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Early Mitochondrial Calcifications in the Rabbit Aorta after Adrenaline

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Summary. Sequential fine structural changes were assessed in the aortic media of rabbits shortly after the intravenous injection of a massive single dose of adrenaline. Slight changes first appeared at 1 hour and by 6 hours marked changes were present which included accumulation of Alcian-stainable material in the intercellular spaces and alterations in mitochondria of smooth muscle cells. The mitochondria were swollen and showed decreased matrix density together with the presence of electron dense microcrystalline inclusions. Electron diffraction studies showed that these inclusions were composed by apatite-like crystals. The mechanism(s) involved and the possible pathological role of the mitochondrial calcification are discussed.

Introduction

In human pathology, calcification in the vessel wall is a prominent feature of several arterial diseases, including atherosclerosis, Mönckeberg sclerosis and syphilitic aortitis. Calcifications of the aorta and other large elastic arteries may be induced in animals by a number of experimental procedures; the most effective seems to be the administration of adrenaline to rabbits. Adrenaline-induced arterial lesions in the rabbit were first described by Josué in 1903 and usually are situated in the aortic arch and upper portion of the thoracic segment of the aorta. In light microscopy the lesions are typically located in the inner third of the media and are mainly represented by death and necrosis of smooth muscle cells, accumulation of PAS-stainable intercellular material and calcification. Deposition of calcium is currently considered as a late developmental stage following necrosis of the arterial smooth muscle cells.

The present investigation describes the early changes induced in the aortic smooth muscle cells of the rabbit by adrenaline. The aortae of rabbits treated with a single massive dose of the drug were examined by light and electron microscopy shortly after treatment and the sequential changes before the appearance of gross calcification are described. As shown below, early mitochondrial electron-dense inclusions were observed in smooth muscle cells of the media before the appearance of overt necrotic alterations. The nature, significance and possible mechanism(s) of the mitochondrial change will be discussed.

Material and Methods

Adult rabbits of either sex fed a standard, semisynthetic diet and weighing about 2000 g were used as experimental material. Four rabbits without treatment were used as controls. Sixteen animals received a massive single dose of adrenaline hydrochloride (500 μg/kg body

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weight) during a period from 2 to 30 minutes and were killed at intervals in groups of four rabbits from 1 to 72 hours later, namely on the 1st hour and on the 6th, 24th and 72th hour after the end of treatment. An additional group of four animals was similarly treated with adrenaline at lower dosage level (50 μg/kg body weight) and sacrificed as above. Adrenaline hydrochloride was administered intravenously as freshly dissolved $1^0/_{00}$ solution in physiological saline. From some animals blood samples were taken immediately before the injection and at the sacrifice; blood levels of calcium, inorganic phosphate, sodium and potassium were determined with an atomic flame absorption spectrophotometer.

At necropsy the aortae of the control and adrenaline-treated rabbits were carefully dissected from the underlying fibrofatty tissue and opened ventrally. For each animal three samples were taken from the aortic arch and from the thoracic and abdominal aorta for light and electron microscopic studies. For light microscopy, specimens were fixed in 10% buffered formalin and embedded in paraffin; 5 μ-thick sections were stained with the conventional histological methods, including von Kossa stain for calcium, Alcian-method for mucopolysaccharides and Verhoeff stain for elastin. For electron microscopy, small pieces were processed immediately on removal from the animal. Samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodilate buffer (pH 7.4) for two hours at 4° C, dehydrated in graded alcohols and embedded in Epon or Epon Araldite. Some blocks after glutaraldehyde were postfixed in Millonig's osmium tetroxide at 4° C for two hours. Ultrathin sections were cut with a Porter-Blum ultramicrotome, stained partly by lead citrate and uranyl acetate and partly by lead hydroxide, and viewed through a Siemens Elmiskop 1A electron microscope. Ultrathin unstained sections were processed with H_2O_2 2 vol. and formic acid 2% for histochemical studies as shown below. For electron diffraction studies unstained sections were employed. Selected area electron diffraction was performed at 80 kV; MgO crystals were employed to calibrate the diffraction camera. Further details of the technic will be found elsewhere (Steve Bocciarelli, 1973, 1973a).

Results

A. Light Microscopic Observations

The examinations of hematoxylin and eosin stained sections of the aorta from rabbits killed 1, 6 and 12 hours after the end of treatment revealed no abnormalities. In animals killed on the 24th hour after adrenaline, small intercellular accumulations of Alcian-positive material were occasionally found in the inner layers of the media beneath an apparently normal intima; smooth muscle cell necrosis and calcium deposits were not observed. In the animals killed later, besides a more intense intercellular and interfibrillar accumulation of Alcian-stainable material, groups of smooth muscle cells in the innermost layers of the media were occasionally seen showing nuclear picnosis and cytoplasmic degenerative changes. Besides, there was a separation or loosening of the medial elastic components; the internal elastic lamina appeared distorted and sometimes frayed and interrupted. Von Kossa stained sections did never reveal, even in animals sacrificed 72 hours after treatment, calcium deposits in the aortic tissue.

Lesions could be identified much earlier with 1 μ Epon-embedded sections than with the standard paraffin sections. Within 12 hours after the end of adrenaline administration lesions of the internal elastic lamina and of isolated smooth muscle cells were found. Such cells showed a sequence of alterations which were only partially identified in semithin sections, but electron microscopy showed these and other changes in greater detail.

B. Electron Microscopic Observations

With the electron microscope alterations in the various components of the aorta were observed and will be considered separately. The changes reported below

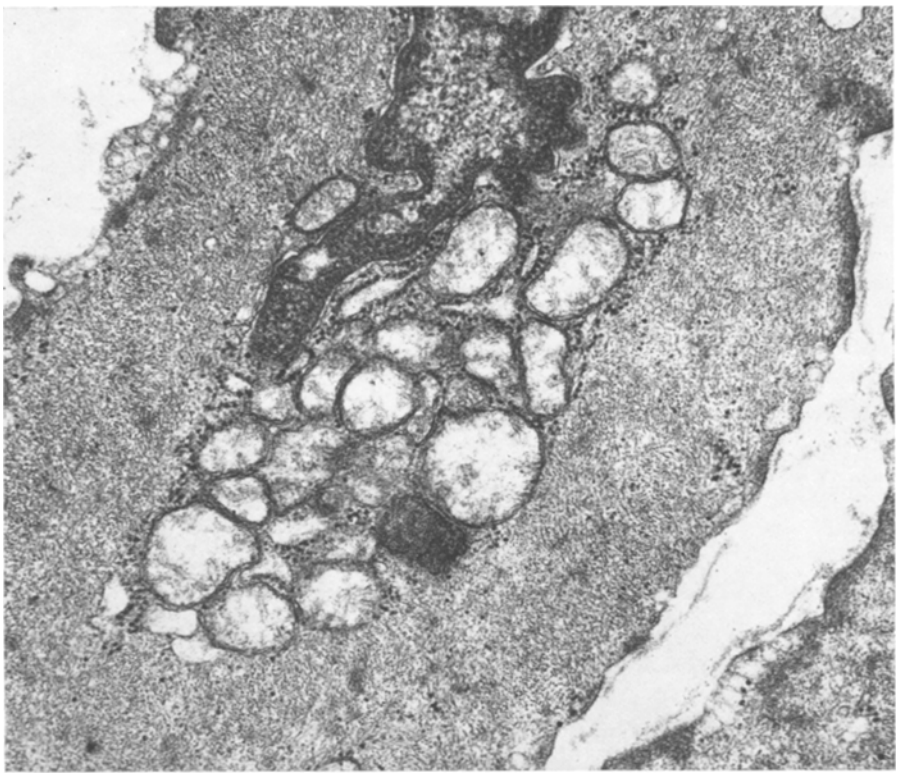

Fig. 1. Portion of a smooth muscle cell from the aortic media of a rabbit killed 6 hours after the administration of adrenaline. Mitochondria appear swollen, with less dense matrix and some disorganization of the cristae. × 28000

were well marked only in the animals receiving 500 μg/kg body weight of adrenaline; actually, it was exceptional to find changes after 50 μg/kg of the drug.

Mitochondria. In the smooth muscle cells of the aortic media mitochondrial alterations were the most prominent and earliest lesions seen and consisted of various kinds of changes of their fine internal structure. In the aorta of the normal, untreated rabbit the smooth muscle cell mitochondria may be spherical but are usually cigar-shaped structures about 0.2 to 0.25 μ in diameter and 0.5 to 0.8 μ long. As in other tissues, they are bound by a double membrane and contain prominent cristae. These cristae sometimes, but not always, run perpendicularly to the long axis. Mitochondria are most abundant near the ends of the nucleus where they often extend out in long chains. Only occasionally dense bodies are seen in the rather dense matrix of some of them.

In the animals killed 1 hour and 6 hours after adrenaline, enlargement and decreased matrix density of mitochondria were common findings. Swollen, rounded and partially vacuolated mitochondria appeared to be mainly concentrated in a paranuclear position. The appearance of one or more electron lucent areas in the matrix resulted in a distortion of the cristae. Swelling, disruption of cristae and loss of matrical density were the earliest changes observed (Fig. 1).

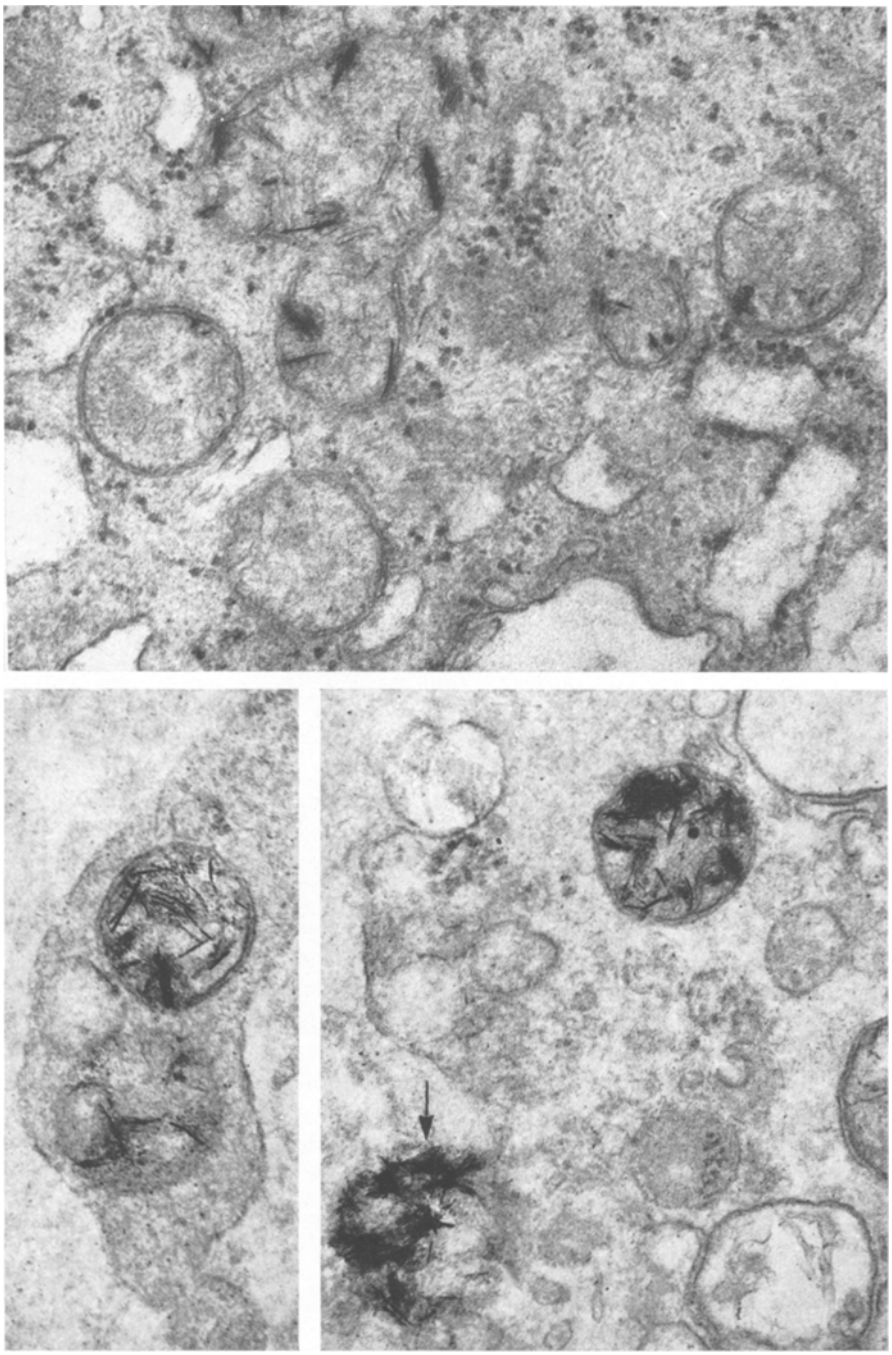

Fig. 2. Needle-shaped microcrystalline inclusions in the swollen mitochondrial matrix of a smooth muscle cell. Aortic media of a rabbit killed 12 hours after the administration of adrenaline. Arrow indicates a disrupted mitochondrion. × 55000

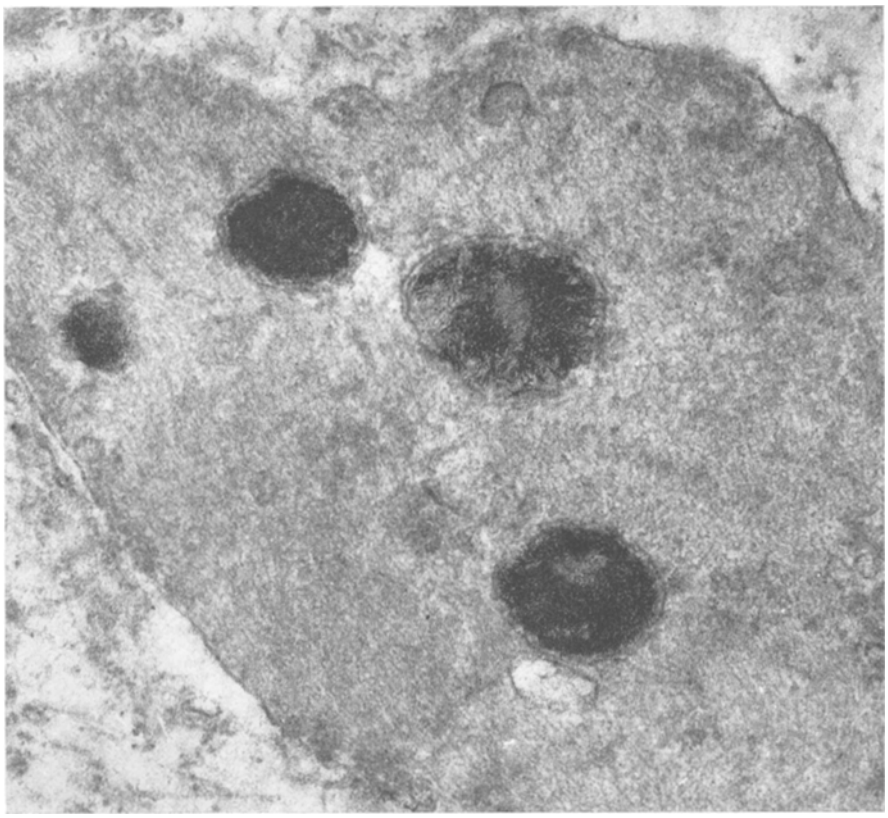

Fig. 3. Part of smooth muscle cell from the aortic media of a rabbit sacrified 12 hours after the administration of adrenaline. Clumps of microcrystals are scattered in the mitochondrial matrix. × 66000

In the animals killed 12 hours and later after treatment more striking alterations were observed. Many mitochondria in some smooth muscle cells contained various numbers of electron dense inclusions. These densities were of two forms and differed sharply from the dense bodies only exceptionally seen in the mitochondria of the normal aorta. One form of inclusions consisted of one or more elongated, needle-shaped microcrystals in part in close proximity to the outer leaflet of the cristae and in part randomly scattered in the mitochondrial matrix (Figs. 2 and 3). The other form of inclusions, less frequently seen, was represented by electron-dense material between the cristae concentrated in prickles and rods up to roundish granules exhibiting a central lucent core (Fig. 4). In high-power micrographs these granular inclusions did not appear as amorphous densities; instead, they appeared to be composed by clumps of microcrystalline needles.

The microcrystalline and granular intramitochondrial inclusions were denser than the mitochondrial membranes and were present in both stained or unstained sections of tissue which had been fixed in glutaraldehyde with or without postosmication. In postosmicated sections, treatment with H_2O_2 2 vol. did not modify

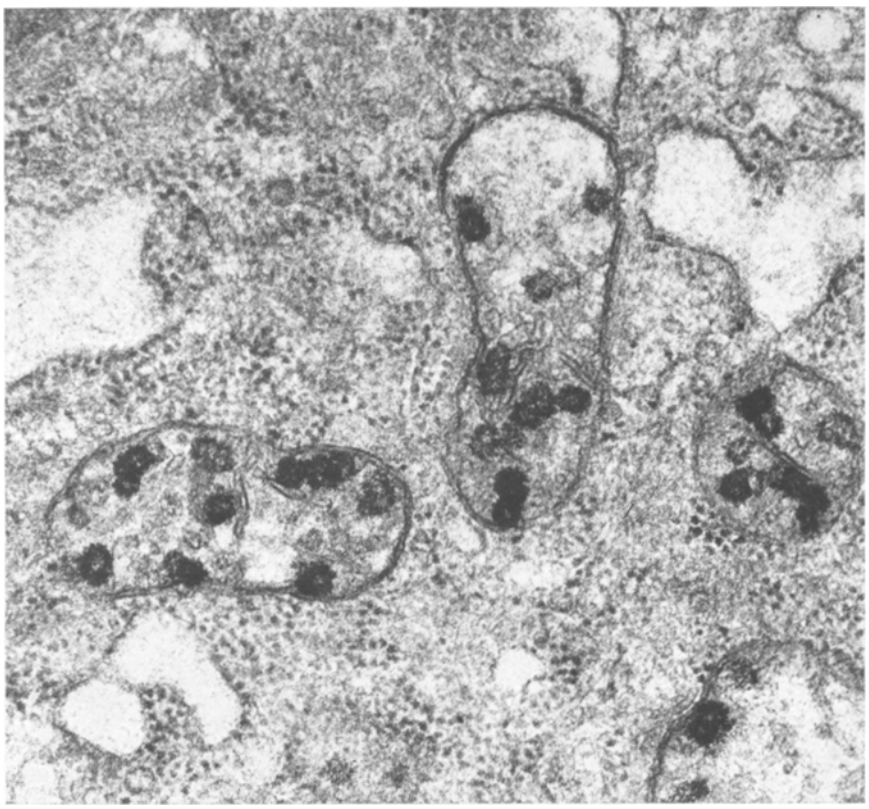

Fig. 4. Intramitochondrial granules in a smooth muscle cell from an animal killed 72 hours after the administration of adrenaline. At higher magnification the granules appear as clusters or aggregates of needle-shaped crystals. × 65000

their electron density. Conversely, a short-term treatment of sections with formic acid 1% was followed by a complete disappearance of the dense inclusions leaving at their place an electron lucent material (Fig. 5).

Selected area electron diffractograms gave results which varied with the amount of microcrystals. Where small intramitochondrial clusters were present, there were no diffraction patterns. Instead, where large numbers of microcrystals were clumped together, an unmistakable pattern of cristallinity was obtained (Fig. 6). In particular in such areas eleven diffraction rings were observed with a mean value for crystal spacings (d_{hkl}) of 3.61, 3.44, 2.81, 2.30, 1.97, 1.88, 1.77, 1.49, 1.27, 1.20 and 1.14. The diffraction ring showing the more striking contrast was that with the d value of 2.81.

Many smooth muscle cells containing intramitochondrial inclusions showed moderate dilatation of the channels and vesicles of the endoplasmic reticulum and slight changes of the myofibrillar component. Glycogen A granules were as normally present. In the same cell only some mitochondria appeared to be calcified;

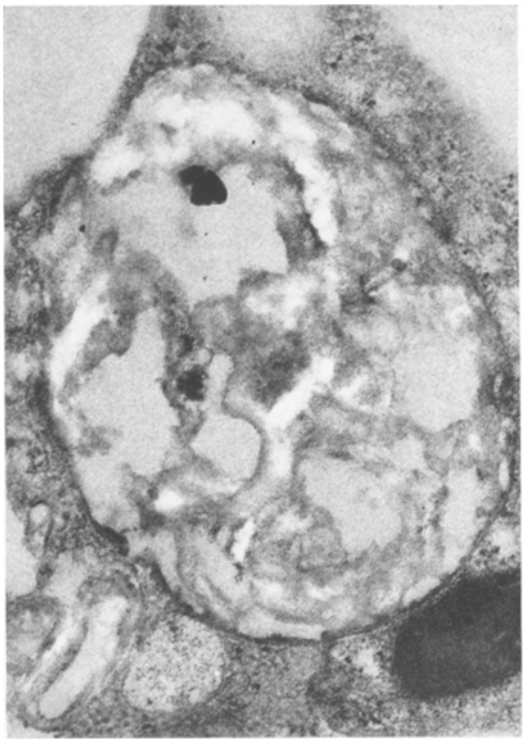

Fig. 5. This section is from the same rabbit as in Fig. 3. After formic acid treatment the intramitochondrial dense inclusions partly disappeared leaving at their place an electron lucent material. ×50000

other mitochondria contained either no crystals or very few. The electron-dense material eventually filled the entire mitochondrion, leading to a disruption of its membranous sac. Altered mitochondria matted together formed large osmiophilic masses of calcium. Neither release of dense mitochondria through rupture of muscle cells, nor phagocytose by macrophages was ever observed. In no instance extrusion of mitochondrial inclusions in the surrounding intercellular ground substance was seen.

Intercellular Components. In all adrenaline-treated animals the spaces between cells and fibers were constantly widened and filled with an electron lucent material. This material was particularly abundant in the subendothelial space and in the innermost layers of the media. In the rarefied areas the regular arrangement of smooth muscle cells and elastica was lost, elastic fibers appeared fragmented and collagen fibers tended to be coarsely bundled. The material filling the interstitial spaces appeared to be partly structureless and of a very low electron density, and partly arranged in tubular patterns of a moderate electron opacity as shown by Fig. 7.

Additional Findings. Besides the above reported mitochondrial and interstitial changes, the medial smooth muscle cells of the aorta showed various kinds of alterations, mainly regressive in nature, which appeared to be present only in

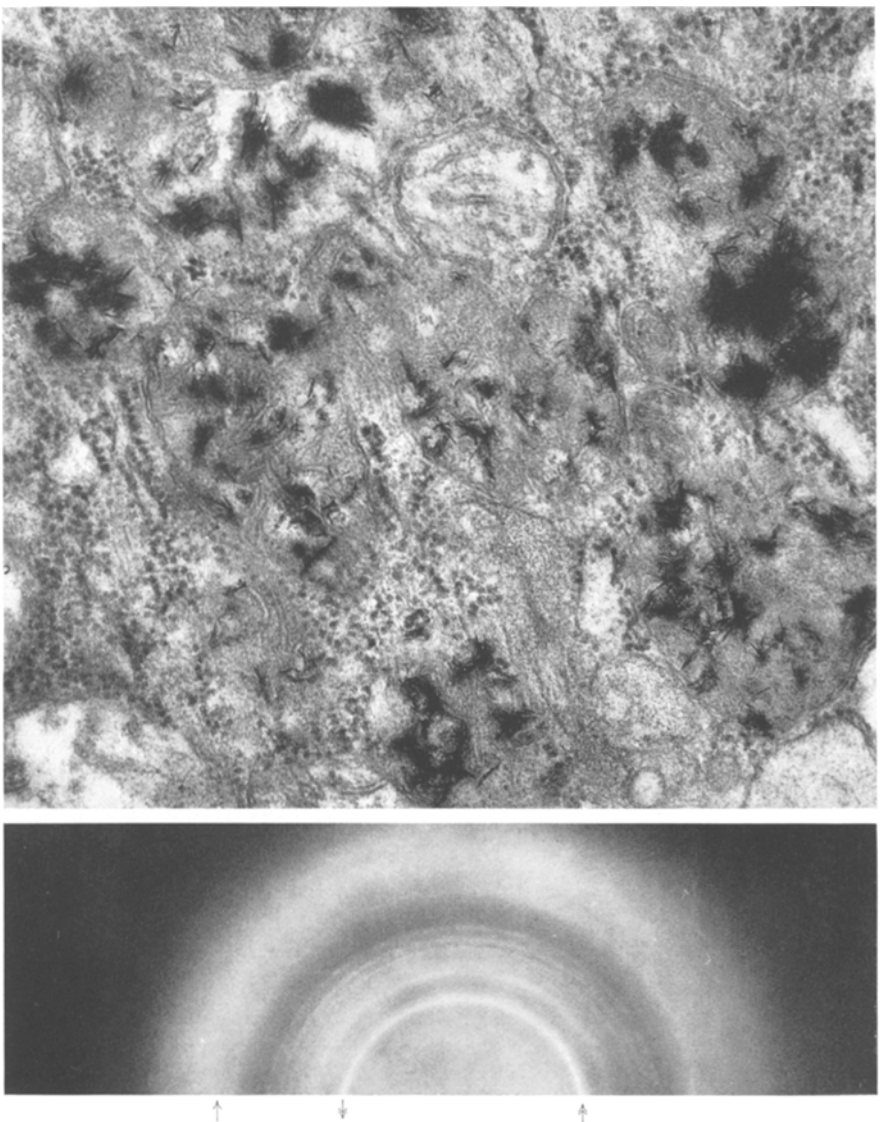

Fig. 6. Electron diffractogram obtained from the calcified area illustrated. Crystalline masses produced by adrenaline give an electron diffraction pattern consistent with apatite patterns obtained by x-ray diffraction of bone. Arrows indicate the most significant ring (see the text). Selected area × 65000

some animals. These include 1) a decrease of cytoplasmic matrix and of myofilaments with their attachment devices, 2) autophagic vacuoles containing cell organelles undergoing various degrees of degenerative breakdown (Fig. 8), 4) ghost bodies (Fig. 9), 5) modified amounts of glycogen, 6) disorganization of endoplasmic reticulum and disruption of sarcolemma. It should be noted that no

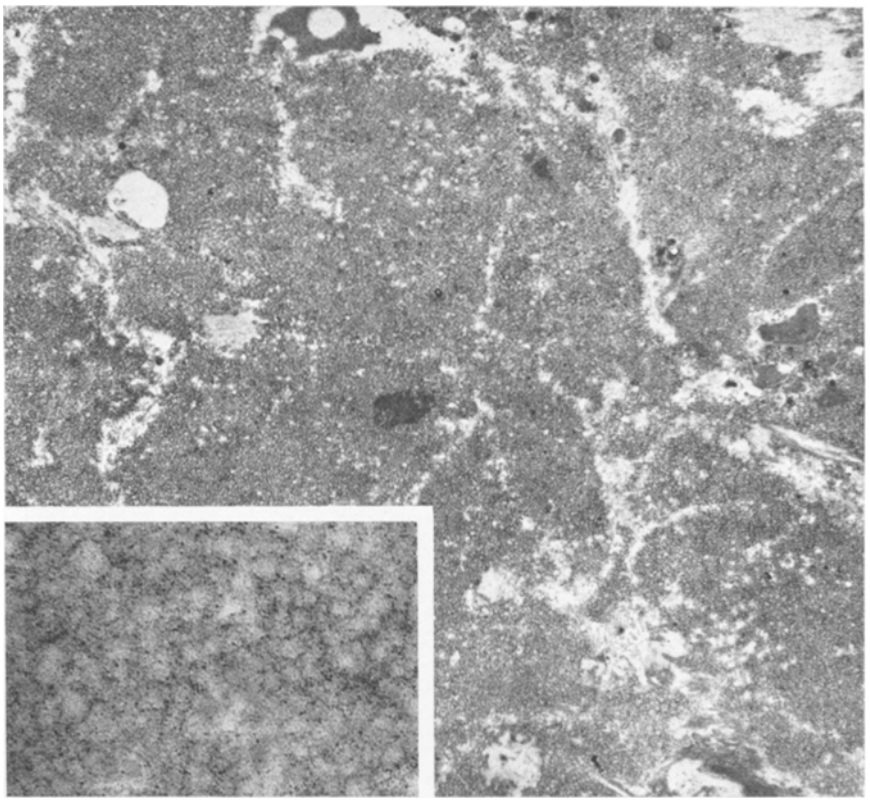

Fig. 7. Portion of the aortic media of a rabbit killed 24 hours after the injection of adrenaline. Increased amounts of intercellular ground substance associated with regressive changes of the smooth muscle cells. × 8000. In the inset it appears that the increased intercellular substance shows in part a tubular pattern. ×50000

clear-cut relationship was ascertained between the mitochondrial calcifications and the alterations reported above. In particular, calcified mitochondria or their remnants were never observed in autophagic vacuoles or in ghost bodies.

Blood Electrolytes. The results of the blood electrolytes determinations are reported in the Table 1.

Table 1

	Controls	Adrenaline			
		before treatment	after 24 hours	before treatment	after 72 hours
Ca^{++} mg%	14.2	12.7	14.0	14.2	14.6
Po_3^{--} mg%	5.9	6.9	5.6	5.8	6.2
Na^{+} mEq/l	170	73	130	127	135
K^{+} mEq/l	5.5	3.1	4.0	4.1	4.2

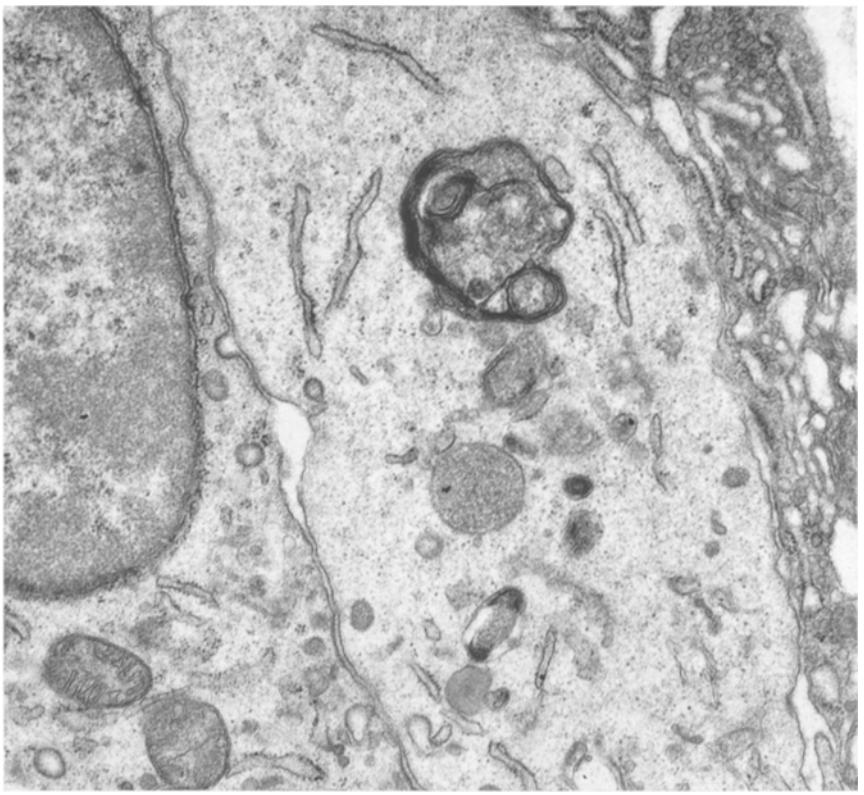

Fig. 8. Autophagic vacuoles in a degenerating smooth muscle cell. ×24000

From the Table 1 it appears that adrenaline administration at 24 and 72 hours do not affect significantly the blood levels of potassium, sodium, calcium and inorganic phosphate.

Discussion

Light microscopy studies carried out by several investigators have shown that a prominent feature of the adrenaline-induced arteriopathy of the rabbit is the appearance of calcium deposits in the tunica media of the large elastic arteries (Josué, 1903; Erb, 1905; Oester, 1959; Bertelsen, 1964; Cavallero *et al.*, 1972). Currently one believes that calcification is a late outcome of cell necrosis and death. This concept describes a passive role to the precalcified tissue. Conversely, in the present study it was shown that after a massive single dose of adrenaline, before the appearance of overt degenerative and necrotic changes, early intramitochondrial electron dense bodies occur in the smooth muscle cells of the aortic media; in selected area electron diffractograms it was ascertained that the electron opaque material shows crystalline apatite-like features.

From the results reported above, the sequence of acute events which occur in the aortic wall of rabbits after intravenous administration of a massive dose of

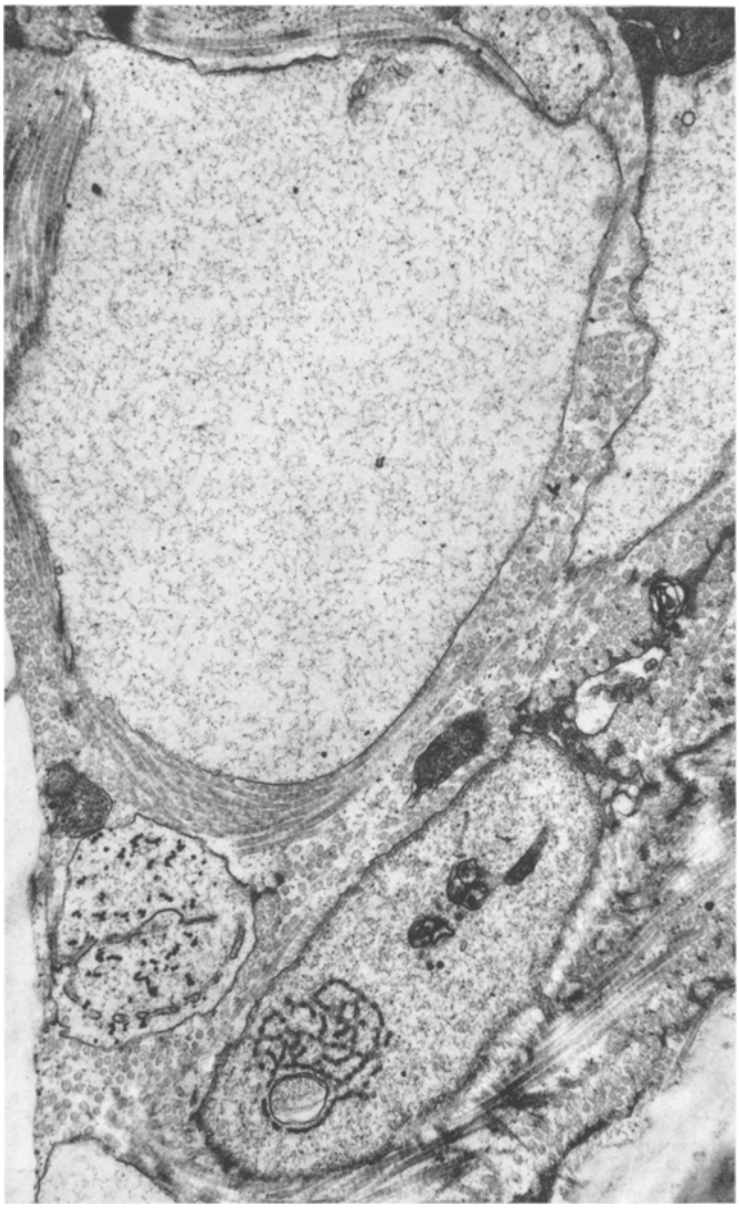

Fig. 9. "Ghost bodies"; some of them contain degenerating organelles. ×18000

adrenaline can be summarized as follows. In electron micrographs the earliest change seems to be an intercellular and interfibrillar accumulation of fluid in the subendothelial space and in the innermost layers of the media, shortly followed by important changes of the mitochondria of the medial smooth muscle cells. The fluid accumulating in the subendothelial space is represented in part by an

electron-lucent amorphous, edematous material and in part by a material of moderate electron density arranged in tubular structures, probably mucopolysaccharide in nature. However, the main changes involve the mitochondria; these consist of a loosening of the matrix, disruption of the cristae, widening of the intercristal spaces and deposition of electron-dense bodies, partly granular in structure and partly microcrystalline. Later, degenerative changes of the smooth muscle cells and electron dense opacities even in the interstitial spaces may be observed.

Several studies indicate that mitochondria of various organs have the ability to accumulate *in vitro* divalent cations under a variety of circumstances. Studies of metal distribution in isolated cell fractions have generally implicated mitochondria as one site of divalent ion accumulation. Isolated mitochondria are known to contain relatively large amounts of calcium and magnesium and to be able to accumulate these ions, strontium, barium and manganese (Brierly *et al.*, 1962; Varington and Murphy, 1962; Peachey, 1964; Greenawalt *et al.*, 1964; Lehninger, 1970). Fine structural studies on dog myocardium during autolysis have shown early mitochondrial changes consisting of enlargement and decreased matrix density together with the presence of large dense granules, considered to be calcium or divalent cations (Herdson *et al.*, 1969) and similar findings have been previously reported for the liver subjected to autolysis (Trump *et al.*, 1965). Strontium accumulation by sarcoplasmic reticulum and mitochondria in vascular smooth muscle incubated *in vitro* prior to fixation was observed by Somlyo and Somlyo (1971).

Intramitochondrial electron dense inclusions of various kinds, believed to be calcium deposits, have been reported to occur *in vivo* in several experimental conditions including intoxications, dietary deficiencies, ischemic injury and hypercalcemic states. In the liver mitochondria of rats poisoned with CCl_4, Reynolds (1963) observed electron-dense masses resembling deposits of mycrocrystals and suggests they were a form of apatite, the phosphate being derived from the degradation of mitochondrial organophosphates. Accordingly, the unusually high calcium content of the livers of rats poisoned with CCl_4 has been shown to be due to an increase of mitochondrial calcium. Intramitochondrial electron-dense inclusions, probably calcium salts, were noted in the myocardium of rats poisoned with Plasmocid (D'Agostino, 1963) and after combined administration of 9α-fluorocortisol and monobasic sodium phosphate (D'Agostino, 1964). Similar changes were found after administration of low doses of isoproterenol (Bloom and Cancilla, 1969). In this connection, it is of interest that isoproterenol-induced myocardial necrosis is not mediated exclusively by flooding of heart muscle with plasma-derived calcium; actually propranolol, at doses which completely suppress the increase in myocardial calcium after isoproterenol, does not completely prevent necrosis. Thus, at least partly, the intramitochondrial calcium appears to be due to release of calcium sequestered in intramyocardial compartments (Bloom and Davis, 1972). Intramitochondrial dense bodies, considered to be calcium, were observed in the myocardium of magnesium-deficient (Heggtveit *et al.*, 1964), as well as in potassium-deficient rats (Molnar *et al.*, 1962; Maurat *et al.*, 1965).

In dog myocardium irreversibly damaged by ischemia lasting 30 minutes or longer, it was found that the myocardial cells store intramitochondrial granules which became increasingly prominent with more prolonged ischemia (Jennings

et al., 1965; Herdson *et al.*, 1969). Studies on electrolytes of the damaged myocardial mitochondria revealed that magnesium, potassium and nitrogen were decreased, while the calcium and phosphate content was increased (Jennings *et al.*, 1970). Further microincineration analysis of thin section studied by electron microscopy has shown that the calcium ions are localized within granular intramitochondrial dense bodies (Shen and Jennings, 1972). Ghidoni *et al.* (1969) in bovine hearts that fibrillated for prolonged intervals, besides a massive subendocardial damage, have observed in the myocardial cells mitochondrial filled with electron-dense microcrystals which resembled calcium-apatite crystals.

In two patients showing intracellular myocardial mineralization after vascular surgery it was found that the principal ultrastructural site for the deposition of calcium salts was within mitochondria; the crystalline appearance of the intramitochondrial deposits suggested the presence of hydroxyapatite (D'Agostino and Chiga, 1970). Ultrastructural studies of the myocardium of a hypoxic infant indicated that there was extensive calcification of mitochondria as characterized by intramitochondrial deposits of needle-shaped dense crystals resembling those of hydroxyapatite (Lin, 1972).

Mitochondrial calcifications of kidney tubular cells have been induced experimentally in the rat by parathormone or vitamin D overdosage (Caulfield and Schrag, 1964; Scarpelli, 1965). The parathormone-induced masses of needle-shaped crystals in the mitochondria of the kidney tubular cells showed the diffraction pattern of apatite. Vitamin D overdosage induces intramitochondrial calcifications of the myocardial cells in form of needle-shaped crystals and roundish aggregates of finely granular inorganic substance; electron diffractograms showed that the former were apatite-like crystals while the latter gave no indication of crystallinity (Bonucci and Sadun, 1973). After vitamin D, small bowel epithelia show mitochondria containing calcium deposits which, in contrast, appear as amorphous, structureless electron-dense granules (Sampson *et al.*, 1970). Finally, it is of interest that intramitochondrial electron dense granules were observed in neoplastic cells of a malignant melanoma of the human uveal tract (Egeberg and Jensen, 1970) and that intramitochondrial needle-shaped microcrystals, associated with calcium deposits in shadow cells and in the interstitial spaces, were observed by us in the basophil tumoral cells in one case of Malherbe's calcifying epithelioma (unpublished data).

As regards the mitochondrial calcifications in the arterial smooth muscle observed by us, questions naturally arise as to 1) the mechanism(s) involved in mitochondrial calcification after adrenaline, 2) the form in which the metal is retained and 3) the pathological significance of such a change. As to the point 1 it has been suggested that the isoproterenol-induced mitochondrial calcifications of heart muscle might be related to the uncoupling effect of this compound on mitochondrial oxidative phosphorilation. It is very likely that this mechanism is also operating in the adrenaline-induced calcifications. Owing to a lack of transformation of ADP to ATP, phosphate groupings might be available to the mitochondrion for binding intramitochondrial calcium ion in inorganic precipitable compounds. In keeping with this suggestion it has been proposed that myocardial necroses occurring shortly after isoproterenol administration might be caused by excessive levels of calcium within the heart muscle cells.

This could occur through a reduction in cellular ATP levels brought about both by calcium activation of actomyosin ATP-ase (Katz, 1970) and by uncoupling of oxidative phosphorilation (Carofoli and Lehninger, 1971). The uncoupling of oxidative phosphorylation occurs as calcium is taken up by mitochondria and deposited within them in form of dense granules (Greenawalt *et al.*, 1964). Cardiac necrosis and calcification in dietary magnesium deficiency has been also related to an uncoupling of oxidative phosphorylation (Heggtveit *et al.*, 1964).

Another possible explanation of our findings might be related to changes of blood potassium induced by adrenaline. Actually in *in vitro* experiments it was found that potassium ion may influence calcium movements in rabbit aortic smooth muscle (Goodman *et al.*, 1972). However, from the data reported above it appears that short-term adrenaline treatment does not cause consistent changes of blood potassium as well as of the blood levels of calcium, inorganic phosphate and sodium.

To date we cannot say whether catecholamines are capable to interfere with the activity of the blood level of the specific biological inhibitors which, according to Howard *et al.* (1967), may be implicated in calcium deposits.

In isoproterenol experiments it was shown that mitochondria of the heart muscle cells are flooded by calcium *within minutes* after the administration of a necrogenic dose of the drug and consequently it was postulated that this compound exerts an effect on the proportional distribution of calcium among the subcellular organelles (Bloom and Davis, 1972). Instead in our experiments, as shown before, intramitochondrial calcium deposits become evident later, *within hours* after intravenous adrenaline. This discrepancy may be explained on the basis of a lower metabolic activity of the mitochondria of smooth muscle cells inducing lower movements of intracellular calcium ions or on the basis of a slower uncoupling effect of adrenaline on the oxidative phosphorylation of the smooth muscle cells. Whether the calcium deposits represent accumulations of substances already present in the arterial tissue or whether they develop from substances brought to the medial smooth muscle by the circulating blood, is not established by this experiment. However, it is of interest that intramitochondrial electron-dense granules, considered to be calcium, were observed in mouse liver and in dog myocardium subjected to autolysis (Trump *et al.*, 1965; Herdson *et al.*, 1969); obviously these findings clearly indicate that, with autolysing tissues, the former hypothesis is true. Accordingly no evident increases of plasma calcium and inorganic phosphate were found in our electrolyte determinations.

It is of interest that in our experiment only individual smooth muscle cells showed calcified mitochondria and that not all the mitochondria in these cells did load with calcium. Similar findings have been reported by others working on mitochondrial calcifications in different kinds of experiments.

These findings raise the important question whether, as suggested by Matthews (1973) there are enzymic differences between the mitochondria that load calcium and those that do not.

As regards point 2, our electron diffraction studies clearly indicate that calcium is retained within the mitochondria as apatite-like crystals. Similar results have been reported by others in their observations on other experimental models (Caulfield and Schrag, 1964; Bonucci and Sadun, 1973). We cannot say

why usually mitochondrial inclusions occur as scattered microcrystals, while in less numerous cases the microcrystals appear aggregated in granules. We can only suggest that this might be due to a different composition of the binding sites for calcium. Actually it seems likely that calcium is deposited upon an organic component, but to date it is not established whether it is protein, phospholipid or an acid polysaccharide.

The precise pathological significance of the mitochondrial calcification of arterial smooth muscle must await further study, for which, it is hoped, the work presented here can provide a baseline. In this connection it is of interest that normal aorta is capable to accumulate calcium and phosphate when incubated in serum of the same species (Schiffmann and Martin, 1962; Martin *et al.*, 1963) and that hydroxyapatite has been shown to be a component of aortic atherosclerotic plaques (Carlström *et al.*, 1953). As to the adrenaline arteriopathy, smooth muscle necrosis and calcification of the arterial wall are the main distinctive features and are generally believed to be closely correlated. However, from our results, it is not clear whether such a direct relationship exists between smooth muscle calcium uptake and necrosis; instead, it seems that there are imperfections in this relationship. Thus, it seems very likely that factors other than mitochondrial calcification could be etiologically related to smooth muscle necrosis; electron microscopic data reported above indicate that calcium might play only an indirect role in this process. Similarly, no definite conclusions can be drawn from this report as to whether intramitochondrial calcifications are the precursors of the later appearing, more extensive calcifications of the elastic lamellae; for elucidating this point further work is needed.

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