ELECTRICAL, MECHANICAL AND ULTRASTRUCTURAL EVENTS IN HEARTS PERFUSED WITH MEDIA DEFICIENT IN VARIOUS CATIONS

A Thesis
Presented to the
University of Manitoba

In Partial Fulfillment of the Requirements
for the Degree

MASTER OF SCIENCE
in
PHYSIOLOGY

by
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August, 1973

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ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. N. S. Dhalla for providing the facilities for carrying out the research contained in this report as well as for his advice and encouragement in the preparation of this manuscript. Most especially I wish to acknowledge his trust in permitting me to present my own interpretation of this work in spite of his frequent comment of: "I would not have said that."

I cannot adequately express my appreciation of the patience and understanding shown by my wife, Mary Anne, during the course of my studies.

I would also like to thank Pat Migliore for sacrificing so much of her own time during the typing and preparation of this manuscript.

This study was supported by grants to Dr. N. S. Dhalla from the Medical Research Council and the Manitoba Heart Foundation.

ABSTRACT

Isolated rat hearts were perfused for various intervals with Ca^{++} -free, Na^{+} -free, or K^{+} -free media. The mechanical and electrical functions and ultrastructure of these hearts were studied both during deprivation of these ions and during reperfusion with normal medium. In hearts perfused with Ca^{++} -free medium, both contractile force and dF/dt declined to zero within 30 seconds. Failure of contractility was associated with a decrease in time to peak tension and an increase in time to ½ relaxation. Recovery of contractile force on reperfusion with normal medium was dependent upon the duration of the Ca^{++} -free perfusion and appeared to be related in a negative manner both to an increase in resting tension during Ca^{++} -deprivation and a further increase in resting tension upon reperfusion with Ca^{++}. No ultrastructural changes were observed within 3 minutes of Ca^{++} -free perfusion, whereas separation of intercalated discs was noted after 5 minutes or longer and upon reperfusion with normal medium after 3 minutes of Ca^{++} -free perfusion. Longer intervals of Ca^{++} -free perfusion of 10-40 minutes resulted in detachment of the basement membrane from the sarcolemma, contracture, and alterations of the mitochondria and sarcoplasmic reticulum. Reducing the Na^{+} concentration of the Ca^{++} -free medium delayed failure of contractility and

upon restoration of Ca^{++} after 3 minutes of Ca^{++} -free perfusion the recovery of contractile force was greatly augmented and the separation of the intercalated discs was prevented. Reducing the Mg^{++} concentration of the Ca^{++} -free medium also delayed failure of contractility but did not affect recovery. Reducing the K^{+} concentration prevented recovery of contractile force after 3 minutes of Ca^{++} -free perfusion.

Perfusion of hearts with Na^{+} -free medium resulted in a temporary increase in both contractile force and dF/dt followed by a decline to zero within 25 seconds. Failure of contractility was associated with an increase in both resting tension and time to ½ relaxation, but little change in time to peak tension. Electrical activity of the hearts disappeared shortly after contractility. No changes in ultrastructure were apparent even after 30 minutes of Na^{+} -free perfusion. The time course of failure of contractile force was unaffected by the Ca^{++} concentration of the Na^{+} -free medium. The magnitude of the initial increase in contractile force varied directly with the K^{+} concentration of the Na^{+} -free medium, and failure of contractility was greatly delayed by reduced K^{+} concentration. Recovery upon reperfusion with normal medium was associated with a period of ventricular fibrillation the length of which varied with the duration of the Na^{+} -free

perfusion, and was inversely related to the Ca/K concentration ratio of the Na^+ -free medium.

Perfusion of hearts with K^+ -free medium resulted in an initial dF/dt related decline in contractile force followed by a simultaneous increase in contractile force and time to peak tension, dF/dt remaining depressed. Contractility failed abruptly after 10-11 minutes of K^+ -free perfusion. Hearts fixed after 5-8 minutes of K^+ -free perfusion and prior to failure of contractility revealed a swelling of the sarcoplasmic reticulum but otherwise normal ultrastructure, whereas hearts fixed after 14-15 minutes of K^+ -free perfusion showed frequent areas of contracture in which mitochondria are swollen as well. Recovery of contractile force upon reperfusion with normal medium followed a pattern opposite to that which occurred during K^+ -free perfusion, increasing greatly initially then falling again to a low level before returning towards the control level. Reperfusion with normal medium following 8 minutes or less of K^+ -free perfusion resulted in a complete recovery of contractile force within 20-30 minutes. Similar reperfusion after more than 8 minutes resulted in severe contracture.

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I. INTRODUCTION AND STATEMENT OF THE PROBLEM

The proper functioning of the heart is considered to depend upon the cellular architecture and the integrity of fine subcellular components, and any derangement in the ultrastructure of the heart can be conceived to be associated with myocardial dysfunction. Ultrastructural damage in heart may occur at the level of the myofibrils, mitochondria, sarcotubular system, sarcolemma, and the intercalated discs. A wide variety of such changes are known to occur in various types of failing hearts, however a cause-effect relationship between ultrastructural changes and failure of heart to develop contractile force has not been clearly established. This has been mainly due to the difficulties in assessing myocardial function and examining myocardial ultrastructure simultaneously under *in vivo* conditions.

Various cations, particularly calcium, sodium, and potassium are known to have a profound effect upon myocardial contractility. Scattered reports are also available in the literature in which ultrastructural damage has been shown to occur on perfusing hearts with medium lacking these cations. It was therefore considered of interest to examine the time course of ultrastructural changes as well as the ability of the heart to develop contractile force on perfusing hearts with Ca^{++} -free, Na^{+} -free, and K^{+} -free media. The recovery

of the mechanical activity and the ultrastructure of the cation-depleted hearts were also studied by perfusing these hearts with normal medium. Since sodium, potassium, and calcium are known to have effects which are interdependent, the failure and recovery of hearts due to the absence of a particular ion was also investigated by altering the composition of the other ions in the medium. In some experiments surface electrical activity of the myocardium was also monitored. Isolated rat hearts perfused with aerobic Krebs-Henseleit solution were employed in this study and the myocardium was fixed at the desired contractile state for electron microscopic examination. It is our belief that the information gained in this study may have some bearing on the role of electrolytes in the genesis and prevention of heart failure.

II. REVIEW OF LITERATURE

Research on the role of sodium, potassium, and calcium ions in myocardial function dates back to the work of Ringer who first investigated the requirement of the isolated frog heart for the inorganic cations normally present in blood(1). He reported that there was an absolute requirement for calcium for the maintenance of contractility; contracture occurred in the absence of potassium, while the heart arrested in diastole in the presence of high potassium. In a later study (2) he reported that the positive inotropic influence of calcium was antagonized by sodium and potassium. A few years later Gross(3), found that injection of large doses of calcium chloride into the perfusion cannula caused mammalian hearts in vitro to fibrillate. In the course of studying the substrate requirements of the isolated frog heart Locke and Rosenheim(4) found that cessation of the contractile activity upon omission of calcium from the perfusion fluid resulted in a decrease in glucose utilization, and simultaneous omission of potassium resulted in an even greater reduction in glucose utilization. Furthermore, Clark(5) demonstrated that if the osmotic pressure is maintained by addition of sucrose, the concentration of sodium chloride can be reduced to between 0.1 and 0.2% (17-34 mM) without impairing the contractile activity of the heart. Within the range 0.2 to 0.7% sodium

chloride the amplitude of contraction decreases as the sodium concentration is raised, whereas when sodium chloride is omitted the heart is arrested in systole. The force of contraction was found to increase by increasing the ratio of Ca^{++}:Na^{+} + K^{+} in the perfusion medium. These early studies clearly indicated the involvement of the cations, Na^{+}, K^{+} and Ca^{++} in the cardiac mechanical and metabolic activities, and that the functional roles of these ions are interdependent.

The excellent delineation by Hodgkin and Huxley(6,7,8,) of the roles of sodium and potassium ions in the manifestation of action potentials in nerve fibers has been extended and modified by Noble(9) to apply to cardiac action potentials and thus the critical importance of these two ions as regards the electrical behaviour of the heart can be interpreted. A large body of information dealing with the effects of extracellular electrolyte concentrations on the shape and duration of the cardiac action potential has been surveyed by Surawicz and Gettes(10) resulting in the following generalizations: (a) increased extracellular potassium shortens the duration of the action potential while low potassium causes a marked increase in the action potential duration, and (b) large decreases in sodium concentration (of the order of 85-100 per cent) result in decreased upstroke velocity and overshoot leading to a loss of excitability. Stanley and Reiter(11) have reported sodium and calcium to

have an antagonistic effect upon the action potential duration in guinea pig papillary muscle. This conclusion was based upon the finding that an increase in calcium or a decrease in sodium concentration produced shortening of the action potential duration. Furthermore, the maximum action potential duration occurred at 140 mM sodium and 1.2 mM calcium, becoming somewhat shorter if calcium is further reduced.

Simultaneous recordings of both the mechanical and electrical changes in the perfused rat heart made by Mines(12) showed that the effects of alterations in Ca concentration were not associated with changes in the electrical activity. Particularly it was demonstrated that the mechanical activity was abolished while the electrical activity remained unaltered on perfusing the heart with Ca^{++} -free medium. Daly and Clark (13) confirmed this observation and also demonstrated that deficiency of potassium or sodium ions as well as excess of calcium ions in the perfusion medium increased contraction of the heart. The interaction of sodium and calcium ions with regards their effects upon contractile force was quantified by Wilbrandt and Koller(14) who found that the strength of contraction in the frog heart corresponded in a precise manner to changes in the ratio of the calcium concentration to the square of the sodium concentration in the perfusion fluid.

The widely accepted view that extracellular calcium is

directly involved in the activation of contraction following excitation is largely the result of the following observations: (a) an increase in the external calcium concentration has an effect on myocardial contractility similar to that of increased frequency of contraction (Bowditch staircase)(15); (b) external calcium concentration directly influences the contracture tension of KCl depolarized frog ventricle without changing the amount or time course of the depolarization(16); (c) application of calcium after the onset of depolarization results in an increased force of contraction during a single heart beat(17); (d) the rate of action of calcium suggests that the external calcium is in equilibrium with a small and rapidly exchangeable calcium store involved in tension changes (18); (e) calcium entry into cardiac tissue is facilitated by depolarization of the membrane(19); (f) an increased uptake of calcium is associated with contractures initiated by changes in the external Na^+ and K^+ concentration(20); (g) calcium content of frog ventricles perfused with K^+ -free Ringer solution increases linearly to twice the normal value, coinciding with a gradual development of contracture which could be reversed by EDTA(21: (h) calcium exchangeability increases on stretching cardiac muscle, and is decreased in the hypodynamic state(22); (i) in the beating heart both influx and efflux of labeled calcium show an increase which

is of the order of 10-20 times during activity as compared to the heart at rest(23); (j) during contraction entry of calcium increases considerably, and the uptake of calcium by atrial cells is closely correlated with the strength of contraction when either the rate of stimulation or the external calcium concentration is changed(24).

Similarly, the influence exerted by external sodium and potassium concentration on contractility would appear to be largely the result of their effect on the movement of calcium between the extra- and intracellular compartments. Strongly convincing evidence for this is provided by the observations that every known feature of the action of calcium ions on contraction can be simulated by a reduction of the external sodium concentration(25,26). Furthermore, when either sodium or potassium are reduced or omitted from the external fluid the rate of influx of calcium increases and the rate of efflux of calcium is diminished(19,27-31). Decreased sodium or potassium concentrations or increased calcium concentration have been shown to result in an augumentation of the increase in contractile force due to stretch(32). However, it should be noted that the mechanisms by which external sodium and potassium concentrations affect calcium movements are largely unknown.

It has been shown that calcium influx occurs during

excitation which contributes to the cardiac action potential (33,34). The calcium involved in cardiac muscle has been shown to arise from two sources(35-40), one of the sources being extracellular in origin whereas the second is intracellular and probably constituted by the membranes of the sarcoplasmic reticulum as in the case of skeletal muscle (41). Explanations of the inotropic effects of external sodium and potassium which focus on the first aspect of calcium movement include direct competition between sodium and calcium ions at the cell membrane level(25,26) and changes in the action potential duration affecting the time course of the calcium entry phase(42). Explanations have also been offered which assume an intracellular site of action for sodium and potassium as well. These include a competition between sodium and calcium for intracellular membrane binding sites whereby changes in intracellular sodium can affect the availability and size of the intracellular calcium pool(43). It is also proposed that intracellular sodium acts upon the intracellular membranes leading to increased calcium sequestration, whereas potassium results in labilization of intracellular calcium stores(44). These suggestions are difficult to evaluate at present because of insufficient data concerning the relative contribution of intra- and extracellular calcium to the activation of contraction as well as on the proposed intracellular sites of sodium and potassium action.

The possibility that calcium may have a functional role

other than in excitation-contraction coupling is suggested by the discovery that calcium concentration is an important factor in determining the tendency for fibrillation to occur in heart(3,45,46). Recent research has clearly demonstrated the importance of calcium in stabilizing biological membranes and determining their permeability characteristics(47). Yokoyama et al(48) and Muir(49) succeeded in separating individual cardiac muscle cells from each other at the intercalated discs using calcium-free solutions and calcium chelators, thus implicating calcium in intercellular adhesion in cardiac muscle. Weiss et al(50) perfused rabbit hearts with calcium-deficient solutions and found, in addition to irreversible depression of contractility and excitability, light microscopic evidence of myofibrillar damage. Muir(51,52) conducted a more extensive investigation of the ultrastructural changes occuring during calcium-free perfusion of isolated rat hearts and reported, in addition to separation of myocardial cells at the intercalated discs, separation of the basement membrane from the plasma membrane. The myofibrillar damage reported by Weiss et al(50) on light microscopic examination was not confirmed by Muir(52) in hearts perfused with calcium-free medium, but hearts perfused with very low calcium concentration (0.1 or 0.2 mM) for extended periods went into a severe contracture and showed myofibrillar damage.

Zimmerman and Hulsmann(53) introduced the term "calcium-

paradox" for the observation that following perfusion of the isolated rat heart for more than three minutes with Ca^{++} -free medium, reintroduction of the normal calcium-containing medium resulted in loss of contractility and electrical activity. Under this condition loss of cell contents, including myoglobin, high energy phosphates, potassium, lactate dehydrogenase, and creatine phosphokinase was also noted. In a later study, Zimmerman et al(54) found the calcium-paradox" to be associated with extensive morphological damage as well. Lee and Visscher(55) confirmed the findings of Zimmerman and Hulsmann(53) concerning permeability changes and loss of cell contents in the calcium-paradox phenomenon. Muir(56) has also reported that, upon exposure to calcium, calcium depleted hearts undergo severe contracture and extrusion of mitochondria.

The cardiac ultrastructural changes due to depletion of ions other than calcium have not been studied extensively. Legato et al(29) have reported a swelling of the sarcoplasmic reticulum, attributed to calcium accumulation, to occur following a 75% reduction in external sodium concentration. Emberson and Muir(57,58) studied the ultrastructure of isolated rat hearts perfused with hypo- and hyperkalemic solutions. It was found that potassium-free perfusion resulted in severe contracture and changes in the appearance

of the mitochondria while perfusion with low potassium solutions resulted in some disintegration of the thin filaments without any other alteration of ultrastructure. Perfusion with very high potassium concentrations (12-70 mM) resulted in a variety of morphological changes including distension of the transverse tubules, disintegration of thin filaments, and development of contracture. Thus the present study was undertaken in order to examine in a systematic manner the changes, both structural and functional, which occur following depletion of isolated rat hearts of sodium, potassium, or calcium. Special emphasis was laid on determining whether and to what extent the changes in electrical and mechanical activities of the heart can be correlated with changes in cardiac ultrastructure. Experiments were also carried out in which hearts were depleted of each ion with media containing altered concentrations of the other ions for the sake of clarifying the nature of the interactions of these ions.

III. METHODS

Male albino rats weighing 300-350 g were decapitated, the hearts quickly removed and arranged for perfusion by conventional Langendorff technique. The perfusion medium was Krebs-Henseleit solution containing 120 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.25 mM $CaCl_2$, 1.20 mM $MgSO_4$, and 8 mM glucose. The perfusion solution, pH 7.4, was continuously gassed with a mixture of 95% O_2 and 5% CO_2, and the perfusion temperature was maintained at 37.5°C. The perfusion rate was maintained at about 10 ml/min. In an initial series of experiments it was established that contractile force, heart rate, rate of rise of contractile force and surface electrical activity of the heart became stable within about 10 minutes following commencement of perfusion, and did not change thereafter for a period of two hours. In all subsequent experiments hearts were allowed to equilibrate for 20 minutes with the control medium before commencing perfusion with modified solutions.

In perfusion solution where $CaCl_2$ or $MgSO_4$ were omitted, no corrections were made for osmolarity whereas when the concentrations of NaCl or KCl were reduced an equivalent amount of sucrose was substituted. In some of the initial experiments, the Ca^{++} chelating agent ethylene-diamine-tetra-acetic acid (EDTA) was added to the Ca^{++} -free solutions. No differences

were discernable between hearts perfused with Ca^{++} -free medium in the presence or absence of EDTA. In experiments where most of the NaCl content of the Ca^{++} -free perfusion medium was replaced by sucrose, EDTA was again used to chelate any Ca^{++} contamination possibly originating in the added sucrose. Once again, no difference in results was observed to occur with or without the addition of EDTA.

For the Na^{+} -free medium, NaCl and $NaHCO_3$ were replaced by sucrose, and the pH was adjusted using KOH; where the potassium concentration was also varied, the amount of sucrose added was adjusted accordingly. For the K^{+} -free medium, KCl was replaced by sucrose and KH_2PO_4 was replaced by an equivalent amount of NaH_2PO_4; where the sodium content was also reduced, NaCl was replaced by sucrose as well.

The contractile force was recorded on a Grass polygraph via a force displacement transducer (fT.03) connected to the apex of the heart. The times for peak tension and ½ relaxation were calculated from the polygraph tracings of the contractile force obtained at the maximum speed of the recorder (100 mm/sec). The rate of rise of the developed tension (dF/dt) was monitored by differentiating the signals for the contractile force. Surface electrical activity of the heart was monitored by attaching platinum electrodes to the right atrium and the base of the left ventricle. The difference in potential

between these two electrodes was amplified and recorded on the Grass polygraph.

Ultrastructural studies were performed on hearts fixed by perfusion for 2-3 minutes with 0.1 M phosphate buffer (pH 7.4) containing 1 or 2% glutaraldehyde. Small pieces were then dissected out and allowed to continue fixing in 1 or 2% glutaraldehyde for 1 hour, washed overnight in 0.1 M phosphate buffer and post-fixed for 1 hour with 1% OsO_4. Tissue specimans were then dehydrated in a graded ethanol series and embedded in either Epon 812 or Araldite 502. Sections were made on a Porter-Blum MT-II ultramicrotome using glass knives, stained with Reynolds' lead citrate stain, and examined using a Zeiss EM 9S electron microscope.

IV. RESULTS

A. Structural and Functional Changes in the Isolated Rat Heart Following Perfusion with Ca^{++} -Free Medium:

Perfusion with Ca^{++} -free medium caused both contractile force and dF/dt to decline to zero within 30 seconds (Figure 1). During this time there was no change in the surface electrical activity, suggesting that a complete dissociation of electrical and mechanical events had occurred. The decline in contractile force was observed to be associated with a decline in time to peak tension development as well (Figure 2). The diastolic resting tension is also not noticeably altered during this period (Figure 1), whereas the time to ½ relaxation increased dramatically as shown in Figure 2.

The ability of the heart to recover contractility upon reperfusion with Ca^{++} -containing medium was inversely related to the duration of the Ca^{++} -free perfusion. The recovery declined rapidly between first and five minutes of Ca^{++} -free perfusion and there was no recovery of contractility thereafter (Figure 3). The resting tension was seen to rise steadily during the first 3 minutes of perfusion with Ca^{++} -free medium, and then plateaued (Figure 4). Within the first minute of Ca^{++} -free perfusion, the reperfusion with Ca^{++} -containing medium resulted in a return of the resting tension towards the control level. Longer periods of perfusion with

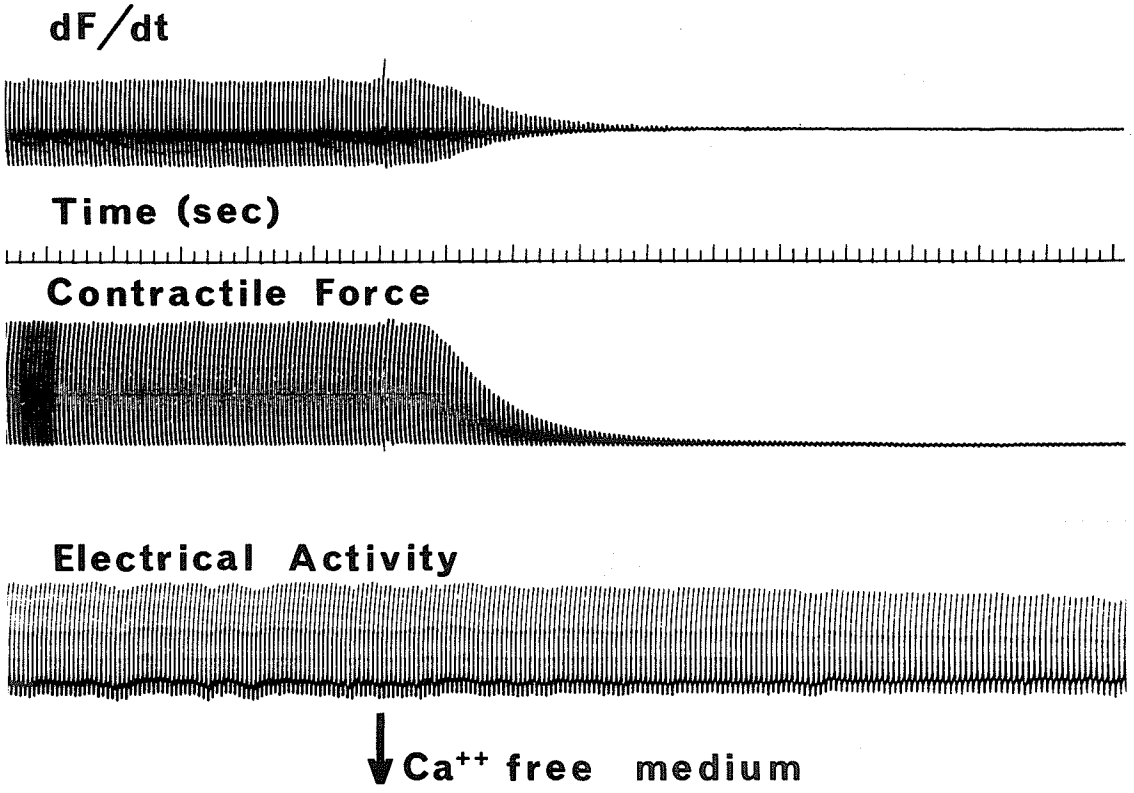

Figure 1: Typical recording of contractile force, dF/dt, and surface electrical activity of an isolated rat heart perfused with Ca^{++} -free medium.

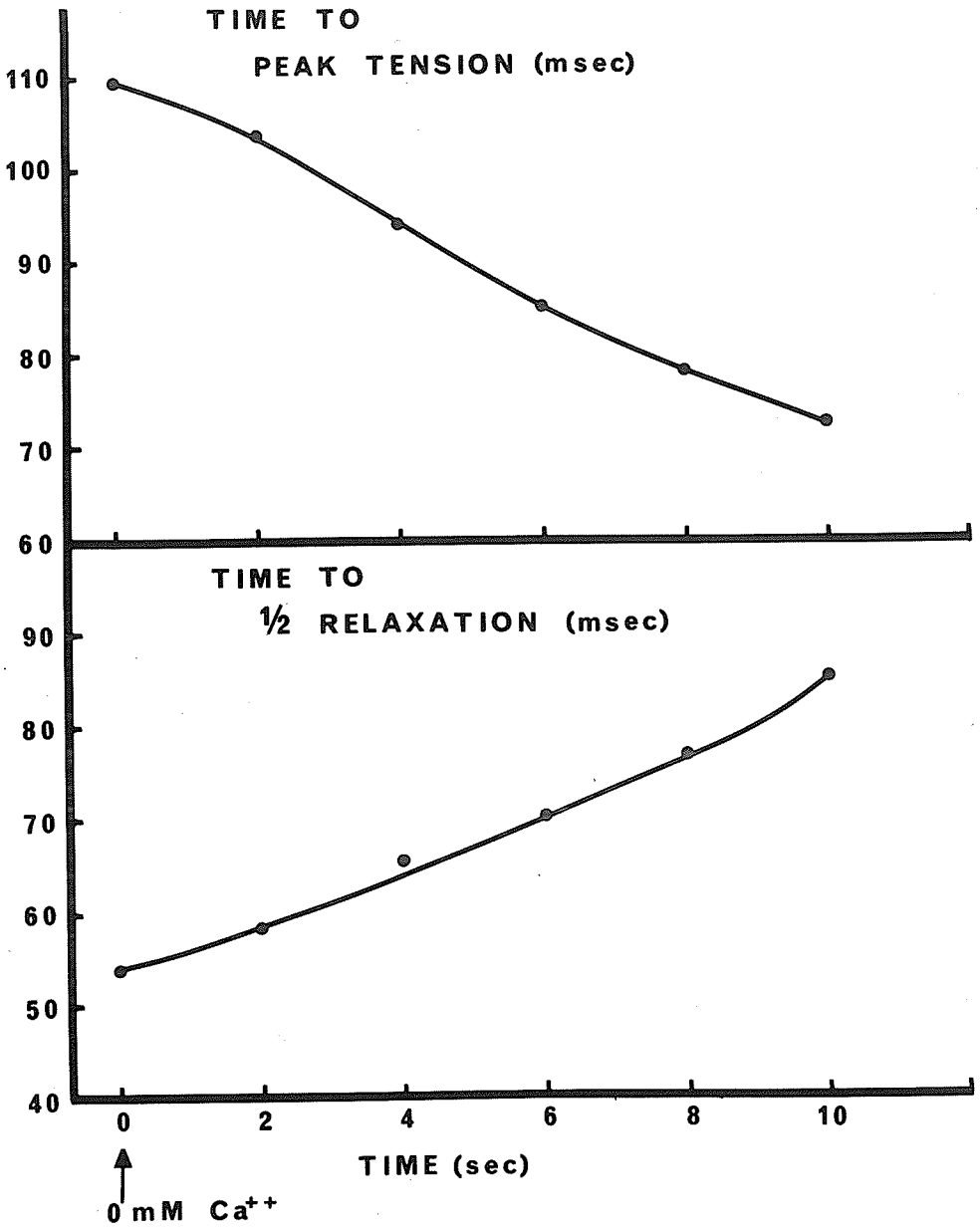

Figure 2: Upper panel - Effect of Ca^{++} -free perfusion on the time to peak tension in isolated rat hearts. Lower panel - Effect of Ca^{++} -free perfusion on the time to ½ relaxation in isolated rat hearts. Each value is a mean of 6 experiments.

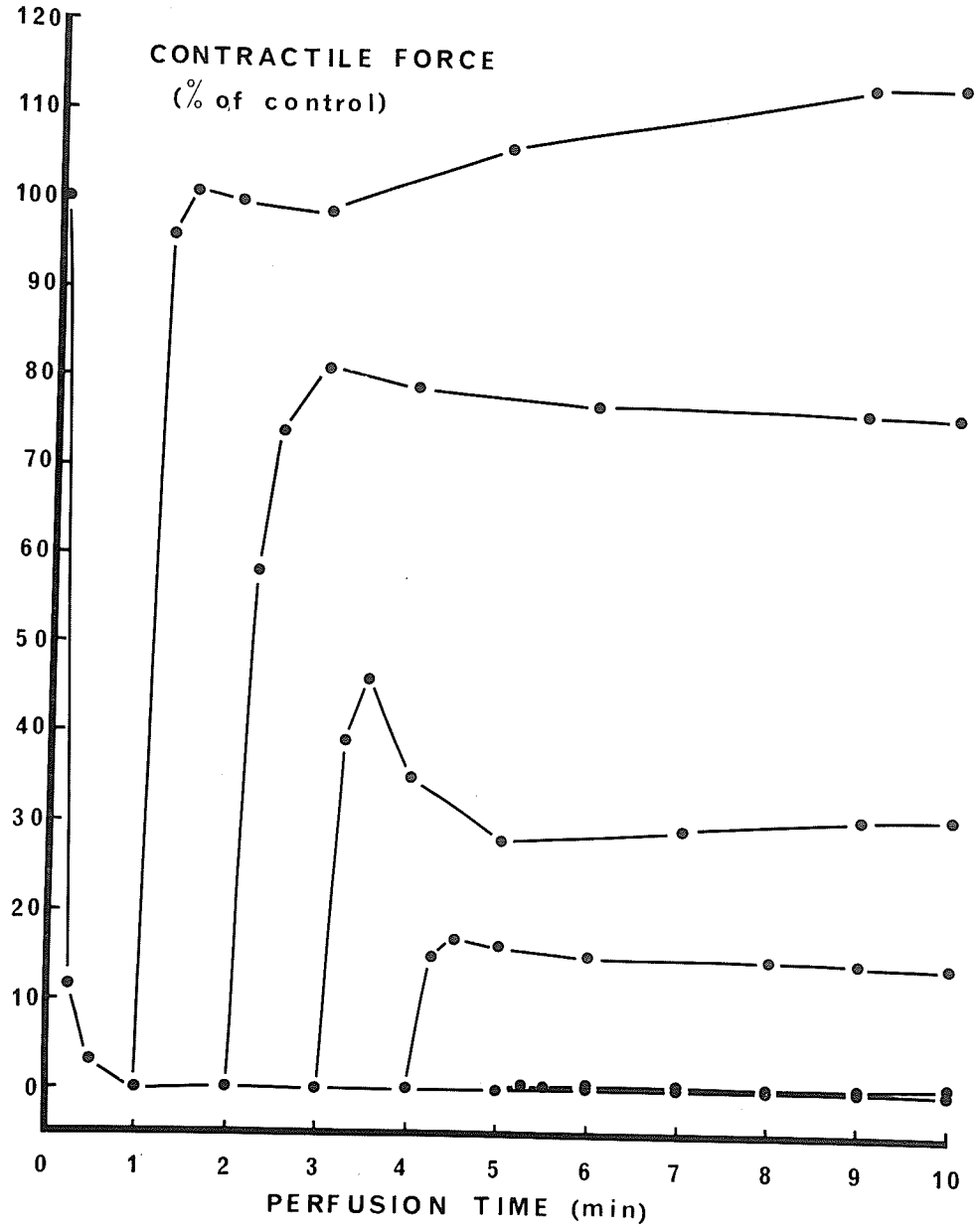

Figure 3: Effect of various intervals of Ca^{++} -free perfusion on the ability of isolated rat hearts to recover contractility upon reperfusion with 1.25 mM Ca^{++}. Each value is a mean of 6 experiments.

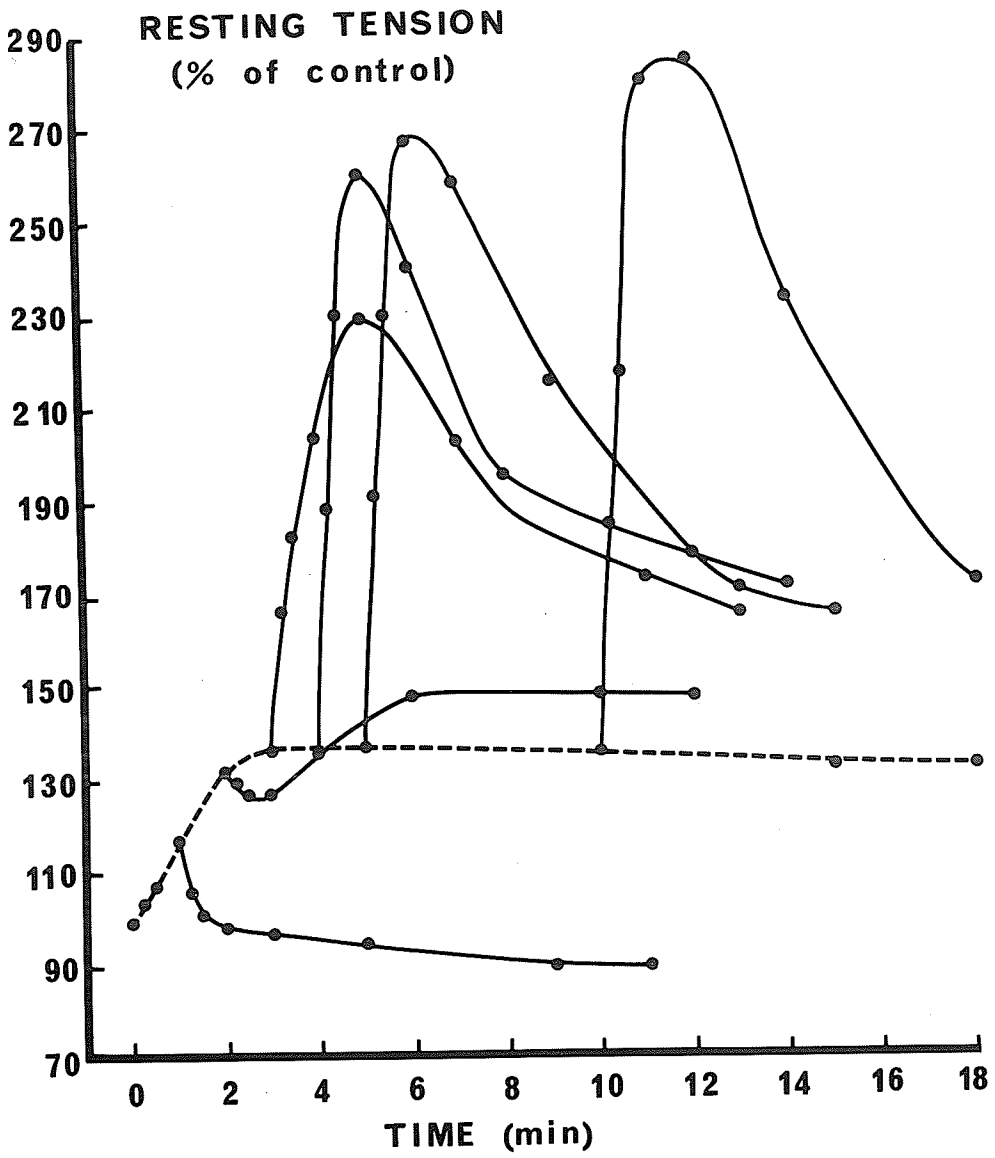

Figure 4: Effects on the resting tension of isolated rat hearts of Ca^{++} -free perfusion and of reperfusion with 1.25 mM Ca^{++} following various intervals of Ca^{++} -free perfusion. ●----● resting tension during Ca^{++} -free perfusion; resting ●——● tension during recovery. Each value is a mean of 6 experiments.

Ca^{++} -free medium resulted in a greatly elevated resting diastolic tension on reperfusion with calcium, which the hearts were unable to maintain at the maximum (Figure 4). The surface electrical activity steadily diminished in amplitude but remained regular for about 3 minutes after commencing Ca^{++} -free perfusion (Figure 5). After 3-4 minutes the surface electrical activity became very irregular and its amplitude declined towards zero in a subsequent 10 to 12 minutes. If reperfusion with Ca^{++} -containing medium was begun during the first 3 minutes of Ca^{++} -free perfusion the amplitude of the electrical activity returned towards the control level, whereas reperfusion with Ca^{++} -containing medium following more than 3 minutes of calcium deprivation resulted in a recording of depressed and irregular amplitude (Figure 5).

Three hearts each were fixed for ultrastructural studies following 1, 2, 3, 5, 10 and 40 minutes of Ca^{++} -free perfusion as well as following 10 minutes of reperfusion with Ca^{++} -containing medium subsequent to Ca^{++} -free perfusion for 3, 5, and 10 minutes duration. Sections taken from these hearts were compared with those from hearts perfused for similar periods with the control medium. Electron microscopic examination of hearts fixed after 1, 2, or 3 minutes of Ca^{++} -free perfusion did not reveal any ultrastructural alterations

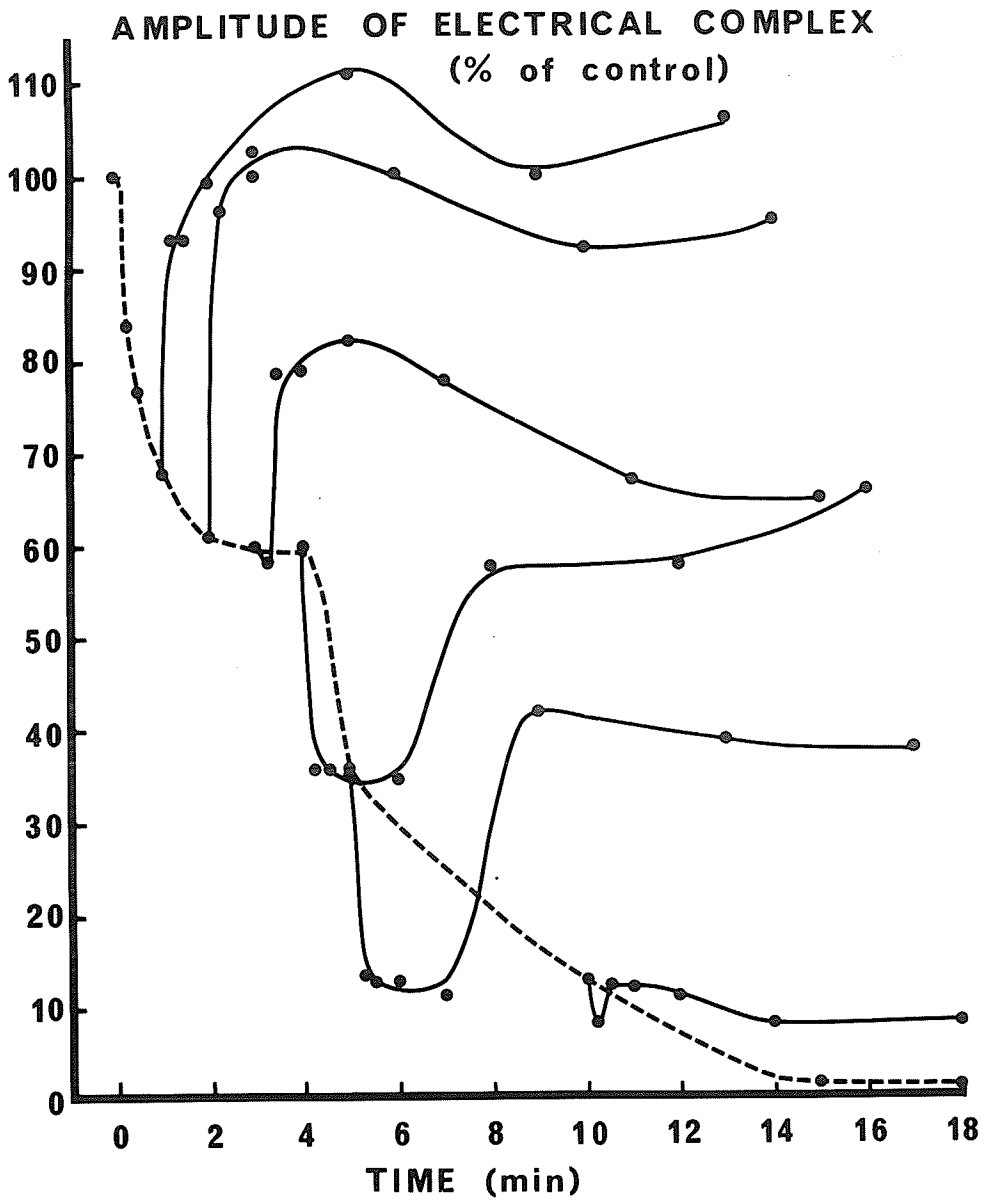

Figure 5: Effects on the amplitude of the surface electrical activity of isolated rat hearts of Ca^{++} -free perfusion and of reperfusion with 1.25 mM Ca^{++} following various intervals of Ca^{++} -free perfusion. ●– – –● amplitude of electrical activity during Ca^{++} -free perfusion; ●——● amplitude of electrical activity during recovery. Each value is a mean of 6 experiments.

as compared with the control (Figure 6). Ca^{++} -free perfusion for 5, 10, or 40 minutes, however, showed various degrees of damage to the cell ultrastructure. The basement membrane, which normally appears as a layer of diffuse material closely adherent to the sarcolemma, became widely separated from the plasma membrane within 40 minutes; only intermittant points of contact remained (Figure 7). Following 40 minutes of Ca^{++} -free perfusion, both mitochondria and sarcoplasmic reticulum were swollen. In some areas adjacent mitochondria appear to have become joined, the intramitochondrial spaces being continuous for considerable distances, and the mitochondria contained numerous small vesicular spaces. Large smooth surfaced vesicles occurred frequently among these mitochondria (Figure 8). As perfusion time with Ca^{++} -free medium was increased from 5 to 40 minutes, there was progressive appearance of contracture of the sarcomeres in some areas until at 40 minutes much of the heart was observed to be in a state of contracture to some degree (Figure 9). In areas where contracture of the sarcomeres was most severe the structure of the mitochondrial cristae appeared disrupted. The most striking and consistent observation was the appearance of progressively more distinct separations of the intercalated discs in hearts perfused for longer than 5 minutes with Ca^{++} -free medium (Figures 10 and 11). No separations

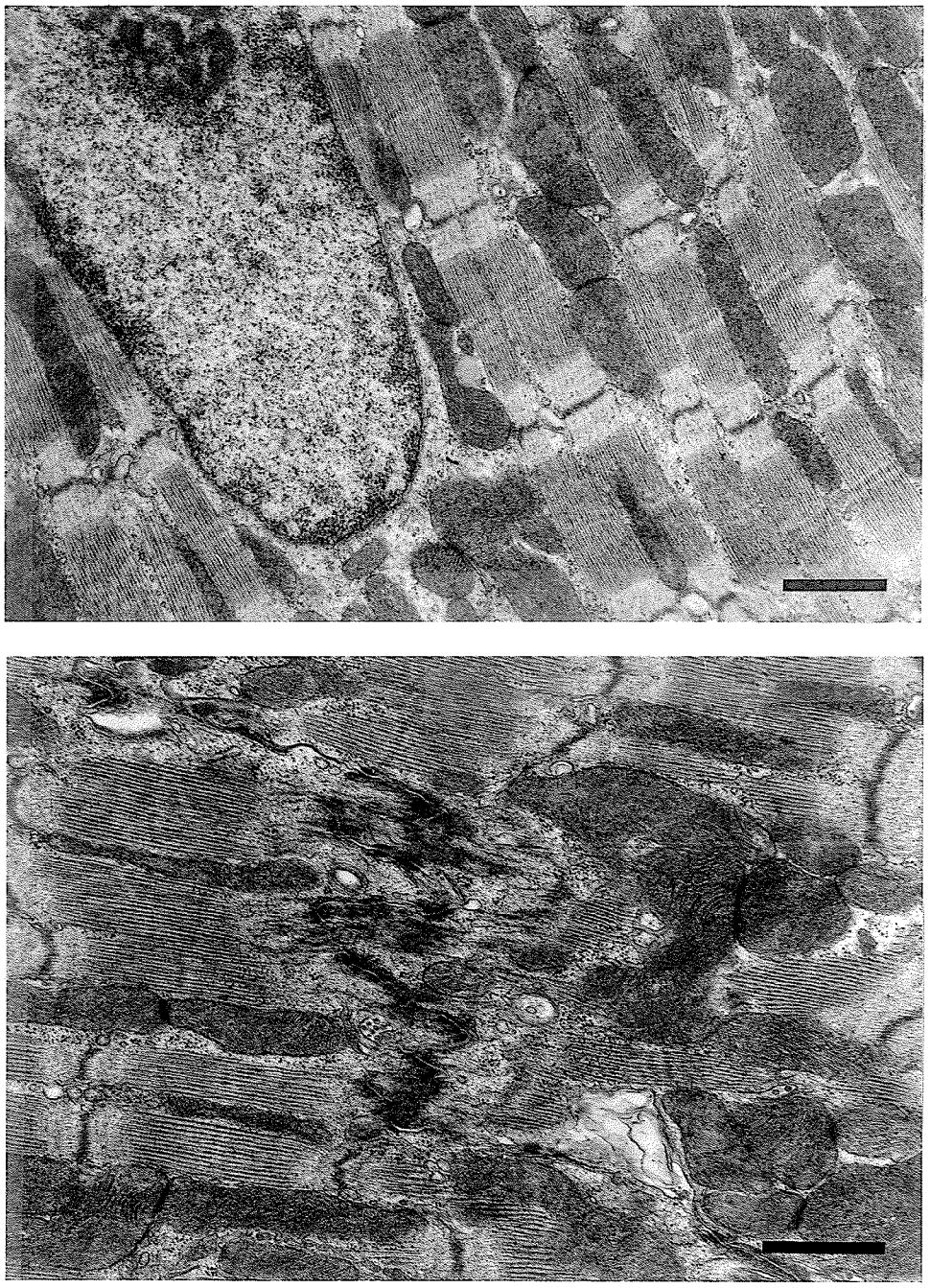

Figure 6: Electron micrographs of typical sections from isolated rat hearts perfused for 3 minutes with Ca^{++} -free medium. Black line indicates one micron.

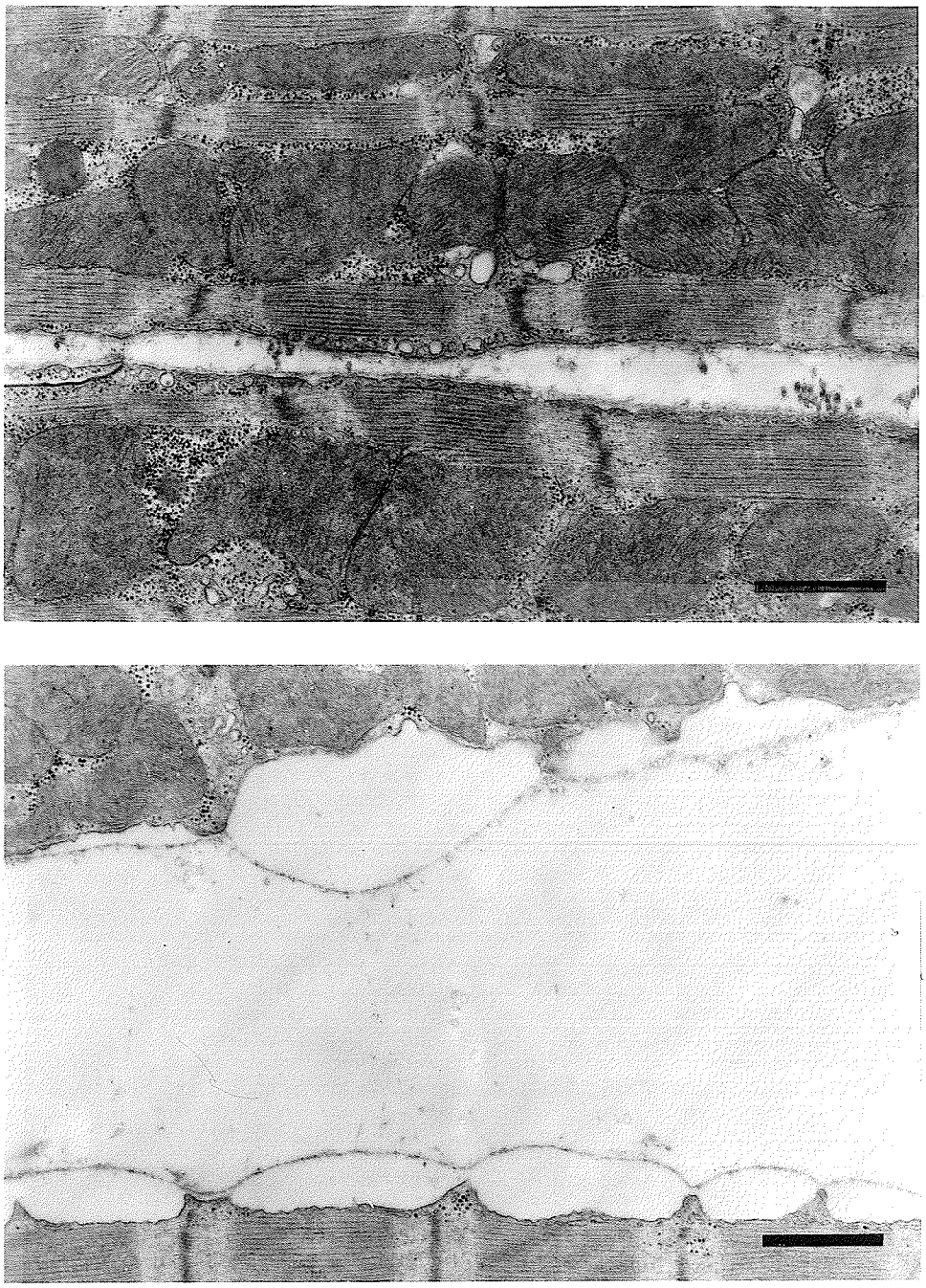

Figure 7: Upper panel - Electron micrograph of a section from an isolated rat heart perfused with 1.25 mM Ca^{++}, showing the normal relationship of the basement membrane to the sarcolemma. Black line indicates one micron. Lower panel - Electron micrograph of a section from an isolated rat heart perfused with Ca^{++} -free medium for 40 minutes, showing the separation of the basement membrane from the sarcolemma. Black line indicates one micron.

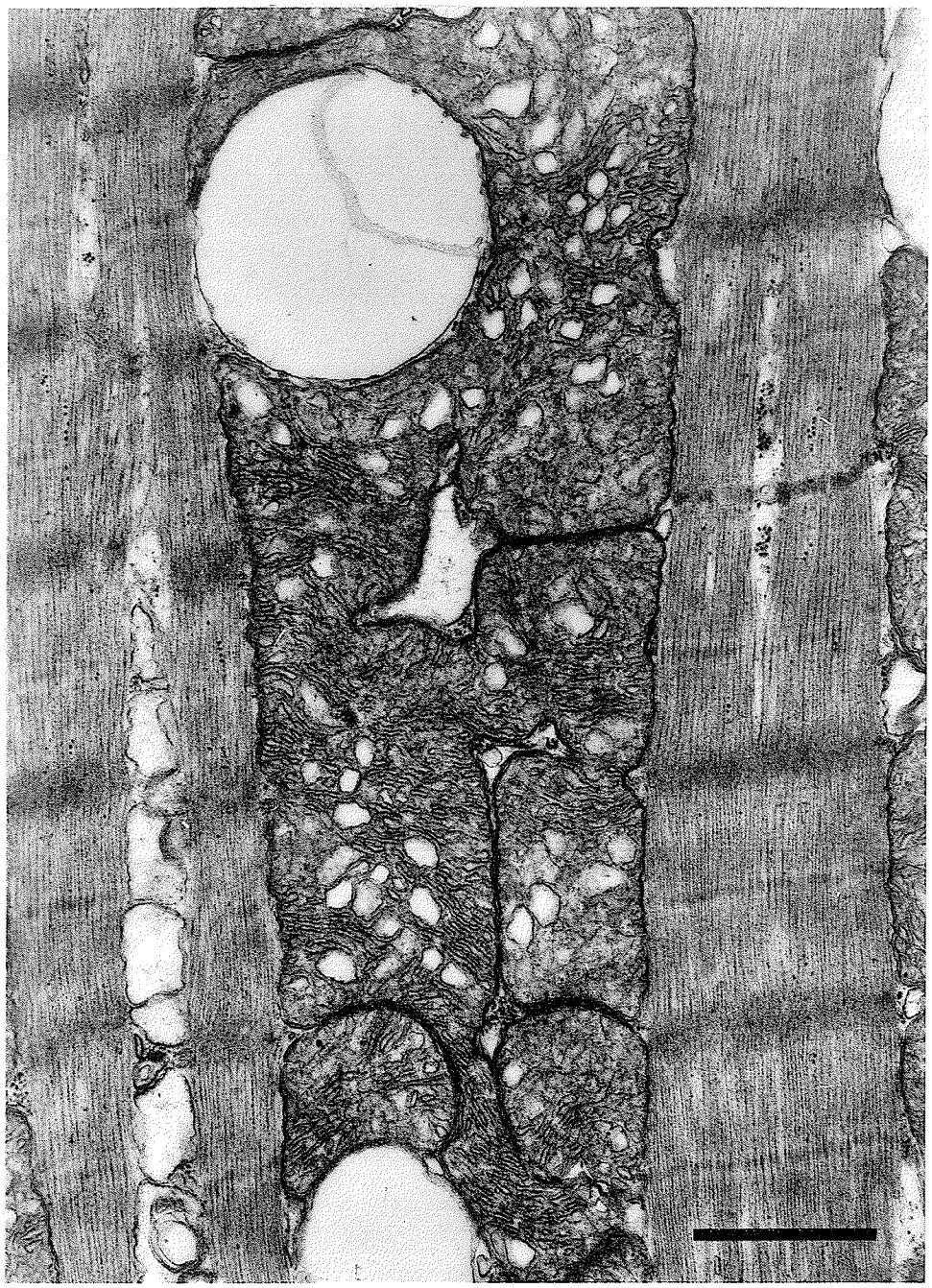

Figure 8: Electron micrograph of a section from an isolated rat heart perfused with Ca^{++} -free medium for 40 minutes, showing swollen sarcoplasmic reticulum and giant mitochondrian. Black line indicates one micron.

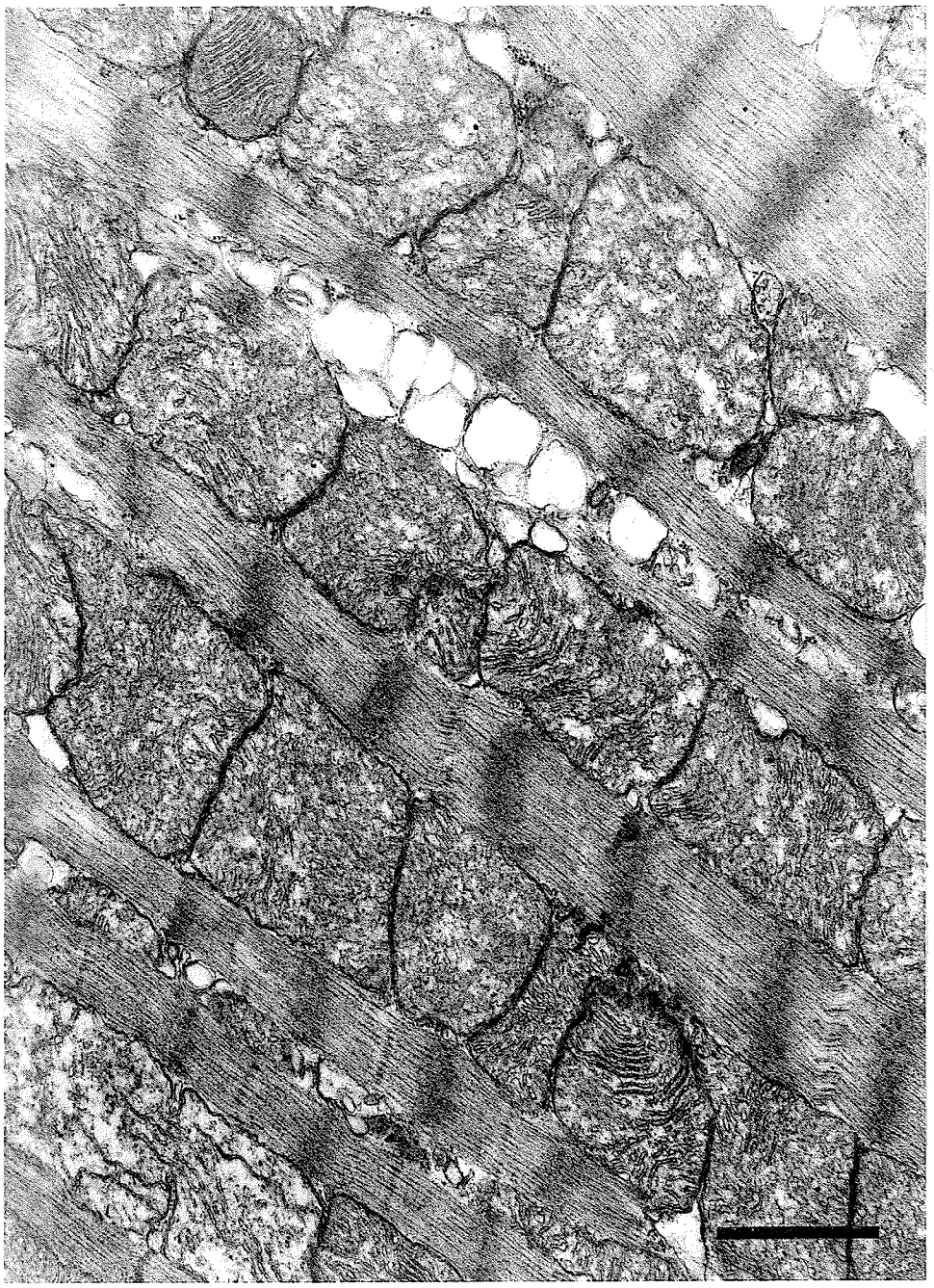

Figure 9: Electron micrograph of a section from an isolated rat heart perfused with Ca^{++} -free medium for 40 minutes, showing swollen and damaged mitochondria, swollen sarcoplasmic reticulum and contracted sarcomeres. Black line indicates one micron.

Figure 10: Electron micrograph of a section from an isolated rat heart perfused for 10 minutes with Ca^{++} -free medium, showing an early stage in separation of the intercalated disc. Black line indicates one micron.

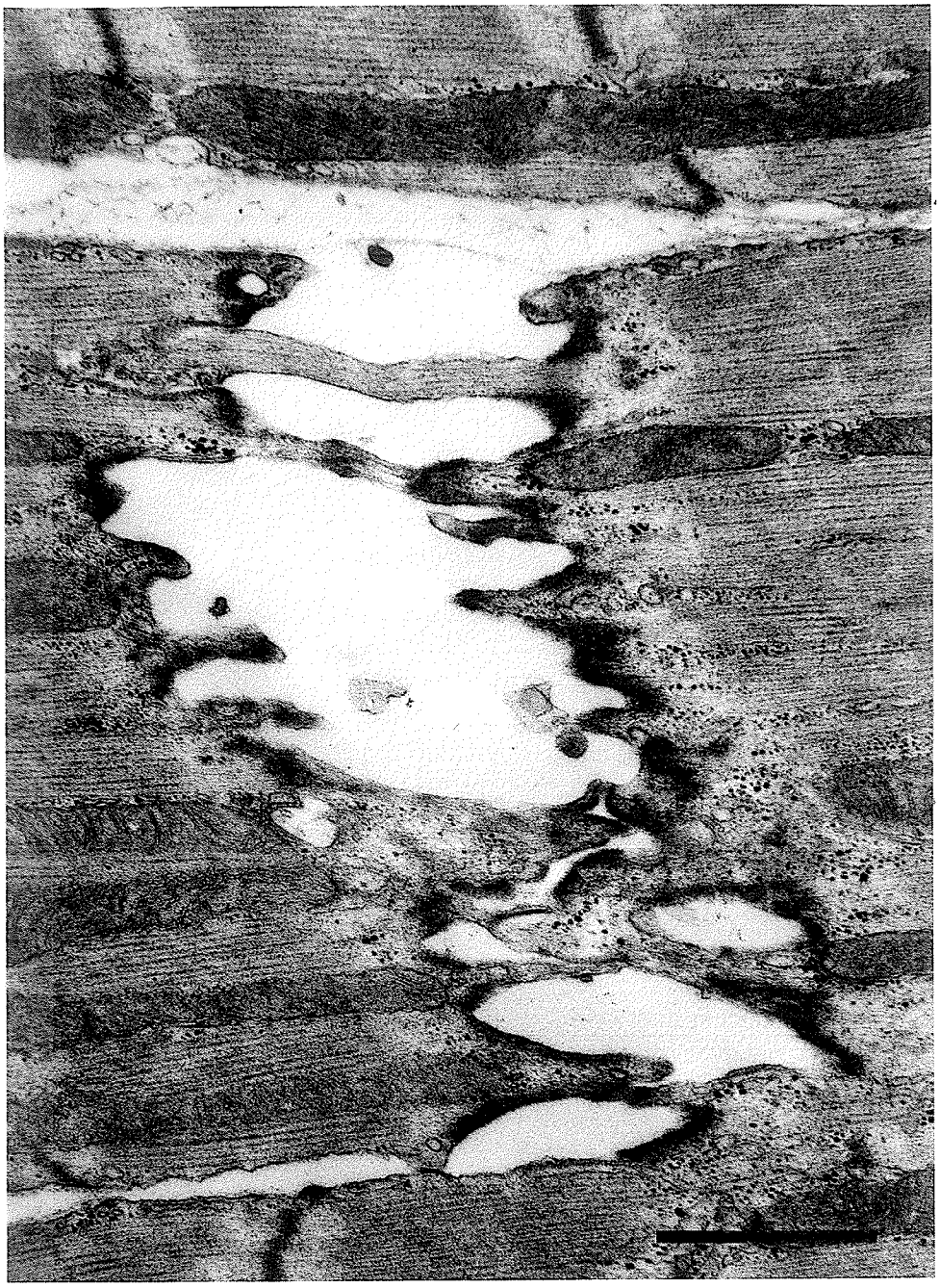

Figure 11: Electron micrograph of a section from an isolated rat heart perfused for 40 minutes with Ca^{++} -free medium, showing an advanced degree of separation of the intercalated disc. Black line indicates one micron.

of the intercalated discs were observed in 3 minutes of Ca^{++} -free perfusion; however, reperfusion with Ca^{++} -containing medium after 3 minutes of Ca^{++} -free perfusion resulted in marked separation of the intercalated discs (Figure 12). Hearts reperfused with Ca^{++}-containing medium following 5, 10 or 40 minutes of calcium deprivation showed extensive ultrastructural damage such as complete separation of myocardial cells, dramatic contracture with considerable disruption of contractile elements, and swollen mitochondria in which aggregates of an electron dense material are seen to occur and the cristae are ruptured (Figure 13).

B. Influence of Na^{+}, K^{+} or Mg^{++} on the Effects of Ca^{++} -Free Medium on the Heart

As described above, the perfusion of rat heart with Ca^{++} -free medium caused a complete failure of the heart to contract in about 30 seconds. A 75% reduction in sodium concentration of the Ca^{++} -free medium was found to augment the contractile force and dF/dt during the first 30 seconds and resulted in measurable rythmic contractions for almost 2 minutes (Figure 14). Omission of magnesium from the Ca^{++} -free medium similarily delayed failure of contractility, whereas an 85% reduction in potassium concentration had no beneficial effect (Figure 14). Upon reperfusion of hearts with 145 mM sodium and 1.25 mM calcium following a 3 minute

Figure 12: Electron micrograph of a section from an isolated rat heart perfused for 3 minutes with Ca^{++} -free medium followed by perfusion for 10 minutes with medium containing 1.25 mM Ca^{++}, showing distinct separation of the intercalated disc. Black line indicates one micron.

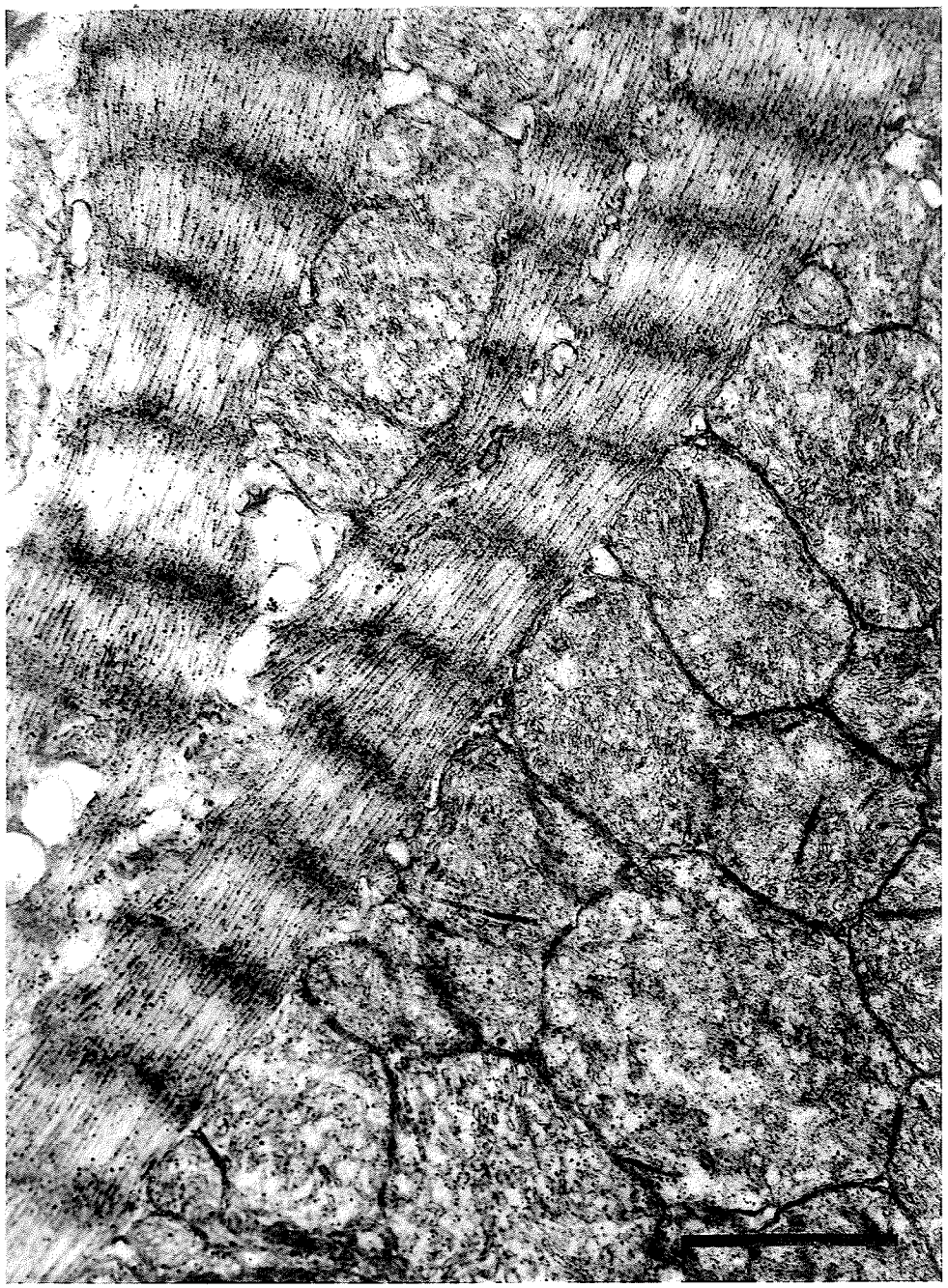

Figure 13: Electron micrograph of a section from an isolated rat heart perfused for 10 minutes with Ca^{++} -free medium followed by perfusion for 10 minutes with medium containing 1.25 mM Ca^{++}, showing contracture and disruption of contractile elements, and swollen mitochondria with ruptured cristae and aggregates of electron dense material. Black line indicates one micron.

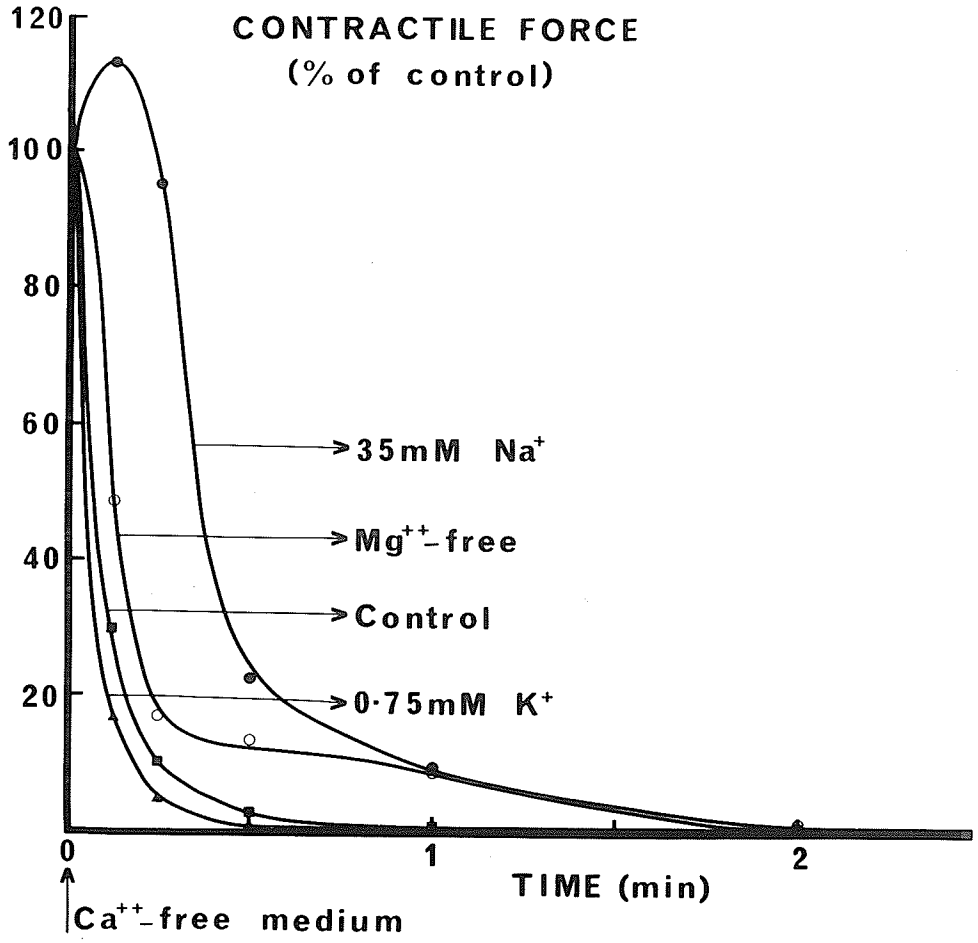

Figure 14: Effects on contractile force of perfusion of isolated rat hearts with Ca^{++} -free medium in which the concentration of Na^{+}, K^{+} or Mg^{++} has been varied. ■—■ normal Na^{+}, K^{+} and Mg^{+} concentrations in Ca^{++} -free medium; ●—● 35 mM Na^{+} in Ca^{++} -free medium; ▲—▲ 0.75 mM K^{+} in Ca^{++} -free medium; ○—○ 0 mM Mg^{++} in Ca^{++} -free medium. Each value is a mean of 6 experiments.

perfusion with Ca^{++} -free medium containing 35 mM sodium the contractile force rapidly returned to control levels, whereas in hearts perfused with Ca^{++} -free medium containing normal sodium concentration, the contractile force recovered to little more than 20% of the control level (Figure 15). While the omission of magnesium from the Ca^{++} -free medium did not appreciably alter the recovery of contractile force, reduction of potassium concentration to 0.75 mM was quite deleterious to the ability of the heart to recover contractility (Figure 15).

Electron microscopic examination of four hearts reperfused for 10 minutes with normal perfusion medium following a 3 minute perfusion with Ca^{++} -free medium in which the sodium concentration was reduced to 35 mM disclosed that this modification prevented separation of the intercalated discs. As can be seen from Figure 16, the ultrastructure of these hearts appeared to be quite normal in every respect.

C. Structural and Functional Changes in the Isolated Rat Heart Following Perfusion with Na^{+} -Free Medium

Perfusion of the isolated rat hearts with Na^{+} -free medium resulted in an initial period of increased contractile force with a peak 40% above the control values occuring after approximately 6 seconds and a subsequent decline in contractile force with failure of contractility occuring at about 25 seconds (Figure 17). Figure 18 shows a recording of

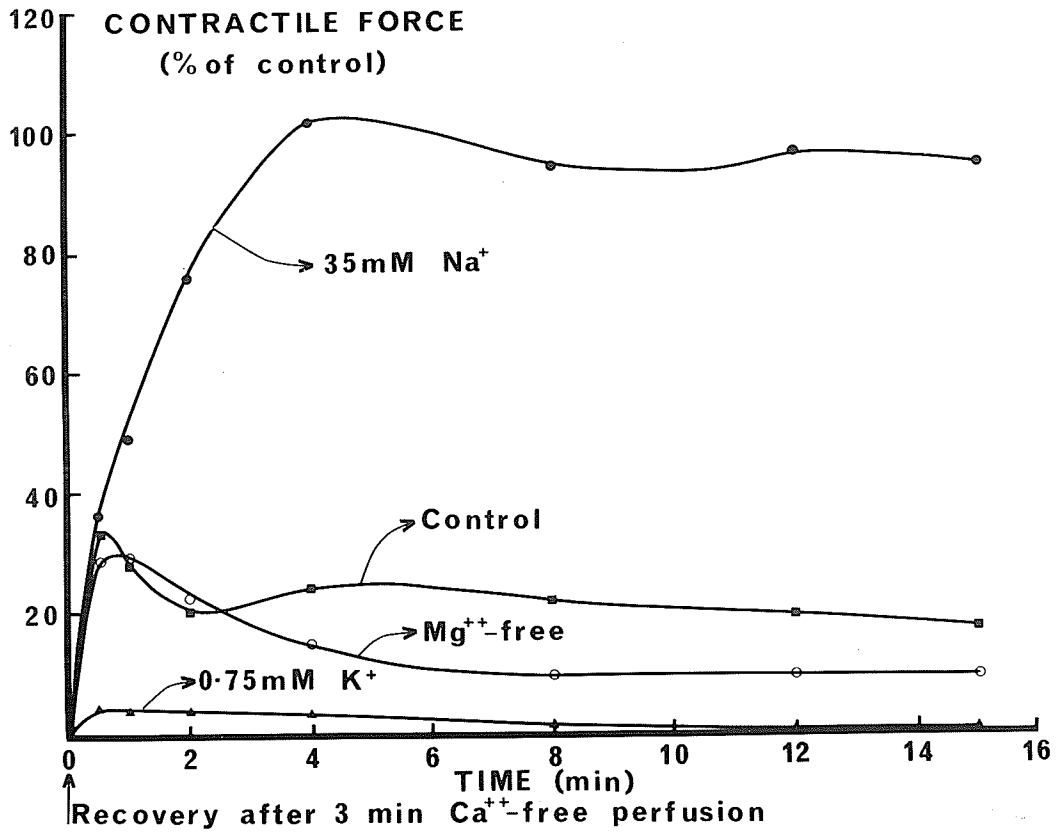

Figure 15: Effects of perfusion for 3 minutes with Ca^{++} -free medium in which the concentration of Na^{+}, K^{+} or Mg^{++} has been modified on the ability of isolated rat hearts to recover contractility upon reperfusion with control medium. ●—● 35 mM Na^{+} in Ca^{++} -free medium; ▲—▲ 0.75 mM K^{+} in Ca^{++} -free medium; ○—○ 0 mM Mg^{+} in Ca^{++} -free medium. Each value is a mean of 6 experiments.

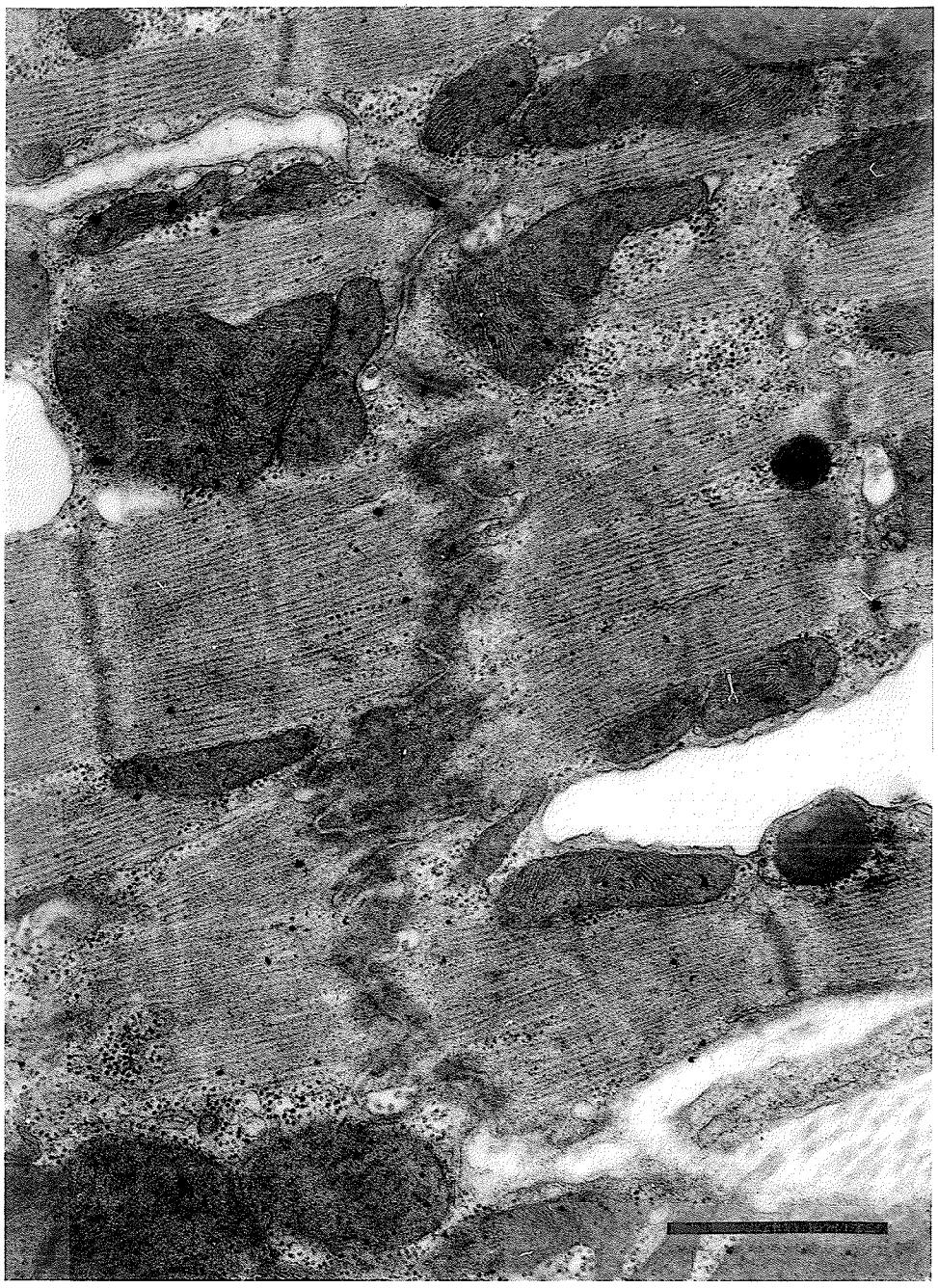

Figure 16: Electron micrograph of a typical section from an isolated rat heart perfused for 3 minutes with Ca^{++} -free medium containing 35 mM Na^{+} followed by perfusion for 10 minutes with medium containing 1.25 mM Ca^{++} and 145 mM Na^{+}, showing apparently normal ultrastructure of the heart cells and intercalated disc. Black line indicates one micron.

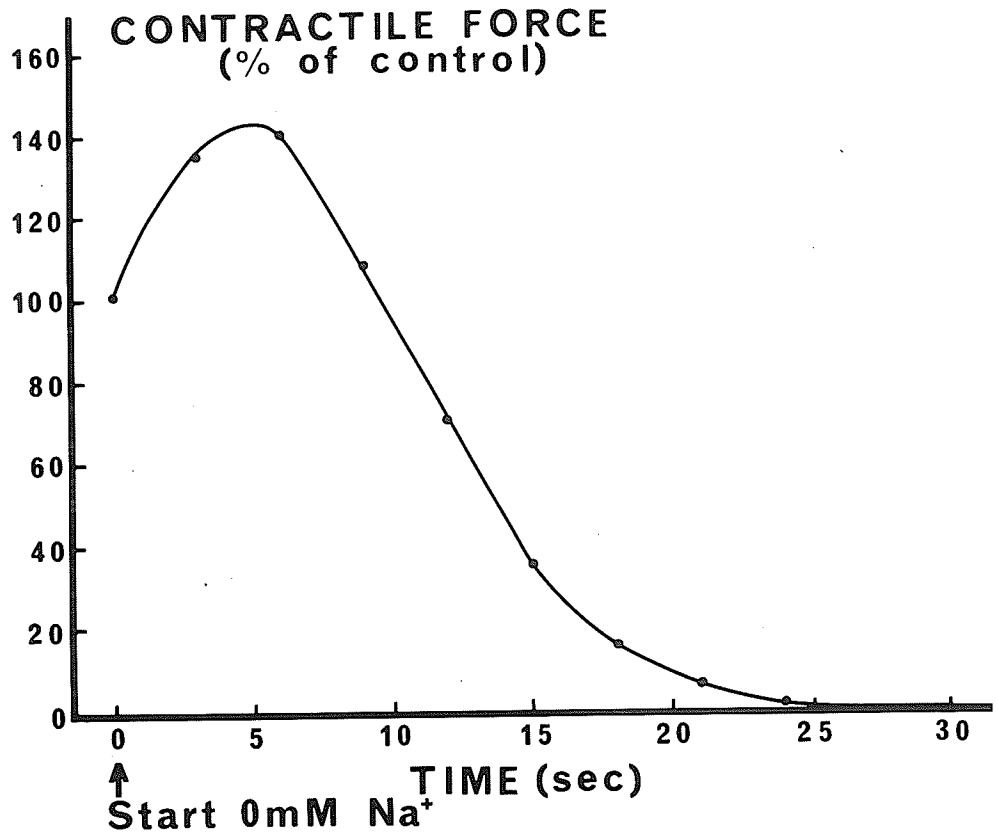

Figure 17: Effect of Na^+ -free perfusion on the contractile force development of isolated rat hearts. Each value is a mean of 24 experiments.

contractile force, dF/dt, and surface electrical activity from a typical experiment in which the heart was perfused with Na^+ -free medium. From this recording it can be seen that both the initial increase and the subsequent decline of contractile force are associated with parallel changes in dF/dt. An increase in resting diastolic tension occurred during failure of contractility. It can also be seen from Figure 18 that the electrical activity of the heart gradually disappeared. During initial events there was little change in time to peak tension (Figure 19); however, a 20% increase in time to ½ relaxation occurred following about 4 seconds of Na^+ -free perfusion.

Twelve hearts were perfused for various periods of time from 1 to 30 minutes with Na^+ -free medium and subsequently fixed for ultrastructural studies. Electron microscopic examination revealed no changes even after 30 minutes of perfusion with Na^+ -free medium (Figure 20).

Failure of contractility was found to be completely and rapidly reversible if reperfusion with Na^+ -containing Krebs-Henseleit solution was begun within the first few minutes of perfusion with Na^+ -free medium. Reperfusion with Na^+ -containing medium following longer periods of Na^+ -free perfusion ranging from 10 to 30 minutes resulted in an initial delay before the hearts began to contract. During partial

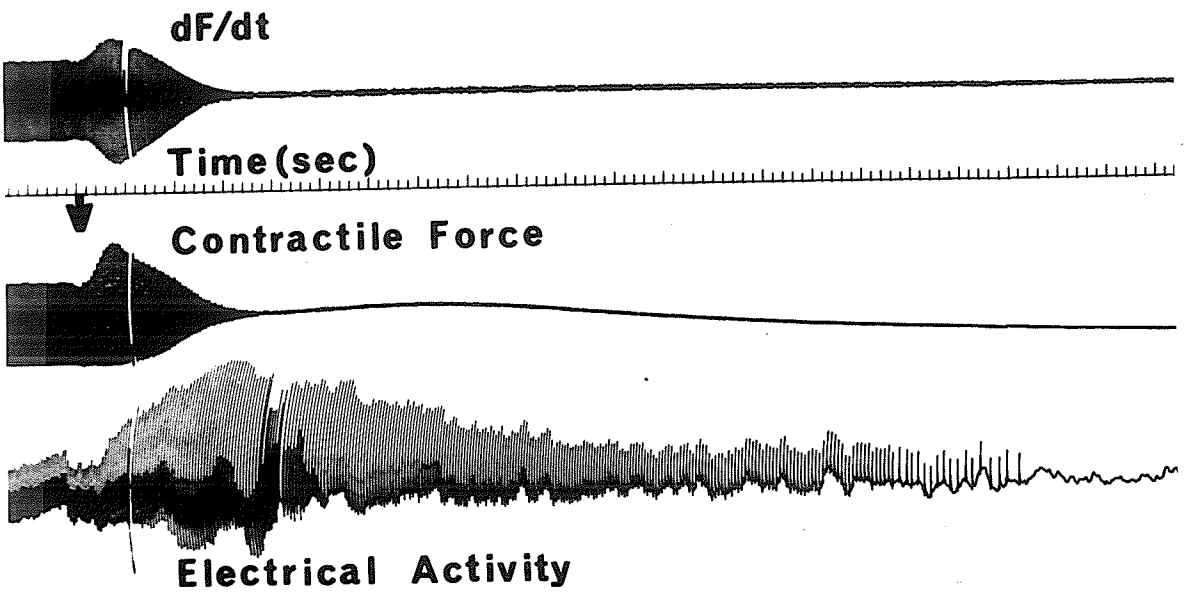

Figure 18: Typical recording of contractile force, dF/dt, and surface electrical activity of an isolated rat heart perfused with Na^+ -free medium.

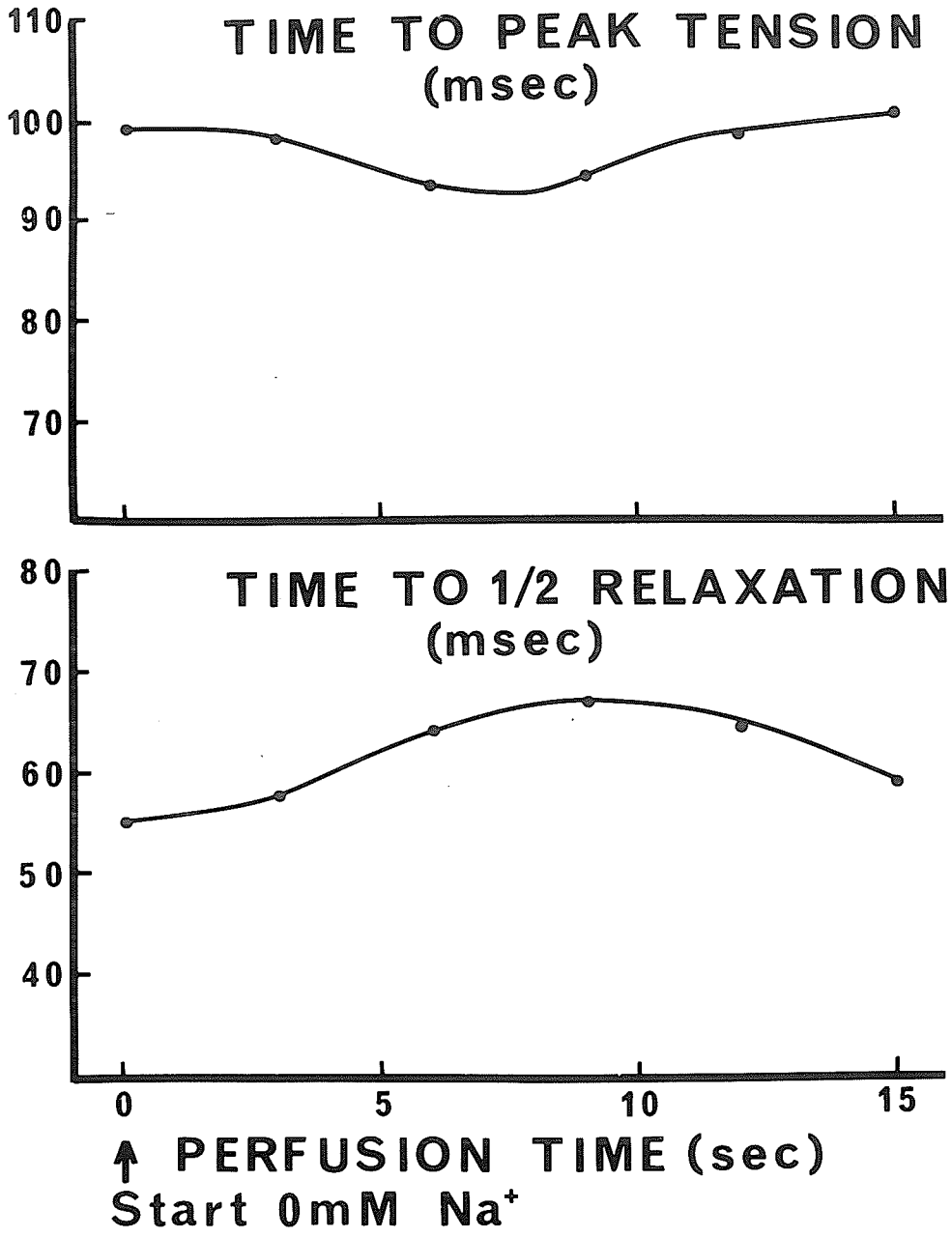

Figure 19: Upper panel - Effect of Na^+ -free perfusion on the time to peak tension in isolated rat hearts. Lower panel - Effect of Na^+ -free perfusion on the time to ½ relaxation in isolated rat hearts. Each value is a mean of 6 experiments.

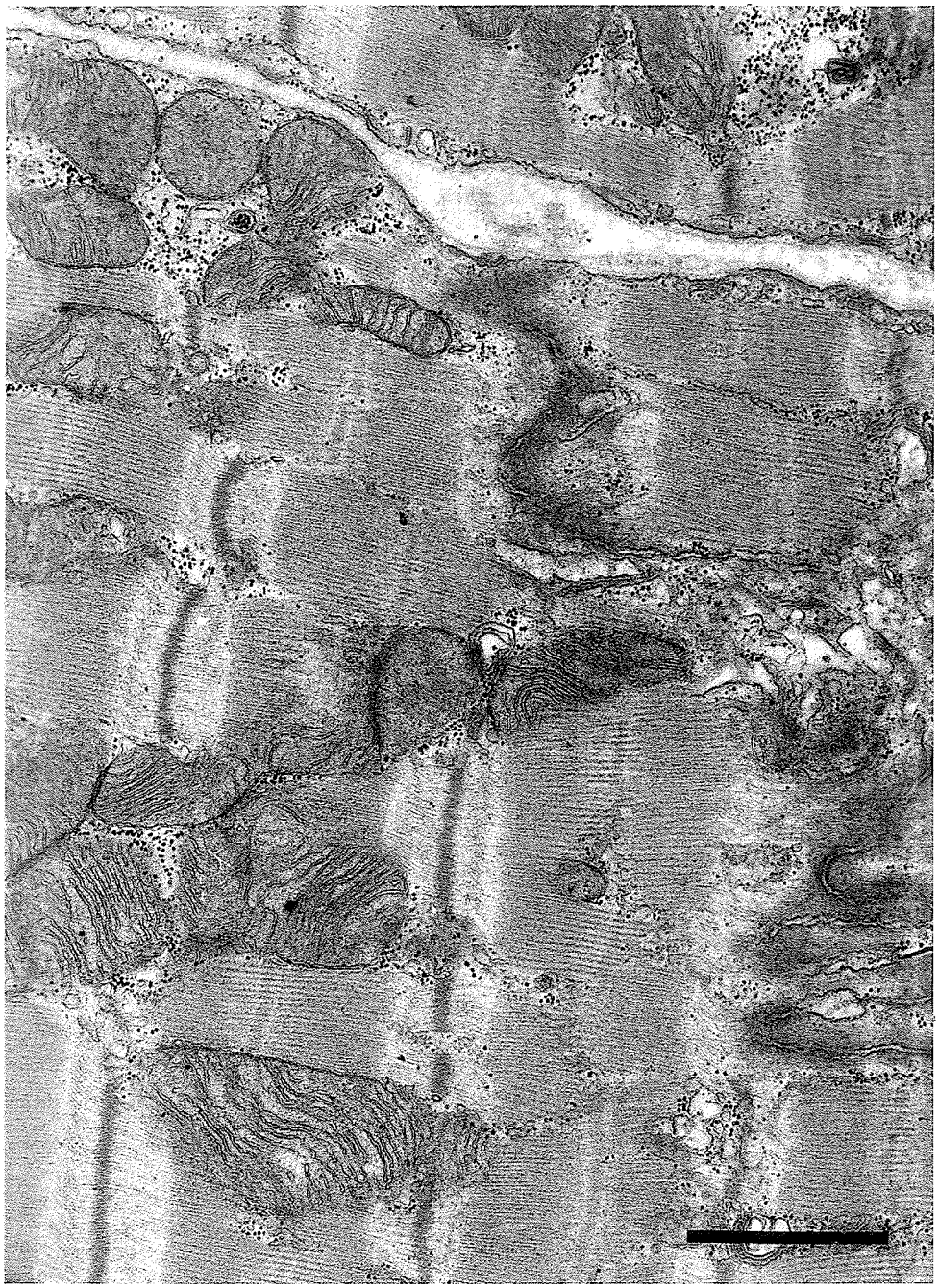

Figure 20: Electron micrograph of a typical section from an isolated heart perfused for 30 minutes with Na^+ -free medium, showing apparently normal ultrastructure of the myocardium. Black line indicates one micron.

recovery of contractile force there were variable periods of ventricular fibrillation, which began 3 to 4 minutes after the onset of recovery (Figure 21). The extent of recovery of the contractile force was rarely less than 50-60% of the control values.

D. Influence of Ca^{++} or K^{+} on the Effects of Na^{+} -Free Medium on the Heart

In experiments where hearts were perfused with Na^{+} -free medium in which the calcium concentration was varied, the time-course of failure of the heart to develop contractile force was not altered appreciably (Figure 22). On reperfusing the hearts with control medium after ten minutes of perfusion with Na^{+} -free medium containing 0.3 mM Ca^{++}, an enhancement of recovery was seen in comparison with hearts perfused with sodium-free medium containing 1.25 mM calcium (Figure 22). No fibrillation occurred during recovery of hearts perfused in the presence of 0.3 mM calcium. Reperfusion following 10 minutes exposure to sodium-free medium in which the calcium concentration was 3 mM resulted in a delayed spontaneous beating and reduced contractile force development which terminated in fibrillation within 6 to 7 minutes (Figure 22).

Varying the potassium concentration of the Na^{+} -free medium resulted in initial effects on contractile force which were reverse of the usual response (13) to variations in

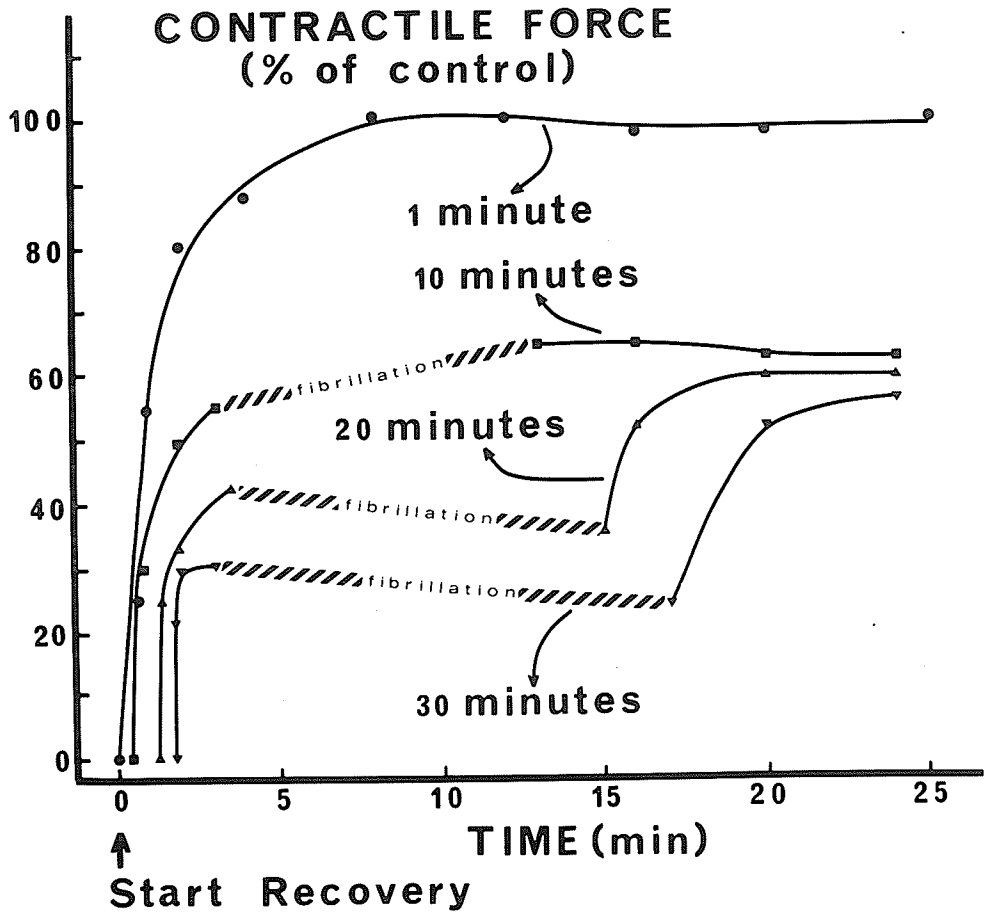

Figure 21: Effect of various intervals of Na^+ -free perfusion on the ability of isolated rat hearts to recover contractility upon reperfusion with Na^+ -containing medium. ●——● 1 minute of Na^+ -free perfusion; ■——■ 10 minutes of Na^+ -free perfusion; ▲——▲ 20 minutes of Na^+ -free perfusion; ▼——▼ 30 minutes of Na^+ -free perfusion. Each value is a mean of 6 experiments.

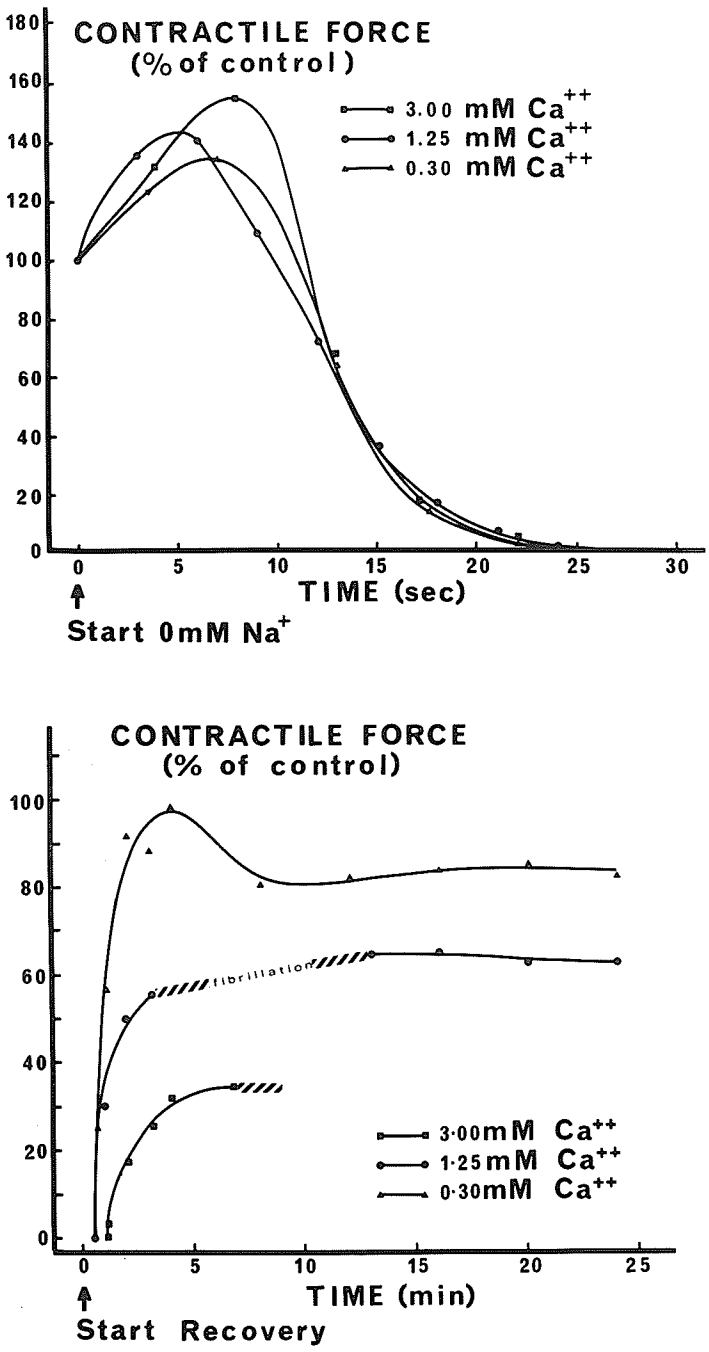

Figure 22: Upper panel - Effects on contractile force of perfusion of isolated rat hearts with Na^+ -free medium in which the Ca^{++} concentration has been varied. Lower panel - Effects of perfusion for 10 minutes with Na^+ -free medium in which the Ca^{++} concentration has been varied on the ability of isolated rat hearts to recover contractility upon reperfusion with control medium. ■—■ 3 mM Ca^{++} in Na^+ -free medium; ●—● 1.25 mM Ca^{++} in Na^+ -free medium; ▲—▲ 0.3 mM Ca^{++} in Na^+ -free medium. Each value is a mean of 6 experiments.

potassium concentration in the presence of sodium (Figure 23). The time course of failure due to Na^+ -free perfusion in the presence of 2 mM potassium was greatly prolonged in comparison to that in the presence of 6 or 12 mM potassium. When reperfusion was begun after 10 minutes perfusion with sodium-free medium, the duration of fibrillation was found to decrease as the potassium concentration in the Na^+ -free medium was increased (Figure 23). Both elevated (12 mM) and reduced (2 mM) potassium concentrations in the Na^+ -free medium resulted in a reduced level of recovery of contractile force on reperfusion with control medium.

E. Structural and Functional Changes in the Isolated Rat Heart Following Perfusion with K^+ -Free Medium

Perfusion of isolated rat hearts with aerobic K^+ -free medium alters contractility in at least three distinct phases (Figure 24). Upon switching to perfusion with K^+ -free medium the contractile force initially declined by 40 per cent then increased again usually to a value about 10 per cent above the control level within two minutes, and subsequently became irregular with alternating weak and strong contractions gradually declining in magnitude. The heart failed to generate contractile force abruptly after about 10 minutes of perfusion with K^+ -free medium. Prolonged perfusion with K^+ -free medium resulted in contracture (results not shown). Figure 25

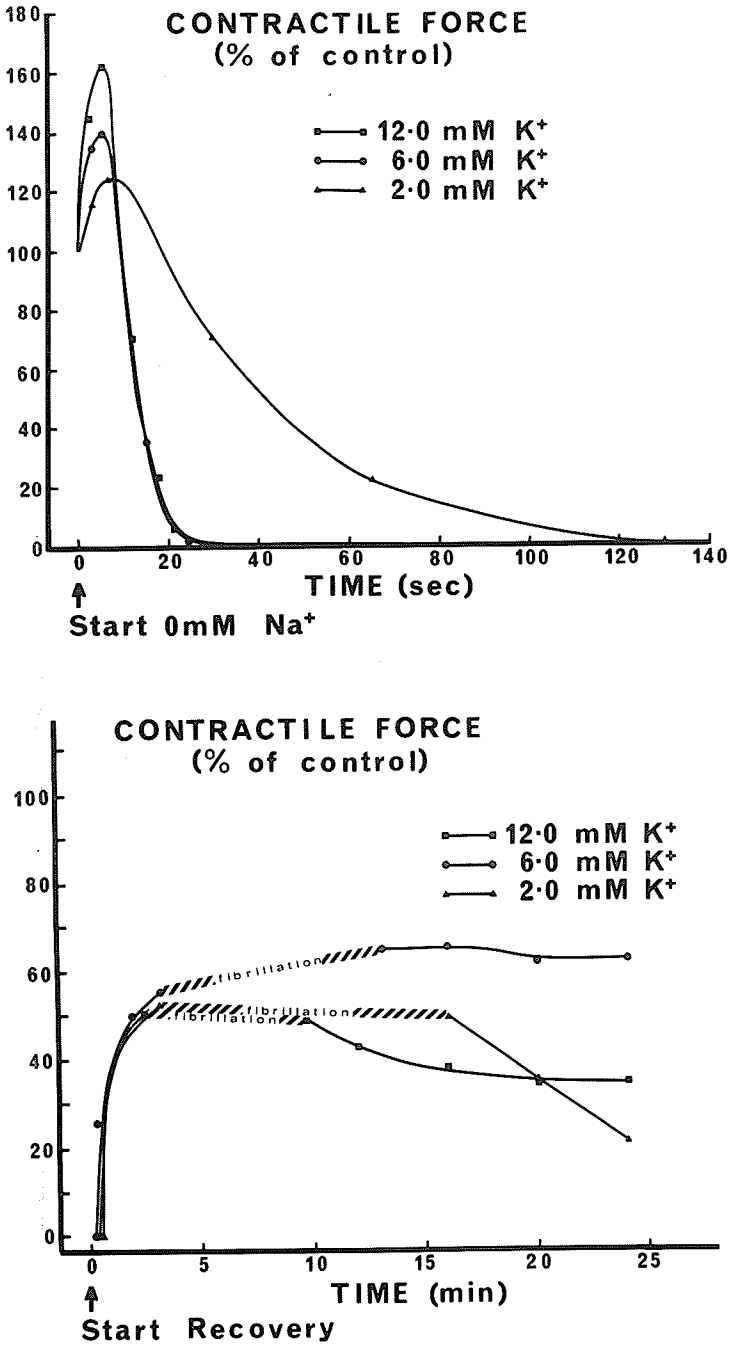

Figure 23: Upper panel - Effects on contractile force of perfusion of isolated rat hearts with Na^+ -free medium in which the K^+ concentration has been varied. Lower panel - Effects of perfusion for 10 minutes with Na^+ -free medium in which the K^+ concentration has been varied on the ability of isolated rat hearts to recover contractility upon reperfusion with control medium. ■—■ 12 mM K^+ in Na^+ -free medium; ●—● 6 mM K^+ in Na^+ -free medium; ▲—▲ 2 mM K^+ in Na^+ -free medium. Each value is a mean of 6 experiments.

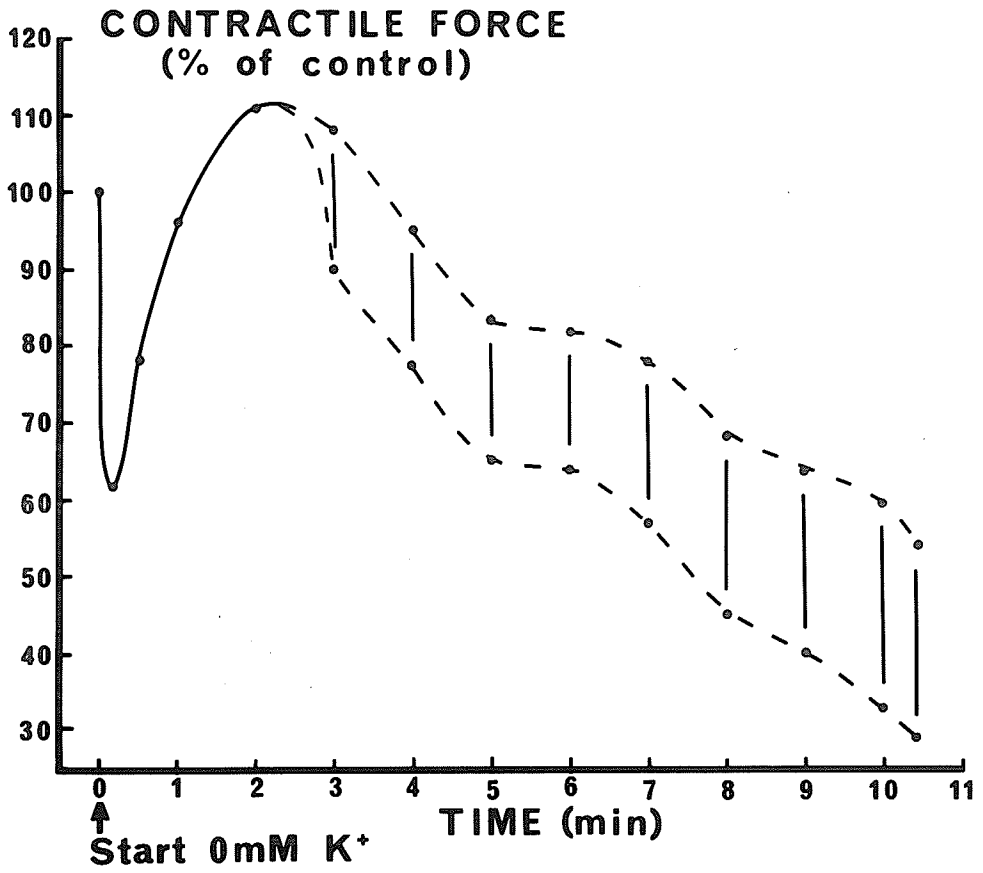

Figure 24: Effect of K^+ -free perfusion on the contractile force development of isolated rat hearts. The upper and lower interrupted lines represent the contractile force developed in alternate strong and weak contractions, respectively. At the terminal points there was an abrupt failure of contractility. Each value is a mean of 8 experiments.

shows a recording of the contractile force, dF/dt, and surface electrical activity from a typical experiment in which the heart was perfused with K^+ -free medium. From this recording it can be seen that dF/dt declined immediately after starting the perfusion with K^+ -free medium and increased somewhat subsequently but remained depressed noticeably below the control level until it disappeared. It can also be seen from Figure 25 that the pattern of electrical activity of the heart was changed upon perfusion with K^+ -free medium, although it was not affected initially. An increase in time to peak tension was observed to coincide with the phase in which contractile force was increased, but decreased thereafter to near its initial value upon perfusion with K^+ -free medium (Figure 26). Furthermore, an increased time to ½ relaxation was seen which subsequently returned to near its initial value.

Three hearts each were fixed for ultrastructural studies following perfusion with K^+ -free medium for 5-8 minutes, when the contractile force was reduced and irregular, as well as at 14-15 minutes perfusion with K^+ -free medium, by which time the hearts had ceased to beat and appeared to be in a state of strong contracture. Thin sections from these hearts were compared with hearts perfused with control medium for similar periods. A marked swelling of the sarcoplasmic reticulum was characteristic of hearts perfused for 5-8 minutes with K^+ -free

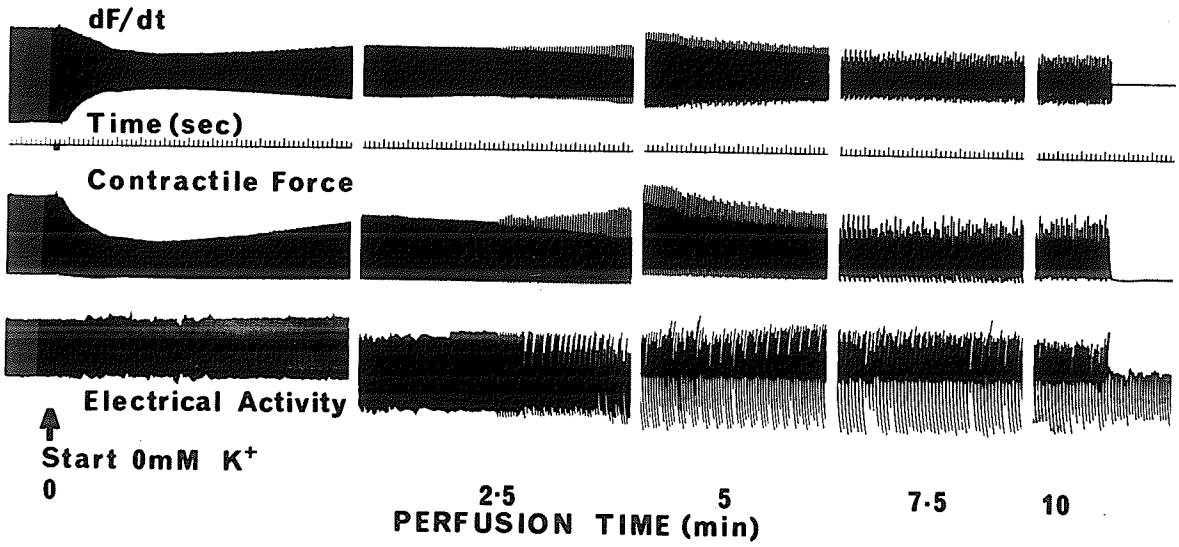

Figure 25: Typical recording of contractile force, dF/dt, and surface electrical activity of an isolated rat heart perfused with K^+ -free medium.

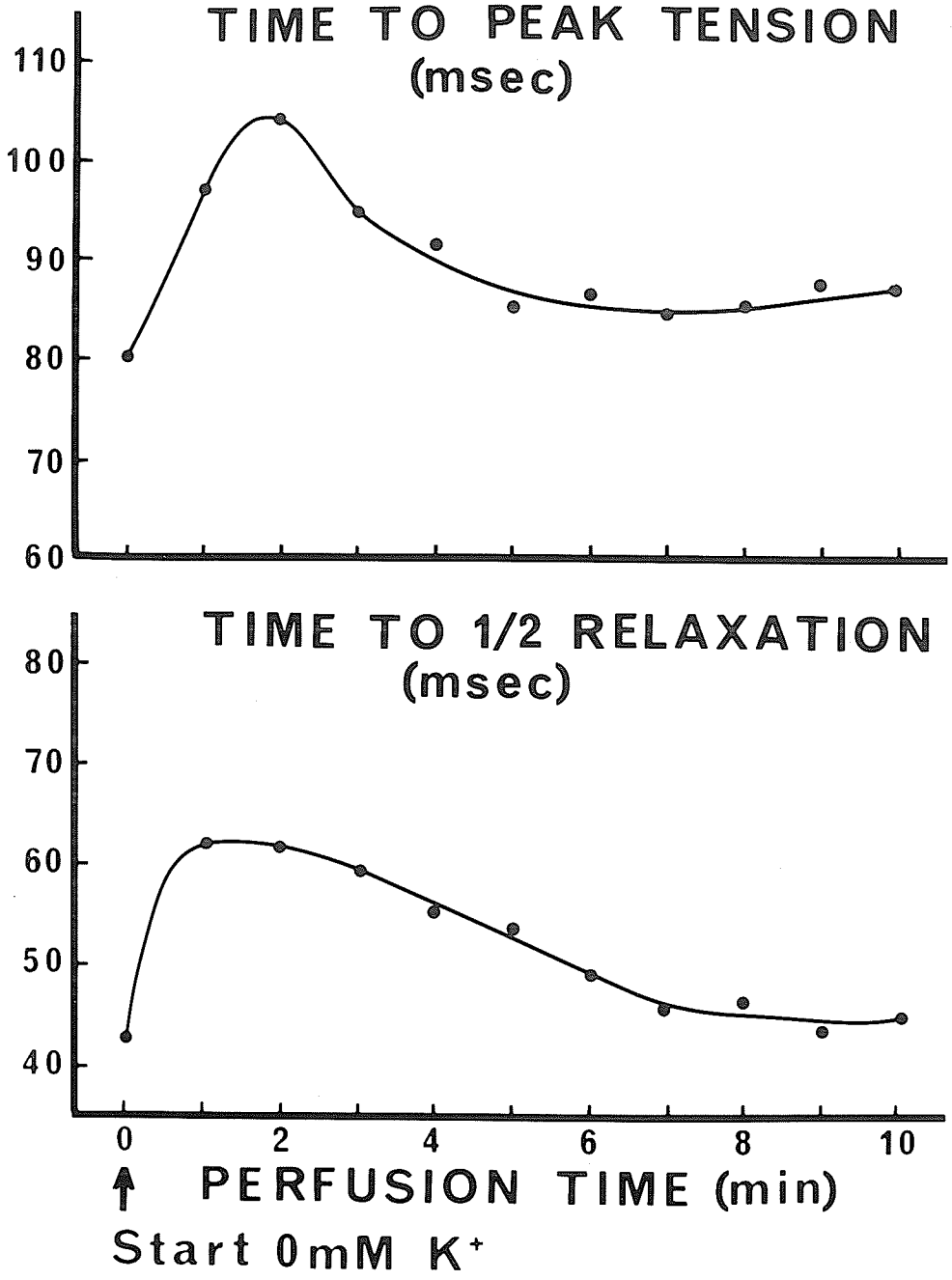

Figure 26: Upper panel - Effect of K^+ -free perfusion on the time to peak tension in isolated rat hearts. Lower panel - Effect of K^+ -free perfusion on the time for ½ relaxation in isolated rat hearts. Each value is a mean of 6 experiments.

medium; the contractile elements, mitochondria, and other membranous structures appeared normal (Figure 27). Electron microscopic examination of hearts fixed following 14-15 minutes of K^+ -free perfusion revealed frequent areas of the myocardium in contracture, and the mitochondria in these areas (Figure 28) were seen to be swollen and to have a much less dense matrix than normally seen in control preparations.

Reperfusion with control medium after longer than 8 minutes perfusion with K^+ -free medium resulted in contracture. Contractile force was fully recoverable within 20 to 30 minutes if reperfusion with control medium was begun at 8 minutes or earlier after starting perfusion with K^+ -free medium (Figure 29). An initial period of fibrillation frequently occurred upon return of control medium in hearts perfused for 6 to 8 minutes without potassium. It was interesting to note that the effects of potassium replacement on the recovery of contractile force also involved three phases, which were reverse of the phases observed during potassium depletion. Typically, there occurred first a dramatic increase in contractile force, which then fell to a low level, and finally increased again slowly toward the control level (Figure 29). Ultrastructural examination during recovery of the K^+ -depleted hearts is in progress.

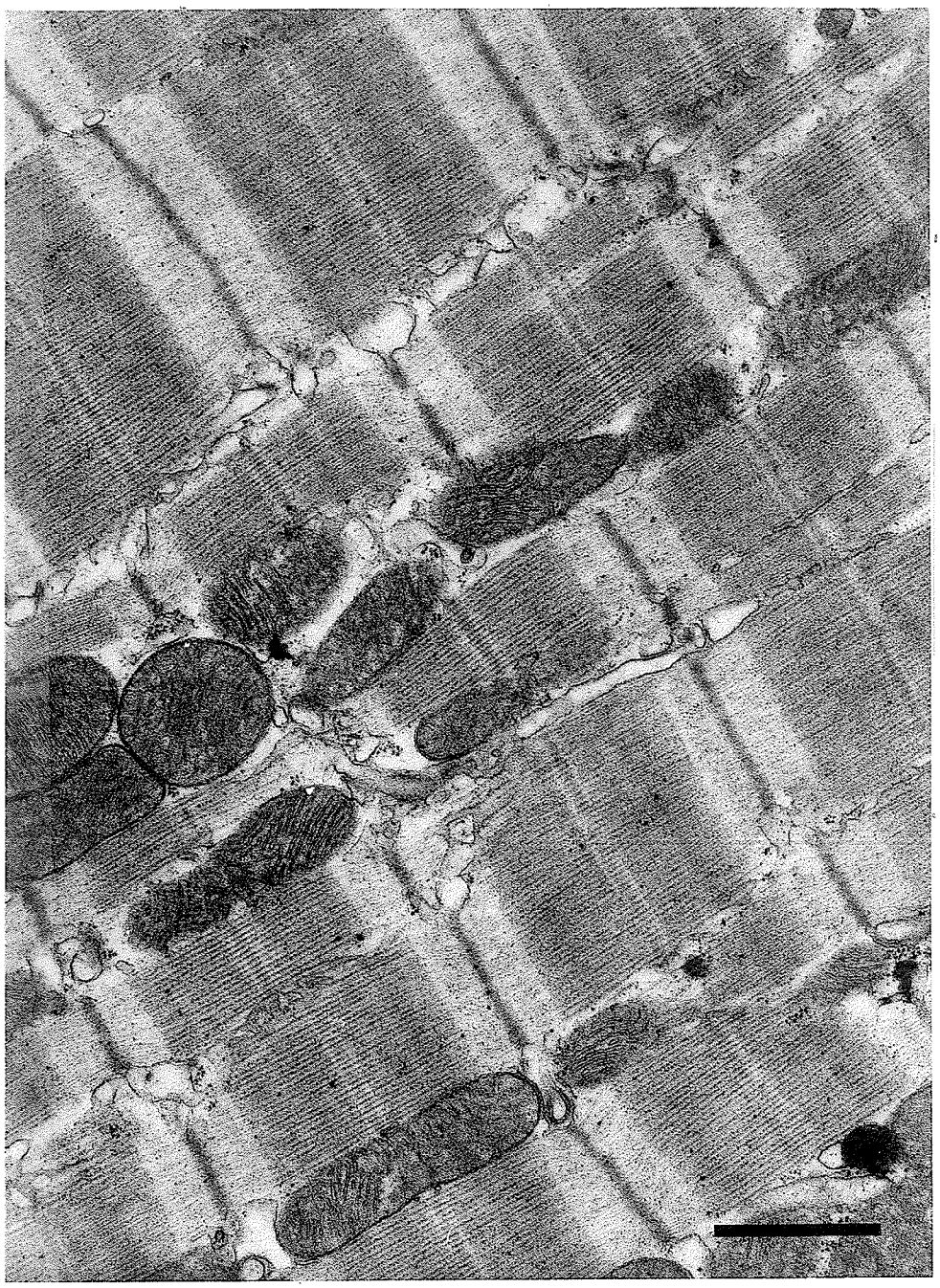

Figure 27: Electron micrograph of a section from an isolated rat heart perfused for 5 minutes with K^+ -free medium, showing swelling of the sarcoplasmic reticulum. Black line indicates one micron.

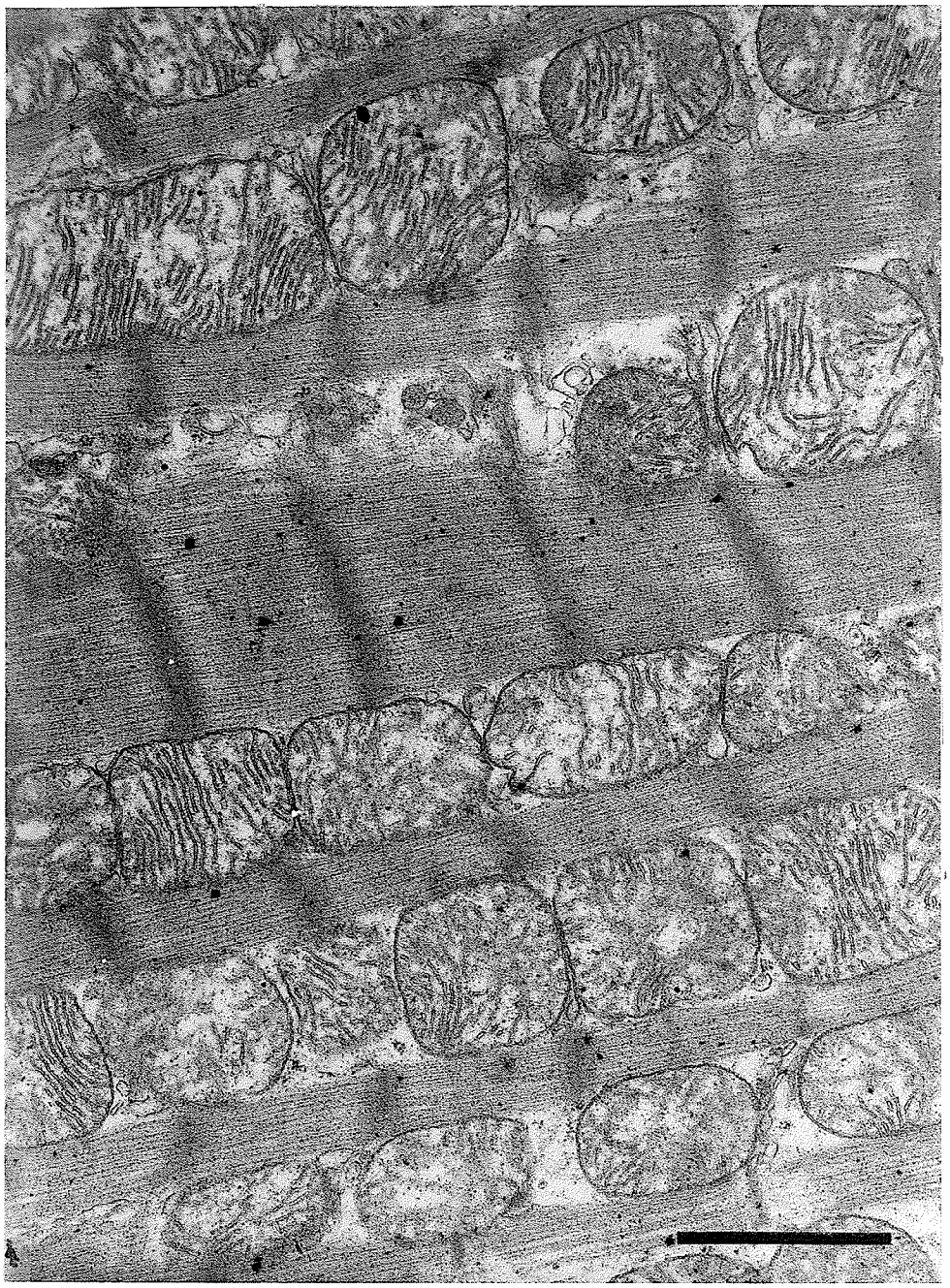

Figure 28: Electron micrograph of a section from an isolated rat heart perfused for 15 minutes with K^+ -free medium, showing sarcomeres in contracture and swollen mitochondria in which the matrix is less dense than is usually seen. Black line indicates one micron.

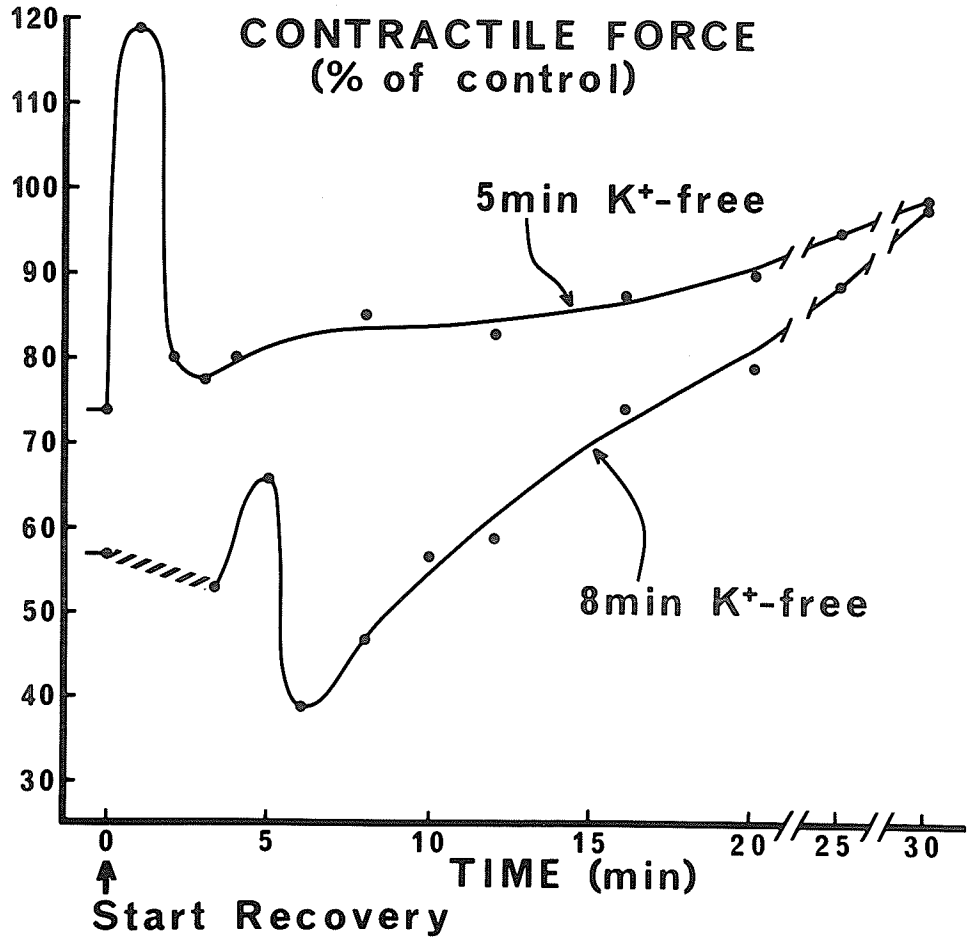

Figure 29: Effect of K^+ -free perfusion for 5 minutes or 8 minutes on the contractile force development of isolated rat hearts upon reperfusion with K^+ containing medium. ////// represents fibrillation. Each value is a mean of 6 experiments.

F. Influence of Ca^{++} or Na^{+} on the Effects of K^{+} -Free Medium on the Heart

In experiments where hearts were perfused with K^{+} -free medium in which the sodium concentration was reduced to 35 mM, rapid decline of contractility and onset of fibrillation within 1-2 minutes were observed (results not shown). It should be noted that this sodium concentration has been reported to be sufficient for continued function in the rat heart(32). Variation of calcium concentration in the K^{+} -free medium affected both the phases of increased and decreased contractile force associated with potassium depletion. The initial decline in contractile force was enhanced by reducing the concentration of Ca^{++} in the K^{+} -free medium whereas this phase was reversed on increasing the amount of Ca^{++} (Figure 30). The time of abrupt disappearance of contractile force was decreased on increasing the concentration of Ca^{++} from 0.3 to 3 mM in the K^{+} -free medium.

Reperfusion of hearts with control medium after 5 minutes perfusion with K^{+} -free medium containing only 35 mM sodium resulted in rapid recovery of the contractile force but the initial large increase in contractile force was not seen (Figure 30). Similar reperfusion after 5 minutes with K^{+} -free medium containing 3.0 mM calcium resulted in the development of irreversible contracture (results not shown). On the other hand, recovery was greatly enhanced following

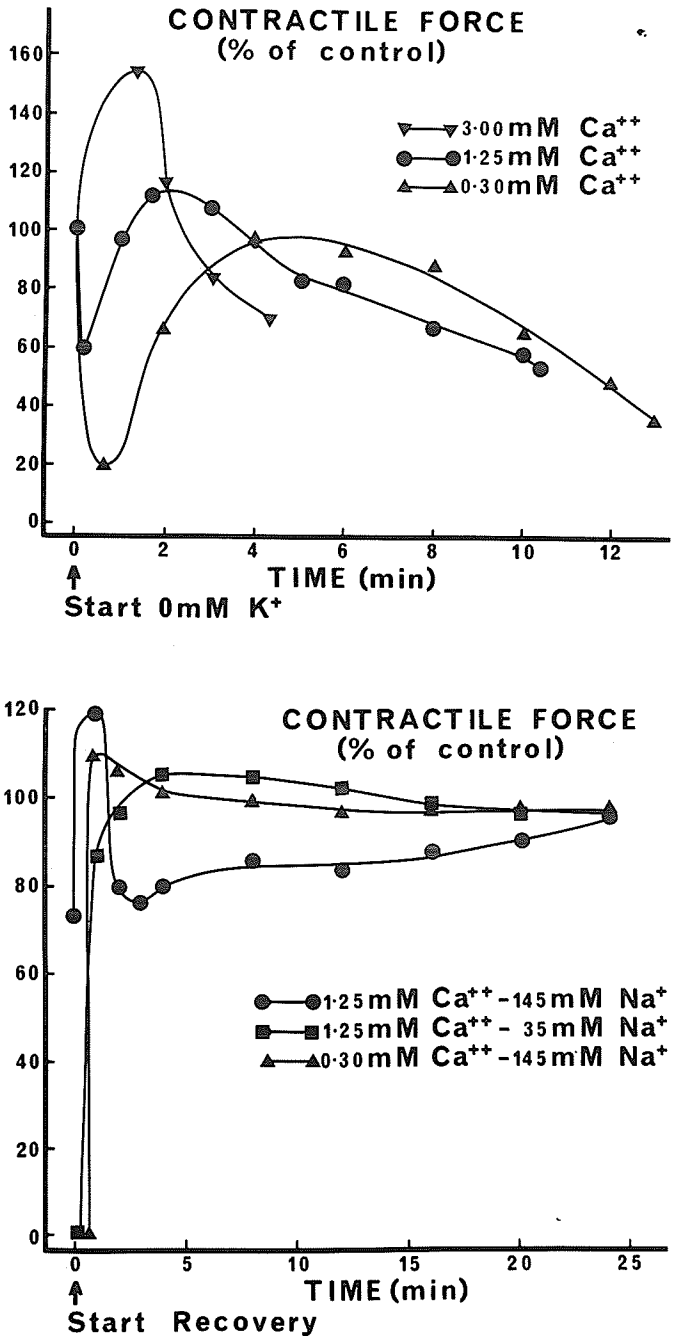

Figure 30: Upper panel - Effects on contractile force of perfusion of isolated rat hearts with K^+ -free medium in which the Ca^{++} concentration has been varied. Lower panel - Effects of perfusion for 5 minutes with K^+ -free medium in which the Ca^{++} or the Na^+ concentration has been lowered on the ability of isolated rat hearts to recover contractility upon reperfusion with control medium. ▼—▼ 3 mM Ca^{++} in K^+ -free medium; ●—● 1.25 mM Ca^{++} in K^+ -free medium; ▲—▲ 0.3 mM Ca^{++} in K^+ -free medium; ■—■ 35 mM Na^+ in K^+ -free medium. Each value is a mean of 6 experiments.

perfusion for 5 minutes with K^+ -free medium containing 0.3 mM calcium. In this case a slight initial increase in contractile force occurred, but the contractile force did not fall again before returning to the control level (Figure 30).

V. DISCUSSION

A. Calcium Depletion

The major consequences of Ca^{++} -free perfusion in order of occurence are failure of excitation-contraction coupling, loss of intercellular adhesion, and disruption of functionally important intracellular structures. Uncoupling of the electrical and mechanical events may be due to removal of a labile calcium component occurring within 30 seconds of Ca^{++} -free perfusion; the reversibility of these effects suggests that this calcium-component can be readily replenished. The decline and disappearance of contractility due to Ca^{++} -free perfusion can be readily understood in terms of membrane calcium fluxes. The decline in dF/dt is predictable with regard to decreased calcium entry during each excitation-contraction cycle which results in diminished active state intensity. In view of the close relationship between time to peak tension and action potential duration(59) along with the observations that the action potential duration decreased on reducing the calcium concentration below 1.2 mM(11), it is probable that the decline in time to peak tension in our experiments is a consequence of shortening of the action potential duration. The significance of the increased time to ½ relaxation is not clear at this time; however, it can be considered to be due to some alteration in the "calcium

pump" mechanisms in the myocardial cells.

The period of increased contractile force and the delay in failure of contractility which occurred when the Ca^{++} -free medium contained only 35 mM sodium is probably a direct result of a diminished rate of depletion of intracellular calcium brought about by well established Na^{+} - Ca^{++} antagonism. Contractile force has been shown to be proportional to the ratio of the calcium concentration to the square of the sodium concentration in the interstitial fluid(14). Upon switching the heart to perfusion with a solution containing no calcium and a drastically reduced amount of sodium, the interstitial fluid is being simultaneously depleted of sodium and calcium. In such a situation the square of the sodium concentration will initially decrease more rapidly than does the calcium concentration. The resulting increase in the contractile force probably reflects increased calcium entry. Furthermore, it has been shown by Reuter and Seitz(30) that efflux of calcium from the myocardium is highly dependent upon the extracellular calcium and sodium concentrations. The removal of either of these ions will decrease the rate of calcium efflux, and the effects of removal of both will be synergistic. Such a delay in depletion of the intracellular calcium content would of course also explain the enhanced recovery of hearts perfused for 3 minutes with Ca^{++} -free medium containing a

reduced amount of sodium.

The sudden decline in amplitude of the surface electrical activity following about 4 minutes of Ca^{++} -free perfusion suggests that some defect has occurred in the generation or conduction of excitation. This is further substantiated by the changes in amplitude of the electrical activity associated with recovery after about 4 minutes of calcium deprivation. Although there is no clear evidence of ultrastructural damage in hearts fixed after 3 to 4 minutes of Ca^{++} -free perfusion, in hearts fixed after reperfusion with normal medium following 3 minutes of Ca^{++} -free perfusion there are quite obvious separations at the intercalated discs. It appears likely that intercellular connections at the intercalated discs are critically weakened after 3 to 4 minutes of calcium deprivation, and that the shortening of adjacent cells when contractions resume during recovery can cause these weakened discs to become separated. Also consistent with this is the observation that the resting tension curve of the heart perfused with Ca^{++} -free medium plateaus after about 3 minutes. The increase in resting tension may reflect a gradual shortening of the myocardial cells, in which case the plateau probably represents a failure of intercellular adhesive forces at the intercalated discs. The inability of the hearts to maintain the contracture tension which develops upon reperfusion with

calcium containing medium represents the same process of separation of the individual cells. At any rate these observations are consistent with the well documented "calcium paradox" phenomenon(53,54).

Significant structural damage to the myocardium associated with prolonged Ca^{++} -free perfusion has been reported in detail by earlier workers(49-52). Our observations are essentially confirmatory in nature. However, in the present study it has been shown that alterations of mechanical parameters occur much earlier than the ultrastructural changes due to the Ca^{++} -free perfusion. A steady increase in the resting tension of the arrested heart, a steady decline in the ability of the heart to recover contractile force, and the development of a progressively greater degree of contracture upon calcium replacement are all closely related to the duration of the Ca^{++} -free perfusion. Although these effects could not be correlated with any particular ultrastructural alterations, these studies suggest dramatic alteration of intracellular structures involved in the regulation of heart function and metabolism. Thus the inability of these hearts to recover may be associated with impaired functions of mitochondria, sarcoplasmic reticulum, and myofibrils. These observations clearly support the role of calcium in the maintenance of cardiac ultrastructure in

addition to its well known participation in the process of excitation-contraction coupling.

B. Sodium Depletion

The increase in contractile force occuring immediately after starting perfusion with Na^+ -free medium is in agreement with earlier reports employing low concentration of sodium. Such an effect is probably the result of an increased rate of calcium entry into the cell with each excitation-contraction cycle. It is well known that at least part of the calcium involved in the activation of contraction in the heart enters the cell from the extracellular fluid rather than from intracellular stores(15-24,33,34), and low sodium concentrations have been shown to result in a net gain of tissue calcium (25-27,31). The concept of increased calcium entry is further supported by the observation that the magnitude of the contractile force increase varies directly with the calcium concentration of the Na^+ -free medium. Furthermore, the increase in contractile force was associated with a simultaneous increase in dF/dt, which is considered to reflect an increased rate of calcium made available to the contractile elements per unit time(43).

Legato et al(28) have demonstrated a dilation of the sarcoplasmic reticulum following perfusion of dog papillary muscle with medium containing one-quarter of the normal sodium

concentration. This dilation was attributed to excessive calcium accumulation by the sarcoplasmic reticulum brought about by the increase in calcium influx in the presence of reduced sodium. However, in the present study no ultrastructural changes were observed upon perfusing hearts with Na^+ -free medium. The discrepancy in these observations may be due to the fact that Legato et al have employed a medium containing low sodium which is sufficient to maintain excitability. On the other hand in our experiments excitability persists for only about 25 seconds. Since the increased calcium influx is thought to occur during membrane depolarization, the quantity of additional calcium entering via the sarcolemma and accumulated in the sarcoplasmic reticulum over such a brief period of time might well be expected to be less than that required to produce osmotic swelling of these tubules.

The decline and disappearance of contractile force which follows the initial period of increased contractile force might be expected to be associated with a decreased time to peak tension, since low sodium concentration has been shown to result in a shortened action potential(59), and time to peak tension is frequently related to action potential duration (60). By contrast we have observed little change in time to peak tension in our experiments. As the usual relationship between action potential duration and time to peak tension

is secondary to the relationship of time to peak tension to active state duration, and thus to the availability of free calcium, our results suggest that some factor other than action potential duration is operating to maintain free calcium for a longer period of time. The decline of both dF/dt and contractile force suggests that as sodium depletion progresses calcium entry becomes restricted, thus ruling out the possibility that active state duration is prolonged because of an increase in the rate of calcium influx. On the other hand both an increase in time to ½ relaxation and an increase in resting tension occur at this time. It is possible that these effects are a result of an alteration in the intracellular calcium sequestering mechanism. Such an alteration could easily result in a prolonged contraction time even though the action potential duration decreases. This would suggest that extracellular sodium may, either directly or indirectly, participate in the regulation of the calcium pumping mechanisms of the heart. It is interesting to note that Arora(44) has arrived at a similar conclusion with regards the presence of sodium ion and calcium sequestration in frog atria. Although he suggests that sodium may normally function as an activator of the calcium pump in quantities small enough for contamination to have interfered with previous <u>in vitro</u> attempts to identify a relationship between

sodium and the calcium sequestering ability of microsomes (61,62), it is also possible that the calcium pumping activity of the sarcolemma is regulated by Na concentration. This would be consistent with the reduced efflux of Ca from myocardium perfused with low sodium concentration(27,30).

Recovery of hearts from Na^+ -free perfusion of longer than 5 minutes duration was found to be associated with a period of ventricular fibrillation. Since the duration of this period of fibrillation is proportional to the duration of the sodium-free perfusion, and can be altered by varying either the Ca^{++} concentration or the potassium concentration of the Na-free medium it is possible that some relatively slow redistribution of either or both of Ca^{++} and K^+ occurs in the absence of Na^+. In this connection it should be noted that alterations in the Ca/K ratio are known to result in ventricular fibrillation(45,46,63).

The observation that higher potassium concentrations of the Na^+ -free medium enhanced the period of increased contractile force was of great interest as this is in contrast to the negative inotropic effect usually associated with increased extracellular potassium concentration. In view of the present findings, it does not appear likely that the usual effect of potassium concentration is due to any direct effect of K^+ on the contractile system, even if the inotropic effects

of potassium concentration were assumed to be entirely masked in our experiments by the positive inotropic effect of reduced sodium concentration. Rather it appears more likely that the negative inotropic effect of increased potassium is mediated by sodium or calcium movements which do not occur in a situation where the interstitial fluid is being rapidly depleted of sodium.

C. Potassium Depletion

Most of the effects of K^+ -free perfusion on cardiac ultrastructure and contractile activity appear to be secondary to changes in the movement of calcium between the extra- and intracellular compartments. This is reflected by the observation that mechanical parameters such as developed tension, dF/dt, time to peak tension, and time to ½ relaxation, which are the consequences of interaction of calcium and the contractile elements were altered on perfusing the heart with K^+ -free medium. When perfusion with K^+ -free medium was started the first effect was a decrease in contractile force associated with a decrease in dF/dt; shortly thereafter both contractile force and time to peak tension increased greatly while dF/dt remained depressed. Since changes in dF/dt are considered to reflect changes in the rate at which calcium becomes available to the contractile elements(43), and since the decline in dF/dt is evident immediately upon changing the

heart to perfusion with K^+ -free medium, reduced extracellular potassium may be causing a reduction in the influx of calcium during excitation. Furthermore, this initial dF/dt related decline in contractile force during K^+ -free perfusion was eliminated by increasing the calcium concentration and exaggerated by reducing the calcium concentration. This first phase in which contractile force was reduced did not last long and although the rate of force development remains depressed, contraction time increased sufficiently for contractile force to exceed the control level, probably as a result of increased action potential duration(64-67).

In experiments on hearts perfused with Na^+ -free medium we generated a situation in which the effects of altering the potassium concentration on contractile force were the opposite of what is usually observed. The mechanisms by which the inotropic effects of altered potassium concentrations occur have never been clearly defined. In light of the dual effects on rate and duration of force development which occur during K^+ -free perfusion, it is possible to explain both of these phenomena. Under most conditions the effect of variation of the potassium concentration on action potential duration(10) probably leads to changes in contractile force by altering the contraction time. When the potassium concentration was altered in a medium containing no sodium the shortening of

the action potential due to omission of sodium prevented this effect from occuring, and the effects of potassium concentration on the rate of force development predominated.

In spite of the fact that the rate at which calcium enters the myocardial cells is probably reduced during K^+ -free perfusion, it is not unlikely that, as the action potential duration increases, prolongation of the calcium entry phase results in a net increase in intracellular calcium content. A distinct swelling of the sarcoplasmic reticulum was observed in hearts perfused with K^+ -free medium for 5 to 8 minutes which is similar to that reported by Legato et al (29) after perfusion of dog papillary muscle with low sodium or high calcium concentrations and considered to be due to calcium accumulation. Furthermore, Thomas(21) has shown that K^+ -free perfusion of frog ventricles results in a steady accumulation of calcium by the myocardium. Thus it is probably calcium accumulation which is responsible for the development of contracture seen to occur after prolonged K^+ -free perfusion.

The initial dramatic increase in contractile force observed upon recovery of hearts following K^+ -free perfusion also appears to be dependent upon the availability of calcium to the contractile elements, since it is greatly reduced by lowering the calcium concentration of the potassium deficient

medium. The fact that this phase is absent in recovery after perfusion with low-sodium K^+ -free medium, in which calcium entry may have been limited by early failure of electrical and mechanical activity, suggests that calcium taken up during potassium free perfusion is important in producing this effect. In this regard it is interesting to note that although recovery of contractility upon reperfusion with control medium did not occur when the calcium concentration of the K^+ -free medium was 3 mM, there appeared to be an enhancement of recovery when the sodium concentration of the K^+ -free medium was reduced to 35 mM, in spite of the fact that the ratio $[Ca] : [Na]^2$ is considerably higher in the latter case. This may also be due to early cessation of electrical and mechanical events serving to limit calcium accumulation.

From the results of this study it would appear that potassium may be an important regulator of calcium fluxes, and therefore, of contractility.

VI. CONCLUSIONS

In this study we have examined the effects upon the structural and functional integrity of the isolated heart of depletion of Na^+, K^+, and Ca^{++}. From the results obtained the following conclusions are made:

1. The most immediate consequence of calcium depletion is reversible interruption of excitation-contraction coupling.
2. The disappearance of electrical activity which occurs several minutes later is probably due to failure of intercellular adhesion at the intercalated disc, for maintenance of which calcium is apparently essential.
3. Intracellular membranous structures such as mitochondria and sarcoplasmic reticulum are altered by severe calcium depletion.
4. As depletion of calcium progresses there occurs a gradual increase in the resting tension of the myocardium which culminates in the development of contracture and a disruption of the contractile filaments similar to that occuring in connection with the "calcium paradox".
5. The susceptibility of the heart to the "calcium paradox" phenomenon coincides with the increase in resting tension during calcium-free perfusion.

6. The influence of altered sodium and potassium concentrations on calcium movements between intra- and extracellular compartments is sufficient to dramatically alter the course of development of the effects of calcium depletion.
7. During the course of sodium depletion and prior to the disappearance of contractility there is an apparent reduction in the effectiveness of the relaxation system of the heart. Thus sodium is implicated as a possible regulator of cardiac calcium pumping mechanisms.
8. Sodium depletion has no effect on cardiac ultrastructure.
9. In the absence of extracellular sodium, or at least in the absence of repeated excitation a tendency for fibrillation develops, probably associated with a redistribution of calcium and potassium.
10. Potassium depletion results in structural changes suggestive of excess calcium accumulation and ultimately results in the development of irreversible contracture.
11. Extracellular potassium concentration affects both the rate of contractile force development and the duration of the contraction but in opposite directions, with decreased extracellular potassium causing a reduction of dF/dt and an increased time to peak tension.

12. The structural and functional changes associated with potassium depletion are probably secondary to effects upon the exchange of calcium across the sarcolemma.

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