MEAT ASSAY

Hydroxyproline as an Index of Connective Tissue in Muscle

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A method for the determination of hydroxyproline as an index of connective tissue in muscle is presented and which employs the use of acid and alkaline hydrolyzates with or without preliminary separation of fibrous proteins of muscle tissue. Acid hydrolysis of muscle tissue in presence of stannous chloride was superior to alkaline hydrolysis. A method is suggested for determining collagen and elastin. Connective tissue of *longissimus dorsi* muscle had 12.39 ± 0.40% hydroxyproline and consisted of 84% collagen and 16% elastin. Fat had a tendency to destroy tryptophan during alkaline hydrolysis. Tyrosine and tryptophan content was 1.024 ± 0.047% and 0.330 ± 0.020%, respectively.

THE ESTIMATION OF connective tissue is desired in many studies involving muscle or other tissues. In Ohio, investigations have been under way for some time on the relation of biochemistry and physiology of muscle to quality attributes of meat. This paper summarizes data from three years' experience using the method of Neuman and Logan (*13*) for determining hydroxyproline as an index of connective tissue in muscle.

For the estimation of connective tissue in meat, Husaini *et al.* (*8*) modified a gravimetric procedure of Lowry, Gilligan, and Katersky (*10*) which was based upon the insolubility of collagen and elastin in 0.1*N* sodium hydroxide at room temperature. However, this method has the disadvantage that some of these proteins may be lost during extraction (*9*), particularly when the tissues are rich in fat.

Based on the uniquely high hydroxyproline content of collagen Neuman and Logan (*13*) developed a method for determining this amino acid as a means of estimating the amount of collagen and elastin in a mixture of proteins. The method depends upon the oxidation of hydroxyproline with alkaline hydrogen peroxide to a product that forms an intense red color with *p*-dimethylaminobenzaldehyde.

This method has been rechecked and made suitable for use in studying beef and the procedure has been applied on both acid and alkaline hydrolyzates of muscle. When alkaline hydrolyzates are used, tryptophan and tyrosine interfere to some extent. In acid hydrolyzates this is true only with tyrosine. To prevent the formation of humin during the acid hydrolysis, stannous chloride was used as suggested by Hlaziwetz and Habermann (*7*).

Recently Neuman and Logan (*14*) reported the use of aqueous urea for the extraction of nonfibrous proteins from tissues containing a small amount of collagen, such as muscle, before acid hydrolysis. However, this technique offered no advantages in the investigation reported here.

Experimental

Preliminary Experiments When the method of Neuman and Logan (*13*) was applied to known samples of hydroxyproline, the relation between hydroxyproline and absorbance using an Evelyn photoelectric colorimeter with the 540 mμ filter was made more uniform by changing the sodium hydroxide from 2.5*N* as described by these authors to 3.5*N* (Figure 1). These results are not in accord with those reported by Baker *et al.* (*1*).

In applying this method to muscle it was necessary to establish the optimum time for tissue hydrolysis. This was established for acid hydrolysis as follows: Samples of 100 mg. of alkali insoluble proteins obtained by the method of Husaini *et al.* (*8*) were placed in 100-ml. round-bottom flasks with 10 ml. of 6*N* hydrochloric acid and refluxed for varying periods of time. They were neutralized with 6*N* sodium hydroxide (Hydrion paper, pH 7), filtered, and diluted so that 1 ml. was equivalent to 100 γ of connective tissue. Alkaline hydrolysis was carried out in the same manner except that 15 ml. of 3*N* sodium hydroxide was used. Hydroxyproline was determined on each hydrolyzate using curve *A*, Figure 1. The results, given in Table I, indicate that the optimum time of hydrolysis of the connective tissue with respect to hydroxyproline was 7 hours using acid and 10 hours using alkali, and that there was some destruction of hydroxyproline during hydrolysis when either acid or alkali was used. These facts were taken into account and corrected for in the preparation of a standard curve for the hydroxyproline determination as indicated below.

Six 20-mg. samples of hydroxy-L-proline were accurately weighed into 100-ml. round-bottom digestion flasks. Three samples were subjected to the acid hydrolysis treatment (7 hours) and three to the alkali treatment (10 hours). After cooling, the contents of each flask were neutralized, diluted to 100 ml., and filtered.

Table I. Variation of Hydroxyproline with Time of Hydrolysis

Acid Hydrolysis, 6N HCl		*Alkaline Hydrolysis, 3N NaOH*	
Time in hours	*Hydroxyproline, %*	*Time in hours*	*Hydroxyproline, %*
3	8.83	2	8.61
4	9.03	4	9.27
6	9.70	6	9.58
7	12.10	8	9.69
8	11.44	10	11.70
9	10.03	12	11.24
12	9.61	16	9.69
16	7.41		
24	5.88		

Aliquots of each of the six were further diluted so that the concentrations of 5, 10, 15, and 20 γ of hydroxyproline per 1 ml. were obtained. One milliliter from each of the diluted samples was then taken for the determination of hydroxyproline. These values were then compared to the values found for known hydroxyproline without the hydrolysis treatment. The results are shown in Figure 2. Each point on the curves represents an average of the triplicate determinations and these curves illustrate the destructive effects of the hydrolysis

treatments. From the average of 12 measurements for each of the acid and alkali treatments, partial destruction of hydroxyproline was found to be 5.84 ± 2.49% (standard deviation) during the acid, and 9.25 ± 2.98% during the alkaline hydrolysis.

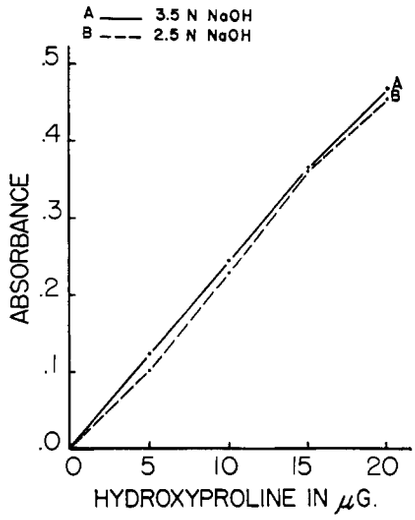


Figure 1. Effect of sodium hydroxide concentration upon the color development

Evelyn photoelectric colorimeter, Filter No. 540

In order to check the accuracy of the method and the necessity of using the corresponding correction for the destructive effect of the hydrolysis treatment, the hydroxyproline content of gelatin has been determined because its content of this amino acid is known.

Three 100-mg. samples of gelatin were hydrolyzed with 10 ml. of 6*N* hydrochloric acid and another three samples with 15 ml. of 3*N* sodium hydroxide. One milliliter of each hydrolyzate containing 100 γ of the standard gelatin was taken in duplicate for the hydroxyproline determination. The amount of hydroxyproline in micrograms per 1 ml. was then established using the standard curves given in Figure 2, curve *B* being used for the acid hydrolyzates and curve *C* for the alkaline hydrolyzates. For the acid hydrolysis procedure the gelatin was found to contain 14.42 ± 0.25% hydroxyproline and for the alkali hydrolysis a value of 14.41 ± 0.30% was obtained.

This evidence indicates the adequacy of the correction factor for loss of hydroxyproline during hydrolysis and gives values for hydroxyproline in gelatin which agree with those of McFarlane and Guest (*11*), Dakin (*4*), and Bergmann (*2*). However, Neuman and Logan (*13, 14*) gave the hydroxyproline content for gelatin and collagen of 13.6 and 13.5 ± .24%, respectively. This can be explained by the fact that Neuman and Logan (*13, 14*) did not apply a necessary correction for the decomposition of hydroxyproline during acid hydrolysis. When no correction for loss was made in data obtained in this laboratory a value of 13.58% resulted. Consequently, the factor for the conversion of hydroxyproline into its equivalent of collagen (gelatin) will be 6.94 rather than 7.46 as given by Neuman and Logan (*13*).

Application of Method To Muscle Hydrolyzates The experimental material used was the *longissimus dorsi* muscles of cattle and sampling was performed by the method described by Husaini *et al.* (*8*).

Acid Hydrolysis Of meat Eight grams of water-meat slurry (1 to 1), 3 ml. of water, 10 ml. of 12*N* hydrochloric acid, and 0.7 gram of stannous chloride (approximately ¾ of the weight of the proteins) were refluxed for 7 hours. The hydrolyzates were transferred quantitatively into 100-ml. volumetric flasks, neutralized by 6*N* sodium hydroxide, brought to pH 8 with saturated sodium carbonate, cooled in an ice-water bath, filled to the mark, and after standing for a 0.5 hour filtered through a Büchner funnel to remove the stannous hydroxide precipitate. The addition of sodium carbonate is necessary to prevent the formation of stannite ions in the alkaline medium and to facilitate the removal of stannous ions in the form of stannous hydroxide. The slight alkalinity (approximately pH 8) is of importance in preventing the precipitation of tyrosine. (An exact knowledge of the tyrosine content in the hydrolyzates is necessary for making a correction on hydroxyproline). The hydrolyzates obtained were transparent and slightly yellow in color.

Preparation of Standard Curve A standard curve for hydroxyproline was prepared for acid hydrolysis in the presence of stannous chloride in a manner similar to that described. The color development was performed as before except that the absorbance was determined at 560 mμ using a Beckman DU spectrophotometer. The results are shown in Figure 3 and show that the acid stannous chloride hydrolysis treatment causes 13.5 ± 1.41% lower hydroxyproline values. This loss is twice that previously found using hydrochloric acid alone. Apparently stannous chloride affected the destruction of hydroxyproline on hydrolysis or adsorbs some of this amino acid on subsequent removal from the hydrolyzate as stannous hydroxide.

Determination of Hydroxyproline In Hydrolyzates The meat hydrolyzates were filtered and 1 ml. of the hydrolysate, representing 40 mg. of fresh meat, was taken in duplicate for the color development. The apparent absorbance obtained had to be corrected for the interference of tyrosine and the color of the hydrolyzate before final reading of the corresponding hydroxyproline concentration could be read from the standard curve *B*, Figure 3.

Tyrosine Correction Before this correction could be applied an exact knowledge of this amino acid in the meat was necessary. Therefore, tyrosine was determined by the method of Folin and Ciocalteu (*5*) on samples from 36 different animals.

Tyrosine was subjected to the acid hydrolysis treatment as applied to the meat. Absorbance measurements were made at 740 mμ using both treated and untreated tyrosine standards. The results in Figure 4 show that tyrosine is partially destroyed by acid hydrolysis. This amounts to 9.4 ± 5.4% in average of 30 determinations.

The tyrosine content of the 36 muscles investigated was found to be quite constant, 1.024 ± 0.047% on the wet basis, and 4.40% on the fat and moisture-free basis. This value has been obtained using curve *B*, Figure 4, and is in accord with that given by Block and Bolling (*3*). However, the actual amount of tyrosine present in the hydrolyzates is 9.4% (relative) less—i.e., 0.928% on the wet basis (curve *A*, Figure 4)—and this value was used for making correction in hydroxyproline determinations.

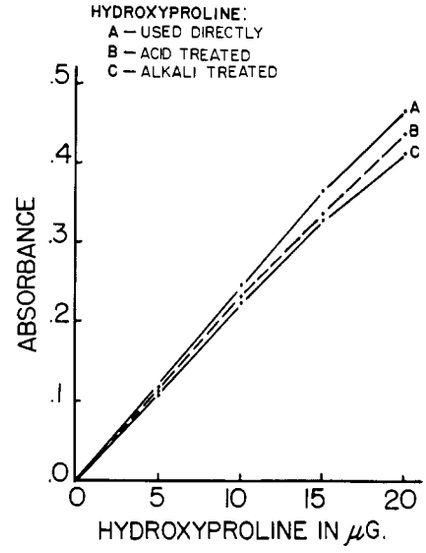


Figure 2. Effect of acid and alkali hydrolysis upon hydroxyproline

Evelyn photoelectric colorimeter, Filter No. 540

Neuman and Logan (*13*) reported that tyrosine developed 1.5% as much color as hydroxyproline. However, in these studies the value of 1.3% was found. These tyrosine corrections were made as indicated in the example—20 γ of hydroxyproline (Figure 3) develop the absorbance of approximately 0.300. It follows that in 1 ml. of the hydrolyzate representing 40 mg. of meat the correction for tyrosine in apparent absorbance is:

$$\frac{0.300}{20} \times \frac{1.3}{100} \times \frac{0.928}{100} \times 40{,}000 =$$

0.072 absorbance unit

Acid meat hydrolyzates were slightly yellow and this amounted to an additional correction of 0.010 ± 0.003 absorbance unit per each 40 mg. of fresh meat represented in 1 ml. of the hydrolyzate.

Accordingly, the apparent absorbance at 560 mμ, as obtained by the determination of hydroxyproline by the method employed, was corrected in the amount of 0.082 absorbance unit per 40 mg. of fresh meat represented in 1 ml. of the hydrolyzate. This represents total correction for acid hydrolysis.

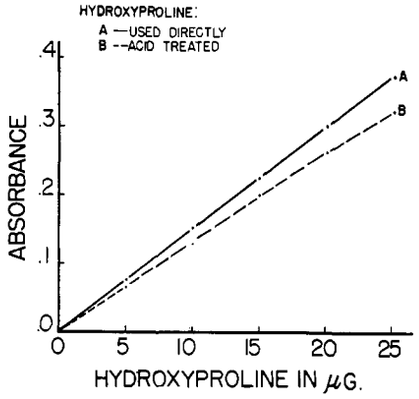


Figure 3. Formation of color with hydroxyproline

Beckman DU spectrophotometer, 560 mμ

Alkaline Hydrolysis Of Meat Alkaline hydrolysis of beef was used during the first year of this study. At that time it was considered that the difficulty encountered during the acid hydrolysis due to the humin formation would be hard to the overcome. It is of interest to compare both methods of hydrolysis. The following procedure has been used for hydroxyproline determination:

Eight grams of water-meat slurry (1 to 1) was weighed into a 50-ml. long-tapered centrifuge tube; acetone was added to make 40 ml. The mixture was stirred thoroughly, let stand for 0.5 hour at room temperature, centrifuged, and the supernatant liquid removed. The tube was dried for 2 hours at 80° C. to remove acetone. The contents were transferred to 100-ml. round-bottom flasks using 20 ml. of 3*N* sodium hydroxide. After refluxing 10 hours the hydrolyzates were neutralized to pH 6.5 to 7.0 and diluted to 50 ml. This dilution was necessary to obtain an appropriate concentration for tryptophan determination. The silicic acid precipitate was removed by two filtrations. Before the estimation of hydroxyproline the original hydrolyzates were diluted, so as to contain 20 mg. of fresh meat per 1 ml. The intensity of color was measured at that time by the Evelyn photoelectric colorimeter, filter No. 540. The apparent absorbance obtained was corrected for the interference of tryptophan, tyrosine, and the color of the hydrolyzate, before final readings of hydroxyproline concentrations could be taken from curve *C*, Figure 2.

Tryptophan Correction The colorimetric method by Graham *et al.* (*6*) has been used for the quantitative determination of tryptophan. The colorimetric readings were made immediately using filter No. 540, and reagents as a blank. Curve *A*, Figure 5 was obtained by using the standard tryptophan solutions directly, while the curve B represents tryptophan after the alkaline hydrolysis treatment and shows that approximately 17% of the tryptophan was destroyed during alkaline hydrolysis. Tryptophan contents of the samples were computed using curve *B*. The amount of tryptophan found in 30 different carcasses was 0.330% ± 0.020 on the wet basis and 1.44% on the fat and moisture free basis. These results are in full agreement with those given by other authors (*3*), and show a relatively constant value for tryptophan in meat.

Tryptophan was found to develop 0.6% as much color with the reagents used for the determination of hydroxyproline as hydroxyproline; the value given by Neuman and Logan (*13*) is 0.7%. However, the actual amount of tryptophan present in the hydrolyzates was 17% less, (curve *A*, Figure 5)—i.e., 0.330 × 0.83 = 0.274%. This value was used in correcting for the interference of tryptophan. This interference amounted to 0.008 absorbance unit per milliliter of alkaline meat hydrolyzate containing 20 mg. of fresh meat.

Total Correction This is the sum of the hydrolyzate color, and the interference for tyrosine and tryptophan which can be calculated as indicated above. Consequently, for a solution representing 20 mg. of meat per milliliter, this amounts to 0.004, 0.059, and 0.008, respectively, or a total of 0.071 absorbance unit. The hydroxyproline concentration per 1 ml. of the hydrolyzate was then found by reading the point corresponding to its corrected absorbance on the standard curve *C*, Figure 2. Per cent of hydroxyproline in the meat was then calculated.

Conversion of Hydroxyproline into Connective Tissue Hydroxyproline may be converted to its equivalent of connective tissue provided the amount of this amino acid in the connective tissue is known, and if it is assumed that the ratio of elastin to collagen of *longissimus dorsi* muscle does not change from sample to sample.

Connective tissue (alkali-insoluble proteins) of meat were prepared by extraction of meat slurry three times with 0.1*N* sodium hydroxide; the residue was washed with distilled water until neutral to phenolphthalein. The residue was washed twice with alcohol-ether mixture (1 to 1), and dried in air at 105° C. for 6 hours. The resulting white flakes were pulverized, and hydroxyproline was determined using the alkaline hydrolysis procedure. Because tryptophan was absent it is presumed that only connective tissue proteins were present in each case.

The average of 5 different samples showed that alkali-insoluble proteins contained 12.39 ± 0.40% of hydroxyproline. Thus, the factor for the conversion of hydroxyproline to its equivalent connective tissue was 8.07.

Results and Discussion

Comparative Study Of Acid and Alkaline Meat Hydrolysis Acid as well as alkali can be used for the preparation of the hydrolyzates for the determinations of the amino acids in question. The alkaline hydrolysis of meat can be used conveniently when tryptophan determination is one of the main objectives of the study. However, in the case of the hydroxyproline determination the following difficulties were encountered:

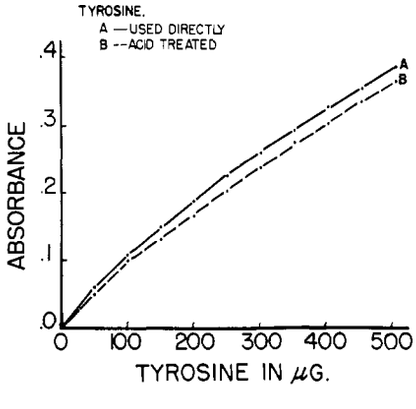
Alkali attacks the hydrolysis flasks.

After neutralization of the hydrolyzates a large amount of the silicic acid gel was precipitated. Some of the hydroxyproline could be absorbed and removed together with the silicic acid from the hydrolyzates.

In order to remove all the silicic acid, hydrolyzates must be brought to pH 6.5 which is near the isoelectric point of tyrosine and some of the tyrosine may be removed together with the silicic acid. This loss might be not equal to all samples. Consequently, applying the same correction for the interference of tyrosine for all samples may not be justified.

Figure 4. Formation of color with tyrosine by Folin reagent

Beckman DU spectrophotometer, 740 mμ



An additional correction for the interference of tryptophan had to be made.

The additional step of acetone treatment of the meat slurry before its hydrolysis is necessary to remove fat, so as to avoid foaming during hydrolysis. Furthermore, fat promotes the destruction of tryptophan. During the course of this work it was found that in 30 samples investigated about 8% of the tryptophan was destroyed when fat was present during hydrolysis. Furthermore, the amount of tryptophan destroyed was directly related to the amount of fat present. A negative correlation coefficient (−0.564) between the percentage of fat and that of tryptophan was highly significant (*15*).

When the acid hydrolysis is employed all these difficulties are avoided. It is only essential that the amount of stannous chloride added to the hydrolysis mixture be not less than $^3/_4$ of the amount of the protein (dry basis) present; otherwise, some humin will form.

Because fat does not interfere, the acid hydrolysis procedure using stannous chloride can be considered superior to the alkaline method. However, the accuracy of both methods was comparable when all analytical precautions for each method were taken into account.

Accuracy of Method

The formation of color is proportional to the hydroxyproline concentration up to 20 γ of the amino acid per milliliter. At higher concentrations the color development is slightly reduced. The reproducibility of color formation is within ± 2%, thus confirming the original results given by Neuman and Logan (*13*). However, the absolute level of the color density for the same concentration of hydroxyproline has been found to fluctuate when determined at different times. Similar results have been reported recently by Baker *et al.* (*1*). To eliminate this difficulty known hydroxyproline standards must be run simultaneously with each set of the unknowns. Triplicate standard hydroxyproline (15 γ per milliliter) which have been subjected to the acid hydrolysis treatment can be recommended since this approximates the concentration of hydroxyproline in 1 ml. of the hydrolyzate representing 40 mg. of muscle.

For the stability of the color formed the purity of the *p*-dimethylaminobenzaldehyde is essential. The purification procedure of Neuman and Logan (*13*) can be simplified by dissolving the substance in warm ethyl alcohol, chilling, filtering, drying the crystals in air at 65° to 70° C., then redissolving in alcohol, precipitating by adding ice water, filtering, and drying in a desiccator. In 30 minutes a solution of absorbance about 0.250 lost only approximately 2 to 3% of its color. It is recommended that no more than 6 samples in duplicate plus three samples of the standard hydroxyproline be run simultaneously, and to start color measurement after 5 minutes of cooling of the samples in a water bath.

The greatest single error encountered in this study is that of sampling. Duplicate and triplicate sampling of the tissue is very important for often the most careful grinding will not overcome these errors.

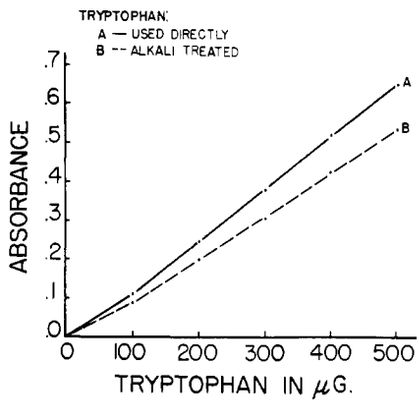


Figure 5. Formation of color with tryptophan

Evelyn photoelectric colorimeter, Filter No. 540

The reproducibility of the sampling has been checked on 36 *longissimus dorsi* muscles. Two samples from each were taken for the acid hydrolysis and two determinations from each hydrolyzate were made, thus giving four single determinations for each hydroxyproline value. In the 36 muscles the standard deviation from the mean for each muscle ranged from 14.0% to 1.1%.

The reproducibility can be improved by direct hydrolysis of a larger sample of muscle without preparation of the slurry. For example, using 10 grams of ground meat, 8 ml. of water, 1.8 grams of stannous chloride, and 15 ml. of 12*N* hydrochloric acid reduces sampling errors to about 2% on a relative basis.

Recently Baker *et al.* (*1*) have shown that when the Neuman and Logan procedure is applied to an amino acid mixture where the ratio of other amino acids to hydroxyproline exceeds 50 to 1, color development was appreciably lower. Furthermore, the authors found that the development of color increases as the concentration of copper sulfate increases especially when other amino acids were present.

Since the ratio of other amino acids to hydroxyproline in 114 different muscle hydrolyzates ranged from 300 to 1200 to 1, it was important to know the decrease in color development. In order to evaluate this error absorbances were determined for a meat hydrolyzate (containing other amino acids in the ratio of 600 to 1), for a standard hydroxyproline solution (5 γ per milliliter), and for the hydrolyzate plus the hydroxyproline. Eight similar experiments using 0.01*M* copper sulfate indicated an error of −11.7 ± 0.7% in color development. Whereas this correction is necessary on muscle hydrolyzates no correction was necessary on gelatin hydrolyzates where the ratio of other amino acids to hydroxyproline was less than 10 to 1.

Baker *et al.* (*1*) found also that by increasing the copper sulfate concentration from 0.01*M* originally used to 0.03 to 0.05*M* the color given by hydroxyproline alone increased very little, but, in the presence of other amino acids, an appreciable increase in color resulted. This error was studied by varying the copper sulfate concentration from 0.01 to 0.08 *M* and determining color formation for standard hydroxyproline, muscle hydrolyzate, and hydrolyzate plus the hydroxyproline. It was found that 0.05 *M* copper sulfate gives the most desirable results, and gives only a −7.5 ± 1.4% error instead of −11.7% noted above.

Probability and theory of errors (*12*) when applied to the method of hydroxyproline determination in muscle as discussed here indicate that the over-all accuracy will vary from ± 3.2 to ± 5.7% of the value found.

A series of experiments were carried out to determine the hydroxyproline lost by the method of Lowry, Gilligan and Katersky (*10*) as modified by Husaini *et al.* (*8*). Some connective tissue may have been lost by suspension in the aqueous phase and by occlusion with the fat. Consequently, determination of hydroxyproline is preferred over previous methods for estimating connection tissue (collagen).

Calculation of Relative Amounts of Collagen and Elastin

Connective tissue consists largely of collagen and elastin, and occasionally a third fibrous protein, reticulin, may be present. In muscle only collagen and elastin are present (*14*), and the relative amounts of each may be calculated, provided the hydroxyproline contents of collagen, elastin, and the connective tissue are known.

Neuman and Logan (*13*) have shown that the hydroxyproline content of collagen from various mammalian or avian sources is constant within ± 2% (relative). Elastin contains 1.5 to 2.3% of hydroxyproline (*13, 14*), depending on the source. The hydroxyproline content of a sample of connective tissue has been found to contain 12.39 ± 0.40% hydroxyproline.

These data permit calculation of the collagen and elastin contents of the connective tissue of the *longissimus dorsi* muscle of cattle as 84% and 16%, respectively.

This method of determining the collagen and elastin may be applied to other animal tissues. Detailed data on the hydroxyproline and connective tissue of muscles of cattle will appear elsewhere.

Summary

The method of Neuman and Logan has been adapted to the determination of hydroxyproline as an index of connective tissue in muscle.

The procedure may be used on alkaline or acid hydrolyzates directly without preliminary separation of the fibrous proteins of the muscle tissue.

Acid hydrolysis of muscle tissue in presence of stannous chloride has been found superior to the alkaline hydrolysis for hydroxyproline determinations.

In determining hydroxyproline, corrections must be made for destruction during hydrolysis of tissue, the interference of tyrosine or tryptophan and the color of the hydrolyzate in color formation, and the fact of other amino acids present in the hydrolyzate may depress color development.

A method for determining relative amounts of collagen and elastin in a connective tissue is suggested.

The connective tissue (alkali insoluble proteins) of the *longissimus dorsi* muscle of cattle was found to be 12.39 ± 0.40% hydroxyproline and to consist of 84% collagen and 16% elastin.

During the course of this investigation it was observed that fat tends to destroy tryptophan during alkaline hydrolysis of tissue.

The tyrosine and tryptophan content of the *longissimus dorsi* muscle of cattle was found to be relatively constant 1.024 ± 0.047% and 0.330 ± 0.020%, respectively.

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FRUIT PECTIN

Determination of a Soluble Pectin in Apples

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METHODS FOR THE DETERMINATION of the pectin content of natural materials have been, in most cases, very tedious and not applicable to small amounts of material. They have been critically discussed by McColloch (*11*).

It was found necessary to examine the methods as a basis for a study of the relation of maturity of apples to the soluble pectin content. Methods based on precipitation of calcium pectate have been used more frequently, perhaps, than others, but are tedious. The evolution of carbon dioxide in the Tollens-Lefèvre reaction (*10*) would seem to be most specific for polyuronic acid material, but is not conveniently applicable to large numbers of samples. After the work reported here was well advanced, the colorimetric carbazole method of Stark (*16*) appeared.

The authors have modified the calcium pectate method so as greatly to expedite its use, have developed a photometric method based on the results of Ikawa and Niemann (*6*), and have compared the results obtained by these methods with the uronic acid content as determined by applying the carbon dioxide method to apples.

Extraction of Soluble Pectin from Apples

In order to remove all the soluble pectin from apples, Carré and Haynes (*3*) found it necessary to carry out 60 to 80 water extractions. This is obviously impractical when a considerable number of samples are under consideration, and more recent investigators have used four or five extractions (*1, 5, 12*). McColloch (*11*) described a "water-soluble" pectic fraction which he defined by the method of preparation. This involved two 2-hour extractions with water at 30° C. of the alcohol-insoluble portion of the sample. Carré and Haynes reported that as much as 20% of the pectin may remain behind after 10 extractions with cold water.

In view of such results, the whole procedure of repeated extractions seems of dubious validity, and it is not unlikely that the pectin appearing as "soluble" under these circumstances is different from that actually in solution in the apple. While the chemical nature of protopectin has not been established, it is known to be easily rendered soluble, and 80 extractions would clearly entail very long contact with water. Other unknown changes to pectic materials might also occur during such extensive treatment.

In the ripe apple, less than 4% of the edible material is insoluble in water (*9*). The solids in solution include pectin, so that the pectin content of a sample of the solution present in the apple may be considered to represent the dissolved pectin content of the whole edible portion of the apple within a small margin of error. Information on the composition of unripe apples is not extensive. Widdowson (*19*) found 3 to 7% alcohol-insoluble material in fresh apple tissue in June, with the level decreasing thereafter. This would include practically all the water-insoluble material, as well as soluble pectin, and perhaps other water-soluble polyoses. During ripening, starch is converted to sugar, but the maximum level of starch in McIntosh apples has been reported as only 1.5 to 2% in late July and early September (*9*).