Chemical Composition of the Rabbit Aorta during Development

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ABSTRACT
Changes in both the relative and the absolute amounts of collagen and elastin in segments of the aortic wall of New Zealand white rabbits (1-130 weeks old) were determined by chemical analysis. As in other mammals, elastin was the major component of the wall of the thoracic aorta although the proportion of collagen increased along the abdominal aorta and into the more distant arteries. Data on the absolute amounts of collagen and elastin per aorta showed that collagen and elastin deposition was most rapid during the early postnatal weeks. Although this deposition slowed in later weeks, it continued in both segments of the aorta throughout the period of this study. The proportion of the wall mass attributable to collagen and to elastin increased very rapidly during the first postnatal weeks and then, after 20 weeks, declined. The results of the present study indicate that there is a significant increase in some unidentified nonscleroprotein component within the aortic wall of older rabbits.

KEY WORDS collagen elastin nonscleroproteins arteries media

Harkness et al. (1) have observed that the scleroprotein composition of the canine aortic wall varies at different anatomical sites; elastin is the major component in the thoracic aorta and collagen is the predominant component in the abdominal aorta and its major peripheral branches. They (1) have also found this pattern in the aortic walls of young puppies of unspecified age. Several subsequent studies (2-6) have shown that this pattern of scleroprotein distribution within the walls of the major arteries is common to many mammalian species and some birds. However, these studies have revealed a number of differences between species. To what extent this pattern of scleroprotein distribution is genetically determined or to what extent the developing arterial system differentiates in response to locally acting hemodynamic and physical stresses is unknown. Only one previous study—an investigation of the rat aorta—has detailed the changes with age in scleroprotein composition of different arterial segments (7). The present study describes the changes in both the relative and the absolute amounts of collagen and elastin in segments of the wall of the aorta and its major branches in healthy rabbits from postnatal development to maturity.

Methods
One hundred and twenty-one New Zealand white rabbits (1-130 weeks old) of either sex were studied. The rabbits were fed standard rabbit food pellets, fresh carrots, and tap water.

DETERMINATION OF BLOOD PRESSURE
Mean arterial blood pressure was determined on restrained unanesthetized rabbits under quiet conditions by percutaneous puncture of the femoral artery as described by Goldblatt (8).

PREPARATION OF VESSELS
The rabbits were killed by cervical fracture. The anterior chest wall was removed, and the abdominal cavity was opened to expose the aorta and its main branches. These vessels were excised as a unit, and loose adventitial tissue was removed by blunt dissection. The vessels were opened longitudinally, washed free of blood with physiologic saline, and blotted gently between filter papers. The arterial branch orifices were excised from the aorta, which was then divided at the level of the upper border of the crura of the diaphragm into thoracic and abdominal portions. All of the thoracic aorta distal to the aortic valveular ring was used; the lower limit of the abdominal aorta was the aortic bifurcation. The two portions were weighed separately.

CHEMICAL ANALYSES
For chemical analyses, the thoracic and abdominal aortas were cut into segments. Transection of the thoracic aorta immediately below the insertion of the ductus arteriosus separated the thoracic descending aorta from the ascending aorta and the aortic arch. The thoracic...
Descending aorta was then divided into three segments of equal length, which will be referred to as the upper, the middle, and the lower thoracic segments. The abdominal aorta was also divided into three segments of equal length. The common carotid arteries, as defined anatomically, were used whole. The common and external iliac arteries were taken together as the vessels between the aortic bifurcation and the inguinal ligament and will be referred to as the iliac arteries. The axial tissue available. Samples were pooled so that enough abdominal aorta was also divided into three segments of equal length. The common carotid arteries, as defined anatomically, were used whole. The common and external iliac arteries were taken together as the vessels between the aortic bifurcation and the inguinal ligament and will be referred to as the iliac arteries. The axial tissue available. Samples were pooled so that enough tissue was available for gravimetric quantification of the elastin residues. To measure the residues reliably, samples weighing more than 0.1 g (fresh weight) were needed. In most instances, samples of fresh tissue weighing between 0.2 g and 0.5 g were available for each estimation. Estimations were made on vascular segments from 19 groups of rabbits.

**DETERMINATION OF COLLAGEN AND ELASTIN**

Each tissue sample was minced finely with scissors in a tared weighing bottle and weighed (wet weight). It was then dried to constant weight in a vacuum over phosphorus pentoxide (dry weight). The dried samples were repeatedly extracted with ether to remove fat and then dried again to constant weight (dry fat-free weight). The dry fat-free weight was used as the most reproducible reference standard for purposes of comparison.

Collagen was extracted from the segments as gelatin by autoclaving in distilled water for 6 hours at 30 psi. After hydrolysis in 6N hydrochloric acid, the collagen contents of the extracts were determined by colorimetric assay of hydroxyproline. A modification of the procedure of Neuman and Logan (9) was used. This procedure has been described in detail elsewhere (procedure 1, Jackson and Cleary [10]). A hydroxyproline content of 14.4% for mammalian collagens was assumed for the collagen hydroxyproline from all segments from newborn, young, and old rabbits.

Weighed samples of dried elastin were hydrolyzed in 6N hydrochloric acid in sealed tubes by autoclaving for 4 hours at 40 psi. The hydroxyproline contents of the hydrolysates were determined colorimetrically as previously described.

To establish the reliability of the colorimetric assay for hydroxyproline with these tissues and to exclude the possibility of interference with color development by some component in the aortic gelatin and the elastin hydrolysates from different segments and the same segments at various ages, recovery experiments were carried out after known amounts of pure hydroxyproline had been added to the hydrolysates.

**HISTOLOGY**

Vessels were removed from 1-, 20-, and 58-week-old rabbits. After preparation for chemical analysis, segments were sectioned for histological examination. The amount of adventitia remaining on each segment was checked, and histological appearance was compared with chemical data. Sections were stained with hematoxylin and phloxine, with aldehyde fuchsin, hematoxylin, and light green, and with aldehyde fuchsin, hematoxylin, and picro-indigo-carmine combinations (5).

**Results**

**BLOOD PRESSURE**

Mean arterial blood pressure rose steadily from an initial value of 30 mm Hg in 1-week-old rabbits. Adult values (90–100 mm Hg) were attained by the age of 10–12 weeks. Thereafter, blood pressure showed only minor variations about this value up to 130 weeks of age. These findings are very similar to those reported by Bauer (11) and Dawes et al. (12).

**REPRODUCIBILITY OF CHEMICAL ANALYSES**

Duplicate hydroxyproline determinations agreed to within ±1.5%, and recovery of hydroxyproline added to hydrolysates of both collagen and elastin from different vascular segments and from the same segment from different age groups was within the range of 100 ± 2%. The reproducibility of collagen determinations on tissues from any one vascular segment was better than ±2%. The autoclave procedure extracted more than 98% of the collagen hydroxyproline from all segments from newborn, young, and old rabbits.

For elastin determinations, the size of the tissue sample affected the reproducibility. For those samples with a wet weight of 100–500 mg, elastin content was reproducible to within ±2%. For the samples containing only small amounts of elastin, e.g., abdominal aorta segments from young rabbits, replicate elastin analyses fell within a wider range of ±5%.

**PATTERNS OF SCLEROPROTEIN DISTRIBUTIONS**

The typical distribution of collagen and elastin along the aorta and its main branches in New Zealand white rabbits is represented in Figure 1. The values for the ascending aorta–aortic arch segments have been omitted from the figure in the interest of clarity. The elastin content of these segments was identical to that in the remainder of the thoracic aorta, but the collagen content was slightly less than that in the thoracic descending aortic segments. The proportion of the dried defatted vessel wall mass composed of both elastin and collagen was relatively constant along the aorta but declined in the iliac and more distant “muscular” arteries.

The collagen and elastin contents relative to
total scleroprotein content changed considerably along the arterial system. Elastin was predominant throughout the thoracic aorta. In the proximal half of the abdominal aorta, the elastin content decreased and the collagen content increased. These changes continued into the distal aorta and peripheral branches, in which collagen was the predominant component. The carotid arteries also showed a preponderance of collagen.

CHANGES IN SCLEROPROTEIN CONTENT WITH AGE

Although the pattern of distribution of collagen and elastin shown in Figure 1 was found in the major arteries of rabbits at all ages examined, there were variations during development in the contents of collagen and elastin in both thoracic and abdominal aortic segments. These changes are shown in Figure 2A and B.

Weeks 1–20.—For both the thoracic and the abdominal segments of the aorta during weeks 1–20, a progressive increase occurred in the proportion of the wall mass composed of collagen. Expressed algebraically, the collagen content of the thoracic aorta increased in this period according to the equation

Aortic collagen (thoracic)  

\[ = 0.20 \times \text{age (weeks)} + 15.5. \]  

(1)

The increase was significant \((P < 0.001)\). The increase in the collagen content of the abdominal aorta is described by the equation

Aortic collagen (abdominal)  

\[ = 0.29 \times \text{age (weeks)} + 24.4. \]  

(2)

Again, the increase was significant \((P < 0.001)\).

The changes in elastin content of both the thoracic and abdominal segments of the aorta during weeks 1–20 are more difficult to describe than are the changes in collagen content. In both segments, a sharp rise in the elastin content occurred in the first 4 weeks. Although the elastin content of the thoracic aorta attained its highest value by the fourth week, the elastin content of the abdominal aorta appeared to continue to increase until a maximum was reached at about the twentieth week (the same time the collagen maximum occurred). This latter increase can be described by the equation
Aortic elastin (abdominal)

$$= 0.05 \times \text{age (weeks)} +32.1 \quad (3)$$

The increase was significant ($P < 0.01$).

**After 20 Weeks.**—Figure 2 shows that the collagen and elastin contents of both thoracic and abdominal aortic segments declined after 20 weeks and that the rates of these decreases were greater after 66 weeks of age. Linear regressions were fitted to the data for collagen content; the equations describing the changes after 20 weeks are as follows.

Aortic collagen (abdominal)

$$= -0.05 \times \text{age (weeks)} + 32.74 \quad (4)$$

The decrease was significant ($0.01 < P < 0.025$).

Aortic collagen (thoracic)

$$= -0.029 \times \text{age (weeks)} + 20.41 \quad (5)$$

The decrease was significant ($P < 0.001$).

Linear regressions were also fitted to the data for elastin content. Because the onset of the decline in the percent of elastin present was not clear-cut, the regression equations were calculated for the data obtained after 18 weeks. Inclusion of the additional values between 18 and 20 weeks will tend to reduce rather than increase the extent and the significance of the decline in elastin values. Nevertheless, a highly significant decrease in the elastin proportions in both the abdominal and the thoracic aortic segments occurred. These changes are described by the following equations.

Aortic elastin (abdominal)

$$= -0.079 \times \text{age (weeks)} + 43.62 \quad (6)$$

The decrease was significant ($P < 0.001$).

Aortic elastin (thoracic)

$$= -0.044 \times \text{age (weeks)} + 51.95 \quad (7)$$

Again, the decrease was significant ($P < 0.001$).

The possibility that the elastin content of the thoracic aorta decreased after the fourth week was also considered. However, little change in the slope of the regression equation resulted; the decrease in elastin content remained highly significant ($P < 0.001$).

**TOTAL SCLEROPROTEIN CONTENT AND AGE**

As a result of the changes in collagen and elastin contents, changes in the proportion of the aortic wall mass composed of scleroproteins (elastin plus collagen) occurred with age. In the early postnatal weeks, total scleroprotein content increased rapidly in both the thoracic and the abdominal segments of the aorta. The increase in the abdominal segment was of greater magnitude and occurred over a longer period of time than that in the thoracic segment of the aorta. Following this initial postnatal phase, scleroprotein content remained relatively stable in both segments for some months, after which it declined in both segments.

**NONSCLEROPROTEIN CONTENT AND AGE**

The changes in total scleroprotein content can be looked at in another way. The proportion of the wall mass not composed of collagen and elastin can be considered as the nonscleroprotein content. Obviously, a rise in scleroprotein content means a commensurate fall in nonscleroprotein content and vice versa. Figure 3A and B graphically shows the changes in the proportion of the aortic wall mass composed of materials other than collagen and elastin. (These graphs show the inverse of the changes in contents of collagen plus elastin.) The changes in the postnatal phase were prominent. The nonscleroprotein content of both segments of the aorta increased after 60 weeks of age. Because few data points were available after 60 weeks, regression lines were fitted to the data after 20 weeks of age. Both aortic segments showed a significant increase ($P < 0.005$ for each) in nonscleroprotein contents after the twentieth week. The rate of increase in the proportion of nonscleroproteins was even greater after the first year. The increases might be greater in the abdominal segment than they are in the thoracic segment.

**ABSOLUTE AMOUNTS OF ELASTIN AND COLLAGEN**

The changes with age in the absolute amounts of elastin and collagen are shown separately in

![Figure 3](http://circres.ahajournals.org/)

**FIGURE 3**

Changes with age in the proportion of the wall of rabbit aorta segments composed of nonscleroprotein material (material other than collagen and elastin). A: Changes in the thoracic segment of the aorta. B: Changes in the abdominal segment of the aorta. Results are expressed as a percent of the dry fat-free weight (D.F.F.WT.) of the whole segments analyzed.
Figure 4A and B. In the thoracic aorta, elastin and collagen accumulated at relatively constant rates with a ratio of approximately 3:1 throughout the first 20 weeks of life. Thereafter, the rate of increase in both components slowed considerably. However, the deposition of both components in this portion of the aorta continued throughout the duration of this study. In the period after 20 weeks of age, the increases in elastin and collagen had a ratio of about 1.9:1. In the abdominal aorta, the initial phase of rapid elastin and collagen accumulation at a ratio of approximately 4:3 appeared to be of shorter duration, and the absolute mass of both components leveled off after 8-12 weeks. Again, in this segment of the vessel, the deposition of both components continued at a slow rate throughout the period of the study; the ratio of the increase in elastin to that in collagen was approximately 1.4:1.

HYDROXYPROLINE CONTENT OF ELASTIN

The mean value for the hydroxyproline content of dried elastin samples was 1.67 ± 0.09% (SE) (10 estimations).

HISTOLOGY

Examination of histological sections from different levels of the vessels prepared for chemical analyses revealed that the removal of adventitial tissue was uniformly complete throughout the thoracic aorta. In the abdominal aorta, a thin rind of residual adventitia remained. This rind was composed mainly of collagen-staining material with a few fibroblasts and occasional elastic-staining fibers. There was no obvious change with age in the proportion of aortic adventitial residue. Throughout the aorta, the intima consisted of a single layer of endothelial cells and a small amount of subendothelial connective tissue. Therefore, the main mass of the vessel wall, as analyzed, consisted of the media.

A 20% reduction in the number of medial elastic lamellas between the thoracic descending aorta and the lower half of the abdominal aorta was observed. Concurrently, the elastic lamellas became thinner and the smooth muscle elements in the lower abdominal portion appeared to increase. No pathological changes were observed in either the media or the intima of these vessels.

The amount of adventitial tissue remaining on the more peripheral muscular arteries was relatively greater and represented between 10 and 15% of the wall thickness.

Discussion

This study indicated that the pattern of scleroprotein distribution in wall segments along the rabbit aorta and its major branches is similar to that reported for other mammals. The histological examination revealed that the changes in the distribution of scleroproteins along the vessels occur in aortic segments that consist almost entirely of tunica media.

The importance of the scleroproteins in the aortic wall relates to their influence on the physical properties of the vessel. Elastin is readily extensible and rubberlike, but collagen is much stiffer and inextensible. The reduction in elastin content and the increase in collagen content of the aortic wall as it becomes more distant from the heart is associated with an increase in stiffness of the more peripheral portions of the arterial tree (13, 14). This nonuniformity of chemical composition and physical properties is in turn associated with changes in the contour of the arterial pressure pulse and with an increase in the rate of transmission of these pulse waves as they pass into the more peripheral arteries (15).

The data from the present study on New Zealand white rabbits showed that, despite the low arterial blood pressure and the relative immaturity of the cardiovascular system at birth and in the early postnatal weeks in this species, the adult pattern of scleroprotein distribution is already established by the end of the first postnatal week (when our first observations were made). Additional observations on newborn rabbits of another breed (Cleary, unpublished data) have shown that the pattern of scleroprotein distribution at birth is very similar to that of the 1-week-old rabbits used in the present study.
Although the actual pattern of scleroprotein distribution along the aorta does not change, extensive changes in the relative amounts of the individual components do occur during the portion of the life-span considered in this study. In the early postnatal weeks the proportions of both collagen and elastin in the two aortic segments increase (Fig. 2). These increases in relative contents of the scleroproteins correspond to the phase of rapid deposition of collagen and elastin in the aortic wall segments during the first 15–20 weeks of life (Fig. 4). After this initial phase, collagen and elastin continue to accumulate at a reduced rate, at least throughout the remainder of the period of this study. The ratios of the rate of accumulation of elastin to that of collagen are different in the two aortic segments, but for each segment very little change in this ratio occurs with further increase in age.

Our observation of a significant continuing decrease in the proportion of the wall mass attributable to collagen after 20 weeks of age is surprising because of the well-known "sclerosis," or increase in collagen, reported to occur in the aortic media of older animals of many species (16–18). Saxton (19) has shown that aging in rabbits is associated with progressive replacement of aortic smooth muscle cells by collagen; our own histological studies on these vessels appear to be consistent with this view.

However, it is obvious from our chemical data that this explanation is not sufficient. On a whole-organ basis, there is chemical evidence of continuing collagen and elastin deposition with increasing age; yet, simultaneously, collagen and elastin concentrations in both aortic segments decrease. Because the chemical findings are in opposition to the usually accepted interpretations, we have meticulously ruled out possible sources of error in our chemical assays.

Erroneous collagen values could result from contamination of the vascular gelatins by some substance(s) which interferes with color development in the hydroxyproline assay. The accumulation with age of a substance which suppresses color development or the decrease in a substance which enhances it could produce an apparent but false fall in collagen content. Both of these possibilities were excluded in this study by the demonstration that recovery of pure hydroxyproline added to vascular gelatins from young, mature, and old rabbits was complete and within the range of 100 ± 2%. Furthermore, an apparent but false decline with age in collagen content could result from incomplete extraction of gelatin from the older vessels. We found that more than 98% of the total hydroxyproline (other than that in the alkali-insoluble elastin) was present in the gelatins from young, mature, and old rabbit aortas extracted using the autoclave procedure. There was no systematic difference in the proportion of unextracted hydroxyproline with age; thus, this source of error was also excluded.

Finally, it should be noted that the collagen results were obtained by assuming that the hydroxyproline content of vascular collagens does not vary with age. Although to our knowledge there is no direct evidence for this assumption in rabbit aorta, there is considerable evidence from other tissues and from other species to support such an assumption. Hydroxyproline content does not vary with age in bone collagen in fowls (20), in tail tendon and skin collagens in rats (21), and in lung and skin collagens in humans (22). Some workers (23) have reported an increase with age in the hydroxyproline content of collagens from lung and tail tendon in rats. If these latter findings are valid and applicable to rabbit aorta collagens, they would indicate a decrease with age in collagen content even greater than that proposed in the present study. We know of no report indicating that the hydroxyproline content of collagen from any one soft tissue in a single species decreases with age. Thus, the decrease in collagen content observed by chemical assay in the aging rabbit aorta appears to be a real one, and some alternative explanation will have to be found for the histological observations.

Cliff (24) has attempted to reconcile biochemical analyses showing a 10% increment in collagen content in rat thoracic aortic segments between the ages of 4 months and 3 years (5) with the very obvious sclerosis seen histologically and electron microscopically in the aged rat aorta. He found that the distribution of collagen in the rat aortic media alters with advancing age so that, instead of being arranged in large well-localized bundles, it is spread diffusely and randomly throughout the medial elastic network. Cliff (24) has suggested that this change in distribution could account for an apparent increase in collagen in histological sections.

Although the continuing increase with age in the absolute amount of collagen in the aortic wall might have been predicted (probably for inaccurate reasons) from histological examination, neither histological appearance nor previous reports suggested the continuing and even greater increase with age in aortic elastin. Saxton (19) has reported an increase in the number of fine elastic fibrils.
between the elastic membranes of the aorta in old rabbits and a concurrent reduction in the thickness of the elastic lamellas themselves. Similar observations have been made by Cliff (24) in rats up to 36 months of age.

Furthermore, the conclusion that elastin is biologically inert and has a small rate of turnover in adult and aging animals (25–28) is widely accepted. However, the experiments from which these conclusions were derived measured the incorporation and the specific activity of $^{14}$C-glycine in insoluble aortic elastins in adult rats and adult rabbits. Since radioactive glycine was readily incorporated into these insoluble elastins, there must have been a continuing elastin synthesis at the time. The failure to detect a decrease in the specific activity of glycine in aortic elastins was probably a function of the short time interval over which the observations were made, i.e., 9 days in the rats and 28 days in the rabbits.

These same criticisms cannot be applied to the findings of Walford et al. (27). These workers also studied the changes in the specific activity of $^{14}$C-glycine in rat aortic elastin, but the radioactive glycine was incorporated during the rapid growth phase of young rats and the elastin specific activity was measured in rats killed at repeated intervals up to 930 days. The specific activity of the elastin fell rapidly during continued growth until the age of 120 days, after which it remained constant. This otherwise excellent experiment is subject to the criticism that the method used to purify the insoluble elastin does not apply uniformly at all ages. Hass (29), who was largely responsible for reviving interest in the method used by Walford et al. (27), showed that a longer extraction time (36–72 hours) was necessary to remove collagen from human vascular elastic tissue in older vessels. This same stipulation might apply to rat vessels and until this possibility is investigated, the results of Walford et al. (27) must also be accepted with reservations.

Results from two reports by Wolinsky (30, 31) show an increase in elastic tissue in the aortas of normal rats between the ages of 28 weeks and 18 months. Unfortunately, no observations were made on rats of intermediate ages; thus, it is impossible to say definitely that elastin synthesis was still occurring at 18 months of age and to comment on the changes in older rats.

Our own results clearly indicate continuing elastin accumulation with increasing age in the aortic wall of the rabbit, even though relatively harsh methods of purification were used.

Our observations on the changes in collagen and elastin in the aortic wall of the rabbit highlight the fact that examination of the changes in the content of collagen and elastin relative to the whole wall mass not only can give an incomplete understanding of the changes in collagen and elastin metabolism but also can actually lead to an erroneous interpretation. This fact can be clearly seen by examining the changes in the rabbit aorta after 20 weeks of age. The results for the changes in the absolute amounts of collagen and elastin per aorta show that both components continue to accumulate throughout the period of the study, although the percent of both relative to wall mass is declining. Wolinsky (30) has already drawn attention to this important observation in his study of the effects of hypertension on rat aorta. Whenever possible, quantification of absolute amounts of aortic components in identical vascular segments should be performed if metabolic changes are to be interpreted.

The demonstration of continuing accumulation of collagen and elastin in the aorta in the face of reductions in collagen and elastin content expressed as a percent of the dry fat-free weight indicate that some other nonscleroprotein component is accumulating in the aortic wall at a rate faster than the combined rates of elastin and collagen deposition. The electron microscopic data of Cliff (24) for thoracic aortas of aging breeder rats show an accumulation of granular, tubular, and vesicular debris lying free within the extracellular space. This material appears to be derived from degeneration and necrosis of medial cells. Although the female breeder rat has an increased susceptibility to aortic disease, similar changes might occur in the aging rabbit aorta. Such changes could account for some of the increase in the nonscleroprotein content with advancing age. Our own data do not permit speculation about the nature of this debris; however, they do enable us to exclude the possibility that the material is derived from collagen, since we have demonstrated that hydroxyproline does not accumulate in the residue after collagen extraction.

Our attempts to analyze the relative importance of genetic and hemodynamic influences in the determination of the scleroprotein distribution pattern in the relatively immature, low-pressure system of the young rabbit have been inconclusive. The presence at birth of the adult pattern of scleroprotein distribution along the aortic wall appears to favor genetic determination. Even so, we have demonstrated that the accumulation of
both elastin and collagen in the aortic wall reaches its highest rate at 1–3 weeks of age and begins to taper off after 10 weeks. This phase of most rapid scleroprotein deposition predates by 2–3 weeks the period of most rapid increase in total body weight in this breed of rabbits and corresponds very closely to the period of increasing blood pressure. Additional information relevant to the question of hemodynamic influences can probably be obtained from experiments designed to alter the hemodynamic stresses in the rabbit aorta during the period of rapid vascular growth.

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References
