J. Anat. (1980), **131**, 4, pp. 611–620
With 4 *figures*
Printed in Great Britain

The roles of hyaluronic acid, collagen and elastin in the mechanical properties of connective tissues

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(*Accepted* 9 *April* 1980)

INTRODUCTION

Biomechanical testing of connective tissues is usually done on samples of whole tissue. The results thus reflect the combined biomechanical properties of the various components of tissue – in particular those of the fibres and those of the ground substance. Knowledge of the mechanical properties of the individual component is important for understanding the connection between the functional properties and the molecular structure of the tissue. One way of investigating this subject is to study the effect of enzymic degradation of the tissue components on the mechanical properties. This principle has been applied by some authors, but with somewhat variable results (Partington & Wood, 1963; Hoffman, Grande & Park, 1977).

One crucial point in the use of enzymic degradation is the specificity (purity) of the enzyme preparations. A testing procedure has been devised by which one may detect enzymic impurities capable of influencing the mechanical properties of collagen: films of purified, reconstituted collagen extracted from skin were produced and treated with the various enzymes; the biomechanical properties of the films were tested before and after the enzymic treatment; only those enzymes which failed to alter the properties of the collagen films were considered usable for the degradation of the ground substance in the whole tissue samples.

In the present study the production of collagen films, the evaluation of ground substance degrading enzymes by the use of such films, the use of specific enzymes on whole tissue and the mechanical properties of skin and aorta after enzymic treatment are described.

MATERIALS AND METHODS

Production of collagen films

The collagen was prepared from rat skin (freed of hair and muscle), homogenized and extracted with sodium citrate buffer (0·067 M, pH 3·7, 4 °C). The supernatant was collected by centrifugation (50000 *g* for 30 minutes) and the acid soluble collagen purified by double precipitations through adding NaCl until a 20 % solution was reached, followed by dialysis against demineralized water, lyophilization and storage at −20 °C. For moulding into collagen films, lyophilized collagen was brought into solution (1 % w/v) in citrate buffer (0·067 M, pH 3·7, 4 °C) by stirring for 24 hours. The solution was cleared by centrifugation (50000 *g* for 15 minutes, 4 °C) and dialysed against phosphate buffer (0·005 M, pH 7·4, 4 °C) containing NaCl (0·14 M). Moulding chambers made of polyethylene (25 × 25 × 20 mm) covered with dialysis membranes were filled with measured amounts of the collagen solution.

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The chambers were then carefully immersed in a large volume of phosphate buffer (0·005 M, pH 7·4, 4 °C, constant stirring) containing NaCl (0·14 M), placed exactly horizontally and dialysed through the membrane. The temperature of the phosphate buffer was then increased by 2·5 °C every 30 minutes until it reached 29 °C which resulted in a subsequent aggregation of the collagen. The chambers were then immersed over night in demineralized water (29 °C) for dialysis. The membrane was removed from the chambers, which were placed horizontally on a metal plate and kept at 29 °C with air constantly passing the surface of the collagen gels to dry them into films. These were removed from the chambers and stored at −20 °C. Collagen films were rehydrated in Ringer's solution (pH 7·4, 22 °C) and strips, 2 mm wide, were punched out, washed in distilled water and used for the treatment with enzymes.

Test of the ability of enzymic preparations to influence the mechanical properties of collagen films

Strips of collagen films were immersed in buffers containing the enzyme solution. Strips from the same collagen film immersed in buffers only served as control. After the enzymic treatment the strips were washed in Ringer's solution, mounted between two clamps with a jaw space of 3.5 mm, immersed in Ringer's solution and tested in a materials testing machine in the same way as described for aorta and skin specimens. The following enzymic preparations were tested: (*a*) α-amylase from *B. subtilis* (Sigma), 0·4% in a sodium phosphate buffer (0·22 M, pH 5·4, 22 °C) for 6 hours. (*b*) Hyaluronidase from bovine testes (Sigma), 10000 units per 100 ml sodium acetate buffer (0·1 M, pH 5·4, 37 °C) containing NaCl (0·15 M) for 6 hours. (*c*) Collagenase from *Clostridium histolyticum* (Sigma), 4 mg per 100 ml in a 'tris' buffer (0·03 M, pH 7·5, 37 °C) containing $CaCl_2$ (0·005 M) and NaCl (0·2 M) (Reed, 1976). (*d*) Elastase from hog pancreas (Sigma), 10 mg per 100 ml in a carbonate buffer (0·05 M, pH 8·8, 37 °C) for 6 hours.

Procedure for the enzymic treatment and biomechanical test of specimens from aorta and skin

The enzymic preparations of hyaluronidase and collagenase, buffer solutions and temperatures employed for treatment of tissue specimens were the same as those used for treatment of collagen films.

Aorta specimens

Ring-shaped specimens, 2 mm wide, were punched out between the origins of intercostal arteries from the thoracic aorta from twenty male Wistar rats, 120 days old. The specimens were washed in Ringer's solution, weighed and divided at random into five groups, which were submitted to enzymic treatment: (1) collagenase (4 mg per 100 ml) for 2 hours; (2) corresponding buffer control; (3) hyaluronidase (10000 units per 100 ml) for 6 hours; (4) corresponding buffer control; (5) intact control. In an attempt to obtain more profound decomposition of the collagenous structures of thoracic aorta with collagenase, aorta specimens were prepared in the same way from ten male Wistar rats, 360 days old. The ring-shaped specimens from these were divided at random into three groups and submitted to enzymic treatment: (6) collagenase (10 mg per 100 ml) for 6 hours; (7) corresponding buffer control and (8) intact control. Specimens from the intact control groups were immersed in Ringer's solution (pH 7·4, 22 °C) for 30 minutes before testing. After enzymic treatment specimens were washed in Ringer's solution, mounted between two hooks

and immersed in Ringer's solution to which papaverine chloride was added to relax the smooth musculature of the vessel wall. The specimens were then loaded with a constant deformation speed of 10 mm per minute until breaking, while load and deformation were continuously registered and signals fed to a *x-y*-recorder.

Skin specimens

Strip specimens of dorsal skin, 2 mm wide, were punched out transversely with a dice. The specimens were randomly divided into three groups: (1) hyaluronidase (10000 units per 100 ml) for 6 hours; (2) corresponding buffer control and (3) intact control. The cross sectional area of each specimen was estimated by means of an optical micrometer, measuring width and thickness to the nearest 0·1 mm, before mounting in clamps with a jaw space of 3·35 mm. The enzymic treatment was then carried out while the specimen remained untouched in the clamps. The starting position of the clamps was maintained in order to avoid changes in original length which might be brought about by increased laxity during the enzymic or buffer treatment. After treatment, the specimens were loaded until breaking, while recording load and deformation. The specimens from the intact control group were only washed in Ringer's solution and then tested biomechanically.

The effect of collagenase treatment on aortic specimens was controlled by measuring the hydroxyproline content (Grant, 1964) of the collagenase-treated specimens and corresponding buffer controls and also by measuring the hydroxyproline content of the enzyme and buffer solution used for each specimen. The effect of hyaluronidase treatment was ascertained by determining the content of uronic acid (Thunell, 1967; Cifonelli, 1976) of the hyaluronidase-treated specimens and corresponding control.

Growth of micro-organisms was inhibited by adding ampicillin (1 mg per 100 ml) to the various solutions.

Calculations

Correlation between mechanical parameters and specimen quantities

Correlations between the maximum load value and the direct measure of cross sectional area (width × thickness) of each skin specimen and the hydroxyproline content were calculated. For aorta the correlation between the maximum load value and wet weight of each specimen was calculated.

Computation of 'stress'–strain curves and parameters

'Stress' values for skin specimens were calculated as load values normalized to the measured cross sectional area. For aorta specimens the 'stress' values were calculated from the load values by normalizing to wet weight of each aortic specimen. Strain values were obtained by expressing deformation values in units of original specimen length. The load–deformation curves were read into a calculator by a digitizer and transformed to 'stress'–strain curves by recording the 'stress' values for each strain increment of 1 %. From the resulting data, group mean curves with S.E.M. were calculated. The following parameters were calculated from load–strain curves:

F_{max}: maximum load value
$\epsilon_{F_{max}}$: strain at maximum load
$\tan \beta$: tangent of the angle between the linear region of the load–strain curve and the x-axis

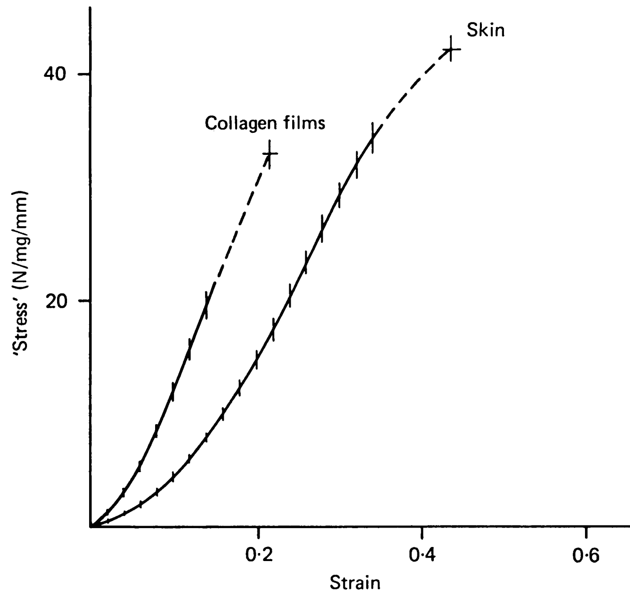

Fig. 1. 'Stress'–strain diagrams for reconstituted collagen films and skin from which they were produced. 'Stress' values were calculated by normalizing load values to collagen content (mg/mm) of each specimen. S.E.M. for the 'stress' values are given by bars.

and in addition from 'stress'–strain curves:

σ_{max}: maximum 'stress' value

$\epsilon_{\sigma_{max}}$: strain at maximum 'stress' (same as $\epsilon_{F_{max}}$)

tan α: tangent of the angle between the linear region of the 'stress'–strain curve and the x-axis.

Statistical analysis

Statistical analysis was performed by using Student's t-test after ascertaining homogeneity of variances. In the case of inhomogeneity of variances, the Wilcoxon two-sample test (Sokal & Rohlf, 1969) was used. Differences were regarded as statistically significant if $2P < 0{\cdot}05$.

RESULTS

The mechanical properties of the collagen films differed from those of the rat skin from which the film collagen was extracted in being stiffer but with less strength and extensibility (Fig. 1). The hyaluronidase preparation used did not significantly change the mechanical properties of the collagen film while α-amylase significantly weakened it (Fig. 2) and collagenase totally decomposed the collagen films. Therefore the hyaluronidase was chosen for degradation of ground substance and collagenase for decomposition of collagen in whole tissue. Elastase weakened the collagen films significantly (Fig. 2) and was therefore not used for treating aorta and skin specimens.

Hyaluronidase treatment (10000 units per 100 ml for 6 hours) of aorta specimens decreased their content of uronic acid with no change in their biomechanical

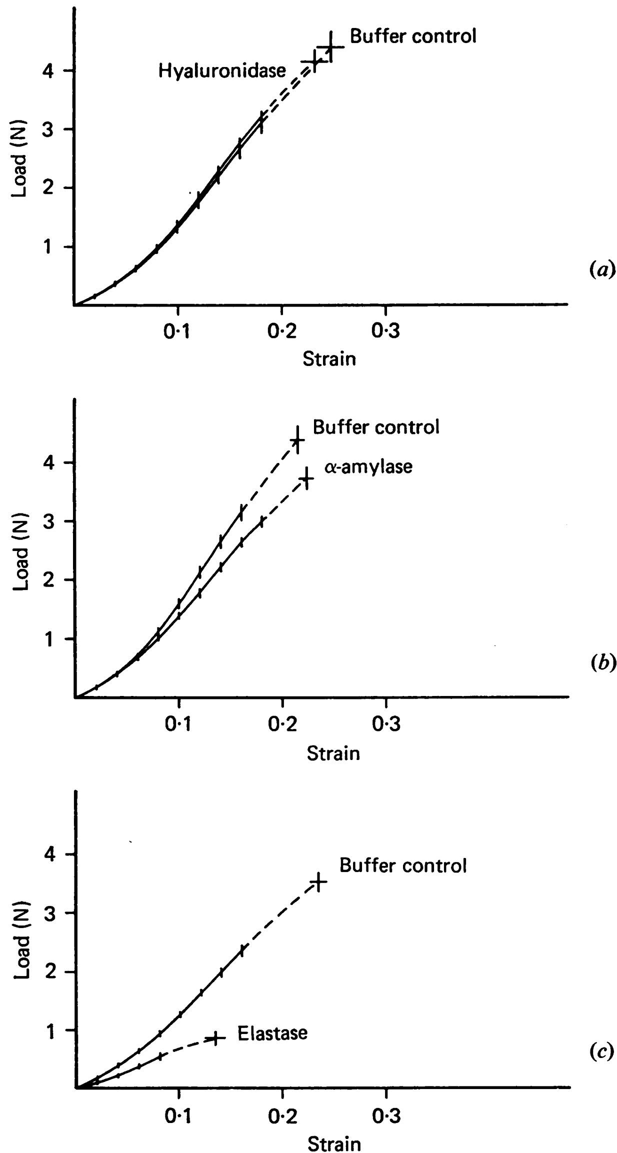

Fig. 2. Load–strain diagrams for collagen films treated with enzymic preparations: (*a*) hyaluronidase; (*b*) α-amylase and (*c*) elastase. S.E.M. for the load values are given by bars.

properties (Table 1; Fig. 3*a*). Collagenase treatment (4 mg per 100 ml for 2 hours) of aorta specimens resulted in a decrease of their hydroxyproline content (29 ± 1 μg) compared with specimens immersed in only the buffer (32 ± 1 μg per sample, mean ± S.E.M., $2P < 0{\cdot}05$). The hydroxyproline released by collagenase treatment was recovered in the enzyme buffer ($3{\cdot}4 \pm 0{\cdot}2$ μg per sample). In the biomechanical tests this collagenase treatment of aorta specimens resulted in decreased values of maximum 'stress' and elastic stiffness and also decreased maximum load value (Table 2). The toe-part of the curve, however, was not influenced by this treatment (Fig. 3*b*). Collagenase treatment (10 mg per 100 ml for 6 hours) decreased the

Table 1. '*Stress*'-*strain and load–strain parameters for specimens of thoracic aorta treated with hyaluronidase* (10000 *units*/100 *ml*) *for* 6 *hours*

	Uronic acid after treatment with hyaluronidase (μg/sample)	n	$\epsilon_{\sigma_{max}}$	σ_{max} (N/mg/mm)	tan α (N/mg/mm)	F_{max} (N)	tan β (N)
Intact control	21·8	16	0·780	1·91	6·46	1·74	5·92
	(1·0)		(0·020)	(0·18)	(0·43)	(0·15)	(0·37)
Buffer control	21·3	9	0·869	1·78	5·53	1·81	5·51
	(0·6)		(0·041)	(0·13)	(0·45)	(1·17)	(0·30)
Hyaluronidase	13·5*	9	0·821	1·70	5·50	1·66	5·40
	(0·9)		(0·013)	(0·14)	(0·37)	(0·13)	(0·33)

Means with S.E.M. in parentheses.

*2P < 0·001 for hyaluronidase against buffer control.

Table 2. '*Stress*'-*strain and load–strain parameters for specimens of thoracic aorta treated with collagenase* (4 *mg*/100 *ml*) *for* 2 *hours*

	n	$\epsilon_{\sigma_{max}}$	σ_{mxa} (N/mg/mm)	tan α (N/mg/mm)	F_{max} (N)	tan β (N)
Intact	16	0·780	1·91	6·46	1·74	5·92
		(0·020)	(0·18)	(0·43)	(0·15)	(0·37)
Buffer control	9	0·775	1·85	6·59	1·58	5·65
		(0·024)	(0·12)	(0·25)	(0·12)	(0·32)
Collagenase	9	0·730	1·38*	4·68**	1·21*	4·09**
		(0·035)	(0·14)	(0·29)	(0·12)	(0·20)

Means with S.E.M. in parentheses.

*2P < 0·05 for collagenase against buffer control.
**2P < 0·01 for collagenase against buffer control.

Table 3. '*Stress*'-*strain and load–strain parameters for specimens of thoracic aorta treated with collagenase* (10 *mg*/100 *ml*) *for* 6 *hours*

	n	$\epsilon_{\sigma_{max}}$	σ_{max} (N/mg/mm)	tan α (N/mg/mm)	F_{max} (N)	tan β (N)
Intact control	6	0·74	2·66	9·5	1·94	7·00
		(0·02)	(0·29)	(0·8)	(0·12)	(0·38)
Buffer control	9	0·70	1·82	7·0	1·88	7·08
		(0·02)	(0·18)	(0·8)	(0·19)	(0·65)
Collagenase	9	0·52**	0·41**	2·0**	0·42**	2·1**
		(0·02)	(0·05)	(0·2)	(0·05)	(0·1)

Means with S.E.M. in parentheses.

**2P < 0·01 for collagenase against buffer control.

hydroxyproline content of the aorta specimens from 33·8 ± 0·4 μg to 7·4 ± 1·9 μg per sample and the hydroxyproline released in the collagenase buffer was found to be 36·4 ± 1·1 μg per sample. This more thorough collagenase treatment resulted in decreased strain at maximum 'stress', decreased maximum 'stress' value and elastic stiffness (Table 3). The maximum load value and the steepness of the load–strain

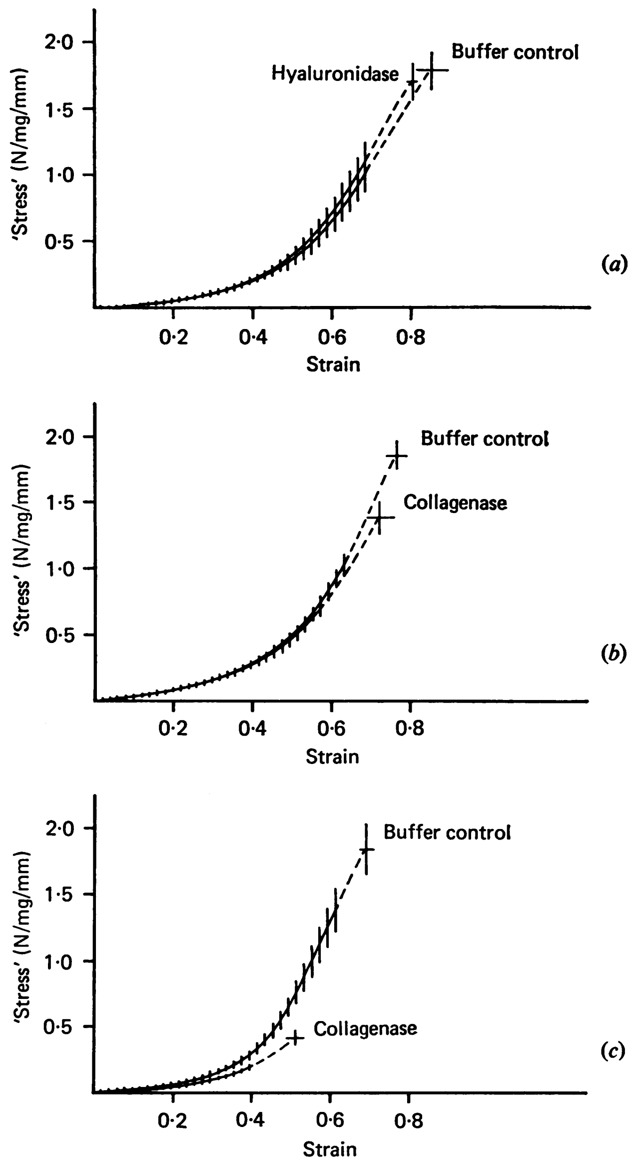

Fig. 3. 'Stress'–strain diagrams for thoracic aorta specimens treated with enzymic preparations: (*a*) hyaluronidase (10000 units/100 ml) for 6 hours; (*b*) collagenase (4 mg/100 ml) for 2 hours and (*c*) collagenase (10 mg/100 ml) for 6 hours. S.E.M. for the 'stress' values are given by bars.

curve were also decreased. The toe-part of the curve was lowered towards the x-axis (Fig. 3*c*).

Hyaluronidase treatment (10000 units per 100 ml for 6 hours) of skin specimens decreased the content of uronic acid by 35%, from 56 ± 4 μg to 36 ± 3 μg per sample, but did not change their mechanical properties (Fig. 4). After collagenase treatment skin specimens were totally disintegrated and possessed no mechanical strength.

Studies of the different measures of the cross sectional area showed that the cross sectional area measured by an optical micrometer was closely correlated, for aorta

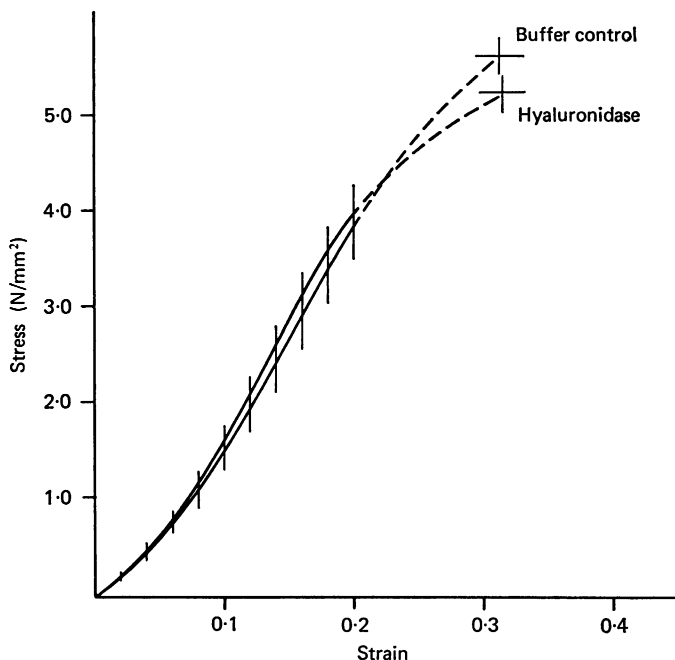

Fig. 4. Stress–strain diagrams for skin treated with hyaluronidase (10000 units/100 ml) for 6 hours. S.E.M. for the stress values are given by bars.

specimens, to wet weight and dry defatted weight and, for skin specimens, to the hydroxyproline content. The correlation coefficient for maximum load and wet weight of aorta specimens was 0·65. For skin specimens the correlation coefficient for maximum load and cross sectional area was 0·86 and hydroxyproline content 0·83.

DISCUSSION

The enzyme preparations used for removal of tissue components must be highly specific when they are used for evaluation of changes in mechanical properties. A preparation must, for example, be capable of degrading parts of the ground substance of skin and aorta while being pure enough not to influence the mechanical properties of the collagen in these tissues. Different preparations of α-amylase and hyaluronidase were therefore tested on films from purified reconstituted collagen extracted from rat skin. The method used produced a collagen film with less strength and extensibility, but increased stiffness compared to the skin from which the collagen was extracted. The gross appearance of the 'stress'–strain curve of the collagen film is not far from that of the skin. The hyaluronidase used in these experiments for degradation of hyaluronic acid, chondroitin and chondroitin sulphates A and C (White, Handler & Smith, 1973) did not significantly influence the mechanical properties of collagen films, while its effect on tissues was verified by a decrease in their content of uronic acid. Hyaluronidase treatment did not change the mechanical properties of aorta nor those of skin.

Collagenase treatment resulted in pronounced alterations in the mechanical properties of aorta, a pronounced decrease of stiffness and strength in the high

strain region of the 'stress'–strain curve and a less pronounced lowering of the curve in the low strain region. Histological studies showed that the elastin fibres of the aorta were not influenced by the collagenase treatment. The effect of collagenase was verified by hydroxyproline assessment. Skin specimens submitted to collagenase treatment were totally decomposed with no possibility of mechanical testing. These results are in agreement with the investigation on aorta of Hoffman *et al.* (1977) and support their findings that collagen in the aorta also contributed to the mechanical strength in the low strain region even though the mechanical characteristics in this region are mainly determined by elastin. We found a pronounced loss of extensibility after collagenase treatment of aorta specimens. This might be due to an abolition of an interaction between collagen and elastin. Lake & Armeniades (1972) removed the collagen from bovine aorta by autoclaving the specimens and studied the mechanical properties of the remaining elastin fibres. They also found a pronounced loss of strength in the high strain region. In the present investigation it would have been of obvious interest to degrade the elastin fibres of aorta followed by a mechanical test. Preparations of elastase were therefore tested on collagen films, but all preparations weakened the collagen film and elastase treatment of aortic specimens was abandoned. Minns, Soden & Jackson (1973) found a decrease in stress level and stiffness of human tendon, aorta and bovine ligamentum nuchae after removal of ground substance by a preparation of α-amylase from *B. subtilis* or by a chelating agent (EDTA). They attributed these changes to a reduced viscosity of the interfibrillar matrix. However, as shown in the present study, α-amylase may influence the mechanical properties of collagen. Minns and co-workers also studied the mechanical properties of elastin fibres and found a marked decrease in stress levels by removal of collagen and other materials in aorta and ligamentum nuchae by formic acid treatment.

Skin and aorta are morphologically and biochemically complex and heterogeneous. When such tissues are submitted to mechanical testing, the load applied to the test specimen is normally expressed in terms of stress, i.e. load per unit cross sectional area. This permits the comparison of results from tests on specimens of different sizes and thus provides more general data concerning the mechanical properties of the tissue analysed and the possibility of comparing the mechanical properties of different tissues. However, for such comparisons to have quantitative meaning, it is necessary that the measure for cross sectional area used in stress calculation be determined by a method which is reasonably accurate and reproducible and without damaging effects upon the specimen. For heterogeneous tissues such as skin and aorta, containing components with such extremely different mechanical properties as fat, elastin and collagen, the direct measure of the cross sectional area is not always the most appropriate measure. Thus the collagen content of each specimen, as an expression of the functional cross sectional area for skin specimens, may be the more interesting measurement since collagen, according to Levenson *et al.* (1965), Vogel (1974) and this study, is the component which is mainly responsible for the mechanical properties of skin. Further, we found that the collagen content of each specimen was just as well correlated to tensile strength as was the direct measure of the cross sectional area. However, when measuring the collagen content of a specimen, the tissue between the clamps has to be isolated for determination of hydroxyproline after the mechanical test. In the case of enzymic treatment of specimens, this in itself may change the hydroxyproline content of the specimens and collagen content cannot be used as a measure of the cross sectional area. We

therefore used the direct measure of the cross sectional area in this investigation. When testing collagen film strips, we were obliged to work with load values because of difficulties in measuring the thickness of the thin collagen film strips. A correct measure of the cross sectional area of the ring-shaped aorta specimens is also difficult to obtain, when damage to and drying up of the specimen has to be avoided. We therefore used the wet weight of each aorta specimen for normalizing load values to 'stress' values.

This study has shown that collagen is mainly responsible for the tensile strength of skin and aorta, while the toe-part of the 'stress'-strain curve of aorta is determined by both elastin and collagen. An interaction between collagen and elastin might be suggested from the pronounced loss of extensibility after collagenase treatment. Hyaluronic acid does not seem to influence the static mechanical properties of skin and aorta.

SUMMARY

Biomechanical testing of connective tissue is usually done on whole tissue. Studies of the mechanical properties of each component of the tissue, however, are important for elucidating the connection between the functional properties and the molecular structure. Enzymic degradation of one component at a time followed by mechanical testing was employed. The specificity of enzymes used was ascertained with mechanical tests on collagen films from purified, reconstituted collagen after enzymic treatment. The study shows that collagen is the component which is mainly responsible for the tensile strength of skin and aorta, while the toe-part of the 'stress'-strain curve of aorta is determined by both elastin and collagen. The hyaluronic acid does not seem to play any role in the static mechanical properties of skin and aorta as evaluated by means of the present method.

This work was supported by grants from the Danish Medical Research Council (project no. 512-8292, 512-10126).

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