PATHOGENESIS OF FATTY AND SCLEROTIC LESIONS IN THE CARDIOVASCULAR SYSTEM OF CHOLINE-DEFICIENT RATS.*

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GROSSLY sclerotic aortic lesions and abnormal microscopic deposits of fat in coronary arteries have been observed in our laboratories in adult rats fed a low-choline diet for periods ranging from 5 to 7 months (Hartroft, Ridout, Sellers and Best, 1952). Subsequently we reported the rapid (2–7 weeks) development of fatty and necrotic lesions in cardiac muscle of young rats fed low-choline diets containing up to 40 per cent lard or beef fat (Wilgram and Hartroft, 1953; Wilgram, Hartroft and Best, 1954*a*). This observation confirmed and extended earlier findings of a somewhat related nature by Kesten, Salcedo and Stetten (1945), who found interstitial myocarditis in young rats fed choline-deficient diets containing 30 to 40 per cent ethyl laurate. Recently we have also reported (Wilgram, Hartroft and Best, 1954*b*) that young rats fed diets low in choline and high in naturally occurring fats, may develop within periods as brief as 17 days visible evidence of aortic sclerosis and microscopically demonstrable deposits of stainable fat in coronary arteries. The incidence of lesions in some of the latter experiments was as high as 90 per cent in the deficient animals. Controls given comparable amounts of the basal diet§ supplemented with choline chloride (0·85 per cent) failed to develop vascular abnormalities in any single instance. The incidence and severity of the vascular and cardiac lesion have been reported in these publications (*loc. cit.*). The purpose of the present paper is to describe and illustrate the pathogeny and pathology of the lesions and to report some recent experimental findings.

METHODS.

Details concerning preparation of the various diets, methods of housing and sacrificing the animals, and other information about the experimental procedures involved, have already been published in previous papers by the authors (Hartroft *et al.*, 1952; Wilgram *et al.*, 1954*a*, *b*).

Histochemical tests were performed on selected tissues to demonstrate cholesterol (Schultz, 1924), phospholipid (Baker, 1944), mucopolysaccharides (McManus, 1946), fatty acids (Fischler, 1904), ceroid (Wilson, 1950), haemosiderin (Perls, 1866) and calcium salts (von Kossa, 1901). The vascular systems of choline-deficient rats were injected with colloidal

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§ The percentage composition of the basal diet fed to young male rats (85–120 g.) was as follows: alcohol-extracted peanut meal 30, washed α-protein 5 (Glidden Co., Chicago, Ill.), casein 5, lard 35, P.D.W. vitamins I, L.P. salts 3, cod liver oil concentrate 0·015, α-tocopherol acetate 0·01, sucrose 21.

suspension of carbon particles* to aid in the visualisation and study of the coronary vasculature. The method employed has been previously described by one of us (Hartroft, 1941) and utilises the preparation of thick cleared slices (200–500μ) as well as thin paraffin sections.

RESULTS.

Young rats fed severely hypolipotropic diets developed acute lesions in the heart and vessels within relatively short periods (sometimes less than 3 weeks), whereas older animals on less severely choline-deficient regimens did not show significant abnormalities in less than 3–4 months. The latter lesions were of a chronic nature and will therefore be described under a separate heading.

Acute lesions of heart and vessels.

Gross evidence of aortic sclerosis was not observed in any of the choline-deficient animals under a period of 17 days. Microscopic deposits of stainable fat, however, have been discovered in vessels (either aortas or coronary arteries) as early as 8 days after the initiation of the experimental regimen.

Fig. 1 illustrates initial alterations in a coronary artery of a young choline-deficient rat which developed the acute form of vascular lesion. Intimal endothelial cells are first affected. The cells become swollen and a diffuse sudanophilia affects the entire cytoplasm. Later, discrete droplets barely capable of resolution under the oil immersion objectives of the light microscope make their appearance. Still later (Fig. 2 and 3) stainable fat droplets are demonstrable in every layer of the arterial wall, including even the adventitia. Young rats fed severely hypolipotropic diets frequently died of either cardiac or renal cortical necrosis before the lesions in coronary arteries or aortas had progressed beyond the stage just described.

Frequently the fat may be more prominent in one layer than in others, and it appears that the distribution is governed largely not only by the amount of fat involved but also by the stage at which the lesion is observed. In the early phases of the disease the fat is in the inner portion of the vessel wall. Sometimes increased basophilia in the arterial wall has been noted (suggesting early and abnormal deposition of calcium salts), but in the acute coronary lesions we have not observed frank hyperplastic changes.

Abundant deposits of stainable fat in cardiac muscle cells (Fig. 4) can be demonstrated in a high percentage of the choline-deficient animals, but only rarely, and then in small amounts, in the controls. Fatty muscle cells subsequently became swollen, vacuolated and necrotic. Studies of the cardiac vasculature with the aid of ink injections showed (Fig. 5) that the muscle degeneration and necrosis were not ischaemic in origin, because capillaries in the regions of the necrosis were still patent. With necrosis and lysis of affected muscle cells, their fat droplets escape into the interstitium where they are engulfed by macrophages. In late stages of the lesion, areas of necrosis contain little fat. In the early stages of the inflammatory response to necrosis, polymorphonuclear leukocytes are prominent. At a later stage small round cells become more abundant, and with onset of healing fibroblasts appear and sarcolemmic cells proliferate at the periphery of the lesions.

* Higgin's India ink diluted with two parts distilled water and a few drops of ammonia added to ensure suspension and the whole filtered before use.

Frequently the distribution of muscle degeneration appeared to follow the localisation of the coronary arterial lesions, but this association was not found to be the case always.

In the aorta, intimal endothelial cells become sudanophilic in the initial stages of the lesion. Later there is degeneration and necrosis of the underlying media, associated with fatty change and eventual deposition of calcium salts (Fig. 6 and 7). Inflammatory reaction in the necrotic regions of the media is minimal or absent. The proliferative response in the intima is achieved by endothelial cells. Macrophages and foam cells were found only in very rare instances. In the late stages of the lesion (see below) intimal fibrosis may develop.

Chronic cardiovascular lesions.

Chronic changes were not observed in mature rats (180–200 g.) under a period of five months. The gross appearance of a typical lesion in an aorta is shown in Fig. 8. In the heart, minute flecks representing fibrosis could sometimes be observed with the aid of a hand-lens.

The coronary arteries showed eccentric intimal thickening in the form of small plaques which sometimes encroached on the lumen (Fig. 9). In these vessels, sites of lipid deposition were restricted to intima and media. In some instances the amount of lipid was less than that observed in the acute stages described above, suggesting that although the abnormal fat may have initiated the lesions, it had not remained *in situ* over the entire experimental period.

Fatty deposits in the myocardium in chronically choline-deficient rats were rarely encountered. Focal areas of myocardial fibrosis had developed, however, in a large number of the animals. The amount of collagen varied, and sometimes stained only palely with connective tissue stains. In many instances proliferated sarcolemmal cells were a more prominent feature than fibrosis *per se*. No constant pattern of distribution for the fibrotic changes could be discerned.

The microscopic features of the aortas in the rats subjected to chronic choline deficiency were not essentially different from those of the late stages of the acute changes described above. In many instances fractures of the medial bars of calcification were encountered, sometimes in various stages of callus formation and healing. In the most advanced cases, the entire thoracic aorta was converted into a rigid dilated pipe.

Histochemistry of hearts and aortas.

The lipid deposits were subjected to the series of histochemical tests recommended by Lison (1953) for the identification of deposits of sudanophilic fat in sections. In the acute lesions the lipid was positive to tests for neutral fat and fatty acids, and negative to tests for phospholipid, cholesterol and cholesterol esters. Deposits of calcium salts were present in acute lesions of both hearts and arteries, including aortas. Tests for ceroid were uniformly negative.

PAS (McManus) stains on hearts and aortas did not yield any significant increase of mucopolysaccharides in the necrotic areas of the cardiac muscle, but in aorta and coronary arteries, where the choline-deficient regimen led to hyperplasia of the inner layer of these vessels, mucopolysaccharides showed up intercellularly in the form of diffuse plaques as well as subendothelial nodules (Fig. 9 and 10).

Iron.—Of interest was a positive haemosiderin reaction throughout the entire

wall of some of the coronary arteries and within the inner layer of the aorta in acute and chronic choline-deficient experiments (Fig. 11 and 12). The Prussian blue reaction appeared rather diffusely throughout and no red blood cells or débris from their breakdown were to be found in the tissues specified above.

Frequently iron pigment also appeared in granular form within the cyto-architecture of many intact cardiac muscle fibres. Similar pictures have been seen in heart muscle and coronary vessels in haemochromatosis (Granick, 1954), although the mechanism of iron deposition in this latter disease is likely to be different from that operating in our experiments. There is no grossly visible haemolysis in our choline-deficient animals, and whether a bone-marrow depressed by the impaired kidney in choline deficiency cannot utilise the amount of iron offered, or whether the fatty choline-deficient liver cannot store iron in sufficient amounts and the surplus is partly deposited in cardiac muscle and coronary arteries, awaits further investigation. Iron balance studies might show if changes of the " intestinal mucosal block " are responsible for an increased iron absorption with subsequent deposition of iron in the cardiovascular system. Careful laboratory investigation was able to rule out artefacts in staining technique (calcium, formol haematin).

It is interesting that Salmon and Copeland (1954) described an increase in iron-positive pigment in the liver and kidney of choline-deficient chickens.

Lesions in organs other than hearts and aortas.

The pathology of the hepatic, renal, testicular and ocular lesions in choline-deficient rats has been described elsewhere (Hartroft, 1948, 1950 ; Hartroft and Burns, 1949 ; Hartroft and Ridout, 1951). The young animals almost consistently showed the classical gross and microscopic lesions of the haemorrhagic renal syndrome first described by Griffith and Wade (1939). The possible relation between the acute cardiovascular damage and these changes in other organs, especially those in the kidneys, will be discussed.

Experimental Variations.

Hormones.—Female rats are very resistant to the induction of cardiovasculorenal lesions in choline deficiency. However, by the simultaneous administration of androgens (5 mg./150 g. body wt.) and growth hormone (0·1 mg./150 g.) it is possible to produce renal and cardiovascular damage as described above in 80 per cent of females.

Cholesterol.—Firstbrook (1950), Moses and Longabaugh (1950) and Duff and Meissner (1951) have all demonstrated that the addition of choline to *normal* diets of rabbits given high doses of cholesterol has no inhibitory effect on the development of atheromatous changes. A surplus of choline has therefore no beneficial effect on this type of vascular degeneration. It should be noted that the amounts of cholesterol employed in the experiments of these authors were large and the basal diets already contained abundant lipotropic factors.

Our choline-deficient diets which were routinely employed for the production of cardiovascular disease contained no added cholesterol and the constituents in the diet were relatively free of this lipid. Cholesterol serum levels in choline-deficient rats were lower than in the choline-supplemented controls (Ridout,

Patterson, Lucas and Best, 1954). However, cholesterol supplements given to choline-deficient rats, which were otherwise resistant to the induction of cardiovasculorenal lesions (female rats, older males) produced the changes in 75 per cent of the animals. Cholesterol given to choline-supplemented rats had no ill effect whatsoever, although the cholesterol serum levels were again higher than in the cholesterol-supplemented choline-deficient group. All animals were pair-fed. Thus it appears that dietary cholesterol aggravates the effects of choline deficiency on the cardiovasculorenal system of choline-deficient rats, although it is by no means essential for the production of these lesions. This aggravating effect of cholesterol on the lesions (liver, kidney, heart and vessels) is not accompanied by an elevation of serum cholesterol.

DISCUSSION.

Pathogenesis.—Observations indicate that cardiovascular degeneration in choline-deficient rats is initiated by the appearance of abnormal deposits of stainable fat at the sites of the lesions wherever they may be. In heart muscle they are followed by swelling of affected myocardial cells with their eventual necrosis, lysis and replacement by fibrosis. In coronary arteries, fatty intimal cells may eventually undergo eccentric hyperplasia; the media may thicken concentrically and the supporting fibrous tissue in the adventitia may proliferate, particularly in mature animals with chronic lesions. In the aorta, intimal hyperplasia of fatty endothelial cells develops, accompanied by medial degeneration, necrosis and calcification. The mid-portion of the media is most frequently affected and we have previously presented evidence to suggest that interference with the nutrition of elasto-muscular tissue deep in the aortic wall may be one of the responsible factors (Hartroft *et al.*, 1952). The concept that abnormal deposits of fat in parenchymal cells may eventually lead to irreversible structural lesions has been proposed as a cause of the characteristic change in livers and kidneys of choline-deficient rats. In the liver, over-distension of hepatic cells by fat leads to cirrhosis (Hartroft, 1950), and in the kidney abnormal lipid precedes either bilateral cortical necrosis or atrophic regression of tubules (Hartroft, 1948). The evidence suggests that there may be but few sites in the body other than adipose tissue, wherein appreciable degrees of fat storage may occur for long without damaging the tissues or organs involved. We do not know why abnormal fatty deposits should be harmful to the cells in which they accumulate. In the cardiovascular lesions in choline-deficient rats, abnormal fat deposits certainly constitute the initial stage in their pathogenesis.

Aetiology.—Elsewhere we have discussed the evidence concerning the aetiology of the initial fatty deposits (Wilgram *et al.*, 1954*a*, *b*). Cardiac necrosis is probably independent of renal damage and is a direct effect of choline deficiency on heart muscle of young rats, because if the animals are fed diets containing ethyl laurate or trilaurine as the source of fat, massive cardiac necrosis may prove fatal before renal damage has developed. The relation between vascular and renal damage is not yet clear. Renal lesions produced by surgical methods in dogs (McCormick and Holman, 1949) or nephrotoxic agents administered to rats (Wissler, Collins, Schroeder and Soules, 1953; Lehr and Churg, 1952) are associated with vascular lesions not unlike those we have seen in choline-deficient rats. But the lipid component of the vascular lesions produced directly by renal damage is not as prominent as in those vessel changes we have encountered in choline deficiency.

Furthermore, Copeland (1954, personal communication) has observed vascular lesions in choline-deficient chickens and dogs identical with those in the rats, but unassociated with significant degrees of renal abnormalities. Artom (1954) has found that choline injected into choline-deficient rats a few hours before removing slices of their livers, kidneys and hearts enhances the rate of fatty acid oxidation in these tissues, compared with non-injected choline-deficient controls similarly observed. These observations of Copeland and of Artom taken together suggest that the cardiac and coronary damage in hypolipotropic rats may eventually prove to be a direct result of insufficient supplies of dietary choline and its precursors. The aortic lesions however, seem in part at least to depend on renal damage. The choline-deficient kidneys may be impaired in their capacity to excrete calcium and phosphorus and the retention of these minerals may conceivably lead to metastatic calcifications. We feel at present that this mechanism, known clinically as secondary hyperparathyroidism in chronic renal disease, together with choline deficiency *per se* as an equally important factor, may be responsible for the development of aortic lesions in choline-deficient rats. On the other hand, even if vascular lesions in our rats should prove to be entirely secondary to the renal damage induced by choline deficiency, the experiments may be of some interest in that the whole lesion-complex has been produced by brief periods of a *dietary deficiency alone*, rather than by the introduction of toxic agents or by surgical manipulations.

SUMMARY.

The pathology and pathogenesis of cardiovascular lesions in choline-deficient rats of various ages are described. Coronary arterial lipoidosis, grossly evident aortic sclerosis and myocardial necrosis have been observed in both acute and chronic forms. Choline supplements added to the basal diet prevented the development of comparable lesions in controls. Deposits of stainable fat initiated the induction of cardiovascular lesions by diets low in choline.

The sequence of changes in hearts and arteries is described. Lipid appeared within a few days in cardiac muscle cells that subsequently rapidly underwent swelling and necrosis. Intimal fat in coronary arteries eventually led to eccentric hyperplasia of endothelium and luminal narrowing. In the aorta, fatty deposits in endothelial cells were followed by formation of intimal plaques. The underlying media became necrotic and calcified.

There is some evidence that cardiac muscle necrosis and coronary lipoidosis may be independent of renal damage induced in rats by choline deficiency. Aortic lesions may be secondary to the kidney changes, but the possibility has not been excluded that choline deficiency is also directly implicated in the aortic lesions.

A positive haemosiderin reaction was obtained in the walls of coronary arteries and aortas as well as within intact cardiac muscle fibres. The reaction was diffuse throughout the vascular tissues and granular in the cardiac muscle. It was not dependent on local lysis of erythrocytes or artefacts. Its explanation is not yet known.

Androgen and growth hormone, administered simultaneously, induced cardiovasculorenal lesions in choline-deficient animals which were otherwise resistant to their development.

The addition of cholesterol to the choline-deficient diet greatly aggravated the ill effects of choline deficiency on the cardiovasculorenal system, without any accompanying elevation of serum cholesterol. Cholesterol given to choline-supplemented controls had no ill effect.

The clinical significance of these results is not apparent, but the rapid production of cardiovascular lesions in rats by a dietary deficiency alone may offer another useful experimental approach to the problems of coronary arterial disease and arteriosclerosis in man.

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EXPLANATION OF PLATES.

FIG. 1.—Initial alterations in a coronary artery of a young choline-deficient rat are shown. Tiny fat droplets (black in photograph) appear in the intima, the cells of which are swollen and weakly sudanophilic. Frozen section, Oil Red O stain. × 300.

FIG. 2.—Lipoidosis is more advanced in this coronary artery of a choline-deficient rat than that illustrated in Fig. 1. The intima is swollen and strongly sudanophilic. Fat droplets and globules appear in the media and even in the adventitia. Frozen section, Oil Red O stain. × 350.

FIG. 3.—In this advanced lesion in the coronary artery of a young choline-deficient rat large masses of lipid appear throughout the entire wall of the blood vessel. Frozen section, Oil Red O stain. × 350.

FIG. 4.—Stainable fat is deposited in cardiac muscle fibres in an area of acute necrosis. The high-power inset shows the fat droplets arranged in the pattern of the cardiac muscle striation. Frozen section, Oil Red O stain. × 30, × 280.

FIG. 5.—India ink was injected into the coronary arteries of this young choline-deficient rat. The cardiac muscle fibres are swollen, vacuolated and necrotic, but the capillaries in this area are still patent to the injection fluid. Haematoxylin-eosin stain, × 400.

FIG. 6.—Fat is deposited (early stage) within the medial layer of the aorta of a young rat fed a severely hypolipotropic diet. The site of this lesion is near the mouth of one of the two coronary arteries. Frozen section, Oil Red O stain, × 300.

FIG. 7.—In later stages of aortic lesions in young choline-deficient rats as shown here, fat deposition has largely been replaced by gross calcification mainly located in the media of the aortic wall. Frozen section, Oil Red O stain, × 35.

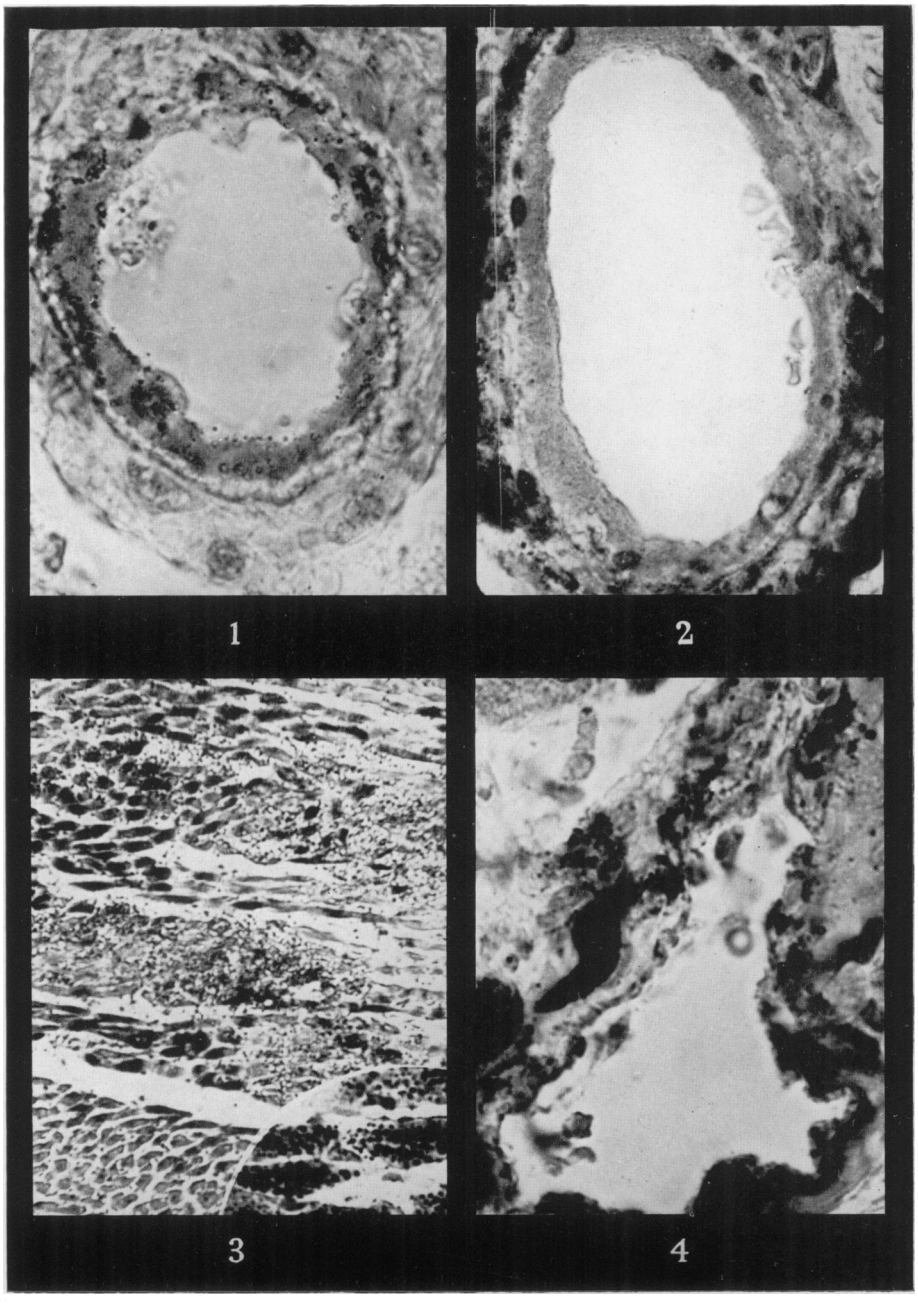
FIG. 8.—This aorta of a mature choline-deficient animal resembles a stick of bamboo because of medial sclerosis.

FIG. 9.—Eccentric intimal thickening and plaque formation has occurred in this coronary artery of a mature rat, suffering chronic choline deficiency. A subendothelial nodule is well demonstrated by this stain for mucopolysaccharides, (PAS). × 320.

FIG. 10.—Subendothelial fibrosis, hyperplasia and diffuse medial swelling of an aorta of a chronically choline-deficient animal is illustrated. The intercellular substance shows consolidation and separates the medial layers. Periodic acid stain, × 100.

FIG. 11.—Haemosiderin is deposited in coarse granules within intact cardiac muscle fibres in a young choline-deficient animal. There is no red cell breakdown visible in this section. Haemosiderin stain, × 1000.

FIG. 12.—The entire intima and media of this coronary artery of a mature choline-deficient rat stain diffusely throughout. Artefacts or post-mortem changes have been excluded. No erythrocyte breakdown is apparent in this section, stained by the Prussian blue reaction for haemosiderin, × 350.



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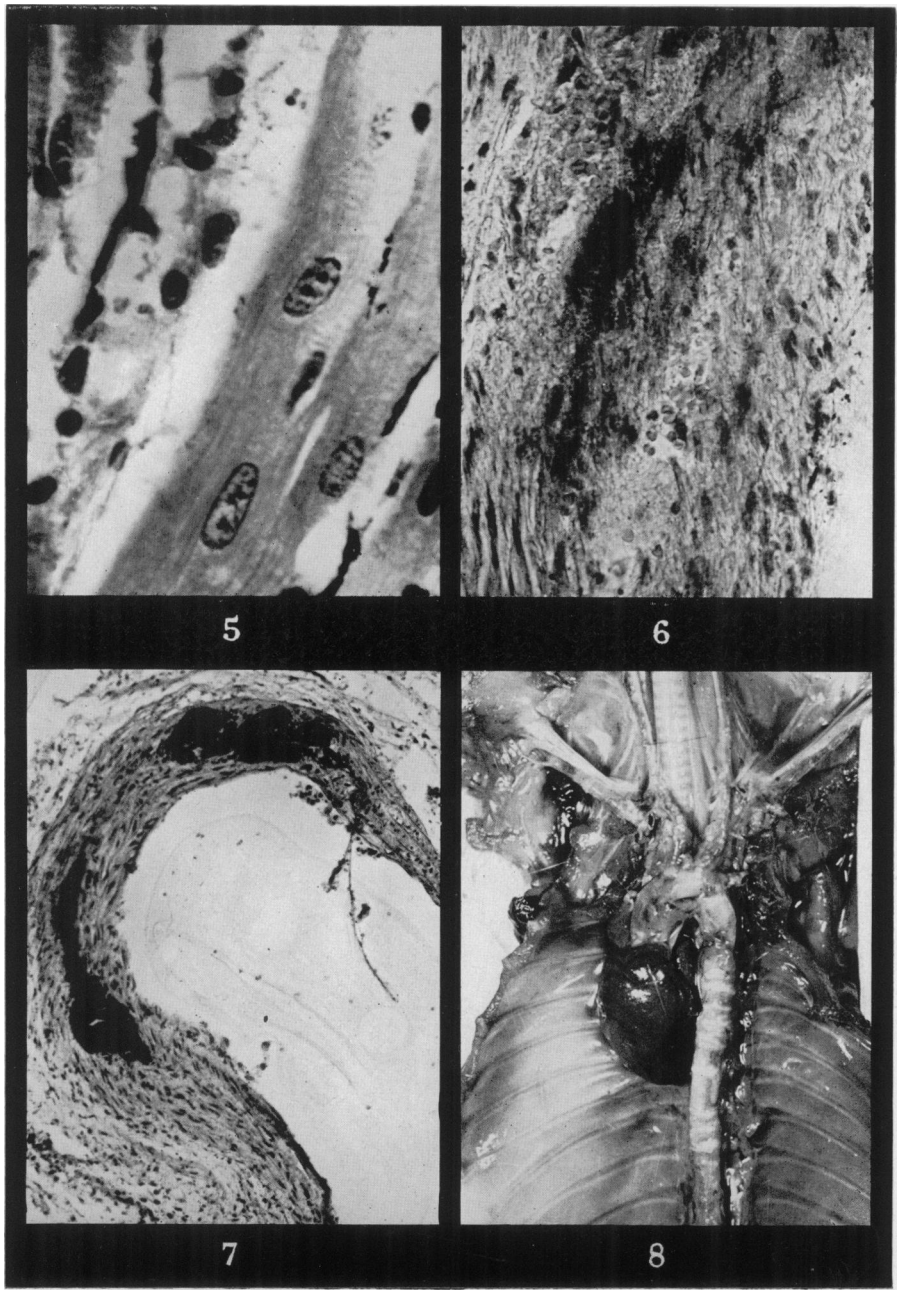

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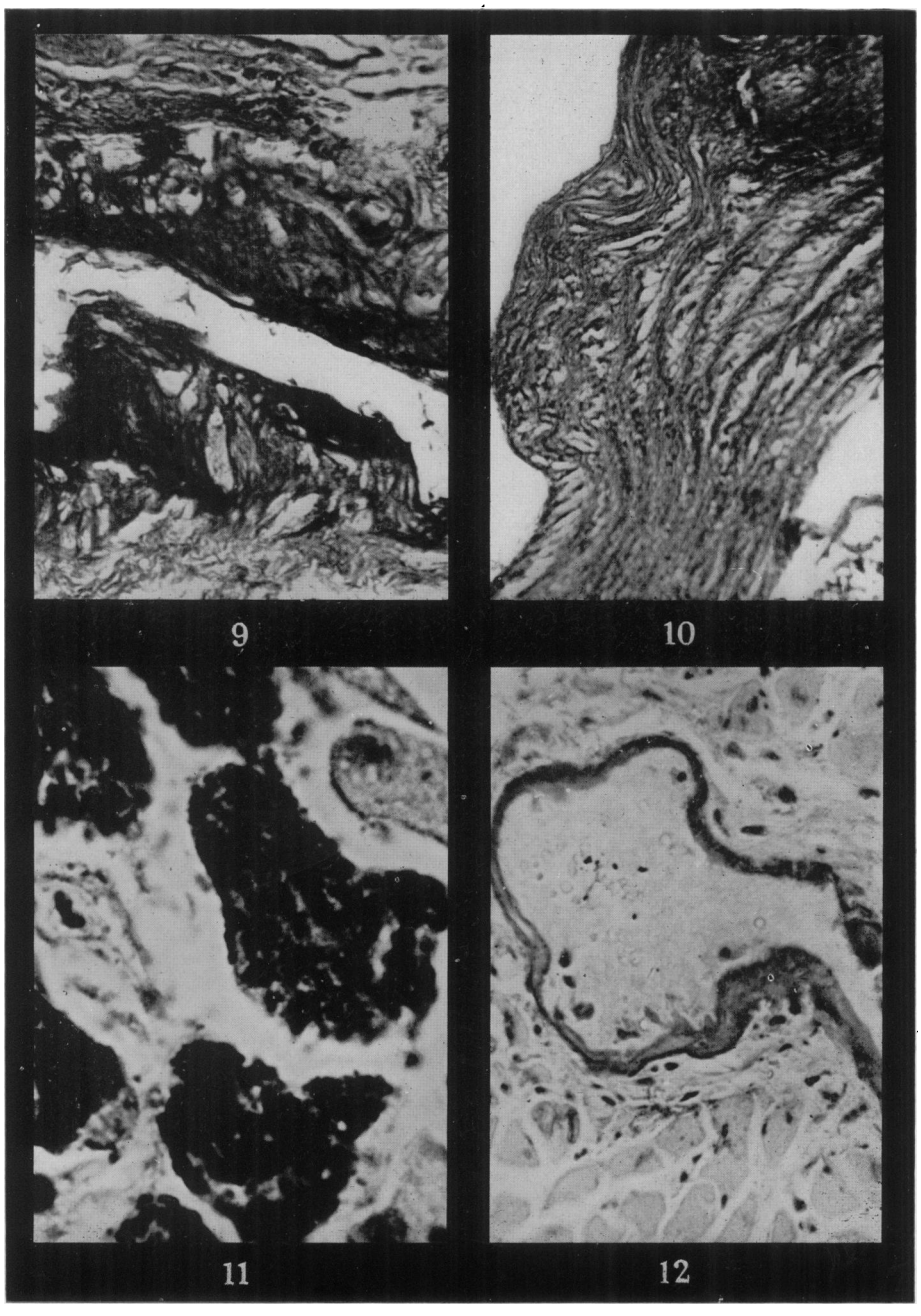

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