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The effects of divalent cations on the ultrastructure of the perfused rat heart

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INTRODUCTION

The isolated perfused heart is a profitable tool in biochemical, physiological and pharmacological investigations, but little use has been made of this system in experimental histology. Jennings, Marchesi & Florey (1962) used the perfused rat heart to study the transport of particles across capillary endothelium and Davies, Francis, Wood & Johnson (1956) dissociate the roles of the nerve and Purkinje fibres in the conduction of the cardiac impulse in the isolated dog heart. In the present paper the ultrastructure of the perfused rat heart is defined and the effects of varying the divalent cation content of the perfusing fluid are studied.

It is now established that calcium ions are necessary to stabilize the intercellular cement materials in plants and invertebrate animals. The early work of Herbst (1900) and Chambers (1938) on echinoderm larvae and Gray (1926) on mussel gill epithelium are reviewed with many more recent studies by Rinaldini (1958) and Weiss (1960). However, the role of divalent cations in maintaining intercellular adhesion in vertebrates is less clear, since, in these species, the normal control of the internal ionic environment excludes simple deprivation experiments. Although methods for isolating mammalian tissue cells for culture (Moscona, 1952; Paul, 1965) usually recommend mincing in calcium- and magnesium-free solutions, the effectiveness of this procedure does not seem to be proven experimentally. Moscona, Trowell & Willmer (1965) review the evidence and conclude that the role of these ions in preserving cell contact becomes less important in adult tissues. Most experiments on mammalian tissues depend on the application of chelating agents such as ethylene diamine tetra-acetic acid (Versene, EDTA), either directly to blocks of tissue or by intravascular perfusion. Because of their unspecific chelation and possible direct toxic effects, chelating agents are not employed in the present work, where the changes in structure due to the simple deprivation of calcium and other divalent cation ions are observed.

Cardiac muscle is known to be composed of individual cells, fused at their intercalated discs (Sjöstrand & Andersson-Cedergren, 1960; Muir, 1965), and this tissue is used here to determine the role of calcium in maintaining cellular adhesion at the discs. The experiments are performed on isolated perfused rat hearts by varying the constitution of the perfusing medium while simultaneous recordings are made of the contractile force of the ventricle and the electrocardiograph. Any consequent structural change can then be examined after fixation of the specimen by intravascular perfusion with any selected fixative.

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The omission of calcium from the perfusate inhibits the contraction of cardiac muscle (Ringer, 1883), but, although no mechanical contraction is occurring, an almost normal electrocardiograph continues in the absence of this cation (Locke & Rosenheim, 1907; Mines, 1913). The presence of a sinus rhythm electrocardiograph, even if not accompanied by a contraction, indicates that impulse conduction throughout the myocardium is continuing, and that any effect of calcium removal on intercellular impulse conduction would be made obvious by changes in the pattern of this excitation.

A preliminary report of this investigation is available (Muir, 1966).

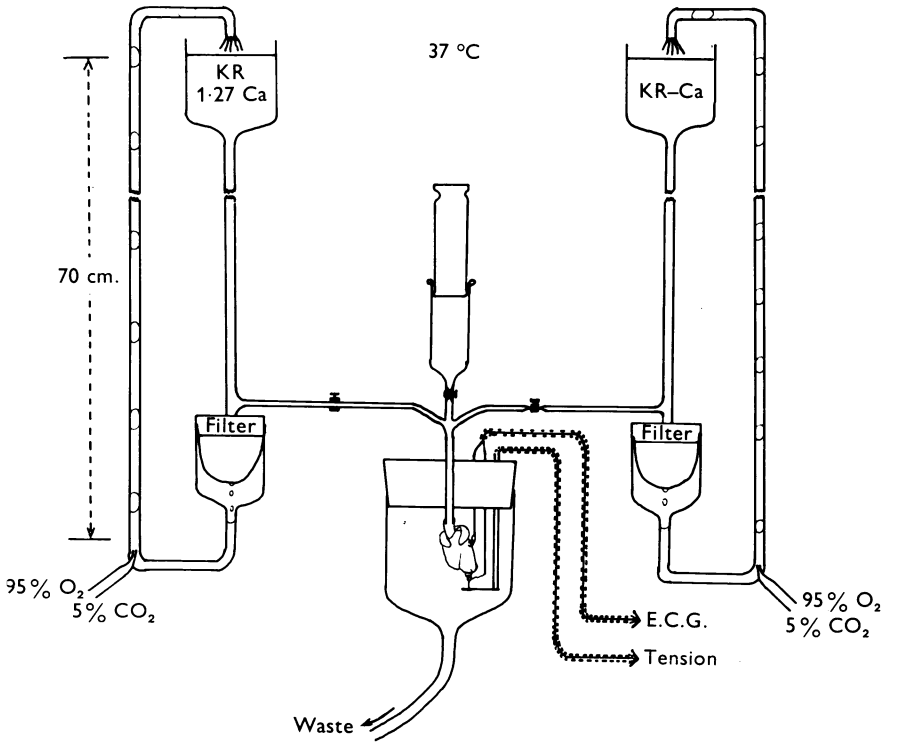


Fig. 1. Apparatus diagram.

MATERIAL AND METHODS

Hearts from adult white rats, weighing 200–350 g, are used in this study. All specimens are obtained by the following *in vitro* perfusion technique.

The apparatus (Fig. 1) consists of a pair of hydrostatic columns either of which can supply their oxygenated, filtered perfusing solutions at 37 °C to a single warm chamber containing the heart. The effluent solution from the heart can be measured and analysed; it is not recirculated. The electrocardiograph is recorded directly from clips on the right atrium and left ventricle and connected to one channel of a Solartron CD 1014 oscilloscope with a deflexion of 10 mV/cm. The other channel receives the amplified output from a strain gauge (Electro-Physiological Instruments) attached

to the apex of the left ventricle. Permanent recordings are made by photographing the screen or by an ink-writing electroencephalograph.

Under chloroform anaesthesia, the thorax is opened and the pericardium and thymus gland dissected away from the heart and great vessels. The venae cavae and pulmonary vessels are cut and the aorta divided at the outlet of the right brachiocephalic artery; a polythene cannula, 2 mm diameter, is tied into the proximal part of the aorta, care being taken not to reach or damage the aortic valve cusps. If this valve is made incompetent, the hydraulic pressure of the column is instantly and constantly applied to the inner wall of the left ventricle, thus occluding the coronary circulation in the deeper layers of the wall of the left ventricle. Hence, after fixation, the middle layer of the wall of this ventricle is found unfixed, the inner layer being fixed by diffusion from the cavity. When the cannula is positioned correctly above the aortic valve, the heart slowly distends with perfusing solution as the blood is washed from the capillaries into the chambers of the heart; only these specimens are used and, as an additional precaution, the right ventricular wall is examined since this is well perfused even if the aortic valve is damaged. Care is taken to avoid air bubbles in the cannula and the time from cutting the vessels to established perfusion must not exceed 30 s. The clips for the strain gauge and electrocardiogram are attached and the heart placed in the 37 °C chamber. At least 5 min perfusion with the control medium is always allowed before an experimental solution so that a constant rate and strength of beat can be established and recorded.

The perfusing solutions are of the Krebs-Ringer type; the formula for its inorganic salts is given in a previous paper (Muir, 1965), which recommends the use of this solution as a medium for washing out the coronary vessels prior to fixation, and as a diluent for the fixing agent. For this purpose, a pure electrolyte solution is satisfactory, since the perfusion need not continue for more than 5 min before fixation. Hearts undergoing prolonged perfusion with this solution show some weakening of the beat after 1 h, and cease to beat within $2\frac{1}{2}$ h. Thus it was found necessary in the longer-term experiments now reported to add glucose (80 mg/100 ml) to the balanced salt solution so as to main a strong regular beat for more than 6 h.

The solutions are prepared with Analar grade reagents (B.D.H., Poole, England) in distilled water; the molar concentrations of their components are given below (mM/l):

Control (KR 1·27 Ca)

Na^+	143·5	Cl^-	125·8
K^+	5·9	HCO_3^-	24·8
H^+	1·2	HPO_4^{2-}	1·18
Mg^{2+}	0·61	SO_4^{2-}	0·61
Ca^{2+}	1·27	Glucose	4·44

The osmolarity of this solution, assuming complete dissociation, is 309·3 milliosmoles. All experimental solutions are adjusted to an osmolarity of between 305 and 315 mosm with NaCl.

Calcium-free (KR − Ca). Omit the 1·27 mM-Ca^{2+}. Contamination of the sodium chloride, potassium chloride and sodium bicarbonate reagents with Ca^{2+} and Mg^{2+} is stated to be less than 0·005, 0·005 and 0·01 % respectively. Analysis of the KR − Ca

solution on an atomic absorption spectrometer shows that it contains 0·0052 mM-Ca^{2+}, i.e. 0·4 % of its concentration in KR 1·27 Ca.

Calcium-reduced (KR 0·5 Ca), (KR 0·1 Ca), etc., contain these millimolar concentrations of Ca^{2+}.

Magnesium-free (KR − Mg). Omit the 0·61 mM-$MgSO_4$ from the control solution.

Magnesium- and calcium-free (KR − Mg − Ca). Omit the 0·61 mM-$MgSO_4$ and the 1·27 mM-Ca^{2+}.

Strontium-replaced (KR − Ca + Sr). 1·27 mM-Sr^{2+} as $SrCl_2$ is added to KR − Ca.

Barium-replaced (KR − Ca − $MgSO_4$ + Ba). Omit the 0·61 mM-$MgSO_4$ and 1·27 mM-Ca^{2+} and add 1·27 mM-Ba^{2+} as $BaCl_2$.

Fixation at the end of the experiment is by either 1 % osmium tetroxide (39 mmol/l) or 2 % glutaraldehyde (200 mmol/l) made up in whichever solution is then perfusing the heart. The fixative, at 37 °C, is injected from a syringe into the cannula; 10 ml of fixative is sufficient for the adult rat heart.

The right ventricular wall is removed and cut through its whole thickness into 2 × 2 mm squares. In this form the osmium-fixed material remains in the fixative for 1–2 h. Glutaraldehyde-fixed tissue is transferred to the osmium fixative for a similar period of osmication. The tissues are embedded in Araldite and cut from their epicardial surface on a Porter Blum microtome equipped with a glass knife. Sections one micrometre (μm) thick, mounted on slides, are stained with toluidine blue/pyronin (Ito & Winchester, 1963) for light microscopy. Sections for the electron microscope are mounted on Athene 483 grids, without a supporting membrane, and stained with uranyl acetate/lead citrate (Reynolds, 1963) before examination in an A.E.I. EM6 microscope.

RESULTS

Control hearts perfused with KR 1·27 Ca

No change in the electrocardiograph or strength of the beat is detected in a group of twelve hearts after continuous perfusion for 2–6 h with the control medium. The rate of flow through the coronary circulation varies from 350 to 450 ml/h. As the experimental material is perfused for less than 2 h, three of the control specimens were fixed after 2 h perfusion. The structure of the muscle fibres in these hearts is the same as after shorter perfusion periods (Muir, 1965), where the structure is shown to correspond with that found in fresh unperfused hearts after many different types of immersion fixation (Moore & Ruska, 1957; Stenger & Spiro, 1961). The main difference in structure is the maintenance, after intravascular fixation, of all the myofibrils at similar sarcomere length of 2·1–2·3 μm. Intercalated discs are easily recognized in the plastic-embedded light-microscopy specimens (Fig. 2) as a line or staircase of dark plates crossing the fibres at the junction between adjacent cardiac muscle cells. At the very edges of the blocks, where they are cut from the right ventricular wall, some of the fibres are damaged and fragmented, otherwise all the fibres are intact.

The characteristic features of the sarcolemma and the junctional region between cells at an intercalated disc are shown in Fig. 3. The sarcolemma consists of a well-defined, narrow, osmiophilic layer, the plasma membrane, which is separated from the diffuse material of the basement membrane by a gap of about 15 nm. The base-

ment membrane continues from cell to cell, with the two plasma membranes turning in at the junction to traverse the fibre axis. The relationships between these membranes at the discs are described fully in an earlier paper (Muir, 1965); myofibrillar insertion plaques, desmosomes and a quintuple-layered membrane junction are indicated in Fig. 3. A higher magnification of such a junction between the membranes shows that the centre of the membrane junction appears as a row of dots, with a spacing of about 9 nm. As other views show a central line and since the periodicity of the dots is considerably smaller than the section thickness, it is possible that the dots represent linear densities on the outer lamellae of the coherent plasma membranes.

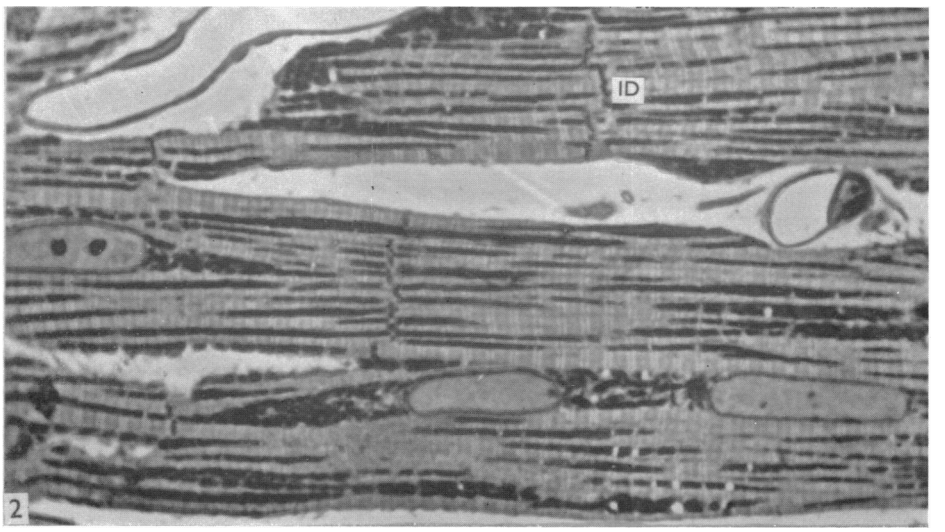
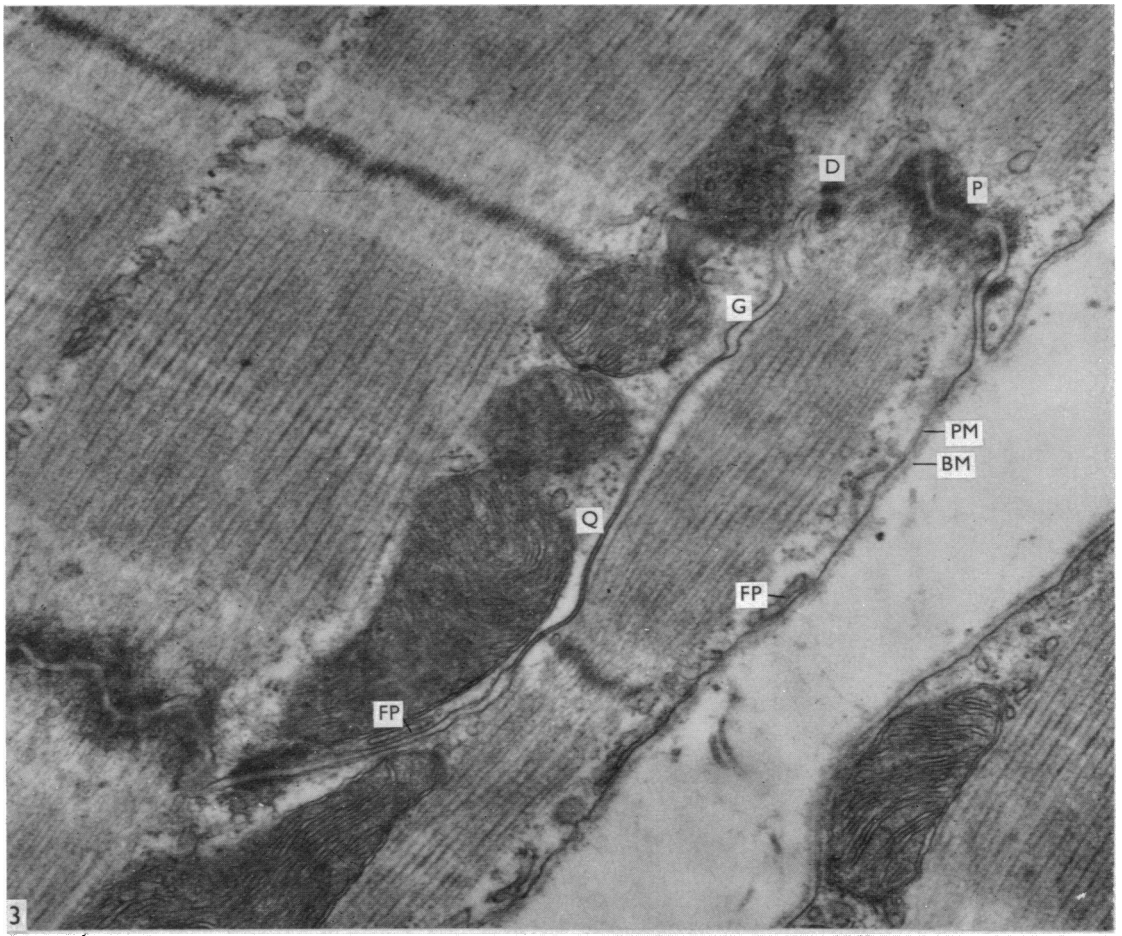


Fig. 2. Light micrograph of right ventricular rat cardiac muscle. The lower cell contains two nuclei and numerous mitochondria, in the perinuclear sarcoplasm and between the myofibrils. The myofibrils are all preserved in a similar state of contraction, sarcomere length 2·2 μm. The intercalated discs (ID) mark the junctions between adjacent cells. Between the branching muscle fibres, the capillaries are distended and empty due to perfusion. KR 1·27 Ca for 90 min. Osmium fixation. Araldite. Toluidine blue/pyronin. × 1350.

The sarcoplasm contains a very large number of ovoid mitochondria. Their long axes are parallel to the myofibrils and, although they occupy most of the space between the myofibrils, they rarely cross the plane of the Z discs. A profuse and complex arrangement of membrane tubules surrounds the myofibrils and has an organized relationship to the banding pattern of the myofibrils (Figs. 5, 6). Close to the level of the Z discs, a relatively large T-tubule, with a diameter of about 0·25 μm, forms a network across the fibre, occasionally communicating by longitudinal branches with the corresponding tubular network near the level of the next Z disc. A plexus of finer tubules, diameter 60 nm, embraces the myofibrils to form the sarcoplasmic reticulum. The plexiform arrangement of these tubules is uniform throughout the length of the sarcomere. Flattened saccular extensions from the sarcoplasmic reticulum are applied as footplates to the surface of the T-tubules.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P
G
PM
BM
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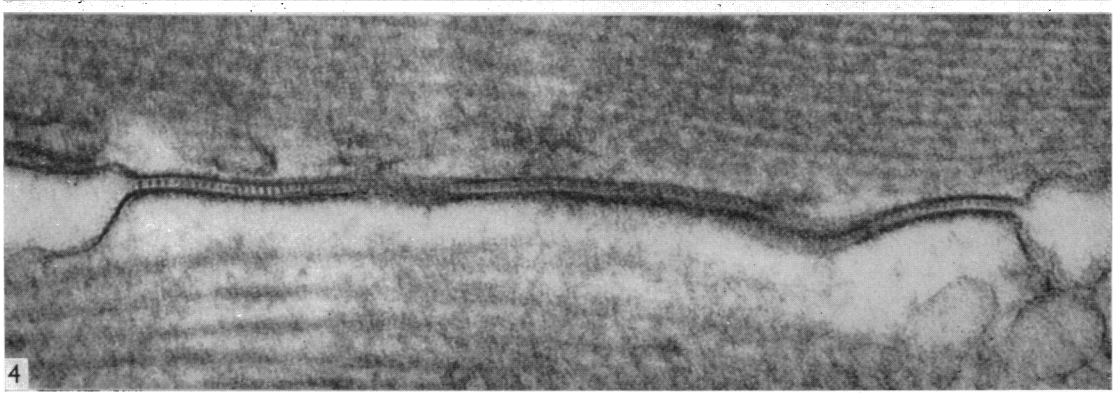
FP
3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Other footplates are applied to the deep surface of the sarcolemma on both sides of the Z discs and to the plasma membranes forming the intercalated discs (Fig. 3). In the discs, it is noted that the footplates are applied to the plasma membrane only at unspecialized areas of the membrane which abut onto an intercellular space. Each footplate is about 30 nm thick and its lumen is bisected by a faint central line. The wall of the T-tubules is formed by a single membrane less than 2 nm thick; there is no lining of basement membrane as on the surface plasma membrane (Fig. 5). Spherical granules which have a darker outer layer are occasionally seen in the lumen of the T-tubule (Fig. 6).

Calcium-deprived hearts (brief perfusion with KR–Ca)

Within 1 min of transferring the heart to the calcium-free medium, ventricular contraction ceases, although the electrocardiograph continues with a normal amplitude and approximately half the normal rate (Fig. 7, ii). After fixation in this flaccid state (Fig. 8) no changes in the general structure or the membrane relationships at the intercalated discs are observed (Fig. 4). In particular it is noted that the membranes and luminal contents of the sarcoplasmic reticulum and the T-tubules are the same as in the control heart (Fig. 8).

Calcium-restored hearts (KR 1·27 Ca after a brief period of KR − Ca)

When the calcium-containing solution reaches the flaccid ventricle there is an immediate restoration of movement with a short period of irregularity and doubling of the beat before the electrocardiograph and its now associated mechanical contraction return to normal (Fig. 7, iii). The structure of these calcium-restored specimens is, as might be expected, the same as in the control and calcium-deprived hearts.

Calcium-exhausted hearts (prolonged perfusion with KR − Ca)

If the motionless heart is left perfusing with the calcium-free medium for a variable but considerable time, the sinus rhythm of the electrocardiograph (Fig. 7, ii) suddenly changes into a flutter, then a fibrillation, before all recordable electrical activity ceases (Fig. 7, iv). Restoration of calcium (KR 1·27 Ca) does not now produce any movement or return of the electrocardiograph. A series of fifteen hearts were perfused with the control solution for periods varying from 5 to 40 min before transferring to the calcium-free medium. After transfer, the mean time for development of a flutter in thirteen of these specimens was 12·1 min (range 3–31 min); the remaining two specimens showed a gradual loss of amplitude of the sinus rhythm until the activity ceased after 72 and 90 min. The period of perfusion on the control

Fig. 3. Electron micrograph of an intercalated disc from a control specimen. The basement membrane (BM) continues from cell to cell, but the paired plasma membranes (PM) turn inwards to transect the fibre. The plasma membranes pass through myofibrillar insertion plaques (P), desmosomes (D), quintuple-layered fusions (Q) and unspecialized regions (G) during their course. Footplates (FP) of the sarcoplasmic reticulum are applied to the sarcolemma and the unspecialized membranes of the discs. KR 1·27 Ca for 12 min. Osmium fixation. × 33000.

Fig. 4. Quintuple-layered membrane fusion showing the 9 nm periodicity of the central densities. Normal structure taken from a calcium-deprived specimen. KR − Ca for 3 min. Osmium fixation. × 133000.


FP
T
BM
Z
SR2
T1
SR1
FP
FP
T
Z
5
6

medium does not affect the time required for electrical changes to develop when subsequently perfused with the calcium-free solution. The quantity of calcium-free solution necessary to produce the changes of calcium exhaustion obviously varies with the time range given above, the average volume being 170 ml.

Twelve specimens were fixed, 5–20 min after the electrical activity had ceased, with the fixative dissolved in KR – Ca. All these specimens show a marked change in myocardial structure. Light-microscopic examination of the plastic-embedded specimens shows that the muscle fibres have separated into isolated segments (Fig. 9).

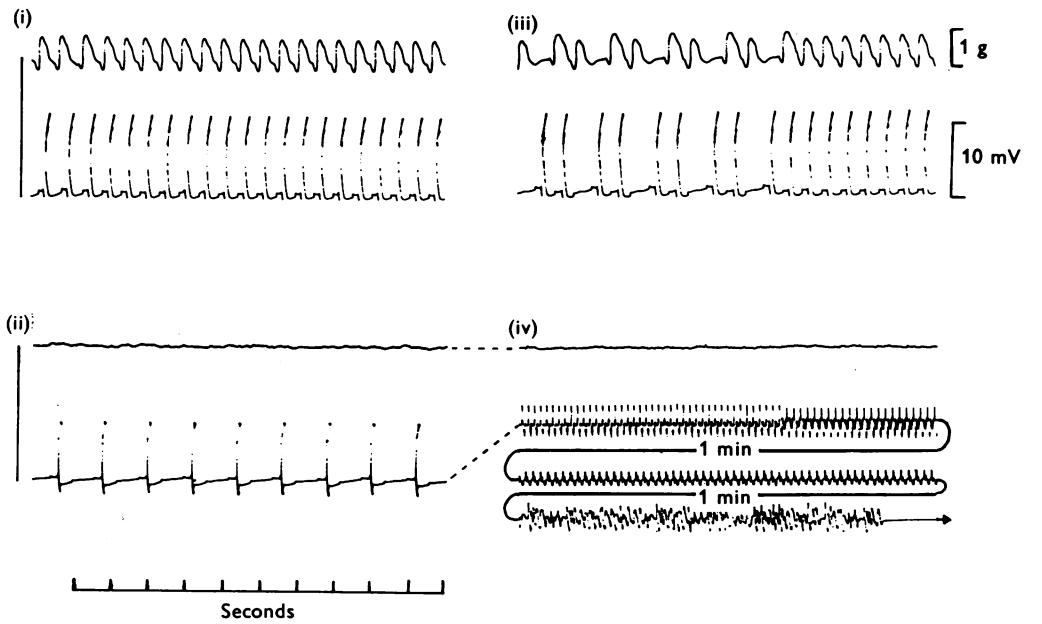
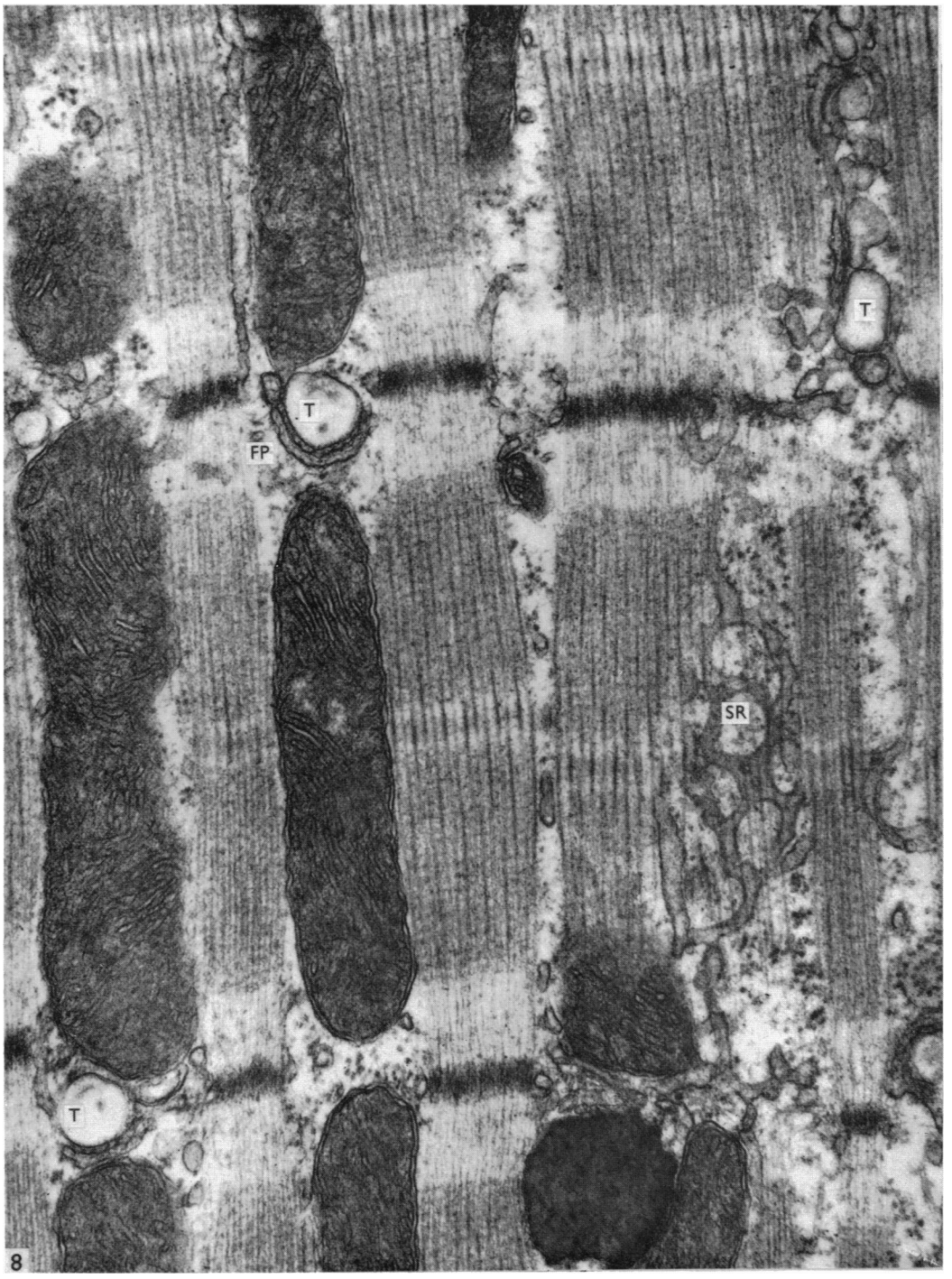


Fig. 7. Recordings of the contractile force (upper traces) and electrocardiograph (lower traces). (i) Control KR 1·27 Ca; (ii) calcium-deprived KR – Ca; (iii) calcium-restored KR 1·27 Ca after KR – Ca for 5 min; (iv) calcium-exhausted KR – Ca for 24 min.

Although a few intercalated discs can be recognized, most have disappeared. Dark plaques at the end of the muscle segments suggest that the segmentation is due to separation of the discs; this is confirmed by low-power electron micrographs (Fig. 10). The crenated dense plaques receiving the insertion of the myofibrils are seen at the end of each segment; in the example shown the complementary patterns on the ends of the two segments are apparent. This is not always observed owing to movements

Fig. 5. Longitudinal section of a control fibre showing the arrangement of internal membranes. The T-tubules (T) close to the level of the Z discs occasionally communicate with those at the level of the next Z disc (T_1). The basement membrane (BM) does not line the T-tubules. The sarcoplasmic reticulum is seen in surface view (SR_1) and in transverse section (SR_2) and, at the arrows, it extends to form footplates (FP) applied to the T-tubules. KR 1·27 Ca for 7 min. Glutaraldehyde fixation. × 42 000.

Fig. 6. A T-tubule, embraced by a footplate from the sarcoplasmic reticulum, contains some spherical granules in its lumen. KR 1·27 Ca for 8 min. Osmium fixation. × 80 000.


T
T
FP
SR
T
8

of the isolated segments at right angles to the plane of section. All the transverse fissures across the fibre axis examined by electron microscopy show these features of split intercalated discs. It is therefore justifiable to refer to the segments as cardiac muscle cells.

Examination of the discs shows various stages in the dissolution of cellular continuity. These stages can be classified as follows:

Stage I. Increase in the width of the gap between the unspecialized membranes in the junctional region. This change is seen in control specimens fixed in glutaraldehyde.

Fig. 9. Light micrograph of a longitudinal section of the myocardium from a calcium-exhausted specimen. The muscle segments are separated but not contracted. Most of the intercalated discs have disappeared, but an apparently normal one is shown in the uppermost fibre (ID). KR – Ca for 32 min. Osmium fixation. Araldite embedding. Toluidine blue/pyronin. × 1350.

Stage II. Loss of intercellular material, i.e. reduced electron density, between the myofibrillar insertion plaques and desmosomes.

Stage III. Separation of opposed myofibrillar insertion plaques and desmosomes, with retention of quintuple-layered membrane fusions.

Stage IV. Rupture of the surrounding basement membrane, complete dissociation of the cells visible by light microscopy. Only the fused membranes of the quintuple-layered junction remain unseparated, both being attached to one of the separate cells.

Normal discs, or even ones showing only stage I of separation, are not seen in the

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Fig. 8. Longitudinal section of the myocardium from a calcium-deprived heart (cf. Figs. 5, 6). The T-tubules contain granules in their lumina and have footplates (FP) from the sarcoplasmic reticulum applied to their outer surface. The plexiform arrangement of the sarcoplasmic reticulum (SR) extends the whole length of the sarcomere and at the lower end continues into the next sarcomere. KR – Ca for 6 min. Osmium fixation. × 45000.

calcium-exhausted specimens. Fig. 11 shows a stage II separation, with a disc of normal thickness and a clear zone between the opposed plaques. Separation of the myofibrillar insertion plaques (P) and desmosomes (D) is evident in Fig. 12, with the quintuple-layered junction (Q), running parallel to the myofibrils, being unaffected. This is classed as a stage III separation. The persistent adherence of the quintuple-layered junction is shown in Fig. 13 where a slender peninsula of one cell has been drawn out by the separation.

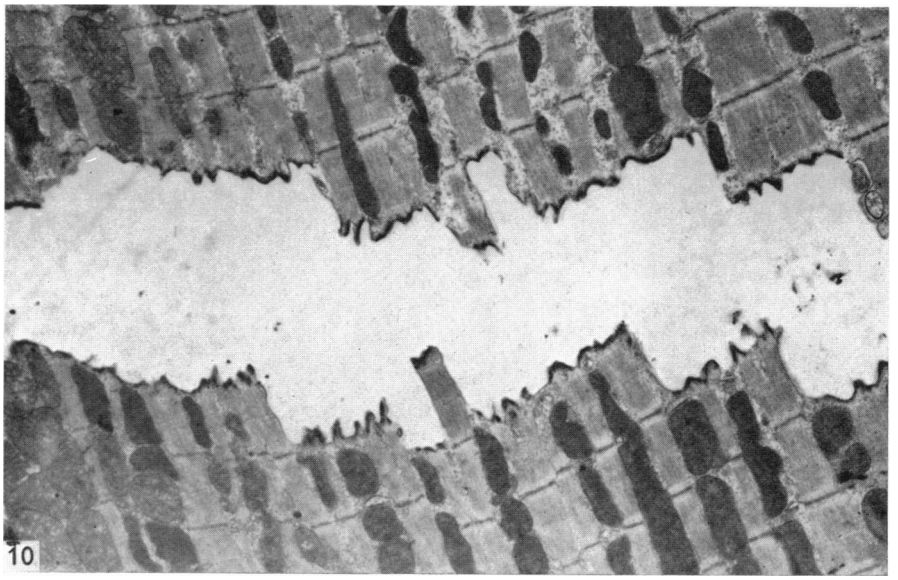


Fig. 10. Low-power electron micrograph of a longitudinally sectioned intercalated disc in a calcium-exhausted specimen. The dense plaques receiving the insertion of myofibrils are seen at the ends of each cell. The two cells could fit together to reconstitute an intercalated disc. KR−Ca for 31 min. Osmium fixation. ×5300.

The ends of the dissociated cells, stage IV, are mostly covered by the plasma membrane (Fig. 14) and the dense material on the cytoplasmic side of the plasma membrane receives the myofilaments of the sarcomere. In places, however, a quintuple-layered membrane fusion is observed (Fig. 15), the outer layer being originally a part of the other cell forming the disc. It is not surprising, then, that at the ends of some cells there are small areas bereft of their plasma membrane covering.

Separation from its neighbours does not produce any marked changes in the structure of the isolated cell: the myofibrils are fixed at rest length (Figs. 9–14); mitochondrial structure is normal (Figs. 12, 14) with a retention of the dense granules in the intramitochondrial matrix in these calcium-exhausted specimens. At the sarcolemma along the sides of the cells, the basement membrane component is found to deviate from the plasma membrane for short distances (Fig. 16). The endothelial cells of the capillaries do not separate from each other, the quintuple-layered membrane junctions between these cells at the terminal bars (Muir & Peters, 1962) remaining intact.

Calcium-reduced hearts (KR 0·5 Ca, KR 0·25 Ca, KR 0·2 Ca and KR 0·1 Ca)

Reduction of the calcium concentration to 0·5 mM-Ca^{2+} noticeably diminishes the strength of the ventricular contraction, but the sinus rhythm and the associated contraction continue for more than 2 h. With the K^+ concentration of 5·9 mM used in all these experiments, a Ca^{2+} concentration of 0·25 mM just maintains a perceptible beat which does not weaken, and there is no change in the electrocardiograph during a 90 min perfusion. The structure of the myocardial cells and the intercalated discs is normal after these perfusions with 0·5 and 0·25 mM-Ca^{2+}. Perfusion with 0·2 mM and 0·1 mM-Ca^{2+} causes the beat to diminish in strength, vanishing within 10 and 2 min respectively. In each case the electrocardiograph is not maintained at its original amplitude or regularity. After 22 min perfusion with 0·1 mM-Ca^{2+}, stage I and II separations are frequently found (Fig. 17). Longer perfusion with this weak calcium solution does not, however, produce the well-preserved isolated cells seen after the calcium-free perfusion. Instead, all the separated cells are in a severe contracture. The nature and causes of this contracture, which is induced by even low calcium concentrations on separated cells, will be the subject of another paper.

Magnesium-deprived hearts (KR − Mg) (KR − Mg − Ca)

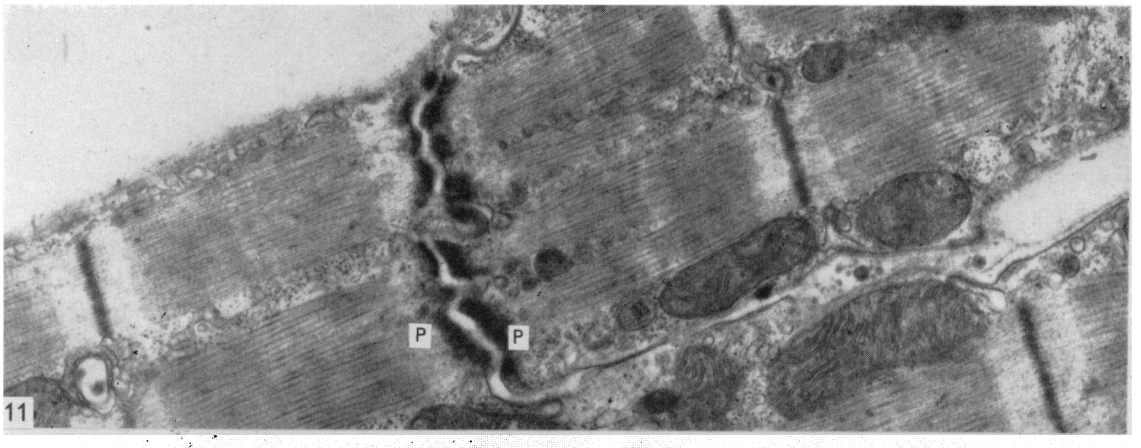
In the presence of calcium, deprivation of magnesium does not produce any detectable change in the strength of the beat. In one of four specimens occasional extrasystoles occur, otherwise normal function is maintained for at least 2 h. No change in the structure of these specimens is observed and no signs of disc separation are found.

If calcium and magnesium are omitted, the functional changes and cell separation are identical to those observed in calcium exhaustion. The variability of the time needed to produce cell separation after perfusing with the calcium-free medium makes it impossible to determine whether the additional omission of magnesium has accelerated the process.

Strontium and barium (KR − Ca + Sr) (KR − Ca − $MgSO_4$ + Ba)

An equimolar concentration of strontium will not maintain the beat or the electrocardiograph; both these functions fail within 5 min of transfer, which is appreciably longer than the beat continues after the transfer to a calcium-free medium. In the absence of an electrocardiograph it is impossible to observe the electrical changes which may signal dissociation of the cells, but two specimens which were perfused with KR − Ca + Sr for 31 min (210 and 230 ml) show that the cells are separated. Fig. 13 illustrates a stage III separation from one of these specimens. The completely isolated cells are all in the state of contracture observed after perfusion with low concentrations of calcium.

Owing to the insolubility of barium sulphate the magnesium sulphate is omitted in the experiments designed to determine whether barium can replace the role of calcium at the intercalated discs. In these hearts the physiological response and the anatomical changes are the same as after strontium replacement.



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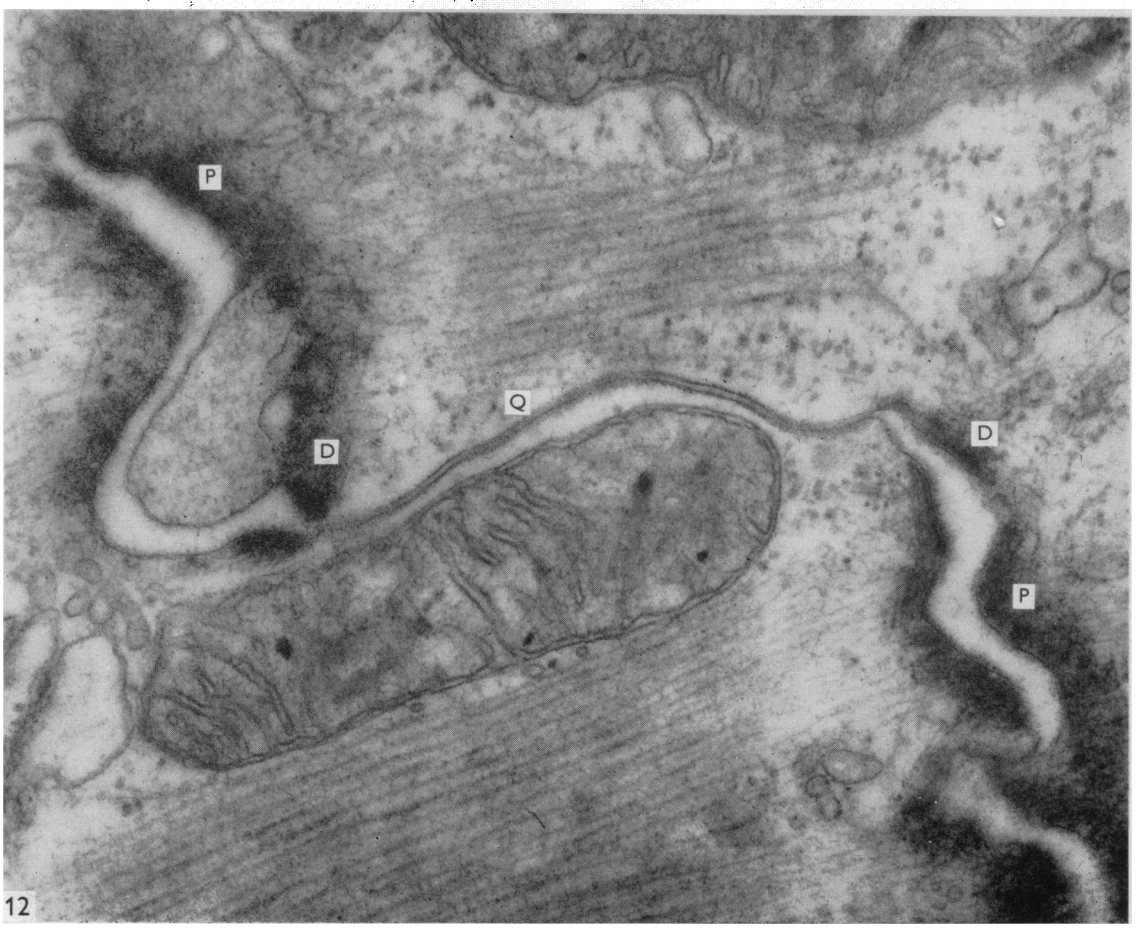

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Fragmented cardiac muscle

At the edges of all blocks, mechanical damage produces some transverse fragmentation of the fibres. Large numbers of such breaks can be produced by rough handling of fixed material. By light microscopy it is difficult to distinguish these transverse fissures from separated discs. However, careful study can establish the absence of the dense insertion plaques for the myofibrils which are visible on the separated cells in calcium-exhausted specimens. Electron microscopy shows the difference very clearly (Fig. 18); it is seen that the break occurs through the I bands on one or other side of

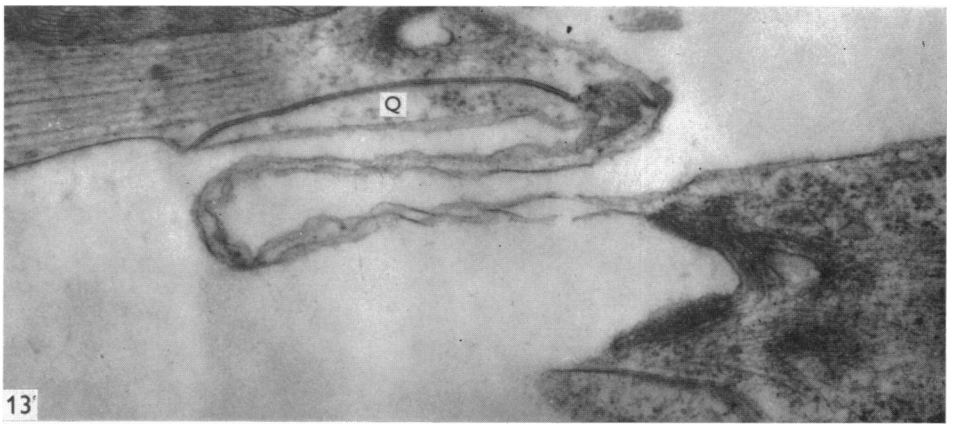


Fig. 13. Stage III separation. The cells are well apart from each other, but contact is maintained by a long extension of the right-hand cell which ends as an expansion forming a quintuple-layered junction (Q) with the other cell. KR−Ca+Sr for 31 min. Osmium fixation. ×41 000.

the Z disc. As in the case of intercalated discs, these breaks need not pass straight across the fibres. They often move up or down one or two sarcomeres during their passage. The I band seems to be the weakest part of the myofibril structure in studies on isolated myofibrils (Aronson, 1965). Fragmentations observed in control specimens do not pass through the intercalated discs, although they are seen to pass through the I band adjacent to the disc, indicating the strength of the normal intercellular adhesion.

DISCUSSION

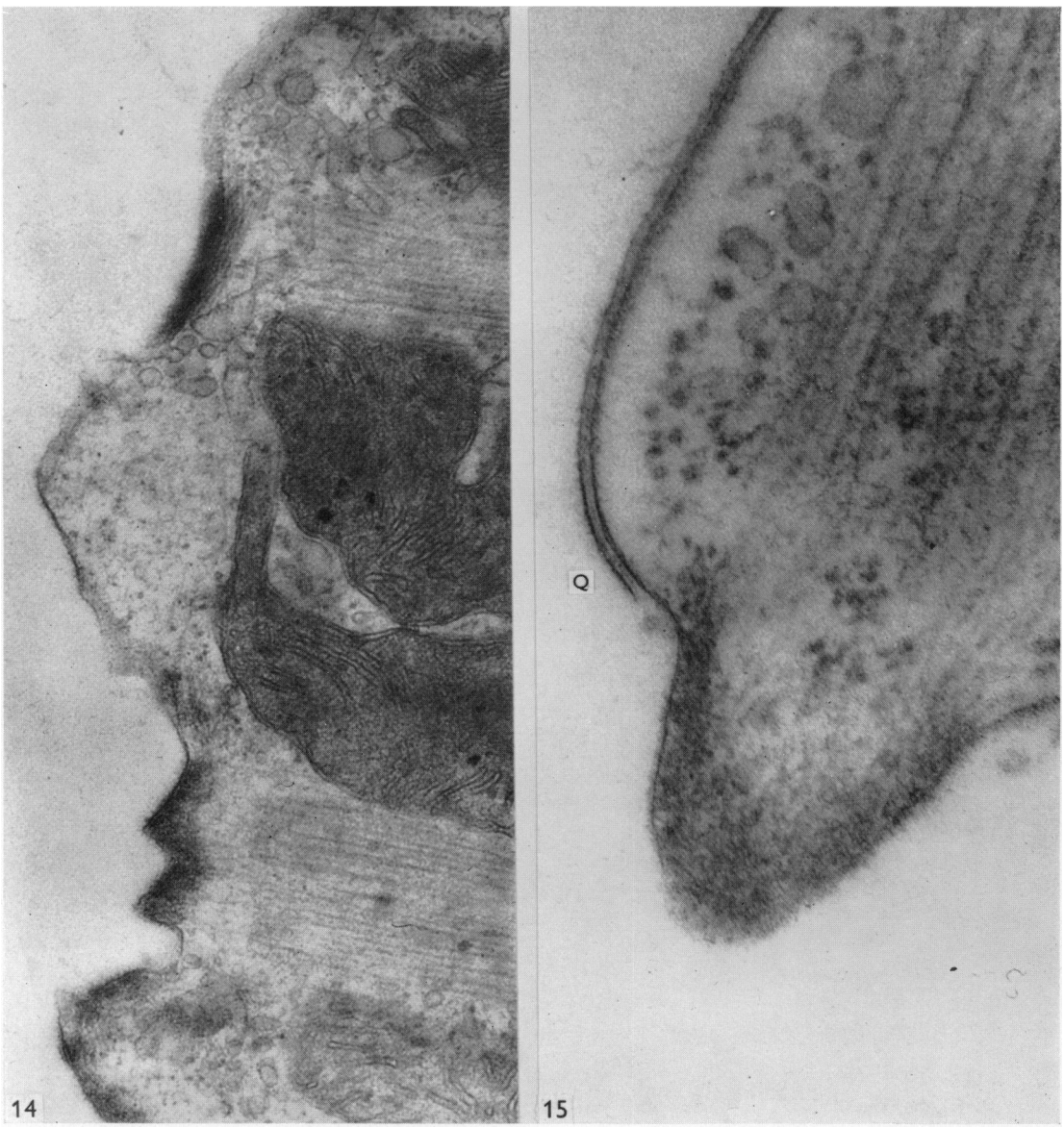
The observations reported have some relevance to three separate problems, namely, the role of the sarcoplasmic reticulum in contraction-excitation coupling, the importance of divalent cations in maintaining intercellular adhesion in mammalian tissues, and the nature of impulse transmission across the intercalated discs of the heart.

Fig. 11. Stage II separation of an intercalated disc. The myofibrillar insertion plaques (P) have not separated from each other, but the intervening material is much paler than in control discs (cf. Fig. 3). KR−Ca for 32 min. Osmium fixation. ×22 000.

Fig. 12. Stage III separation. The myofibrillar insertion plaques (P) and the desmosomes (D) are split and the clear space between them is wider than normal. The quintuple-layered membrane fusion (Q) is not affected. KR−Ca for 26 min. Osmium fixation. ×80 000.

Sarcoplasmic reticulum

The continuing electrocardiograph, in the absence of calcium ions, shows the persistence of surface electrical changes at the sarcolemma, while the failure to contract demonstrates that calcium is necessary either for myofibrillar shortening or as



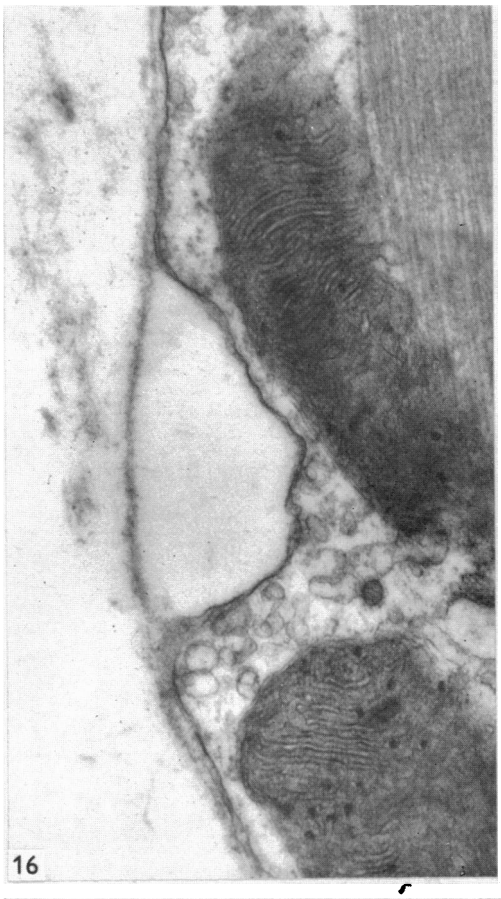
Figs. 14, 15. Stage IV separation. Two myofibrillar insertion plaques in Fig. 14 are separated by unspecialized plasma membrane. The quintuple-layered membrane junction (Q) in Fig. 15 shows that its left-hand component has been derived from the neighbouring cell. KR−Ca for 32 min. Osmium fixation. Fig. 14, ×90000. Fig. 15, ×125000.

part of the linkage between the surface events and the myofibrils. These two possibilities are not exclusive and the precise mode of action of calcium is widely discussed (Frank, 1964; Nayler, 1965). Most suggestions involve either an increase in calcium permeability of the T-tubule membrane during excitation or the release of calcium from the membranes of the sarcoplasmic reticulum. The present evidence indicates that any changes in the structure of these organelles are not revealed by current electron microscope techniques. In twitch and slow fibres from frog skeletal muscle, kept in Ringer's solution for some time before fixation with glutaraldehyde, Page (1965) illustrates small dense particles in the T-tubules, which are soluble in EDTA. The T-tubules of rat cardiac muscle contain some spherical granules in the normal and calcium-depleted specimens, but no small dense particles are seen either in these tubules or in the sarcoplasmic reticulum.

The disposition of the internal membranous system in the sarcoplasm of these perfused rat hearts resembles the descriptions given for immersion-fixed hearts from a variety of species: rat (Porter & Palade, 1957), bat (Fawcett, 1961), dog (Lindner, 1957), sheep (Simpson & Oertelis, 1962), cat (Fawcett, 1965), rabbit and human (Nelson & Benson, 1963). A notable species difference in the arrangement of the T-tubule seems to emerge from the present study and those quoted above, as frequent continuities between the T-tubule and the sarcolemma are reported and illustrated in sheep, cat, rabbit and human hearts, but not in the rat (Porter & Palade, 1957) or bat heart (Fawcett, 1961). Karnovsky (1964) mentions this discrepancy in his account of cholinesterase activity in rat myocardium and, in the present study, only occasional dubious communications are observed although they are frequently seen in similar samples of cat and rabbit myocardium. Simpson (1965), in a report received after the completion of this study, confirms the presence of communications between the T-system and sarcolemma in ox, sheep and guinea-pig, but is also unable to find convincing continuities in the rat heart. Another difference is that in many animals other than rat the T-tubule is lined by a structure resembling the basement membrane of the sarcolemma. Such a lining is not seen in the present preparations or by Porter & Palade (1957), and it is not visible in the many published pictures of rat myocardium.

Intercellular adhesion

The experiments indicate that about 0·25 mM-Ca^{2+} is necessary to maintain intercellular adhesion in rat cardiac muscle fibres. None of the other divalent cations tested can replace calcium in this function. The bonds between adjacent plasma membranes separated by the usual intercellular gap of 15 nm and the bonds between desmosomal and myofibrillar insertion plaques all seem to depend on calcium, but the quintuple-layered membrane fusions are apparently not disrupted under these conditions. It is possible that if these membranes separate they might not be recognizable as the remains of junctions; but the absence of partially split junctions of this type, their persistence after all other connexions have separated, and the presence, on the ends of completely separate cells, of a normal complement of these junctions make it likely that such cellular unions are not dissociated by the absence of calcium. Such fused membrane junctions are also retained by cardiac muscle cells isolated by calcium chelation and violent agitation (Muir, 1965). On the surface of liver cells, isolated by similar methods, Leeson & Kalant (1961) illustrate in their fig. 12, but



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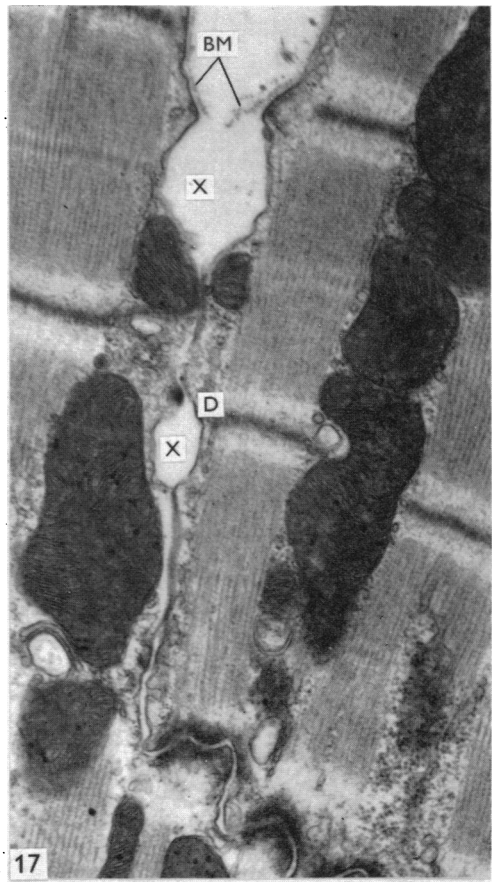

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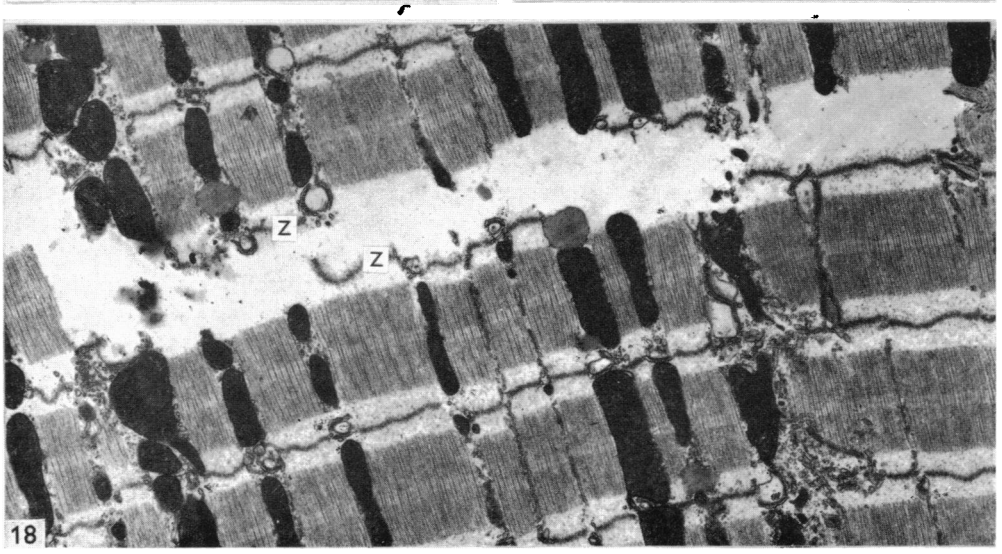

18

do not describe, residual portions of formerly adjacent cells sticking to the plasma membrane by similar close junctions.

Calcium combines readily with connective-tissue ground substance (Aldrich, 1958) and such a reaction may be involved at intercellular adhesions, but the same results would be obtained if calcium ions are necessary to maintain the configuration of the intercellular substances without actual combination. The ease with which the calcium can be removed favours the latter interpretation. Biochemical studies on the efflux of $^{45}Ca^{2+}$ from cardiac muscle (Nayler, 1965) show that calcium is contained in four distinct kinetic compartments, a vascular phase, fast and slow components of an exchangeable phase, and a non-exchangeable residue. In experiments with guinea-pig atria (Winegrad & Shanes, 1962), dog papillary muscle (Langer & Brady, 1963) and toad ventricle (Nayler, 1963), the half time for the removal of the fast component ranges from 4 to 6·5 min. Thus, this component, which is thought to reside in the cell membrane (Nayler, 1965), would be almost completely removed by 12 min perfusion; that is the average time for the interruption of the electrocardiograph after calcium deprivation. The slowly exchangeable fraction varies from 55 to 168 min, a time course which makes it less likely that this fraction is concerned in intercellular adhesion. Since there is a non-exchangeable residue, the term calcium-exhausted refers only to the physiological and structural state produced by prolonged calcium deprivation. The intramitochondrial granules which persist in the 'calcium-exhausted' cells are likely to contain calcium phosphate (Greenawalt, Rossi and Lehninger, 1964).

Other studies on the role of calcium in intercellular adhesion in mammalian tissues do not seem to establish the important role of this element. Simple deprivation in calcium-free balanced salt media is employed by Yokoyama, Jennings & Wartman (1961) on fragments of dog myocardium; the subsequent mincing and homogenization make it impossible to demonstrate the effect of the medium on the cell junctions. Berry & Simpson (1962) obtain isolated liver cells by perfusing the organ with a calcium-free buffered medium containing 400 mM sucrose, but the cells show extensive mitochondrial and plasma membrane damage and the intercellular junctions are not specifically described.

Many reports refer to the effects on cellular adhesion of chelating agents such as ethylene diamine tetra-acetic acid (EDTA, versene). Anderson (1953) gives a method for isolating hepatic parenchymal cells by perfusion with EDTA and other chelating agents, and a number of descriptions of the fine structure of livers treated by such methods are available. These descriptions vary; Coman (1954) and Bucciolini &

Fig. 16. The sarcolemma, from a calcium exhausted heart, shows a separation between its basement membrane and plasma membrane components. KR−Ca for 32 min. Osmium fixation. × 60000.

Fig. 17. Separation of a connexion between the lateral surfaces of myocardial cells in a calcium reduced specimen. The unspecialized membranes have separated at X, rupturing the basement membrane (BM). A desmosone (D) has sheared apart and the intercalated disc at the base shows a stage II separation. KR 0·1 Ca for 32 min. Osmium fixation. × 20000.

Fig. 18. A fractured myocardial fibre showing the split passing through the I bands on either side of the Z discs. To the left of the field the split ascends one sarcomere to continue its course across the fibre. KR 1·27 Ca for 5 min. Osmium fixation followed by trauma. × 9300.

Marsilii (1961) observe gross cellular damage, while Leeson & Kalant (1961) consider that the slight damage shown is due to fixation and embedding artefacts. These authors all agree that EDTA perfusion induces cellular separation, but Branster & Morton (1957) do not report any increase in yield of cells after chelation, and Easty & Mutolo (1960) deny any effect on hepatic cellular adhesion, suggesting that the role of calcium in cellular adhesiveness in mammalian tissues has been overemphasized. Myocardial cells are separable after chelation (Yokoyama, Jennings & Wartman 1961; Muir, 1965), but these cells show considerable damage, due to either EDTA or the violent procedures of separation; the physiological effects of EDTA on the heart are not reported. Forte & Nauss (1963) demonstrate that the normal transmucosal potential difference across isolated frog gastric mucosa is lost rapidly after applying chelating agents, and electron microscopic examination of this material shows separation of desmosomes in the mucosa (Sedar & Forte, 1964). These effects are stated to be reversible with recombination of the desmosomes (Sedar & Forte, 1964) and a return of the transmucosal potential (Forte & Nauss, 1963) after restoration of calcium.

Studies with chelating agents can be criticized because the agent itself may produce effects apart from its chelating action. Also, the chelation involves all divalent and many other cations, so the relative importance of one element, calcium, cannot be shown by these methods.

Impulse transmission

Although it is tempting to assume that the sudden loss of the sinus rhythm in the electrocardiograph, with the subsequent flutters and fibrillation, signals the dissociation of cardiac muscle cells, the evidence presented only shows that prolonged calcium deprivation produces an irreversible cessation of the normal propagated impulse and that, after fixation and embedding, these specimens show intercellular separation. The electrocardiographic changes could be due to a change in the surface sarcolemma which impairs its conduction properties, and which is unrelated to the loss of adhesion at the intercalated discs. It may not be only a coincidence that the concentration of calcium necessary for intracellular conduction and contraction is the same as that required for survival of the electrocardiograph. Intracellular microelectrode recordings during the period when overall activity is ceasing could show whether the electrical activity of single cells is also affected.

If, however, the rhythmic activity and conduction properties of each myocardial cell are retained, then the electrocardiographic changes must be due to a failure to transmit across the discs. This failure could be due to intrinsic, undemonstrable chemical changes in the component membranes or to actual physical separation. If normal intermembranous relationships are maintained, it might be expected that restoration of calcium would rebind the cells with or without a return of the electrocardiograph. No such restoration is observed, so it is likely that some separation precedes fixation. However, the clear separation of the cells with rupture of the adherent membranes in the quintuple-layered fusions is probably due to the shrinkage forces of fixation and embedding, since no other force acts on these flaccid ventricles. So, prior to fixation, the cells are likely to be joined by their quintuple-layered membrane junctions and, with the reservations defined above, this interpretation does not support the current suggestions that these junctions are the sites of

electrotonic intercellular transmission (Eccles, 1964; Dewey & Barr, 1964; Robertson, Bodenheimer & Stage, 1964).

Single, growing, beating cardiac muscle cells can be obtained from neonatal rodent hearts (Harary & Farley, 1963), a facility which seems to be lost with maturity. The present method of loosening intercellular adhesion could produce single cells from adult hearts which have been subjected to minimal physical or chemical damage and which might be capable of independent existence. However, the failure to split the quintuple-layered membrane adhesions raises a difficulty; for, remaining with one cell, they must damage the plasma membrane of the neighbour. Conceivably, one cell could acquire all the junctions from its two neighbours, but if these junctions do act as low-resistance pathways from cytoplasm to cytoplasm, even this fortuitous event would not preserve the ionic integrity of the isolated cell.

SUMMARY

The ultrastructure of the adult rat heart, after intravascular perfusion with an oxygenated balanced salt solution, is described. During *in vitro* perfusion the electrocardiograph and ventricular contraction are recorded.

Modifications in the divalent cation content of the perfusing solution produce the following effects:

(1) Brief omission of Ca^{2+} uncouples contraction from excitation but does not produce any change in the structure of the T-tubule, sarcoplasmic reticulum or membrane relationships at the intercalated discs.

(2) Prolonged omission of Ca^{2+} disturbs and then destroys any recordable electrocardiograph. The component cells of these hearts are separated at the intercalated discs. Only the quintuple-layered membrane fusions at the discs remain intact.

(3) A concentration of 0·25 mM-Ca^{2+} is necessary to maintain intercellular adhesion and Mg^{2+}, Sr^{2+} and Ba^{2+} cannot replace Ca^{2+} in this function.

The significance of these observations in relation to excitation-contraction coupling, intercellular adhesion and the role of membrane fusions in cardiac impulse conduction is discussed.

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