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CALCIUM-RELATED CHANGES IN THE ULTRASTRUCTURE OF MAMMALIAN MYOCARDIUM

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Summary.—Isolated rat hearts, perfused with Ca^{2+}-free medium for 5 min, were perfusion-fixed for ultrastructural studies. Increased pinocytotic activity and characteristic changes in the staining of the intercalated disc coupled with conspicuous staining of the middle lamina in the tight junctions in these hearts indicated an alteration in the sarcolemmal activity as well as cell–cell relationship. The intracellular effects of Ca^{2+}-free perfusion were indicated by the presence of an active Golgi body as well as the loss of heterogenic staining of the nucleoplasm. The latter appears to be a direct consequence of Ca^{2+} depletion of the cell, as heterogeneity reappeared upon reintroduction of the calcium in the perfusion medium. The extent of structural damage upon reintroduction of calcium was dependent upon the extracellular calcium concentration. These observations suggest that perfusion with Ca^{2+}-free medium causes some membrane changes in the heart which probably make it more vulnerable to the reintroduction of calcium. Furthermore, the structural damage observed upon reintroduction of calcium appeared to be related to the amount of calcium overload.

IT is now well documented that successive perfusion of heart with Ca^{2+}-free and Ca^{2+}-containing medium results in structural damage as well as in the derangement of metabolic functions (Zimmerman and Hülsman, 1966; Zimmerman *et al.*, 1967; Muir, 1968; Yates and Dhalla, 1975; Boink *et al.*, 1976; Lee and Dhalla, 1976). This model of paradoxical effects of calcium is of great interest to pathophysiologists and cardiologists, since heart to a certain degree is exposed to these conditions in situations such as the occurrence of ischaemia and development of collaterals, as well as in the interruption of circulation to the heart during open-heart surgery followed by restoration of flow (Sharma *et al.*, 1975; Hearse, 1977). The damage during calcium paradox has been attributed to the massive influx of calcium upon reperfusion. Ca^{2+} overload has also been suggested as the cause of catecholamine-induced myocardial necrosis (Bloom and Cancilla, 1969; Fleckenstein *et al.*, 1973; Bloom and Davis, 1974). The irreversible injury to the myocardial cell in reperfusion of ischaemic myocardium after 40 min of coronary occlusion is also probably due to Ca^{2+} overload (Shen and Jennings, 1972*a*, *b*; Jennings *et al.*, 1975). However, increase in calcium from 1·25 mM to 5 mM in the extracellular fluid, which would also increase intracellular calcium, failed to cause any structural damage (Tomlinson *et al.*, 1974). Further, it has been shown that perfusion of rat hearts for 20 min with Na^+- or K^+-free medium causes a dramatic increase in the myocardial Ca^{2+} content (Dhalla *et al.*, 1976); however, the structural damage to the myocardium was observed only in the hearts perfused with K^+-free medium (Singal *et al.*, 1978). Thus, the mode of introduction of Ca^{2+} overload appears to

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be an important determinant of structural damage.

Readily apparent gross structural damage of the myocardium due to Ca^{2+} depletion of the hearts has been observed only after a prolonged exposure to the Ca^{2+}-free medium (Weiss, Surawicz and Rubenstein, 1966; Muir, 1967; Tomlinson *et al.*, 1974). However, for the occurrence of calcium paradox, an exposure of 5 min to Ca^{2+}-free solution has been found to be enough, indicating that some structural and/or functional changes are introduced in the myocardial cell during this Ca^{2+}-free perfusion. The present study reports some hitherto undescribed morphological changes in rat hearts exposed to Ca^{2+}-free medium for only 5 min, at which time heart is unable to recover the contractile force upon reperfusion with Ca^{2+}-containing medium (Yates and Dhalla, 1975). Further, the effects of graded increase in the intracellular Ca^{2+} overload on the cellular structures has also been examined without changing the mode of its introduction. For this purpose rat hearts were perfused with Ca^{2+}-free medium for 5 min followed by 5 min reperfusion with a medium containing different concentrations of calcium (0·05, 0·5, 1·25 and 4·0 mM). This procedure has been shown to cause a concentration-dependent increase in myocardial Ca^{2+} content (Alto, Singal and Dhalla, 1978).

MATERIALS AND METHODS

Male albino rats weighing 300–350 g were decapitated and the hearts were quickly arranged for coronary perfusion with the conventional Langendorff technique. The perfusion medium was Krebs–Henseleit solution containing NaCl, 120 mM; $NaHCO_3$, 25 mM; KCl, 4·8 mM; KH_2PO_4, 1·2 mM; $MgSO_4$, 1·2 mM; $CaCl_2$, 1·25 mM; and glucose, 8 mM. The perfusion solution, pH 7·4, was continually gassed with a mixture of 95% O_2 and 5% CO_2 and the perfusion temperature was maintained at 38°. The hearts were perfused in an open system and were punctured at the tip to avoid fluid accumulation in the ventricles. The perfusion rate was kept at about 10 ml/min. The hearts were allowed to equilibrate for 20 min with control medium before starting perfusion with modified solutions. This duration of equilibration has been found to be enough for the stabilized functioning of the heart (Yates and Dhalla, 1975).

At the end of each experiment the hearts were fixed by perfusion with 0·1M phosphate buffer (pH 7·4) containing 2% glutaraldehyde for 2–3 min. Small pieces of the left ventricle were dissected out and allowed to stand in the buffered glutaraldehyde for 1–2 h for further fixation at 4°. The tissue pieces were washed overnight in the cold phosphate buffer containing sucrose, postfixed for 1 h with 1% osmium tetroxide, dehydrated in a graded alcohol series and embedded in Epon 812 according to the method of Luft (1961). Sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined using a Zeiss electron microscope.

RESULTS

The ultrastructural details of the rat heart fixed after 30 min perfusion with Krebs–Henseleit medium are shown in Fig. 1a–c and are quite similar to those reported by Muir (1967). However, in control hearts of our experiments the intercellular space was seen to be dilated in those portions of the intercalated disc running parallel to the myofibrils (Fig. 1c). In some of the sections membrane vesicles were also apparent in these clear portions of the intercalated disc. Small spherical granules seen in the lumen of T-tubules (Fig. 1c) have also been noticed by others (Muir, 1967).

Effects of Ca^{2+}-free perfusion

Hearts were perfused with Ca^{2+}-free medium for 5 min, at which time the hearts were in a flaccid state. These flabby hearts were processed for electron microscopic examination. There was no gross damage of the ultrastructure in these hearts; however, some morphological alterations in the cells were apparent (Fig. 2a–e). A dramatic increase in the subsarcolemmal vesicles was noticed and some of these vesicles were seen in continuity with the intercellular space (Fig. 2a). Golgi bodies showed increase in the number of cisternae and the associated vesicles (Fig. 2b). Apart from their juxtanuclear occurrence, the Golgi bodies

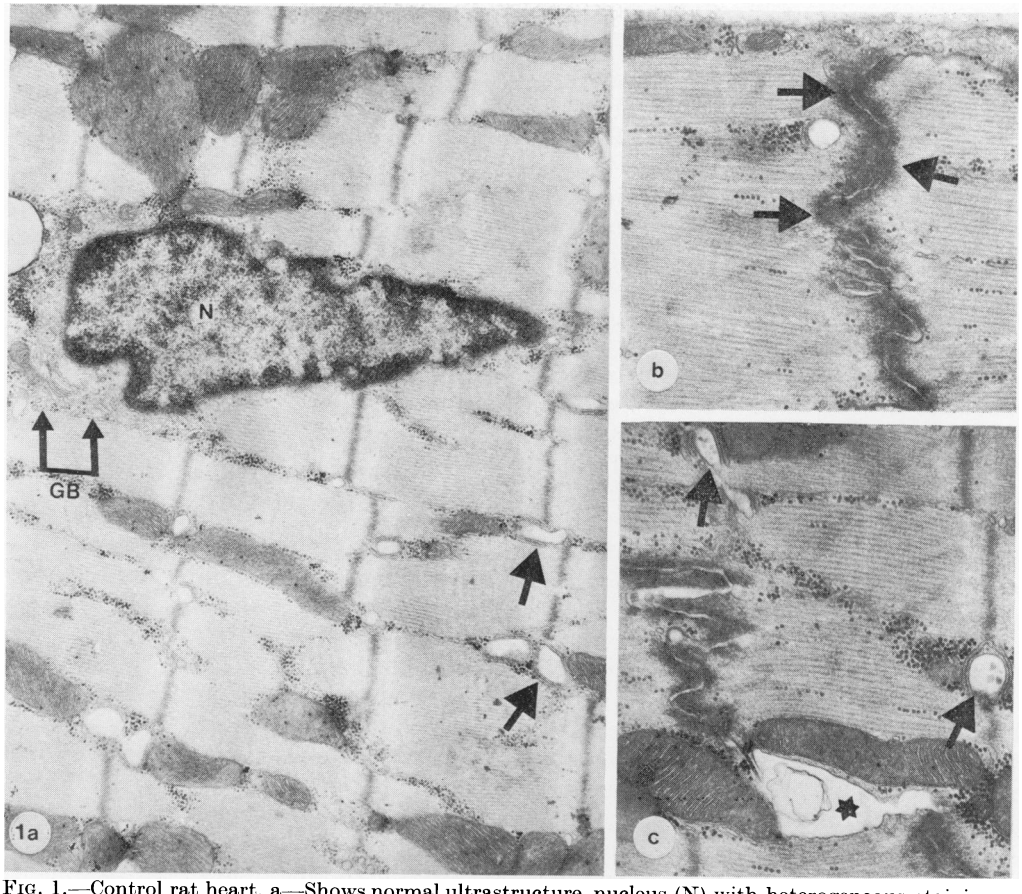

FIG. 1.—Control rat heart. a—Shows normal ultrastructure, nucleus (N) with heterogeneous staining of the chromatin, Golgi bodies (GB), and transverse tubules flanked by the sarcoplasmic reticulum (arrows). (×13,000). b—Shows cell-surface material; the intercalated disc (arrows) has electron-dense material on the cytoplasmic side. (×15,000). c—Shows dilation of the intercalated disc between parallel fibres (star). Note presence of granules in the lumen of T-tubules (arrows). (×15,500). (Uranyl acetate and lead citrate.)

were also noticed elsewhere in the cytoplasm. The nucleoplasm which was heterogeneously stained in the control hearts (Fig. 1a) had lost this characteristic appearance after Ca^{2+}-free perfusion (Fig. 2b). It should be mentioned that nuclear chromatin in the non-muscle cells in the same preparation was normally stained and was heterogeneous in appearance. The intercalated disc had lost the electron-dense staining material on the cytoplasmic side (Fig. 2c). The separation of the intercalated disc between the parallel myofibres and the presence of membranous structures in the intercellular space of these hearts (Fig. 2c) was also seen in the control hearts (Fig. 1c). The middle lamina in the pentalaminar arrangement of the tight junctions in the intercalated disc was conspicuous owing to intense staining (Fig. 2d, e). Other ultrastructural details such as distribution of glycogen, appearance of cell-surface material, myofilaments, mitochondria and sarcoplasmic reticulum were quite comparable to those of control hearts.

Effects of reperfusion with different concentrations of Ca^{2+}

Hearts perfused with Ca^{2+}-free medium for 5 min were reperfused for 5 min with Krebs–Henseleit solution containing dif-

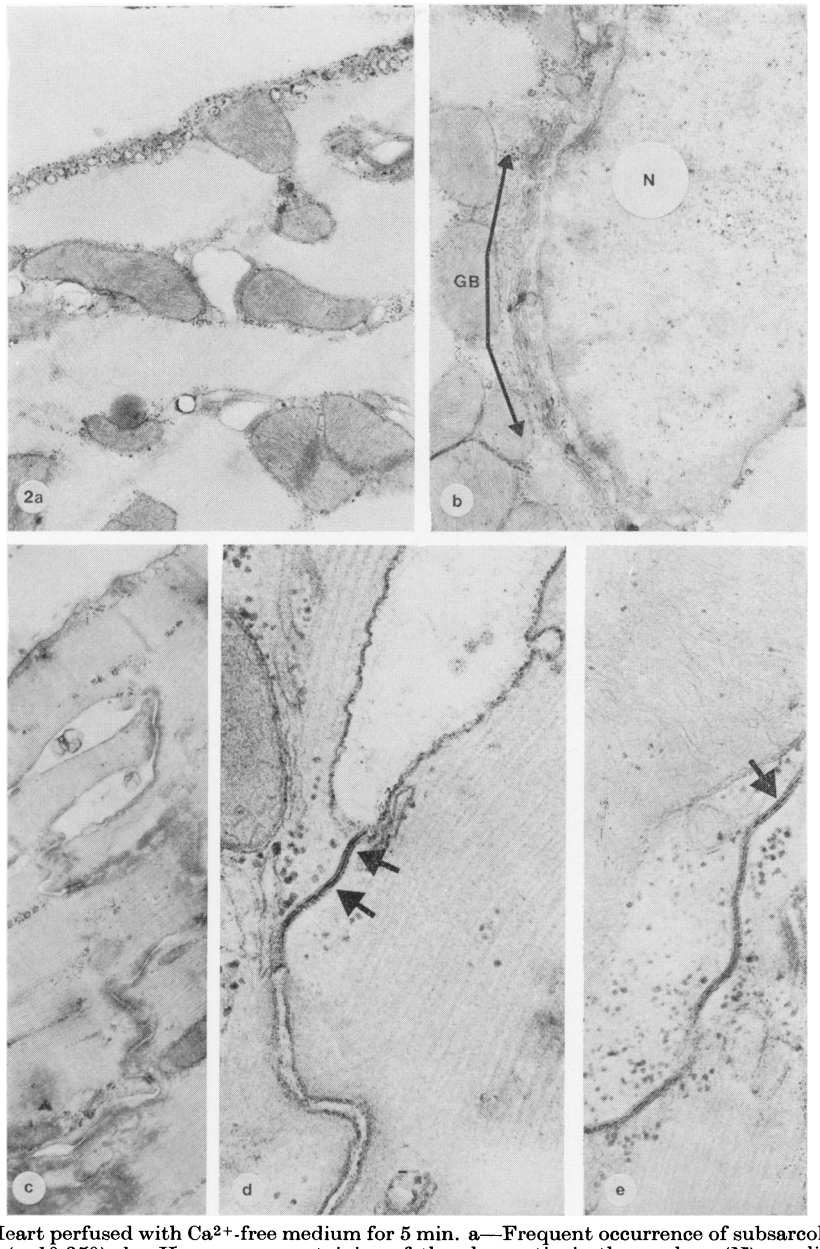

FIG. 2.—Heart perfused with Ca^{2+}-free medium for 5 min. a—Frequent occurrence of subsarcolemmal vesicles. (×10,350). b—Homogeneous staining of the chromatin in the nucleus (N); proliferated juxtanuclear Golgi body (GB). (×13,125). c—Intercalated disc showing reduced electron-dense material on the cytoplasmic side. (×13,125). d and e—Intense staining (arrows) of the middle lamina in a tight junction. (×46,125). (Uranyl acetate and lead citrate.)

ferent concentrations of calcium (0·05, 0·5, 1·25, or 4 mM Ca^{2+}) and were then perfusion-fixed for electron microscopic examination. Reperfusion of the Ca^{2+}-deprived hearts with a medium containing 0·05 mM Ca^{2+} resulted in only minor structural alteration (Fig. 3a, b). The frequency of occurrence of subsarcolemmal vesicles was reduced in comparison to that of Ca^{2+}-deprived hearts. The staining of nucleo-

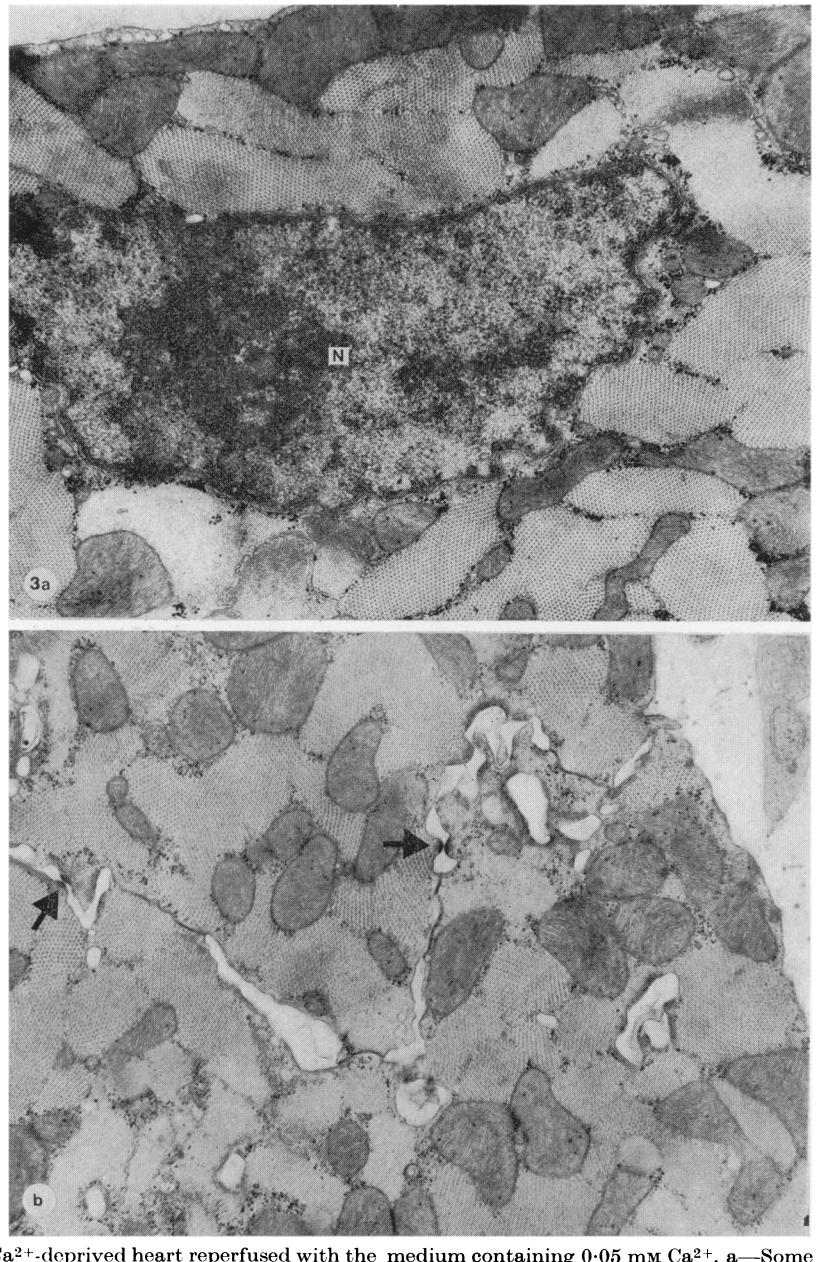

FIG. 3.—Ca^{2+}-deprived heart reperfused with the medium containing 0·05 mM Ca^{2+}. a—Some heterogeneity in the nucleus (N) has reappeared. b—Intercalated disc in a transverse view; some separation of desmosomes (arrows) can be seen. (Uranyl acetate and lead citrate ×12,930.)

plasm was weakly heterogeneous (Fig. 3a). The intercalated disc had tight and gap junctions; however, some widening of the gap between membranes in the desmosomes was noticed (Fig. 3b). Other ultrastructural details were unchanged and comparable to those in the Ca^{2+}-deprived hearts.

When the Ca^{2+}-deprived hearts were reperfused with a medium containing 0·5 mM Ca^{2+}, the structural damage to some of the fibres was quite pronounced

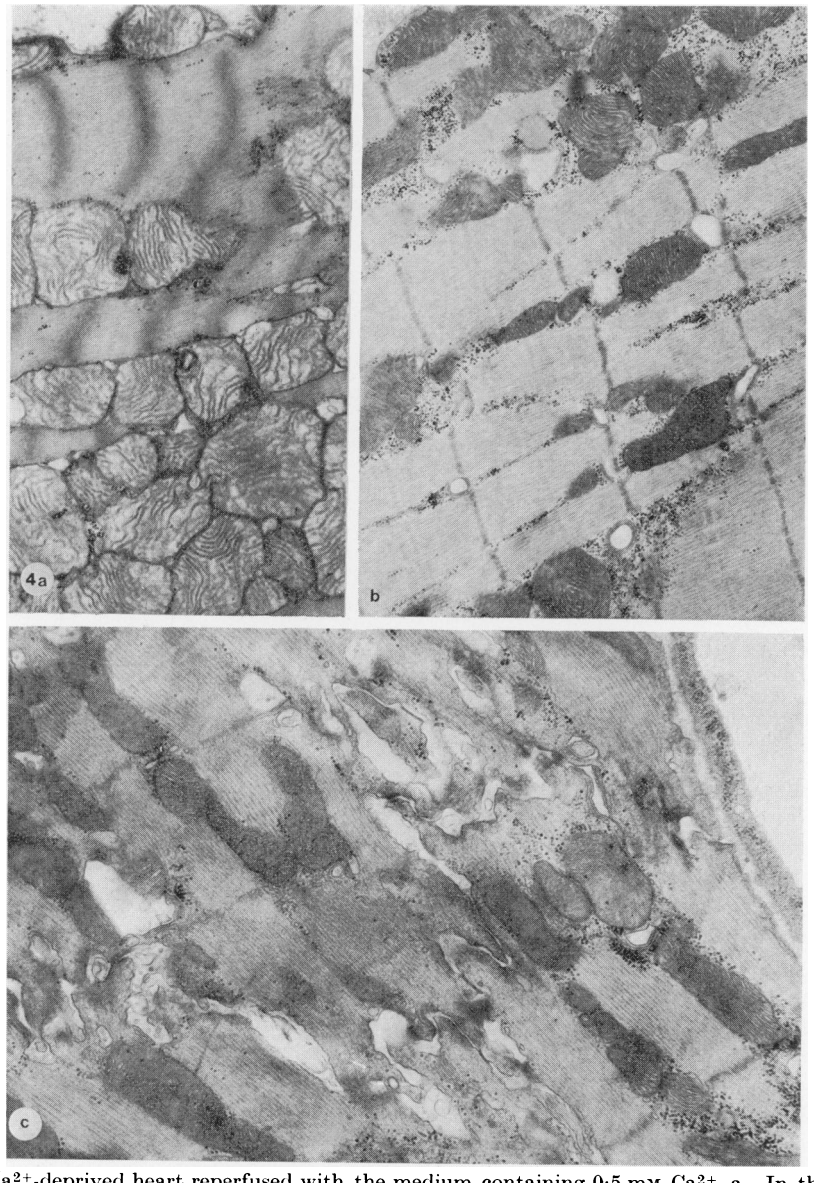

FIG. 4.—Ca^{2+}-deprived heart reperfused with the medium containing 0·5 mM Ca^{2+}. a—In this type of damage contraction bands, swollen mitochondria and disruption of cristae can be seen. (×10,660). b—This micrograph is also from the same thin section as that of Fig. a. Here sarcomeres are contracted but rest of the ultrastructural details look normal. (×10,660). c—A progress in the separation of the intercalated disc is apparent irrespective of the extent of damage to the myocardial cell. (×13,125). (Uranyl acetate and lead citrate.)

(Fig. 4a). In these fibres the contraction bands were apparent and the mitochondria were swollen with some disruption of their cristae. However, other myocardial cells in the same section had only contracted sarcomeres in otherwise normal-looking ultrastructure (Fig. 4b). The appearance of the nucleoplasm in both damaged and so-called undamaged cells was heterogeneous and quite comparable. The intercellular gap in the intercalated disc was increased and the area of membranes in

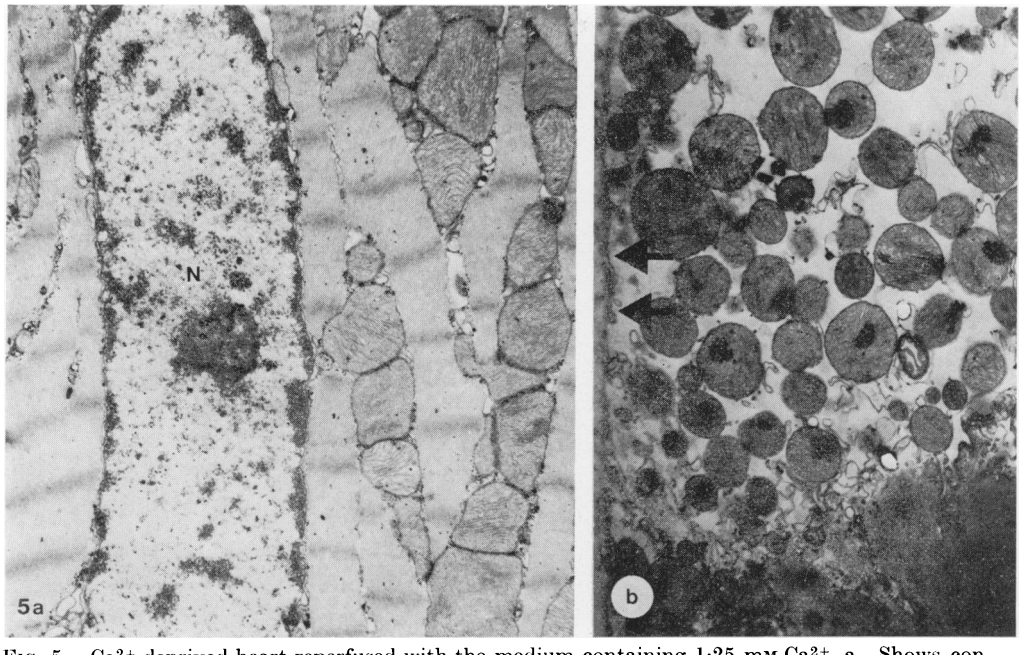

FIG. 5.—Ca^{2+}-deprived heart reperfused with the medium containing 1·25 mM Ca^{2+}. a—Shows contraction bands, swollen mitochondria and heterogeneous staining of the chromatin in the nucleus (N). b—extensive necrosis of the myocardial cell: myofilaments have fused into a mass, accumulated mitochondria are still bounded by the sarcolemma (arrows), formation of membranous inclusions and cell debris is apparent. (Uranyl acetate and lead citrate ×13,500.)

the specialized junctions was reduced (Fig. 4c). The pentalaminar and septalaminar arrangements in the tight and gap junctions respectively were no longer apparent. Furthermore, these ultrastructural changes in the intercalated disc were noticeable in the whole myocardium, irrespective of the extent of damage to the myocardial cell. The Golgi bodies were less frequent in these hearts. The presence of cell-surface material was apparent.

Reperfusion of Ca^{2+}-deprived hearts with a medium containing 1·25 mM Ca^{2+} resulted in extensive damage to some of the fibres (Fig. 5a, b). The myofilaments in some of these badly damaged fibres had changed into a mass of electron-dense material and mitochondria were seen accumulated on the sides (Fig. 5b). Contraction bands and swollen mitochondria were also apparent (Fig. 5a). Accumulation of glycogen granules had occurred among myofibrils as well as near the intercalated disc. The structural details in some other fibres were well maintained in as much as myofilaments, mitochondria, sarcoplasmic reticulum and Golgi bodies were still apparent and normal-looking. These so-called mildly damaged fibres showed dilation of the T-tubules and contraction of sarcomeres. In both badly damaged and slightly damaged fibres the separation of the cells at the intercalated disc was apparent, with some focal contacts still remaining. The nucleoplasm was heterogeneous in appearance.

Reperfusion of Ca^{2+}-deprived hearts with 4 mM Ca^{2+} also caused extensive damage to most of the muscle fibres (Fig. 6a–d). Swollen mitochondria were grouped on the side and myofilaments were seen as a mass (Fig. 6a). In extreme cases of damage, abortive membrane structures loaded with glycogen were present in the intercellular space (Fig. 6c). These abortive vesicles in some cases also contained mitochondria. Some of the fibres, on the other hand, still had discernible though contracted sarcomeres,

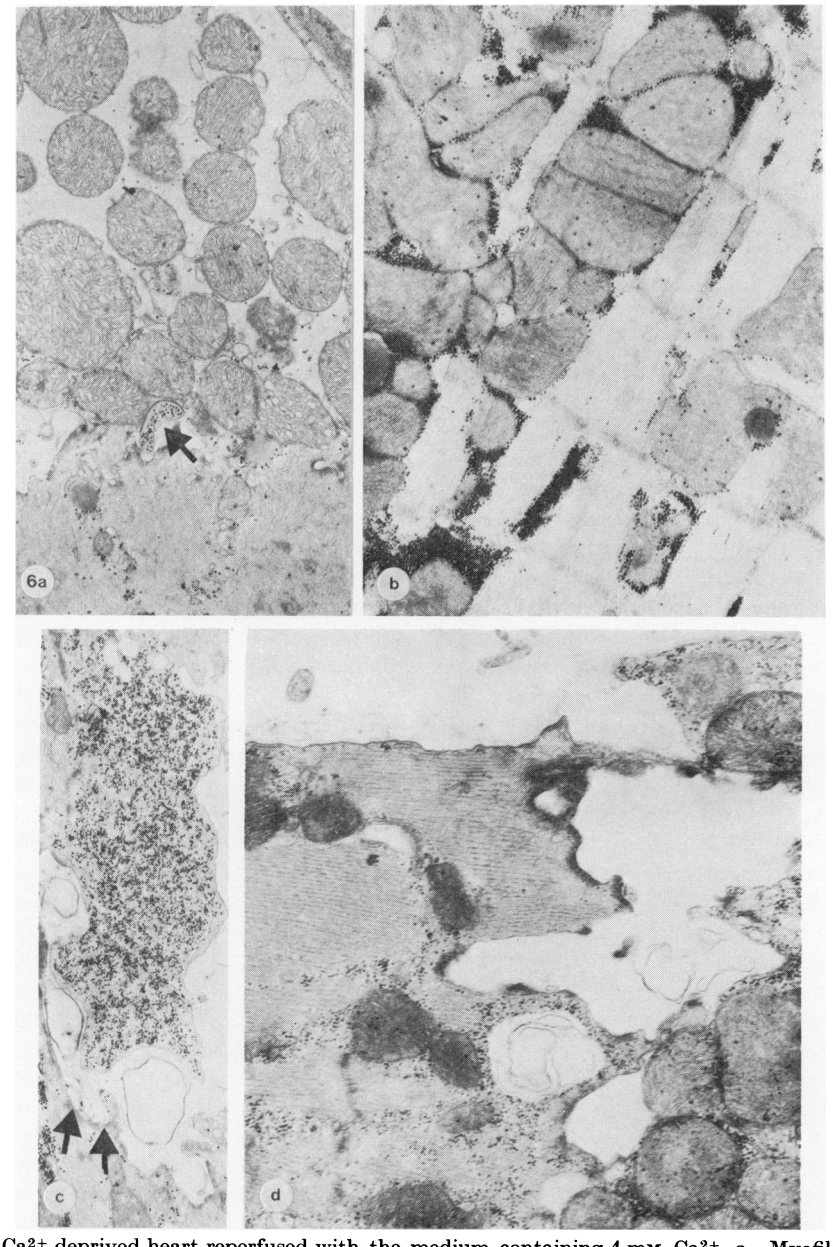

FIG. 6.—Ca^{2+}-deprived heart reperfused with the medium containing 4 mM Ca^{2+}. a—Myofilaments have changed into a mass, mitochondria are swollen and membrane-bound glycogen is also apparent (arrow). b—Shows glycogen accumulation, swollen mitochondria, contracted sarcomeres. c—Membrane-bound glycogen accumulated in the intercellular space. Arrows mark the separated intercalated disc. d—Shows accumulation of mitochondria as well as glycogen near the separated intercalated disc. (Uranyl acetate and lead citrate $\times$ 10,225.)

and showed swollen mitochondria, glycogen accumulation and dilated T-tubules (Fig. 6b). More or less complete separation of the intercalated disc was apparent (Fig. 6d). Nucleoplasm had a heterogeneous appearance.

DISCUSSION

Ever since the suggestion that some changes in the heart occur during perfusion with Ca^{2+}-free medium, which make it sensitive to the reinstitution of calcium supply (Zimmerman and Hülsmann, 1966; Zimmerman *et al.*, 1967), attempts have been made to find and define structural changes due to Ca^{2+}-free perfusion. However, light microscopic study of the hearts perfused with Ca^{2+}-free medium for 1 h in the same report (Zimmerman *et al.*, 1967) did not reveal any structural changes and electron microscopic examination demonstrated only a dilated transverse tubular system. Other studies have revealed separation of the intercalated discs after 10–50 min perfusion with Ca^{2+}-free medium (Muir, 1967; Tomlinson *et al.*, 1974; Yates and Dhalla, 1975) and separation of the basal lamina from the sarcolemma was observed after 40 min perfusion with Ca^{2+}-free medium (Tomlinson *et al.*, 1974). The present study clearly demonstrates that even 5 min perfusion with Ca^{2+}-free medium has both sarcolemmal and intracellular effects as is indicated by the increased pinocytotic activity and altered intracellular details. The intense staining of the middle lamina of the tight junction as well as the loss of electron-dense material from the cytoplasmic side of the intercalated disc after Ca^{2+}-free perfusion may also indicate perturbation of the membrane structure and may be related to the decreased stability of the junction. This may explain the reported separation of cells upon continued perfusion with Ca^{2+}-free medium and will also influence electrical coupling of the cells. Study of this area with the freeze-fracture technique will provide better insight into this membrane perturbation.

The loss of staining of nuclear chromatin in the Ca^{2+}-deprived hearts appears to be due to lack of calcium, as the characteristic appearance of the chromatin returned upon the reinstitution of calcium supply. This contention is further supported by the fact that the intensity of chromatin staining increased with an increase in the concentration of calcium in the reperfusion medium. The lack of any change in the chromatin staining of the non-myocardial cell nuclei in response to Ca^{2+}-free perfusion may indicate a muscle specificity of this effect of calcium deprivation.

It has been shown that reperfusion of Ca^{2+}-deprived hearts with a Ca^{2+}-containing medium results in the increase of intracellular calcium (Dhalla *et al.*, 1976). Further, this increase in intracellular calcium is directly related to the amount of calcium present in the reperfusion medium under similar experimental conditions (Alto *et al.*, 1978). It is then conceivable that increase in structural damage in response to increase in the Ca^{2+} concentration of the reperfusion medium is related to the amount of intracellular Ca^{2+} overload.

Separation of intercalated disc upon the reinstitution of calcium supply in calcium-deprived hearts observed in this study has also been reported by others (Zimmerman *et al.*, 1967; Yates and Dhalla, 1975). However, the present observations reveal that separation of the cells upon resumption of the calcium supply occurs in stages and appears to proceed in the following manner. The visible process is initiated by widening of the gap between the paired membranes in non-specialized areas of the intercalated disc. This is followed by the separation of desmosomes which is also accompanied by a reduction in the area of membranes engaged in the tight and gap junctions. Eventually separation of these junctions also takes place, thus completing cell–cell separation. A similar mode of cell separation at the intercalated disc has been reported in rat hearts subjected to prolonged Ca^{2+}-free perfusion (Muir, 1967). This may mean that separation of the

intercalated disc, once triggered, would proceed on more or less similar lines irrespective of the stimulus. It should be noted that cell damage due to Ca^{2+} overload on the other hand is dependent on the mode of its introduction (Singal *et al.*, 1978).

The occurrence of 2 extremes in the myocardial cell damage in the same heart in response to the same amount of Ca^{2+} overload is intriguing, but the chances of these results being an artefact are negligible for 2 reasons: (a) the level of separation of the intercalated discs in any heart reperfused with a medium containing 0·5 mM or more calcium was quite comparable, indicating that conditions of the experiment were equally generated in all areas of the heart; and (b) the intracellular response such as dilation of the T-tubules and nuclear staining were also comparable in the whole myocardium. The observation may suggest that the cellular response to a similar Ca^{2+} overload is different. In this regard it should be mentioned that differences in membrane permeability of the myocardial cells in a coronary-occluded dog heart are known to occur (Ashraf, White and Bloor, 1978). However, it is also possible that all the cells are destined to reach the same state of damage and what we are looking at is the different levels of advancement in cell necrosis.

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