein concentrations in the range of 0.4 to 2% and used to compute the intrinsic viscosities.

RESULTS

Effect of Concentration of Guanidine Hydrochloride and Temperature on Activity of Pepsin—Fig. 1 shows the effect of guanidine at pH 3.4 and 37° on the activity of pepsin. It is apparent that the enzyme in 3.0 M guanidine hydrochloride is only slowly inactivated. However, an appreciable loss of activity occurs if the concentration of the salt is raised to 5.0 M. In 6.0 M guanidine almost complete inactivation has taken place after 30 minutes of contact with the reagent. The loss of activity was the same whether the enzymic assay was conducted in the presence or absence of the reagent and is independent of the protein concentration when the latter was varied from 0.5 to 2.5%.

In a second series of experiments, a concentration of 4.0 M guanidine hydrochloride, pH 3.4, was maintained but the temperature was lowered from 37° to 30° and 25°, respectively. The rate of inactivation decreased considerably. Thus at 37°, the first order rate constant, $k$, is $1.58 \times 10^{-4}$, at 30°, $k$ is $3.06 \times 10^{-4}$ whereas it decreases to $1.50 \times 10^{-4}$ min$^{-1}$ at 25°.

From these results it can be concluded that, as in the case of urea, the inactivation of pepsin in the presence of guanidine salts is irreversible and is a function of the concentration of the reagent, and of the temperature.

Effect of pH—In an attempt to determine whether the proteolytic activity of pepsin is affected by the pH of the solvent, activity measurements were carried out after the protein was exposed to 3.0 and 4.0 M guanidine hydrochloride solutions of various pH values and were compared with the rate of proteolysis of hemoglobin that occurs in aqueous solutions in the pH range of 1.0 to 6.5. As illustrated with the aid of Fig. 2, the rate of

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in the pH range of 4.0 to 5.0 which is the range of maximum stability of the enzyme in acetate buffers. It thus seems that the zone of spontaneous inactivation of the enzyme, namely at pH values more alkaline than 6.0 (4), is shifted to a more acid pH if guanidine salts are present in the reaction mixture. In the case described here, this displacement corresponds to 1.8 pH units. This finding agrees well with that found by Edelhoch (5) who observed a similar shift for pepsin in guanidine hydrochloride below 1.6 M. One should keep in mind, however, that the pH of concentrated guanidine hydrochloride solutions is not strictly comparable with the values measured in aqueous buffers and, therefore, cannot be reduced to a common scale.

Effect of Guanidine Solute on Autodigestion of Pepsin—As previously reported, exposure of pepsin to concentrated urea solutions produces a marked increase in the solubility of the protein in hot 10% trichloroacetic acid. Since the amount of nonprotein material formed was influenced by the pH of the solution, it was attributed to an enzyme-catalyzed autolysis.

Similarly to its behavior in urea, the loss of peptic activity in guanidine hydrochloride solution is also accompanied by the formation of low molecular weight peptides which are soluble in 10% trichloroacetic acid and are dialyzable. In Table I are presented the results of experiments in which the activity of pepsin was measured and compared to the amount of nonprotein material formed after the protein was exposed to 3.0 and 4.0 M guanidine hydrochloride at 37° for 1 hour.

It is apparent from the results given in Table I that the rate of formation of trichloroacetic acid-soluble material parallels the rate of inactivation in the pH range of 1.0 to 4.4. However, after exposure to the reagent for extended periods the loss of enzymic activity exceeds somewhat the formation of low molecular weight peptides. A further point of interest is that at pH values above pH 5.0 where pepsin is inactivated rapidly, only little nonprotein material is formed (see Fig. 3).

Effect of Guanidine Hydrochloride on Other Properties of Pepsin—Having thus demonstrated that pepsin is inactivated if exposed to concentrated guanidine solutions at temperatures above 25°, the intrinsic viscosity of the protein in the presence of this reagent was determined. The results of these measurements

<table>
<thead>
<tr>
<th>pH</th>
<th>3.0 M Guanidine hydrochloride Inactivation</th>
<th>Trichloroacetic acid-soluble material%</th>
<th>4.0 M Guanidine hydrochloride Inactivation</th>
<th>Trichloroacetic acid-soluble material%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2</td>
<td>55</td>
<td>45</td>
<td>100†</td>
<td>not measured</td>
</tr>
<tr>
<td>2.0</td>
<td>10</td>
<td>19</td>
<td>88</td>
<td>62</td>
</tr>
<tr>
<td>2.4</td>
<td>9</td>
<td>9</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>3.1</td>
<td>3</td>
<td>3</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>3.5</td>
<td>1</td>
<td>3</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>4.1</td>
<td>9</td>
<td>7</td>
<td>63</td>
<td>60</td>
</tr>
<tr>
<td>4.4</td>
<td>100†</td>
<td>40</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

* Apparent pH.
† Complete inactivation after 30 minutes.
are given in Table II which also lists those on the optical rotatory properties of the protein reported elsewhere (6, 7).

<p>| Composi-| pH* | φ (g/ml) | [α] | λ* |</p>
<table>
<thead>
<tr>
<th>Solvent</th>
<th>tion</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M acetate</td>
<td>4.64</td>
<td>3.00</td>
<td>63.4</td>
<td>178</td>
</tr>
<tr>
<td>2.4 M Guanidine-acetate</td>
<td>4.44</td>
<td>3.14</td>
<td>62.3</td>
<td>175</td>
</tr>
<tr>
<td>3.0 M Guanidine-acetate</td>
<td>4.44</td>
<td>3.52</td>
<td>62.1</td>
<td>173</td>
</tr>
<tr>
<td>3.0 M Guanidine hydrochloride</td>
<td>3.44</td>
<td>3.54</td>
<td>62.1</td>
<td>173</td>
</tr>
<tr>
<td>4.0 M Guanidine hydrochloride</td>
<td>3.44</td>
<td>3.47</td>
<td>59.7</td>
<td>166</td>
</tr>
</tbody>
</table>

* Apparent pH.

**DISCUSSION**

In this communication it has been shown that the enzymic activity of pepsin in concentrated guanidine hydrochloride solutions depends on a variety of factors, such as concentration of guanidinium ion, pH, temperature, and time of exposure to the reagent. Thus, in 3.0 M guanidine hydrochloride of pH 3.0 to 4.0 at 25°, pepsin is as active as in guanidine-free buffers of the same pH. Moreover, the optical rotatory properties remain unchanged and only a small increase of the intrinsic viscosity occurs. On prolonged exposure to the reagent, however, or at temperatures above 25°, the enzyme is irreversibly inactivated, and in the pH range of 1.0 to 4.4 the loss of activity is accompanied by the appearance of low molecular weight peptides which are a result of the autodigestion of the protein. Pepsin, therefore, differs in its behavior from that of other enzymes, such as ribonuclease, trypsin, chymotrypsin, and papain (8-13). With these proteins, as judged by changes of the optical rotatory properties and of the intrinsic viscosity, high concentrations of urea and guanidine salts bring about an alteration of the secondary structure and their catalytic activity is impaired. Since in the case of pepsin, little effect on the above mentioned properties has been observed, one has to conclude that the folding of this protein differs markedly from that of the other enzymes and that the bonds that maintain the configuration essential for the biological activity of pepsin are not affected.

From the evidence presented here three points become readily apparent which will be discussed with the aid of Fig. 3 in which also recent results obtained with 8.0 M urea are included: (a) The range of maximum stability of pepsin in aqueous solvents extends over a wide pH range, is narrowed to pH 3.0 to 3.5 in 4.0 M guanidine hydrochloride and to pH 3.3 to 4.3 in 8.0 M urea, respectively; (b) Although denaturation of pepsin at extreme acid pH values, i.e., 1.5 has been reported by Northrop (14), the loss of activity in the presence of hydrogen bond-breaking reagents already proceeds rapidly below pH 3.0, and the formation of low molecular weight peptides closely parallels the rate of inactivation and results from the action of intact pepsin on the denatured molecules; and (c) pepsin at pH more alkaline than 6.0 loses its activity spontaneously without the formation of nonprotein nitrogen. If guanidinium ion or urea is present in the reaction mixture, this rapid loss of activity occurs at more acid pH values. It is of interest to note that although the pH zone where inactivation takes place depends on the reagent as well as on its concentration, the maximum rate of inactivation within a small pH interval is independent of the nature of the solvent. Therefore, one may conclude that intramolecular bonds of the same nature are sensitive to the hydroxyl ions of the media and that in the presence of hydrogen bond-breaking reagents the configuration of the pepsin molecules is loosened. Thus, these bonds can dissociate at lower pH values than they do in aqueous solution. Dissociation of hydrogen bonds of the type —COOH . . . . —OOC— may partly account for the effects observed here. One should keep in mind, however, that although urea and guanidinium salts are usually considered to be hydrogen bond-breaking reagents, these reagents may possibly also affect certain types of hydrophobic bonds (15).

In conclusion, we should like to state that although the observations presented in this report do not permit a clear definition of the mechanism and of the factors responsible for the loss of activity, the over all configuration of the molecule is not drastically altered.

**SUMMARY**

1. Pepsin is active in concentrated guanidine hydrochloride solutions of pH 3.0 to 3.5 at 25°. On prolonged exposure to this reagent or on raising the temperature the enzyme is irreversibly inactivated.

2. Although the inactivation at pH 3.0 to 3.5 is slow, it proceeds rapidly outside this pH range. It is accompanied by the formation of low molecular weight peptides resulting from autolysis of the enzyme and the rate of inactivation parallels the rate of autodigestion. At pH values more alkaline than 5.0, the loss of activity is rapid and no nonprotein material is formed.

3. As judged by the small increase of the intrinsic viscosity,
guanidine hydrochloride does not produce a marked unfolding of the pepsin molecule. This is further supported by the observation that no significant changes of the optical rotatory properties, [α] and λ, have been observed in the presence of guanidinium ions.

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