THE DETERMINATION OF HYDROXYPROLINE

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There has been a great need for a satisfactory method for the determination of hydroxyproline to facilitate the study of the composition of proteins. Lang (1) and Waldschmidt-Leitz and Akabori (2) developed a colorimetric estimation involving oxidation to pyrrole with sodium hypochlorite and color formation with isatin or \( p \)-dimethylaminobenzaldehyde. However, oxidation was said to be incomplete and a correction factor was necessary. Dakin (3) and Bergmann (4) estimated hydroxyproline by isolation procedures. These procedures also required the use of large correction factors, as well as relatively large quantities of protein. McFarlane and Guest (5) devised a colorimetric method for hydroxyproline involving sodium peroxide oxidation and color formation with copper and isatin. This method yielded low values according to Devine (6), and in our hands similar results were obtained.

The unique high hydroxyproline content of collagen suggests the desirability of an accurate method for the determination of this amino acid in small quantities as a means of estimating the amount of collagen or gelatin in a mixture of proteins. Because hydroxyproline so far has not been found to be a nutritive requirement for a microorganism, a chemical procedure is required.

A short and simple colorimetric method is here described that is applicable to the determination of hydroxyproline in hydrolysates of 40 to 100 \( \gamma \) of collagen with a reproducibility of \( \pm 2 \) per cent (Table II) and an accuracy of \( \pm 2 \) per cent (Table I) as judged by recovery of hydroxyproline from elastin hydrolysates and from an amino acid mixture simulating collagen. Oxidation, in the manner of McFarlane and Guest with sodium peroxide, yields products that form an intense red color with \( p \)-dimethylaminobenzaldehyde. The intensity of color produced with 5 to 15 \( \gamma \) of hydroxyproline is 4 to 5 times that formed in previous colorimetric procedures. Because of this greater intensity of color and because of a different preliminary treatment of the hydroxyproline solutions, only 1 to 2 per cent as much protein is required as in earlier methods.

In acid hydrolysates of proteins, the only amino acid other than hy-

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dihydroxyproline which gives color under the conditions employed is tyrosine, which yields 1.5 per cent as much color as does hydroxyproline. This interference is so small that it can be corrected from the tyrosine content of the protein. Pure tryptophan gives 0.7 per cent as much color as hydroxyproline but humin formation on acid hydrolysis of proteins eliminates this interference.

Reagents—
Standard solutions of L-hydroxyproline containing 5, 10, and 15 γ of hydroxyproline per ml. Pfannstiehl c.p. hydroxyproline was found to be satisfactory.

- 0.01 M copper sulfate solution.
- 2.5 N sodium hydroxide.
- 6 per cent hydrogen peroxide.
- 3.0 N sulfuric acid.
- 5 per cent p-dimethylaminobenzaldehyde in c.p. n-propanol. The p-dimethylaminobenzaldehyde is recrystallized according to the procedure of Adams and Coleman (7), followed by recrystallization from warm alcohol by adding water and cooling.

Procedure
For the assay of one unknown solution, nine dry 18 X 150 mm. Pyrex test-tubes are employed. With an accurate pipette, the reagents are distributed as follows: Tube 1, 1 ml. of distilled water; Tubes 2 and 3, 1 ml. of standard containing 5 γ of hydroxyproline; Tubes 4 and 5, 1 ml. of standard containing 10 γ of hydroxyproline; and Tubes 6 and 7, 1 ml. of standard containing 15 γ of hydroxyproline. 1 ml. of the solution to be assayed, containing 5 to 15 γ of hydroxyproline, is pipetted into the eighth and ninth tubes.

Into each test-tube is pipetted in succession 1 ml. each of 0.01 M copper sulfate solution, 2.5 N sodium hydroxide, and 6 per cent hydrogen peroxide. The solutions are mixed and shaken occasionally during a period of 5 minutes, and are then placed in a water bath at 80° for 5 minutes with frequent vigorous shaking. The heating and shaking destroy the excess of peroxide. Traces of peroxide which remain will decrease color formation and produce an orange-red hue. The tubes are chilled in an ice and water bath and 4 ml. of 3.0 N sulfuric acid are added with agitation; 2 ml. of p-dimethylaminobenzaldehyde solution are then added with thorough mixing.

The tubes are placed in a water bath at 70° for 16 minutes and then cooled in tap water. The contents are transferred to selected absorption tubes and light transmission is read with an Evelyn photoelectric colorimeter and a No. 540 filter. Tube 1 is used for the blank setting.
The amount of hydroxyproline (measured in micrograms) in 1 ml. of unknown solution is established by finding the point corresponding to its optical density on the standard curve prepared at the same time. Percent of hydroxyproline in the sample of material is determined by the equation

\[
\text{Micrograms of hydroxyproline in 1 ml. of unknown solution} \times 100
\]

Protein hydrolysates are prepared by autoclaving 50 mg. of protein with 1.0 ml. of 6 N HCl in sealed tubes for 3 hours at 50 pounds pressure. The hydrolysates are neutralized, brought to appropriate volume, and filtered if necessary. Assay values were 93 per cent of maximum after 1 hour of hydrolysis. Maximum values were reached after 3 hours and did not decrease after 8 hours hydrolysis. As described in Table I, hydroxyproline is quite stable when subjected to the conditions of hydrolysis for 3 hours.

Because this procedure is intended to be used for the estimation of collagen in mixtures of proteins including animal tissues, the proteins were dried for 16 hours at 108° and weighed in glass-stoppered weighing bottles with precautions to prevent the absorption of moisture. The varied hygroscopic properties of tissues or protein mixtures of unknown composition make this the method of choice. In order that the results might be referred to other preparations of the same proteins, nitrogen determinations were made with a precision of ±0.4 per cent on more than fifty portions of assorted gelatins, collagens, and elastins weighed in this manner.

Gelatins, collagens, and elastins were prepared as previously described (8).
Results

Fig. 1 shows a standard curve prepared with 0 to 20 \( \gamma \) of hydroxyproline. The light absorption is 4 to 5 times that produced by the isatin reaction (5) referred to earlier.

Hydroxyproline was added to hemoglobin, casein, pig aorta elastin, and to a mixture of amino acids corresponding to the composition of collagen save for hydroxyproline. These mixtures, as well as hydroxy-

![Graph showing the formation of color with hydroxyproline.](http://www.jbc.org/)

Fig. 1. The formation of color with hydroxyproline. \( E = 2 - \log \) of per cent light transmission with a No. 540 filter.

proline alone, were subjected to the usual procedure for preparation of hydrolysates (in 6 N HCl, 3 hours at 50 pounds pressure) and hydroxyproline was determined (Table I). Hydroxyproline was completely recovered after autoclaving with elastin, with amino acid mixtures simulating collagen, or by itself in the hydrochloric acid. However, small losses appeared after autoclaving hydroxyproline with 37 times its weight of hemoglobin or casein. These latter hydrolyses may represent conditions
which are more severe than would be encountered in any anticipated use of the procedure because the entire quantity of hydroxyproline is free to react with substances liberated during the hydrolysis of the casein or hemoglobin. It may be presumed that hydroxyproline originally in peptide combination in these proteins would be partially protected during hydrolysis and recovery would be nearer the theoretical value. If the procedure is to be used for the estimation of collagen in tissues, a preliminary separation of the collagen is anticipated.

Guest (9) reported that proline oxidized by sodium peroxide in the presence of copper sulfate gave 10 per cent as much color with p-dimethyl-aminobenzaldehyde as did hydroxyproline. It is possible that the proline was contaminated with hydroxyproline. Three commercial preparations of L-proline were found by us to yield color corresponding to 2.3 to 3.4 per cent hydroxyproline. Upon formation of the crystalline complex with cadmium chloride as described by Kapfhammer and Eck (10), the color formation decreased to 1 per cent hydroxyproline. After recrystallizing the complex four times from hot water by adding alcohol and cooling, removing the cadmium by hydrogen sulfide, and evaporating the remaining solution so that the proline crystallized, the proline gave color corresponding to 0.17 per cent hydroxyproline.

Hydrolysates of proteins (casein, zein, cattle hair, wool) containing a high content of proline gave no indication of color formation due to proline. It is evident that the method here described gives no significant amount of color with proline.

Although three commercial samples of L-proline were contaminated with hydroxyproline, two different commercial samples of L-hydroxyproline were found by microbiological assay with Leuconostoc citrovorum 8081 to be free from proline.

Tyrosine appears to be the only interfering substance commonly encountered in protein hydrolysates prepared as described. Under the conditions of the determination, tyrosine yields 1.5 per cent as high readings as hydroxyproline with a No. 540 filter. The color produced with tyrosine may be differentiated from the hydroxyproline color by its bronze hue with maximum absorption at 500 mμ instead of at 550 mμ, by its much greater stability, and by its greater solubility in amyl alcohol. Casein, lactalbumin, ovalbumin, cattle hemoglobin, fibrin, zein, wool, silk fibroin, cattle hair, chicken epidermal scales, and turtle scutes yielded traces of color that displayed the properties of the color formed with tyrosine by the assay procedure. The absorption with the No. 540 filter corresponded to 0.01 per cent (hemoglobin) to 0.23 per cent (turtle scutes) hydroxyproline. After correction for tyrosine content, the hydroxyproline equivalent was negligible.
Pure tryptophan yields color corresponding to 0.7 per cent hydroxyproline. However, interference of tryptophan present in proteins is eliminated by the formation of humin during acid hydrolysis. To proteins containing large amounts of tryptophan but forming little humin, glucose may be added before hydrolysis to insure destruction of the tryptophan. 20 mg.

### Table II

**Hydroxyproline Content of Gelatins, Collagens, and Elastins**

<table>
<thead>
<tr>
<th>Protein*</th>
<th>Hydroxyproline (gm. per 100 gm. protein)</th>
<th>S. e.†</th>
<th>No. of determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gelatins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacto-difco</td>
<td>13.6</td>
<td>0.144</td>
<td>4</td>
</tr>
<tr>
<td>Eastman purified calf skin</td>
<td>14.4</td>
<td>0.138</td>
<td>6</td>
</tr>
<tr>
<td>“pig”</td>
<td>13.1</td>
<td>0.076</td>
<td>4</td>
</tr>
<tr>
<td>Fish scale</td>
<td>10.3</td>
<td>0.047</td>
<td>4</td>
</tr>
<tr>
<td>Cattle hide†</td>
<td>13.2</td>
<td>0.129</td>
<td>6</td>
</tr>
<tr>
<td>“bone”</td>
<td>13.3</td>
<td>0.087</td>
<td>4</td>
</tr>
<tr>
<td>“Achilles tendon”</td>
<td>13.4</td>
<td>0.166</td>
<td>4</td>
</tr>
<tr>
<td>Pig Achilles tendon</td>
<td>13.5</td>
<td>0.148</td>
<td>6</td>
</tr>
<tr>
<td>Sheep Achilles tendon</td>
<td>13.7</td>
<td>0.145</td>
<td>7</td>
</tr>
<tr>
<td>Chicken tarso-metatarsal tendon</td>
<td>13.5</td>
<td>0.158</td>
<td>4</td>
</tr>
<tr>
<td><strong>Collagens</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cattle tail tendon</td>
<td>13.3</td>
<td>0.112</td>
<td>4</td>
</tr>
<tr>
<td>Rat tail tendon</td>
<td>13.0</td>
<td>0.075</td>
<td>4</td>
</tr>
<tr>
<td>Kangaroo tail tendon</td>
<td>13.0</td>
<td>0.029</td>
<td>4</td>
</tr>
<tr>
<td>Fish skin</td>
<td>9.1</td>
<td>0.029</td>
<td>4</td>
</tr>
<tr>
<td><strong>Elastins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cattle ligamentum nuchae</td>
<td></td>
<td></td>
<td>1.84</td>
</tr>
<tr>
<td>Sheep “aorta”**</td>
<td>0.84</td>
<td>0.014</td>
<td>3</td>
</tr>
<tr>
<td>Cattle “aorta”**</td>
<td>1.92</td>
<td>0.026</td>
<td>4</td>
</tr>
<tr>
<td>Pig aorta**</td>
<td>1.91</td>
<td>0.004</td>
<td>4</td>
</tr>
<tr>
<td>Sheep aorta**</td>
<td>1.46</td>
<td>0.029</td>
<td>4</td>
</tr>
</tbody>
</table>

* These proteins were prepared as previously described (8).
† Standard error = \(\sqrt{\frac{\sum(X - \overline{X})^2}{N(N-1)}}\).
‡ Preparation involved treatment with trypsin and Ca(OH)₂.
§ Preparation involved treatment with 10 per cent NaCl and acetone.
∥ Preparation involved heating 24 hours in water at 100°C.
** Preparation involved heating 40 hours in 40 per cent urea at 100°C.

of glucose were sufficient to eliminate entirely the color produced from 25 mg. of tryptophan added to 50 mg. of casein before hydrolysis. This treatment has no effect on hydroxyproline or tyrosine.

Table II shows the hydroxyproline content of gelatins, collagens, and elastins as determined by the present method. It will be noted that the hydroxyproline content of most gelatins and collagens was 13 to 14 per
cent (13 to 14 gm. of hydroxyproline per 100 gm. of dry weight of protein). These values are within the limits of the "best" values in the literature. The range of these values in the literature is 12.9 to 14.6 per cent of gelatin (1, 3-5, 11). It will be noted that fish skin collagen and fish scale gelatin yielded outstandingly low values. In a previous publication (8) it was reported that fish skin collagen was higher in threonine, serine, and methionine than collagen of higher organisms. It is significant that the hydroxyproline content of fish skin collagen is proportionally lower than in the other collagens. Fish scale gelatin was also found to be high in threonine and methionine and somewhat high in serine (8); the hydroxyproline content again was low.

The elastin from ligamentum nuchae and aorta of cattle contained about 1.9 per cent hydroxyproline, in accordance with the value reported by Stein and Miller (12). However, pig and sheep aorta elastin contained 1.5 per cent hydroxyproline and sheep ligamentum nuchae elastin, 0.8 per cent. Previous assay of other amino acids of pig elastin has revealed several differences in its composition from other elastins (8). At this time it cannot be said whether these differences are due to impurities.

We are indebted to Dr. R. M. Lollar and Dr. P. R. Buechler of the Tanner's Council Laboratory, University of Cincinnati, for the two samples of hide collagen.

SUMMARY

A procedure for the colorimetric determination of hydroxyproline is described.

The hydroxyproline content of four gelatins, eleven collagens, six elastins, and several other preparations is reported.

Gelatin and collagen from mammalian and avian sources contained 13.0 to 14.4 per cent hydroxyproline.

Gelatin and collagen from fish contained 9.2 to 10.3 per cent hydroxyproline.

Elastin from ligamentum nuchae and aorta of cattle contained 1.84 to 1.92 per cent hydroxyproline. Elastins from pig and sheep tissues were lower, possibly owing to impurity.

Several other proteins examined appeared to contain no hydroxyproline.

BIBLIOGRAPHY

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