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Light and Electron-Microscopic Studies on Guinea Pig Hearts after Perfusion with Nitro-Blue Tetrazolium (NBT)*

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Summary. Guinea pig hearts isolated by the Langendorff technique were perfused with a nitro-blue tetrazolium salt solution. In spite of a homogeneous dark-blue staining at the macroscopic examination, light-microscopic studies showed a spotty staining of the myocardial fibers. Since no microscopic alterations were found which could explain the unstained areas, ultramicroscopic studies were undertaken. At ultrastructural level, striking alterations of myocardial cells were observed. They consisted of mitochondrial lesions, alterations of the transversal tubuli, and intercalated discs. Arteriolar narrowing and capillary edema were found, suggesting that pathological changes in the microcirculation could be a possible reason for the spotty staining of the myocardial fibers. Some speculations on the nature and localization of the formazan granules are also reported.

Key words: Heart – Microcirculation – Tetrazolium.

Zusammenfassung. Herzen von Meerschweinchen, die nach der Methode von Langendorff durchströmt worden waren, wurden mit einer Nitro-Blue Tetrazoliumsalzlösung perfundiert. Makroskopisch waren die Herzen ganz dunkelblau verfärbt. Bei der histologischen Untersuchung findet sich dagegen eine fleckförmige Anfärbung der Herzmuskelzellen. An den Gefäßen ergeben sich histologisch außer einzelnen Mikrothromben keine wesentlichen Befunde. Elektronenmikroskopisch lassen sich an den Herzmuskelzellen Mitochondrienveränderungen wie Schwellung, Lyse der Cristae und Rupturen der Außenmembranen, sowie Dilatation der transversalen Tubuli und Dehiszenzen der Glanzstreifen darstellen. Bei der elektronenmikroskopischen Untersuchung lassen sich außerdem Schwellungen der kapillären Endothelzellen sowie Einengungen der arteriolären Lichtungen belegen, die die fleckförmigen

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Niederschläge von Formazangranula, als Folge von Störungen der Mikrozirkulation, erklären könnten. Einzelne Aspekte der elektronenmikroskopischen Struktur der Formazane und deren intrazelluläre Lokalisatión werden beschrieben.

Introduction

Tetrazolium salt solutions are widely employed for detecting recent ischemic myocardial lesions (Sandritter and Jestädt, 1957; Nachlas and Shnitka, 1963; Brody et al., 1967; Lichtig et al., 1973). For this purpose, slices of whole hearts, blocks of myocardium, or even frozen sections are incubated in solutions containing the tetrazolium salt alone or in the presence of co-enzymes and substrates. In anoxic-damaged myocardial tissue, the tetrazolium salt cannot be reduced to formazan granules in the presence of co-enzymes and substrates. The ischemic lesion therefore appeared unstained in contrast to non-ischemic dark-blue stained myocardium. In recent investigations Lichtig et al. (1973) and Roesch et al. (1976) have perfused whole human and canine hearts with tetrazolium salt solutions in order to identify both macro- and microscopically, disturbances of the coronary circulation. This method was not found to be suitable for the simultaneous macro- and microscopic identification of acute ischemic lesions of the myocardium, however, since large, unstained myocardial areas were found, even in normal hearts. In an attempt to clarify the pathogenesis of these unstained areas, isolated guinea pig hearts (Langendorff hearts) were perfused with nitro-blue tetrazolium (NBT) salt solution and examined by light- and electron microscopy.

Material and Methods

Fifteen guinea pig hearts were prepared according to the method of Poche et al. (1967, 1969). After mounting the hearts on the Langendorff apparatus, they were perfused for 30 min with a 37°C warmed Krebs-Henseleit solution (solution A) at a constant pressure of 80 cm water. Following this period, 10 hearts (group I) were perfused for 25 min with solution B at +37°C at a constant pressure of 80 cm water. The solution B consists of a mixture of 500 mg nitro-blue tetrazolium (NBT), 5 mg of nicotinamide-adenine-dinucleotid (NAD), 2700 mg of sodium succinate and 1000 ml of distilled water (−1 part), Sörensen's buffer 1 M (−1 part) and distilled water (−8 parts). The pH of this solution was 7.35. As controls, two hearts (group II) were perfused with solution A only for 55 min and then fixed. To show the effect of NBT salt on the myocardium, three hearts (group III) were perfused with solution A for 30 min and then for another 25 min with solution C (solution B without NBT salt) under the same temperature and pressure. Following this, the hearts were perfused with a 6.25% glutaraldehyde solution in 0.1 M cacodylate buffer at pH 7.4 for 15 min at the same perfusion pressure. The solutions A, B, and C were equilibrated with carbogen (95% O_2 and 5% CO_2) for not less than 30 min before use. During the perfusion the coronary flow and the heart rate were measured at intervals of 5 min. Immediately after the perfusion stop, the hearts were taken from the apparatus and cut transversely in slices of 1 to 2 mm thickness and immersed in the same fixative solution at +4°C for not less than 4 h to several days. Before washing them in cacodylate buffer, the slices were cut into small pieces of 1 mm^3, post-fixed in 1% osmium-tetroxide (OsO_4) for 2 h, washed in Palade buffer, dehydrated in progressive alcohol series, propylenoxide, and embedded in araldite. The semithin and ultrathin sections were cut on a Reichert om U_2 ultramicrotome. For electron microscopy, we selected only blocks showing the highest deposition of formazan granules by light microscopic observations on semithin sections.

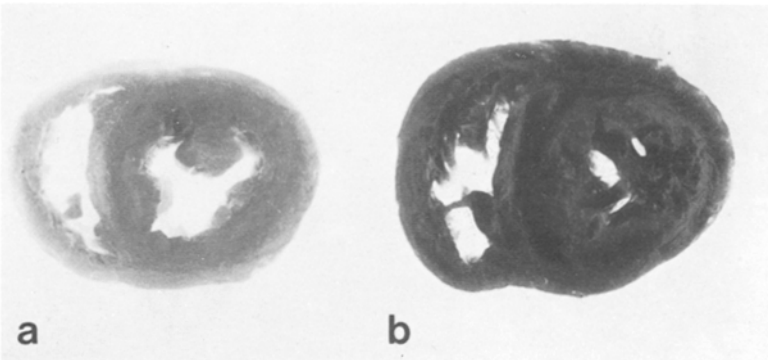

Fig. 1 a and b. Transversal slices from two guinea pig hearts (A4, A8), **a** perfused with Krebs-Henseleit solution, unstained, **b** perfused with NBT salt solution, stained homogeneously dark-blue. ×2.8

Ultrathin sections were mounted on 314 meshes grids and stained with uranyl acetate and lead sodium citrate; some unstained ultrathin sections were examined. The ultrastructural examinations were performed on a Siemens Elmiskop 101 electron microscope at 60 Kv beam. For light microscopy, some slices from the hearts were fixed in 10% buffered formalin, processed in the usual way for embedding in paraffin and sections stained with H&E and elastic-van Gieson. In some cases cryostate sections from NBT-perfused hearts were cut and examined by the light microscope before and after incubation in solution B at +37°C for 25 min.

Results

1. Functional Aspects

All hearts were perfused with Krebs-Henseleit solution (N=15) for a period of 30 min. The perfusion was carried out at a constant pressure of 80 cm water. The coronary flow, which was 16.4 ± 2.3 ml/min at the beginning of the perfusion, dropped slowly to 8.7 ± 0.8 ml/min at the end of the experimental period. At the same time, heart rate decreased slightly from 201 ± 7 to 172 ± 9 beats per min. The hearts, which were perfused with solution A for a further 25 min (group II), showed no significant changes in coronary flow and heart rate. The hearts, which were perfused another 25 min with solution B (group I), however, became arrhythmic and stopped beating within 10 to 105 s after the perfusion had begun. The coronary flow increased to 12.9 ± 3.2 ml/min, because of the cardiac arrest, and dropped very slightly to 11.9 ± 2.2 ml/min at the end of the perfusion period. The hearts that were perfused with solution C (group III) showed results similar to those from group I.

2. Macroscopic Findings

All the hearts perfused with solution B stained more or less intensely dark-blue (Fig. 1 b), in contrast to those perfused with solution A and C, which remained unstained (Fig. 1 a). Some hearts showed dilatated cavities and thinned ventricu-

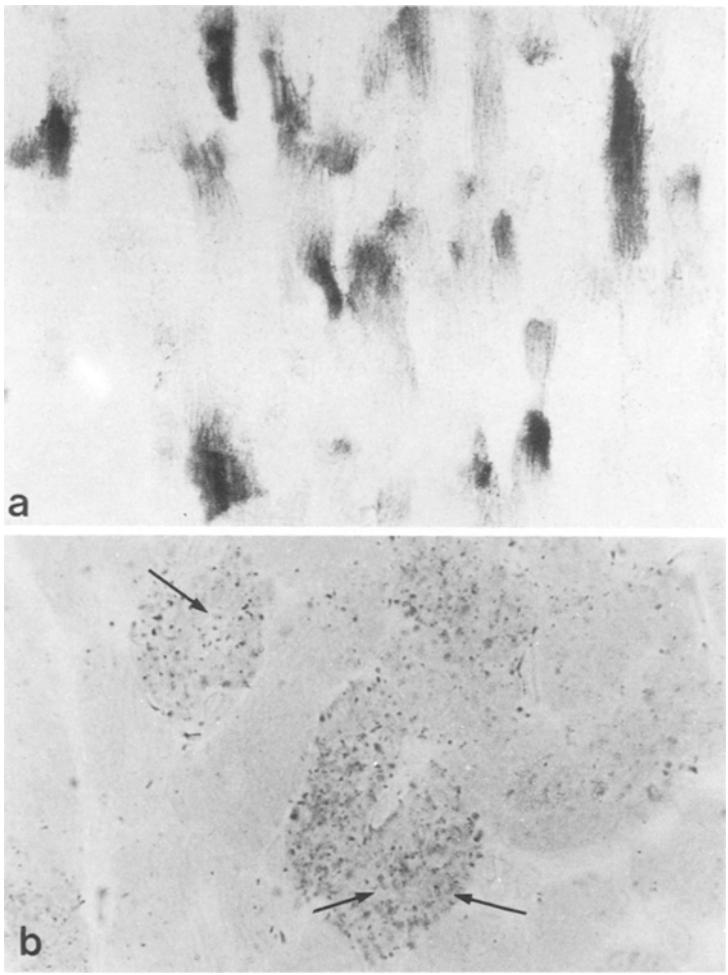

Fig. 2a and b. Light micrograph from guinea pig heart (A15) perfused with NBT salt solution. **a** Intense black-blue stained myocardial fibers or cellular segments, intermingled with others showing less staining. Cryostat section, ×310. **b** Unstained semithin section showing different amounts of formazan granules in almost all myocardial fibers. The reduced NBT salt appeared like black-blue granules of different size, mostly located in or at small, clear spaces (↗). Araldite, ×750

lar walls. The hearts that bear for a short time after beginning perfusion with NBT salt solution displayed a less marked bluish staining than those with longer survival times. No spotty coloration of the hearts was noted at this level.

3. Light-Microscopic Examination

By histologic examination, caryostate sections of NBT-perfused guinea pig hearts showed spotty deposition of formazan granules (Fig. 2a). Semithin sections of myo-

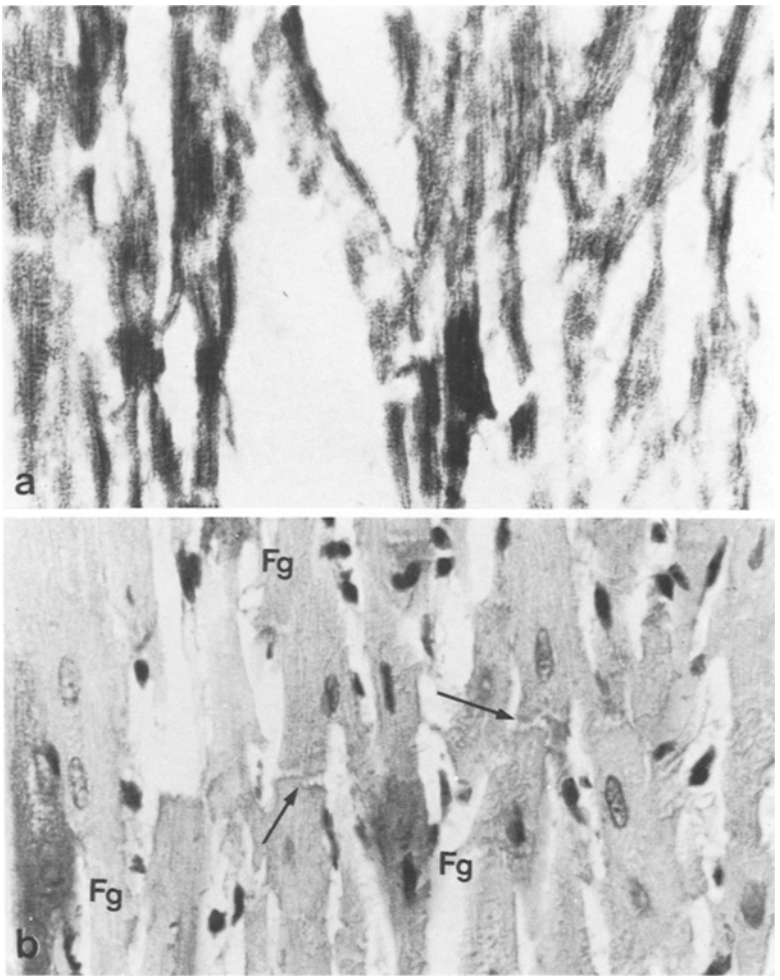

Fig. 3a and b. Light micrograph from guinea pig heart (A15) perfused with NBT salt solution. **a** Cryostat section incubated for 25 min in solution B. The spotty aspect, as seen at Figure 2a, disappeared almost completely. ×310. **b** Paraffin section showing three myocardial fibers with formazan granules (*Fg*) and some widened intercalated discs (↗). H & E, ×500

cardial fibers showed the same spotty staining as observed in cryostat sections (Fig. 2b). Some myocardial fibers showed numerous formazan granules which were rather scarce in other myofibers. When cryostat sections of NBT-perfused hearts were sequentially incubated, complete staining of the myocardial fibers could be seen (Fig. 3a). Unstained areas disappeared even though small differences in the amount of deposited formazan granules were still detectable. When sections of hearts perfused only with solution A (group II) were examined, interstitial edema and fragmentation of some muscle fibers could be observed. In the slices from hearts perfused with solution C (group III), interstitial edema, fragmentation of myocardial fibers, and rarely, widening of the intercalated

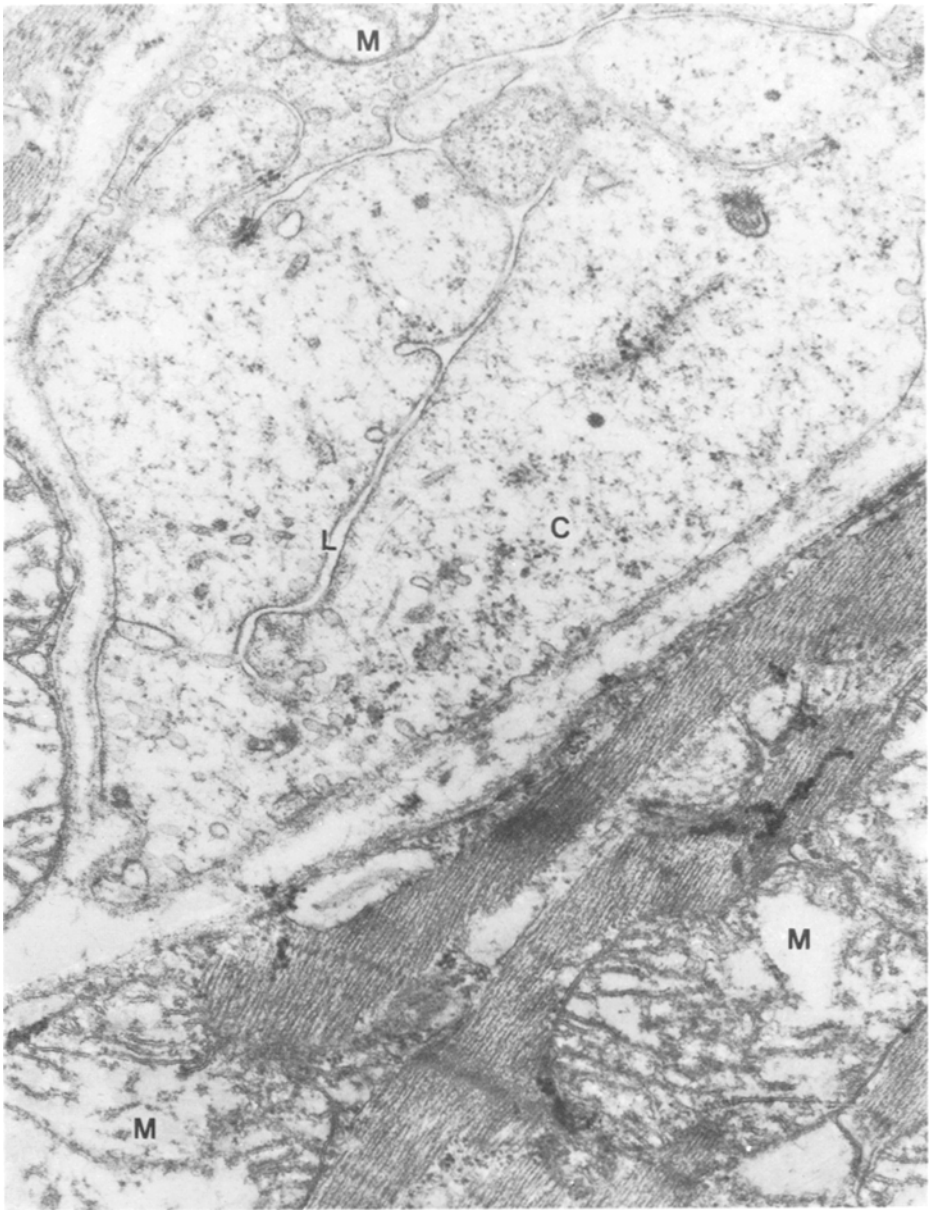

Fig. 4. Electron micrograph from guinea pig heart (A5) perfused with NBT salt solution. The mitochondria (*M*) shows swelling and crystolysis and the capillary (*C*) exhibits edema with luminar stenosis (*L*). Uranyl/lead, ×27,100

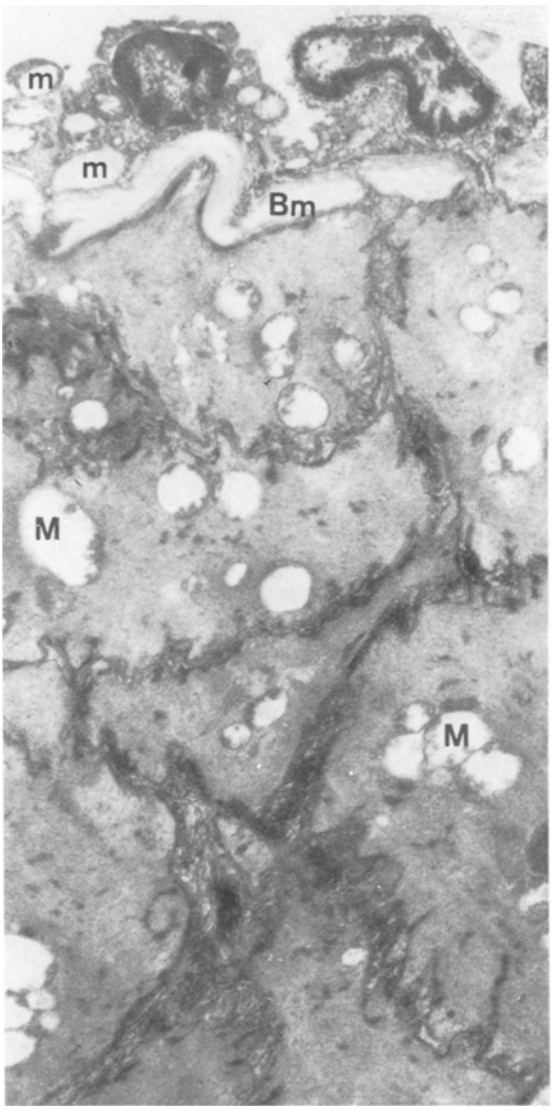

Fig. 5. Electron micrograph from guinea pig heart (A5) perfused with NBT salt solution. Arteriolar wall with damaged mitochondria (*M*) in the smooth muscle and endothelial cells. Note the wavyness of the basement membrane (*Bm*). Uranyl/lead, ×7700

discs, were seen. Slices from hearts perfused with solution B (group I) showed interstitial edema and fragmentation of myocardial fibers. The sarcoplasm was granular in aspect containing small sarcoplasmic vacuoles. Less frequently, contraction bands and widening of the intercalated discs were observed. Sometimes myocardial fibers appeared as homogeneous blue spots (Fig. 3b). Very rarely obstruction of venules and capillaries by microthrombi was seen.

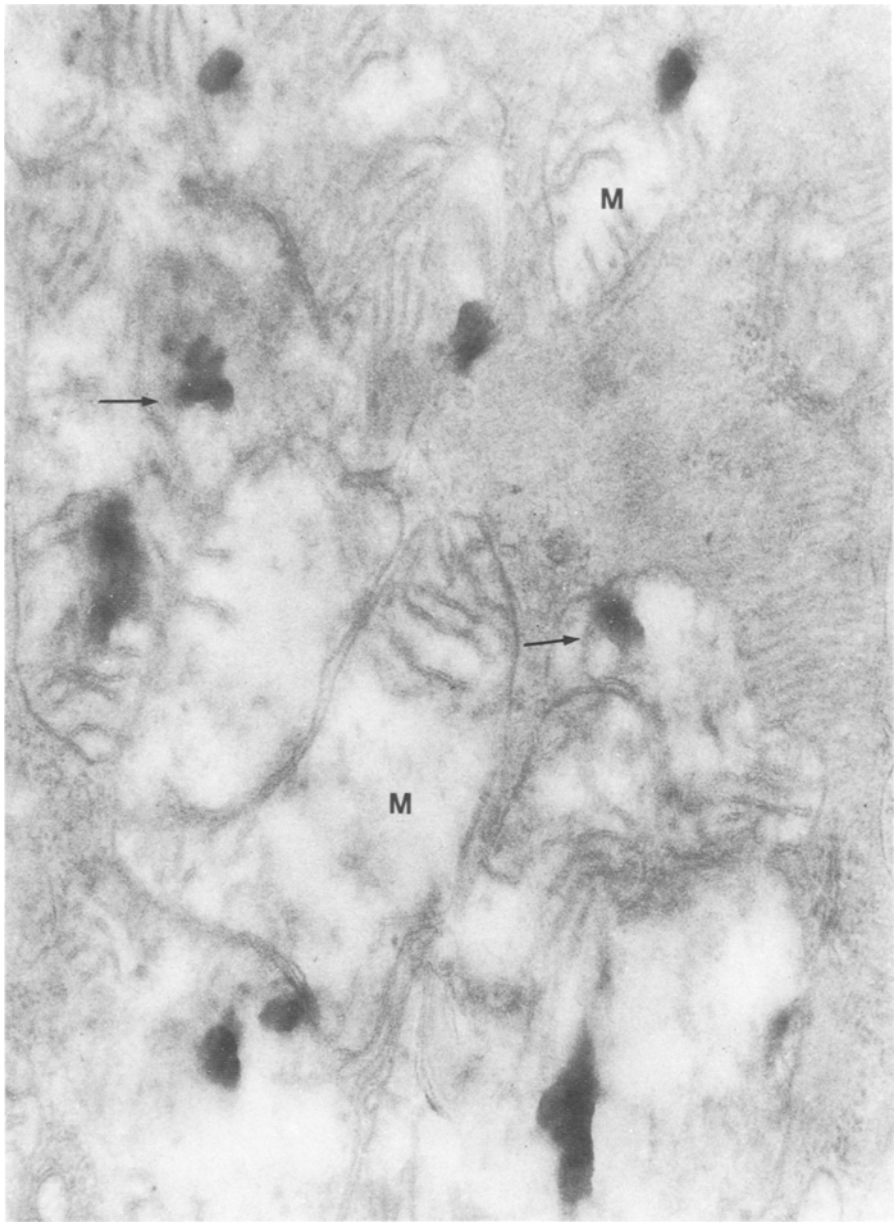

Fig. 6. Electron micrograph from guinea pig heart (A6) perfused with NBT salt solution. Largely disrupted mitochondria (*M*) show deposition of relatively homogeneous, irregular, electron-dense material (↗). No staining, ×48,000

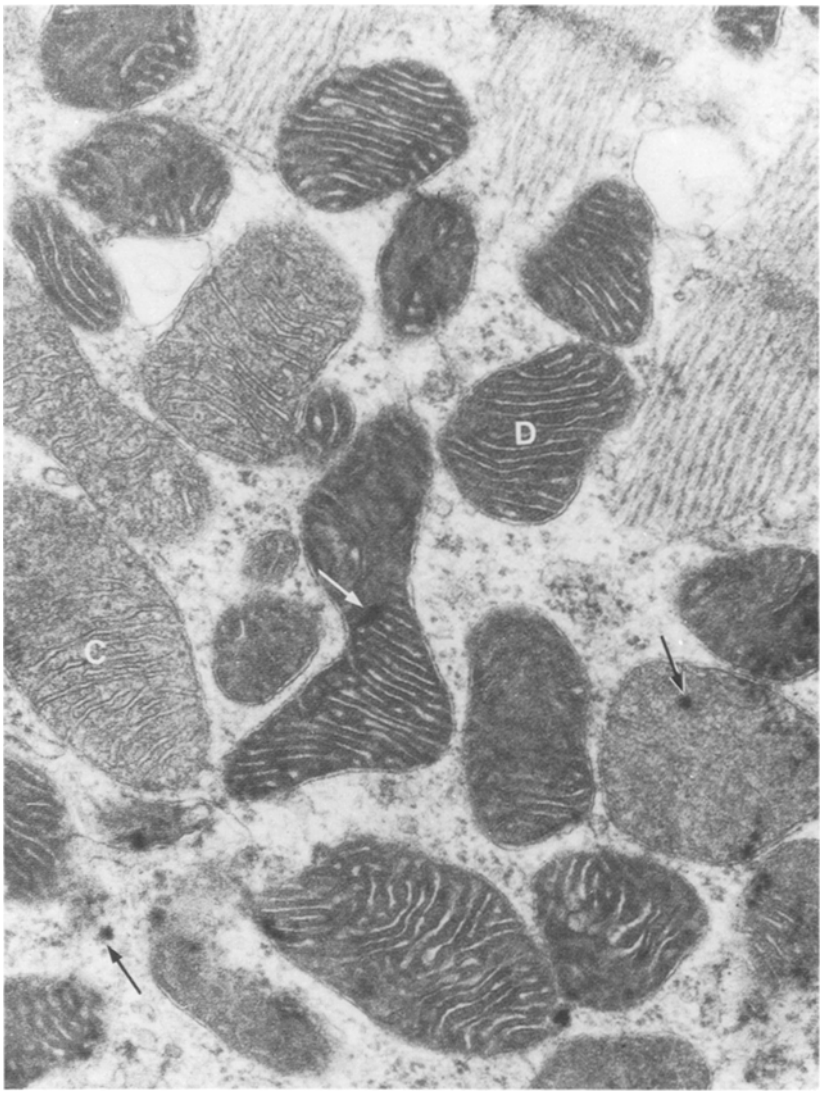

Fig. 7. Electron micrograph of guinea pig heart (A3) perfused with NBT salt solution. Dark (*D*) and clear (*C*) mitochondria with more or less well preserved cristae. Electron opaque material is seen both inside and outside mitochondria (↗). Uranyl/lead, ×37,400

4. Ultrastructural Findings

On electron-microscopic examination the highest degree of cellular damage was observed in hearts which had been perfused with the solution containing NBT salt. The myocardial cells showed dilatation of the sarcoplasmic reticulum, mainly of the transversal tubuli, enlargement of the intercalated discs, contraction bands, and lipid droplets. The most striking alterations were, however,

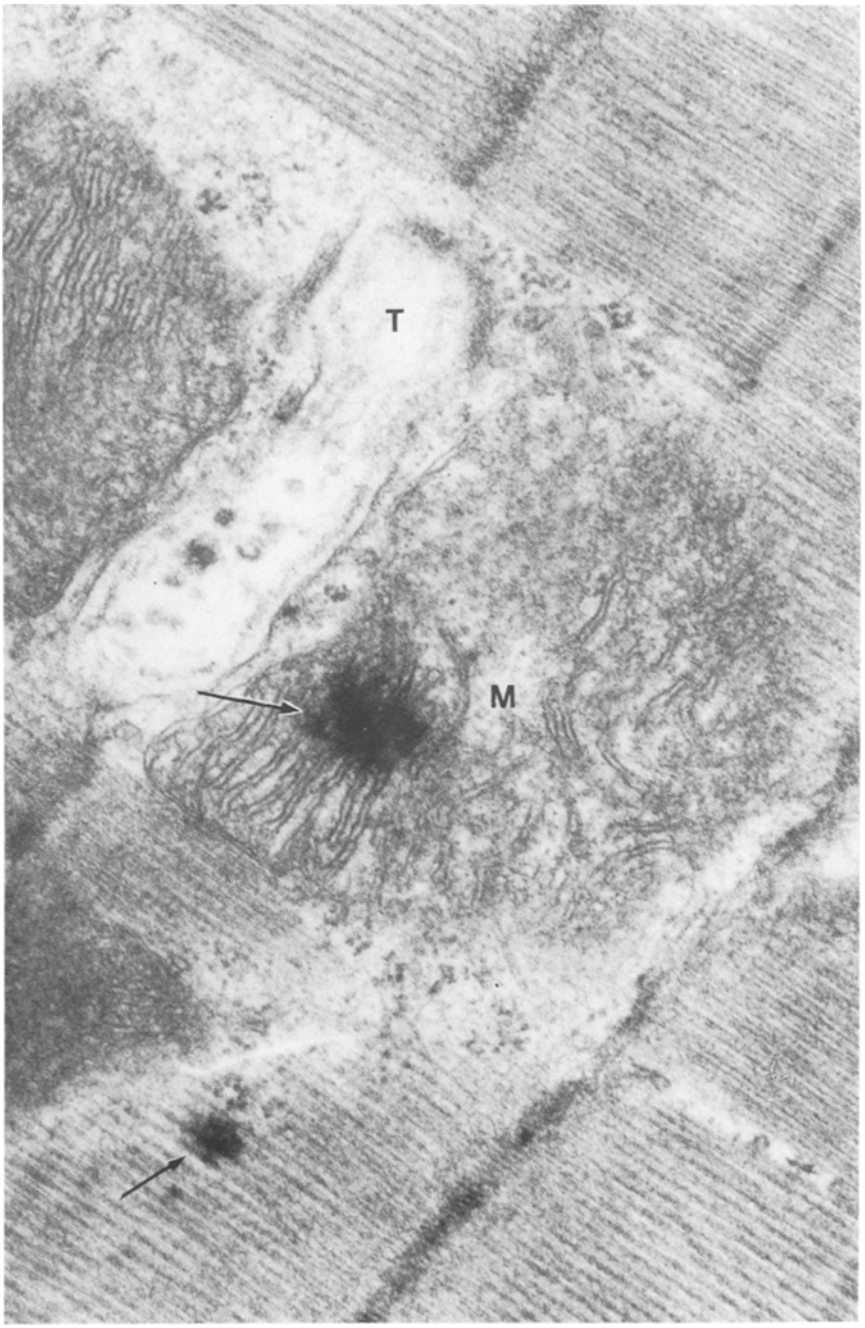

Fig. 8. Electron micrograph of guinea pig heart (A_3) perfused with NBT salt solution. Arrows indicate electron-opaque deposits in a partially disrupted mitochondrium (*M*) and within the sarcomere. Note enlargement of transverse tubule (*T*). Uranyl/lead, × 54,000

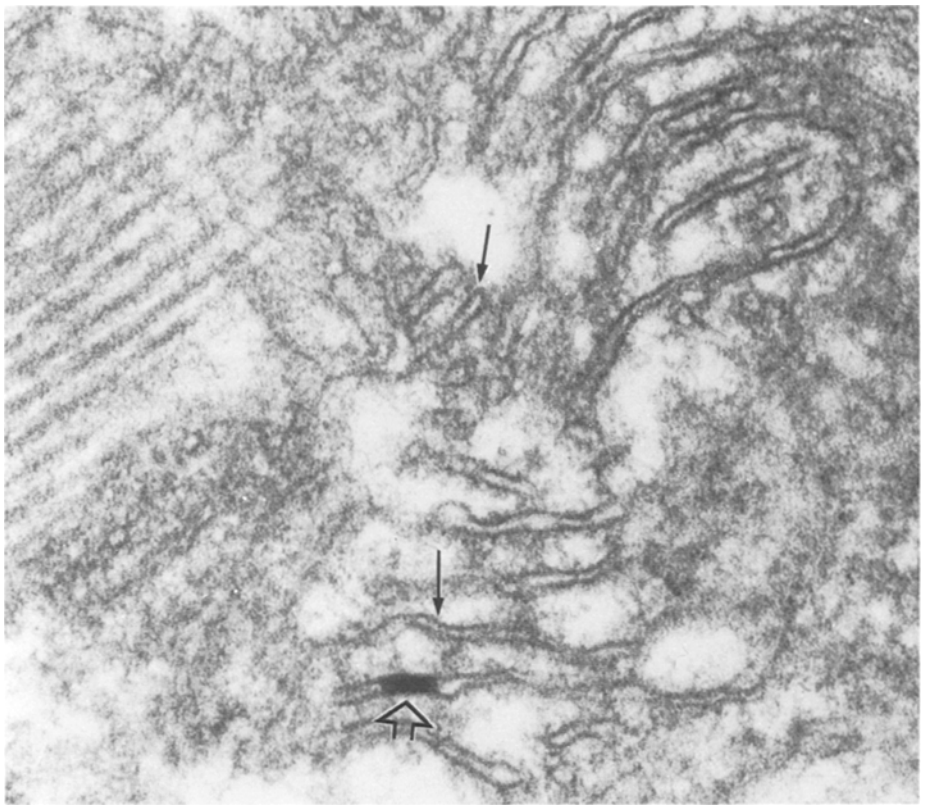

Fig. 9. Electron micrograph of guinea pig heart (A6) perfused with NBT salt solution. Some cristae in disrupted mitochondria showed fine, granular, electron-opaque deposits (↗) on the cristal membranes. In one point there is intra-cristal space with a rectangular deposit of similar material (⇑). Uranyl/lead, ×96000

located in the mitochondria and consisted of swelling, fragmentation of the cristae, clearing of the matrix; frequently, interruptions in the continuity of the mitochondrial limiting membranes were observed, together with variable amounts of irregular, electron-dense deposits. These mitochondrial alterations were found not only in the myocardial cells but in capillaries and arterioles as well. The capillaries showed extreme edema of the endothelial cells, occasionally with complete stenosis of the lumen (Fig. 4). Sometimes arterioles with contraction of the smooth muscle and endothelial cells and waviness of the basement membrane were observed (Fig. 5). The electron-dense deposits were found not only in very damaged mitochondria (Fig. 6) but also in less altered ones (Figs. 7, 8). It was not infrequent to find these deposits in normal mitochondria, at the sarcomeres, in the sarcoplasma, and in the capillary wall. Sometimes electron-dense material, as a fine granular deposit, was found on the membranes of fragmented mitochondrial cristae or in the intra-cristal space (Fig. 9). In a few areas of the hearts perfused with solution B, dark mitochondria intermingled with clear ones were noted; electron-opaque material was not necessarily

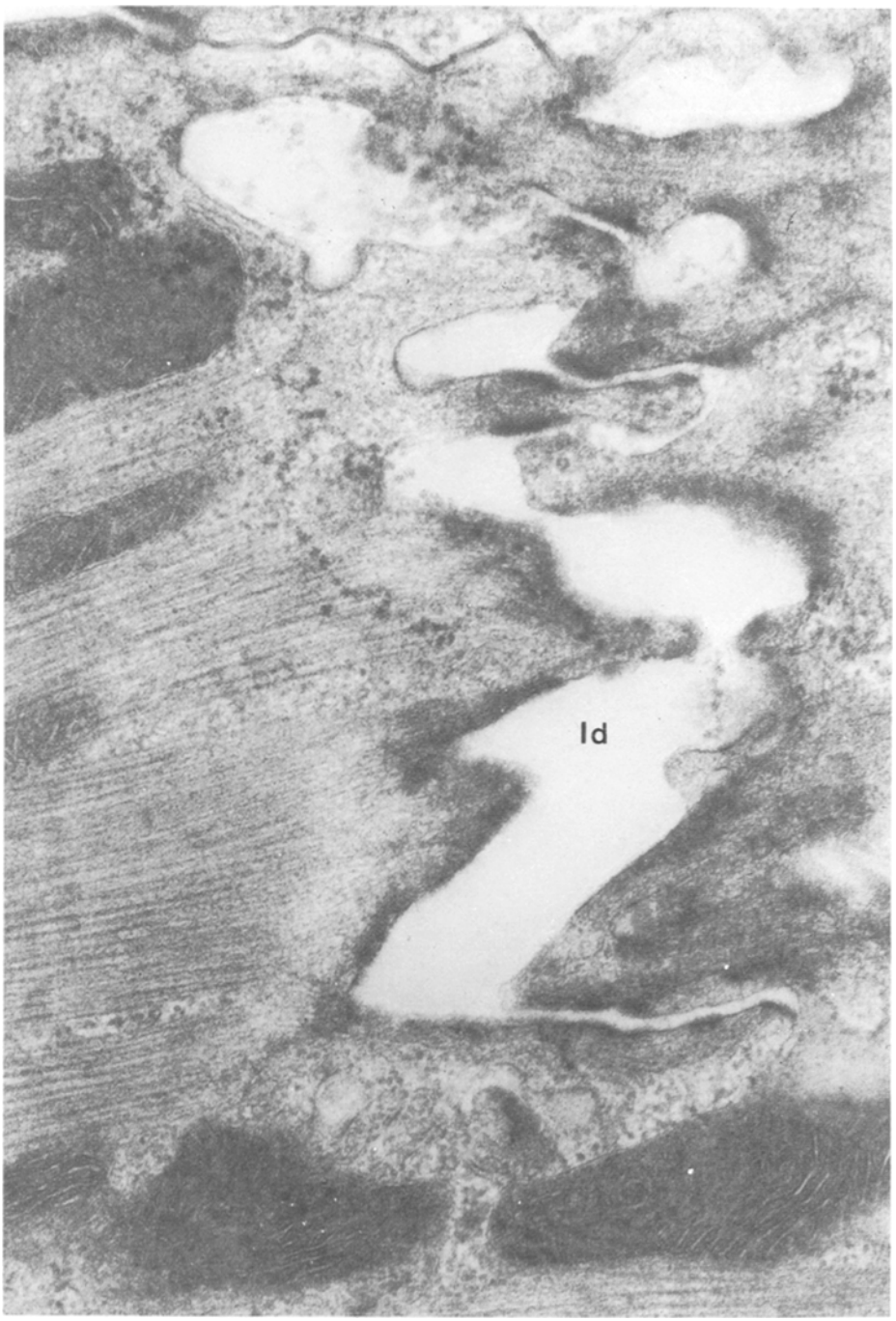

Fig. 10. Electron micrograph of guinea pig heart (A13) perfused with solution C. Moderate enlargement of the intercalated disc (*Id*) and homogeneous aspect of the mitochondria. Uranyl/lead, ×48,000

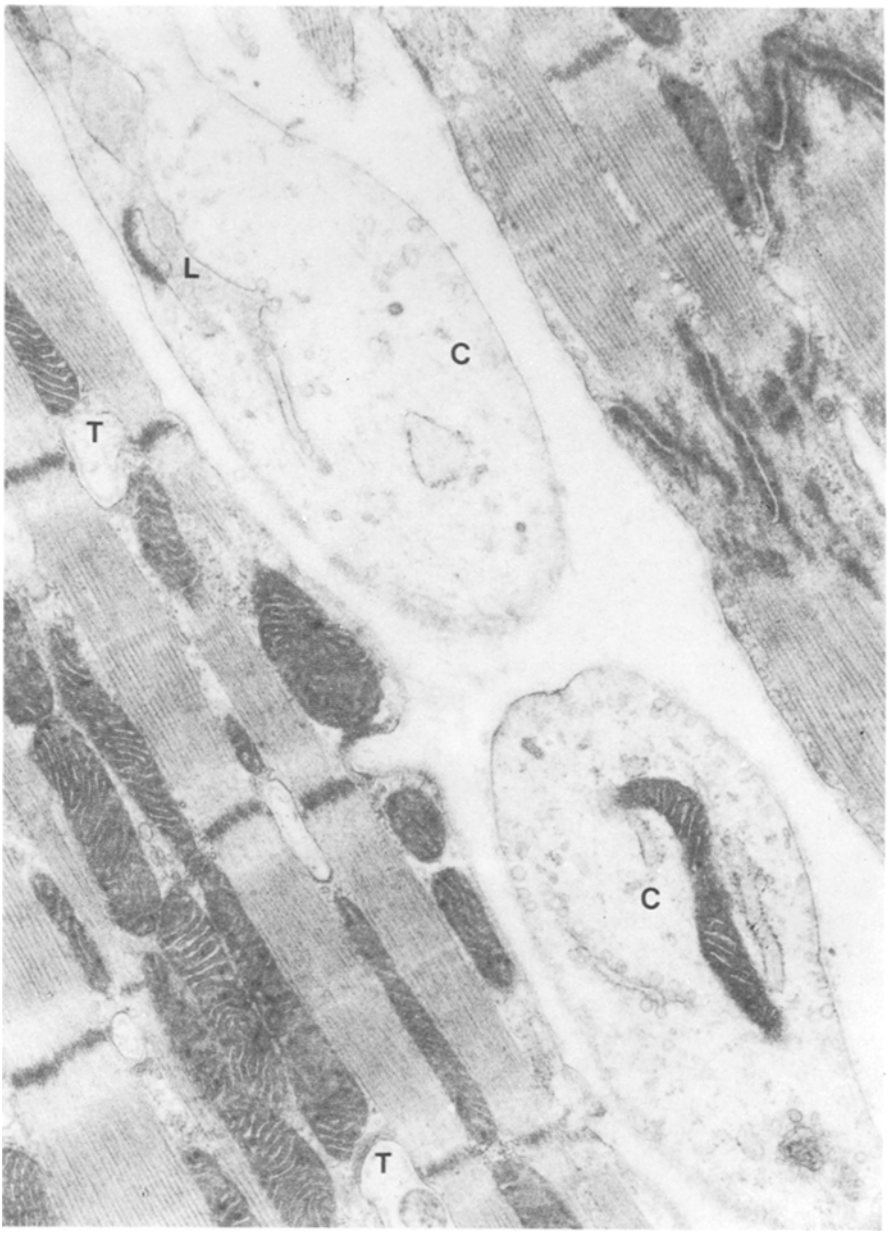

Fig. 11. Electron micrograph of guinea pig heart (A4) perfused with solution A. Capillary (*C*) with swelling of the endothelial cells and stenosis of lumen (*L*). Discrete dilatation of transversal tubuli (*T*). Uranyl/lead, ×23,500

present (Fig. 8). The ultrastructural examination of the hearts perfused with solution C (group III), showed almost identical alterations as described for the NBT-perfused hearts (Fig. 10). The mitochondria showed no significant alterations. The amount of electron-dense material scattered on the cytoplasmic structures was less than in group I. No electron-opaque depositions, like those in Figure 6, were found in the mitochondria. The alterations showed by the hearts perfused with solution A (group II) consisted of dilatation of the sarcoplasmic reticulum, mainly of the transversal tubuli. Sometimes small lipid droplets were observed. The mitochondria, the intercalated discs, the longitudinal tubuli, the sarcomeres, the β-glycogen granules showed no essential modification. Very rarely, swelling of the endothelial cells with stenosis of capillaries was seen (Fig. 11).

Discussion

The validity of a procedure based on the perfusion of coronary arteries for the simultaneous macro- and microscopic identification of reduced tetrazolium salt at the myocardial fibers is based on three fundamental points: First, that the indicator substance, in this case the NBT salt, reaches all the myocardial cells; second, that the indicator be reduced rapidly by the respiratory enzymes present in the myocardial fibers; and third, that the formazan granules formed be identifiable by light- and electron microscopy and do not diffuse from the site of reduction. If these premises are fulfilled, the simultaneous macro- and microscopic, or only the microscopic, identification of ischemic myocardial lesions may be possible. Recently, observations on the perfusion of whole human hearts with the NBT salt solutions has been reported (Lichtig et al., 1973). In spite of some irregular staining observed in these perfused hearts, the method was presented as a diagnostic aid in ischemic heart disease. We subjected this method to a critical evaluation by using human and canine hearts and concluded that this procedure was not suitable for the diagnosis of ischemic heart lesions, since macroscopically unstained areas not corresponding to damaged heart tissue were observed (Roesch et al., 1976). To clarify this problem we perfused isolated beating guinea pig hearts with NBT salt solution. In contrast to our previous results with human and canine hearts, guinea pig hearts examined macroscopically stained homogeneously dark-blue (Fig. 1 b). On light-microscopic examination, however, the myocardial fibers did not stain homogeneously, as macroscopic inspection had suggested. Spotty staining was observed in either paraffin sections (Fig. 3 b), or araldite semithin sections (Fig. 2 b). To rule out the possibility that the formazan granules were washed out during fixation, dehydration and embedding procedures (Egger, 1972), cryostat sections from NBT-perfused hearts were made and examined on light microscopy. Even in these sections intensively stained, stained myocardial cells were observed located close to others with little or no staining (Fig. 2 a). Sometimes, in unstained sections, myocardial fibers showed some fine, bluish shadowing. Generally, unstained myocardial fibers did not exhibit any alterations on light-microscopic examination. The hypothesis that these areas correspond to enzymatically altered cells was investi-

gated by incubating cryostat sections from NBT-perfused hearts in the same solution. After incubation a homogeneous distribution of dark-blue formazan granules was observed (Fig. 3a). This indicates that these unstained myocardial fibers are not damaged enzymatically (O'Brien et al., 1972). It is probable that the solution containing the NBT salt did not reach these cells although by light microscopy microthrombi in venule and capillary lumina were rarely seen, and this finding could not explain these unstained areas. At the ultrastructural level, however, besides the changes already described, we saw, not infrequently, severe edematous swelling of endothelial cells, sometimes with obliteration of the capillary lumen (Fig. 4). Furthermore, the wall of some small arteries showed alterations consisting of contraction of smooth muscle and waviness of the basement membrane, indicative of arteriolar narrowing (Fig. 5). We believe that the unstained areas observed by light microscopic examination of NBT perfused guinea pig hearts may be due, in part, to swelling of capillary endothelial cells, narrowing of the arteriolar lumen by contraction of the arteriolar wall, and, finally, by occasional microthrombi. Even the hearts perfused only with solution A (group II) sometimes showed swelling of endothelial cells (Fig. 11); this suggests that slight ischemia occurs in our experimental conditions.

All the hearts perfused with NBT salt solution stopped beating shortly after perfusion had begun. Since striking mitochondrial alterations were observed in the cardiac cells, in capillaries and arterioles, it suggests that NBT salts have a direct action on the metabolism and structure of mitochondria, as previously reported (Martinez-Rodrigues, 1972; Egger, 1972; Egger, 1973a). Electron-dense deposits were frequently found within or on mitochondrial profiles. As they are present in unstained section as well this rules out the possibility that they might be due to other stainings, for example, lead or uranyl acetate (Fig. 6). Extramitochondrial electron-dense material was frequently encountered in sections of hearts perfused with NBT salt solution (Figs. 7, 8). This was irregularly scattered in the sarcomeres, among the myofibrils, or distributed along the sarcoplasmic reticulum. Similar findings were previously reported by Sedar and Rosa (1961), Wohlrab and Fuchs (1967), and Egger (1973a). Occasionally electron-dense material was found outside the myocardial cells along the capillary wall. We are unable to say whether all this electron-dense material found at mitochondria and on other sarcoplasmic structures consists only of reduced NBT salt. Egger (1973a) identified the osmiophilic material found in damaged mitochondria or in cristae as reduced tetrazolium salt and that outside the mitochondria as lectron-opaque, non-reduced tetrazolium salt or other lipoprotein osmiophilic substances. Alternatively, tetrazolium salt reduced enzymatically in the mitochondria may diffuse into the sarcoplasm, after rupture of the mitochondrial membranes (Sedar and Rosa, 1961; Egger, 1972). Sometimes altered mitochondria showed only fine granular, osmiophilic structures on the membranes of the cristae and rarely intracristal, rectangular-shaped deposits (Fig. 9). Identical observations were made by Sedar et al. (1962), Ogawa and Barrnett (1964), Castelletto de Fanchiotti et al. (1971), Kalima et al. (1972) and Egger (1973). From our studies we can say that the NBT salts were reduced mainly by the succinodehydrogenase system (Martinez-Rodrigues et al., 1972) and that the fine, granular, electron-dense deposits correspond to the ultrastructural localization of this enzyme, as indicated in several reports (Sedar and Rosa, 1961; Sedar et al., 1962; Castelletto de Fanchiotti et al., 1971; Egger, 1973). We agree with Egger (1973) that the topochemical identification of such enzymes, on the cristae, must be done only on relatively well-preserved mitochondria. In very damaged mitochondria, the formazan granules are irregularly distributed and/or joined together (Fig. 6), without an evident relationship to the mitochondrial membranes. Electron-dense material was occasionally found in the mitochondrial structures of hearts not previously treated with NBT salt solution. At present no explanation about the genesis of this material can be offered. Morphologically, it is similar to that reported in ischemic heart mitochondria (Ganote et al., 1975; Iglesia and Lub, 1972; Schwartz et al., 1973).

Our results suggest that perfusion of coronary arteries with NBT salt solution is not a suitable method for identifying ischemic disturbances in the cardiac microcirculation. NBT solution does not seem to be able to reach all the myocardial cells, probably as a consequence of arteriolar narrowing and capillary edema. This results in a spotty staining of myocardial fibers as indicated by light microscopic examinations, supporting our previous suggestion that the macroscopic unstained areas observed in human and canine hearts were due in part to similar changes (Roesch et al., 1976). Furthermore, the localization of the electron-dense material (formazan granules, NBT salt, lipoproteic structures, calcium, alone or together) outside of the mitochondria make an identification of ischemic heart lesions difficult at ultrastructural level. The occurrence of capillaries and arterioles, the presence of normally changes inactive capillaries (Hellberg et al., 1971), and the non-specific localization of the formazan granules in the heart structures constitute serious obstacles to the identification of ischemic microcirculatory disturbances by this method.

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