THE DETERMINATION OF COLLAGEN AND ELASTIN IN TISSUES

By ROBERT E. NEUMAN* AND MILAN A. LOGAN

(From the Department of Biological Chemistry, College of Medicine, University of Cincinnati, Cincinnati)

(Received for publication, May 21, 1950)

In the study of the occurrence and distribution of collagen in normal and pathological tissue and its formation and development in the embryo there has been need for an accurate method to determine this substance in small amounts of tissue. Spencer, Morgulis, and Wilder (1) devised a method based on conversion of collagen to gelatin followed by the determination of the nitrogen of the gelatin precipitated with tannic acid. There is no assurance that only gelatin is extracted and precipitated under the given conditions. Later work (2) has indicated that excess nitrogenous material is measured by this method.

Lowry, Gilligan, and Katersky (2) developed a gravimetric procedure involving extraction of soluble non-collagenous substances with 0.1 N NaOH at room temperature and conversion of collagen to gelatin by autoclaving; collagen was determined by difference in the dry weight of the residues. It was necessary to assume that different tissues behaved alike under the standard conditions and that the various fractionating procedures specifically effected the separation of the different components of the tissues. Abercrombie and Johnson (3) obtained an extract of nerve tissue by autoclaving according to the procedure of Lowry *et al.* (2) and calculated collagen from the nitrogen content of the extract. Elastin has been determined by weighing the fraction of tissue resistant to solution in 0.1 N NaOH at 100° (2).

A simple method is described here for determining collagen in small amounts of tissue with a precision and accuracy usually better than ±5 per cent. In tissues containing but little collagen much of the soluble protein is separated from the tissues with the aid of 20 per cent urea solution. (Tissues containing large amounts of collagen do not require this preliminary separation.) The collagen is converted to soluble form (gelatin) by hydrolysis with water in an autoclave and thus extracted from elastin and other substances not dissolved by the process. The collagen content is estimated from the hydroxyproline content of acid hydrolysates of the extract.

* This work was done during the tenure of a Life Insurance Medical Research Fellowship.

As previously shown (4), the hydroxyproline content of collagen from various mammalian or avian sources is 13.5 ± 0.24 per cent and apparently is present in significant amounts in no other protein except elastin.

Elastin, which contains 1.5 to 2.3 per cent hydroxyproline, depending on the source, is estimated from the hydroxyproline content of the residue remaining after extraction of the collagen.

Procedure

The collagen content of tissues is expressed as per cent of dry fat-free weight. To 100 to 1000 mg. of finely minced tissue are added 15 ml. of acetone. After 6 hours or more the acetone is decanted and replaced by 15 ml. of fresh acetone which is allowed to stand 6 hours or more. This is followed by extraction with 15 ml. of ether for 12 to 16 hours. The residue is dried to constant weight at 108°.

For the extraction of collagen, 5 to 100 mg. portions of dry fat-free tissue high in collagen (skin, aorta, chordae tendineae, bone) are accurately weighed and placed in 15 ml. centrifuge tubes with 4 ml. of water. The tubes are stoppered with cotton and gauze plugs and autoclaved 3 hours at 15 pounds pressure. The solution is centrifuged until clear if necessary and transferred to 18 × 150 mm. test-tubes. The residue is washed with 4 ml. of water which, after centrifuging, is transferred to the test-tube. The residue in the centrifuge tube is again autoclaved with about 4 ml. of water for 3 hours. In the meantime, the supernatant in the test-tube is evaporated almost to dryness by directing a stream of air into the tube placed in a boiling water bath. The supernatant from the second autoclaving and two washings with hot water are transferred to the test-tube and then evaporated to dryness.

To prepare hydrolysates of the extracted collagen, 1.0 ml. of 6 N HCl is added for each estimated 50 mg. of extracted protein. The tubes are sealed and autoclaved 3 hours at 50 pounds pressure. The hydrolysates are neutralized with NaOH solution, diluted to appropriate volume (35 to 120 γ of hydrolyzed collagen per ml.), and filtered. Hydroxyproline is determined according to the procedure previously described (4).

Tissues low in collagen (muscle, kidney, spleen) require a preliminary extraction of non-collagenous proteins. This preliminary extraction is accomplished by grinding 100 to 1500 mg. of the fresh tissue (dry fat-free weight being determined from other samples of the tissue) with sand and 20 per cent urea solution in a mortar. The mixture is suspended in about 40 to 45 ml. of urea solution at room temperature in a 50 ml. centrifuge tube for 1 hour with occasional stirring. The tubes are centrifuged and the supernatant is discarded. The residue is washed three times with 45 ml. of water, and transferred to a 15 ml. centrifuge tube with the aid

of about 10 ml. of water which is removed by centrifugation and discarded. The residue is stirred with 4 to 5 ml. of water and autoclaved. The subsequent procedure is as described above.

The hydroxyproline content of collagen preparations from different mammalian and avian sources has been found to be 13.4 ± 0.24 per cent (4). Hydroxyproline may be converted to its equivalent of collagen through multiplication by the factor 7.46. The percentage of collagen of a tissue sample may be expressed by the relation

$$\frac{\text{Micrograms hydroxyproline in 1 ml. hydrolysate}}{\text{Micrograms tissue represented in 1 ml. hydrolysate}} \times 7.46 \times 100$$

As a measure of protein other than collagen remaining in extracts used for estimation of collagen, the tyrosine content of hydrolysates of the extracts was determined with the aid of the Folin-Ciocalteau phenol reagent according to the method of Heidelberger and MacPherson (5). The Millon-Lugg procedure as modified by Block and Bolling (6) yielded essentially similar values. The hydroxyproline values of extracts and residues for collagen and elastin estimations were corrected for the color contributed by tyrosine.

To determine elastin, the residues remaining from the collagen determination (which had been autoclaved for two 3 hour periods and washed after each autoclaving) are autoclaved for a third 3 hour period and again washed with 8 ml. of water. The residue is then hydrolyzed for 3 hours at 50 pounds pressure with 6 N HCl, and the hydroxyproline determined. The values are corrected for tyrosine present.

The per cent of elastin is calculated from the expression (micrograms of hydroxyproline)/(micrograms of sample) × factor × 100. The factor is 66.7 for pig elastin (1.50 per cent hydroxyproline), 52.3 for beef elastin (1.91 per cent hydroxyproline), and 43.4 for rat elastin (2.30 per cent hydroxyproline).

Collagen in tissues is contained in tendons, membranes, or in fibrils forming a network within structures. The content in a muscle may be expected to increase as the locations of the samples taken for analysis approach the fibrous attachments.

Comparison of the collagen content of different tissues necessitates the analysis of representative samples of entire organs or careful definition of the location of the sample taken to represent a constituent part.

Most of the analyses reported in this paper were carried out to test the reproducibility of the procedure on small contiguous samples of similar gross appearance from muscles, or organs of large animals, or minced portions of the entire organs of small animals.

From the muscles of large animals, areas near the attachments were

avoided and membranous surfaces discarded to obtain uniform samples of tissue. Similarly, portions of apparently uniform beef or pig kidney cortex or liver tissue were dissected. On the other hand, the entire ventricles, liver lobes, and spleen of the rat were used for analysis.

Results

The collagen content of samples of tissue determined by the present procedure is recorded in Table I. The values ranged from 85 per cent collagen content for beef chordae tendineae to 0.22 per cent for rat brain. The average deviation from the mean of the determinations was usually less than ±5 per cent of the mean. The correction for tyrosine in the majority of samples was less than 1 per cent and never greater than 3 per cent of the collagen value.

Also in Table I is shown the elastin content of samples of tissues as determined by the present procedure. The values ranged from 57 per cent for pig aorta to 0.5 per cent for rat kidney cortex. Liver, brain, and myocardium did not contain measurable amounts of elastin. The average deviation from the mean of the values was usually less than ±5 per cent of the mean. Tyrosine corrections were 2 to 20 per cent of the elastin values.

DISCUSSION

Autoclaving several different tissues (except bone and teeth) for 2 to 4 hours resulted in the extraction of 93 to 98 per cent of the collagen. Autoclaving for two 3 hour periods removed 99 per cent or more of the collagen, and the extracts from an additional 3 hours contained less than 1 per cent as indicated by hydroxyproline determinations. Tissues were autoclaved for two 3 hour periods (rather than one 6 hour period) and the supernatant liquid decanted after each autoclaving to insure complete removal of any gelatin which might be held in the insoluble residue.

Undecalcified bone and teeth retained 5 to 9 per cent of their hydroxyproline after 6 hours and 3 per cent after 12 hours of autoclaving. The total hydroxyproline extracted, as well as that retained in the residues, could be obtained from the determinations made on hydrochloric acid hydrolysates of the whole tissue.

Autoclaving of whole tissues removes proteins which contain tyrosine along with the collagen. Because tyrosine produces 1.5 per cent as much color as does hydroxyproline in the determination, it is advisable to remove much of the extraneous protein from tissues which contain but little collagen by a preliminary extraction with 20 per cent urea solution.

Tissues high in collagen or containing but small amounts of other pro-

TABLE I

Collagen and Elastin Content of Tissues

Tissue	Collagen* (uncorrected)	Tyrosine correction	Collagen* (corrected)	Average deviation from mean	Elastin* content	Average deviation from mean
	per cent	*per cent of total color*	*per cent*	*per cent*	*per cent*	*per cent*
Beef aorta (arch)	23.2 (4)†	0.60	23.1	2.1	39.8 (2)†	1.1
" " " ‡					37.8 (2)	0.50
Pig " "	16.1 (4)	0.65	16.0	3.1	57.1 (1)	
" " " ‡					53.4 (4)	4.2
Rat " "	25.7 (2)	0.40	25.6	2.3	47.7 (2)	0.33
" " " ‡					47.0 (2)	1.38
Beef bone (tibia)§	24.2 (4)	0.11	24.2	0.43		
Rat " (femur)§	15.1 (4)	0.15	15.1	2.5		
Beef chordae tendineae (large)	84.8 (5)	0.25	84.6	0.78	4.88 (1)	
Pig chordae tendineae (large)	77.1 (2)	0.27	76.9	3.5	3.69 (1)	
Beef liver‖	1.99 (4)	1.12	1.97	9.0	0 (2)	
Pig " ‖	2.49 (5)	1.21	2.46	5.1	0 (2)	
Rat " ‖	0.65 (3)	2.15	0.64	5.1	0 (2)	
Beef kidney cortex‖	5.30 (4)	0.39	5.28	2.8	1.65 (2)	2.7
Pig " " ‖	3.82 (5)	0.43	3.80	3.9	0.58 (2)	2.8
Rat " " ‖	3.35 (3)	0.57	3.33	0.99	0.45 (2)	6.0
Beef muscle (shoulder)‖	2.14 (4)	2.90	2.08	2.3		
Rat " (abdominal)‖	5.83 (3)	0.98	5.77	4.1		
Beef myocardium (left ventricle)‖	1.99 (5)	2.96	1.93	2.8	0 (2)	
Pig myocardium (left ventricle)‖	2.24 (5)	2.86	2.18	2.2	0 (2)	
Beef myocardium (right ventricle)‖	3.82 (4)	1.56	3.76	3.4	0 (2)	
Pig myocardium (right ventricle)‖	3.45 (2)	2.00	3.38	0.90	0 (2)	
Rat ventricle‖	3.00 (3)	1.44	2.96	2.7	0 (2)	
Beef spleen‖	3.12 (4)	0.75	3.10	2.9	4.55 (2)	5.1
Pig " ‖	2.42 (4)	0.82	2.40	6.1	1.25 (2)	8.0
Rat " ‖	3.52 (2)	0.57	3.50	0.85	0.55 (2)	15.0
Rat brain (cerebrum)‖	0.22 (2)	1.80	0.22	2.3	0 (2)	
" duodenum‖	12.0 (2)	0.38	12.0	0.0		
" lung‖	11.3 (2)	0.44	11.3	1.7	4.89 (2)	1.04
" stomach (cardia)‖	23.7 (3)	0.40	23.6	4.6	1.64 (3)	12.9
" " (pylorus)‖	13.9 (3)	0.52	13.8	6.5	1.27 (2)	2.8
" teeth (incisor)§	10.8 (2)	0.16	10.8	1.4		
" skin (side)	67.8 (4)	0.26	67.6	1.54		

TABLE I—*Concluded*

Tissue	Collagen* (uncorrected)	Tyrosine correction	Collagen* (corrected)	Average deviation from mean	Elastin* content	Average deviation from mean
	per cent	*per cent of total color*	*per cent*	*per cent*	*per cent*	*per cent*
Dog skin¶	64.5 (2)	0.34	64.3	0.46		
Guinea pig skin¶	72.3 (2)	0.21	72.1	0.41		
Human skin**	72.1 (3)	0.25	71.9	0.69		

* Based on dry fat-free weight.

† The figures in parentheses indicate the number of samples.

‡ Based on weight of residues from autoclaving and NaOH treatment.

§ Autoclaved for 12 hours.

‖ Extracted with 20 per cent urea before autoclaving.

¶ Kindly supplied by Dr. Wm. A. Altemeier, Department of Surgery, University of Cincinnati College of Medicine.

** Kindly supplied by Dr. R. R. Suskind, Department of Dermatology, University of Cincinnati College of Medicine.

tein made soluble by autoclaving (skin, bone, aorta, chordae tendineae) do not require preliminary extraction.

Tyrosine-containing proteins were present in extracts made by autoclaving whole tissues of low collagen content (muscle, liver, spleen, brain, kidney) in amounts sufficient to produce color equivalent to 2 to 9 per cent of that from the hydroxyproline. After preliminary extraction with 20 per cent urea solution, the tyrosine remaining usually accounted for much less than 2 per cent of the total color formation, except in the case of muscle tissue, especially heart muscle, in which the remaining tyrosine color amounted to almost 3 per cent of the total (Table I).

The hydroxyproline content of the residues obtained after urea extraction were found to be equal to that of the whole tissues tested (Table II).

Preliminary extraction of heart muscle tissue overnight with 0.1 N sodium hydroxide did not remove appreciably greater amounts of the extraneous tyrosine-containing proteins than did urea. This extraction furthermore introduced the necessity of a tedious extraction of the NaOH from the residue which had been made viscous by the swelling of the nucleates. Occasionally, low values for collagen were obtained either as a consequence of mechanical losses or through the dissolution of part of the collagen by the alkali.

It is not presumed that the precision of the elastin determination by this or any other procedure available at present is equal to that of the collagen analyses. There is good evidence that elastin is measured by it

with adequate reproducibility to indicate changes which may occur in tissues under normal and pathological conditions.

The hydroxyproline content of samples of elastin prepared by different procedures, although small, has been found to be essentially similar within the limits of the analytical method. For instance, the hydroxyproline contents of pig and beef aorta were respectively 1.50 to 1.91 per cent, whether the tissues were autoclaved for three 3 hour periods and washed after each autoclaving and then heated at 100° for 30 minutes with 0.1 N NaOH or heated at 100° for 40 hours with urea solution.

Previous procedures for the determination of elastin rested on the assumption that the residue after extraction with 0.1 N NaOH contained all

TABLE II

Comparison of Collagen Content Found in Tissues with and without Preliminary Extraction with Urea Solution

Tissue	Tyrosine correction, per cent total color		Collagen found,* per cent	
	Sample A†	Sample B‡	Sample A†	Sample B‡
Beef spleen	5.7	1.0	3.34	3.29
Pig "	6.9	0.1	3.25	3.17
Beef kidney cortex	7.0	0.7	6.82	7.03
Pig " "	7.0	0.7	3.27	3.44
Beef myocardium, left ventricle	6.9	2.8	2.16	2.19

* Corrected for tyrosine color.

† Sample A, whole tissue.

‡ Sample B, extracted with 20 per cent urea solution.

the original elastin and no other substances, because the elastin was estimated from the weight of the residue.

Treatment of myocardium (residues) with 0.1 N NaOH at 100° for 30 minutes left a weighable residue which, however, contained no hydroxyproline and therefore was not elastin. Similar treatment of kidney residues, in which the elastin present is known to be in fine fibrils, produced results which were considerably lower than those obtained from the hydroxyproline content of thrice autoclaved samples. It is reasonable to presume that the lower values determined after NaOH treatment may have been due to partial solution of the fine fibrils by the NaOH.

In the procedure described here, it is only necessary to assume that collagen is separated quantitatively from the elastin. Extraneous proteins which contain no hydroxyproline do not influence the results.

The amount of hydroxyproline contained in the third extraction after autoclaving would increase the determined elastin value of aorta 1 per

cent, and as much as 20 per cent in kidney in which the elastin content is small (0.6 per cent) and the collagen content relatively high (4 per cent). The amount removed in each extraction (90 per cent of the amount in the residue) corresponds to that expected on a clear separation of an insoluble moiety from a soluble portion. Consequently the error due to retained collagen in the precipitate can be estimated to be not greater than 2 per cent. After the third autoclaving of liver, brain (cerebrum), and myocardium, a residue remained which was comparable in amount to that obtained from other tissues. These residues contained no measurable amount of hydroxyproline and therefore insignificant amounts of elastin, results which are in keeping with the histological examination of these tissues.

SUMMARY

1. A colorimetric method of determining the collagen content of tissues is described.

2. Means are provided for estimating the effectiveness of separation of collagen from tissue by autoclaving.

3. A method of estimating the elastin content of tissue is described.

4. The collagen content of different tissues of the cow, pig, and rat, and of human, guinea pig, dog, and rat skin as determined by the present method is reported.

5. The estimated elastin content of different tissues is reported.

BIBLIOGRAPHY

1. Spencer, H. C., Morgulis, S., and Wilder, V. M., *J. Biol. Chem.*, **120**, 257 (1937).
2. Lowry, O. H., Gilligan, D. R., and Katersky, E. M., *J. Biol. Chem.*, **139**, 795 (1941).
3. Abercrombie, M., and Johnson, M. L., *J. Neurol., Neurosurg. and Psychiat.*, **9**, 113 (1946).
4. Neuman, R. E., and Logan, M. A., *J. Biol. Chem.*, **184**, 299 (1950).
5. Heidelberger, M., and MacPherson, C. F. M., *Science*, **97**, 405 (1943).
6. Block, R. J., and Bolling, D., Amino acid composition of proteins and foods, Springfield (1945).

THE DETERMINATION OF COLLAGEN AND ELASTIN IN TISSUES

Robert E. Neuman and Milan A. Logan

J. Biol. Chem. 1950, 186:549-556.