CHEMICAL AND MORPHOLOGICAL STUDIES ON THE *IN VITRO* CALCIFICATION OF AORTA

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ABSTRACT

After a lag period, rat aortas incubated in rat serum *in vitro* accumulated substantial quantities of calcium and phosphate. Examination of the tissue by x-ray diffraction, microradiography, electron diffraction, and electron microscopy indicated that the calcium-phosphate phase which formed was hydroxyapatite and that the crystals were localized almost exclusively in elastin. Selective elimination of various components of the aorta with proteolytic enzymes indicated that the presence of elastin was required for mineralization. Collagen fibers did not appear to be required for the initiation of calcification, nor did they seem to undergo appreciable calcification in the time periods studied. Analysis of the initial lag period suggested that at least two changes occurred in serum prior to the mineralization of this tissue. Inhibitors of the reaction were destroyed, and the level of dialyzable calcium was elevated owing to its release from serum protein.

It is well known that the calcium content of various blood vessel walls increases with age (1) and that hydroxyapatite is a major component of aortic plaques (2). However, the processes which are involved in this deposition of calcium salts are still not understood. Various theories have been advanced to explain the initial events, such as injury to the vessel wall (3), infiltration of lipids into the medial region (4), or depletion of mucopolysaccharides from the tissues involved (5). Studies by Rosenheim and Robison (6), as well as Sobel and his collaborators (7), have demonstrated that normal aortas will accumulate calcium and phosphate when incubated in a synthetic medium.

Recently we have observed that substantial quantities of calcium and phosphate are deposited in rat aorta during incubation of this tissue in rat serum (8). This finding has provided a useful system for studying various factors involved in the *in vitro* calcification of this tissue.

EXPERIMENTAL

Serum and the ascending arch of the aorta were obtained from mature male Sprague-Dawley rats (200 to 300 gm). Penicillin (5 mg), streptomycin sulfate (50 mg), and 2 ml (approximately 1,200,000 CPM) of a carrier-free calcium^{45} chloride solution were added to 100 ml of serum. Ultrafiltrates of the serum containing antibiotics and calcium^{45} were prepared at 4°C using an LKB Ultrafilter (LKB-Produkter AB, Stockholm, Sweden). 5 ml of 1 M Tris (pH 7.4) were added to 100 ml of serum prior to ultrafiltration in order to maintain constant pH. In most experiments each sample of aorta was incubated with 1 ml of serum at pH 7.4 and 37°C in a Dubnoff shaker in a stoppered Erlenmeyer flask. The serum solution was replaced at 24 and 48 hours with portions of the original solution. Under these conditions, negligible numbers of microorganisms were detected in smears of serum. When antibiotics were omitted there was a heavy growth of microorganisms, and calcification of the aorta did not occur.

The aortas were removed after various intervals of

incubation, washed in several liters of distilled water, air dried, weighed, and then placed in 5 ml of 0.12 M HCl. Aliquots of this solution were removed for calcium (9), phosphate (10), and calcium^{45} assay. Calcium^{45} was measured in a low background (1.6 CPM) gas flow counter. Calcium^{45} uptake has been expressed as counts per minute per dry weight of aorta.

X-ray diffraction patterns were obtained from air-dried aortas which were ground to a fine powder.

In order to localize the mineral deposits, rat aortas which had been incubated in serum for 1 and 72 hours were fixed in buffered 1 per cent OsO_4 and embedded in butyl-methyl methacrylate. To facilitate orientation, contact microradiographs of thick sections containing large pieces of tissue were first made. Subsequently the blocks were trimmed so as to contain mineralized areas (in the 72 hour samples) or comparable unmineralized regions (in the 1 hour samples). Thin sections were cut on an LKB Ultratome, stained with uranyl acetate, and examined in a Siemens Elmiskop I.

In experiments designed to identify components of the aorta that are essential for calcification, aortas were treated with various enzymes prior to incubation. Enzymatic digestions were carried out in saline at pH 7.4 and 37°C for 24 hours. The activities of the enzymes were assayed qualitatively against Azocoll (11), orcein-impregnated elastin (12), and rat tail tendon. Trypsin (Worthington), protease (a crude bacterial product obtained from Nutritional Biochemical Corp.), pronase (*Streptomyces griseus* protease obtained from California Biochemical Corp.), and collagenase (obtained from Dr. P. Gallop) all rapidly digested Azocoll. Only elastase and pronase digested orcein-impregnated elastin. Only collagenase digested rat tail tendon.

RESULTS

After a lag period of at least 24 hours there was a marked increase of calcium, phosphate, and calcium^{45} in the aortas (Fig. 1). About 15 per cent of the calcium in the serum was fixed by the aortas during a 72 hour incubation. Exchange of serum calcium^{45} with calcium bound to the aorta was found to be negligible in comparison with the large increase of calcium^{45} in the tissue due to the deposition of a mineral phase of calcium phosphate. Consequently, the calcium^{45} uptake was used in some experiments as a measure of calcification. Little difference in the degree of mineralization was found between pH 7 and 8, while below pH 7 mineralization did not occur (Fig. 2A). A marked dependence of calcification on temperature was also noted (Fig. 2B). Both these effects have been observed in other systems involving the deposition of mineral on a matrix (13).

In order to determine the nature of the mineral phase forming on the aorta, samples incubated

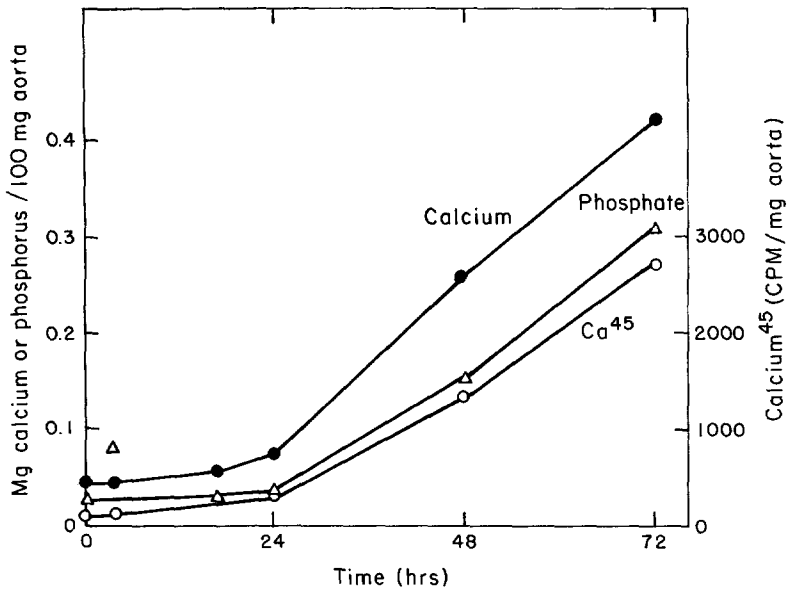

FIGURE 1

Uptake of calcium, phosphate, and calcium^{45} by aorta from serum.

for 1 and 72 hours were examined by x-ray diffraction. There was no indication of a crystalline mineral phase in samples incubated for 1 hour, but samples incubated for 72 hours gave a diffraction pattern similar to that of hydroxyapatite (Fig. 3).

Microradiographic studies of uncalcified aortas showed that the elastic laminae were uniformly more radiopaque than the other tissue components. In the 72 hour samples this difference was enhanced, with some of the elastic membranes

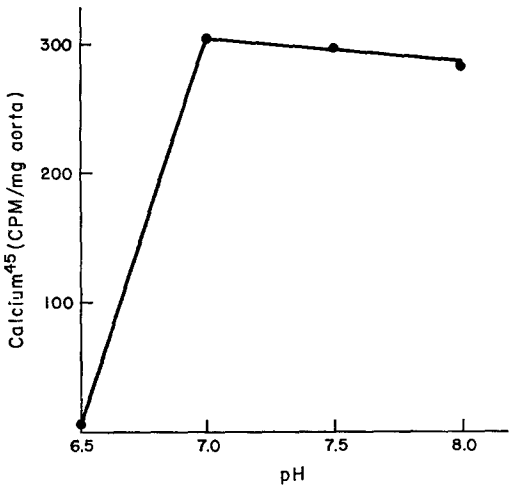

FIGURE 2A

Effect of pH on the uptake of calcium[45].

demonstrating a considerable increase in x-ray absorption (Fig. 4).

Electron microscopic studies of such regions (Figs. 5 and 6) revealed the presence of electron-opaque deposits in the elastic laminae, which were identified as hydroxyapatite by selected area electron diffraction. The crystals in these deposits appeared to be flat plates (Fig. 7), and there were no indications of preferred orientation. The elastin itself appeared as homogeneous bands surrounded for the most part by collagen fibers (Figs. 7 and 8). Cellular elements between elastin bands were lacking, undoubtedly owing to degeneration of the cells during the incubation period. Considerable variation was seen in the degree of mineralization in different regions of the elastic laminae. In some instances only single small foci were found (Figs. 5 and 7), while in others the laminae were quite solidly calcified (Figs. 6 and 8). As judged from the peripheral distribution of the foci in areas which were least mineralized, it appeared that calcification was initiated along the outer edges of the laminae rather than in the center (Figs. 5 and 7). No difference in the distribution of mineral was found between stained and unstained sections.

In most of the sections, crystalline material was present only in the elastin. Occasionally, however, crystals were observed on collagen fibrils adjacent to heavily calcified elastic laminae (Fig. 8).

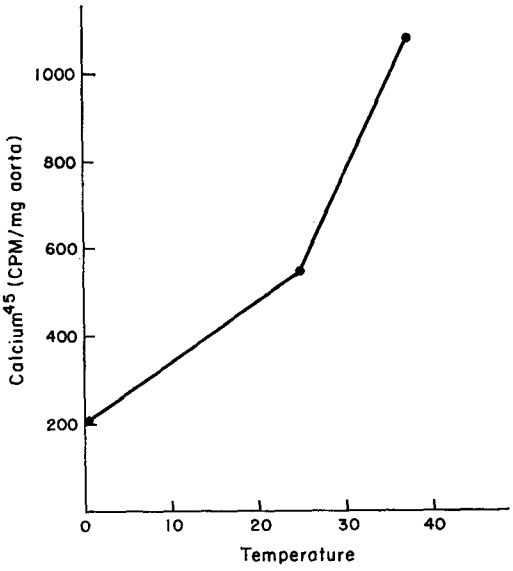

FIGURE 2B

Effect of temperature on the uptake of calcium[45].

In order to identify the components of the aorta that are involved in the initiation of calcification, aortas were treated with various enzymes prior to incubation (Table I). Pretreatment of the aortas with collagenase, trypsin, protease, and hyaluronidase did not reduce the uptake of calcium[45] by aorta. Trypsin and protease increased the uptake of calcium[45] per milligram of aorta by reducing the amounts of non-essential components. The actual uptake of calcium[45] per aorta in these samples was similar to that in the 72 hour control. Both elastase and pronase treatment prevented mineralization, as indicated by a negligible calcium[45] uptake as compared with the 72 hour control. These results indicate that the *in vitro* calcification of aortic tissue is dependent upon the presence of a certain component of the

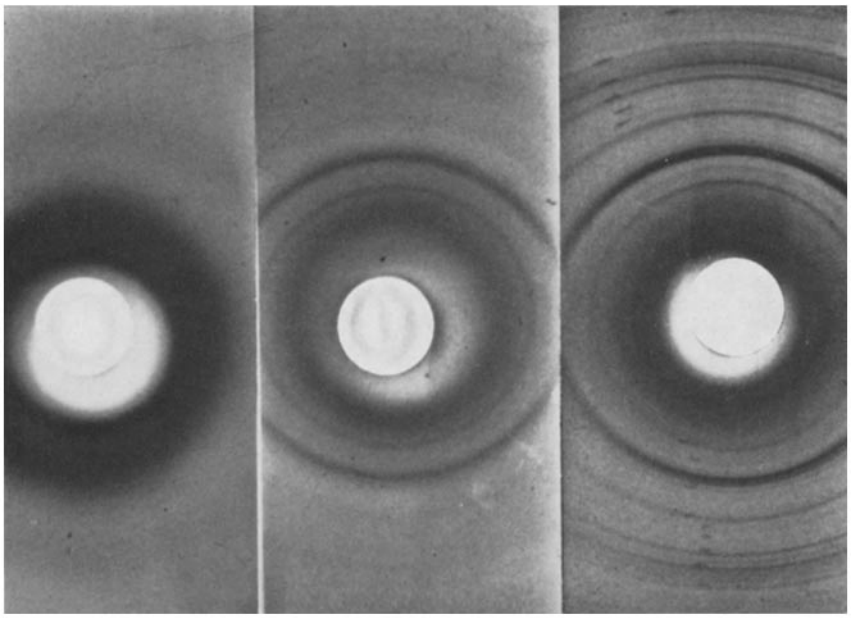

FIGURE 3

X-ray diffraction patterns from: *left*, aorta incubated for 1 hour; *center*, aorta incubated for 72 hours; *right*, hydroxyapatite standard obtained from powdered tooth enamel.

matrix, presumably elastin. In its absence other elements of the matrix cannot serve as substitutes.

Serum Alterations

After the defining of some features of the general system, attention was next directed toward the lag period which preceded calcification. Experiments were performed to determine whether mineralization could be accelerated by incubating the serum at 37°C prior to the addition of aortic tissue. In order to test this hypothesis, serum containing antibiotics and calcium[45] was incubated at 37°C for 24 hours. Fresh serum was collected on the following day. At this time aortas were incubated with varying proportions of fresh and aged serum. 24 hours later some aortas were removed for analysis and the remainder were resuspended in the original aged serum or in freshly collected serum.

The uptake of calcium[45] by the aortic tissue was markedly greater from the aged serum than from the fresh (Fig. 9). This was particularly

FIGURE 4

Parallel radiopaque bands seen in contact microradiograph of a cross-section through a rat aorta incubated for 72 hours in rat serum. × 800.

FIGURE 5

Electron micrograph showing early mineral deposits within elastic laminae of aorta incubated for 72 hours. × 5000.

FIGURE 6

Pronounced mineralization of the elastic laminae in another region of the same specimen. × 5000.

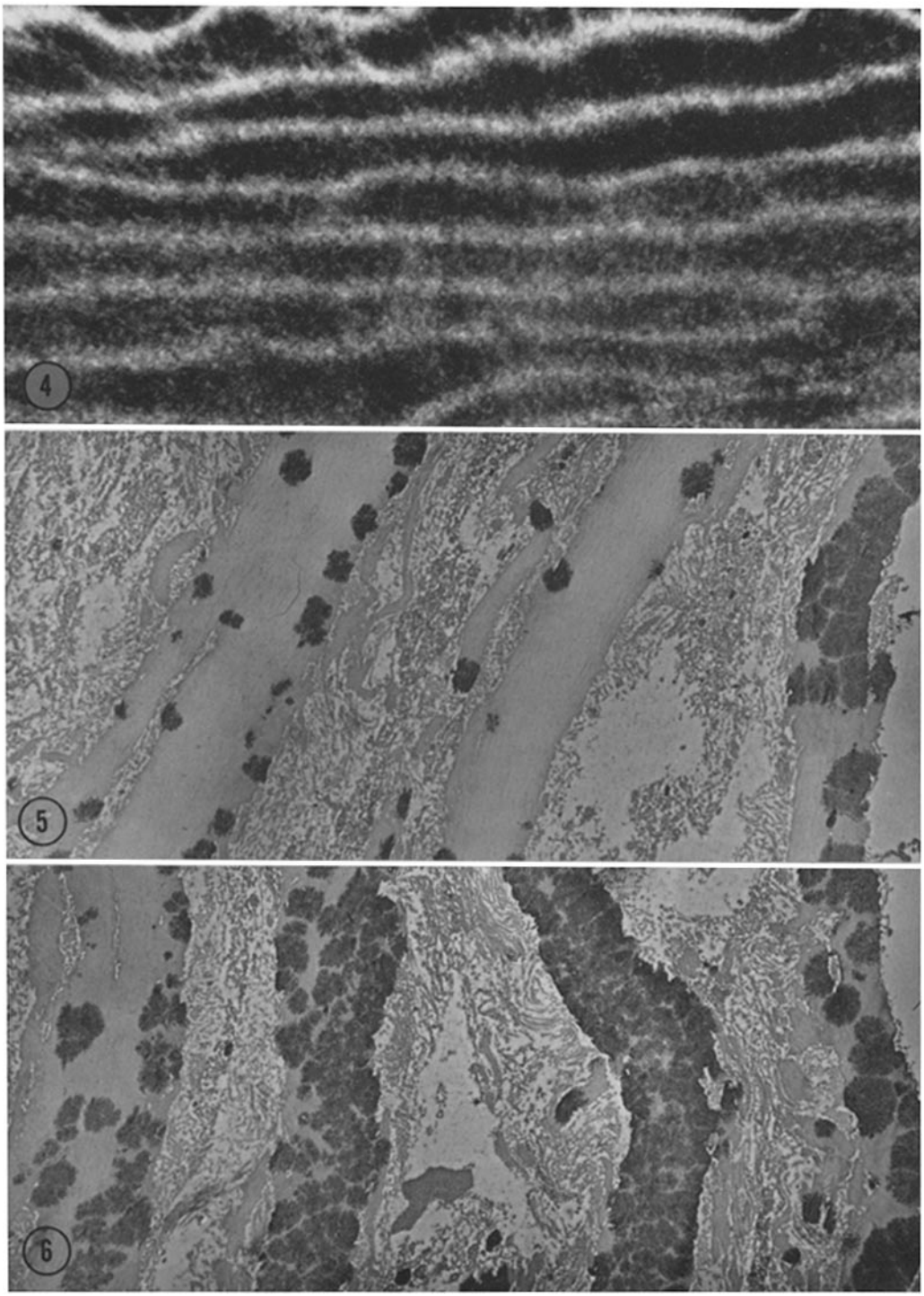

FIGURE 7

Small aggregate of plate-like crystals within an elastic lamina shown at higher magnification. No crystals are evident on the adjacent collagen fibrils. × 64,000.

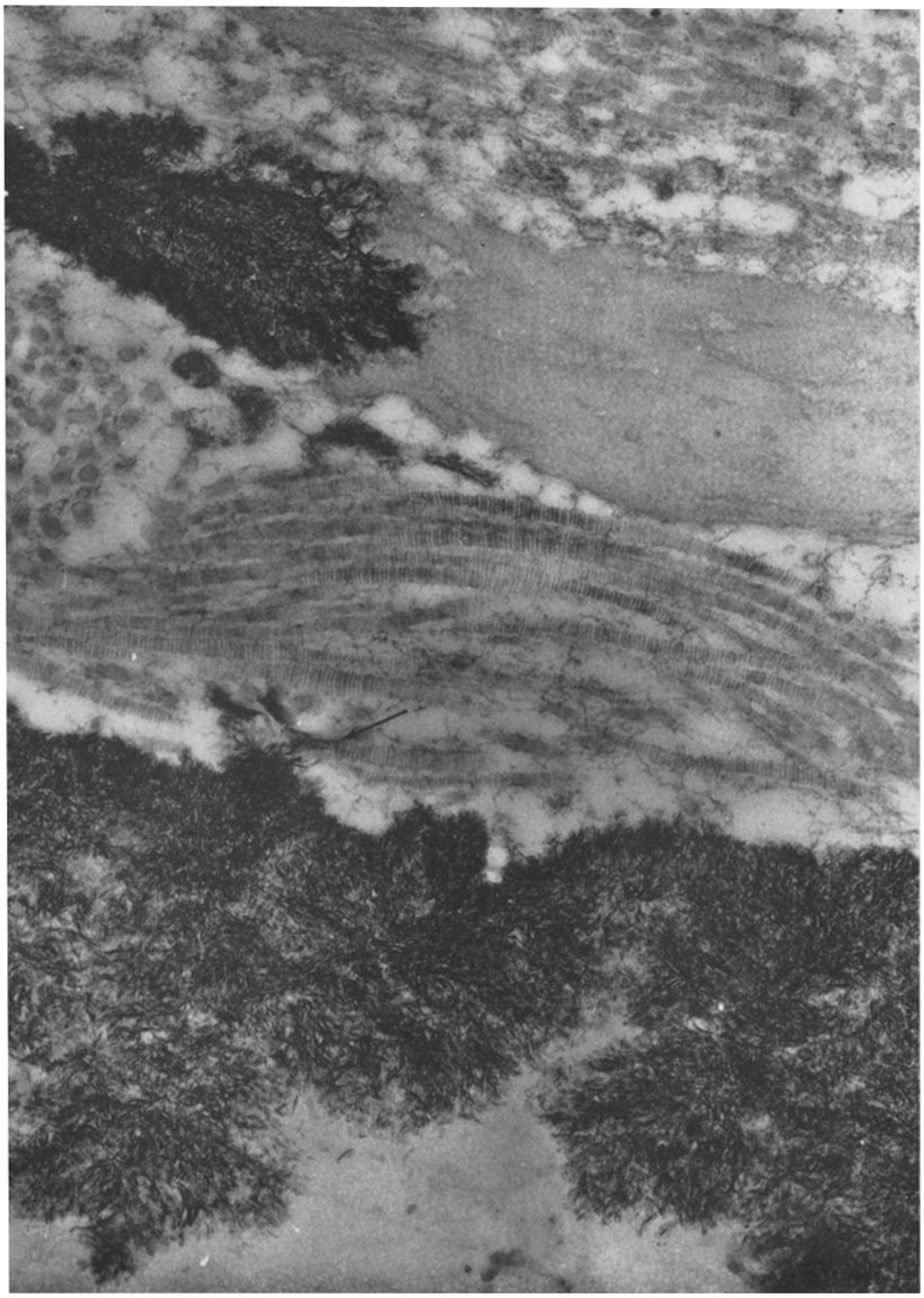

FIGURE 8

Two elastic laminae showing zones of heavy mineralization. A few crystals (arrow) are also found on the intervening collagen fibrils. × 64,000.

TABLE I

Effect of Enzymatic Digestions on the Uptake of Calcium^{45} by Rat Aortas

Pretreatment	Incubation period	Weight	Calcium^{45}*
	hr	*% of control*	CPM/*mg*
None	1	103	34 ± 0.1
None	72	100	2865 ± 178
Elastase	72	43.0	53 ± 7.7
Pronase	72	58.5	308 ± 4.9
Collagenase	72	60.7	2756 ± 128
Protease	72	65.1	4100 ± 320
Trypsin	72	62.4	4750 ± 321
Hyaluronidase	72	102	2460 ± 73

* Five aortas in each group.

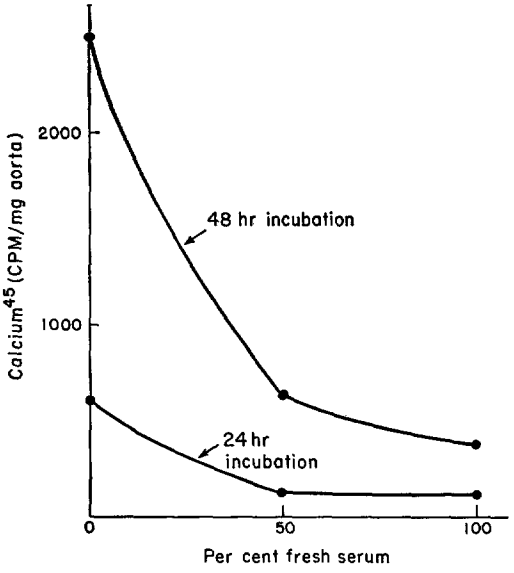

FIGURE 9

Uptake of calcium^{45} by aorta from fresh and aged serum.

evident after 48 hours. Calcium^{45} uptake was depressed in a 1:1 mixture of aged and fresh serum. These results are quite similar to those reported by Fleish and Neuman (14), who found that an ultrafiltrate of serum markedly inhibited the formation of hydroxyapatite induced by collagen. These investigators found that aging the serum or adding alkaline phosphatase promoted the precipitation of hydroxyapatite, and they postulated that a substance(s) capable of preventing calcification was present in serum.

In order to investigate the action of alkaline phosphatase in this *in vitro* system, aortas were incubated in serum and serum ultrafiltrates which had been treated in various ways. The usual large uptake of calcium^{45} by aortas incubated in serum was observed after 48 hours (Table II). During the same period there was one-sixth the uptake of calcium^{45} by aortas incubated in freshly drawn serum. The addition of alkaline phosphatase (Nutritional Biochemicals) to freshly drawn serum greatly increased the calcium^{45} accumulation by aortas, but the level was still below that observed when the aortas were incubated in aged serum. Calcification of the aortas did not occur in an ultrafiltrate prepared from freshly drawn

TABLE II

Uptake of Calcium^{45} by Aorta from Serum and Serum Ultrafiltrates

Incubation fluid	Time	Additions	Ca^{45}
	hr		CPM/*mg*
Aged serum	1		9.2 ± 0.8
	48		377 ± 77
Fresh serum	48		53 ± 10
	48	Alkaline phosphatase (0.5 mg/ml)	268 ± 39
Fresh serum ultrafiltrate	1		8.8 ± 3.0
	72		72 ± 10
	72	Alkaline phosphatase (0.5 mg/ml)	62 ± 15
Aged serum ultrafiltrate	1		7.5 ± 1.9
	72		532 ± 85

serum even when alkaline phosphatase was added. Calcification of the aortas did occur in an ultrafiltrate prepared from preincubated serum. The calcium level in the ultrafiltrates was found to be 5.5 mg per cent in the fresh serum and 7.5 mg per cent in the aged serum. Ninhydrin-reactive material was also elevated above the control in the ultrafiltrate prepared from the pre-aged serum. These results indicate that free calcium levels were rising owing to the destruction of serum proteins by proteolysis.

DISCUSSION

It is evident that the *in vitro* mineralization of rat aorta in serum is dependent both on chemical

alterations in fresh serum and on the integrity of certain components of the matrix. At least two changes occur in the serum prior to mineralization. First, the enhancement of calcification when alkaline phosphatase is added to fresh serum is consistent with the theory presented by Fleish and Neuman (14) that some agent present in serum, an inhibitor of mineralization, is destroyed by the enzyme. However, since ultrafiltrates prepared from fresh serum are not active as calcifying solutions, even when alkaline phosphatase is added, other factors must be involved. These may be related to alterations in the levels of ionized calcium in the serum prior to the *in vitro* calcification of rat aorta. As the classical experiments of McLean and Hastings (15) have demonstrated, only about 50 per cent of serum calcium is ionic and would be expected to participate in the initiation of calcification. Most of the remaining calcium is bound to protein. Piez *et al.* (16) have demonstrated that proteolysis occurs in serum incubated at 37°C. The destruction of serum protein would be expected to increase the level of ionic calcium in the serum. This phenomenon probably occurs in the *in vitro* system under study here. At least, ultrafiltrates prepared from aged serum contain higher levels of both calcium and ninhydrin-reactive material than do ultrafiltrates prepared from fresh serum. This increase in ionic calcium concentration would be expected to promote the deposition of a calcium phosphate phase.

Recent theories have directed a good deal of attention to the role of the matrix in initiating calcification (13, 17, 18). These theories propose that mineral deposition is brought about by a heterogeneous nucleation reaction involving an epitactic interaction between calcium and phosphate and some template on "ordered proteins" such as collagen. In the system under study here, the over-all pattern of mineralization is one of primary calcification within elastin, a protein which is not generally considered to have the degree of order found in collagen, whereas the calcification of collagen occurs only as a secondary phenomenon. Though the explanation for this preferential deposition of apatite is not readily apparent, this observation indicates that the role of "ordered proteins" in the initiation of mineral deposition in the hard tissues may not always be the controlling factor.

There are interesting similarities between the mineralization of aorta in the *in vitro* model system and in the human. Previous observations on human aortic tissue (19, 20) have shown that calcium deposits are usually absent from the aorta of individuals less than 20 years of age, whereas after the age of 20 the incidence and extent of these deposits, in the average specimen examined, rises markedly and continuously with advancing age of the individual. It seems reasonable to propose that this rather sharp demarcation may be related to alterations in the activity of control mechanisms which have previously restricted mineral deposition. In addition, it is of particular interest that attention has already been called to the association of mineral with elastin in human aortic tissue (19, 21). A similar association also exists in the model system.

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