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Changes in the ultrastructure of rat myocardium induced by hyperkalaemia

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INTRODUCTION

A reversible cardiac arrest can be induced by raising the plasma K^+ concentration and this phenomenon has been studied because of its possible clinical application and physiological interest (Melrose, Dreyer, Bentall & Baker, 1955; Helmsworth *et al.* 1959; Miller *et al.* 1961). These workers induced reversible arrest by the intravascular injection of potassium citrate. The effects of hyperkalaemia on the electrical and contractile properties of isolated perfused mammalian hearts are well established. The reports by Walker & Weatherall (1963), Paes de Carvalho & Langan (1963), Page, Goerke & Storm (1964), and Lee, Richman & Visscher (1966*a*, *b*) provide examples. Walker & Weatherall found spontaneous activity in the rabbit heart when the K^+ concentration was < 11 mM/l (normal K^+ concentration is 5·9 mM/l); when the K^+ concentration was between 11 and 30 mM/l there was no spontaneous activity, but activity could be restored by the addition of Ca^{2+} or adrenaline. When the K^+ concentration was between 50 and 70 mM/l addition of Ca^{2+} caused a slow steady contraction. Lee *et al.* (1966*a*, *b*) studied the effects of varying both the Ca^{2+} and K^+ concentrations; in particular, they measured the K^+ concentration required to induce arrest at given Ca^{2+} concentrations and studied the reversibility of the physiological changes induced by the variation of ionic concentrations.

The isolated perfused heart provides a convenient preparation for electron microscopic study because reliable and uniform fixation can be obtained by intravascular injection (Muir, 1967). Hence in the present paper the changes in structure of rat ventricular myocardium induced by varying the K^+ concentration from the normal 5·9–70 mM/l are described and related to the physiological behaviour of the hearts. The normal physiological level of Ca^{2+} concentration of 1·27 mM/l was used in all experiments and with this Ca^{2+} concentration the cardiac arrests produced by K^+ concentrations of 12, 20 and 30 mM/l could be reversed by returning to normal K^+ concentration; this allows the corresponding ultrastructural changes to be investigated. The effects of hypokalaemic solutions are described in another paper (Emberson & Muir, 1969).

MATERIALS AND METHODS

Hearts from adult white rats (weighing 200–350 g) were used in this study. All specimens were prepared by the *in vitro* perfusion technique previously described (Muir, 1967).

The perfusing solutions were of the Krebs–Ringer type and contained glucose. The molar concentrations of the control solution (KR 5·9 K) are given below in mM/l:

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

All specimens were perfused with this control solution for 5 min and a regular strong beat obtained, before the introduction of the hyperkalaemic solutions. The osmolarity of the control solution, assuming complete dissociation, was 309·3 milliosmoles. The hyperkalaemic solutions were prepared by raising the level of KCl in the solution and an osmolarity of between 305 and 315 milliosmoles was obtained by a reduction of the level of NaCl. In the following text the hyperkalaemic solutions have been described by KR followed by the K^+ concentration, e.g. the solution with 12 mM/l K^+ has been described by KR 12 K.

Fixation at the end of the perfusion was by 1 % osmium tetroxide (39 mM/l) made up in whichever solution was then perfusing the heart. The fixative was injected from a syringe into a cannula which itself was set in the aorta. The right ventricular wall was removed and cut through its whole thickness into 2 × 2 mm squares and placed in the fixative for 2 h. The tissues were embedded in Araldite and cut, from the epicardial surface, on a Porter–Blum microtome with a glass knife. Sections 1 μm thick, mounted on glass slides, were stained with toluidine blue/pyronin (Ito & Winchester, 1963) for light microscopy. Sections for electron microscopy were mounted on Athene grids without a supporting membrane and were stained with uranyl acetate/lead citrate (Reynolds, 1963) before examination in an A.E.I. EM 6 microscope.

RESULTS

Perfusion in KR 5·9 K

The *T*-tubules of four hearts perfused for 20 min in control solutions were either round or oval with the long axis parallel to the *Z* discs. The greatest diameter was between 0·1 and 0·6 μm (Fig. 1*a*). The myofibrils, intercalated discs, sarcolemmae, mitochondria and sarcoplasmic reticulum appeared normal.

Perfusion in KR 12 K

Three hearts were perfused in KR 12 K. They did not cease activity suddenly. The ECG slowed from 240/min to 120/min within 3–5 min and then stopped. As long as action potentials continued they were accompanied by powerful ventricular contractions, the strength of which was apparently constant. One of the hearts was perfused for 3 min and was fixed while still displaying a slow ECG. The other two hearts were fixed after 20 min perfusion when they had been inactive for 5 min. The appearance of all three hearts was similar.

As compared to the control hearts there was only one variation in structure. Some of the *T*-tubules were much larger, their diameters being in the range 0·6–1·1 μm (Fig. 1*b*, solid line; Fig. 2). Distended *T*-tubules were particularly common near

intercalated discs. The diameters of the *T*-tubules in a single cell varied considerably but the range of this variation was similar in all the cells of each specimen. Footplates of the sarcoplasmic reticulum were observed on most of the distended tubules, but they did not appear to have enlarged with the increase in size of their companion tubule. Thus they almost surrounded the small *T*-tubules, but lay along a smaller arc of the larger *T*-tubules (Fig. 2).

Two hearts were returned to perfusion by the control solution (KR 5·9 K) after 20 min perfusion by KR 12 K. Within 1 min the ECG and contractility apparently reverted to normal. They were fixed after 20 min perfusion in the KR 5·9 K. A histogram of the size of their *T*-tubules was almost identical to that of the *T*-tubules of control hearts (Fig. 1*b*, broken line).

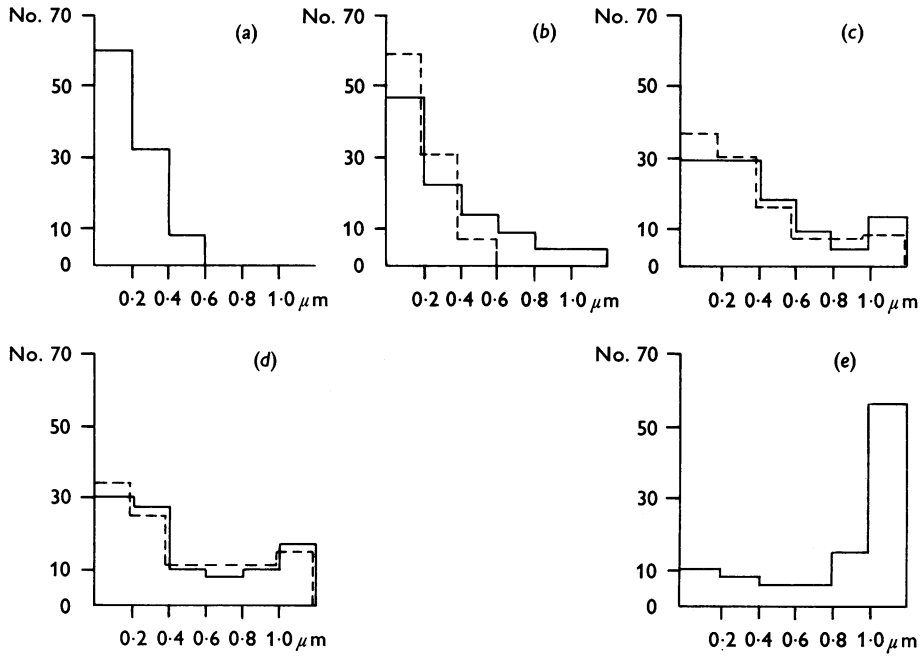
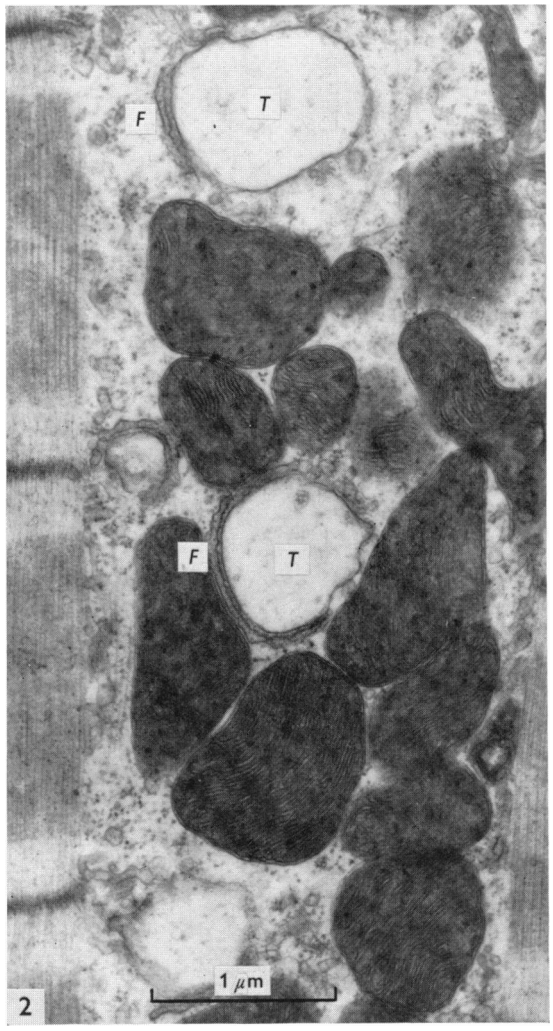


Fig. 1. The solid lines in the histograms show the longitudinal diameters of 100 *T*-tubules after 20 min perfusion in (*a*) KR 5·9 K, (*b*) KR 12 K, (*c*) KR 20 K, (*d*) KR 30 K and (*e*) KR 70 K. The dotted lines on (*b*), (*c*) and (*d*) show the longitudinal diameters of 100 *T*-tubules after 20 min perfusion in KR 12 K, KR 20 K and KR 30 K respectively, followed by 20 min re-perfusion in KR 5·9 K. Equal proportions of *T*-tubules were selected from each block of tissue studied for a particular concentration of K^+. The ordinates of the first five columns represent the number of *T*-tubules whose diameter was between successive lengths on the abscissa and the final column represents the number whose diameter was > 1·0 μm. Each histogram was constructed from data obtained from four hearts.

Perfusion in KR 20 K

Two hearts were perfused for 20 min in KR 20 K. Contractions and recordable electrical activity coincidentally ceased within 3 min of perfusion.

The proportion of the *T*-tubules which was distended was greater than after perfusion in KR 12 K (Fig. 1*c*, solid line). In addition the degree of distension was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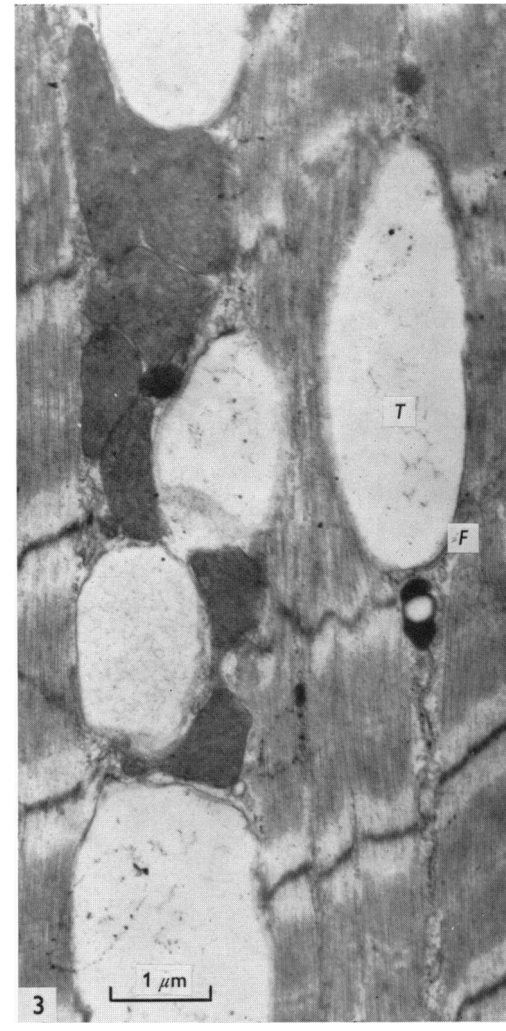
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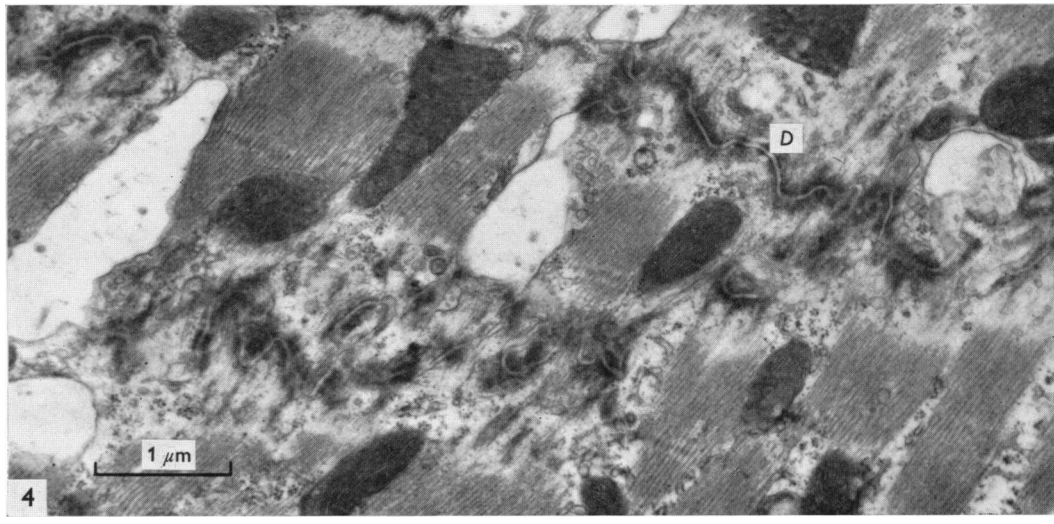
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greater. Many had long diameters, parallel to the myofibrils, of over 1·2 μm and some were longer than a sarcomere (Fig. 3). These large *T*-tubules filled the space between adjacent myofibrils except for that occupied by mitochondria. Some granular and filamentous material was present in the tubules. Footplates were apparent on some distended tubules, even though the sarcoplasmic reticulum was generally inconspicuous in the vicinity of the larger *T*-tubules. Apart from the changes in the *T*-tubules the only other observed abnormality was the presence of contractures in small areas of a few cells.

Two hearts were perfused for 20 min in KR 5·9 K after having been perfused for 20 min in KR 20 K. Apparently normal activity returned within 2 min, an observation which is in agreement with the work of Lee *et al.* (1966*a*, *b*). The histogram of the diameters of their *T*-tubules (Fig. 1*c*, broken line) was similar to that of those only perfused in KR 20 K and quite unlike that of control hearts.

Perfusion in KR 30 K

Four hearts were perfused in KR 30 K. Contractions and the ECG ceased within 30 s. One heart was fixed after 1 min perfusion, one after 5 min perfusion and two after 20 min perfusion. The appearances of all four were similar. The distension of at least a third of the *T*-tubules was the most conspicuous result of the perfusion (Fig. 1*d*, solid line). Distended tubules containing filamentous and granular material were particularly common near discs (Fig. 4). Most of the myofilaments appeared normal, but some were in contracture. The intercalated discs and the sarcolemmae appeared unaffected by the perfusion.

Four hearts were perfused in KR 5·9 K for 20 min, after having been perfused in KR 30 K. After 5 min perfusion an apparently normal ECG coupled with ventricular contractions returned. The histogram of the diameters of the *T*-tubules was very similar to that of those perfused only in KR 30 K for 20 min, and was quite unlike that of control hearts (Fig. 1*d*, broken line).

Perfusion in KR 70 K

All four perfused hearts became inactive within 15 s with this concentration; they were fixed after 20 min perfusion. Distension of the *T*-tubules was most conspicuous. All cells were affected though to varying degree, and the great majority of the *T*-tubules were affected (Fig. 1*e*, solid line), though not all. Gross distension was more common than at lower K^+ concentrations and many tubules were several sarcomeres in length (Fig. 5). The scalloped perimeter of some of these suggested that they were formed by the amalgamation of several tubules (Fig. 5). The tubules contained filamentous and granular material, probably of cytoplasmic origin, which may have entered through ruptured tubules. Footplates, and indeed the sarcoplasmic reticulum

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Fig. 2. Perfusion with KR 12 K: the transverse tubules (*T*) are distended but footplates (*F*) are intact.

Fig. 3. Perfusion with KR 20 K: there is gross distension of the transverse tubules (*T*), but a footplate is intact (*F*).

Fig. 4. Perfusion with KR 30 K: there is distension of the transverse tubules but the desmosomes (*D*) and myofibrillar insertion plaques of the intercalated disc appear intact.

in general, were not in evidence in grossly affected regions. Such regions often occurred near intercalated discs and often the sarcolemma was ruptured in the unspecialized regions. Otherwise the discs appeared intact (Fig. 5).

There was one outstanding feature in the structure of the myofibrils which was not observed at other concentrations; namely, a disintegration of the myofibrils which did not cause contracture, or even severe contraction. Different stages of this disintegration could be observed in any one cell and 80% of all observed cells were

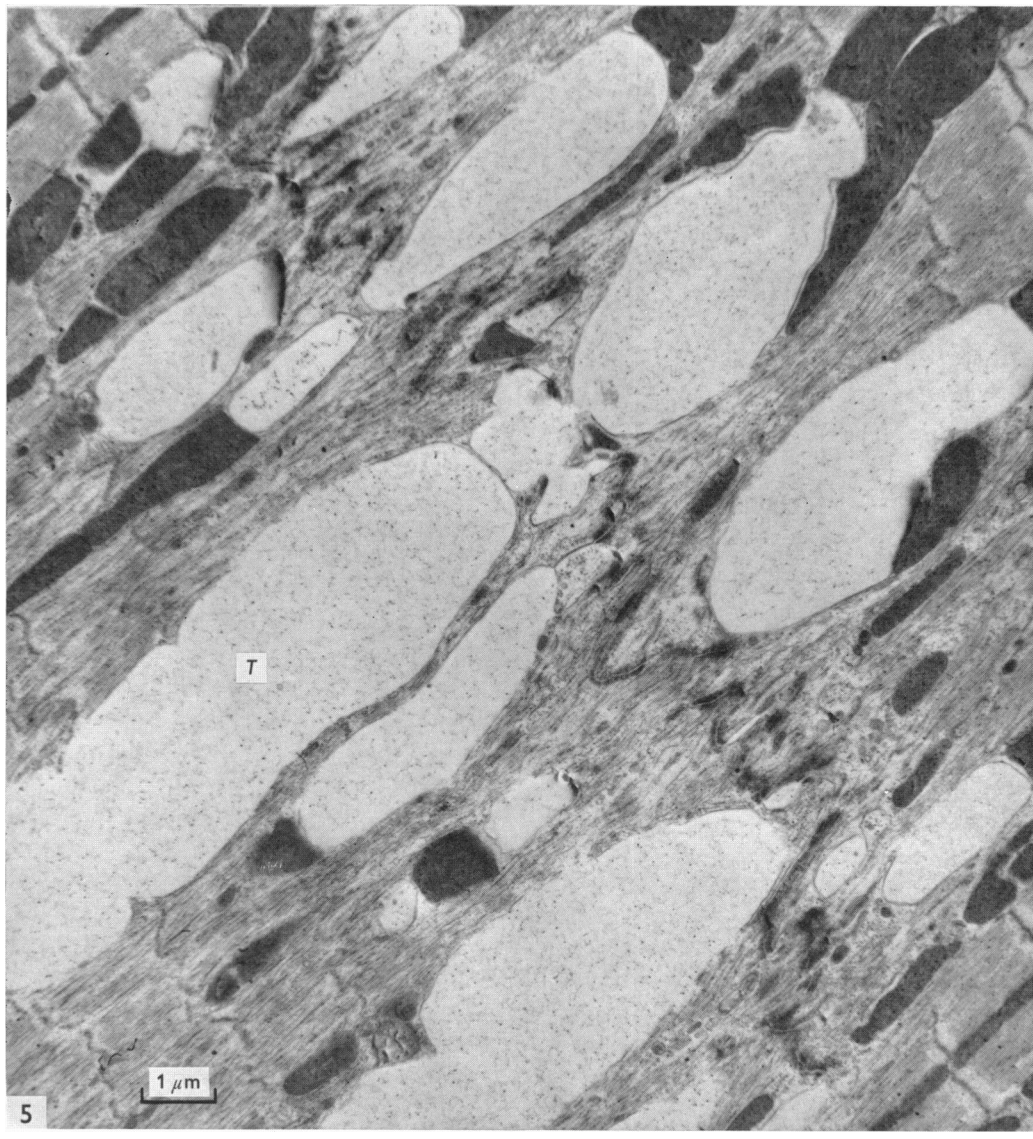


Fig. 5. Perfusion with KR 70 K: the transverse tubules (*T*) are grossly distended and some are scalloped. Some of the myofibrils are partially disintegrated.

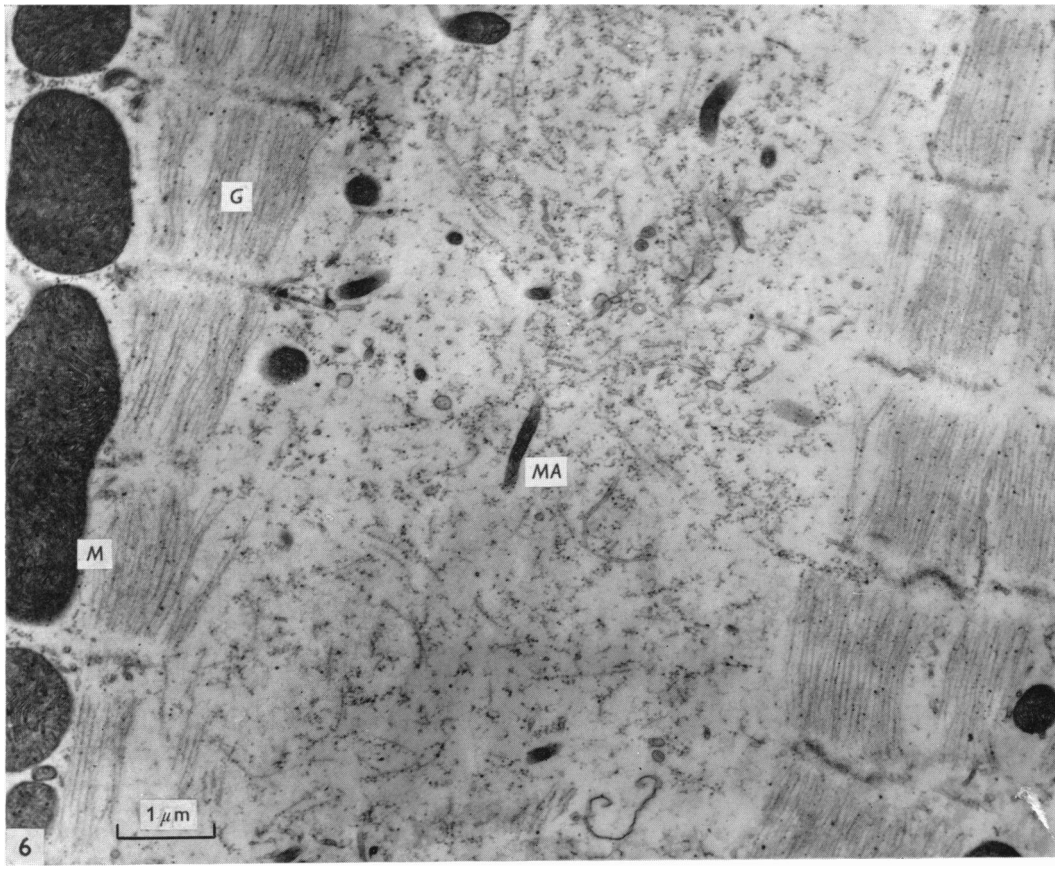
affected. It is convenient to describe the disintegration as occurring in four stages. At stage one, the general form of the myofibrils was still obvious because the thick (*A*) filaments lay approximately parallel to the axes of the myofibrils which were segmented by *Z* discs (Fig. 6). However, in many such myofibrils the thin filaments were not apparent and there was an empty band on either side of the *Z* disc (Fig. 6). Within the region of the myofibrils, there were dense granules many of which appeared in contact with the thick filaments (Figs. 6, 7). At stage two, the *Z* discs were less well defined and many thick filaments were no longer parallel to the myofibrillar axis, but had swung outwards into the sarcoplasm (Fig. 6). At stage three, there was little sign of the individual myofibrils; rather there was a field of preferentially orientated thick filaments (Fig. 7). At stage four, the disintegration has progressed to a disorientated mass of thick filaments, with dense granules apposed to many of these (Fig. 6). The dense granules were not observed in non-disintegrated areas and were present in unstained sections. In the areas of partial disintegration most mitochondria appeared normal (Figs. 5, 6), but in areas of substantial disintegration the mitochondria appeared severely contracted or fragmented (Figs. 5, 6). *T*-tubules were either distended or not observed (Figs. 5–7) and the sarcoplasmic reticulum was fragmented and disorganized (Fig. 7). Where intercalated discs occurred in these regions the relationships of the two cell membranes were normal (Fig. 7). However, thin filaments from the *I* bands had disappeared and the density and thickness of the myofibrillar insertion plaques were reduced (Fig. 7).

After perfusion in KR 70 K for 20 min, four hearts were then perfused in KR 5·9 K. After approximately 5 min perfusion an ECG returned. In two hearts the ECG signal gave a deflection similar to the controls, and the rate was 3/min. In two hearts the rate was 50/min and again the signal was of normal magnitude. The right atrium of each heart contracted with the ECG, but there was no return of ventricular contractions. The ultrastructure of the ventricular myocardium of the four hearts fixed after 20 min re-perfusion in KR 5·9 K did not appear to differ from that of those hearts fixed after perfusion in KR 70 K.

DISCUSSION

As the physiological effects of hyperkalaemia described above in rat hearts were similar to those already reported for the rabbit heart by Walker & Weatherall (1963) and Lee *et al.* (1966*a*, *b*), this discussion is restricted to the structural changes which were observed.

The outstanding feature, consistently found in all perfusions with high K^+ concentration, was the distension of the transverse tubules. This distension was not observed by Miller *et al.* (1961) on hearts removed from dogs after perfusion with potassium citrate concentrations sufficiently high to cause cardiac arrest. However, similar though smaller distensions have been observed in muscle perfused with hypertonic solutions (Hodgkin & Horowicz, 1957; Howarth, 1958; Freygang *et al.* 1964). In this case the solutions used were isotonic and direct osmotic effect could not have been the cause. As the distensions observed after 1, 3 and 5 min perfusion were similar to those observed after 20 min perfusion, at least for KR 12 K and KR 30 K, the distensions appear to occur rapidly rather than by a gradual process.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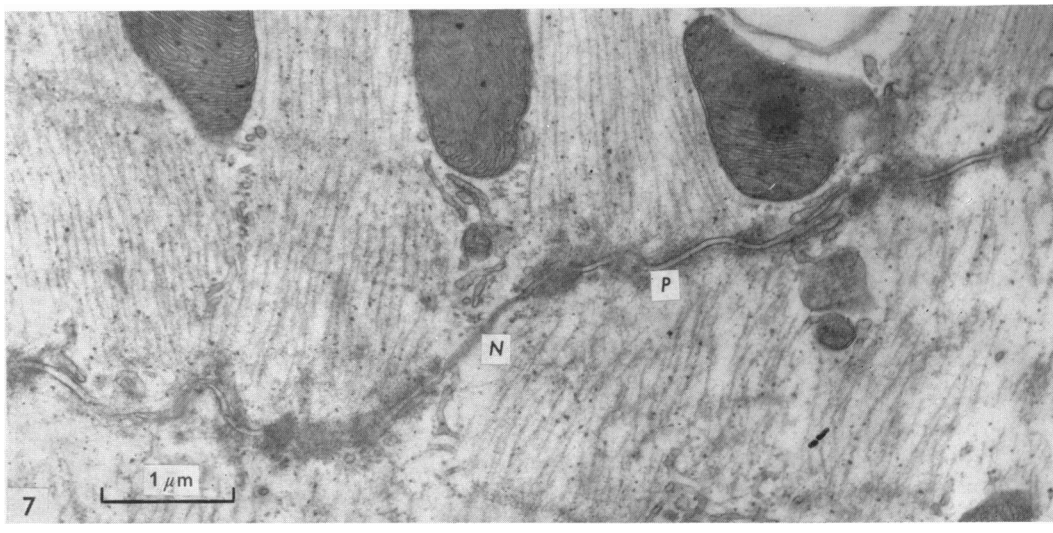
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7

It is possible that complete perfusion of the *T*-system does not occur after loss of normal activity, and thus long-term perfusion is ineffective.

Together with an increase in K^+ concentration, the perfusing solutions were altered by a compensatory decrease in Na^+ concentration to preserve isotonicity. The effects of decreasing the Na^+ concentration of the Krebs–Ringer solution, while preserving the tonicity with ionically inactive agents, have not been studied; so it remains possible that the changes are due to the lower Na^+ concentrations. However, when the K^+ concentration was 12 mM/l, a 100% increase, the Na^+ concentration was decreased by only 4%, and as distensions did occur in this case, the distensions would appear most likely to be due to the relatively large increase in K^+ concentration. As the distensions are not due to osmotic factors, they would appear to be due to a loss of structural integrity due to the increased K^+ concentration. It is possible that the excess of K^+ interferes with interactions necessary for the maintenance of the *T*-tubule system, or that the system expands to accommodate the excess K^+ by providing an increased number of attachment sites or space to reduce the effective concentration. Page *et al.* (1964), from studies of the effect of ouabain and the external K^+ concentration on the transport of cations through the plasma membrane of cat cardiac muscle, suggested that K^+ are attached to the outer surface of the plasma membrane. If the *T*-tubules are continuous with the plasma membrane, as shown clearly in the human, rabbit and sheep hearts (Nelson & Benson, 1963; Simpson, 1965), one might then expect K^+ to be located on the *T*-tubule membrane.

The great variation in the size of the *T*-tubules within a given cell was a feature of these preparations. A similar variation in the degree of distension followed perfusion with hypertonic solutions (Freygang *et al.* 1964). These variations could be due to local variations in the properties of the *T*-tubules, or due to uneven perfusion leading to local variations in K^+ concentration. If the former is the case there is the possibility that the role of any one tubule in the functioning of its sarcomere is not the same as that of other tubules. Thus though it seems reasonable to assume a similar role for each tubule, particularly if they form one complete system, this may not be so.

However, uneven perfusion may be the cause of this observation. Perfusion to the region of the *T*-tubules could occur by two paths; either by absorption of the K^+ through the plasma membrane and passage through the sarcoplasm, or by direct entry into the *T*-tubules. It is not clear why perfusion through the plasma membrane would produce uneven K^+ concentrations at different *T*-tubules, but if perfusion was more readily effected by direct entry and the *T*-tubular system was open to the extracellular space at relatively few points, the wide variation in the effects could be explained. It may be relevant that, in rat myocardium, convincing openings of the *T*-tubules are rarely seen (Simpson, 1965; Muir, 1967).

Fig. 6. Perfusion with KR 70 K: thin filaments are absent from the myofibrils which are partially or substantially disintegrated. There are dense granules (*G*), some of which appear along thick filaments. Some mitochondria (*M*) appear normal but others (*MA*) appear unusually small, elongated and compact.

Fig. 7. Perfusion with KR 70 K: thin filaments are absent from the myofibrils which are partially disintegrated. Dense granules are present, some apparently in contact with thick filaments. Intercellular adhesion at the intercalated disc appears normal, but the myofibrillar insertion plaques (*P*) appear reduced in depth and density. A nexus (*N*) appears normal.

The reversibility of structural changes induced by high K^+ concentrations has physiological and clinical interest. The present experiments have indicated that the *T*-tubule distensions were reversible after perfusion with KR 12 K solutions but not after perfusion in solutions in which the K^+ concentration was 20 mM/l or greater. This was so despite the ECG and contractility apparently returning to normal after perfusion in KR 20 K and KR 30 K, which is consistent with the observations of Lee *et al.* (1966*a*, *b*). Perhaps longer re-perfusion in normal Krebs–Ringer would result in reversal of the distensions, but if it occurs it must take much longer than the initial distension. It is also possible that the observation of the ECG for a longer period would result in the appearance of abnormalities due to the distended tubules. While the present results indicate a threshold K^+ concentration of between 12 and 20 mM/l, above which distensions were not reversible, there is no indication of such a threshold in the size of the *T*-tubules, that is a diameter above which a drastic change in structure occurred. On reperfusion in KR 5·9 K, after perfusion in KR 20 K or KR 30 K, an apparently normal ECG and contractility was obtained, despite extensive distension of approximately 30% of the *T*-tubules. Thus it appears that this distension does not inhibit excitation–contraction coupling by preventing the spread of activity along the *T*-tubules and thence to the sarcoplasmic reticulum, assuming this is the normal mode of activity spread. This evidence supports the conclusion of Howarth (1958) that in distension of the *T*-tubules by hypertonic solutions, the electrical activity can still spread to the myofilaments.

The size and form of the sarcoplasmic reticulum was not affected where the distended tubules were < 1 μm. Freygang *et al.* (1964) found the sarcoplasmic reticulum to be unaffected by hypertonic solutions. Thus either the reticulum is not susceptible to alteration by these procedures or the high K^+ concentrations did not pass into the region of the reticulum, suggesting that diffusion is by way of the *T*-tubules directly.

The primary action leading to the break up of the myofibrils in KR 70 K solutions appears to be the disappearance of the thin filaments and the appearance of dense granules within the region of the myofibrils. It is possible that the granules represent coagulations of the thin filament material. As the Na^+ concentration, in this solution, is only 56% of normal, it is possible that the low Na^+ concentration is a factor promoting this coagulation. Either the high K^+ concentration or the low Na^+ concentration appears to cause links to form between adjacent areas of the thin filaments. As the *Z* disc is apparent in many sarcomeres from which the thin filaments have disappeared, this suggests that the tropomyosin of the *Z* discs differ from that in the thin filaments.

Walker & Weatherall (1963) using K^+ concentrations of 50 mM/l to 70 mM/l found that, on addition of $CaCl_2$, the fibres went into a slow sustained contraction. In this study the great majority of the fibrils had lost all or part of their thin filaments and it is difficult to see how they could support contraction, assuming the validity of the sliding filament theory. However, perhaps sufficient intact myofibrils remained to produce a contraction.

SUMMARY

Changes in the ultrastructure of adult rat ventricular muscle induced by *in vitro* perfusion with hyperkalaemic solutions are described. The following three changes are produced:

(1) When the K^+ concentration was 12, 20, 30 or 70 mM/l, there is distension of the transverse tubules, which is reversible only after perfusion with the 12 mM/l solution.

(2) When the K^+ concentration is 70 mM/l there is a disintegration of the myofibrils, not involving contracture, which is apparently due to coagulation of the thin filaments into granules.

(3) When the K^+ concentration is 20 mM/l or greater, approximately 10% of the myofibrils are in contracture.

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