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Technical methods

Estimation of hydroxyproline by the AutoAnalyser

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A method has been developed for the determination of hydroxyproline by the Technicon AutoAnalyser[1] using a modified version of the Stegemann assay (1958). This involves oxidation of the hydroxyproline with chloramine T; excess chloramine T is destroyed with perchloric acid and the hydroxyproline chromogen is then reacted with p-dimethylaminobenzaldehyde to develop a pink colour. In the present method the absorption is measured at 550 mμ. The reagents used are adapted from the method described by Woessner (1961), in particular the composition of the p-dimethylaminobenzaldehyde solution (P.D.A. reagent) has been changed to avoid precipitation in the transmission tubing. This method has been applied to the determination of collagen and elastin in arteries and connective tissues and the results obtained agree well with those found using a manual method.

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[1]Technicon Instruments Company.

EXPERIMENTAL

REAGENTS Buffer is made up of 50 g. citric acid monohydrate, 12 ml. glacial acetic acid, 120 g. sodium acetate ($3H_2O$), and 34 g. sodium hydroxide. These are dissolved in water to a final volume of 1 litre, the *p*H is adjusted to 6.0 and stored under toluene in the cold.

p-Dimethylaminobenzaldehyde (*P.D.A.*) A 5% solution of p-dimethylaminobenzaldehyde (E. Merck, 3057) is made in n-propanol. The solution should be clear and very pale yellow. Store in a refrigerator.

Perchloric acid 70% Perchloric acid A.R., 27 ml., is diluted to 100 ml. with water.

Chloramine T Chloramine T (1·41 g.) is dissolved in 80 ml. water and diluted with 120 ml. of methyl cellosolve (ethylene glycol monomethyl ether) and 200 ml. buffer; this solution is made up fresh daily.

STANDARDS Solutions of hydroxyproline containing 2, 5, 10, 15, and 25 μg./ml. are made in distilled water containing a small amount of hydrochloric acid (final *p*H about 3).

PROCEDURE The estimations are carried out using the manifold shown in Figure 1. Samples are run at 60 per hour with water cups alternating with the sample cups to give a more efficient wash. A calibration curve is set up from the above standards. The relation between

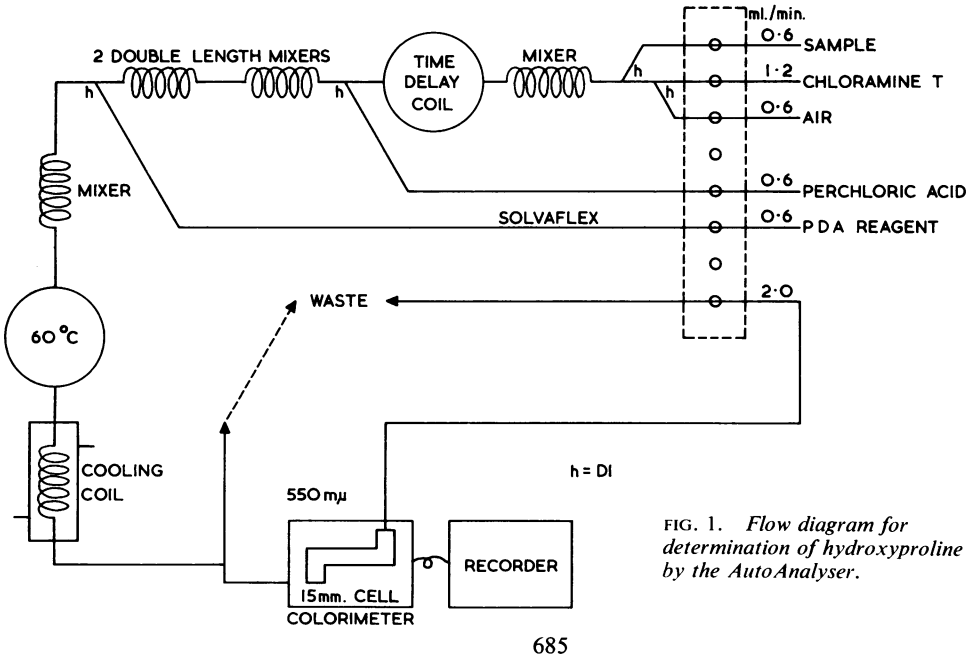

FIG. 1. *Flow diagram for determination of hydroxyproline by the AutoAnalyser.*

TABLE I

COLLAGEN AND ELASTIN CONTENT OF PIG AORTA[1]

Section	*No.*	*Collagen*		*Elastin*	
		Manual	*AutoAnalyser*	*Manual*	*AutoAnalyser*
Arch	4	17·6 ± 1·0	16·5 ± 1·4	47·7 ± 6·6	50·6 ± 3·8
Mid thoracic	4	18·5 ± 2·6	18·0 ± 1·4	45·5 ± 4·4	49·3 ± 3·5
Upper abdominal	4	34·6 ± 6·5	35·5 ± 6·6	28·7 ± 2·6	30·2 ± 5·0
Subrenal	4	32·7 ± 4·0	35·1 ± 5·3	29·2 ± 2·6	29·0 ± 2·8

[1]Results are expressed as percentages of dry fat-free tissue (mean ± S.D.)

optical density and concentration is almost linear in the range 2-20 μg. hydroxyproline and deviates somewhat above this.

Precautions should be taken to prevent p-dimethylaminobenzaldehyde precipitating out in the flow cell and tubing after a run has been completed. This can be avoided by flushing the P.D.A. reagent line with n-propanol and the other reagent lines with 1 N-HCl, finally flushing all the lines with water.

APPLICATION TO DETERMINATION OF COLLAGEN AND ELASTIN IN ARTERIAL TISSUE

Selected pieces of artery are rinsed with saline and freed from adhering connective tissue. The samples are then cut into small pieces (about 2 mm. square) and placed under acetone for several hours. The acetone is then replaced by fresh and, after standing overnight, the acetone is removed and replaced by a mixture of ethyl alcohol and diethyl ether (1:1 by volume). After standing overnight the ether-alcohol mixture is removed and the tissue dried to constant weight in a hot air oven at 50 to 70°C.

COLLAGEN Portions of dry defatted tissue weighing 10 to 20 mg. are placed in stoppered or screw-top glass tubes with 4 ml. of distilled water. The tubes are then heated in an autoclave for six hours at 15 lb. pressure (Neuman and Logan, 1950). The tubes are then removed, allowed to cool, and the aqueous extract containing gelatin is poured into a small beaker (20 to 30 ml.). The residual tissue is washed with about 5 ml. of boiling water and the washing added to the first extract. The solution in the beaker is evaporated to dryness in a hot air oven and the residue taken up in two successive 1 ml. portions of 6 N hydrochloric acid and transferred to a stoppered tube. Hydrolysis is achieved by heating for six to 12 hours at 15 lb. pressure in the autoclave. After cooling the hydrolysate is diluted to 10 ml. with water and filtered if turbid.

To prepare samples for the AutoAnalyser, 1 ml. portions of the diluted hydrolysate are further diluted with 1 ml. of 1 N sodium hydroxide solution and 3 ml. of water. In the case of tissues containing more than 25% of collagen, *e.g.*, skin, a higher final dilution should be used.

In calculating the results it is assumed that:
% collagen = % hydroxyproline × 7·46 (Neuman and Logan, 1950).

ELASTIN After extraction of the collagen the tissue residue is autoclaved for a further six hours with 4 ml. of water to remove any traces of collagen which could interfere with the accuracy of the elastin estimation. The second aqueous extract is discarded and the tissue remaining is hydrolysed with 2 ml. of 6 N hydrochloric acid for six hours at 15 lb. pressure. The hydrolysate is allowed to cool and is then diluted to 10 ml. with water and filtered if necessary.

Portions, each of 1 ml., of the diluted hydrolysate are diluted with 1 ml. of 1 N sodium hydroxide solution before placing in the sample cups.

It is assumed that:
% elastin = % hydroxyproline (not removed by autoclaving) × 66·7 (pig elastin) 52·3 (ox elastin) 43·4 (rat elastin).

The inherent accuracy of the elastin determination is less than that of the collagen estimation owing to the small content of hydroxyproline and the consequent large factor.

RESULTS

A series of samples taken from different parts of the pig aorta were analyzed for collagen and elastin by the autoanalyser method and manually using the method of Neuman and Logan (1950). The results are shown in Table I and good agreement was obtained between the two methods.

SUMMARY

A method for the determination of hydroxyproline by the Technicon AutoAnalyser has been developed and applied to the analysis of arterial tissue for collagen and elastin.

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