The Chemistry of Connective Tissues

8. THE COMPOSITION OF ELASTIN FROM THREE BOVINE TISSUES*

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The morphological form in which elastic fibres appear in different tissues is so variable that some authors have been led to doubt if all the fibre forms, identified histochemically, are composed of a unique fibrous protein (for review see Hall, 1959). The subject is confused by the great variation in the composition of the ground substance surrounding, and sometimes penetrating, elastic fibres in different elastic tissues. Confusion has also resulted from the considerable variation in the ease with which the elastic fibres in different sites may be purified by chemical means. In the present work three typical bovine tissues have been chosen as sources of elastin: ligamentum nuchae, aorta and the elastic cartilage of the external ear. Purification procedures of progressive severity have been applied to these tissues and the composition of the products examined at each step in order to discover if a single protein species, of constant composition and physical properties, results finally from each. Because of the danger that severe extraction procedures will themselves result in changes in the composition and properties, the purified protein preparations have been examined for changes brought about by partial hydrolysis. Such changes would be expected to be revealed by changes in the fluorodinitrobenzene titre and dye-binding capacity.

METHODS

Purification of elastin

Purified elastic-fibre preparations were obtained from three bovine tissues: ligamentum nuchae, aorta and the elastic cartilage of the external ear. In each case the structures were dissected free from adhering tissues, cut into thin strips and extracted with a large volume of 1% (w/v) NaCl in a low-speed mechanical shaker. The treatment was continued until the extract gave a negative test for protein with trichloroacetic acid solution. The strips of tissue were then suspended in water and disintegrated in a high-speed blender. The fibre bundles were recovered by low-speed centrifuging and washed several times with further quantities of water. The washed preparations were

finally defatted with acetone, ethanol and ether, and dried in warm air.

From the dried defatted tissue, samples of purified elastin were prepared by each of the following procedures.

Autoclaved elastin. The tissue, suspended in water, was repeatedly autoclaved at 15 lb./in.² pressure, dehydrated with acetone, and re-autoclaved after being milled in the dry state, according to the procedure described by Partridge, Davis & Adair (1955). The purified samples were free from collagen, as judged by their hydroxyproline content, and contained no extractable fat.

Autoclaved elastin extracted with 0.5N-sodium hydroxide at 25°. Extraction was continued by using a gentle mechanical shaker for three or four periods of 1 hr., or until the extract gave a negative test for protein. With autoclaved ligamentum-nuchae elastin the procedure extracted no more than 2% of the dry weight of the preparation, but with elastin from other sources the amount of material extracted was greater.

Elastin extracted with 0.1N-sodium hydroxide at 98°. The dried defatted tissue was digested with 0.1N-NaOH at 98° for 45 min., according to the procedure of Lansing, Rosenthal, Alex & Dempsey (1952).

Elastin extracted with 88% (v/v) formic acid at 45°. The dried defatted tissue was extracted with 88% (v/v) formic acid in an oven at 45° according to the method of Lloyd & Garrod (1946) and Ayer; Hass & Philpot (1958). Extraction was continued for 48–72 hr. in different samples. The purified elastin fibres were recovered by repeated washing with hot water in a low-speed centrifuge.

Analytical methods

Determination of the rate of alkaline hydrolysis. Samples (0.2 g.) of elastin from the three different tissues, prepared by autoclaving with water (autoclaved elastin) were suspended in a measured volume (100 ml.) of 0.5N-NaOH and the vessels were set up together on a shaker that provided a gentle rocking motion. The assembly was kept in a constant-temperature room at 25°. At intervals the shaker was stopped and small samples were pipetted from the clear supernatant solutions. After 3–7 days the samples had completely dissolved and the clear solutions so formed were used to prepare a standard curve relating the colour yield to protein concentration in the modified biuret method of Lowry, Rosebrough, Farr & Randall (1951). This curve was then used to determine the amount of protein solubilized at intervals during the reaction.

Rate of hydrolysis in formic acid. Elastin purified by extraction with formic acid was used. Samples of dried elastin powder, from ligamentum nuchae, aorta and ear cartilage, were extracted with 88% (v/v) formic acid at 45° for the minimum period to remove collagen and ground

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substance (48 hr.). Weighed samples were then suspended in 88% (v/v) formic acid and placed in an incubator at 45°. The vessels were gently shaken by hand three times each day and small samples of the clear supernatant were taken at intervals throughout the period of reaction (20 days). The amount of protein solubilized at progressive time-intervals was then measured by the method given above.

Determination of ultraviolet-absorption spectra. Samples (100 mg.) of autoclaved elastin from the three sources were each digested with 0.5 mg. of a purified preparation of pancreatic elastase (Banga, 1952) in 0.1M-Na₂CO₃-HCl buffer, pH 8.8. The reaction was continued until dissolution was just complete, when the solution was neutralized to pH 7, boiled, and, when cool, filtered under gravity through Whatman no. 54 paper. The clear solution was then made up to a standard volume (100 ml.) adjusted to 0.1N with respect to HCl. These solutions were used for the comparison of the ultraviolet-absorption spectra of the soluble products from elastin preparations derived from ear cartilage, aorta and ligamentum nuchae.

Determination of N-terminal residues. For the preparation of the dinitrophenyl (DNP) derivatives of elastins and modified elastins, the original reaction conditions described by Sanger (1945) were employed. The insoluble DNP-proteins were hydrolysed by refluxing with 11.4N-HCl for 2 hr. The DNP-amino acids extractable into ether were separated by using the 2-methylbutan-2-ol-phthalate buffer and benzene-water-acetic acid systems of Blackburn & Lowther (1951). The DNP-amino acid spots were eluted quantitatively with water and the amounts present were determined by measuring the extinction at 360 m μ with a spectrophotometer. Corrections were applied for losses of DNP-amino acids during hydrolysis and chromatography on the basis of preliminary trials with pure DNP-amino acids.

Amino acid analysis. The chromatographic procedure was that of Moore, Spackman & Stein (1958). Tryptophan determination was carried out by the method of Horn & Jones (1945).

Electron microscopy

For this work elastin fibres prepared by extraction with alkali without autoclaving were used. Fibre preparations from the three kinds of tissue were disintegrated by ultrasonic treatment (50 kcyc./sec.) according to the procedure of Gotte & Serafini-Fracassini (1963). The opalescent suspension resulting was freed from coarse particles by repeated sedimentation under gravity, and the final stable suspension of microfibrils was applied to collodion film and allowed to dry. The specimens were shadowed with chromium at 20°.

Changes in staining character with partial hydrolysis

Changes in the staining character of elastin after mild treatment with NaOH solution were investigated. Samples of the purified elastin-fibre preparation (obtained by digestion with 0.1N-NaOH at 98° for 45 min.) were extracted with 0.5N-NaOH at 25° for 4 hr. (sample A), 8 hr. (sample B), 24 hr. (sample C) and 48 hr. (sample D). The samples were gently rocked with a large excess of alkali in a constant-temperature room, washed with water and dried.

Five dye solutions, of the composition given in Table 3, were investigated. The dyes used were: orcein (G. T. Gurr

Ltd.; batch 14689); aniline blue (G. T. Gurr Ltd., batch 1248); bromophenol blue (British Drug Houses Ltd., batch 240980); fast green (G. T. Gurr Ltd.); methyl green (G. T. Gurr Ltd., batch 14579). The elastin samples (10 mg.) were first equilibrated with the solvent used for dissolving the dye by shaking with an excess of solvent for 1 hr. at 20°. The solvent was then poured off, replaced by 1 ml. of dye solution and allowed to stain for 24 hr. with intermittent shaking. The fibres were allowed to settle and the dye solutions decanted into 0.5 ml. light-path spectrophotometer cells. The extinction of the dye which had not been taken up by the elastic fibres was measured at the wavelength of maximum absorption for the dye solution used.

RESULTS

Table 1 shows the results of amino acid analysis of material prepared from the three types of tissue by autoclaving at neutrality, by extraction with alkali at 25° or by extraction with alkali at 98°. With the product from ligamentum nuchae, alkali extraction results in a protein of almost identical composition with that produced by autoclaving. The product from aorta, extracted with alkali at 98°, again has a closely similar composition but the autoclaved material has a significantly higher content of aspartic acid, glutamic acid, tyrosine, lysine, arginine and tryptophan, and a significantly lower content of glycine, alanine and valine. This suggests that autoclaved aorta elastic fibres are contaminated by a second protein with a composition very different from that of elastin and that this substance can be removed by treatment with alkali. The attempt to extract autoclaved aorta elastin with cold dilute alkali resulted in considerable purification, but clearly did not remove the whole of the contaminant. The process of autoclaving applied to ear-cartilage tissue gave a product that contained an even higher proportion of acidic and basic amino acids than the autoclaved product of aorta. With this tissue, extraction with alkali at 25° resulted in little change in composition, but again extraction of the autoclaved fibres with alkali at 98° resulted in a considerable decrease in the content of diamino and dicarboxylic acids with a corresponding increase in the content of amino acids with aliphatic side chains. From this it appears that ear-cartilage elastin is contaminated with a protein that can be extracted with alkali only with difficulty. The amino acid analysis of the residue after extraction at 98° is of the same general pattern as that of ligamentum-nuchae and aorta elastin, but the differences are too great to be accounted for by experimental error. Accordingly, attempts were made to continue the alkali treatment in the hope of diminishing the suspected contamination still further.

However, as described below, the product from ear cartilage was exceptionally sensitive to the

Table 1. *Amino acid composition of bovine elastins*

Experimental details are given in the text. The results are expressed as g. of amino acid/100 g. of dry ash-free protein.

Method of isolation	Ligamentum nuchae		Aorta			Ear cartilage		
	Auto-claved	Extracted with 0.1 N-NaOH at 98°	Auto-claved	Autoclaved, then extracted with 0.5 N-NaOH at 25°	Extracted with 0.1 N-NaOH at 98°	Auto-claved	Autoclaved, then extracted with 0.5 N-NaOH at 25°	Autoclaved, then extracted with 0.1 N-NaOH at 98°
Amino acid								
Aspartic acid	1.1	0.7	2.0	1.4	1.0	3.5	3.4	2.1
Serine	0.9	0.9	1.1	1.4	1.0	1.6	1.4	0.9
Threonine	1.1	1.0	1.6	1.5	1.0	1.6	1.3	1.0
Glutamic acid	2.4	2.4	3.4	3.4	2.4	6.4	6.3	4.6
Proline	13.5	15.2	—	12.1	14.0	13.7	12.8	14.3
Glycine	26.7	26.8	23.9	25.2	26.8	22.9	22.9	25.8
Alanine	21.3	22.8	19.9	20.9	22.2	16.8	17.2	20.3
Valine	17.7	18.2	16.0	16.7	—	13.4	13.7	16.0
Methionine	Trace	Trace	0.3	Trace	Trace	Trace	0.6	Trace
Isoleucine	3.8	3.5	4.0	3.6	3.8	3.3	3.0	3.0
Leucine	9.0	8.8	8.8	8.3	9.0	9.9	9.9	9.4
Tyrosine	1.5	1.6	2.3	2.1	1.8	3.3	3.3	2.6
Phenylalanine	6.2	5.5	6.0	5.3	—	5.9	5.8	5.6
Lysine	0.5	0.6	1.2	1.0	1.1	1.8	1.6	0.5
Histidine	0.1	Trace	0.3	0.2	0.1	0.5	0.2	0.1
Arginine	1.3	0.8	1.7	1.0	0.8	2.5	2.4	1.3
Hydroxyproline	1.6	1.5	1.5	1.5	1.8	1.9	1.8	1.8
Tryptophan	0.1	0.1	0.5	0.4	0.1	0.3	0.3	0.3
Amino acid nitrogen	16.8	16.9	16.9	16.6	16.8	16.4	16.1	16.7

hydrolytic action of alkali and further treatment resulted in a loss in weight, which appeared to be caused by the dissolution of the substance of the fibres. Further attempts to purify the product from the ear cartilage by more prolonged alkali treatment were therefore abandoned.

Properties of the isolated products. Because of the observation reported above, that elastic fibres isolated from ear cartilage appeared to be less resistant to alkali than fibres isolated from ligamentum nuchae, a direct comparison was made of the rate of dissolution of fibre preparations from the three sources. The three preparations were made by autoclaving at neutral pH and care was taken to standardize the conditions so that each sample had equal treatment. The rate of solution in 0.5 N-sodium hydroxide at 25° is plotted for each sample in Fig. 1. The reaction shows a marked lag phase in each case, but differences in the rate of solution are very marked. About 10% of the preparation from ear cartilage dissolved in the first hour, but this was presumably due to the extraction of the protein contaminant reported above.

Since the amino acid composition of the three samples is very similar, differences in the rate of alkali hydrolysis must be ascribed either to differences in the mean diameter of the fibres or to configurational differences in the substance of the fibres. To gain information about the first point, the sample of elastin fibres from ligamentum

nuchae was finely ground with water in a mortar. During this process some of the fibres were crushed, but the greater part of the product of grinding consisted of irregular grains with a maximum diameter of about 5 μ . The rate of dissolution in alkali of the ground sample was measured under the same conditions as that of the intact fibres, and the results are shown as the broken line in Fig. 1: there was no detectable difference in rate as a result of the decrease in particle size. A similar experiment was carried out to compare the rate of dissolution of the fibre preparations under acid conditions. The preparations were digested in 88% (v/v) formic acid at 45° for periods up to 20 days. The results (Fig. 2) show that there was little difference in the rate at which the different fibres dissolved.

Ultraviolet-absorption spectra. It was thought that a possibility that might account for the variations in rate of dissolution in alkali could be some change in the number or kind of the cross-linkages between adjacent peptide chains. From the study of soluble proteins derived from elastin Partridge *et al.* (1955) found evidence of the presence of covalent cross-linkages in the fibrous proteins, and Partridge & Davis (1955) deduced that these linkages must be present to the extent of one cross-linkage to about 35 amino acid residues. Partridge & Davis (1955) also pointed out that some 4–5% of the nitrogen in ligamentum nuchae elastin could not be recovered as amino acid

nitrogen, and, further, that soluble proteins derived from elastin have a greater ultraviolet absorption than could be accounted for by their content of tyrosine. Thomas & Partridge (1962) have shown that the cross-linkages in elastin are due to a substance with a heterocyclic ring system that absorbs in the ultraviolet and gives a blue-white

fluorescence. Accordingly, if there are differences in the degree to which elastin samples are cross-linked, the soluble proteins derived from them should show differences in ultraviolet absorption.

Fig. 3 shows ultraviolet-absorption spectra of the three samples of elastin after partial hydrolysis with a purified preparation of elastase. (For clarity the curve for aorta elastin has been displaced upwards by a distance corresponding to 0.1 unit on the extinction scale.) The shapes of the three curves are generally very similar but the absorption due to ligamentum-nuchae elastin is slightly lower than that of the other two samples.

Electron microscopy. Gotte & Serafini-Fracassini (1963) have shown clearly for the first time that the elastic fibres from ligamentum nuchae of cattle are composed of very large numbers of long uniform microfibrils no more than about $10\text{ m}\mu$ thick. These authors prepared their material by treating the tissue with 0.01 N -sodium hydroxide at 95° for 1 hr., and then washing it thoroughly in hot water by using a homogenizer. The purified fibre preparation was then suspended in water and treated ultrasonically at 40 kcyc./sec. Most of the fibres were fragmented into granular particles, but some fibres disintegrated longitudinally into fibrils and fibril bundles. The granular material was separated by sedimentation under gravity, and the more stable suspension of microfibrils was applied to collodion film for examination with the electron microscope. Plate 1 shows electron micrographs of material prepared in this way from (a) ligamentum nuchae and (b) aorta. The preparations were shadowed with chromium at 20° . Both kinds of fibre appear

Fig. 1. Time-course of solubilization of elastin from three bovine tissues. The elastin samples (prepared by auto-claving) were suspended in 0.5 N -NaOH and maintained at 25° with gentle rocking. Experimental details are given in the text. $\circ$, Ear cartilage; $\triangle$, aorta; $\square$, ligamentum nuchae. The broken line refers to a ground sample of ligamentum nuchae (see the text).

Fig. 2. Time-course of solubilization of elastin from three bovine tissues in 88% (v/v) formic acid at 5° . Experimental details are given in the text. $\circ$, Ear cartilage; $\triangle$, aorta; $\square$, ligamentum nuchae.

Fig. 3. Ultraviolet-absorption spectra of digests of elastin prepared from three bovine tissues. The solutions contained 1 mg. of elastin/ml. and were adjusted to 0.1 N with respect to hydrochloric acid. Experimental details are given in the text. A, Aorta; B, ear cartilage; C, ligamentum nuchae. For clarity the curve for aorta elastin is displaced upward by 0.1 E unit.

to be composed of microfibrils of similar diameter, but the fibrils from aorta are not so clean in appearance and seem to be interpenetrated and coated with small amounts of an amorphous substance. Microfibrils of approximately the same diameter and similar appearance were also observed in the suspensions produced by ultrasonic treatment of elastic fibres from bovine-ear cartilage.

Changes induced by the method of isolation. Because of the cross-linked structure of elastin it would be expected that any partial hydrolysis that occurs through the use of alkaline or acidic reagents would give rise to rupture-points in the network owing to hydrolysis of susceptible peptide bonds in the back-bone chains. Each rupture-point should result in the appearance of a new α -carboxyl and a new α -amino group attached to the free ends of the broken chains. Another type of hydrolysis to be expected, particularly with alkaline reagents, concerns the liberation of ammonia from asparagine and glutamine residues. These reactions would give rise to a greater number

of charged groups combined to the network and would be expected to result in a change in the staining character of the fibres and corresponding alterations in other physical properties.

Table 2 shows the results of the reaction of a number of samples of elastin with fluorodinitrobenzene under the conditions used by Sanger (1945). The first three elastin samples were prepared from ligamentum nuchae, aorta and ear cartilage by digestion at 98° with 0.1N-sodium hydroxide for 45 min. The number and kind of *N*-terminal residues detected in the sample from ligamentum nuchae were very similar to those found for the preparation of aorta elastin, and were close to those found by Partridge & Davis (1955) for ligamentum-nuchae elastin prepared by autoclaving at neutral pH. This indicates that the extent of damage by hydrolysis suffered by the samples during treatment with sodium hydroxide for 45 min. is very small. The sample prepared in the same way from ear cartilage showed a slightly higher content of *N*-terminal residues, but as

Table 2. *N*-Terminal residues of bovine elastins

Experimental details are given in the text. The results are expressed as moles of *N*-terminal residues/100 000 g. of protein.

Method of preparation	Ligamentum nuchae. 0.1N-NaOH for 45 min. at 98°	Aorta. 0.1N-NaOH for 45 min. at 98°	Ear cartilage. 0.1N-NaOH for 45 min. at 98°	Ligamentum nuchae. 0.5N-NaOH for 66 hr. at 25°	Aorta. 0.5N-NaOH for 66 hr. at 25°
DNP derivative of <i>N</i> -terminal amino acid					
DNP-aspartic acid	0.01	0.02	0.04	0.02	0.06
DNP-glycine + DNP-serine	0.26	0.22	0.45	5.01	5.80
DNP-alanine	0.04	0.03	0.08	0.30	0.33
DNP-valine	0.03	0.05	0.10	1.10	0.90
DNP-leucine	0.02	0.02	0.07	0.60	0.40
Total <i>N</i> -terminal residue	0.36	0.34	0.74	7.03	7.49

Table 3. *Absorption of dyes from solution by an elastin-fibre preparation from bovine aorta and the same preparation after treatment with dilute alkali for various periods at 25°*

Experimental details are given in the text. The samples (10 mg.) had been treated with 0.5N-NaOH at 25° for: A, 4 hr.; B, 8 hr.; C, 24 hr.; D, 48 hr.

Dye solution	Wave-length (m μ)	Extinction of dye solution	Extinction of supernatant solution after shaking with 1 ml. of dye solution for 24 hr. at 20°				
			Untreated control	Sample A	Sample B	Sample C	Sample D
Orcein [50 mg./l. in 70% (v/v) ethanol containing HCl (0.1N)]	500	0.80	0.42	0.55	0.65	0.63	0.66
Aniline blue [10 mg./l. in 1% (v/v) acetic acid]	600	0.32	0.09	0.10	0.15	0.18	0.16
Bromophenol blue [12.5 mg./l. in Na ₂ CO ₃ -HCl buffer, pH 8.8]	590	1.17	0.33	0.33	0.53	0.62	0.71
Fast green [20 mg./l. in 1% (v/v) acetic acid]	625	0.46	0.23	0.21	0.18	0.10	0.09
Methyl green [100 mg./l. in Na ₂ CO ₃ -HCl buffer, pH 8.0]	630	0.86	0.48	0.46	0.38	0.34	0.24

Plate 1. (a) Electron micrograph showing the fibrillar structure of an elastic fibre from bovine ligamentum nuchae. The elastic fibres were treated with dilute sodium hydroxide at 98° for 1 hr., washed with hot water and disintegrated ultrasonically at 40 keyc./sec. Experimental details are given in the text. The specimen was shadowed with chromium at 20°. (b) Electron micrograph showing a disintegrated fibre from bovine aorta. The preparation procedure was the same as for (a).

mentioned above the sample may still be contaminated with other tissue components. The sample from ligamentum nuchae after treatment at 25° with 0.5N-sodium hydroxide for 66 hr. showed a much higher content of terminal α -amino groups. At this stage in the alkaline hydrolysis some 23% of the elastin had dissolved, and most of the increase in *N*-terminal residues was due to the α -amino groups of glycine and serine; peptides formed by these two amino acids are readily susceptible to hydrolysis. When the elastin from aorta was treated for 66 hr. with cold alkali, the amount dissolved was much higher (44%) but the total content of *N*-terminal amino groups found for the insoluble residue was not much greater than for the similar residue from ligamentum-nuchae elastin.

The uptake of dyes for samples of aorta elastin treated for various times with 0.5N-sodium hydroxide at 25° (sample *A*, 4 hr.; sample *B*, 8 hr.; sample *C*, 24 hr.; sample *D*, 48 hr.) is shown in Table 3. The dyes fall into two groups. The first three dyes, orcein, aniline blue and bromophenol blue, stain the untreated elastin well, but mild hydrolytic damage brought about by the action of dilute sodium hydroxide in the cold results in a progressively lower uptake of the dyes. The second group, fast green and methyl green, are both good dyes for collagen under the conditions used and the staining of unmodified elastin is relatively ineffective. However, as the liberation of free α -carboxyl and α -amino groups progresses, owing to the hydrolytic breakdown of peptide bonds within the network (Table 2), the density of staining increases and more of the dye is removed from the equilibrium solution.

DISCUSSION

The methods at present available for the isolation of elastic fibres are all based on the insolubility of elastin and on its relatively very great resistance to hydrolytic reagents. Elastic-fibre formations in connective tissue are universally accompanied by collagen fibres and by components of the ground substance which is of variable composition in different tissues. Histochemical studies, and studies with the electron microscope, show great differences in the morphological form in which elastin is laid down in elastic tissues with differing function, and also reveal that in most types of tissue there is penetration of elastic formations by collagen fibrils and by mucopolysaccharides and mucoproteins of the ground substance. Accordingly, the ease with which elastin may be purified varies very considerably with the source; but the present paper shows, by the use of extraction procedures of increasing severity, that the end product from different kinds of tissues in the same animal approaches constancy of composition.

In the choice of techniques for the purification of elastin it must be borne in mind that collagen is itself resistant to the action of cold alkali or cold dilute acids, but it is easily solubilized in hot dilute alkali, in warm concentrated formic acid or by repeated autoclaving at neutral pH. The ground substance contains molecules of very high molecular weight, such as protein complexes of the chondroitin sulphates, and these substances do not readily yield to extraction unless the conditions are sufficiently hydrolytic to degrade them (Partridge & Elsdon, 1961). Thus, with tissues such as aorta and the elastic cartilage of the bovine ear, conditions that are effective for the extraction of extraneous substances are likely to result in some degree of hydrolytic damage to the elastin itself.

Elastin is now known to contain interchain linkages of covalent character (Thomas & Partridge, 1962), and, accordingly, if the structure is regarded as that of a cross-linked gel, then hydrolytic damage will result in the appearance of new α -carboxyl and α -amino residues at rupture-points in the network of polypeptide chains. The number of *N*-terminal residues was therefore determined with samples prepared by different methods. Little such damage was detected in samples prepared by the hot-alkali technique of Lansing *et al.* (1952), provided that treatment was not extended beyond 45 min. as recommended by these authors; but a considerable increase in *N*-terminal residues resulted when the treatment with alkali was continued to the point at which part of the elastin dissolved.

Considerable variations in the physical properties of elastins prepared by different methods have been reported in the literature, and it is thought that these differences arise in the main because of the effects of partial hydrolysis in opening the structure of the cross-linked gel and in altering its charge density. These changes are revealed by alterations in the dye-staining character of elastic fibres, in changes in their adsorptive capacity for proteolytic enzymes and in the rate of their digestion by elastases. Variations in the rate of hydrolysis by enzymes due to differences in preparative procedure have been reported by several authors. Grant & Robbins (1957) examined two specimens of elastin prepared by boiling ox aorta for 1 hr. in (a) 1N-sodium hydroxide and (b) 0.1N-sodium hydroxide. The rates of enzymic hydrolysis in (a) and (b) were in the ratio 100:17. The adsorptive capacities of (a) and (b) for elastase were assessed by measuring the loss of soluble enzyme after stirring with a fixed weight of elastin at pH 4.7 for 30 min. at room temperature. Contact with elastin preparation (a) resulted in the loss of 89% of the enzyme activity, whereas contact with (b) resulted in only a 63% loss. In both cases the enzyme could be

eluted from the fibre preparations by treatment with 0.2*N*-acetic acid. Similar experiments were performed in this Laboratory with preparations of ligamentum-nuchae elastin prepared by autoclaving and by boiling with 0.1 *N*-sodium hydroxide and with 1 *N*-sodium hydroxide. The autoclaved elastin was less adsorptive than either of the alkali-treated preparations; but its adsorptive capacity could be progressively raised by subsequent treatment with alkali, and very highly active preparations could be made by severe treatment with boiling sodium hydroxide.

Although it appears that the various types of fibre in different tissues of the same animal are of the same or closely similar amino acid composition, it still remains possible that there are other differences in protein constitution that might account for the various typical morphological forms. In an attempt to display any such differences a number of chemical and physical properties of the isolated fibre preparations have been compared. There were marked differences in the rate of solubilization in alkali, but little difference in the rate of solution in concentrated formic acid was observed. The ultraviolet-absorption spectra of soluble elastins prepared from the three fibre preparations also showed little change, and this seems to suggest that the content of the fluorescent chromogen reported by Thomas & Partridge (1962) was substantially the same. Since this chromogen is believed to act as an interchain cross-linkage in elastin, the result suggests that there is little variation in the content of covalent cross-linkages.

The differences in morphological form observed between different types of elastic fibres appear in a new light when the submicroscopic structure of the fibres is examined in the electron microscope. Gotte & Serafini-Fracassini (1963), using ultrasonic disintegration, showed clearly, for the first time, that the massive fibres of ligamentum-nuchae elastin are corded structures containing many thousands of long regular microfilaments no more than 10 *mμ* thick. Using a similar technique, we have now shown that the membranous elastic material of the aorta and the elastic fibres in the cartilage of the external ear are composed of corded microfibrils of similar appearance. It thus seems that the different types of elastin fibres are differently woven structures made up from the same elementary fibril. The native fibres appear to be homogeneous under the light-microscope, and when purified by autoclaving followed by a short treatment with cold dilute alkali their surfaces appear to be clean and uncontaminated by any amorphous material from the ground substance. Nevertheless, such preparations from aorta or ear cartilage still yield polysaccharide-containing material when digested with alkali at 98° (L. Gotte, unpublished

work). It thus seems that the microfibrils of which the fibres are composed are interpenetrated by mucoproteins of the ground substance. The composition of the ground substance is variable in different elastic tissues, but the material of the microfibrils, which accounts for by far the greater part of the fibres, is a unique cross-linked protein substantially free from polysaccharide, and it is to this protein that the name 'elastin' is properly applied. The observed differences in the rate at which various forms of elastin are solubilized by alkali cannot as yet be explained, but it is possible that the phenomenon may be related to an effect of the density of cording of the microfibrils rather than to differences at the molecular level.

SUMMARY

1. Elastic-fibre preparations were isolated by a number of different methods from three typical bovine tissues: ligamentum nuchae, aorta and the elastic cartilage of the external ear. The isolation procedures were of increasing severity and included autoclaving at neutral pH, extraction with dilute sodium hydroxide at 20° and at 98°, and extraction with 88 % formic acid at 45°.

2. The ease with which other components of the connective tissue were released from the elastin fibres varied with the different tissues, but the purified elastin approached a constant amino acid composition that was the same for each tissue.

3. Electron-microscope studies of the purified fibres after ultrasonic disintegration showed that with each tissue the fibres or membranous formations were composed of elementary microfibrils of about the same diameter (10 *mμ*).

4. The rate at which the fibre preparations were dissolved by dilute sodium hydroxide at 25° differed with different tissues. Similar, but much smaller, differences were observed in the rate of dissolution in 88 % formic acid at 45°.

5. The ultraviolet-absorption spectra of soluble elastin from the three tissues were similar.

6. Samples of elastin purified by prolonged contact with dilute sodium hydroxide showed signs of hydrolytic damage. This was revealed by an increase in the content of free α -amino groups, by a change in absorption capacity for various dyes and by an increase in adsorption capacity of the fibres for proteolytic enzymes.

7. It was concluded that the microfibrils forming the main part of the substance of the elastic fibres found in different tissues of the same animal are composed of a unique protein of invariable composition.

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Biochem. J. (1963) **87**, 351

α -(1 $\rightarrow$ 4)-Glucosans

16. THE MOLECULAR STRUCTURE OF SOME PIG-MUSCLE GLYCOGENS*

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In recent years, increasing attention has been directed to a condition in the muscles of pigs that is superficially characterized by an absence of normal pigmentation and by marked exudation of fluid. This condition, the so-called 'white-muscle disease', has been related to the precipitation and denaturation of sarcoplasmic proteins (Bendall & Wismer-Pedersen, 1962) that occurs when a high temperature and a relatively low pH coincide, e.g. when the rate of fall of pH during post-mortem glycolysis is abnormally high (Ludvigsen, 1954; Wismer-Pedersen, 1959; Lawrie, 1960). The condition appears to affect pigs of the Landrace, Pietrain and Poland China breeds more than those of the Large White breed, which predominates in the United Kingdom (Briskey, Bray, Hoekstra, Phillips & Grummer, 1959; Lawrie & Gatherum, 1962). Notwithstanding possible differences in the glycolytic enzymes, it seemed desirable to investigate the structural characteristics of the muscle glycogen in these circumstances. Glycogens from longissimus dorsi muscles of normal British pigs of the Large White breed and of apparently normal and exudative muscle of American pigs of Poland China, Chester White and Hampshire breeds have accordingly been isolated and examined.

* Part 15: Kjølberg & Manners (1962).

MATERIALS AND METHODS

Preparation of glycogen for analysis. Samples of glycogen were prepared before the onset of *rigor mortis* from longissimus dorsi (lumbar) muscles of Large White pigs (of various ages), obtained in the United Kingdom within 1 hr. of slaughter, as described by Lawrie, Manners & Wright (1959). 'Pre-rigor' glycogens were prepared similarly from Poland China, Chester White and Hampshire pigs, samples of longissimus dorsi muscle being frozen in acetone and solid carbon dioxide immediately after slaughter and sent in the frozen condition by air from Madison, Wis., U.S.A., to the United Kingdom.

Analytical methods. Glycogen was determined by the method of Good, Kramer & Somogyi (1933); results are expressed as percentages of wet weight of tissue. The methods used for the determination of glucose content, iodine-staining, periodate oxidation and β -amylolysis limit were those described by Lawrie *et al.* (1959); the interaction with concanavalin-A and measurement of 'glycogen value' was carried out by the method of Manners & Wright (1962*b*). Average chain length was determined by α -amylolysis, as described by Manners & Wright (1962*a*), except that the relation between the apparent percentage conversion into maltose, P_M , and the degree of branching, $P_{(1\rightarrow6)}$, was obtained from a slightly different equation:

$$P_{(1\rightarrow6)} = 23.4 - 0.20P_M.$$

All analytical results are based on the observed glucose contents, which were all higher than 96.5%.