Various properties of crystalline papain have been under investigation in this laboratory for several years in attempts to understand the specificity, mode of action, and other aspects of the behavior of this enzyme (1). Inasmuch as papain must be in its native state to retain its enzymic activity, a knowledge of the role of the amino acid side chains in maintaining conformation is essential. The difference spectrum obtained by comparison of papain in acid and neutral solution exhibits peaks at 279 and 287 μm (2) and is suggestive of the involvement of the phenolic hydroxyl groups in hydrophobic or hydrogen bonding.

Crammer and Neuberger (3) developed a spectrophotometric procedure to investigate the ionization of phenolic groups in ovalbumin and insulin. Similar investigations have since been performed on poly-L-tyrosine (4), serum albumin (5), ribonuclease (6,7), lysozyme (8,9), chymotrypsinogen (10), trypsinogen (11), myosin, and the meromyosins (12). These studies have shown marked differences in the ionization behavior of phenolic groups in various proteins.

This paper presents an investigation of the behavior of the phenolic hydroxyl groups in papain by means of spectrophotometric titration of this protein in the range from pH 5.5 to 14.

**EXPERIMENTAL PROCEDURE**

Papain was prepared by the method of Kimmel and Smith (13). Ovalbumin, twice crystallized, salt-free, was obtained from Nutritional Biochemicals Corporation. Lysozyme was obtained from Armour and Company (Lot No. 003L1). Fresh stock solutions of twice recrystallized, salt-free lyophilized papain were prepared each day and stored at 4°C. Protein concentration was determined from the absorption at 278 μm in an appropriately diluted solution. The value of ε∞

was found to be 25.0 for anhydrous, ash-free papain. The value for the molecular weight of papain used in all calculations was 20,700. A stock solution of sodium hydroxide was made by diluting a saturated solution with carbon dioxide-free water. The concentration was checked by titration against standard potassium biphosphate. The solutions for measurement were in the concentration range 1 to 2 × 10^-6 M papain, and the pH was adjusted by careful addition of appropriate amounts of acid or base. Sodium chloride was used to adjust the ionic strength of each solution to either 0.01 or 0.2.

Measurements of pH were made with a Radiometer-Copenhagen type TTTT1 autotitrator equipped with a type C electrode. Correction for sodium ion errors was made with the aid of a nomograph supplied with the instrument. Solutions of high pH (in the range 11.5 to 13.0) were NaOH solutions of known molality containing sufficient sodium chloride to bring the ionic strength to 0.2. The pH of these solutions was computed from the activity coefficients obtained from hydrogen electrode measurements by Tanford (14). The Radiometer autotitrator and the Cary spectrophotometer (see below) were both located in a room where the temperature was maintained at 27°C ± 0.2°C.

A Cary model 14 recording spectrophotometer was used for absorption measurements. Cylindrical tightly stoppered cells with a 10-mm light path were employed. The cells were matched with water over the range 400 to 220 μm with the multipots on the instrument.

**RESULTS**

**Ultraviolet Absorption Spectrum of Papain**—The spectrum of papain in neutral solution, immediately after adjustment to pH 12, and immediately after adjustment to pH 13, is shown in Fig. 1, which shows that not all the phenolic hydroxyl groups are ionized at pH 12 (Table I). In fact, the situation appears to be very similar to that described by Crammer and Neuberger (3) for ovalbumin.

It was observed that the absorbancy of papain solutions in the pH range of 12 to 12.5 changes with time. A typical plot of change in absorbancy as a function of time is shown in Fig. 2. The molar extinction at pH 12.2 increases from about 40,000 (obtained within 30 seconds of adjusting the pH) to 51,000 ± 1,000 at the end of approximately 60 minutes at 27°C. Adjustment of a solution from pH 6 to 13 is accompanied by an instantaneous change in the molar extinction from 12,500 to about 50,000. A slow increase in absorbancy at 295 μm is then observed, a final value of 52,500 being reached in about 30 minutes at 27°C. Exposure to normal sodium hydroxide (near pH 14) for 2 or 3 minutes at 30°C results in a final molar extinction of 52,500 at 295 μm.

It was essential to decide whether the time-dependent changes at 295 μm observed above pH 12 were due to slow ionization of a few phenolic groups, or, whether they were due to some other cause such as an increase in light scattering caused by denaturation and aggregation. The ionization of the phenolic groups in tyrosine (3), polystyrene (4), and in a number of proteins (3, 7) is accompanied by a large increase in extinction at 295 μm, a considerably smaller change at 265 μm, and a very small change at 278 μm. It was observed that the wave length dependence of the increase in absorbancy of papain solutions follows that of tyrosine. It thus appears that the changes with the protein are due to a slow ionization of phenolic groups.

A similar situation has been reported for ribonuclease (7). In this protein, a slow ionization of three phenolic groups takes

FIG. 2. Time dependence of the ultraviolet absorption of papain at 295 μ at pH 12.3 and 27°. Papain concentration, 1.8 × 10^{-4} M; light path, 1 cm.

FIG. 3. Ionization of the phenolic groups in papain. —, direct titration; O, ionic strength 0.01; Δ, ionic strength 0.20. ---, reversed titration, reversed after exposure to pH 13 for sufficient time to attain complete ionization equilibrium. ●, ionic strength 0.01; ▲, ionic strength 0.20.

**TABLE II**

<table>
<thead>
<tr>
<th>pH</th>
<th>Conditions*</th>
<th>No. of ionized phenolic groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.8</td>
<td>5 minutes at 27°</td>
<td>11.6</td>
</tr>
<tr>
<td>13.0</td>
<td>30 seconds at 27°</td>
<td>15.0</td>
</tr>
<tr>
<td>14.0†</td>
<td>5 minutes at 39°</td>
<td>17.0</td>
</tr>
</tbody>
</table>

* All solutions were 0.2 M in NaCl.
† Normal sodium hydroxide solution.

Place at pH values greater than 12, the wave length dependence of the process being very similar to that observed with papain. On the other hand, the slow change in absorbancy observed with bovine serum albumin at pH values greater than 12, shows the greatest change at 265 μ, a smaller one at 278 μ and the smallest at 295 μ, and has thus been attributed to a denaturation process (5).

_Spectrophotometric Determination of Tyrosine and Tryptophan Content—_The ultraviolet absorption spectrum of papain in alkaline solution was studied under a variety of conditions. The methods of Goodwin and Morton (15) and of Benche and Schmid (16) were applied to the determination of the tyrosine to trypto-
The tyrosine to tryptophan ratio in lysozyme was also determined under various conditions. A tyrosine to tryptophan ratio of 0.28 ± 0.02 was obtained by the Benche and Schmid method after exposure of lysozyme to 0.1 N NaOH for 5 minutes at 27°C. This ratio changed to 0.49 ± 0.02 after exposure of this protein to 0.1 N NaOH for 17 hours at 27°C.

**Table II**

Tyrosine to tryptophan ratio in papain and ovalbumin

<table>
<thead>
<tr>
<th>Protein</th>
<th>Conditions</th>
<th>No. of determinations</th>
<th>Tyrosine-tryptophan ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Benche and Schmid method</td>
</tr>
<tr>
<td>Papain</td>
<td>pH 13, 27°, 5 min</td>
<td>4</td>
<td>2.70 ± 0.10 2.50 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>pH 13, 27°, 17 hours</td>
<td>4</td>
<td>3.20 ± 0.05 7.15 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>pH 13.5, 39°, 90 min</td>
<td>2</td>
<td>2.95 ± 0.05 2.87 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>pH 13.5, 99°, 180 min</td>
<td>3</td>
<td>3.23 ± 0.15 3.25 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>Denatured at pH 1.7 at 92° for 5 min, then pH 13.5, 39°, 30 min</td>
<td>2</td>
<td>2.80 ± 0.10 2.84 ± 0.17</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>pH 13, 27°, 5 min</td>
<td>2</td>
<td>2.65 ± 0.05 2.85 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>pH 13, 27°, 17 hours</td>
<td>2</td>
<td>2.60 ± 0.05 2.90 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>Denatured at pH 1.7 at 92° for 5 min, then pH 13, 27°, 5 min</td>
<td>2</td>
<td>2.65 ± 0.10 2.85 ± 0.20</td>
</tr>
</tbody>
</table>

Phenolic ratio in papain under various conditions used. In agreement with the results obtained by measuring the Δε at 295 μm, it was found that the true tyrosine to tryptophan ratio of 3.4:1 (indicated by amino acid analysis) was approached only after prolonged exposure of the protein to alkali (see Table II for details). After 24 hours at pH 13 and 27°, or after 3 hours at pH 13.5, a slow ionization of 1 to 2 phenolic groups takes place. Three to four phenolic groups in papain ionize slowly at pH 12 results in the slow ionization of approximately four more phenolic groups. Since the protein which had been exposed to pH values higher than 12 is insoluble below pH 9.5, it would seem that the ionization of these four groups is associated with an irreversible disorganization of the protein molecule. Such a disorganization is also indicated by the fact that the phenolic ionization curve of papain is not reversible once the protein has been exposed to pH greater than 12.

**DISCUSSION**

Of the 17 phenolic groups of papain, 11 to 12 ionize in the pH range 8.5 to 12. The observed pK' for these groups is 10.3 ± 0.1. The following values have been assumed for the calculation of the electrostatic interaction factor, V, and of the intrinsic pK (7). The molecular weight has been taken as 20,700. The radius of the protein sphere including 10% of water of hydration, as 18.9 Å, and of the tyrosine content of papain is highly inaccessible to hydroxyl ions. This behavior is in keeping with the known high stability of papain in alkaline solution (1). The extreme tightness of the secondary structure of at least a portion of the papain molecule is also indicated by the relatively high stability of this protein toward urea and guanidine hydrochloride (17). Since the titration of 11 to 12 of the 17 phenolic groups in papain is normal and reversible, these groups cannot be involved in interactions in which rupture would lead to disorganization of the molecule. It is known that papain retains a high proportion of its enzymic activity after exposure to alkaline solutions at pH values below 12 (1).

As noted above, exposure of papain to pH values higher than 12 results in the slow ionization of approximately four more phenolic groups. Since the protein which had been exposed to pH values higher than 12 is insoluble below pH 9.5, it would seem that the ionization of these four groups is associated with an irreversible disorganization of the papain molecule. Such a disorganization is also indicated by the fact that the phenolic ionization curve of papain is not reversible once the protein has been exposed to pH greater than 12.

**REFERENCES**

Phenolic Hydroxyl Ionization in Papain
A. N. Glazer and Emil L. Smith


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