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Hydroxyproline, Methodological Studies of Analysis

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A modification of the method of Neuman and Logan, according to which hydroxyproline is oxidized by hydrogen peroxide, was compared with a method using chloramine T for oxidation. By the former method consistently somewhat lower results were obtained than by the latter. The discrepancy is discussed in the light of the present and earlier findings.

Hydroxyproline is the amino acid typical of collagen. For this reason it is often used as a measure of the occurrence of collagenous material in various tissues [1-7] and, from a practical point of view, as an index of the quality of meat products. A high content of hydroxyproline indicates inferior quality.[4-7]

Since the publication of the method of Neuman and Logan [8] several modifications have been worked out for the determination of hydroxyproline, They all have the colour-developing reagent, *p*-dimethyl-aminobenzaldehyde, in common. This reagent forms an intense red colour with oxidized hydroxyproline. In particular, the method of oxidizing the hydroxyproline has been subject to modifications. Like Neuman and Logan,[8] Baker *et al.*,[9-11] Martin and Axelrod,[12] Wierbicki and Deatherage ,[13] Miyada and Tappel,[14] Grunbaum and Glick,[15] and Lollar [16] use hydrogen peroxide for the oxidation of hydroxyproline to pyrrole. Stegemann,[17] however, introduced sodium *p*-toluenesulphonchloramide (Chloramine T) instead of H_2O_2.

It was considered appropriate to compare the most recent modification of the methods applying hydrogen peroxide for the oxidation, that is the method of Lollar,[16] and the method of Stegeman,[17] according to which another oxidizing agent is used. The methods differ also in other respects.

EXPERIMENTAL

Material. Animal tissue with a content of hydroxyproline varying over a wide range was investigated, the extremes being skeletal muscle and skin (about ½ and 12 % hydroxyproline in the crude protein, respectively).

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Methods

Hydrolysis. The same hydrolyzates were used in both methods. Hydrolysis was performed by boiling 5 g finely ground material with 50 ml 6 N HCl for 16 h under reflux. Lollar [16] applied the same procedure, although he used 6 N H_2SO_4 instead of HCl in order to be able to determine total Kjeldahl nitrogen in a separate aliquot of the hydrolyzate. Stegemann [17] gives no directions for hydrolysis in his paper, but according to a personal communication he proceeded as indicated above. After hydrolysis the acid was neutralized and a first dilution was made to a total volume of 500 ml. The colour of the solution did not interfere with the results.

According to Wierbicki and Deatherage [13] both acid and alkaline hydrolysis of animal tissue destroys hydroxyproline significantly. To prevent the formation of humin, these authors used $SnCl_2$ in the acid hydrolysis. They found, however, that the stannous chloride effects the destruction of hydroxyproline or that the stannous hydroxide formed, which must be removed, adsorbs some of this amino acid. We have not been able to confirm the reported sensitivity of hydroxyproline to hydrolysis. According to Kadoyama [18] no losses were observed during the hydrolysis of solutions of hydroxyproline without or with various sugars, furfural or levulinic acid for 20 h in 6 N HCl with reflux. When hydrolyzing gelatine under the same conditions a maximum value of hydroxyproline was reached after 65 h but the hydrolysis could be continued for at least 115 h without any effect on the hydroxyproline. The content of proline, however, showed a maximum after hydrolysis for 15 h and then gradually diminished.

The method of Lollar.[16] The method was worked out by Lollar for tannery chemists. It was modified by us to adopt the determination to various tissues with a very wide range of hydroxyproline content.

In the case of skeletal muscle the hydrolyzate diluted to a volume of 500 ml was used without further dilution, while hydrolyzates of skin and connective tissue were further diluted ten-fold. Hydrolyzates of tissues with intermediate contents of hydroxyproline, like unstriated (smooth) muscles, were generally diluted five-fold.

Pipette 0.50 to 2.00 ml (= v ml) of the diluted hydrolyzate and (2.00 − v) ml distilled water in a test tube (18 to 20 mm in diameter and 150 mm long). Add 1 ml 0.01 M $CuSO_4$ solution and mix, then add 1 ml 2.5 N NaOH and mix again. Now add 1 ml 1.8 % H_2O_2 solution. Oxidation is assisted by moderate agitation at 5 min intervals. The tube is observed for slowing down of the evolution of bubbles; a 30 min oxidation period at room temperature seems sufficient. When the oxidation is completed the excess of peroxide is removed by vigorous agitation. Pipette 4 ml 3 N H_2SO_4 into the tube and mix the contents thoroughly. Add 3 ml *p*-dimethylaminobenzaldehyde solution and, after mixing, immerse the tube into a 70°C water bath. After exactly 25 min cool the tube and contents in tap water for 10 min and allow to stand at room temperature for 40 min. The optical density is measured against a blank in a Beckman spectrophotometer at 550 mμ.Analyses were run in duplicate. For calibration test tubes with 0.5, 1.0, 1.5, and 2.0 ml of a solution of hydroxyproline containing 30 mg per 1000 ml 0.001 N HCl were run simultaneously.

This description deviates from that of Lollar essentially in that we applied cooling in tap water instead of ice water, the period before measuring of the colour was extended from 30 min to 50 min after removing the tube from the 70°C water bath, and finally, that measurement of the optical density was performed at 550 mμ instead of 560 mμ.

The method of Stegemann,[17] slightly modified. Dilution of the hydrolyzate was made as directed in the description of the method of Lollar.

Pipette 0.50 to 2.00 ml (= v ml) of the diluted hydrolyzate and (2.00 − v) ml distilled water in a test tube (18 to 20 mm in diameter and 150 mm long). Add 1 ml 0.05 M chloramine-T solution, made from 2.82 g sodium N-chloro-*p*-toluenesulphonamide dissolved in 40 ml water and diluted with 60 ml distilled methyl cellosolve (= mono-methyl ether of ethylene glycol; must give a clear and practically colourless solution when mixed with an equal volume of a 10 % KI solution) and 100 ml acetate-citrate-buffer of pH 6, made from 10.0 g citric acid, 1 H_2O, 2.4 ml glacial acetic acid, 24.0 g sodium acetate, 3 H_2O, and 6.8 g NaOH and water to a total volume of 200 ml (toluene is used as a preservative). After mixing, leave at room temperature for 20 min; then add 2 ml 4 M perchloric acid (133 ml 70 % $HClO_4$ + 233 ml water), mix, leave for 10 min, add 1 ml *p*-dimethylaminobenzaldehyde solution (10 g of the reagent dissolved in 100 ml distilled methyl cellosolve) and mix thoroughly. Heat for exactly 15 min in a 60°C water bath, cool in tap water for

5 min and measure the optical density against a blank at 550 mμ after a further 5 min period, using a Beckman spectrophotometer model B. Analyses were run in duplicate. Calibration was made as previously described in the method of Lollar.

We found it particularly important to maintain exact timing. This is not emphasized in the original description of the method.

RESULTS

The contents of hydroxyproline found in various animal tissues according to the two methods appear in Table 1.

Table 1. Content of hydroxyproline, calculated on a crude protein basis, according to the methods of Lollar [16] and Stegemann.[17] Crude protein = N × 6.25.

Sample No.	Tissue	% hydroxyproline in the protein according to the method of	
		Lollar	Stegemann
1	Skeletal muscle, beef, without visible connective and fatty tissue	0.3	0.3
2	Skeletal muscle, horse, with connective and fatty tissue	2.5	2.7
3	» » beef and pork, as sausage	3.6	3.8
4	» » » » » » »	4.0	4.2
5	Lungs, pig	1.9	2.1
6	» beef	2.4	2.5
7	Udder, cow	5.0	5.6
8	Stomach, pig	3.0	3.2
9	Rumen, beef	2.9	3.2
10	Stomach, horse	5.2	5.4
11	Chitterling (large intestine), pig	2.4	2.6
12	Cap (Caecum), pig	2.6	2.9
13	Bladder, pig	4.8	5.4
14	Fat end (Rectum), beef	5.1	5.7
15	Bung (Caecum), horse	4.1	4.4
16	Cracklings (connective tissue), from wet rendered pig fat	6.4	6.9
17	Neck tendon (ligamentum nuchae), cow	4.3	4.5
18	Diaphragm (connective tissue), beef	7.7	8.0
19	Achilles tendon, beef	10.7	11.8
20	» » »	11.2	12.5
21	Skin (back rind), pig	9.5	10.2
22	» , newly born calves, from fore legs	11.2	12.1
23	» , fattened calves » » »	9.7	10.5
24	» , » » , » » »	10.5	11.3
25	Hide, bull, from neck	10.4	11.2

DISCUSSION

From Table 1 it can be seen that the method of Stegemann gives consistently higher results, on an average 8.1 % (relative) higher than those of the method of Lollar (sample No. 1 excluded); the standard deviation is 2.7 % and the 95 % confidence interval for the mean is from 7.0 to 9.2 %.

Earlier investigations have revealed the difficulty of complete removal of the excess of hydrogen peroxide after oxidation. This causes a reduction of colour development with the reagent. Martin and Axelrod [12] state that this error can be avoided by replacing the heating step by the addition of 0.1 ml M/20 $FeSO_4$-solution. Likewise, Miyada and Tappel [14] modified the procedure for removing the excess of H_2O_2. They found optimal colour development at 80°C after 30 min (Neuman and Logan [8] used 70°C for 16 min). They also introduced 1.5 N H_2SO_4 instead of 3 N, because the colour fades more rapidly at high hydrogen ion concentrations. Grunbaum and Glick [15] found that a 2 % H_2O_2 solution yielded a more intense colour and reduced the interference of tyrosine. They made use of more optimal concentrations of the reagents than Neuman and Logan [8] and introduced manipulative improvements for determinations on microgram amounts of tissue. Lollar [16] reported great variations of the standard curves from one day to another. To get rid of the excess of H_2O_2 he found vigorous agitation to be the only remedy; the value of $FeSO_4$ for this purpose, as stated by Martin and Axelrod,[12] could not be confirmed.

To eliminate the difficulties which seem to be involved with the use of hydrogen peroxide Stegemann [17] radically introduced another oxidizing agent, Chloramine T, the excess of which will be destroyed by acidifying, a step which in any case must be undertaken. Contrary to other methods, the oxidation does not take place in an alkaline solution but in a slightly acid buffer (pH 6). It is claimed that concomitant amino acids — among these tyrosine, which is said to contribute to some although not very significant increase of colour when applying the methods using H_2O_2 — do not interfere.

For constructing the calibration curves the same standard solutions of hydroxyproline were used in the present investigations and these solutions were analyzed in the same way as were the hydrolyzates according to the respective methods. For this reason no discrepancy ought to exist between the methods unless the colour development in the one method is depressed in some way— for instance, by non-removable peroxides formed from hydrogen peroxide and constituents contained in the hydrolyzate — or extra colour is contributed in the other method by reaction between the reagents and substances other than hydroxyproline in the hydrolyzates. In view of the earlier findings referred to it seems likely than the results obtained according to the method of Stegemann are more reliable than those of the method of Lollar.

It may be mentioned, however, that Piez *et al.*[19] recently reported the occurrence of small amounts of 3-hydroxyproline (besides the usual 4-hydroxyproline) in collagenous tissue like skin and tendon. It could be assumed that the reason for the discrepancy between the two methods is a different behaviour of the reagents towards 3-hydroxyproline.

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