Virchows Arch A (1987) 410:383–390

Virchows Archiv A

Interstitial collagens and ageing in human aorta

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Summary. Types I and III collagen were identified in four segments of human aorta using pepsin and cyanogen bromide digestion followed by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Collagen is not uniformly distributed in the different segments of the arterial wall. Collagen type I is always the major collagen present. With ageing collagen type III decrease in quantity from the heart to the distal portion of the aorta. Histologically the elastic tissue is more altered in the lower abdominal section of aorta than in the arch. This study allowed a correlation between morphological observations and biochemical changes.

Key words: Collagen types – Ageing – Human aorta

Introduction

Collagen is an important structural component of all blood vessels. Several types of collagen have been isolated in arteries. The two interstitial species, collagens types I and III have been well characterized by Chung and Miller (1974) Epstein et al. (1975) Trelstad (1974) and by Leushner and Haust (1986). Small amounts of collagen type IV have been isolated from human and bovine aorta by Mayne et al. (1980) and by Trelstad (1974). Recently type V collagen was identified in the intima and media of aorta (Aumailley 1978; Bornstein and Sage 1980; Morton and Barnes 1982). Type VI collagen was first isolated from human intima by Chung et al. (1976). The results of analysis of the amounts of type I and III collagens have been controversial (McCullagh and Balian 1975; Morton and Barnes 1982; Rauterberg et al. 1977), they vary according to the techniques used and different studies cannot easily be compared. However it is now considered that collagen type I is the predominant species in normal tissue, representing around two thirds of the total of the two (Barnes 1985).

Immunohistochemical staining of human aorta sections with specific antibodies prepared against type I and type III collagens suggested that the localization of these collagens may differ, type III collagen being located near the elastic lamellae while type I collagen is located closer to the surface of the smooth muscle cells (Gay et al. 1975; Gay and Miller 1978; McCullagh et al. 1980).

Ageing is an important factor in bringing about changes in aortic collagen (Bartos and Ledvina 1979; Shekhonin et al. 1985; Berry et al. 1972b). The changes in the total percent content of this protein found by different authors in the aorta from various animals are not consistent. Faber and Moller Hou (1952) found that collagen increased with age in abdominal human aorta, in contrast McCloskey and Cleary (1974) reported a decrease of aortic collagen in aged rabbits and Fischer (1976) found that in rat aortas collagen at first increased progressively but later on the values decreased. In a study during normal development in man from 12 weeks' gestation to 3 months post partum Berry et al. (1972a) found "that collagen increased from 27% to 41% of the dried fat free tissue between 12 weeks and term, remaining fairly constant thereafter".

In the present study: 1) we have determined the normal content of collagen and distribution of collagen types in young human aorta (before 50 years) and how does this vary with anatomic site. 2) We have studied if with age (above

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50 years) they are changes in distribution of collagen types within four segments of aorta macroscopically normal without signs of atherosclerosis. 3) We have verified if biochemical changes correlate with histological alterations.

Materials and methods

Tissue specimens. Samples of 22 aortas were obtained at autopsy within 15 h of death from patients deceased for non vascular causes, none of them had hypertension or diabetes mellitus.

Aortas were stored at −20° C. All the aortas were macroscopically normal or with few small fatty streak. Patients were divided into two groups. 1) Eleven subjects before 50 years with mean age of 33 years (20–48). 2) Eleven subjects above 50 years with mean age of 60 years (51–75). The age of 50 years was selected because it is a critical period where there is an acceleration of the occurence of the symptoms of arterial ageing (Bouissou et al. 1976).

Four segments were separated for each aorta from the heart to the terminal part: arch (22 samples), thoracic aorta (7 samples), upper abdominal aorta (7 samples) between diaphragm and kidney arteries and lower abdominal aorta (22 samples) between kidney and iliac arteries.

Samples were washed free of blood and of adherent contamining tissue and fat, the adventitia was stripped and discarded. The media and intima of each sample were weighed then chopped into small pieces and extracted for collagen analysis. Small aliquots were weighed and hydrolysed and collagen content was estimated by a hydroxyproline assay.

Pepsin digestion. Collagen was extracted from the four aortic segments by limited pepsin digestion. The procedure used was described previously by Maurel et al. (1982).

Tissue specimens (4 g wet weight) minced into small fragments less than 2 mm in diameter were extracted for 24 h with 80 ml of 1 M NaCl/0.05 M tris buffer pH 7.2. The extract was removed by centrifugation and the residue extracted with 80 ml of 0.5 M acetic acid for 24 h. Extracts were discarded.

The final residue was suspended in 80 ml of 0.5 M acetic acid solution containing 1 mg of pepsin for 100 mg wet weight for 24 h. The pepsin digest was collected by centrifugation and the residue subjected to another pepsin treatment. The two pepsin extracts were combined and the pepsin solubilized material was fractionated by addition of NaCl to 0.7 M to obtain a 0.7 M precipitate and 0.7 M supernatant. The two isolated fractions were dialysed against 0.1 M acetic acid freeze dried and weighed prior to examination by SDS polyacrylamide gel electrophoresis. Hydroxyproline determination was made for 0.7 M precipitate.

All operations were carried out at 4° C. All centrifugations were performed at 30,000 g and 4° C for 1 h.

The 0.7 M supernatant was purified. The sample freeze dried was suspended and stirred in 0.5 M acetic acid 1.2 M NaCl pH 3.4 for 24 h. The precipitate was redissolved and stirred in 2 M NaCl/0.05 M tris pH 7.4 for 24 h. The supernatant obtained was stirred for 24 h with 4.5 M NaCl. The 4.5 M NaCl precipitate was resolubilised in 0.1 M acetic acid, dialysed, freeze dried weighed and stored for SDS polyacrylamide gel electrophoresis.

Cleavage with cyanogen bromide. Cyanogen bromide digestion was carried out as essentially described by Weber et al. (1977) and by Kirk et al. (1984). It has been made only for two human aortic segments: Arch and lower abdominal aorta.

Tissue samples were suspended in 1 M NaCl/0.05 M tris buffer pH 7.2 for 24 h. The residue removed by centrifugation was extracted with 0.5 M acetic acid for 24 h. The final residue was dispersed in 0.1 M acetic acid and freeze dried.

The lyophylized residue was suspended (at a concentration of 20 mg/10 ml) in 70% formic acid containing cyanogen bromide (10 mg/ml). The cleavage was obtained by a 5 h incubation at 30° C under an atmosphere of N_2. The reaction mixture was diluted 10 fold with distilled water and freeze dried collagen derived peptides in the lyophilised product were estimated by SDS polyacrylamide gel electrophoresis.

SDS polyacrylamide gel electrophoresis. Characterization of collagen fractions was investigated by polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS) buffer on acrylamide gels. The disc gels were run at 2 mA/tube for 120 min. Gels were stained with Coomassie brilliant Blue and destained 2 h in 50% methanol and 7% acetic acid then in 7% acetic acid. Densitometric scanning was performed at 550 nm on a Sebia cello system quick scan densitometer. Two electrophoresis and three densitometric scanning were made for each sample.

Collagen derived peptides arising from the cyanogen bromide treatment were examined on 10% acrylamide gels.

Electrophoretic separation of pepsin digests was achieved on 5% acrylamide gels (Neville 1971). The chains α_1 (I) and α_1 (III) were separated by the interrupted gel electrophoresis system (Sykes et al. 1976). The disc gels were run for 30 min, electrophoresis was interrupted to allow reduction of disulphide bonds with mercaptoethanol and continued for a further 90 min.

Quantification of type I and III collagens. Type I and type III collagens were quantitated (Tajima and Nagai 1980) in pepsin digests. The densitometrically integrated values of α_1 (I) and α_1 (III) bands in stained gels allowed estimation of α_1 (I)/α_1 (III) ratio. Because type I collagen contains two α_1 (I) chains and type III collagen contains three α_1 (III) chains the α_1 (I)/α_1 (III) ratios were multiplied by 1.5 to obtain the type I/type III ratios. Absolute amounts of both collagens in each sample were estimated from the observed values of hydroxyproline content (converted in collagen) and the α_1 (I)/α_1 (III) ratio.

CNBr peptides chosen for quantitation were α_1 (III) CB5 for type III collagen and α_1 (I) CB8 for type I collagen (Kirk et al. 1984).

Five different dilutions of standard collagen were run on gels. From these a standard curve was drawn and a linear relationship was found between the intensity of staining and the quantity applied to the gel. The standard curve was used for each tissue extract to calculate the percentage of type III compared to the total of type I and III collagen.

The student "t" test for differences between means was used for significance of results. Significance was considered present at the 5% level.

Hydroxyproline determination. Samples were hydrolysed in 6 N HCl in sealed tubes at 110° C for 24 h. Following evaporation to dryness in vacuo hydroxyproline was determined by colorimetric procedure (Bergman and Loxley 1963). Each determination was made in duplicate. The amount of collagen per mg of starting material was obtained by multiplying the concentration of hydroxyproline by 7.46 (Neuman and Logan 1950).

Light microscopy. Samples of each segment were immediately fixed in Dubosq fluid, embedded in paraffin and then stained with Verhoeff's iodine ferric hematoxylin (Verhoeff 1908).

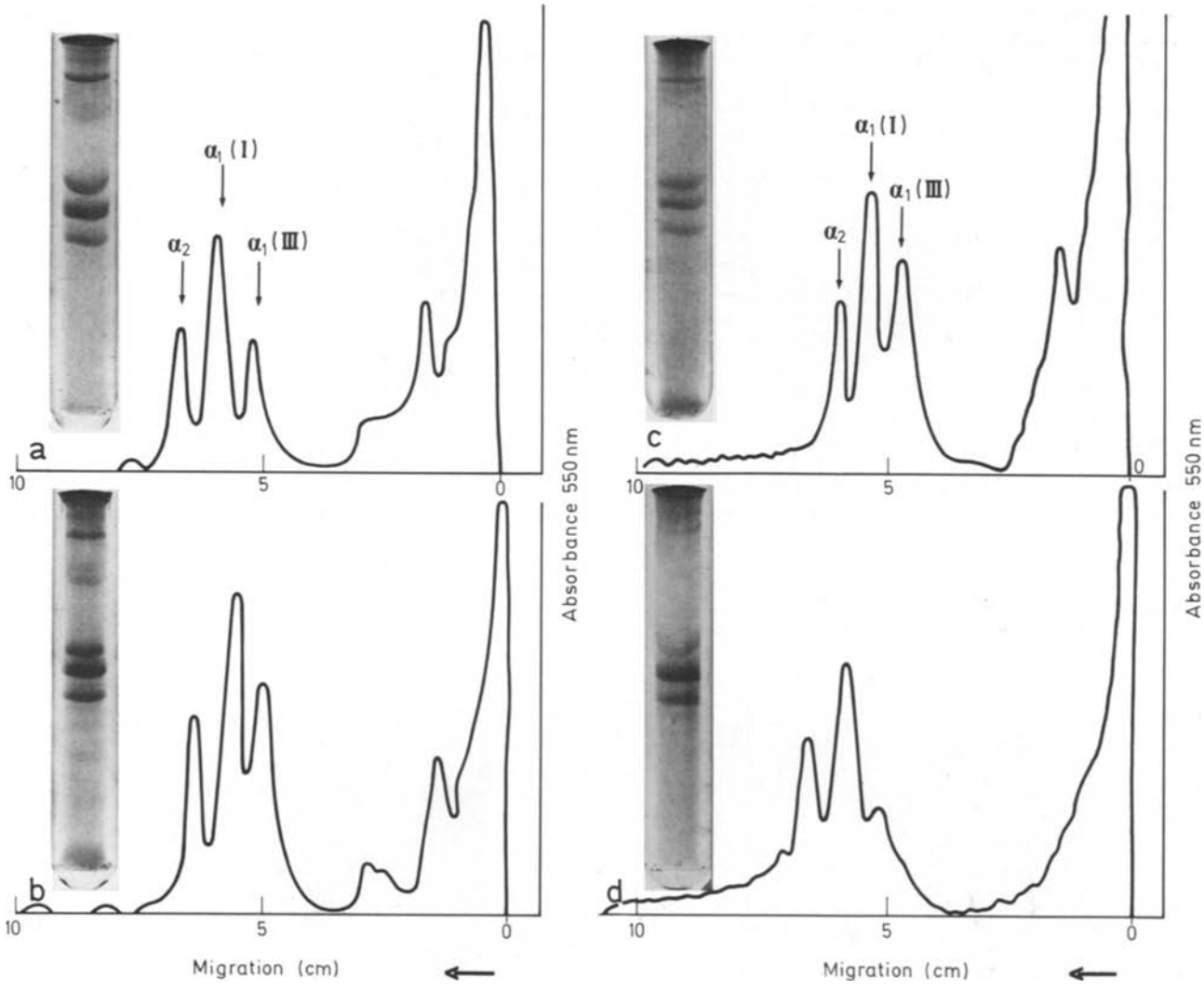

Fig. 1 a–d. Densitometric scans of the 0.7 M precipitate fraction from pepsin digests after SDS polyacrylamide gel electrophoresis with and without reduction with mercaptoethanol **a** arch, 21 years; **b** lower abdominal aorta, 21 years; **c** arch, 66 years; **d** lower abdominal aorta, 66 years

Results

In the 0.7 M precipitate fraction from pepsin digests hydroxyproline determination was made and estimation of collagens types I and III was obtained by polyacrylamide gel electrophoresis.

Densitometric scans and disc electrophoretic pattern of SDS polyacrylamide gel electrophoresis are shown in Fig. 1. Gels revealed a preponderance of type I collagen but a significant proportion of type III, except for the oldest patient, where the amount of type III was markedly decreased in the lower abdominal aorta.

Concentration of collagens types I and III expressed as mg/g freeze dried sample are shown in Table 1. Before 50 years there was more collagen types I and III in lower abdominal segment than in arch ($p<0.01$). With ageing the same variation occured for type I collagen ($p<0.05$) but for type III collagen the measured quantities decreased between arch and lower abdominal segment ($p<0.05$).

In each segment there was no significative change for the two collagens types before and from 50 years except for collagen type III in the lower abdominal segment where the amount decreased with age from 141 mg/g to 82 mg/g freeze dried sample ($p<0.01$). In both groups collagen type I was always present in greater amounts than collagen type III.

The percentage of type III collagen is shown in Fig. 2. Along the same aorta before 50 years

Table 1. Collagens types I and III in the 0.7 M precipitate fraction from pepsin digests in human aortic segments ($n=12$ aortas) (mg/g freeze dried sample ±SEM)

Years	Arch.	Thoracic aorta	Upper abdominal aorta	Lower abdominal aorta
Type I				
<50	330±29	327±100	286±50	442±26 *($p<0.01$)
>50	257±31	245± 62	192±46	364±46 *($p<0.05$)
Type III				
<50	105± 8	99± 17	86±23	141± 6 *($p<0.01$)
>50	125±26	99± 30	59±14	82±15 *($p<0.05$)

* For significance lower abdominal aorta was compared to arch

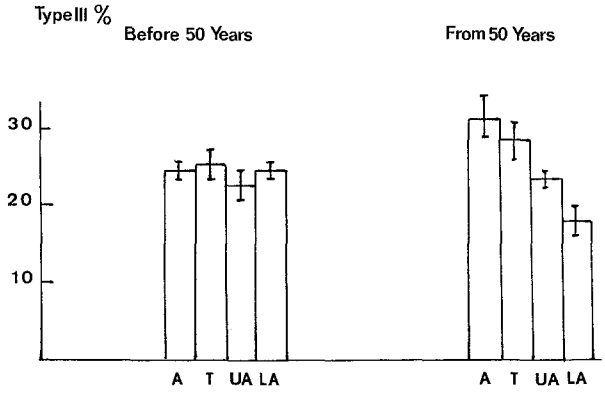

Fig. 2. Collagen type III percentage in the 0.7 M precipitate fraction from pepsin digests in human aortic segments: (*A*) Arch; (*T*) thoracic aorta; (*UA*) upper abdominal aorta; *LA* (lower abdominal aorta)

there was no variation in the concentration of type III collagen, which represented between 20% and 30% of the solubilized collagen. Above 50 years a decrease appeared between the arch and the lower abdominal segment ($p<0.01$).

Changes in the percentage of type III collagen in one fragment occurred with ageing; type III concentration increased in the arch ($p<0.05$) and decreased in lower abdominal segment ($p<0.05$). There was no significant difference for thoracic and upper abdominal aortas.

In the 0.7 M supernatant fraction from pepsin digests, after purification, electrophoresis of 4.5 M precipitate revealed the presence of two major bands migrating slightly slower than α_1 (I) chain. Prior reduction did not alter the mobilities of these

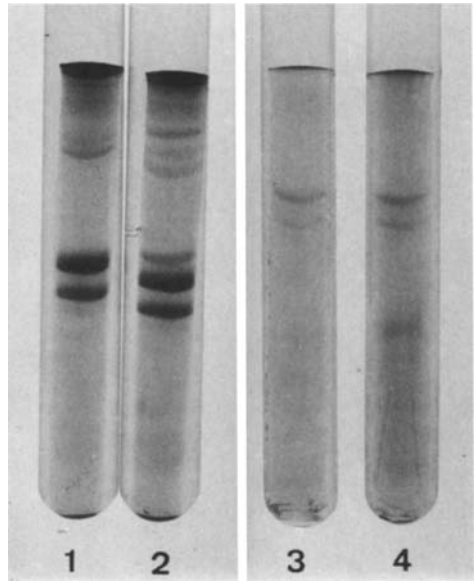

Fig. 3. SDS polyacrylamide gel electrophoresis patterns of pepsin solubilized collagens extracted from the arterial wall. *1* 0.7 M NaCl precipitate, *2* same sample after reduction with mercaptoethanol; *3* 4.5 M precipitate from 0.7 M NaCl supernatant after purification; *4* same sample after reduction with mercaptoethanol

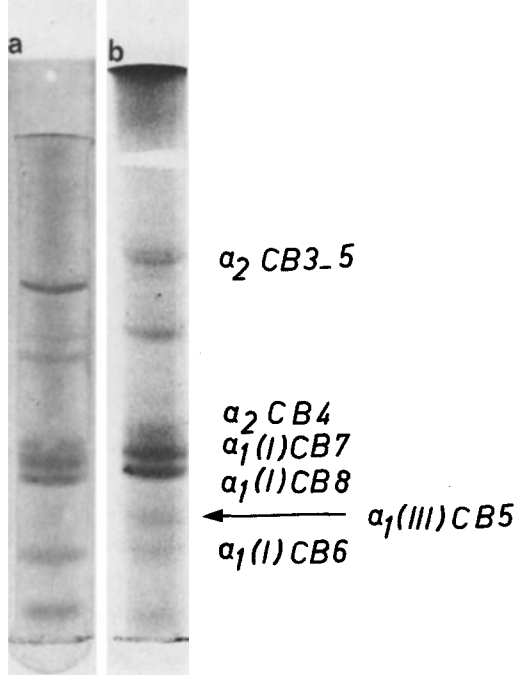

Fig. 4a, b. Electrophoresis of CNBr peptides from **a** type I standard; **b** human aortic segment

bands (Fig. 3). This electrophoretic mobility is similar to that of A or α_2 (V) and B or α_1 (V) chains of type V collagen. This collagen has been identified in aortic tissue (Chung et al. 1976).

Electrophoretic separation of the cyanogen

Table 2. Collagens types I and III in human aortic segments ($n=10$ aortas) by estimation of the CNBr peptides α_1 (I) CB8 and α_1 (III) CB5 separated by SDS polyacrylamide gel electrophoresis (means ± SEM)

	Years	Arch.		Lower abdominal aorta	
Type I (mg/g dry weight)	<50	96 ±7	$\leftarrow p<0.001 \rightarrow$	158 ±7	
	>50	64 ±5	$\leftarrow p<0.05 \rightarrow$	81 ±4	
Type III (mg/g dry weight)	<50	32 ±3	$\leftarrow p<0.001 \rightarrow$	64 ±4	
	>50	19 ±2		20 ±1	
Type III (%)	<50	24.9±0.2		28.8±0.3	
	>50	22.1±0.5		19.8±0.2	$p<0.001$

bromide peptides allowed estimation of collagen types I and III using the peptides α_1 (I) CB8 and α_1 (III) CB5. Densitometric scans of CNBr peptides from type I and type III collagen standards were examined and the positions of individual peptides correlate well with published data (Morton and Barnes 1982; Weber et al. 1977). Examination of the cyanogen bromide peptides obtained from human aortic segments showed that they were derived from a mixture of collagens types I and III. This appears clearly in Fig. 4.

The amounts of collagens types I and III and the percentage of collagen type III in the human aortic arch and lower abdominal aorta before and after 50 years of age are shown in Table 2. These results are comparable with those obtained by pep-

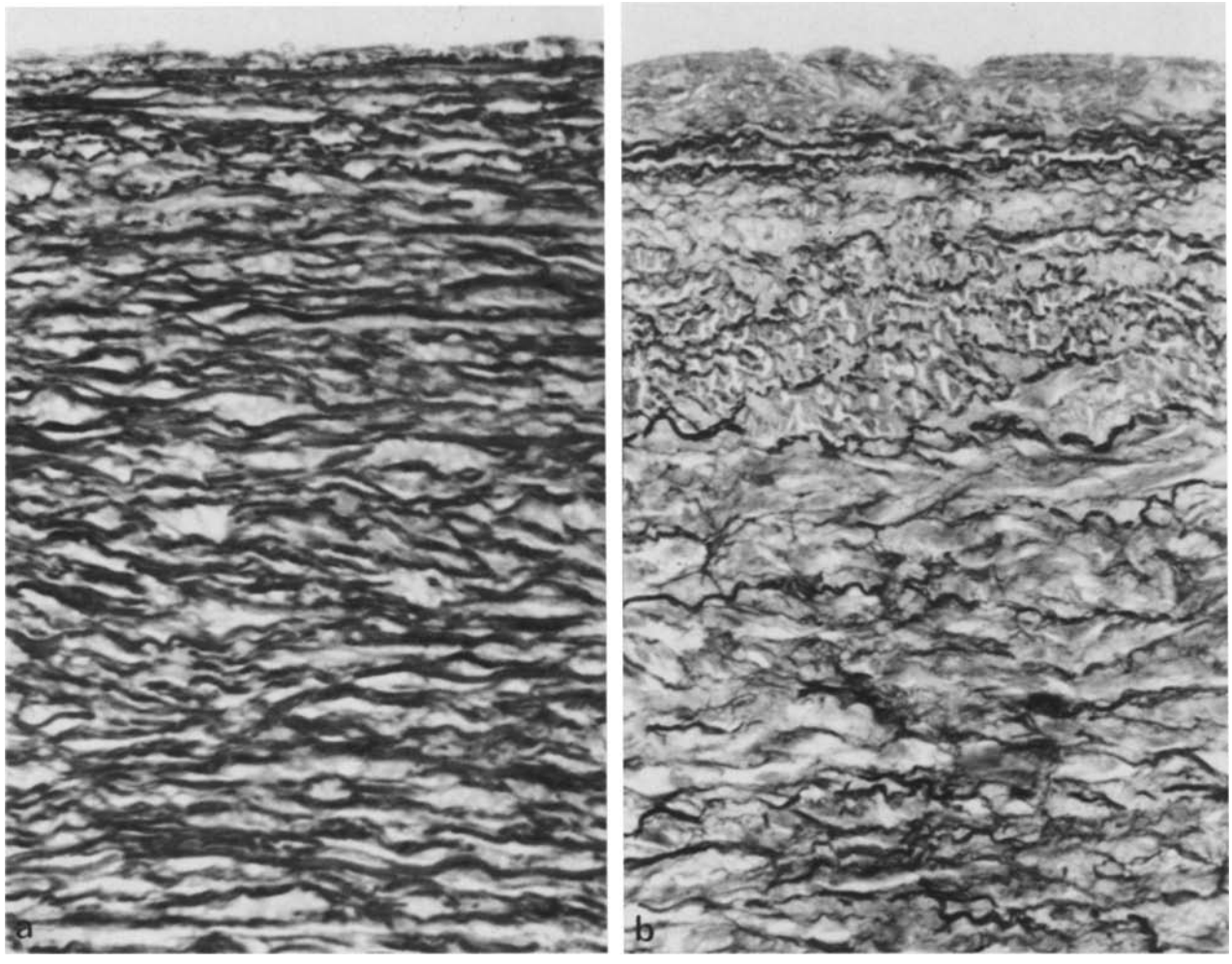

Fig. 5a, b. Normal aorta from a young subject: 21 years; **a** thoracic aorta, close to the heart there is a lot of elastin and these fibres are close together. Little ground substance. Verhoeff ×25; **b** abdominal aorta: elastic fibres are more widely spaced and the interlamellar material become more important. Verhoeff ×25

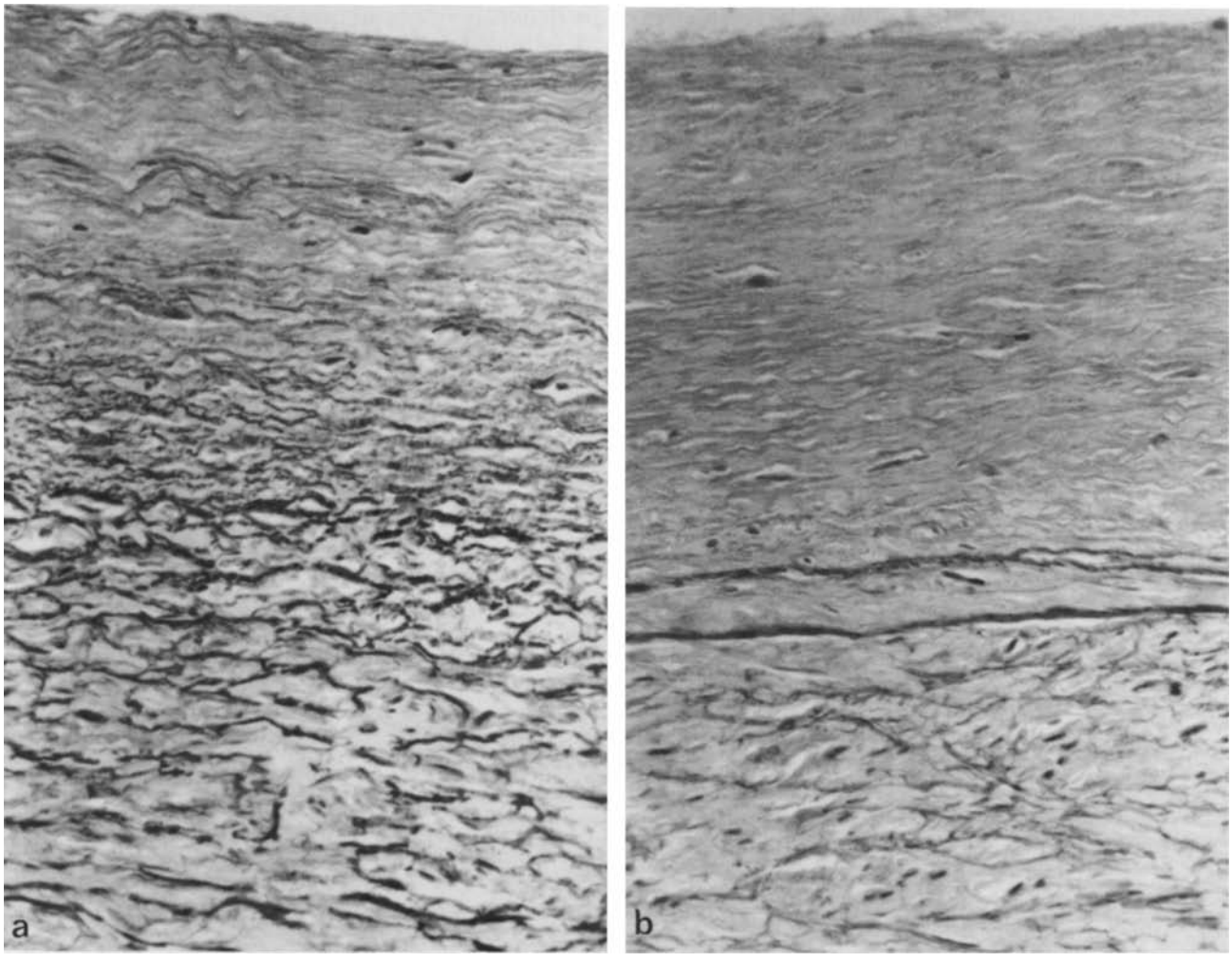

Fig. 6a, b. Aorta from an old subject: 73 years; **a** thoracic aorta. Verhoeff ×25; **b** abdominal aorta: elastin in less abundant than in thoracic segment, ground substance and collagen increase. Verhoeff ×25

sin digestion. There was always more collagen in lower abdominal segment than in arch except for type III after 50 years. For type III, the percentage concentration decreased with age in the lower abdominal aorta, from 29% to 20% ($p < 0.001$).

Morphological observations showed that for normal aorta from a subject under 50 years of age, close to the heart, there were numerous elastic fibres close together and a little ground substance. On descending the aorta the presence of elastic fibres decreased and they were more widely spaced, the interlamellar material becoming more marked (Fig. 5a, b).

With ageing there was a thickening of the intima by destruction of internal elastic fibres and a degradation of the elastic lamellae of the media where the elastic fibres became irregular and fragmented. These modifications were more notable in the abdominal aorta than in the thoracic fragment (Fig. 6a, b).

Discussion

The high insolubility of arterial wall collagen is well known (Borel and Bellon 1985). The method of pepsin digestion has often been used in spite of the fact that this method may not remove all of the collagen from the tissue. The assumption has had to be made that the extracted fraction is representative of the whole. Attempts have been made to improve this method and increase the solubility of aortic collagen. Limited pepsin digestion and dithiothreitol reduction has been used resulting in the extraction of about two thirds of the total collagen of the tissue (Szymanowicz et al. 1982). However with this extraction the technique

of interrupted electrophoresis cannot be used. Another technique using elastase digestion (Faris et al. 1978) leads to complete solubilization of collagen, but the elastase has to be extremely pure to avoid the degradation of collagen. Cyanogen bromide treatment extracts a much greater proportion of total collagen than does pepsin digestion (Hanson and Bentley 1983; Halme et al. 1986).

In these experiments CNBr treatment solubilizes 60%–70% of the total collagen in human aortic segments while with pepsin treatment it is generally around 20%. In spite of this discrepancy in the extractibility of collagen according with the method used we found comparable results.

As indicated by Barnes (1985) collagen type I was found always to be the major collagenous species (Tables 1 and 2). Before and after 50 years collagen type I quantities were always predominant in the arch than in lower abdominal aorta. The same variation occured for collagen type III for young subjects but with ageing the quantity decreased from arch to lower abdominal aorta in pepsin digests (Table 1) and did not changed with cyanogen bromide digestion (Table 2). It is difficult to compare these results with published data because others have generally studied repartition of collagen in different aortic layers (intima-media) and not in aortic fragments. However recently in the aortic arch and in the abdominal aorta of a 30 years old male Hanson and Bentley (1983) have observed the same variation: for type I, 39.4 mg/g wet weight in arch and 52.5 mg/g in abdominal aorta, for type III 23 mg/g wet weight in arch and 37.9 mg/g in abdominal aorta.

Concerning the type III percentage, an important result was identical with both methods: with ageing in the lower abdominal aorta this percentage was significatively decreased from 24% to 18% with pepsin digestion (Fig. 2) and from 29% to 20% with cyanogen bromide treatment (Table 2). This important result allowed us to make a correlation with histological appearance. Two points have to be considered.

1) With ageing morphological lesions appeared first in the abdominal aorta (Sendrail-Pesque et al. 1969). We found variation in collagen types in this segment indicating modification in arterial wall composition. So, in aortic tissue, without lipid accumulation, collagen type III decreases with age in the abdominal aorta. This variation may suggest that collagen ratios play a role in permeability of the arterial wall and suggest that the aortic ageing process is not always related to atheroma.

2) With ageing morphological appearances (Bouissou et al. 1976) showed a marked decrease of elastic fibres in the abdominal portion (Fig. 6b). Evidently, the removal of elastin was in part mediated through elastase and an increase in elastase activity has been shown with age in aortic extracts (Hornebeck et al. 1976). It has also been shown, that type III collagen was more susceptible than type I to neutrophil elastase (Gadek et al. 1980). Finally, immunofluorescent localization of collagen showed that in the media fibres of type III collagen were mainly found as a more or less continuous sheath adjacent to the elastic fibres (Gay et al. 1975; Gay and Miller 1978; McCullagh et al. 1980). This localization and the same enzyme susceptibility may explain the correlation between these histological and biochemical data. The significance of these findings to the in vivo situation in the artery wall remains to be established but the juxtapositioning of elastin and type III collagen and their simultaneous alteration leads one to suspect that their metabolism may be connected.

Acknowledgements. The technical assistance of J. Deloly, D. Divoux and A.M. Caubet is gratefully acknowledged. Type I and III collagens standards were the generous gift of D. Herbage and F. Flandin.

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Accepted October 15, 1986