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Research Article

X-RAY DIFFRACTION STUDIES OF HUMAN AORTIC ELASTIN RESIDUES *

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Abnormal calcification is readily identified both grossly and microscopically in the atherosclerotic plaques of aging human aortic intima (1). Concurrently, and frequently independently, considerable amounts of calcium are deposited in the media. This deposition need not occur in regions of prior lipid deposition (2); indeed, even in the absence of atherosclerosis, the calcium content of aorta has been found to increase with age (3).

Such calcific deposits, mainly from atherosclerotic plaques, have been studied by Carlström, Engfeldt, Engström and Ringertz (4), who tentatively identified the crystallites as hydroxyapatite ($Ca_{10}(PO_4)_6(OH_2)$). Their studies have indicated that the X-ray diffraction powder pattern of grossly visible plaques is essentially similar to that given by bone. Further studies have indicated that the dry mass of human arterial wall is greatest in the media, at a region where elastin is the predominant fiber (5). It appeared possible, therefore, that the hydroxyapatite crystallites might be associated with aortic elastin residue (2).

Elastin is generally separated from the other proteins of aorta by virtue of its marked insolubility in dilute alkali (6), formic acid (7) or hot water (8). Each of these methods has been used to separate elastin residues of relatively similar properties and amino acid composition, although the relationship these products bear to the native protein is by no means clear (7). Such products have been studied by chemical analysis (2) and electron microscopy (9), recently aided by the solubilization of elastin by a relatively specific pancreatic enzyme, elastase (10). The current study was undertaken to demonstrate the presence of crystallites in human aortic elastin residues (AER) by means of X-ray diffraction analysis. Elastin was prepared from aortas obtained at necropsy from individuals aged 8 to 86. In order to demonstrate the possible association of hydroxyapatite with elastin rather than with other connective tissue elements, a form of extraction was employed which would separate elastin on the basis of its insolubility in water at autoclave temperatures.

MATERIALS AND METHODS

Preparation of aortic elastin residues.[1] Human aortas were obtained within 24 hours after death from the Office of the Chief Medical Examiner, New York City. Only samples free of moderate to marked intimal plaque formation were employed. The intima-media portion was stripped from the adventitia as in the preparations of Kirk and Dyrbye (11) at 4° C. Immediately thereafter, this portion (from aortic root to bifurcation of iliac vessels) was diced into 0.5 cm^3 segments, washed 3 times with ice cold physiologic saline (0.9 per cent) by centrifugation in the cold, blotted dry until no further moisture could be observed on Whatman no. 1 filter paper, and weighed. This was designated the *wet weight.* Next, these wet aortic slices (6.0 g) were placed in absolute ethanol (250 ml) and homogenized in the baffled flask (500 ml) of a VirTis 45 homogenizer for 10 minutes at top speed (rated at 45,000 rpm) employing the twin-blade attachment. An ice bath kept the temperature below 10° C. The disintegrated aorta and ethanol were centrifuged at 2,000 rpm for 20 minutes, and the residue washed 3 times each with absolute ethanol and ethyl ether, then dried *in vacuo* over $CaCl_2$ to a constant weight designated as the *dry weight.* (During this pro-

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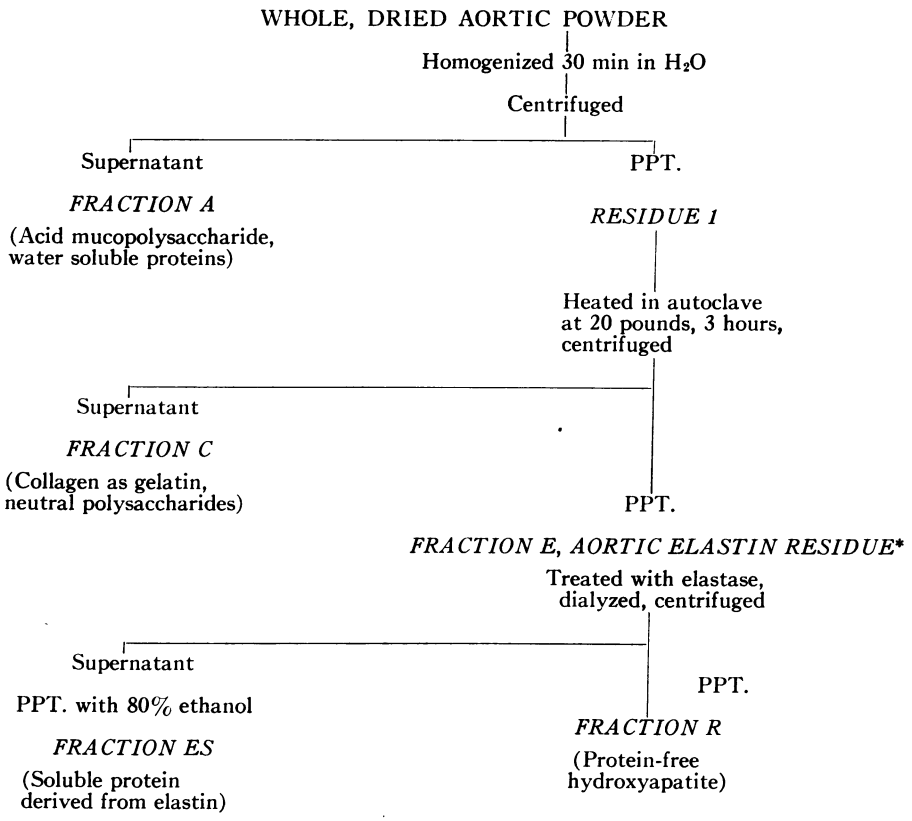
[1] The term "aortic elastin residue" is employed to distinguish the final product from that obtained by Lansing (2). In the present study, sections of intima as well as of media have been employed to include crystallites associated with the elastic membranes of aortic *intima* in the material finally examined by X-ray analysis.



* Used in most X-ray diffraction analyses.

FIG. 1. OUTLINE OF PROCEDURE USED IN PREPARING AORTIC ELASTIN RESIDUES (AER, SEE TEXT).

cedure, designed to dry and defat the specimens, a yellow color was imparted to the ethanol during homogenization, and to the first two ethanol washes.) Designated as *dried aortic powder,* this product, stored at room temperature, was used for further fractionation as late as one year after preparation. To determine whether differences in the nature of aortic elastin residues would appear between dried and fresh aortas, in several early experiments the wet aortic slices (6.0 g) were placed directly in the homogenizer with distilled water (280 ml). They were then immediately carried through the fractionation procedure described below.

Further fractionation[2] of powdered aorta was carried out after portions had been allowed to stand with distilled water (2.0 g per 80 ml) at 4° C for 16 hours to rehydrate (Figure 1). Dried, rehydrated aortic powder (2.0 g) was added to distilled water (280 ml) in the baffled flask (500 ml) of the VirTis 45 homogenizer, which was run at top speed for 30 minutes in a room at 4° C. Constant replenishment of an ice bath kept the temperature of the homogenate below 10° C. This procedure yielded an opaque mixture to which ethanol (560 ml) was added. All subsequent procedures were performed at room temperature (23–25° C). Following centrifugation at 2,000 rpm for 45 minutes, the supernatant solution was filtered through glass wool, and potassium acetate (8.5 g) was added to precipitate polyanionic material (12). The precipitate was washed 3 times each with absolute ethanol and ethyl ether and dried *in vacuo* over $CaCl_2$. Judged by its metachromasia with toluidine blue, and precipitability by polyvalent cobalt salts (13), this product was presumed to contain water soluble acid mucopolysaccharide-protein complexes. Chemical analysis of 10 samples of this fraction (Fraction A, Figure 1) gave values, as per cent, within the ranges: hexosamine 4.5 ± 0.5, nitrogen 11.2 ± 0.8, hydroxyproline 0.0.

The *residue* (Residue 1, Figure 1) was washed 3 times each with ethanol and ether and dried as above. When the weights of Fraction A and the elastin-containing Residue 1 were added, the yield was found to be 87 to 96 per cent. The proportion of the two fractions varied somewhat with age.

The dried *residue* was placed in distilled water (20 ml per g) and heated in an autoclave at 20 pounds for 3 hours, maintaining a temperature of approximately 300° C. Two vol of water were added to the resultant gray suspension which was then centrifuged at 2,000 rpm for 40 minutes. The supernatant solution was filtered, evapo-

[2] For sake of clarity, the fractions containing the final product (AER) have been italicized.

rated over steam to one-fifth the original volume, precipitated with two vol of ethanol, and dried and weighed in the above manner. Several samples of this material, designated Fraction C (Figure 1) yielded upon chemical analysis the following, as per cent: hexosamine 2.4 ± 0.6, nitrogen 13.1 ± 0.8, hydroxyproline 6.9 ± 0.5. Based on the high hydroxyproline and hexosamine concentrations, this fraction probably contained collagen that had been converted to gelatin, and neutral polysaccharides. The *residue* was washed 3 times each with alcohol and ether, and dried as before. This product, designated Fraction E (Figure 1) represented AER. Fractions C and E were obtained in yields of 78 to 92 per cent from Residue 1. AER appeared as a fine white or beige powder which could conveniently be used for X-ray diffraction analysis. The hydroxyproline content of several samples was 1.3 ± 0.3 per cent. While not strictly comparable with elastin prepared by alkali or formic acid treatment, this product most closely resembled the material studied by Partridge, Davis and Adair (8).

Treatment with elastase. It has been shown that treatment of elastin with elastase results in the formation of soluble proteins (14). To demonstrate the intimate association of hydroxyapatite crystallites with elastin, samples of AER were treated with this enzyme. Were such association to exist, the finding of a hydroxyapatite X-ray diffraction pattern would be expected in the ethanol-precipitated, nondialyzable supernatant solution of elastase-treated aortic residue. In such an experiment, two samples (100 mg) of aortic elastin residue (A_1 55–39) were incubated in a 15 ml Erlenmeyer flask with commercially obtained, crystalline elastase[3] (20 mg) previously dissolved in 5.0 ml of barbital buffer, pH 8.6, 0.1 M. Two similar samples without enzyme added to buffer served as controls. After incubation for 18 hours at 37° C in a constant-temperature water bath, the contents of the four incubation vessels were transferred quantitatively to dialysis bags (Visking, 1⅛ inch) with 5 ml of additional buffer. The contents of the bags were dialyzed against distilled water (6 L) with constant stirring for 24 hours at 4° C. Thereafter, the contents were transferred to centrifuge tubes (25 ml), made up to 15 ml with distilled water, and spun at 3,000 rpm for 45 minutes. All residues were washed 3 times each with alcohol and ether and dried as before. All supernatant solutions representing that protein which had been made soluble, but nondialyzable, by such treatment had ethanol added to 80 per cent vol/vol. After addition of 1 per cent KAc, they were allowed to stand for 3 hours at 4° C. The fine precipitates, which formed only in the tubes containing elastase-treated material, were washed and dried as above. It was possible that incomplete centrifugation might leave crystallites in the nondialyzable supernatant solutions of elastase-treated AER. The appearance of crystals in the ethanol-precipitated supernatant solutions, as disclosed by X-ray diffraction analysis, would in such case *not* be due to the specific action of elastase, but to a nonspecific precipitation of crystallites by any protein in the presence of ethanol. To exclude this possibility, 1.0 ml of a 1 per cent human serum albumin (American Red Cross) was added to the dialyzed supernatant solution of similar non-elastase-treated control samples. Following the addition of albumin, alcohol was added with KAc as above, and the precipitate which formed immediately was washed and dried as before. All samples were subjected to X-ray diffraction analysis by the powder method.

[3] Nutritional Biochemicals, Cleveland, Ohio.

X-ray diffraction. X-ray diffraction studies were performed in a Debye-Scherrer powder camera with a 57.3 mm radius. The powdered residue was placed in 0.2 mm Lindermann glass capillary tubes and exposed for 20 hours. Copper radiation ($CuK\alpha = 1.54$ Å), with a nickel filter, and Kodak no-screen X-ray film were used. The patterns were analyzed by measuring the diffraction angles θ corresponding to various interatomic spacings of the crystalline sample. The interatomic spacings d were computed from the Bragg equation

$$n\lambda = 2\ d\ \sin\ \theta$$

where n is a whole number indicating the order of reflections and λ is the wavelength of the X-rays expressed in Å units. The intensities of the diffraction lines were visually estimated and the crystalline diffraction pattern was identified with the aid of the X-ray Powder Data File (formerly known as the A.S.T.M. Card Index System).

It should be emphasized that a typical, sharp diffraction pattern is obtained by the powder method if the crystallites of the sample are not too small (above 1,000 Å) and exhibit a regular three-dimensional periodicity of their atomic structure. Proteins, because of their large and irregular periodicity, and amorphous materials, because of the absence of three-dimensional periodicity, do not give rise to conventional powder diffraction patterns. These materials exhibit only diffuse halos near the primary beam.

Analyses. Nitrogen was determined by the Kjeldahl method, hexosamine by the method of Schloss (15). Hydroxyproline was determined by the method of Troll and Cannan (16). Elemental analyses for calcium and carbon were performed by Dr. Carl Tiedcke of Teaneck, N. J.

RESULTS

Analytic and X-ray studies of whole aortic residues. The analytic and gravimetric data obtained from nine samples of AER are listed in Table I. The last two columns indicate a visual estimate of the relative amounts of protein and crystalline matter observed by X-ray diffraction analysis. Dry weight of the samples was relatively constant, averaging 24 per cent of the wet weight, and did not vary appreciably with age. There was a trend toward a weight increase in AER with advancing age. It is apparent that while the nitrogen content decreased with advancing age, the per-

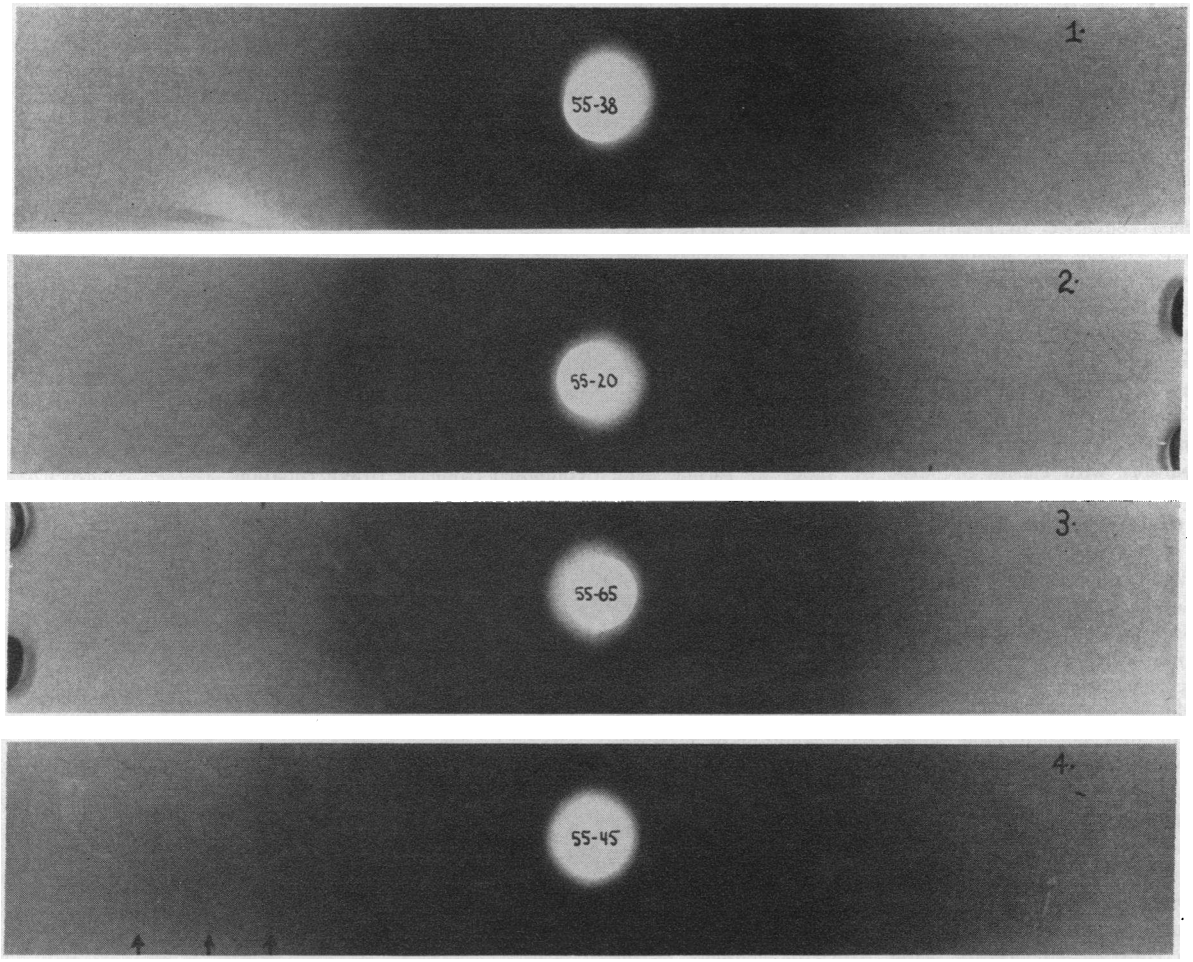


FIG. 2. X-RAY DIFFRACTION POWDER PATTERNS OF HUMAN AORTIC ELASTIN RESIDUES. (CuKα radiation, filter, 20 hours' exposure.) 1, Age 8 years. 2, Age 30. 3, Age 37. 4, Age 41 [arrows indicate the faint crystalline pattern of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$]. 5, Age 80, 24 hour exposure. 6, Age 86, prepared from fresh aorta (see text). 7, Identical with 6, but with superimposed single crystal patterns which could not be identified as hydroxyapatite. 8, Age 86, prepared from dried aortic powder (see text).

centage of calcium rose markedly. Similarly, the carbon content varied inversely with age.

AER obtained from individuals aged 8, 28, 30 and 37 years did not exhibit crystalline diffraction patterns but showed strong, diffuse haloes characteristic of a high protein content (Figure 2, nos. 1–3). This finding was in good agreement with the high nitrogen and carbon content of these samples. The first crystalline pattern was given by the elastin residue extracted from the aorta of a 41 year old woman (Figure 2, no. 4). Despite the strong, diffuse scattering associated with the high protein content of the specimen, a diffraction pattern emerged which could be identified as hydroxyapatite-like. (The techniques employed are not capable of distinguishing unequivocally between hydroxyapatite and closely related apatites and their solid solutions. For instance, fluorapatite and tribasic calcium phosphate give closely similar patterns, but the former may be excluded from human material, and patterns given by tribasic calcium phosphate agree less well with the observed data in Table II. For these reasons, the observed pattern will be referred to as hydroxyapatite.)

In elastin residues prepared from the aortas of individuals aged 63, 80 and 86, the diffuse protein scattering was definitely decreased. Con-

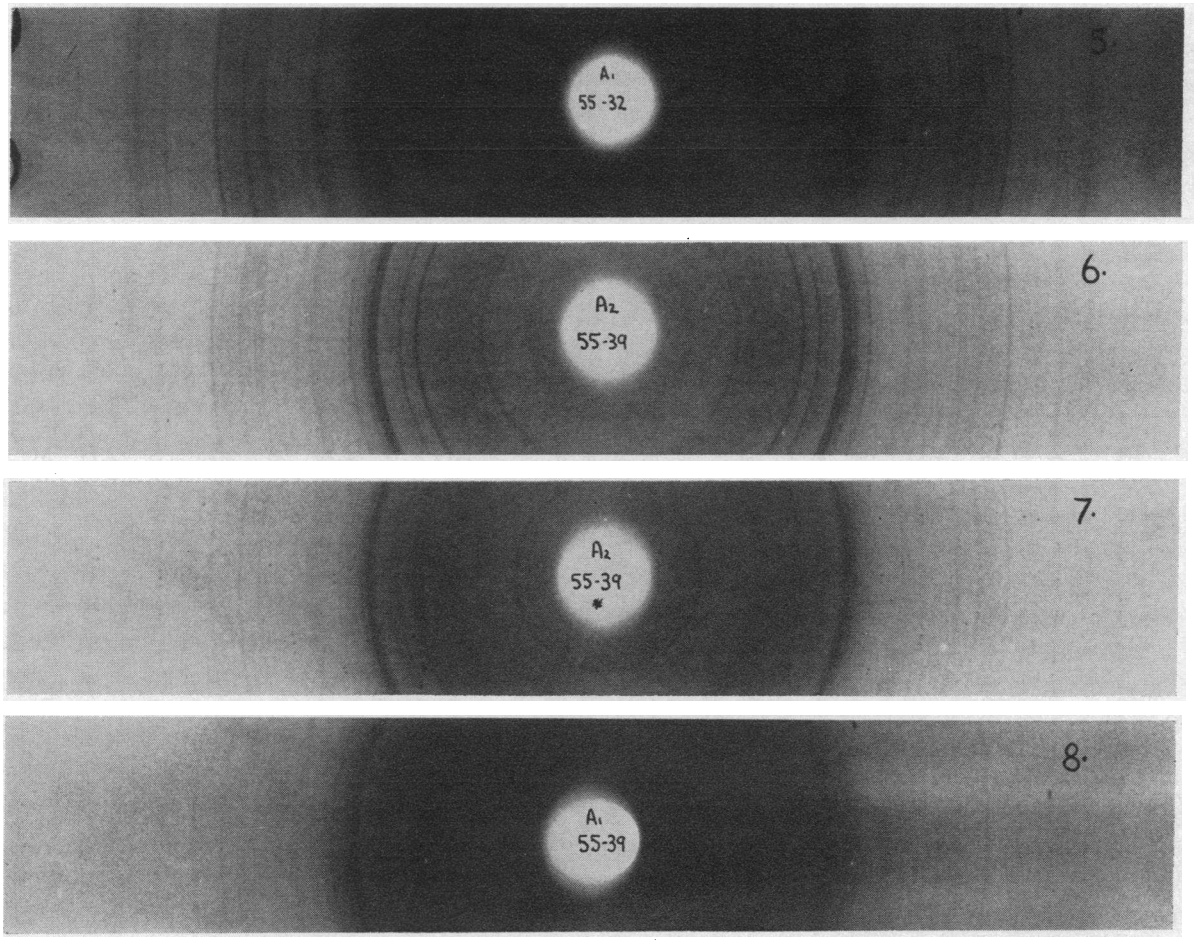


FIG. 2.—*Continued*

TABLE I

Analytic and gravimetric data for nine isolated human aortic residues *

Sample	Age	Sex	Whole aorta	Elastin residue	Analytic values of elastin				X-ray diffraction pattern	
					Nitrogen	Calcium	Carbon	Hexosamine	Protein	Crystal
			% dry weight		%	%	%	%		
55–15	8	M	25.0	54.0	16.3	0.8	49.6		++++	0
55–38	28	M	25.5	48.3	16.8	0.1	49.6		++++	0
55–20	30	M	24.1	49.4	15.8	2.8	47.5	0.2	++++	0
55–65	37	F	24.2	49.0	15.4			0.1	++++	0
55–45	41	F	20.0	53.0	14.0	17.3	48.7	0.1	+++	+
55–46	63	M	23.5	62.9	13.9	10.5	41.5	0.6	++	++
55–32	80	F	28.1	59.0	8.4	31.4	25.5		+	+++
A_1 55–39	86	F	21.0	62.9	9.3	28.2	29.1	0.4	+	+++
A_2 55–39†	86	F	21.0	68.4	10.8	22.4	32.3	0.6	+	++++‡

* The last two columns refer to the relative strength of protein and hydroxyapatite patterns as seen in X-ray diffraction patterns. These are graded from 0 (absent) to ++++ (marked) and were estimated visually (see Figures 2 and 3).

† Prepared directly from sample 55–39 without prior drying.

‡ Single crystal patterns superimposed upon diffraction lines.

TABLE II

d-Spacings calculated from X-ray diffraction analysis (Powder method) of isolated human aortic elastin residues*

hkl	Hydroxyapatite† (Hexagonal $a_0=9.42$ $c_0=6.88$)	Human aortic elastin residues							
		$A_2$55–39	$A_1$55–39	$A_1$55–52	55–45	$A_1$55–39	$A_1$55–39 Fraction D	$A_1$55–39 Fraction R	$A_1$55–39 Fraction ES
100	8.17 VW	7.09	8.38 VW	8.14 VW		8.17 VW	8.28	8.22 VW	
101	5.26 VW	5.18 VW	5.18 W	5.14 M		5.12 VW	5.08	5.09 VW	
200	4.07 VW	4.14 VW				3.99 VW			
111	3.88 VW			3.93 VW		3.85 VW			
201	3.51 VW	3.57 S		3.54 S					
002	3.44 W	3.46 S	3.42 S	3.42 S		3.44 S	3.42 S	3.46 M	3.42 S
102	3.17 VW	3.16 W	3.17 W	3.17 S		3.16 VW	3.15 VW	3.14 VW	3.18 W
210	3.08 VW	3.07 VW	3.11 VW					3.09 VW	
211	2.81 VS	2.84 W	2.85 M	2.86 VS	2.84 VS	2.84 M	2.84 S	2.84 M	2.87 S
112	2.78 S	2.79 VS	2.78 VS			2.79 VS	2.79 VS	2.80 VS	2.79 VS
300	2.72 M	2.71 W	2.71 S			2.74 S	2.70 S	2.73 S	2.71 M
202	2.63 VW	2.63 VW	2.63 VW				2.63 VW	2.63 VW	2.64 VW
301	2.53 VW	2.49 VW	2.53 VW	2.53 M	2.57 VW	2.57 VW	2.59 VW	2.58 VW	2.58 VW
310	2.26 W	2.25 VW	2.25 VW	2.24 VW		2.27 W	2.24 W	2.27 VW	
311	2.15 VW	2.13 VW	2.15 VW	2.15 VW		2.16 VW	2.14 W	2.16 VW	
113	2.07 VW		2.06 VW			2.07 VW	2.06 W	2.07 VW	
303	2.00 VW		2.01 VW	2.00 VW		2.00 VW		2.00 VW	
222	1.94 W	1.95 W	1.94 W		1.98 W	1.94 W	1.94 M	1.95 W	1.94 VW
312	1.89 VW		1.91 VW	1.91 VW		1.91 VW		1.89 VW	
213	1.84 W	1.85 W	1.84 W	1.88 VW		1.84 W	1.82 M		1.84 VW
411, 004	1.72 W	1.72 W	1.72 W	1.72 W		1.71 W		1.70 W	1.72 VW
313	1.61 VW		1.65 VW	1.65 VW	1.60	1.60 VW		1.61 VW	
501	1.59 VW		1.56 VW					1.58 W	
331	1.53 VW	1.54 VW		1.53 W	1.54 W	1.54 VW	1.54 W		

* Intensities estimated visually: very strong (VS) = 100, strong (S) = 70–90, medium (M) = 50–70, weak (W) = 30–50, very weak (VW) = <20.

† X-Ray Power Data File Card no. 9-342 (prepared by P. De Wolff), Techn. Phys. Dientst., Delft, Holland, 1959.

comitantly, the diffraction pattern of hydroxyapatite became more conspicuous (Figure 2, nos. 5–8).

The *d* spacings and corresponding intensities of all samples analyzed are given in Table II. These are compared with a standard diffraction pattern of hydroxyapatite from the X-ray powder data file. It will be seen that virtually all lines are in close agreement with those of the standard pattern. The slight deviations in *d* spacings observed in some of the samples may indicate the presence of solid solutions of hydroxyapatite as postulated by Posner and Stephenson (17).

One preparation derived from an 86 year old woman, (Figure 2, no. 7) exhibited, superimposed upon the powder pattern of apatite, diffraction patterns characteristic of single crystals. The coincidence of the single crystal diffraction spots with lines not belonging to the hydroxyapatite powder pattern indicated that they were of different, not yet identified, origin. All samples that exhibited the diffraction pattern of hydroxyapatite had a considerable calcium content. Since, as indicated above, X-ray diffraction analysis can only indicate the mineral content present in crystalline form, it was not unexpected that no direct correlation could be observed between the total calcium content determined chemically and the intensity of the diffraction lines (compare Tables I and II).

Treatment of human aortic elastin residue with elastase. Treatment of AER with elastase yielded two nondialyzable fractions. Fraction R was a finely powdered, white precipitate with approximately twice the calcium and one-third the nitrogen (in per cent) of the original sample (Table III). X-ray diffraction analysis of this fraction exhibited the highly resolved diffraction pattern shown in Figure 3, no. 10, which was virtually free of the diffuse scattering associated with the presence of protein.

Fraction ES was formed by ethanol precipitation of the nondialyzable supernatant solution resulting from elastase treatment of AER. This material was flocculent and finally fibrous, but en-

tirely white, whereas the original residue was slightly beige. When subjected to X-ray diffraction analysis, in addition to the expected protein scattering, Fraction ES showed a pattern readily identified as hydroxyapatite (Figure 3, no. 11). In order to demonstrate that the hydroxyapatite crystallites in Fraction ES were bound to protein, it was necessary to show that they would not remain in the supernatant fraction if they were unassociated with nondialyzable material. Separation might have occurred if larger crystallites were to precipitate during centrifugation, leaving the finer crystallites to float in the supernatant. Consequently, a diffraction pattern was obtained *without* rotating the specimen. It may be seen in Figure 3, no. 11a, that the lines are just as continuous as in the rotated specimen. This indicated that the crystallite size was of the order of magnitude of 0.5 to 5 μ. Larger crystallites would have given distinct spots of single crystals. Fraction R also yielded a diffraction pattern with continuous lines when the specimen was examined without rotation. This would imply that supernatant and precipitate were not separated on the basis of crystallite size.

Control samples carried through this procedure, save for the substitution of inert buffer for elastase, yielded only one fraction (Fraction D, Tables II and III). This fraction was essentially identical with that of the starting material by chemical and

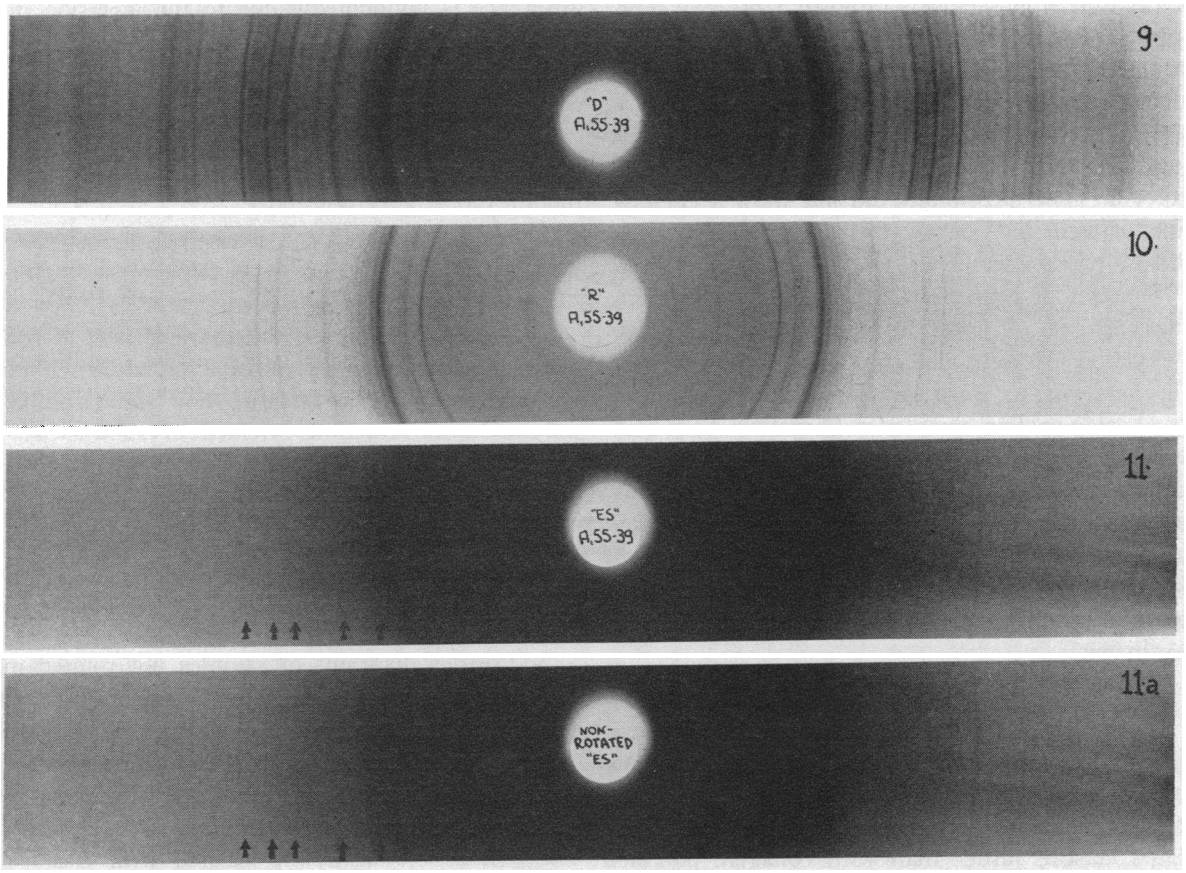


FIG. 3. X-RAY DIFFRACTION POWDER PATTERNS OF HUMAN AORTIC RESIDUES. (CuKα radiation, nickel filter, 20 hrs. exposure.) 9, Elastin residue (A_1 55–39) incubated in barbital buffer and dialyzed (Fraction D, Figure 1). 10, Insoluble precipitate of another sample of A_1 55–39 after elastase digestion and subsequent dialysis. Note the disappearance of the diffuse diffraction halo associated with protein (Fraction R, Figure 1). 11, Ethanol-precipitated, nondialyzable supernatant of sample A_1 55–39 after elastase digestion. Note the characteristic hydroxyapatite pattern, indicated by arrows, and the prominent diffraction halo associated with protein (Fraction ES, Figure 1). 11a, Identical sample as 3. Pattern obtained *without* rotation of specimen. The continuous lines indicate that the crystallite size of the sample is between 0.5 and 5 μ.

TABLE III

Treatment of aortic elastin residue with elastase (sample $A_1$55–39) *

Sample	Symbol	Yield elastin	Calcium	Nitrogen	Carbon	X-ray diffraction pattern	
						Protein	Crystal
		mg/100 mg	%	%	%		
Original elastin	$A_1$55–39		28.2	9.3	29.1	+	+++
Dialyzed elastin	D	89.8	24.5	10.2	31.0	+	+++
Elastase-treated, nondialyzable residue	R	23.1	53.9	3.1	9.8	0	++++
Elastase-treated, nondialyzable supernatant	ES	5.3				++++	+

* After dialysis of the digestion products, centrifugation yielded the fractions R and ES; Fraction ES was reprecipitated with 80% vol/vol ethanol from the supernatant of such a dialyzed digestion mixture (Figure 3). X-ray diffraction intensities are as graded in Table I.

X-ray diffraction analysis (Figure 3, no. 9). The supernatant of this fraction did not yield a precipitate upon the addition of alcohol. When, however, albumin was first added to the supernatant of a similar control preparation, a precipitate readily formed upon the addition of ethanol to 80 per cent vol/vol. This precipitate did *not* show a crystalline pattern, but merely displayed the expected, diffuse protein scattering.

Samples of reprecipitated gelatin formed from the collagen of aorta at autoclave temperature (Fraction C) were also subject to X-ray diffraction analysis, as were samples of acid mucopolysaccharide-protein complexes (Fraction A) which were extracted by the initial homogenization in cold water. No trace of a crystalline pattern was obtained from these fractions, consequently any close structural bond (not disrupted by the isolation procedure) between hydroxyapatite and materials in these fractions appeared to have been excluded.

DISCUSSION

These studies confirm the presence of a hydroxyapatite as the principal crystallite of human aorta. In addition, they provide evidence that this mineral may be closely associated with isolated elastin, rather than with collagen, polysaccharide or lipid. The AER, isolated by the procedure detailed above, showed an increase of calcium and a decrease of nitrogen (expressed as per cent) with increasing age. These findings are in agreement with those of Lansing, Alex and Rosenthal (3), although the yields and percentages of calcium differ from those reported by them. This difference is undoubtedly due to the inclusion in our specimens of intimal calcific deposits. X-ray diffraction analysis of AER has shown a decrease of protein content, coincident with an increase of crystalline hydroxyapatite with advancing age. These changes are probably related to the chemical modifications described above.

The intimate structural association of hydroxyapatite with elastin has been established by the following evidence.

1. Isolated AER gave the characteristic X-ray diffraction pattern of hydroxyapatite, while neither collagen prepared as gelatin, nor water-soluble polysaccharide fractions yielded crystalline patterns.

2. Elastase treatment of AER liberated into the supernatant solution of the digestion mixtures a nondialyzable protein, which, upon ethanol precipitation, showed hydroxyapatite structure on X-ray diffraction analysis.

3. Powder diagrams of samples not rotated in the camera served to exclude the possibility that the crystallites floating in the supernatant solution were separated from those in the precipitate on the basis of size alone. Since insoluble hydroxyapatite could not be found in the aqueous supernatant of suspensions of elastin *not* treated with elastase, intimate bonds between elastin and these small hydroxyapatite crystallites were presumed to exist. Probably these are strong molecular forces between protein and crystal, since they survive autoclave conditions.

4. The coincidental precipitation of small hydroxyapatite crystals floating in suspensions of

dialyzed elastin by another soluble protein such as albumin has not been observed.

Postulation of elastin-mineral association would not exclude a much weaker, yet physiologically significant association of hydroxyapatite with collagen or lipid which would be disrupted by an extraction procedure involving fat solvents and autoclave temperatures. Indeed, were extraction to break such forces, insoluble hydroxyapatite crystallites would be found in the elastin fractions as finally isolated.

Increased crystallinity of aortic elastin with aging may be sufficient to account for the embrittlement thought to be responsible for hemodynamic changes in the aging aorta, whose elastic properties have been attributed to macromolecular aggregation (18). Crystallization in polymeric substances usually diminishes their elastic properties.[4] Examples of this phenomenon are found in natural and synthetic rubbers, as well as in synthetic fibers. Crystallization of such polymers was found to be associated with an increase of stiffness. A tenfold increase in Young's modulus during crystallization was shown in results obtained on unvulcanized rubber as well as on Neoprene (19). Impregnation of fibrous proteins by many small crystallites would thus increase brittleness by the formation of anchor-points for elastin fibers, with a concomitant decrease of their rubber-like mobility. Amorphous deposits do not show these effects, whereas crystallites by means of secondary valence forces (e.g., hydrogen bonds), can establish firm linkages with fibers and thus establish anchor-points (19). In view of these functional considerations, studies of crystallinity may be more fruitful in the study of aging tissues than simple chemical analysis which does not distinguish between crystalline and amorphous deposits of mineral.

Further quantitative studies of the crystal-fiber relationship in blood vessel wall seem appropriate. Since the number and size of crystallites are of vital importance in determining strength and elastic properties of analogous polymer systems, techniques such as low-angle X-ray scattering and Fourier analysis of line profiles (20) should give valuable quantitative information in this regard. Such studies, using the elastin preparations described above, are now in progress.

[4] While this study has been concerned with the deposition and/or formation of mineral crystal upon a polymer, polymers themselves may exhibit crystallinity under appropriate conditions. The resultant loss of elastic properties is essentially similar.

SUMMARY

1. Elastin residues from human aortas have been prepared on the basis of their insolubility in water at autoclave temperature.

2. The crystallites associated with such residues became prominent in aortas from older individuals and were identified as hydroxyapatite by X-ray diffraction analysis.

3. In aortas obtained from young subjects, only a diffuse X-ray scattering characteristic of protein was observed; this scattering decreased with advancing age.

4. These X-ray findings paralleled a decrease in nitrogen and an increase in the calcium content of isolated aortic elastin residues with advancing age.

5. The intimate structural association of hydroxyapatite crystallites with elastin in aortic tissue has been demonstrated by means of elastase. Treatment of elastin residues with this enzyme caused the liberation of a protein into the nondialyzable supernatant solutions of the digestion mixture, which upon ethanol precipitation gave the typical diffraction pattern of hydroxyapatite.

6. Neither collagen nor polysaccharide fractions obtained from human aortas exhibited crystallite patterns.

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