THE EFFECTS OF MANGANESE ON FROG HEART MUSCLE

by

David Ellis

A dissertation submitted for the degree of Doctor of Philosophy in the University of Leicester.

January, 1976

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

MEMORANDUM

This is to certify that the thesis I have submitted in fulfillment of the requirements governing candidates for the Degree of Doctor of Philosophy in the University of Leicester, entitled, "The Effects of Manganese on Frog Heart", is the result of work done mainly by me during the period of registration for the above degree.

David Ellis

David Ellis

ACKNOWLEDGEMENTS

My most sincere thanks are due to Dr. R.A.Chapman, my supervisor, for his help, advice and enthusiasm throughout the course of this work.

I am also grateful to Professor R.Whittam for the provision of the facilities in his department and to Mrs. A.Lear for typing the manuscript.

This work was carried out during the tenure of a research studentship from the Medical Research Council.

CONTENTS

Page

INTRODUCTION

(1) Calcium and muscle contraction

The first indication of the importance of calcium in the contraction of muscle comes from the work of Sidney Ringer in 1883 who reported that calcium was required to sustain the beat of the frogs heart in vitro. The abolition of contractile activity occurs within a few minutes of calcium deprivation and is not accompanied by a loss of the electrical activity of the tissue (Mines,1913). This differs from the case of skeletal muscle where the loss of contractility after calcium removal is associated with the muscle becoming inexcitable. However by artificially depolarising skeletal muscle with potassium-rich solutions it has been shown that contraction is still abolished within two to twenty minutes in the absence of calcium even if the muscle is fully depolarised (Frank, 1962). Calcium was found to be the only ion that produced a significant contraction when injected into frog skeletal muscle (Hielbrunn and Wiercinski, 1947). These results suggested that calcium acts internally to produce activation of the contractile process. Subsequently micro-injection of calcium was shown to produce localised and reversible contraction in single skeletal muscle fibres of frog (Niedergerke, 1955) and crab (Caldwell and Walster, 1963).

A great deal of our present understanding of the mechanism of contractile activation has paradoxically come from studies on the relaxation of muscle. Biochemical studies involving the fractionation of muscle tissue had shown the presence of a "relaxing factor" in the supernatent fraction (Marsh, 1951). This factor was capable of reversing syneresis (the gelation of

actomyosin) and relaxing contracted glycerinated muscle (Bendall, 1953). This relaxing factor was later shown to be granular and not soluble (Kumagai et al, 1955) and was in fact fragments of the sarcoplasmic reticulum of the muscle cell (Ebashi and Lipmann,1962). These granules contain an ATP-dependent calcium pump which is capable of concentrating cytoplasmic calcium 500 times (Hasselbach and Makinose, 1961) or 2,000 times in the presence of oxalate or phosphate (calcium forms an internal precipitate in the presence of these anions).

In skeletal and mammalian cardiac muscle a network of tubules ramifies inward from the cell surface normally at the Z-band region. These tubules, known as the transverse tubular system, are continuous with the sarcolemma and appear to open into the extracellular space. Huxley and Taylor (1958) and Huxley (1959) produced highly localised depolarisation with a micro-electrode on the surface of muscle fibres and observed localised contraction when the micro-electrode was immediately above the entrance to this transverse tubular system. This suggested that the excitation of the muscle fibre sarcolemma was propagated down the tubular system to activate contraction. The close proximity of the calcium loading lateral sacs of the sarcoplasmic reticulum to these transverse tubules suggested that they might be regions from which calcium was released to trigger contraction following depolarisation of the cell membrane (Constantin et al, 1965; Sandow, 1965).

The sarcoplasmic reticulum is much less abundant in cardiac muscle than in skeletal. However, at least in mammalian cardiac muscle it appears to be sufficiently active to fulfil a similar rôle in the relaxation process (Carsten, 1964).

The calcium released into the sarcoplasm to activate contraction appears to act on the troponin molecule in higher animals (Ebashi and Kodama, 1966; Ebashi et al, 1967). Troponin prevents the interaction of actin and myosin and thus prevents contraction. The binding of calcium to troponin is able to lift this repression and thereby permit contraction.

Measurements of the uptake of ^{45}Ca by skeletal muscle cells have shown that with repetitive stimulation the uptake progressively increases (Bianchi and Shanes, 1959). This was later confirmed in cardiac muscle (Winegrad and Shanes,1962; Niedergerke, 1963). The increased calcium influx during stimulation suggested that this calcium might either activate tension directly or induce a calcium release from the sarcoplasmic reticulum (Ford and Podolsky, 1970; Endo et al, 1970). The former possibility i.e. that calcium influx during excitation might produce a significant tension by a direct activation of the myofibrils, is more likely in cardiac muscle as the sarcoplasmic reticulum is less abundant than in skeletal muscle and the smaller cells have a larger surface area to volume ratio. This would be even more prelevant in frog heart muscle where the sarcoplasmic reticulum is less developed (Staley and Benson, 1968; Page and Niedergerke,1972) and the cells are relatively small (average diameter 5μ, Marceau, 1904). The transverse tubular system is absent in frog heart muscle (Staley and Benson, 1968; Chapman 1971) so tension activation probably does not occur by exactly the same route as in skeletal muscle. The calcium entry into frog heart as measured by radioactive calcium fluxes (Niedergerke,1963) indicated that the calcium crossing the membrane during depolarisation was insufficient to completely activate the contractile proteins and therefore suggested the

need for an internal store of releasable colcium. With the introduction of the powerful technique of voltage clamping to cardiac muscle (Rougier et al, 1969; Masher and Peper,1969; Ochi, 1970; Beeler and Reuter, 1970A,B; Tarr,1971) the problem appeared to be near to resolution as an estimate of the calcium inward current during depolarisation seemed possible. Morad and Orkand, (1971), using voltage clamp experiments on frog heart muscle, proposed that this calcium influx was sufficient to completely account for the subsequently observed contraction. However the application of these techniques to frog heart muscle might have been premature because following the criticisms of the methods on theoretical grounds (Johnson and Lieberman, 1971) recent studies indicate that voltage control in such preparations might well be inadequate (Tarr and Trank, 1974).

2. The contraction of frog heart muscle.

Another approach to the study of excitation-contraction coupling and calcium activation of tension is via functional studies i.e. observation of the contractile response of the muscle to variation of the extracellular environment or frequency of stimulation. The contractile tension that frog heart produces was found to be proportional to the ratio $[Ca]_o / [Na]_o^2$ in the bathing solution (Wilbrandt and Koller, 1948). This was later confirmed and the results extended to show that contractures (sustained contractions) produced by depolarisation with potassium-rich solutions also appeared to vary with this ratio (Luttgau and Niedergerke, 1958). This was interpreted as indicating that sodium and calcium competed for binding to an anionic receptor at a site, probably on the surface membrane, and that this receptor was the entry route of calcium into the cell (Niedergerke and Luttgau, 1957).

More recent studies of the contractures produced by a reduction of the bathing sodium concentration has suggested that these contractures and their subsequent spontaneous relaxation could be accounted for by a sodium-calcium exchange across the cell membrane (Chapman, 1974). The contracture tension was found to be proportional to $[Ca]_o^2$ and to $\sqrt[4]{[Na]_o}$. A membrane carrier with two internal and two external anionic binding sites would be in accord with the observed responses. The existence of such a sodium-calcium exchange mechanism was originally suggested in squid giant axon (Baker et al, 1968) and guinea pig heart muscle (Reuter and Seitz, 1968).

Chapman and Niedergerke (1970, A,B,) studied the twitch contraction of frog ventricle in response to an alteration of the extracellular calcium concentration and the stimulus rate. These and subsequent studies (Chapman,1971; Chapman and Miller, 1974) suggested that during the normal twitch contraction the calcium that activates tension originates from three sites in series in the muscle. These three calcium sites or compounds were termed Ca_I, Ca_{II}, and Ca_2 (Chapman,1971). All three appeared to be in equilibrium with extracellular calcium but the rate of change of concentration of calcium at the individual sites in response to an alteration of the external calcium concentration was very different. These were determined by observation of the rate of decline of twitch tension in response to a reduction of the extracellular calcium concentration. This decline exhibited three approximately exponential phases. The rate of change of Ca_I was equivalent to the rate of interstitial solution exchange. Thus Ca_I was suggested to be at the outer surface of the cell membrane and that a physical counterpart of a change

of Ca_I could be a change in the calcium which entered the cell during the action potential. Ca_{II} and Ca_2 could correspond to intracellular calcium binding sites with Ca_2 (the slowest changing component) possibly equivalent to the sarcoplasmic reticulum or mitochondria. The fairly rapidly changing Ca_{II} could be situated at or near the inner surface of the cell membrane (Chapman, 1971). Studies on contractures produced in frog heart by sustained depolarisation with potassium-rich solutions indicated that the tension that the muscle produced varied with the $[Ca]_o^3$ (Chapman, 1971). These results, suggesting a cooperative action of three calcium ions added further support to the hypothesis of three calcium compounds or sites being involved in contraction produced by depolarisation of the cell membrane.

3. Manganese as an inhibitor of calcium fluxes

The transition element manganese was found to inhibit the calcium dependent action potential of crayfish muscle (Fatt and Ginsburg,1958). Subsequently, antagonism by manganese of calcium activation has been demonstrated in mammalian heart (Kleinfeld and Stein, 1968) and smooth muscle (Nonomura et al, 1966), barnacle muscle (Hagiwara and Takahashi,1967), frog skeletal muscle (Oota et al,1972), frog neuromuscular junction (Meiri and Rahamimoff, 1972) and the calcium uptake associated with excitation of squid giant axon (Baker et al, 1973). The influence of manganese on frog heart muscle is of considerable interest because of the apparently large dependence on external calcium of both excitation (Niedergerke and Orkand, 1966) and contraction (Ringer 1883; Wilbrandt and Koller, 1948). The over-shoot of the action potential has been reported to decrease

in the presence of manganese (Hagiwara and Nakajima, 1965), consistent with the entry of calcium at least being partially responsible for the depolarisation phase of the action potential (Niedergerke and Orkand, 1966). The twitch contraction of frog heart is rapidly inhibited in the presence of manganese as are the contractures produced by a reduction of the bathing sodium concentration (Chapman and Ochi, 1971). However the contractures produced by depolarisation with potassium-rich solutions were found to be less sensitive to manganese inhibition. It had been proposed that an internal store exists in frog heart from which calcium can be released to promote contraction in a manner similar to that in skeletal muscle. Therefore if manganese can act as a simple inhibitor of calcium entry then useful information might be obtained on the source of the calcium that activates contraction in frog heart i.e. to differentiate between external and internal sources of calcium activator. With this in mind the effect of manganese (and various other transition elements) on the contractile activity of frog heart has been more thoroughly investigated. A preliminary study of the effects of manganese on the electrical properties of the tissue has also been carried out.

PART 1. SOME EFFECTS OF DIVALENT CATIONS ON THE ELECTRICAL PROPERTIES OF THE HEART

METHODS (1)

(1) The preparation and the experimental chamber.

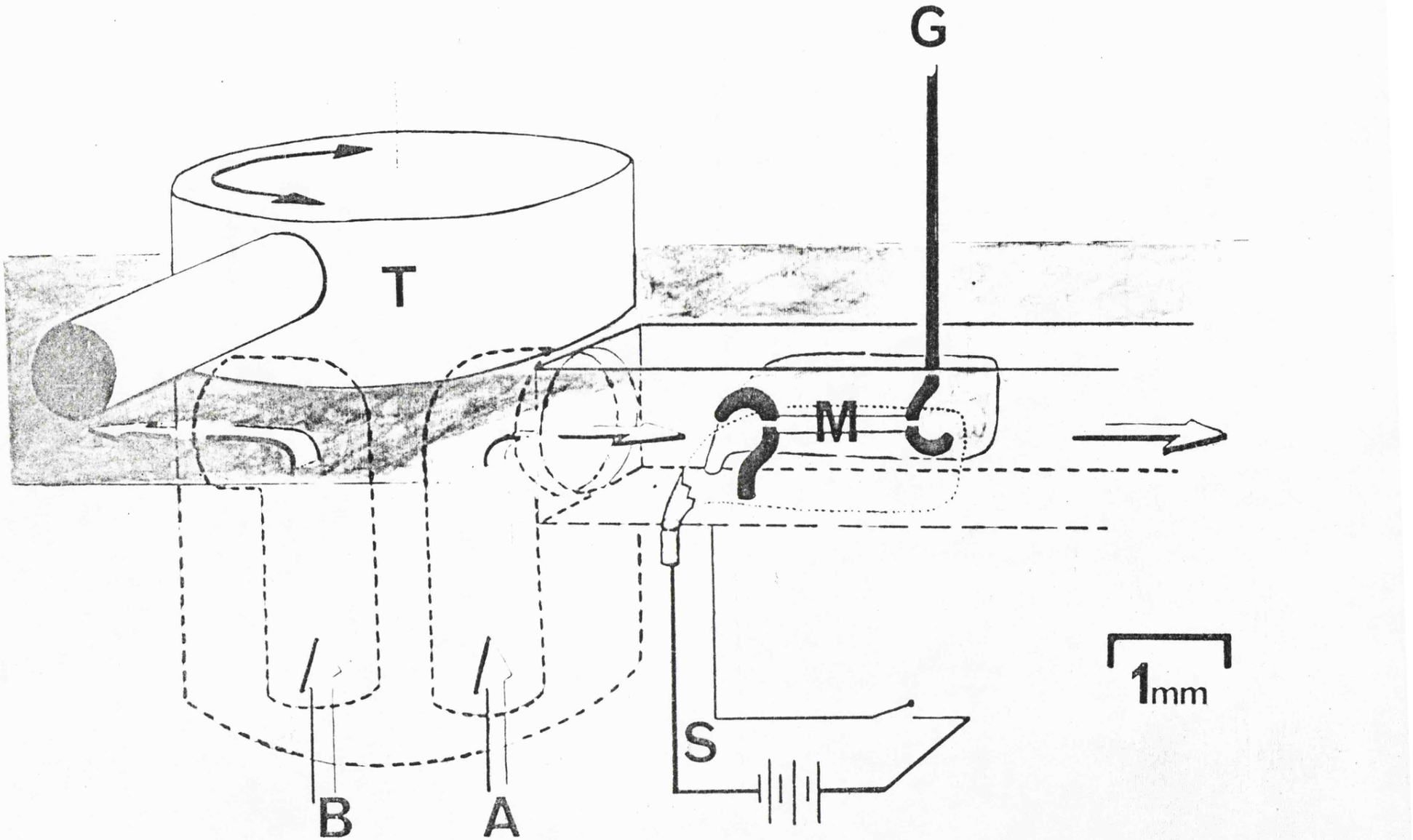
The experiments were performed in a constant temperature room ($20 \pm 1^{\circ}C$) on hearts from adult frogs (Rana pipiens). The frogs were maintained in a $4^{\circ}C$ room in running water at $8^{\circ}C$ until used.

After pithing the C.N.S. the hearts were removed and immediately immersed in normal Ringer solution (see Table I, solution A) containing 1 mM calcium. Following removal of the auricles the ventricle was flushed out with Ringer to remove all traces of blood. The ventricle was pinned down and opened out and a strip of tissue approximately 3 x 1 mm was removed. A loop of fine slik thread (40 μ diameter) was tied to each end of the strip which was then transferred to the experimental chamber (Fig.1). This is a modified Hodgkin-Horowicz dish as described by Chapman and Tunstall (1971) and further modified to permit field electrical stimulation (Chapman, 1973). One end of the muscle, (M), was tied to a hook set into the bottom of the channel, the other end being tied to the hook of the tension transducer, (G), a piezo-resistive strain gauge, Endevco Corp. 8107-2.

Solutions flowing over the muscle were changed with a three way tap (T). Two additional preliminary taps (not shown) permit a rapid switch to any of five different solutions. The exchange time for solutions in the channel has been estimated to be 10 - 90% complete in 0.4 seconds (Chapman and Tunstall, 1971) at a flow rate of 20 mls/min. This flow rate was used

Figure 1.

Muscle (M) situated in perfusion channel. One end of the muscle is tied to the force transducer (G), the other end being tied to a hook set into the base of the perfusion channel. Solutions flowing over the muscle are changed by means of the 3-way tap (T). Field electrical stimulation is produced by an isolated stimulator (S) via two platinum plate electrodes set into the sides of the perfusion channel


G
T
M
1mm
S
B
A

for contracture experiments (Part 2) but a slightly slower rate (15 mls/min) was used for micro-electrode experiments as the less turbulent flow facilitated the maintenance of the micro-electrodes within the cells. The rapid solution exchange time ensured that the response time of the muscle was not limited to any great extent by the time required for change of perfusate.

The solutions were contained in Marriot bottles (not illustrated) in order to maintain a constant pressure head and thus a constant flow rate. Solutions were gassed with oxygen except when actually being used for contracture measurements. A few seconds prior to switching solutions the new solution was allowed to flow to waste through the tap to prevent the muscle being exposed to stale solutions.

The preparation was stimulated by a Devices isolated stimulator connected to two small platinum plates set in the sides of the channel on either side of the preparation. Thresholds for twitch stimulation were normally in the range 1.5 - 4.5 V for 20msec pulses. The rate of stimulation was determined by a Devices digitimer. The tension transducer output was displayed on a Tektronix 502A oscilloscope and a Southern Instruments U.V. chart recorder (1 KHz galvanometers).

(2) Intracellular measurements

Cell resting and action potentials were measured with conventional 3 M - KCl - filled micro-electrodes (8 - 20 MΩ). Measurements were made either between two micro-electrodes or between one micro-electrode and an Agar KCl: Ag Cl_2: Ag indifferent electrode at the end of the channel as shown in

Figure 2. The high impedance differential preamplifier unit utilised a Burr-Brown operational amplifier (input impedence 10^{12} ohms, source current $< 10^{-12}$ A, and with a set gain of 10).

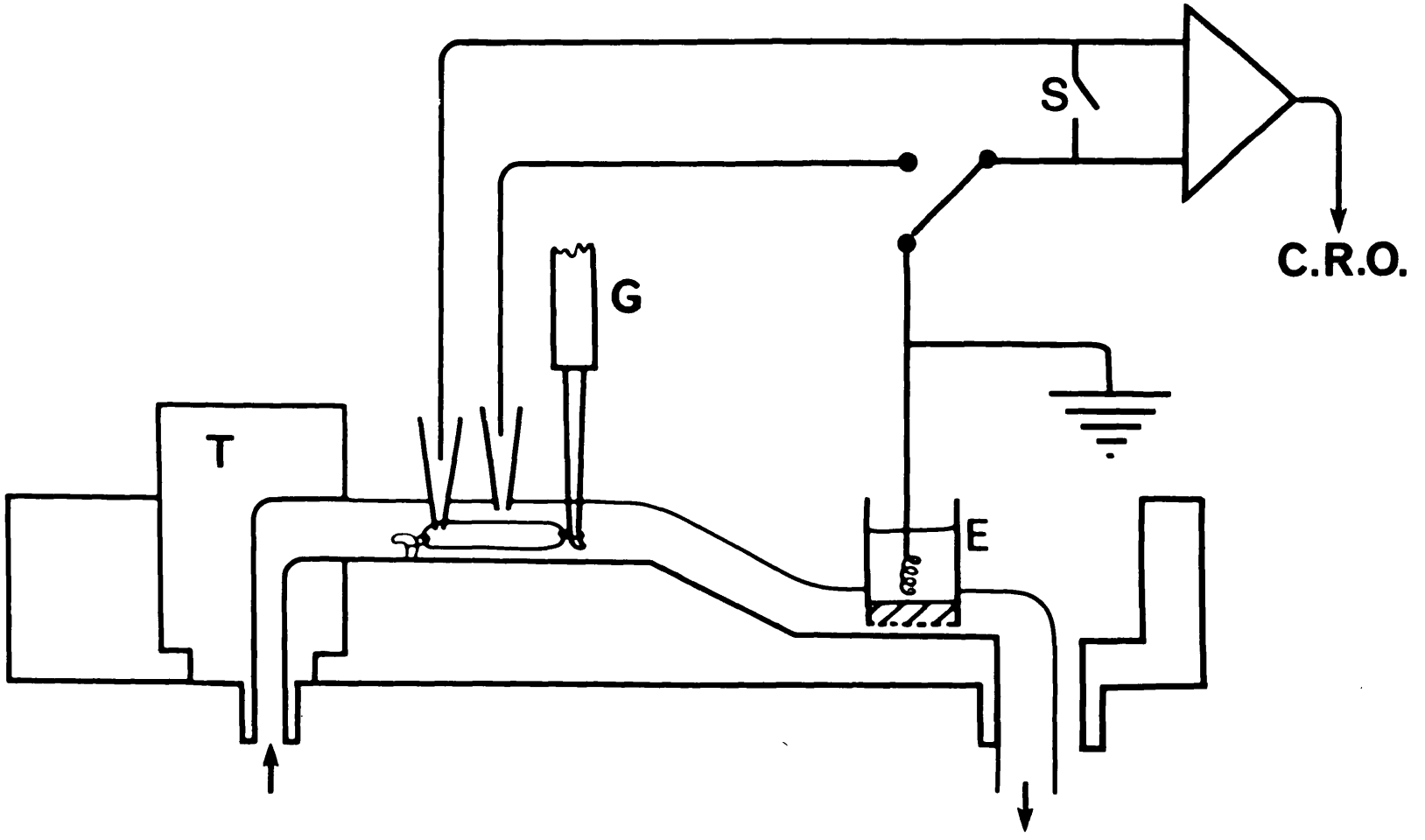
Tip potentials were estimated by closing switch S (Fig.2.) The asymmetry of the system Ag:Ag Cl_2:KCl:RingerKCl Agar:$AgCl_2$:Ag was normally ≤ 3mV and was regularly checked throughout the experiment by breaking a micro-electrode on the bottom of the dish thus eliminating the contribution of the tip potential to the measured potential. Thus micro-electrodes with low tip potentials (≤ 5 mV) were selected. In some experiments the micro-electrode filling solution was acidified to pH 2 as this appears to reduce tip potentials (Wann and Goldsmith,1972). Such precautions were necessary as high tip potentials lead to underestimation of the resting membrane potential (Adrian,1956) and any change in tip potential accompanying the change of solution would lead to erroneous measurements.

When two micro-electrodes were used with one intracellular, the indifferent electrode was positioned as close as possible to it extracellularly to minimise interference and the electrical stimulus artifact.

The signal output was displayed on a Tektronix 502A oscilloscope for photography and subsequent optical enlargement and measurement. Where resting potentials were to be measured the signal output was also displayed on a U.V. chart recorder so that any slow drift of resting potential, indicative of inadequate penetration, could be easily detected.

Figure 2.

Intracellular measurement of electrical activity. Solutions are changed by tap (T). Contractile tension of the muscle is measured by the force transducer (G). Electrical recording is differential between either two micro-electrodes or between one micro-electrode and a Ag:Ag Cl:KCl Agar bridge. Tip potentials of the micro-electrodes were estimated by closing switch (S) (see text for details).


S
C.R.O.
G
T
E

(3) Solutions

All solutions were made up with A.R. grade chemicals (Fisons Ltd.), where available. Glass distilled water was used for Ringer solutions and double glass distilled water for solutions to which no sodium was to be added. Contamination levels of calcium and sodium were determined by atomic absorption spectroscopy and were found to be 3 - 5 μM and 1 - 2 μM respectively.

The pH of all solutions was carefully checked before use. This was adjusted to pH 7.3 except where indicated in the text.

The composition of the various solutions are listed in Table I. Manganese and other divalent cations were normally added without osmotic adjustment in order to avoid a reduction in the sodium concentration. In some experiments where high concentrations of divalent cations have been employed an equiosmotic equivalent of Tris-chloride has been added to the 'control' solutions for direct comparison. Those cases have been specifically stated in the text.

Atropine and caffeine were obtained from B.D.H. Pronethanol was a gift from I.C.I. and D-600 a gift from Knoll A.G. Calcium chloride was normally added as its 1 M solution (B.D.H. volumetric standard, analar grade).

Tris has been used as a buffer in all solutions. Phosphate and bicarbonate buffering is excluded because of the low solubility product of manganese phosphate and hydroxide.

Solution A plus 1 mM calcium has been referred to in the text as normal Ringer.

TABLE I

Concentrations expressed as m - moles / litre

	Solution	NaCl	KCl	Tris HCl	Glucose	LiCl	Sucrose	Hydrazine Cl
A.	Normal Ringer	117	3	3	5	0	0	0
B.	Na-Free, Tris substitute	0	3	132	5	0	0	0
C.	" " Li "	0	3	2	5	117	0	0
D.	" " Sucrose "	0	3	2	5	0	210	0
E.	" " Hydrazine "	0	3	2	5	0	0	117
F.	100 mM K Ringer	117	100	2	5	0	0	0
G.	0.6 mM K Ringer	117	0.6	4.4	5	0	0	0
H.	200 mM K 50% Na	58.5	200	2	5	0	0	0
I.	0 mM K 50% Na	58.5	0	225	5	0	0	0

The calcium concentration was normally 1 mM but was varied as stated in the text. The pH of all solutions was adjusted to 7.3

(4) General procedures

Following removal of the preparation from the heart the muscle was exposed to 1 mM calcium Ringer for one hour at a stimulus rate of 4 per minute at 2.5 x threshold. This period allowed almost complete development of the hypodynamic state (Clark, 1919; Chapman and Niedergerke,1970A) during which time the muscle becomes less sensitive to calcium. Responses of the muscle after this time tend to be relatively stable for several hours.

Exposures to manganese containing solutions were kept as short as possible with intervening recovery period of 15 - 20 minutes in normal Ringer solution.

RESULTS (1)

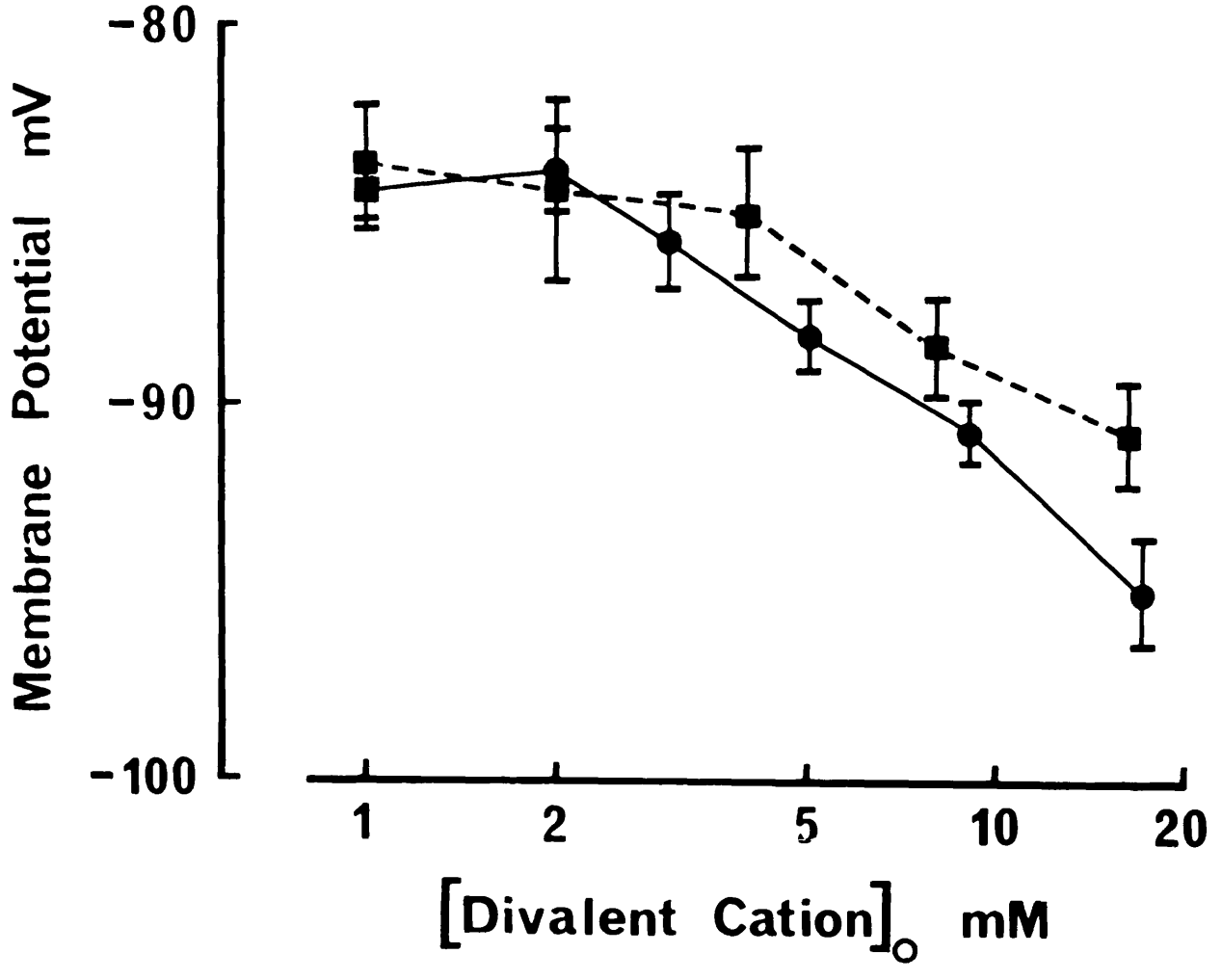
(1) The cell membrane potential and manganese

The effect of various external concentrations of manganese on the cell resting potential is illustrated in Figure 3. The resting potential has been plotted as a function of the total external divalent cation concentration i.e.calcium plus manganese. The calcium concentration was maintained at 1 mM throughout. Manganese addition produced a hyperpolarisation equivalent to 10.8 mV for a ten-fold increase in the external divalent cation concentration over the range 1 to 16 mM. The change in membrane potential when expressed simply as a function of the external manganese concentration (i.e. ignoring the calcium) shows a shallower relationship, the hyperpolarisation being 7.4 mV for a ten-fold change in the manganese concentration.

Takeya and Reiter (1972) found similar hyperpolarisations induced by manganese in rabbit ventricle (7.7 mV between 1 and 10 mM manganese and 12.8 mV between 2.5 and 25 mM). In frog skeletal muscle, Chiarandini and Stefani (1973) reported a 7.4 mV hyperpolarisation on addition of 10 mM manganese to the tibialis anticus longus muscle and 6.5 mV by the addition of 20 mM manganese to the sartorius muscle. Hyperpolarisation is often produced by divalent cations, so much so that Shanes (1958) termed these hyperpolarisations a stablising effect on the membrane. It is therefore not surprising that increasing the calcium concentration produces similar changes to that of manganese on the resting potential (Fig.3. squares joined by dashed line). Over the range 4 to 16 mM calcium the regression line for the points shows a hyperpolarisation equivalent to 10.5 mV for a ten-fold increase in the calcium concentration.

Figure 3.

Resting membrane potential changes produced by the addition of manganese (circles joined by solid line) or calcium (squares joined by dashed line) to normal Ringer. Concentrations are expressed as the total divalent cation concentration of the solution i.e. calcium plus manganese. All manganese solutions contained 1 mM calcium. The ventricle strips were stimulated at a rate of 1 min^{-1} and were exposed to each test solution for 25 minutes and then returned to normal Ringer for 60 min. before the next application of a test solution was made. Eight to twelve acceptable micro-electrode penetrations and hence resting potential measurements were made in each test solution in each of eight experiments. Points shown are the mean of the experiment means, the vertical bars indicate $\pm$ one S.E. of this mean.


-80
-90
-100
Membrane Potential mV
1
2
5
10
20
[Divalent Cation]o mM

At low calcium concentrations the relationship is less steep. This confirms the findings of Niedergerke and Orkand (1966, their Fig.3) where a 4.9 mV hyperpolarisation was found for a ten-fold increase in calcium concentration over the range 0.1 to 10 mM, the relationship being shallower at low calcium, and steeper at high calcium concentrations. However, Ware (1961) also using frog ventricle, reported a 5.4 mV increase in membrane potential for an increase in calcium concentration from 1.08 to 3.24 mM i.e. equivalent to an 18 mV change for a ten-fold increase in calcium concentration. This larger increase may have been due to the high rate of stimulation ($\geqslant$ 20 min^{-1}) or the lower potassium concentration (1.88 mM) in his experiments. In frog sartorius muscle Jenerick and Gerard (1953) found a hyperpolarisation equivalent to 11 mV for a ten-fold increase in calcium concentration.

Over the range of concentrations of calcium and manganese tested in these experiments (i.e. up to 17 mM) there appears to be little difference between the extent of hyperpolarisation produced by calcium or manganese. The curve for calcium is shifted slightly to the right of that of manganese i.e. calcium appears to be slightly less effective at producing membrane hyperpolarisation.

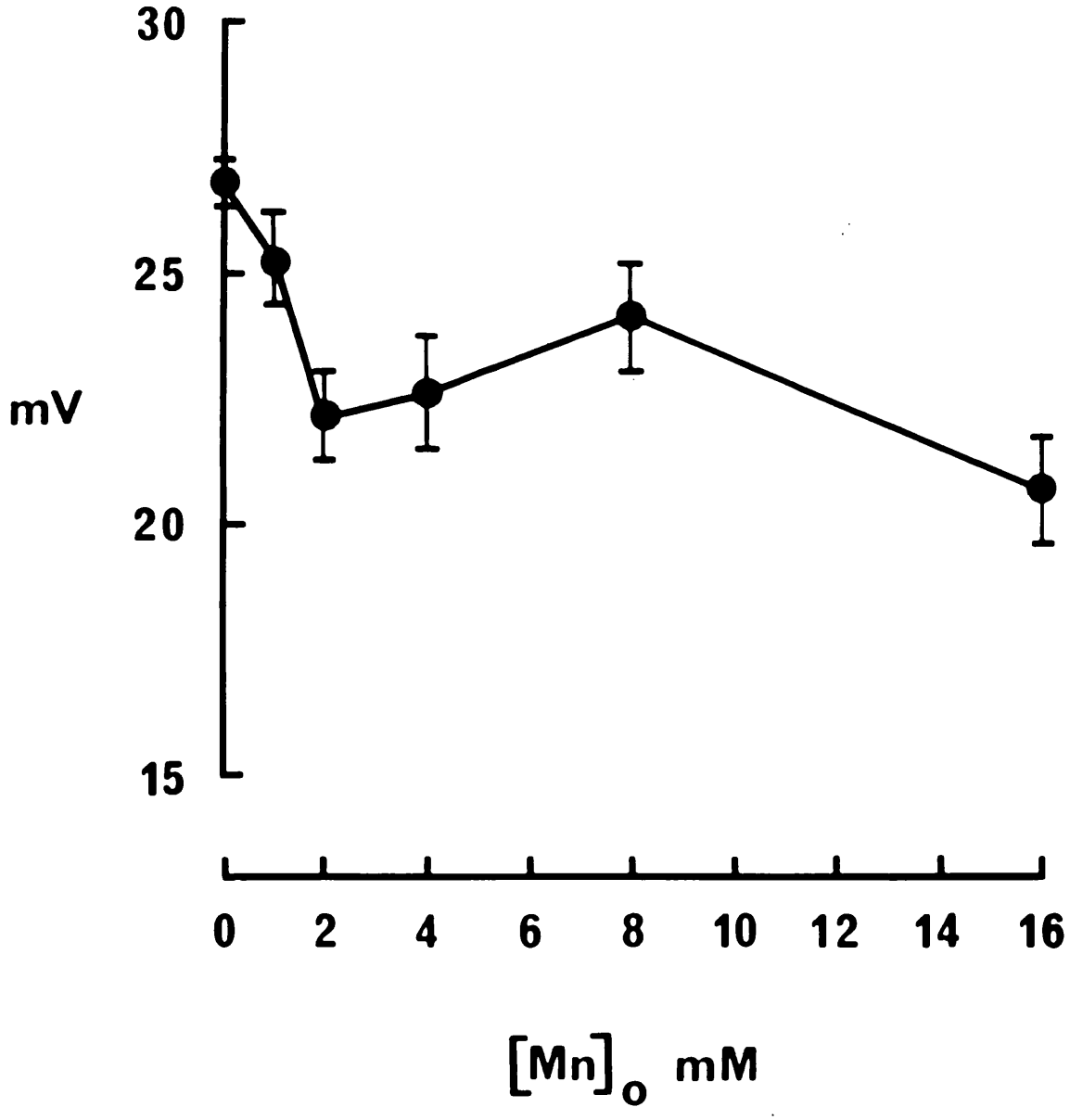
(2) The overshoot of the action potential and manganese

Hagiwara and Nakajima (1965) showed that manganese reduced the overshoot of the action potential in frog ventricle. This was interpreted in terms of an inhibition of calcium influx during the plateau region of the action potential.

I have confirmed Hagiwara and Nakajima's findings and a decrease in overshoot in the presence of manganese is illustrated in Fig.4. A decrease of approximately 5 mV occurred when 2 mM

Figure 4.

Changes in the overshoot of the action potential produced by manganese. The muscles were exposed to the same experimental procedures as for the membrane potential measurements described in connection with Figure 3. The results shown are the means of four to eight penetrations in each solution in each of four experiments. The length of the vertical bars indicate $\pm$ one S.E. of the mean.


30
25
20
15
mV
0
2
4
6
8
10
12
14
16
[Mn]o mM

manganese was present. However, higher manganese concentrations did not produce correspondingly greater decreases in the overshoot unless very high concentrations were employed (16 mM or larger). The decrease in overshoot at these very high concentrations is possibly related to the large increase in threshold and a subsequent inability to produce maximal stimulation of the muscle, an effect that could be associated with the hyperpolarisation produced by manganese as will be discussed later.

Ochi (1970, and personal communication) has suggested that under certain conditions manganese appeared to be able to substitute for calcium as a carrier of slow inward current in guinea pig heart muscle. In the present experiments there was little further decrease in overshoot between 2 and 16 mM manganese (and even an increase in some preparations). This could have been the result of a significant manganese influx during the action potential i.e. manganese acting as a charge carrier during the plateau phase of the action potential. This would suggest that manganese is similar to barium and strontium which are believed to be capable of acting as charge carriers during the cardiac action potential (Masher, 1973; Pappano and Sperelakis, 1969; Vereecke and Carmeliet, 1971).

(3) Submaximal stimulation in the presence of manganese.

Figure 5 shows that the action potential duration and overshoot in the presence of manganese are very dependent upon the degree of stimulation of the preparation. A decrease in action potential duration and overshoot was produced by submaximal stimulation i.e. by a decrease in stimulus intensity (Fig. 5A) or stimulus duration (Fig.5B). Similar findings were reported by Babskii and Donskikh (1973) and Goto and Brooks (1969), also in frog ventricle.

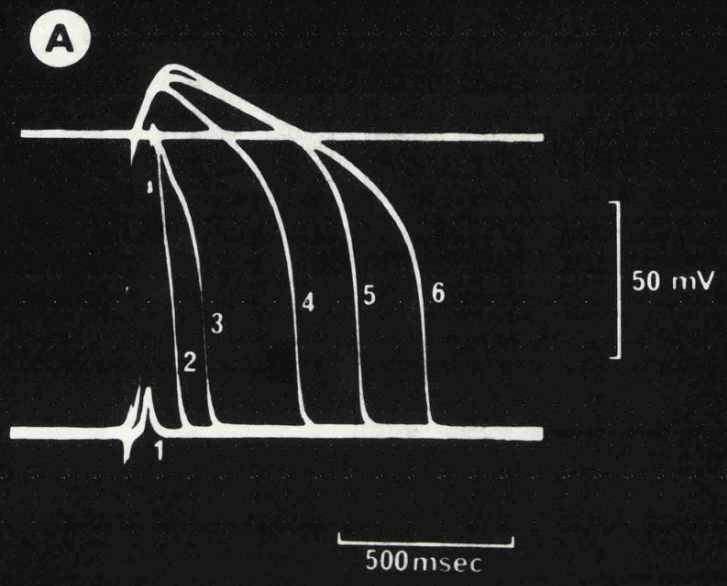
Figure 5.

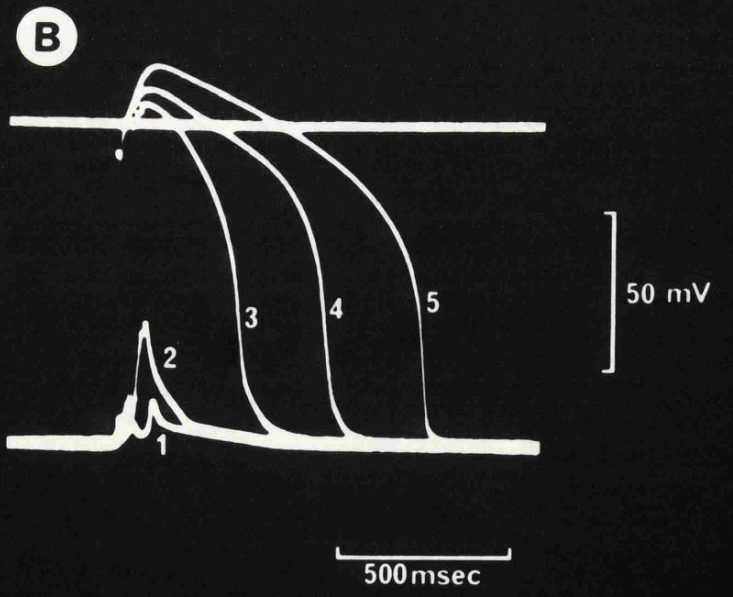
The effects of submaximal stimulation on the action potential in the presence of 8 mM manganese.

A. This shows the superimposed responses to variation of the stimulus voltage at constant stimulus duration (4msec) during continuous impalement of a single cell. Stimulus strength was 2.2, 2.7, 3.2, 4.3, 10.8 and 14.5 volts for responses 1 to 6 respectively.

B. With continuous impalement of another cell in the same preparation the responses to variation of stimulus duration at constant stimulus strength (4.3 volts) are superimposed. Stimulus duration was 3, 3.5, 3.75, 4 and 5 msec for responses 1 to 5 respectively.

The sequence in which the responses were produced in A and B involved alternation between high and low values of stimulus strength and duration. The calcium concentration was 1 mM throughout.


A
1
2
3
4
5
6
50 mV
500 msec


B
1
2
3
4
5
50 mV
500 msec

The results suggest that if maximal stimulation (which was facilitated by the use of field stimulation in these experiments) is not used then measurements of these action potential parameters would show a much greater dependence on the manganese concentration. Studies involving conducted action potentials are therefore likely to show larger decreases in the overshoot and action potential duration for a given manganese concentration. This fact could account for the smaller reduction of these parameters by manganese as measured in the present experiments when compared with the results of Hagiwara and Nakajima (1965).

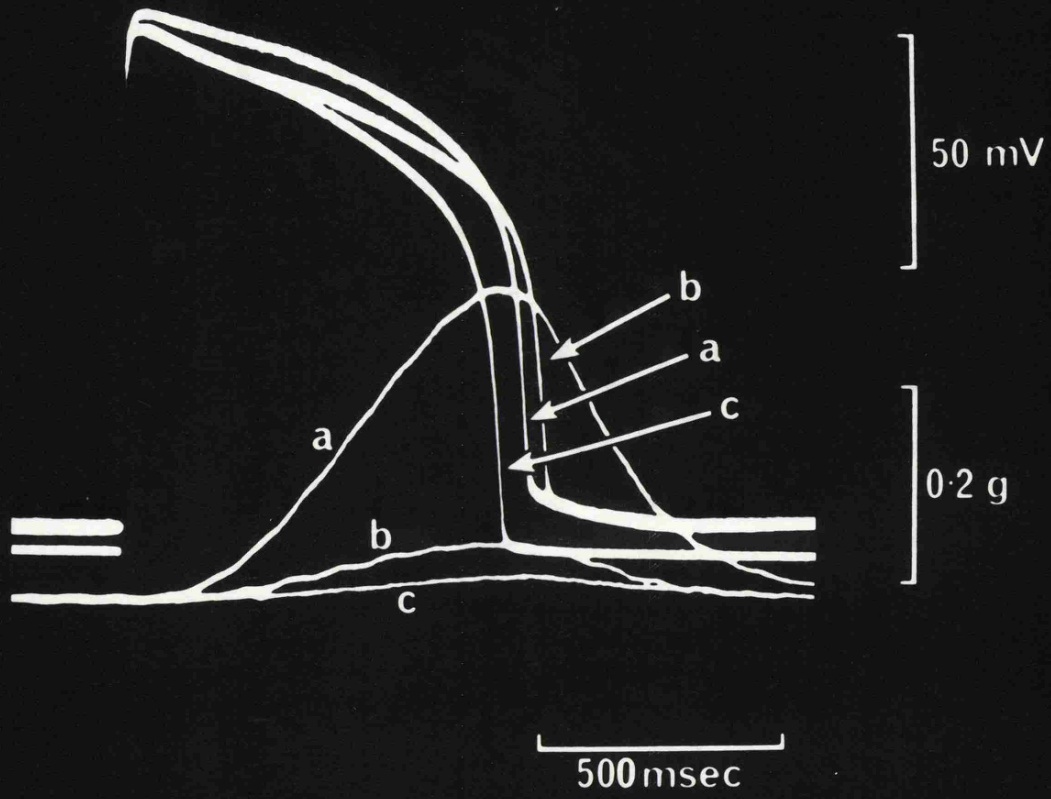
Goto, Abe and Kawata (1961) have suggested that the plateau component of the action potential is dependent upon the excitation of neighbouring cells. If this is true, then where most of the cells in the preparation are producing an action potential as a result of maximal stimulation, the action potential observed in an individual cell will appear with the normal prolonged plateau phase. Whereas when the stimulus intensity is sufficient to excite only a small number of cells in the preparation the action potential appears as a short spike with a reduced or with no plateau component. A similar explanation could be advanced to account for the observation that the action potential observed in extremely thin auricular trabeculae exhibits only a short spike with little or no plateau component (Cl. Leoty - personal communcation).

(4) <u>The action potential duration and manganese.</u>

Manganese produced a biphasic effect on the action potential duration similar to that reported by Takeya and Reiter (1972) in rabbit ventricle. Figure 6 illustrates the changes in

Figure 6.

Biphasic change in the action potential duration produced by 2 mM manganese (continuous impalement of a single cell). (a) control action potential and twitch in normal Ringer. (b) action potential and twitch after 15 seconds in manganese Ringer. (c), the responses after 15 minutes in the presence of manganese. The calcium concentration was 0.5 mM and the stimulus strength was maintained at six times the threshold throughout. Stimulation rate was 4 min^{-1}.


50 mV
0·2 g
500 msec
a
b
c
a
b
c

duration produced by the addition of 2 mM manganese. Prior to the decrease in the duration, a transient increase occurred. This transient increase is smaller and of a shorter time course the higher the manganese concentration employed.

Figure 7 illustrates the time course of the changes in the action potential duration and resting potential during a long single impalement in the presence of 8 mM manganese.

In similar experiments with other transition elements nickel produced only large increases in action potential duration (2.5 times with 8 mM nickel) whereas cobalt only produced action potential shortening.

An increase in the calcium concentration can also produce a decrease in action potential duration in frog ventricle (Niedergerke and Orkand, 1966) but normally an increase in duration was observed. In toad ventricle Fukuda (1972) found only shortening of the action potential in increased calcium concentrations. These changes are possibly related to the hyperpolarisation observed in solutions with high divalent cation contents.

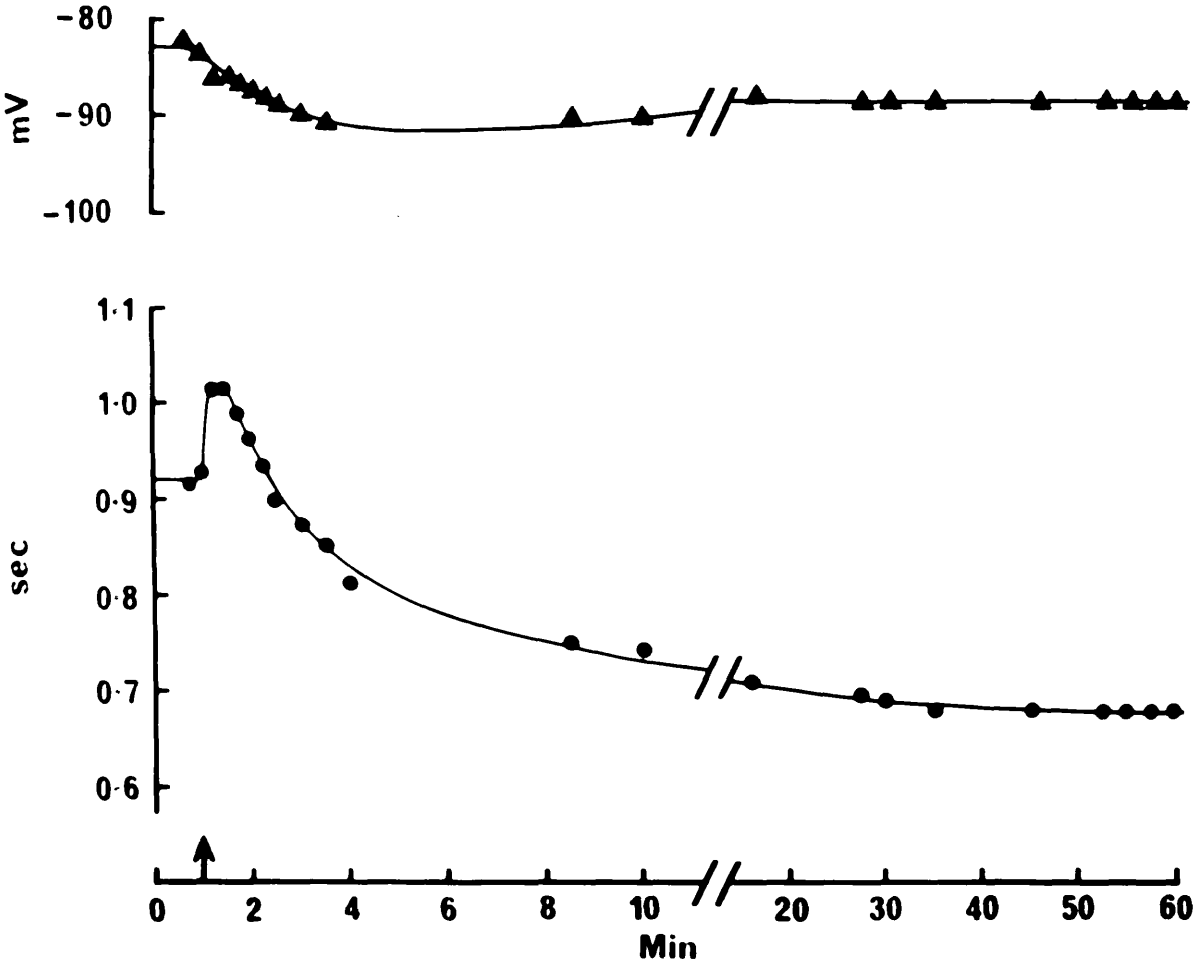
(5) The effect of manganese on the threshold for contraction

Manganese produces large increases in the contraction threshold in frog heart, the threshold being increased two to three times by 8 mM manganese. This is presumably a reflection of the ability of manganese to prevent full generation of the normal action potential at low stimulus intensities as described above.

Figure 8 illustrates a comparison between the effects of calcium (Fig.8 left) and manganese (Fig.8 right) on the threshold of frog heart strips. The threshold being determined

Figure 7.

Time course of changes in the resting potential and action potential duration produced by the addition of 8 mM manganese at a point corresponding to 1 minute on the time axis. Continuous impalement of the same cell. The stimulus rate was 4 min^{-1} and the calcium concentration was 1 mM. Note change of scale on the time axis.


-80
mV
-90
-100
1·1
1·0
0·9
0·8
0·7
0·6
sec
0
2
4
6
8
10
20
30
40
50
60
Min

as that stimulus intensity just sufficient to produce a measurable contraction. Figure 8 also compares the effect of divalent cations on the twitch threshold in auricle (circles) with that of ventricles (triangles).

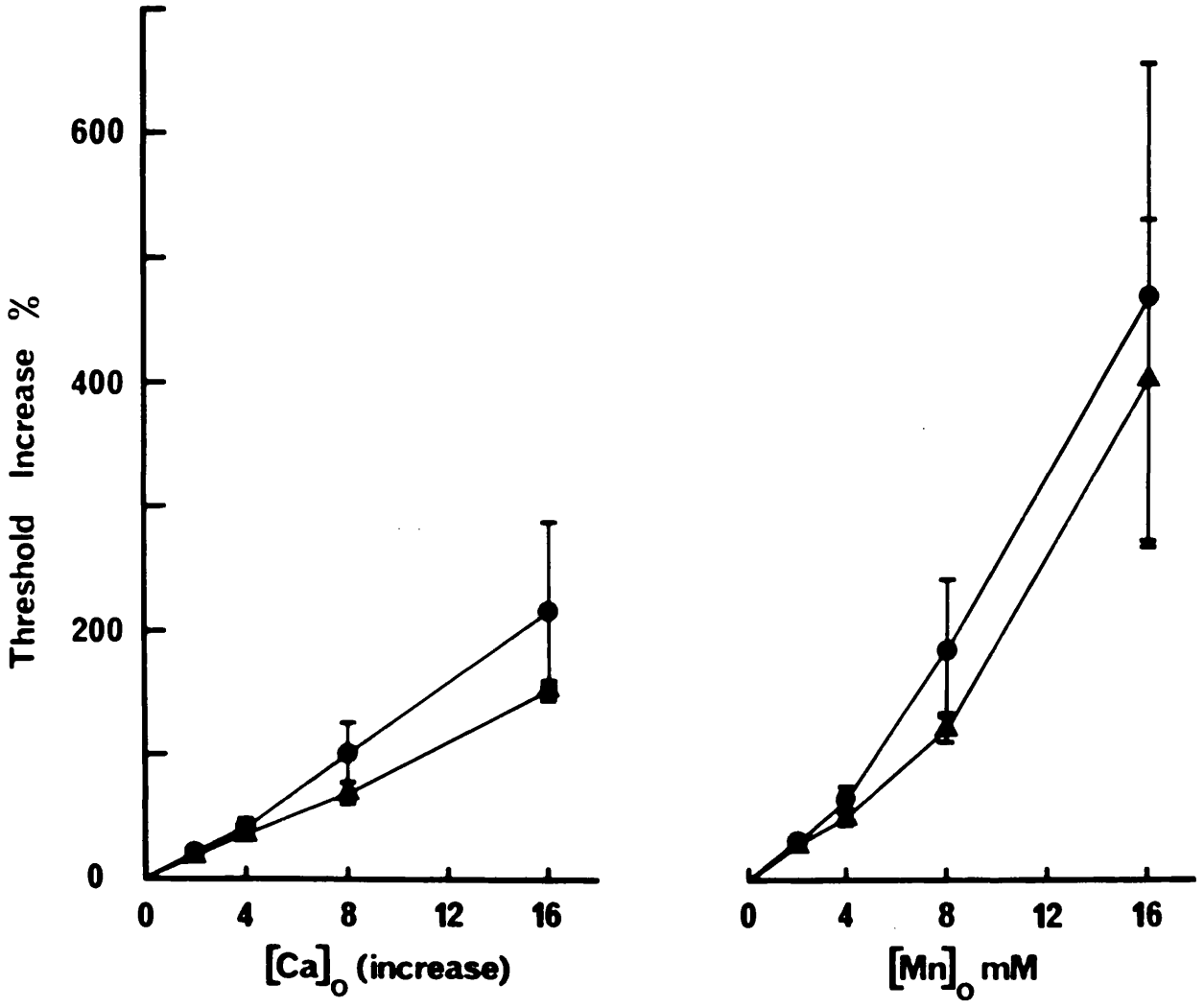
No significant difference was found between the threshold increases produced by addition of calcium and manganese to auricle compared with ventricle strips. This is to some extent reassuring because both auricle and ventricle preparations have been used in the various parts of this work. These experiments also illustrate the similarity in the effects produced by manganese andthose of calcium. Indeed a direct paired t-test of the threshold increases produced by manganese and calcium in the same hearts showed a significant difference between calcium and manganese only in auricle and only then at the 5% level.

Thus both manganese and calcium produce increases in twitch threshold, manganese producing slightly greater increases. It would seem possible that the threshold increase could have resulted from the increase in membrane potential produced by the divalent cations as described earlier. This was tested by lowering the potassium concentration in the perfusion solution to 2 mM. This produces a hyperpolarisation which can be estimated from the data of Luttgau and Niedergerke (1958) as 4.0 mV, Chapman (1973) as 6.8 mV, or Graham, Bennet and Ware (1969) as 8.2 mV (stimulation rate 24 min^{-1}). Thus the hyperpolarisation produced by 2 mM potassium is approximately equivalent to the hyperpolarisation produced by 8 mM manganese (= 6.3 mV, Fig.3) or 8 mM calcium (= 4.7 mV Fig.3). However 2 mM potassium was found to produce only 25-40% of the threshold

Figure 8.

Effects of the addition of manganese or calcium on the stimulation threshold for contraction in auricle (circles) and ventricle (triangles) strips. The stimulus duration was 5 msec at a rate of 12 min^{-1}. The threshold increase was measured 3 min after the addition of calcium or manganese and is expressed as a percentage of the threshold voltage in normal Ringer (1mM calcium). The threshold in normal Ringer was measured before and after every 'test' solution. The preparations were stimulated through a 100 K ohm series resistor, mean stimulating current in normal Ringer was 0.212 mA in auricle and 0.104 in ventricle strips

The results shown are the means ($\pm$ S.E. of mean) of four paired experiments, the auricle and ventricle strips being obtained from the same hearts and were selected as having approximately the same dimensions.


Threshold Increase %
600
400
200
0
0
4
8
12
16
[Ca]o (increase)
0
4
8
12
16
[Mn]o mM

increase produced 8 mM manganese or calcium. At higher concentrations of divalent cations an even smaller contribution of hyperpolarisation to the threshold increase would be anticipated because of the logarithmic relationship between the membrane potentialand the divalent cation concentration.

Alternative explanations for the extra increase in threshold could include the small increase in osmolarity caused by the addition of the divalent cation. However the addition of 12 mM Tris-chloride was found to produce no increase in threshold. It therefore seems unlikely that the extra increase in threshold could be due to an osmotic effect.

The involvement of the release of endogenous transmitter chemicals by manganese or high calcium is ruled out because the increased threshold in these solutions was not reduced by atropine (1.2×10^{-5}M) or pronethanol (12 μ gm/ml).

It would seem therefore that manganese (and calcium) produces an increase in threshold in frog heart which is only partly accounted for by a hyperpolarising effect. Frankenhaeuser and Hodgkin (1957) found that an increase in the calcium concentration produced a positive shift of the membrane potential at which the peak sodium conductance occurs in the depolarisation of the squid axon. This was suggested by A.F.Huxley to be due to calcium binding to negatively charged groups on the outer surface of the membrane. This would change the electrical field within the membrane and thus could decrease the sodium conductance changes associated with the membrane depolarisation. In frog heart this would alter the threshold for electrical stimulation and thereby the threshold for contraction. The large threshold increases in manganese

may therefore be accounted for by this charge screening effect of the divalent cations, an idea supported by the results of Hagiwara and Nakajima (1966) who found a decrease in the maximum depolarisation rate of the action potential occurred in frog ventricle in relatively high concentrations of manganese (8-10 mM).

DISCUSSION (1)

(1) Divalent cations and the membrane potential

Increased calcium concentrations have been shown to produce an increase in resting membrane potential in frog myelinated nerve (Schmidt, 1960), frog skeletal muscle (Jenerick and Gerard,1953), rat diaphragm (Gossweiler et al, 1954) cockroach giant axon (Narahashi,1960), lobster giant axon (Dalton, 1958) and frog heart (Niedergerke and Orkand,1966; Ware,1961). However, increased calcium was without effect on the membrane potential of mammalian Purkinje fibres (Weidmann,1955) and mammalian papillary muscle (Hoffman et al,1956).

In the present experiments the addition of calcium or manganese has been shown to produce a membrane hyperpolarisation in frog ventricle. The increase in membrane potential was equivalent to approximately 10.5 mV for a ten-fold increase in the divalent cation concentration over the range 4 - 17 mM. At lower concentrations the relationship was less steep. Manganese addition produced slightly more hyperpolarisation than equivalent concentrations of calcium over the whole range.

A possible cause of the hyperpolarisation in solutions with raised divalent cation concentrations could be an increased potassium conductance of the membrane. Such an increase could also decrease the action potential duration (Figure 6) and reduce the overshoot (Figure 4). However in frog skeletal muscle manganese has been shown to increase the effective membrane resistance (Chiarandini and Stefani,1973). This was interpreted in terms of a decreased potassium

conductance. (Chiarandini and Stefani found an increased membrane resistance by manganese only in potassium depolarised muscle. The increase in normal Ringer was not significant. However, as they pointed out, their technique could not easily have detected a 50% reduction of the potassium conductance in normal Ringer. They therefore assumed that a decreased potassium conductance had occurred but that it could not easily be demonstrated).

An alternative mechanism proposed for the calcium hyperpolarisation observed in frog skeletal muscle was a decrease in the membrane sodium conductance (Adrian and Freygang,1962), thus allowing the membrane potential to more closely approach the potassium equilibrium potential. This therefore appears to be a more likely explanation for the observed hyperpolarisation.

(2) Action potential changes in the presence of manganese

Manganese was found to produce a decrease in the overshoot of the action potential (Hagiwara and Nakajima,1966). This has been confirmed in these experiments. However smaller decreases in overshoot were found, the effect being almost maximal in 2 mM manganese. Above 16 mM manganese lower overshoots were observed. Experiments have shown that these probably resulted from the concommitent increase in threshold in manganese - Ringer yielding graded responses and preventing full excitation of the muscle. The results indicate that measurements of the overshoot and the action potential duration are dependent upon the stimulus intensity in the presence of manganese. Similar graded cardiac action potentials have

been observed in manganese (Babskii and Donskikh,1973), in manganese and hypertonic solutions (Goto and Brooks,1969), in magnesium and hypertonic solutions (Ohshima, 1969) and in increased potassium concentrations (Tritthart et al, 1973). Thus in these conditions where the conduction velocity is slowed, the action potential becomes graded and appears to split into two components, a short initial spike and a longer plateau depolarisation phase.

Such results might provide indications of the method of action potential generation in frog heart i.e. does the initial spike represent the fast sodium current, and the early plateau phase the slower calcium current observed in voltage-clamp studies? Goto and Brooks (1969) suggested that the initial spike triggers the plateau response and that twitch contractions only occur with the appearance of the plateau. This could be interpreted as suggesting that the calcium inward current assumed to be associated with contraction only occurs during the plateau phase. Alternatively, the short duration of the spike depolarisation might be insufficient to trigger an internal release, or a transmembrane flux, of calcium for contraction. No distinct threshold for the plateau response was found in the present experiments, the response being always graded. As the viability of voltage clamping cardiac tissue, and even the existence of a slow inward current has been questioned (Johnson and Lieberman, 1971), further speculation along these lines seems fruitless.

An alternative explanation for the appearance of the plateau could be that although the action potential response

from a single cell is a short spike, the plateau might be generated by neighbouring cell interaction as a result of the syncytial nature of cardiac tissue. Thus neighbouring cells could act as a large current source for the plateau component. This might account for the shorter action potential in atrial tissue which has a more regular cable-like structure than the "spongy" ventricle. The much branched ventricular tissue, together with the slower conduction velocity, would facilitate a re-enterant collision of excitation (Goto, Abe and Kowata,1961) thus prolonging the action potential. The rather slow subthreshold local responses (Figure 5a1, 5b1, 5b2) might further support this suggestion.

Perhaps the most widely accepted explanation for the prolonged plateau of cardiac action potentials has been summarised by Noble and Tsien (1969). The plateau is considered to be dependent upon delayed potassium currents responsible for repolarisation. High divalent cation concentrations could thereby produce action potential shortening by reducing the delay of onset of these currents. The membrane hyperpolarisation or other electric field changes within the membrane might produce these changes.

The biphasic changes in action potential duration in manganese are not easily explained. Niedergerke and Orkand (1966) found inconsistent effects on the action potential duration in increased calcium concentrations, i.e. sometimes an increase and sometimes a decrease. A shortening of the action potential was found in cardiac muscle of rat (Coraboeuf

and Vassort 1967), rabbit (Takahashi and Holland,1969) and guinea pig (Stanley and Reiter,1965) whereas a prolongation was found in dog heart (Kleinfeld et al, 1966). Takeya and Reiter (1966) found biphasic changes of duration with manganese in rabbit heart similar to those reported here. They suggested that low manganese concentrations produce prolongation whereas higher concentrations produce shortening. They therefore postulated that biphasic changes were due to a diffusion delay in the muscle i.e. low extracellular manganese concentrations were initially experienced by the cells in the preparation thus producing an increase in action potential duration. Later the extracellular manganese concentration effectively increased as its equilibration across the muscle proceeded thereby producing action potential shortening. In the present experiments no prolonged increase in duration in manganese was observed in the lowest concentrations employed (1mM). However, such an explanation cannot be ruled out altogether.

(3) Excitability changes produced by calcium and manganese.

The experiments on the threshold for contraction show an approximate equivalence in the responses of auricle compared with ventricle, and between the effects of calcium and of manganese.

Slight differences between auricle and a ventricle might be anticipated in view of the disparity in their respective ion content e.g. Walker and Ladle (1973) found a significant difference between the internal potassium activities of the two tissues. Fukuda (1972) found a significant difference

between the calcium content of toad auricle and ventricle. The observed structural differences (Miller,1974) might also be expected to affect the threshold for stimulation and subsequent propagation of excitation.

Increased manganese concentrations produced slightly greater increases in threshold than did increased calcium. This might be anticipated in view of the somewhat larger hyperpolarisation observed in manganese solutions (Figure 3).

Similar increases in threshold by increased calcium were found by Fukuda (1972) in toad heart i.e. approximately a 250% increase in 7 - 10 mM calcium compared to approximately a 200% increase in 17 mM.calcium in these experiments. The larger threshold increases would be expected in the experiments of Fukuda as whole auricle or whole ventricle were used. Thus a larger decremental current spread would be anticipated.

The hyperpolarisation produced by increased calcium or manganese was sufficient to account for only 25 - 40% (less at high concentrations) of the threshold increase in these solutions. Thus a stabilising effect of the divalent cations (Frankenhaeuser and Hodgkin 1957) must be invoked to explain the extra increase in threshold. This might occur by a direct binding of calcium (or manganese) to anionic membrane sites or by a charge shielding effect i.e. a build up of a layer of divalent cations adjacent to the negatively charged cell membrane (Gilbert and Erhenstein,1969). Both effects could hinder the approach, or binding of, sodium to the membrane. This would in effect reduce the sodium concentration local to the membrane. Alternatively changes in the electric

field within the membrane, as a consequence of the presence of extra divalent cations, could influence the gating of the sodium current of the action potential.

The upstroke of the action potential appears to result from a rapid inward sodium current (Hodgkin and Huxley, 1952 A,B,C; Brady and Woodbury,1960). Manganese has been shown to slow the maximum upstroke velocity of the action potential in frog ventricle (Hagiwaraand Nakajima,1966;Coraboeuf and Vassort,1968) as does calcium (Ware,1961; Niedergerke and Orkand,1966). Thus despite the membrane hyperpolarisation in high manganese and calcium solutions a decrease in the peak sodium conductance probably occurs. However the decrease in overshoot in manganese is unlikely to result solely from a decreased sodium conductance. Niedergerke and Orkand (1968) found the overshoot to be highly dependent upon the external calcium concentration in frog heart and much less so on the sodium concentration. They found an 18.3 mV change in overshoot for a 10-fold change in calcium concentration. Thus the decrease in overshoot in manganese is more likely to have been due to an inhibition of transmembrane calcium movement. As was suggested earlier, manganese might be able to substitute for calcium to some extent as a charge carrier in this process (see also Ochi, 1970). If this is the case then the observed effect of manganese on the overshoot might be the resultant of two opposing influences, (a) a decrease in overshoot produced by the inhibition by manganese of calcium entry and (b) an increase in overshoot due to a manganese inward current. These two processes could give a curve of the kind seen in Figure 4. However it would seem that many

of the other effects observed in manganese and high calcium solutions are best accounted for by assuming a decreased resting sodium conductance, and a reduction of the peak sodium conductance associated with the depolarisation of the action potential.

PART 2. MANGANESE AND THE CONTRACTION OF THE HEART.

METHODS (2)

(1) The preparation

The preparation chosen for contracture experiments was the thin auricular trabecula. This has the advantage that it is much thinner (1 - 2 mm long with a diameter of 40 - 100 μ) than ventricular strips so that solutions can equilibrate more rapidly through the preparation. Thus the rapidly developing contractures, produced by the various test solutions, and their subsequent spontaneous relaxation will be less limited by the time for diffusion in the extracellular spaces. Auricular trabeculae are also much more robust and more easily isolated than the thinnest ventricular trabeculae.

(2) General procedures

The isolation procedure and the mounting of the preparation was identical to that described in Part 1 for ventricle strips except that greater care is required with auricular trabeculae in view of the small dimensions.

The auricular trabecula can be considered as being approximately equivalent to a single skeletal muscle fibre in cross sectional area but is shorter in length. Response times would be anticipated to be slightly longer in the auricular trabecula because of its multifibre nature i.e. diffusion is required for solutions to obtain access to the surface membranes of central fibres. The maximum contractile

forces obtained with auricular trabeculae are approximately 3,000 times the wet weight of the tissue. This is somewhat less than that obtained with single skeletal fibres and is probably related to the lower proportion of contractile proteins in cardiac muscle (Katz, 1970).

Reproducible contractile responses can be obtained from undamaged preparations for up to 10 hours. Damaged preparations normally show a rapid deterioration in the size of the contractile response and can therefore be abandoned at an early stage in the experiment.

To compensate for any unidirectional drift in contractile forces a "mirror image" procedure (Chapman and Niedergerke, 1970A) was adopted for the sequence in which the contractures were carried out i.e. contracture solutions were applied in a given sequence (normally alternating between large and small responses), the sequence was then performed in the reverse direction.

(3) Tension measurements.

The muscle length was adjusted to produce maximum twitch tension at the beginning of the experiment. This was checked again after an hour long equilibration period.

At the end of all experiments the muscle was gently blotted and weighed on a torsion balance.

The tension transducer (Endevco 8107-2) allows small tension measurements (compliance 3.2×10^{-4} mm/mg, equivalent to 0.6% change of resting length for a force of 20 mg). The transducer forms two arms of a Wheatstone bridge circuit

energised by a Southern Instruments M.1271 bridge power supply. The transducer output, after amplification by a Southern Instruments M.1266 d.c. amplifier, was fed to a U.V. chart recorder and a Tektronix 564 B storage oscilloscope. Tension and its first time differential (obtained by an R-C network with a variable time constant) were simultaneously displayed on the U.V. recorder using 1 KHz galvonometers. The differentiating circuit was calibrated by ramp voltages obtained from an oscilloscope time base. The transducer was calibrated with a series of known weights.

An Ampex F.M. tape recorder enabled a permanent record of most of the experiments to be obtained. The illustrations were obtained by replay of the recorded experiments.

(4) Statistical analysis

Where possible means are calculated ($\pm$ S.D. or S.E.). These and regression lines and their correlation coefficients were calculated with the aid of a Wang programmable calculator. Similarly K_m and V_{max} values for plots of the Lineweaver-Burk type were obtained by use of the calculator to give "best lines" and intercepts by a least squares regression analysis.

RESULTS (2)

(1) Effect of manganese on the twitch contraction

Addition of manganese to the solution perfusing frog heart muscle produces a rapid inhibition ($t_{\frac{1}{2}}$ = 3 - 5 sec) of twitch tension (Figure 9, first arrow). There is a slight recovery of twitch tension and a slowly developing contracture appears with longer periods in manganese containing solution (as described by Chapman and Ochi,1971). On return to normal Ringer lacking manganese (second arrow) a larger contracture develops and twitch tension is potentiated. This contracture relaxed fully after a further 10 - 12 minutes in normal Ringer. The contracture in manganese solutions is not due to depolarisation. There is in fact a significant hyperpolarisation at this time (as described in Part I). The contracture presumably results from an increased sarcoplasmic calcium concentration. If it is assumed, as is widely accepted, that manganese inhibits calcium influx into cells then the contracture development appears somewhat anomalous.

The rapid inhibition of twitch tension of manganese would suggest a superficial site of action e.g. the cell membrane. The twitch contractions in frog heart are very sensitive to the external calcium concentration (Chapman and Niedergerke, 1970), so it would not be unreasonable to assume that the rapid inhibition of tension results from a decreased transmembrane calcium influx.

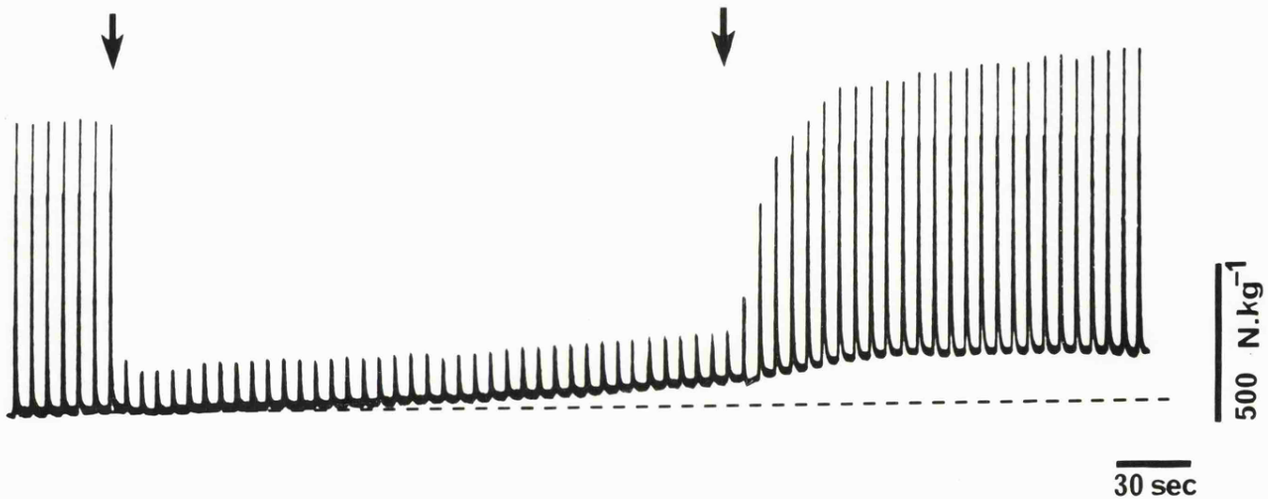
The duration of the action potential controls the duration of contraction in cardiac muscle (Kavaler,1959). Therefore a possible cause of the decrease in twitch tension in manganese might appear to be the shortening of the action potential

Figure 9.

Manganese inhibition of twitch contractions. Record shows contractile tension produced by a regularly stimulated ($4\ min^{-1}$) frog atrial trabecula. 8 mM manganese was added at the first arrow and removed after 10 minutes at the secon

The dashed line gives the resting tension of the muscle. The deviation from resting muscle tension indicates the development of a contracture.

Stimulus strength was maintained at 6 x threshold. Calci concentration was 1 mM.


500 N.kg^{-1}
30 sec

duration. However this could only partially contribute to the tension decline as electrical differentiation of the tension signal shows that manganese produces a large decrease in the rate of rise of twitch tension but only a small decrease in the time to peak tension (the latter being proportional to the action potential duration).

A study of the effects of manganese on the twitch contraction is made difficult by the complicating factors of (1) contracture development in manganese Ringer (2) biphasic changes in the duration of the active state (3) variation of action potential duration with stimulus intensity and (4) threshold increases. In an attempt to circumvent these problems the effect of manganese on the contracture responses (sustained contractions) induced by high potassium (Chapman and Tunstall 1971) and low sodium (Chapman,1974) solutions have been studied. High potassium solutions produce a sustained depolarisation in frog heart muscle (Niedergerke, 1956) thereby eliminating any problems associated with changes in the action potential duration.

(2) Manganese and the potassium-rich contracture

Figure 10 illustrates the results of a typical experiment on the effect of various manganese concentrations on the contracture induced by 100 mM potassium solutions. The triangles and circles represent contracture tension in 4 and 2 mM calcium respectively.

In the absence of manganese the application of 100 mM potassium Ringer produces a contracture proceeded by a twitch contraction as the muscle is depolarised. This twitch

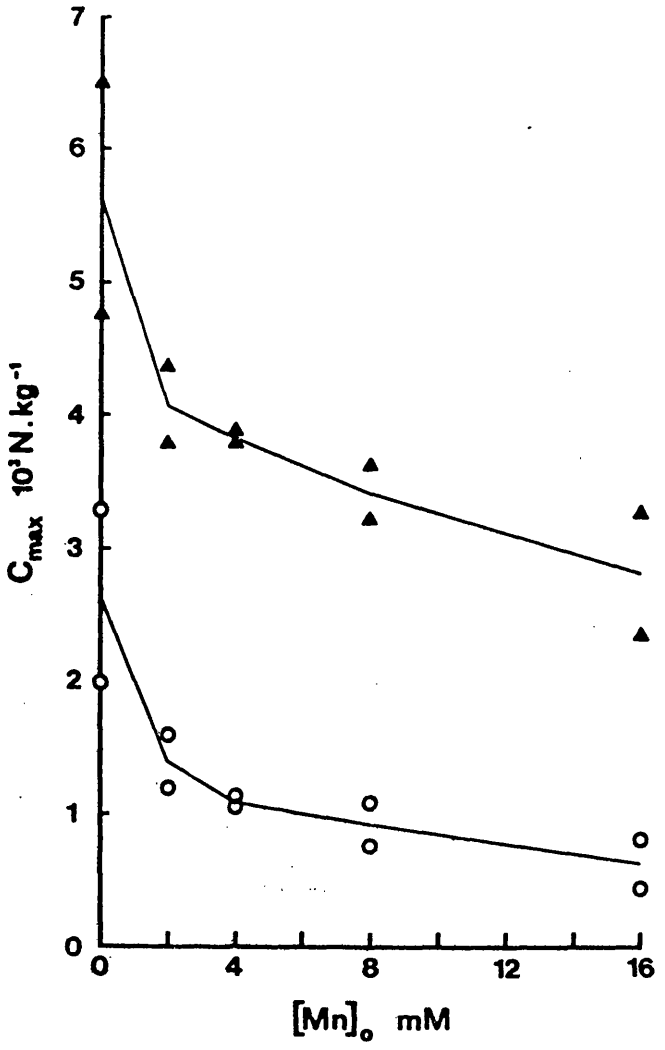
Figure 10.

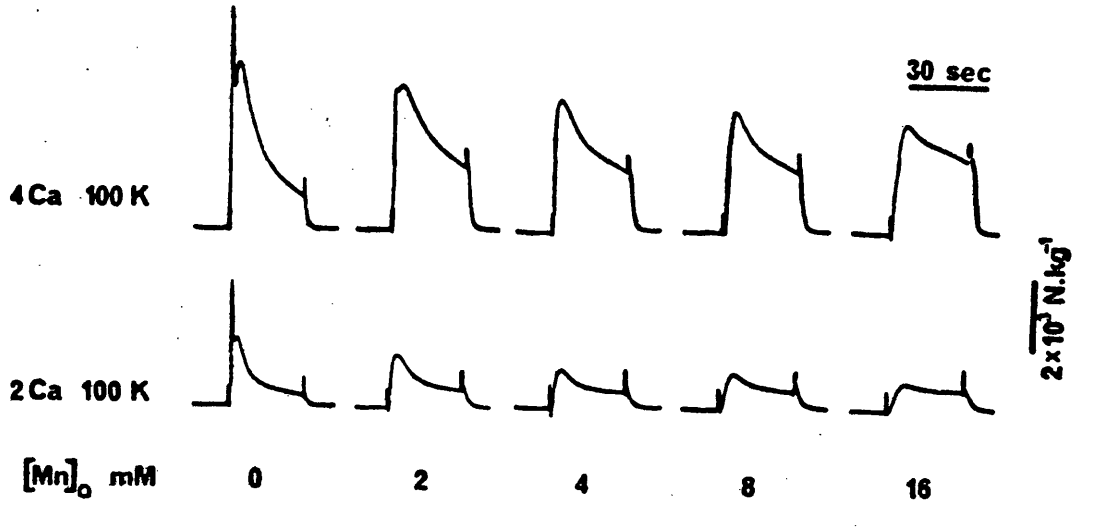
Manganese inhibition of the potassium-rich contracture.

Top: graph shows the maximum contracture tension produced by 100 mM potassium Ringer (Solution F, Table 1) in the presence of 4 mM (triangles) and 2 mM (circles) calcium.

Bottom: Tension recordings of the contractures. The regular upstrokes on the tension recordings at the beginning and end of each contracture are solution changing artifacts and indicate the time of addition and time of removal of the 100 mM potassium Ringer. Manganese was added 30 second prior to the contracture inducing solutions containing manganese to permit its extracellular equilibration. After each contracture the muscle was allowed to recover in normal Ringer solution for 16 minutes while being stimulated at a rate of 4 min^{-1} . The stimulator was switched off during contracture measurements, the contracture solution being introduced 15 seconds after the preceeding twitch. This general procedure was adopted for all the contracture experiments illustrated in this work.

Temperature 23°C.


7
6
5
4
3
2
1
0
C_{max} 10^3N.kg^{-1}
0
4
8
12
16
[Mn]$_o$ mM


30 sec
4 Ca 100 K
2 Ca 100 K
2x10^3 N.kg^{-1}
[Mn]$_o$ mM
0
2
4
8
16

contraction is greatly inhibited in manganese solutions but the potassium-rich contracture is much less sensitive to manganese inhibition.

Prior to the application of contracture inducing solutions containing manganese the muscle was pretreated for 30 seconds, with an equal concentration of manganese in normal Ringer. The procedure was employed in all the contracture experiments. Longer pretreatments were not employed because of the complicated responses observed after long periods in manganese (as described earlier). Pretreatment periods of 30 seconds in manganese solutions were sufficient to produce maximal inhibition of subsequent contractures. Almost complete equilibration of manganese in the extracellular space was possible with this pretreatment time as the half-time of solution exchange in these preparations has been estimated as approximately 3 seconds (Chapman and Tunstall, 1971).

There was quite a large inhibition of the potassium-rich contracture tension in the range 0 to 4 mM manganese but little further inhibition in the range 4 to 16 mM (Figure 10). The small decrease in tension between 4 and 16 mM manganese could be partly due to hyperpolarisation of the cell membrane caused by manganese combating the extent of the potassium depolarisation (Table 2 gives values of the membrane potential measured in normal Ringer and in potassium-rich solutions with and without 8 mM manganese. Manganese produced a significant hyperpolarisation in potassium-rich solutions although the hyperpolarisation was smaller than in normal Ringer). In order to avoid reducing the sodium concentration and thus producing contracture potentiations (Luttgau and Niedergerke, 1958;

TABLE 2

Solutions	Resting Potential mV	Standard error of mean mV	Number of cells	Significance of hyper-polarisation P
3K 1Ca	82.6	0.6	51	< 0.001
8Mn 3K 1Ca	88.1	0.9	35	
15K 1Ca	59.2	0.6	44	< 0.001
8Mn15K 1Ca	63.0	0.7	43	
100K 1Ca	20.3	0.5	40	<0.02
8Mn 100K 1Ca	22.0	0.5	41	

Effect of manganese on the membrane potential in four ventricle strips in normal and potassium-rich Ringer. Potassium-rich solutions were hypertonic. 85 mM Tris chloride was added to solutions containing 15 mM potassium to equate the tonicities. Concentrations are expressed as mM/litre.

Chapman and Tunstall,1971) manganese was added without osmotic balancing i.e. manganese solutions were slightly hypertonic. High tonicity produces a small decrease of the potassium-rich contracture tension (Chapman and Tunstall, 1971) but the slight hypertonicity of the manganese solutions is unlikely to have contributed greatly to the decreased tension in the range 4-16 mM manganese.

Low concentrations of manganese have a large effect on the twitch contraction and on contractures induced by low-sodium solutions (described later). Both of these latter types of contraction appear to be highly dependent upon the influx of extracellular calcium into the cells. The experiments with potassium-rich solutions could indicate that some of the calcium inducing these contractures is released from calcium stores within the muscle by depolarisation as approximately 4 mM manganese could be sufficient to inhibit much of the transmembrane calcium influx. Alternatively the potassium-rich solution could increase the permeability of the membrane to calcium thereby producing contracture development even in the presence of high concentrations of manganese. The effect of manganese over a range of potassium concentrations (and thus depolarisation levels) was studied to investigate this further.

(3) Effect of manganese on the tension-depolarisation curve.

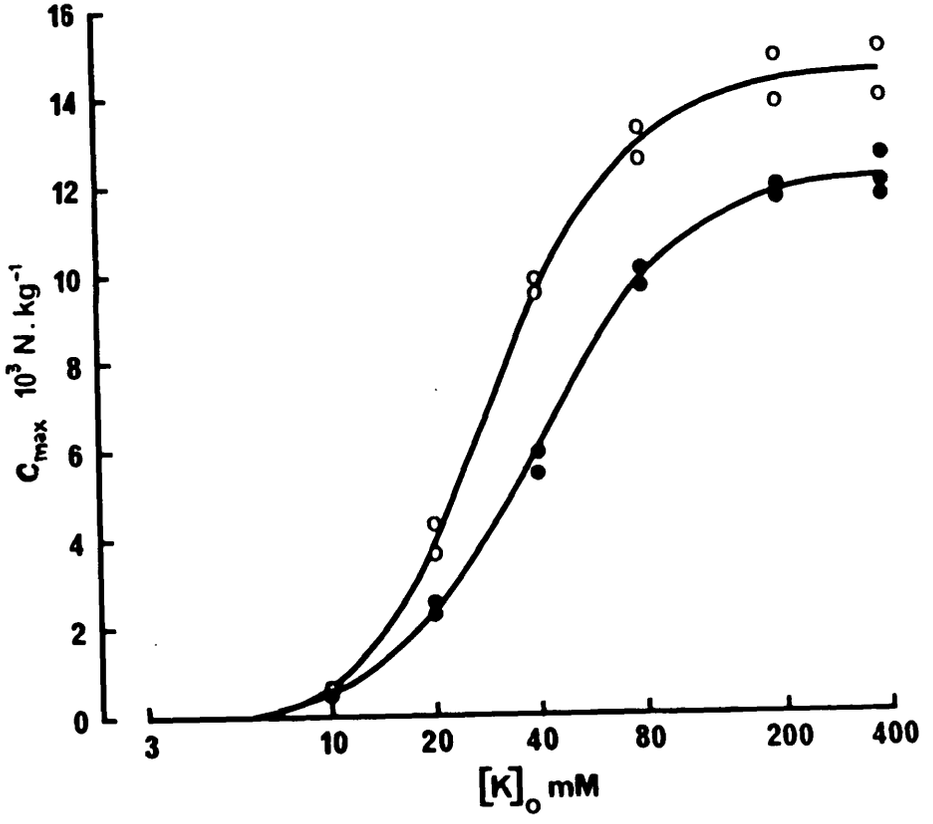
Figure 11 illustrates an experiment on the effect of manganese on the contractures produced by various potassium concentrations. Closed circles indicate contracture tensions in the presence of 8 mM manganese, open circles tension in the absence of manganese. In this experiment the sodium

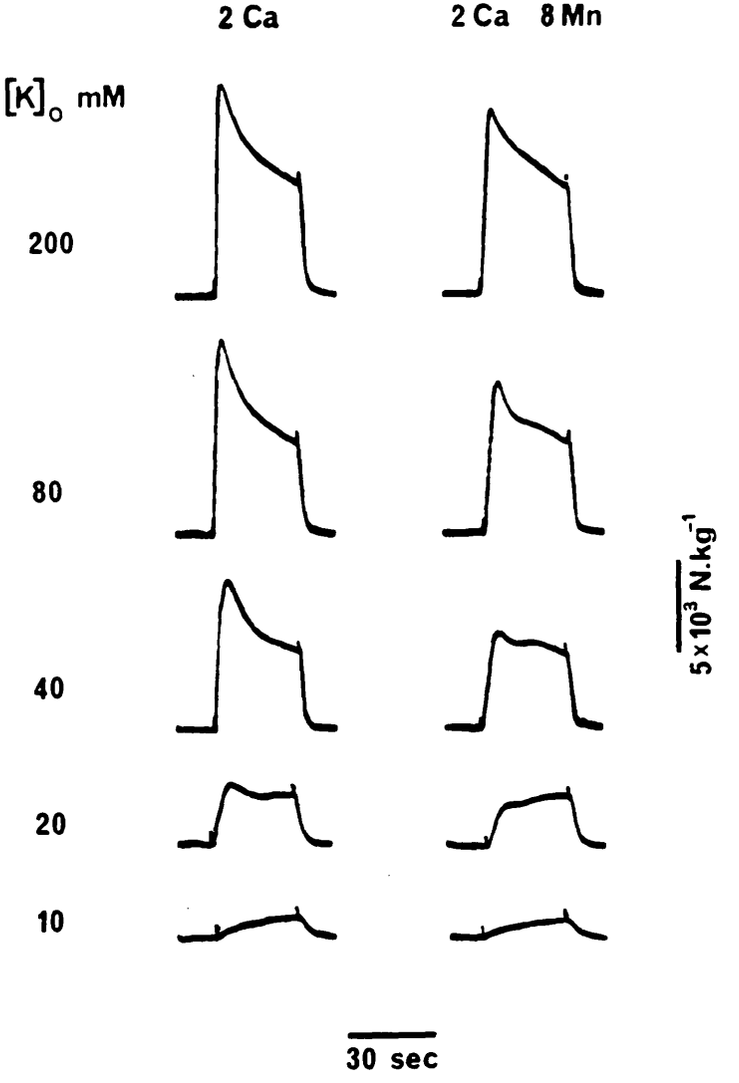
Figure 11.

Contractures produced by depolarisation with potassium-rich solutions.

Left: Maximum contracture tensions produced by depolarisati with various potassium-rich solutions in the presence (close circles) and absence (open circles) of 8 mM manganese. The contracture solutions were produced by mixing solutions A and I (Table 1) so that the muscle was exposed toequi-hypertonic (by approximately 280 m.osmoles) contracture solutions. The 400 mM potassium solutions were produced by adding solid potassium chloride to solution H and were therefore not equihypertonic to the other solutions.

Right: Contracture recordings from the experiment illustrat on the left. Manganese was applied for 30 seconds prior t those contracture solutions that contained manganese. The calcium concentration was maintained at 2 mM throughout the experiment.


16
14
12
10
8
6
4
2
0
C_{max} 10^3 N.kg^{-1}
3
10
20
40
80
200
400
[K]$_o$ mM


2 Ca
2 Ca 8 Mn
[K]$_o$ mM
200
80
40
20
10
5x10^3 N.kg^{-1}
30 sec

concentration had been reduced by 50% to permit the use of higher potassium concentrations without excessive increase in tonicity. Luttgau and Niedergerke (1958) found that the membrane potential in 50% sodium solutions did not differ significantly from that in 100% sodium. Both changed by approximately 11 to 12 mV for a doubling of the potassium concentration over the range 10 to 100 mM. The extent of the depolarisation at the highest potassium concentration in Figure 11 is difficult to estimate. These points are therefore included only as a qualitative comparison.

The presence of manganese in the bathing fluid did not appear to shift the threshold for contracture development to higher depolarisation levels as it does in skeletal muscle (Stefani and Chiarandini,1973). However, as pointed out by Lindly et al (1973), measurements of thresholds in multi-fibre preparations are complicated by the presumed Normal distribution of the thresholds in the population of fibres so that there may be no distinct threshold for potassium-rich contractures either in the presence or absence of manganese. Therefore any change of threshold will be difficult to discern unless the change is very large.

In skeletal muscle manganese shifts the tension-depolarisation curve to higher depolarisation levels (Edwards and Lorkovic,1967; Chiarandini and Stefani,1973). In frog heart muscle however there does not appear to be a simple shift. The contracture inhibition by manganese was not overcome even with the largest depolarisations. In other experiments the tension-depolarisation curve was shifted to more negative levels by the use of higher calcium or lower sodium concentrations (Niedergerke,1956) so that a lower depolarisation would be required to produce a given

tension. However the inhibition by manganese could still not be overcome by large depolarisations. This presumably reflects the extent to which the potassium-rich contracture is dependent upon external calcium in skeletal compared with cardiac muscle. In skeletal muscle the apparent inhibition of the potassium-rich contracture merely indicates that an increased depolarisation is required to produce a given tension. In frog heart muscle a significant portion of the potassium-rich contracture appears to depend upon calcium influx. The inhibition of this influx by manganese cannot be overcome by increased depolarisation levels.

Further evidence that the potassium-rich contracture in cardiac muscle has a greater dependence on external calcium than skeletal muscle comes from the fact that increased calcium causes a simple shift of the tension-depolarisation curve to more positive potentials in skeletal muscle (Luttgau,1963; Constantin, 1968) similar to the manganese effect. However in frog heart increased calcium potentiates the potassium-rich contracture and shifts the tension-depolarisation curve to more negative potentials (Niedergerke,1956).

A more accurate representation of the tension produced at the various depolarisation levels (Figure 11) can be made by applying a small correction which compensates for the hyperpolarising effect of the manganese. This correction is too small to greatly affect the curve at high potassium concentrations but a crossover of the curves at very low potassium concentrations may occur (observed in 5 of 6 experiments).

These results have shown that the tension induced by depolarisation with potassium-rich solutions is not very sensitive to manganese inhibition. However, tension is proportional to the ratio $[Ca]/[Na]^2$ in frog heart (Willbrandt and Koller, 1958). This could mean that the inhibition caused by sodium is so large as to hide the effects of manganese especially if the affinities for manganese and sodium are similar because the latter is present in such higher concentrations. Therefore the effects of manganese on the contractile response in solutions with a low sodium content has been investigated.

(4) The low-sodium contracture and sodium substitutes

Reduction of the sodium concentration in the solution bathing frog heart muscle results in the development of a large contracture (Luttgau and Niedergerke, 1958; Chapman, 1974). This occurs in the absence of membrane depolarisation. An initial investigation was carried out to try to obtain the most appropriate sodium replacing ion. Figure 12 illustrates contractures induced by the total replacement of sodium by Tris, lithium or sucrose. Tension was produced rapidly in each case and was followed by a complete spontaneous relaxation of tension within approximately 3 minutes. The peak tensions produced by sodium removal were similar but the rate of rise of contracture tension was normally slightly slower in lithium.

Figure 13 shows an experiment which compared responses in lithium (squares) and in Tris (circles) at various sodium concentrations. The contracture solutions were produced by

Figure 12

Contractures produced by the removal of sodium from the normal Ringer. Sodium was replaced by Tris, lithium or sucrose (solutions B,C and D respectively, Table 1) The calcium concentration was 1 mM throughout.

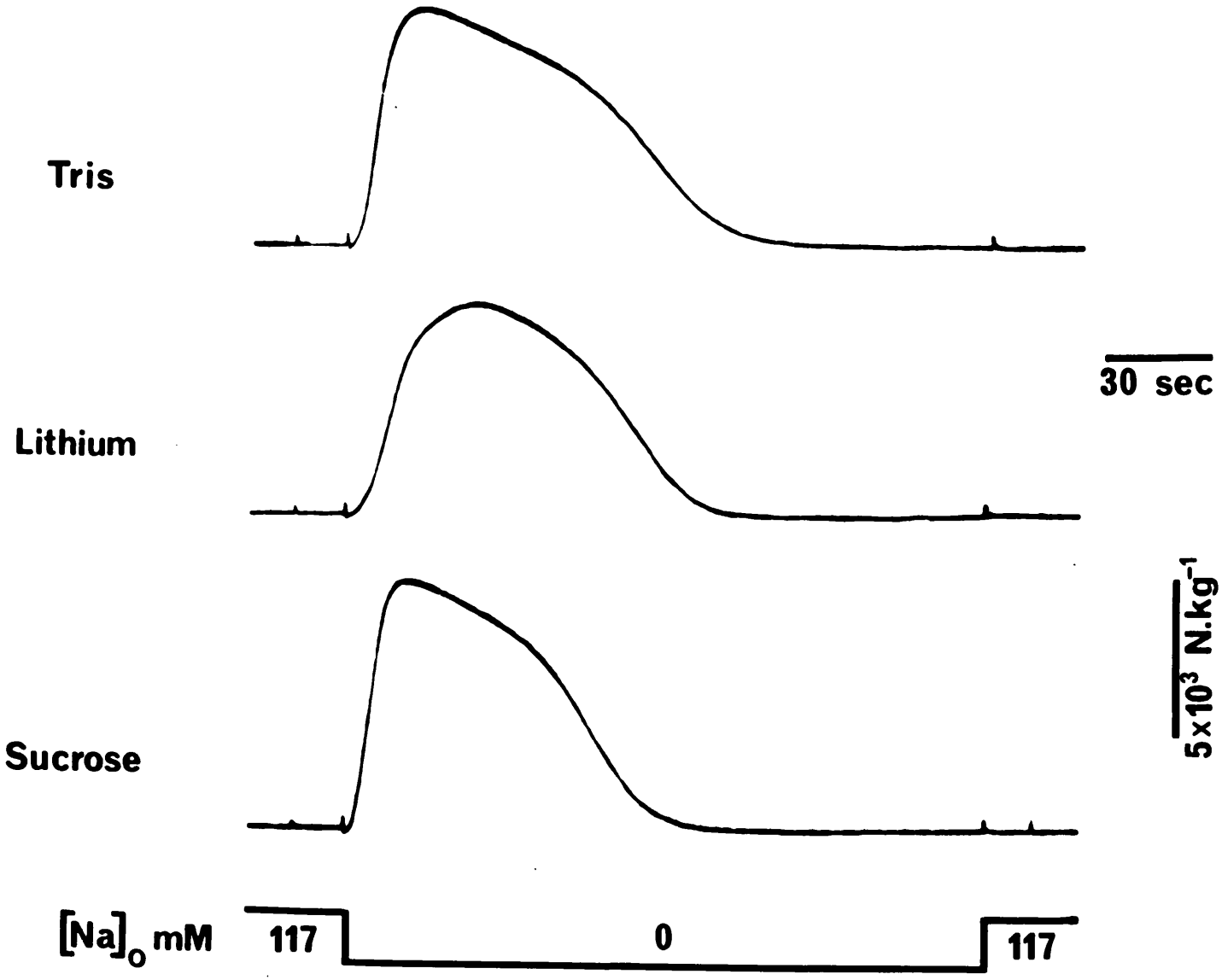

Tris
Lithium
Sucrose
30 sec
5x10³ N.kg⁻¹
[Na]o mM
117
0
117

Figure 13.

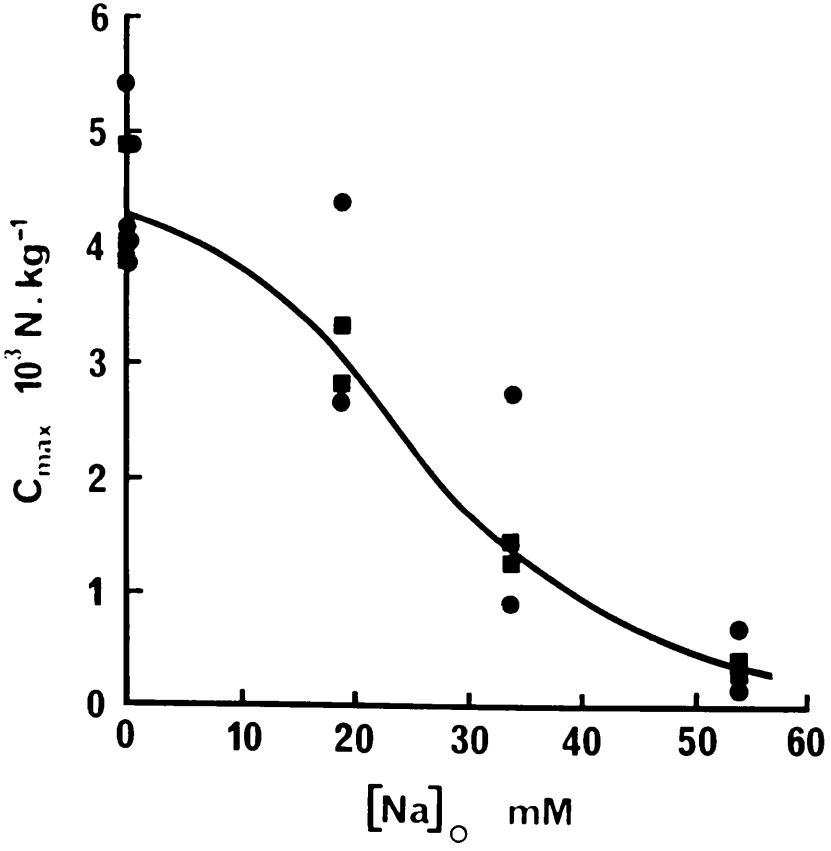
Comparison of the substitution of lithium and Tris for sodium.

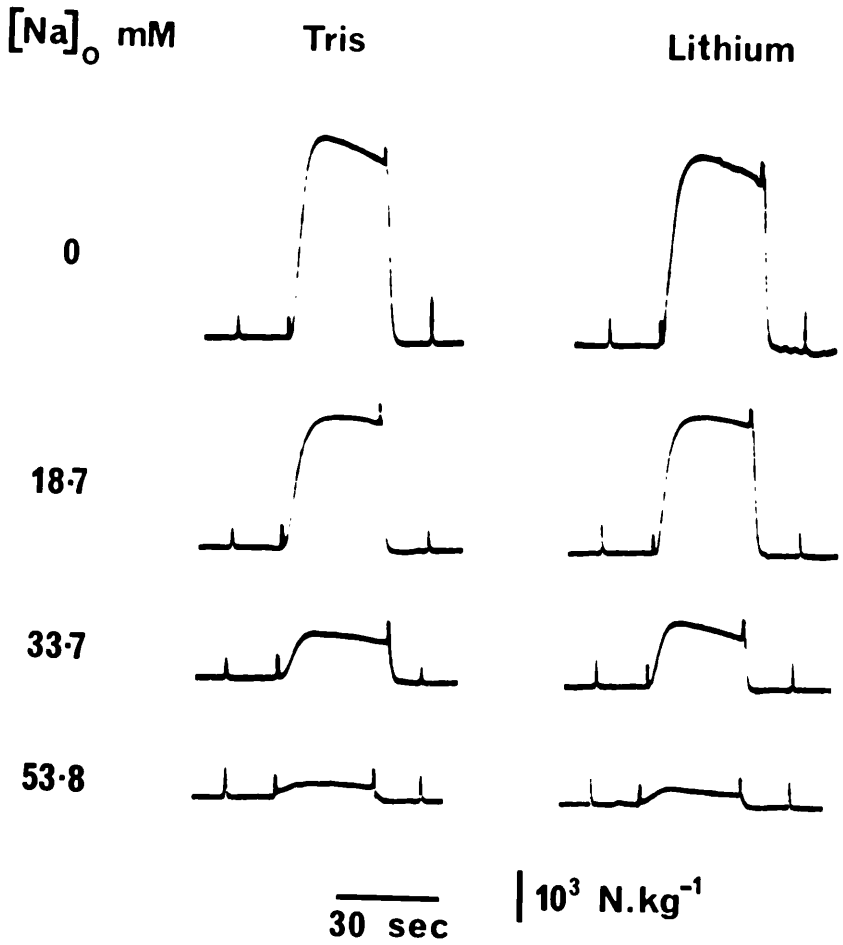
Left: Maximum contracture tensions produced by low-sodium solutions with Tris (circles) and lithium (squares) substitution.

Right: Contracture recordings. The contractures were not allowed to relax spontaneously, the muscle being returned to normal Ringer after 30 seconds in the low-sodium solutio The reintroduction of sodium produced a rapid return to the resting tension.

In the normal Ringer the muscle was electrically stimul (rate 4 min^{-1}) and the resultant twitch contractions can be seen on the tension records.

Calcium concentration was 1 mM.


6
5
4
3
2
1
0
0
10
20
30
40
50
60
C_{max} 10^{-3} N.kg^{-1}
$[Na]_o$ mM


$[Na]_o$ mM
Tris
Lithium
0
18·7
33·7
53·8
30 sec
10^3 N.kg^{-1}

mixing solutions A and C, and A and B respectively (Table 1) for the two sodium replacing cations. The rather large spread of tensions was due to a slow decrease in contracture tension throughout the course of this experiment. A "mirror image" contracture sequence (see Methods) was employed, the responses to lithium substituted low-sodium solutions being sandwiched between those produced by Tris substitution of sodium. These experiments show that there is very little or no difference between the responses of the muscles when either of these two cations are used to replace sodium.

Hydrazine when used by Hille (1971,1972) was found to be able to substitute for sodium as a current carrier through the sodium channel in the Node of Ranvier of myelinated nerve. Figure 14 shows a comparison between the contracture responses obtained by Tris and by hydrazine replacement of sodium. (Hydrazine chloride was produced by acidification of hydrazine hydrate with hydrochloric acid). Hydrazine replacement of sodium produced contracture tensions 10 to 30% greater than those produced by Tris, lithium or sucrose replacement. Spontaneous relaxation was slowed in hydrazine solutions. Figure 15 illustrates an experiment comparing the effects of hydrazine (triangles) with Tris (circles) replacement of sodium. The responses are clearly potentiated with hydrazine substitution. This potentiation of the zero-sodium contracture by hydrazine is possibly related to the potentiation of the twitch response by hydrazine in normal Ringer (Matoba, 1973). In the present experiments a small contracture could be produced simply by the addition of 10 - 20 mM hydrazine to normal Ringer.

Figure 14.

Contractures produced by the total replacement of sodium by Tris (top) and hydrazine (bottom), solutions B and E respectively in Table 1. 1 mM calcium.

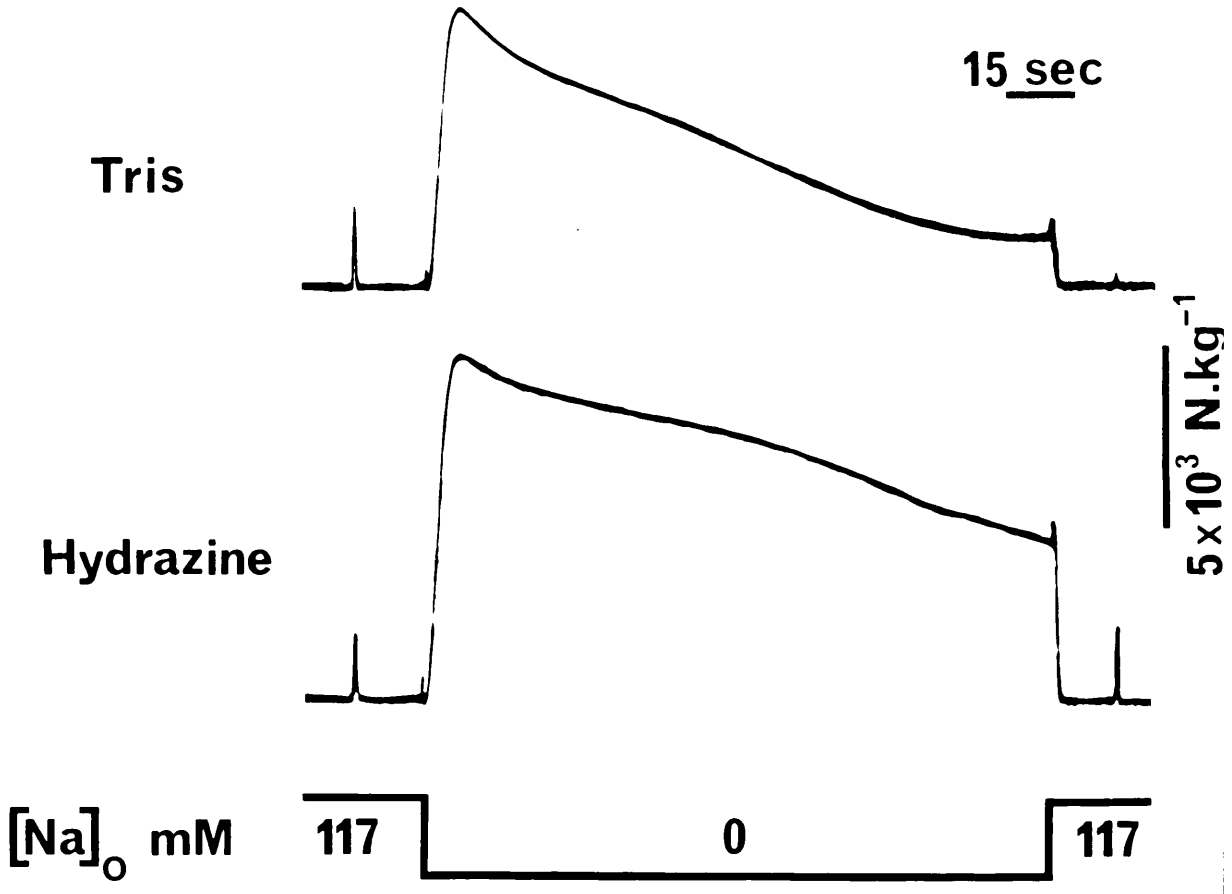
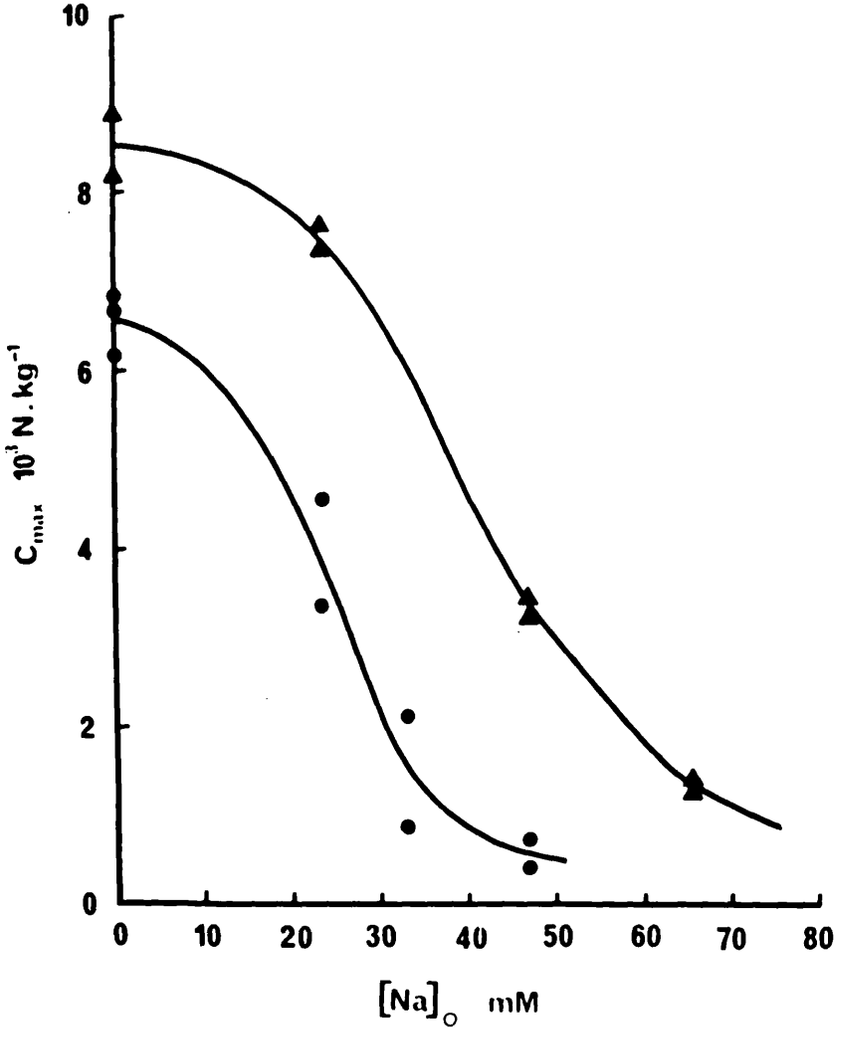

15 sec
Tris
Hydrazine
5x10³ N.kg⁻¹
[Na]o mM
117
0
117

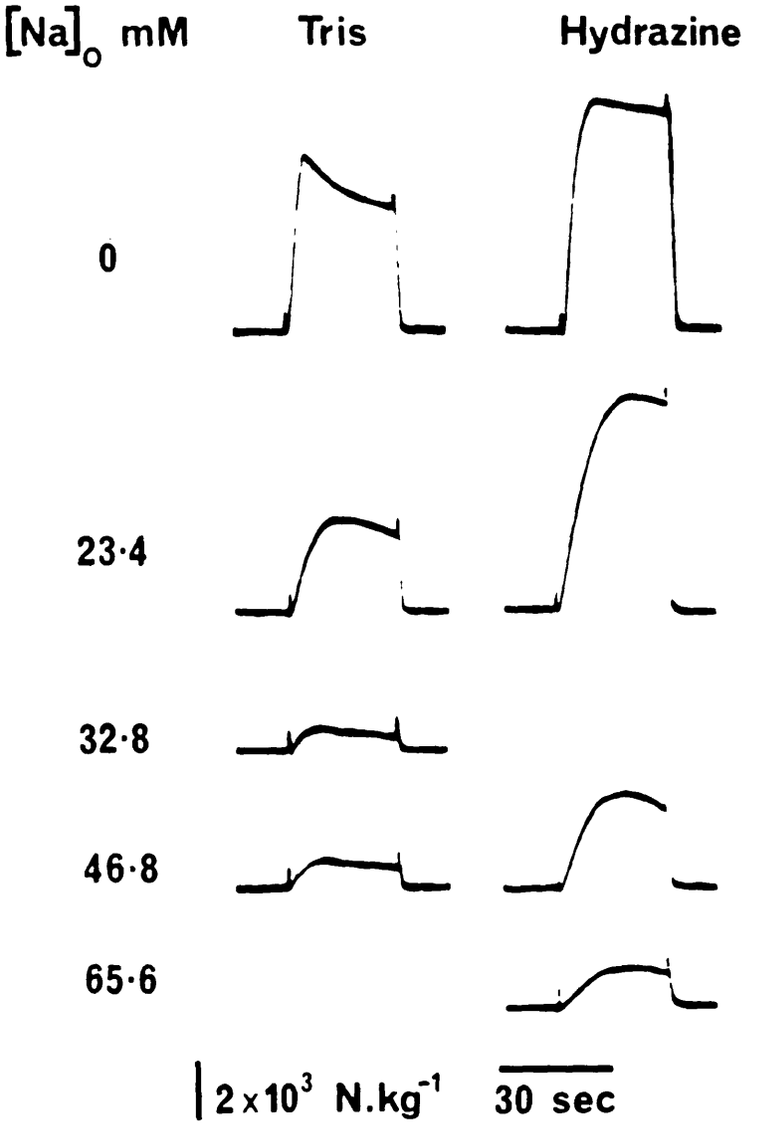
Figure 15.

Comparison of contractures produced by substitution of Tris and hydrazine for sodium.

Left:- maximum contracture tensions produced by low-sodium solutions with Tris (circles) and hydrazine (triangles) substitution.

Right:- Contracture recordings. Solutions were produced by mixing A and B, and A and E (Table 1) for Tris and hydrazine substitution respectively. 1 mM calcium.


10
8
6
4
2
0
0
10
20
30
40
50
60
70
80
C_{max} 10^{-3} N.kg^{-1}
$[Na]_o$ mM


$[Na]_o$ mM
Tris
Hydrazine
0
23·4
32·8
46·8
65·6
2 x 10^3 N.kg^{-1}
30 sec

Replacement of sodium by magnesium produces much smaller contractures than any of the substitutes described above (less than 20% of the contracture tension produced with the Tris substitution for sodium), indicating that magnesium has a weak inhibitory effect on the response to reduced sodium concentrations.

In order to study the antagonism between calcium and manganese it is necessary to find a sodium substitute that shows little or no calcium antagonism. All of the sodium substitutes tested have some drawbacks: sucrose is non-ionic and thus membrane potential changes are likely due to the low ionic strength of the Ringer; Tris appears to be able to penetrate the cell membrane in its unionised form and could thus participate in intracellular reactions in a similar manner to other primary amines (Mahler, 1961); lithium accumulates intracellularly and is apparently not removed by the sodium pump (Keynes and Swan, 1959), lithium also has some sodium-like effects (described later); choline can only be used in heart muscle in the presence of atropine, and has to be well purified prior to use or toxic effects are apparent; hydrazine has clear potentiating effects under most conditions some of which are not readily reversible.

Tris-chloride was finally chosen as the best sodium replacer because it had the fewest drawbacks as a sodium substitute. In experiments with Tris the heart was able to sustain repeatable responses over long periods (up to 10 hours). Tris had the added advantage that it could be used as a buffer in all the solutions employed without precipitation or complexation of the divalent cations used. This was the

sodium substitute used in most of the experiments to be described but the responses were normally checked to be reproducable in at least one other of the above substitutes.

(5) Inhibition of the sodium-free contracture by manganese.

Figure 16 (top trace) shows the normal contracture produced by removal of sodium. The middle three traces illustrate that the addition of 8 mM manganese (between the arrows) produced a rapid decrease in contracture tension. Removal of manganese permitted a return of tension almost to the level prior to manganese addition. This was followed by the normal spontaneous relaxation. The bottom trace shows that pretreatment with manganese for 30 seconds before the removal of sodium (also in manganese) almost completely blocks the contracture response.

Figure 17 shows the effect of various manganese concentrations on the sodium-free contracture. The tension recordings (right) show that the contracture response in manganese becomes biphasic. Low manganese concentrations slow the rate of rise of the contracture, increase the time to peak contracture tension and decrease that tension. Higher manganese concentrations cause the contracture to split into two distinct components, an initial phasic response and a later tonic tension. The contracture responses are completely blocked in high manganese concentrations.

There was a wide variation in the sensitivity of the low-sodium contracture to manganese inhibition e.g. 2 mM manganese produced 69$\pm$ 26% (S.D.) inhibition. Some of this variation appeared to be due to differences in the batches of frogs

Figure 16.

Inhibition of the tension induced by perfusion with low sodium solutions by the addition of manganese at various times during the contractures. Top recording:- Normal sodium-free contracture in the absence of manganese. Middle three recordings:- 8 mM manganese was added for various periods of time (between the arrows) Bottom recording:- 8 mM manganese was applied for 30 seconds prior to the sodium-free solution (also containing manganese). Tris was substituted for sodium; the calcium concentration was 1 mM throughout. 20°C.

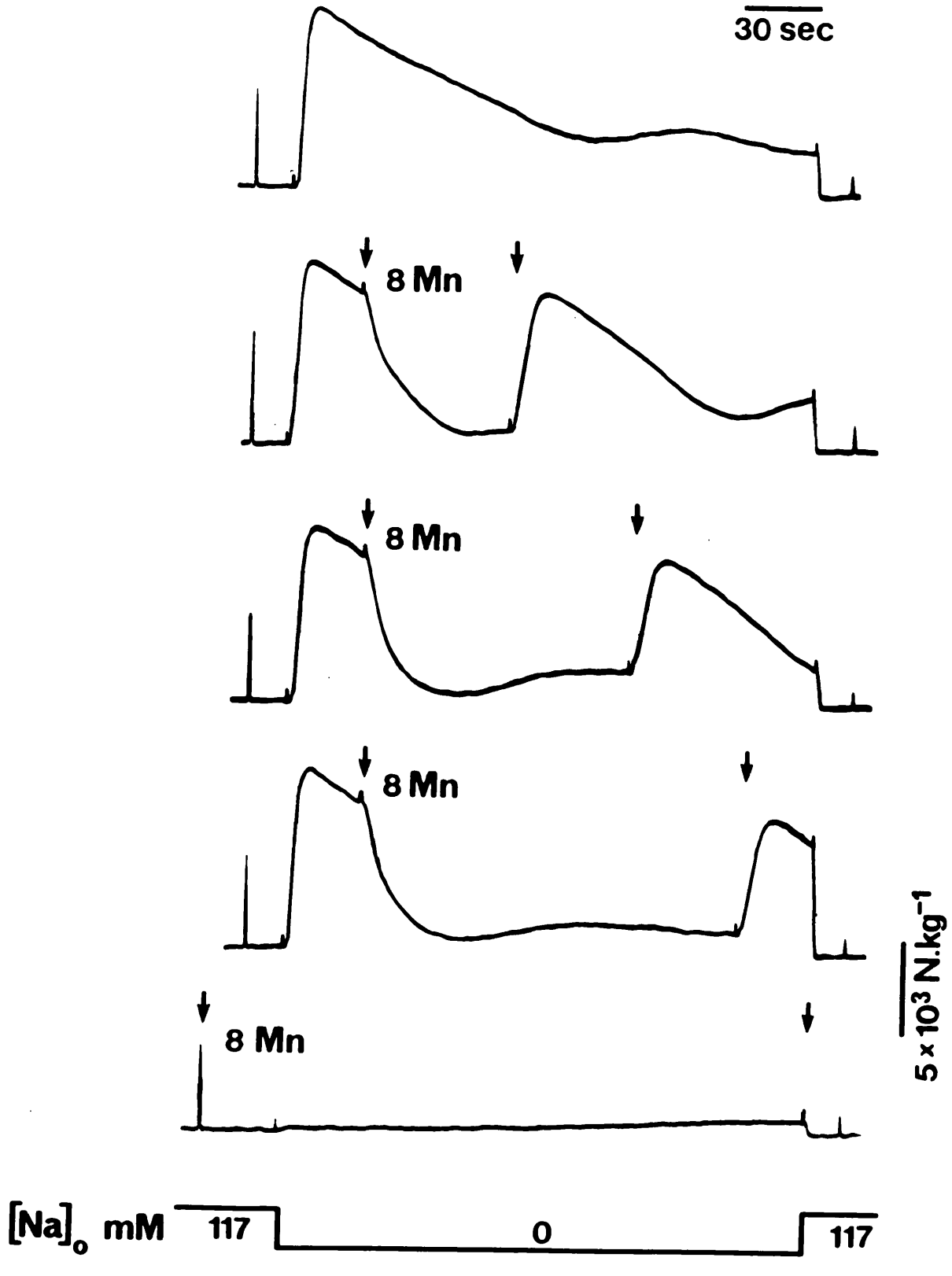
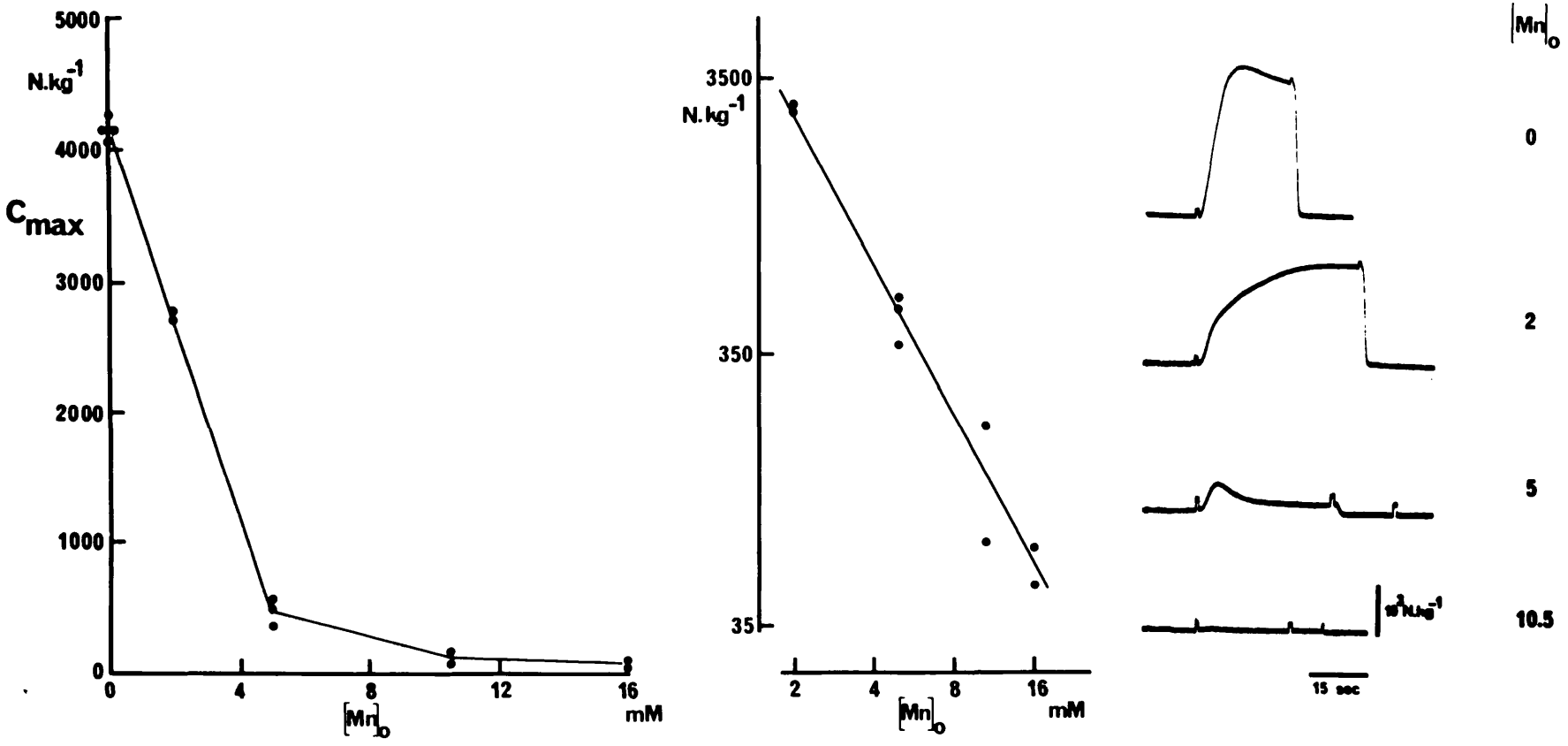

30 sec
8 Mn
8 Mn
8 Mn
8 Mn
5 x 10³ N.kg⁻¹
[Na]o mM
117
0
117

Figure 17.

Dose-response curve for manganese inhibition of the sodium-free contracture. Maximum sodium-free contracture tension (Tris substitution) in the presence of various concentrations of manganese is expressed on linear coordinates (left) and log-log coordinates (middle). Slope of the log-log plot was 1.87, coefficient of correlation 0.8820. 1 mM calcium throughout.


5000
N.kg-1
4000
Cmax
3000
2000
1000
0
0
4
8
12
16
[Mn]o
mM
3500
N.kg-1
350
35
2
4
8
16
[Mn]o
mM
[Mn]o
0
2
5
10.5
15 sec

used. The experiments were performed on both summer and winter frogs and these have significantly different internal ionic contents, particularly for sodium (Novotny and Bianchi 1973) and calcium (de Boer, 1918). Another possible source of the variability was that although the frogs were obtained from the same supplier they originated from different populations, tropical and sub-tropical.

There is a steep relationship between the concentration of manganese and the inhibition of the low-sodium contracture. When plotted on log-log coordinates (as in Figure 17) a straight line relationship resulted and in ten experiments had a mean slope of - 1.97$\pm$ 0.32 S.D. Three experiments with nickel had a mean slope of - 2,07. The corresponding value for cobalt was -1.80 (two experiments). In most respects the inhibition produced by cobalt and nickel appeared similar to that of manganese. However nickel and cobalt tended to produce slightly greater inhibition of the initial phasic response of the contracture and less of the tonic tension (Figure 18).

Cadmium produced a similar inhibition of the low-sodium contracture but was approximately ten times more effective at producing inhibition than manganese.

(6) <u>The membrane potential in sodium-free and manganese solutions</u>.

The inhibition of the low-sodium contracture by manganese is unlikely to have resulted from an effect on the membrane potential. Table 3 gives the results of resting potential

Figure 18.

Sodium-free contractures induced in the presence of 4 and 12 mM manganese (left) or nickel (right). The bar over the contracture recordings gives the duration of exposure to manganese or nickel solutions. Note the greater inhibition of the twitch contraction by nickel than by manganese but for the sodium-free contracture response there is a larger inhibition by manganese than by nickel of the tonic tension of the contracture and vice versa for the phasic contraction. The calibration bar (right) gives the size of the uninhibited sodium-free contracture. Calcium concentration was 1 mM.

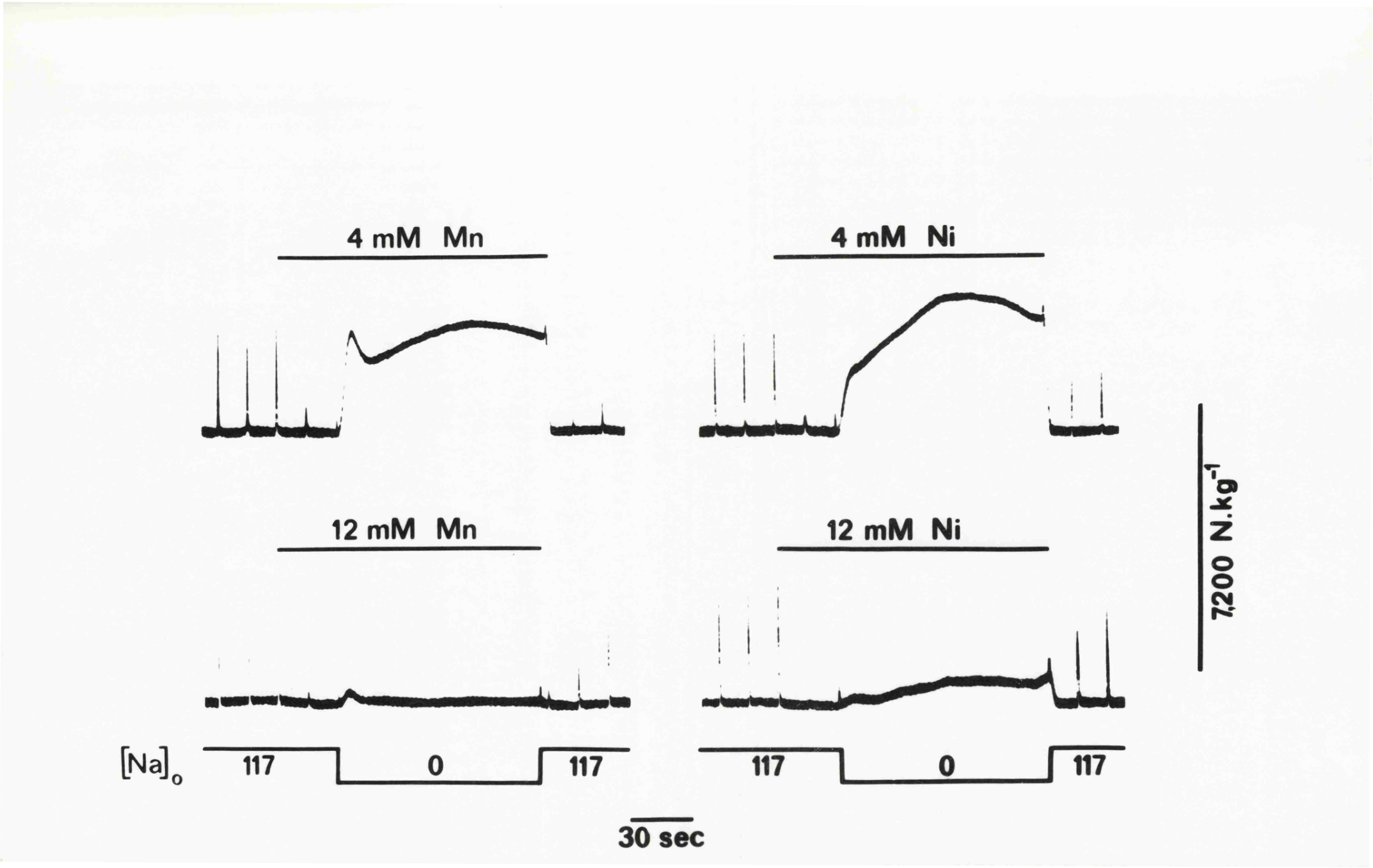

4 mM Mn
4 mM Ni
12 mM Mn
12 mM Ni
7,200 N.kg-1
[Na]o
117
0
117
117
0
117
30 sec

TABLE 3

	Normal Ringer	Sodium-free Ringer	Sodium-free Ringer 8mM manganese	8 mM manganese Ringer
Resting potential (mV)	82.2	86.6	89.8	88.6
Standard Error(mV)	0.6	0.8	1.2	0.7
Number of Cells	47	33	33	33

Students t-test for significant difference between

Sodium-free and Normal Ringer	$P < .001$
Sodium-free, 8 mM manganese & Normal Ringer	$P < .001$
Sodium-free, 8 mM manganese & Sodium-free Ringer	$P < .05$
8 mM manganese Ringer & Normal Ringer	$P < .001$

The results given are the mean membrane potential (mV) as measured from 4 ventricle strips. Concentration of calcium was 1 mM in all solutions. Sodium was substituted by Tris in the sodium-free solutions.

measurements in four ventricle strips. The sodium-free solutions produced an increase of resting potential (see also Chapman,1974). Manganese produced a slight further hyperpolarisation. However, such changes should have little inhibitory effect on the contracture response because similar hyperpolarisation by low potassium solutions (see Figure 26) permit large low-sodium contractures.

(7) The dependence of the low-sodium contracture on the calcium and manganese concentrations.

The manganese inhibition of the sodium-free contracture was further investigated by changing the calcium concentration at various manganese concentrations as is illustrated in Figure 19 (open circles in 5 mM manganese, closed circles in 2 mM manganese). The experiments were performed over the lower tension range of the muscles (normally less than 15% of the maximum contracture tension) where any cooperativity in the calcium transport system can be studied (Chapman,1971). The mean slope of log-log plots of nine similar experiments in which the points fitted a straight line with a coefficient of correlation of greater than 0.90 was 2.10 ± 0.21 S.E.

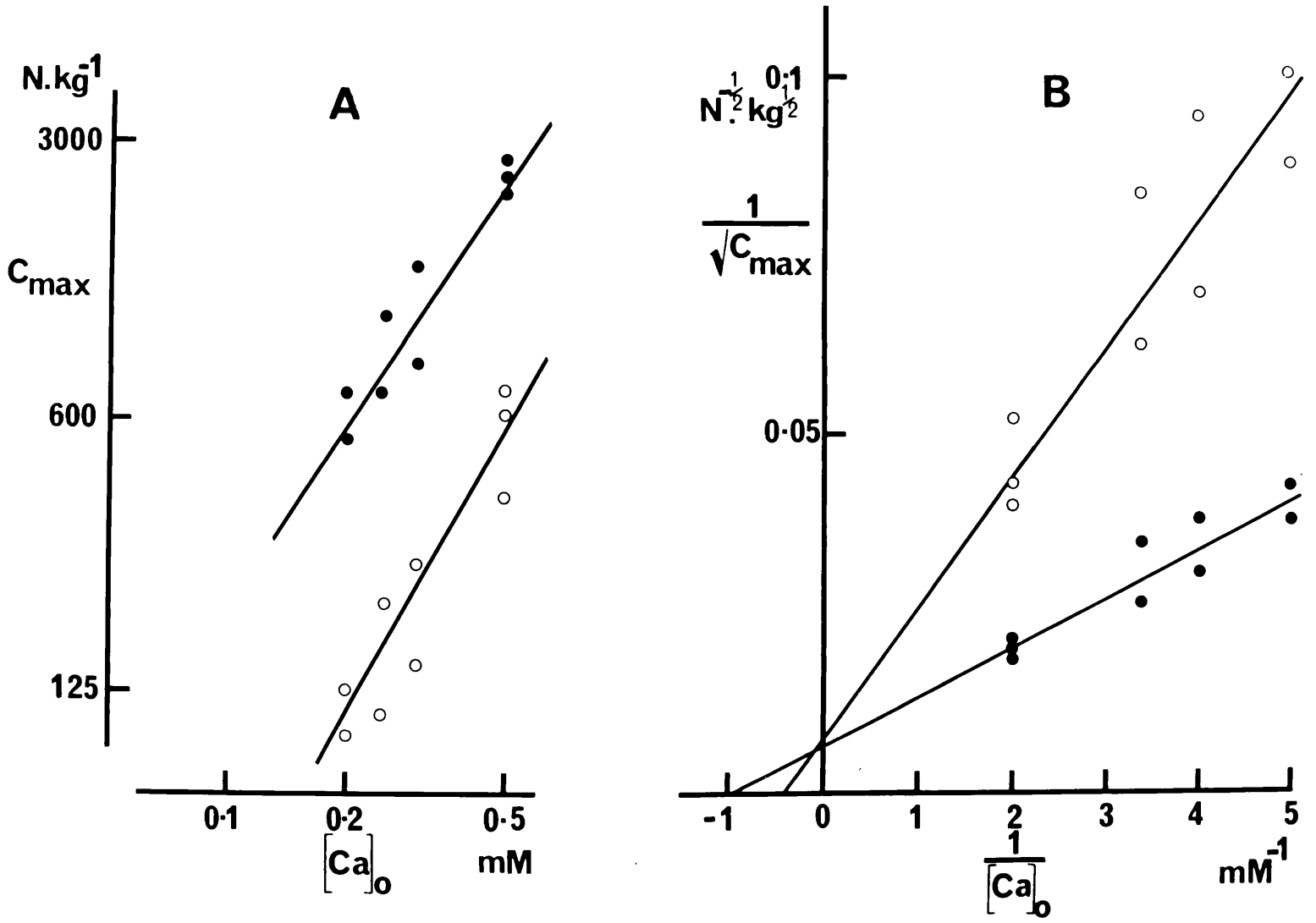
It can be seen from Figure 19 that an x-fold increase in manganese concentration is approximately equivalent to an x-fold reduction in calcium concentration. Thus the ratio $[Ca]_o/[Mn]_o$ for equal tension production was measured at various tension levels. The mean ratio was found to be 0.98 ± 0.08 S.E. (13 experiments). The proximity of this ratio to one indicates that under the conditions of these experiments tension is proportional to $[Ca]_o/[Mn]_o$.

Figure 19.

Maximum sodium-free contracture tensions produced by various concentrations of calcium in the present of 2 mM (filled circles) and 3 mM (open circles) manganese.

A. A logarithmic plot of the maximum contracture tensions in various calcium concentrations. The straight lines have been drawn according to equation 7 with $n = 2$, $\alpha = 1$, $K_i = 2.0$ and $\omega = 141$.

B. A double-reciprocal plot of the same results as in A. The continuous lines are regression lines.


N.kg⁻¹
A
3000
C_{max}
600
125
0·1
0·2
0·5
$[Ca]_o$
mM
$N^{-\frac{1}{2}}kg^{\frac{1}{2}}$
0·1
B
$\frac{1}{\sqrt{C_{max}}}$
0·05
-1
0
1
2
3
4
5
$\frac{1}{[Ca]_o}$
mM^{-1}

A reasonable explanation for the inhibition of contracture tension by manganese would be a competitive binding by manganese at a site normally occupied by calcium. The normal methods of testing for competitive inhibition in enzymic reactions have been applied in these experiments. Expression of the results in a manner similar to the Lineweaver-Burk plot suggests that the inhibition by manganese is of a competitive nature (Figure 19, right). Here the reciprocal of the square root of contracture tension (of the results on the left of the figure) have been plotted against the reciprocal of the calcium concentration. The square root function has been used because of the power relationship between tension and calcium concentration.

The lines drawn through the points in 2 and 5 mM manganese have a common intercept with the y - axis. This suggests a competitive antagonism between calcium and manganese at a site which can lead to tension production.

The power relationship observed between tension and the calcium and manganese concentrations indicates that tension is proportional to $[Ca]_o^2$ and $\sqrt{[Mn]_o}$ (since the slope of log tension versus the log of the calcium concentration was approximately + 2 and log tension versus the log of the manganese concentration was approximately -2). This is in accord with the results of Chapman (1974) where by alteration of the sodium concentration the strength of the contracture produced was found to vary by the $[Ca]_o^2$ and the $\sqrt[4]{[Na]_o}$.

The antagonism between calcium and sodium in frog heart has been analysed (Luttgau and Niedergerke,1958; Chapman,1974)

by assuming that both cations bind to an anionic receptor site according to the equation,

$$Ca + Na_2 R \rightleftharpoons Ca R + 2 Na \qquad (1)$$

A similar antagonism between calcium and magnesium has been studied at the frog neuromuscular junction (Jenkinson, 1957; Dodge and Rahimimoff, 1967). In this case the calcium and magnesium were assumed to bind to a receptor site X such that,

$$Ca + X \rightleftharpoons Ca X \qquad (2A)$$
$$Mg + X \rightleftharpoons Mg X \qquad (2B)$$

Although the models are very similar, they differ in that the receptor site or molecule is assumed to be able to exist free as X in the latter case. In the model presented for frog heart the receptor R can only exist in combination with calcium or sodium (or manganese or any other inhibitor). The predictions of both models have been assessed in order to estimate their applicability to the responses observed in the present experiments in frog heart muscle.

In the second model, if the normal nonmenclature for enzyme inhibition is applied, and X_o is the total concentration of enzyme (receptor) bound and unbound and IX is the concentration of enzyme bound to the inhibitor (e.g. manganese), then from equations similar to 2A and 2B,

$$[Ca]([X_o] - [CaX] - [IX]) = Km\,[CaX] \qquad (3A)$$
$$[I]([X_o] - [CaX] - [IX]) = Ki\,[IX] \qquad (3B)$$

where Km is the

dissociation constant of CaX (equation 2A) and Ki the dissociation constant (inhibitor constant) of IX (equation 2B). Thus assuming the total number of receptor sites (X_o) is finite.

$$[Ca\ X] = \frac{\omega\ [Ca]}{[Ca] + Km\left(1 + \frac{[I]}{Ki}\right)} \qquad (4)$$

where ω is a constant. Tension is assumed to be proportional to $[CaX]^n$ i.e. n is the number of CaX molecules required to generate tension at the unit level.

Equation 4 is the usual equation of simple competitive inhibition in an enzyme process where $[Ca]$ corresponds to the substrate concentration and $[CaX]$ corresponds to the rate at which the reaction proceeds (tension production in this case).

Thus in a Lineweaver-Burk plot of the type illustrated in Figure 19 the intercept with the x-axis is equivalent to $\frac{1}{Km\left(1 + \frac{[I]}{Ki}\right)}$ i.e. the reciprocal of the apparent Km.

If, $\beta = Km\left(1 + \frac{[I]}{Ki}\right)$

then $$Ki = \frac{Km\ [I]}{\beta - Km}$$

so that at two inhibitor concentrations $[I_1]$ and $[I_2]$ there will be two lines intercepting the x-axis at $\frac{1}{\beta_1}$ and $\frac{1}{\beta_2}$

Thus $Ki = \frac{Km\ [I_1]}{\beta_1 - Km}$ and $Ki = \frac{Km\ [I_2]}{\beta_2 - Km}$

$$\therefore \quad \frac{Km\ [I_1]}{\beta_1 - Km} = \frac{Km\ [I_2]}{\beta_2 - Km}$$

which gives, $$Km = \frac{[I_1]\beta_2 - [I_2]\beta_1}{[I_1] - [I_2]} \qquad (6)$$

From experiments where manganese can be considered to be the inhibitor (I) the Km has been calculated by substitution of values of I and β into equation 6. The mean value of the Km was -0.07 ± 0.36 S.E. (6 experiments, where all points fitted straight lines with a coefficient of correlation greater than 0.90). A negative Km is of course not feasible thus we would be forced to conclude that Km is very close to zero. From the values of the Km so obtained the Ki can be calculated from equation 5. The mean value was found to be 0.66 ± 0.59 S.E. ($n = 6$).

The low value of the Km (i.e. close to zero) suggests a tight binding of calcium to the receptor. The binding of manganese (as indicated by the Ki value) appears somewhat less strong.

As these results, indicate tight binding of the cations to the receptor they could justify the use of the first model (equation 1) adopted for frog heart i.e. that the receptor can only exist in combination with a cation. The cations can thus be envisaged as undergoing exchange reactions with

the receptor in a manner similar to the processes that occur in the operation of an ion-exchange resin.

From equation 1 Chapman and Tunstall, (1971) derived the equation,

$$[CaR] = \frac{\omega [Ca] \quad K_{Ca}}{[Na]^2 + [Ca] \; K_{Ca}} \qquad (6A)$$

where ω is a constant and K_{Ca} is the equilibrium constant of the reaction. They found that the contracture responses could be fitted by the equation,

$$C_{max} = \propto \left(\frac{\omega \; [Ca]_o \; K_{Ca}}{[Na]_o^2 + [Ca]_o \; K_{Ca}} \right)^{n} \qquad (7)$$

where C_{max} is the maximum contracture tension and ω and $\propto$ are proportionality constants. The value of n was found to be 3 for contracture tension produced by depolarisation of the muscle.

Equation 7 has to be modified in the presence of manganese. If,

$$Ca + Na_2R \rightleftharpoons CaR + 2Na \quad \text{(with equilibrium constant } K_1)$$

and it is assumed that the interaction of manganese with the receptor is similar then,

$$Ca + Mn\,R \rightleftharpoons CaR + Mn \text{ (with equilibrium constant } K_2)$$

then,

$$[Na_2\,R] = \frac{[Ca\,R] \quad [Na]^2}{[Ca] \quad K_1} \qquad (7A)$$

$$\text{and} \quad [Mn\ R] = \frac{[Ca\ R]\ [Mn]}{[Ca]\ K_2} \qquad (7B)$$

It is assumed that

$$Na_2\ R + Mn\ R + Ca\ R = 1$$

i.e. R is finite with a total concentration of 1,

then

$$\frac{[Ca\ R]\ [Na]^2}{[Ca]\ K_1} + \frac{[CaR]\ [Mn]}{[Ca]\ K_2} + [Ca\ R] = 1$$

$$[Ca\ R] = \frac{1}{1 + \frac{[Na]^2}{K_1[Ca]} + \frac{[Mn]}{K_2[Ca]}}$$

Thus

$$C_{max} = \propto \left[\frac{\omega\ [Ca]}{[Ca] + \frac{[Na]^2}{K_1} + \frac{[Mn]}{K_2}} \right]^{n} \qquad (8)$$

As tension was found to be proportional to $[Ca]_o^2$, $\sqrt[4]{[Na]}$ and $\sqrt{[Mn]}$ for low-sodium contractures then n is assumed to equal 2.

From equation 8,

$$\sqrt{C_{max}} = \frac{\sqrt{\propto}\ \omega\ [Ca]}{[Ca] + \frac{[Na]^2}{K_1} + \frac{[Mn]}{K_2}}$$

This equation is similar to that describing a rectangular

hyperbola. Thus a Lineweaver-Burk plot can be applied. A plot of $1/\sqrt{C_{max}}$ versus $\frac{1}{[Ca]}$ gives a line with an intercept on the x-axis of $\frac{1}{\frac{[Na]^2}{K_1} + \frac{[Mn]}{K_2}}$

If $$\lambda = \frac{[Na]^2}{K_1} + \frac{[Mn]}{K_2} ,$$

for experiments similar to that shown in Figure 19 the sodium concentration will be constant (and near to zero) at the time of the tension measurements. Thus at two manganese concentrations, Mn_1 and Mn_2, the difference between the two intercepts on the x-axis $\frac{1}{\lambda_1} - \frac{1}{\lambda_2}$ will give

$$\lambda_1 - \lambda_2 = \frac{Mn_1}{K_2} - \frac{Mn_2}{K_2}$$

Thus $$K_2 = \frac{Mn_1 - Mn_2}{\lambda_3}$$ where $\lambda_3 = \lambda_1 - \lambda_2$

The mean value of the equilibrium constant K_2 has been calculated for the same six complete experiments (of the type shown in Figure 19). The mean value of $K_2 = 3.36 \pm 2.10$ S.D. (range 1.48 - 7.16).

One complete experiment with nickel gave a $K_2 = 1.77$ and another using cadmium produced a $K_2 = 0.37$.

The lines in Figure 19 (left) have been drawn according to the equation,

$$C_{max} = \alpha \left(\frac{\omega \left[Ca\right]_o}{\left[Ca\right]_o + \frac{\left[Mn\right]_o}{K_2}} \right)^{n}$$

with $n = 2$, $\alpha = 1$, $K_2 = 2.0$ and $\omega = 140$.

Not all the experiments were as successful as that illustrated in Figure 19. This was probably due to two major causes, (1) potentiation of the later contractures in a series and (2) a slightly greater power relationship than is given with $n = 2$. The potentiation of successive contractures is probably related to the potentiated twitch response and contracture development after long periods in manganese Ringer. Although manganese solutions were applied for only short periods (45 - 90 seconds) with 15 to 20 minute intervening periods in normal Ringer, some reduction in the inhibition produced by manganese was observed. This unidirectional effect was compensated for to some extent by using the "mirror image" procedure for the contracture sequence (described in Methods section). The decreased inhibition could be a consequence of another action of manganese possibly intracellular because evidence will be presented in Part 3 that suggests that manganese is taken up readily by the cells and that its efflux in manganese-free solutions is relatively slow.

The mean slope of the plots of log contracture tension

versus log calcium concentration was 2.10$\pm$ 0.62 S.D. This conflicts with the results of Chapman (1974) where the mean slope was found to 1.79$\pm$ 0.26 S.D. However in the latter case the experiments were performed in various low-sodium solutions and in the absence of manganese. As will be discussed later the absence of sodium in the present experiments could account for this apparent discrepancy. The steep relationship between tension and the calcium concentration in these experiments means that a plot of the reciprocal of the square root of contracture tension versus the reciprocal calcium concentrationdid not always adequately compensate for the power relationship. Indeed a better fit for some other experiments was obtained with the reciprocal of the cube root of contracture tension. However the calculated value of K_2 was not significantly different in these other experiments. Thus in three experiments where the cube root of contracture tension was used, K_2 = 3.29$\pm$ 2.27 S.D.

In Figures 20 and 21 an experiment is illustrated where the sodium, manganese and calcium concentrations were all varied. Figure 20 (left) shows the contracture tension produced by various sodium concentrations in the absence of manganese (closed circles), in 2mM manganese (triangles) and 4 mM manganese (open circles). The calcium concentration was maintained at 1 mM throughout. Figure 20 (right) shows the low-sodium contracture tensions from the same experiment in the absence of manganese in 1 mM calcium (closed circles) and 0.5 mM calcium (open circles). The raising of the manganese concentration and the reduction of the calcium concentration can be seen to have different effects. Manganese

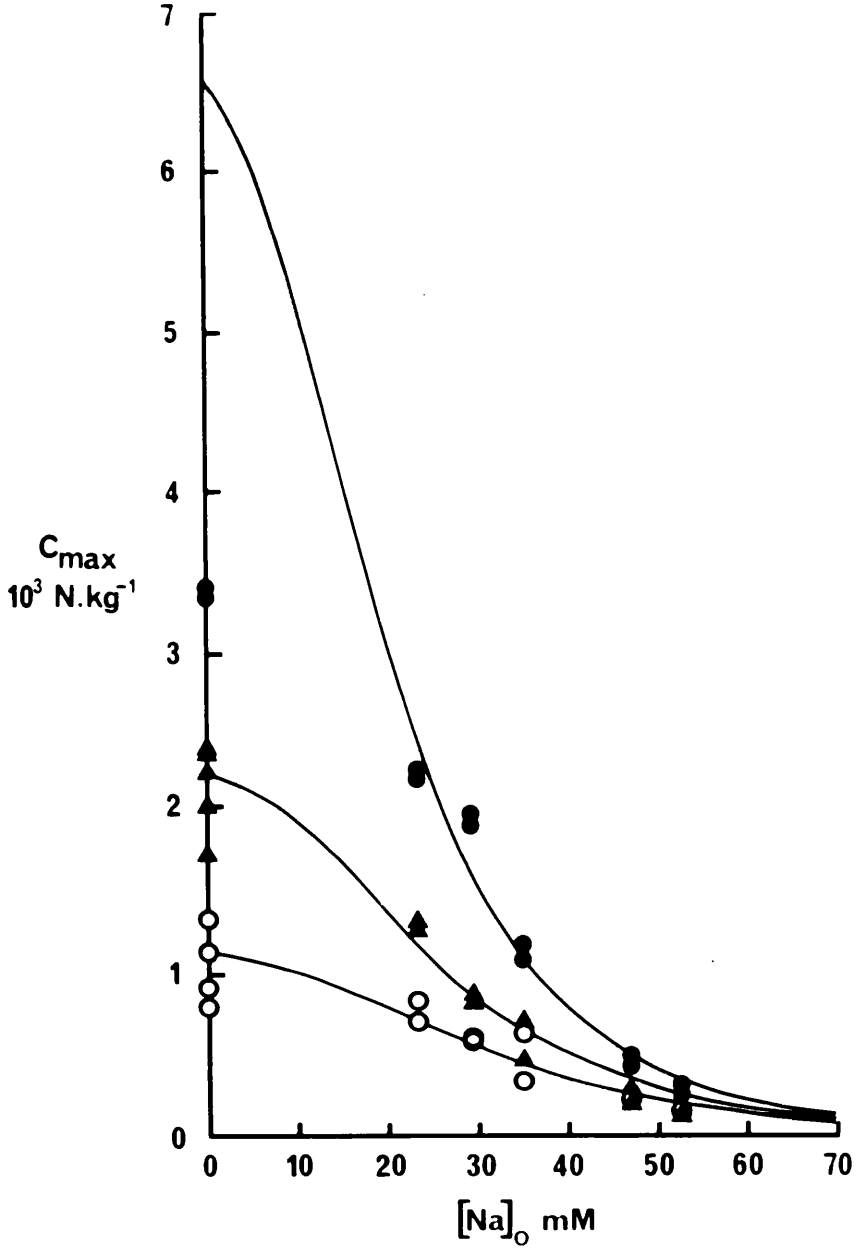
Figure 20.

Maximum contracture tensions produced by low-sodium solutions with variation of the manganese concentration (left) and calcium concentration (right).

Left: Maximum contracture tensions produced in the absence of manganese (filled circles) are compared with the respons in 2 mM manganese (filled triangles) and 4 mM manganese (open circles). 1 mM calcium throughout.

Right: Maximum contracture tensions produced in 1 mM calcium (filled circles) are compared with the responses i 0.5 mM calcium (open circles). All contractures in the absence of manganese.

The solid lines have been drawn according to equation (Page 50) with $K_1 = 0.85$, $K_2 = 2.85$, $\alpha = 1.5$ and $\omega = 4,320$ (i.e. the maximum contracture tension predicte by the Lineweaver-Burk intercept with the tension axis in Figure 21 right).


C_{max}
10^3 N.kg^{-1}
7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
$[Na]_o$ mM

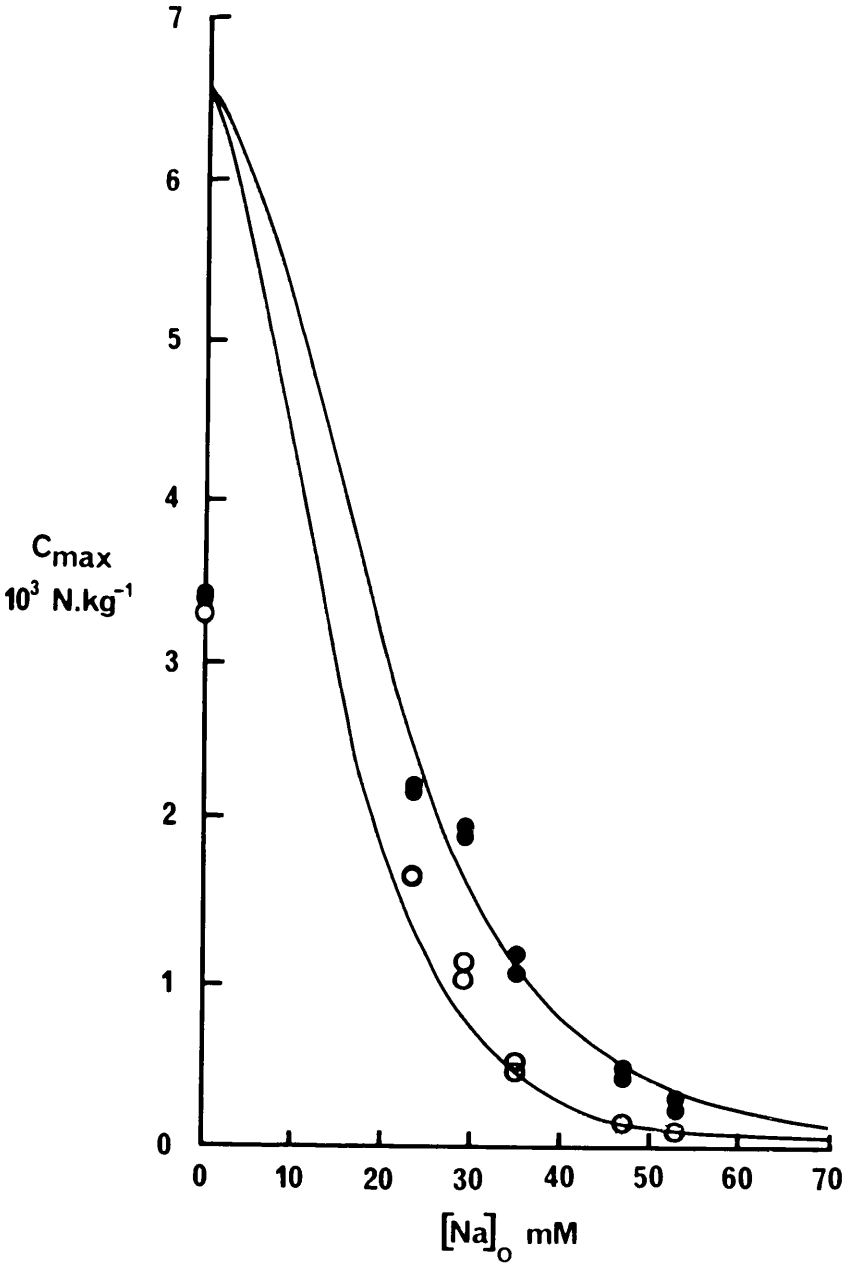
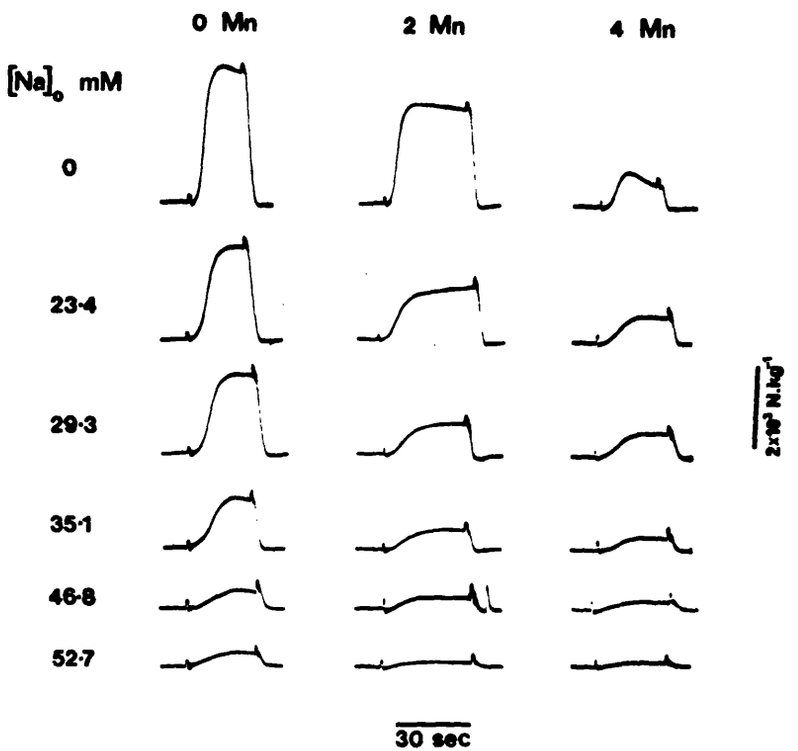
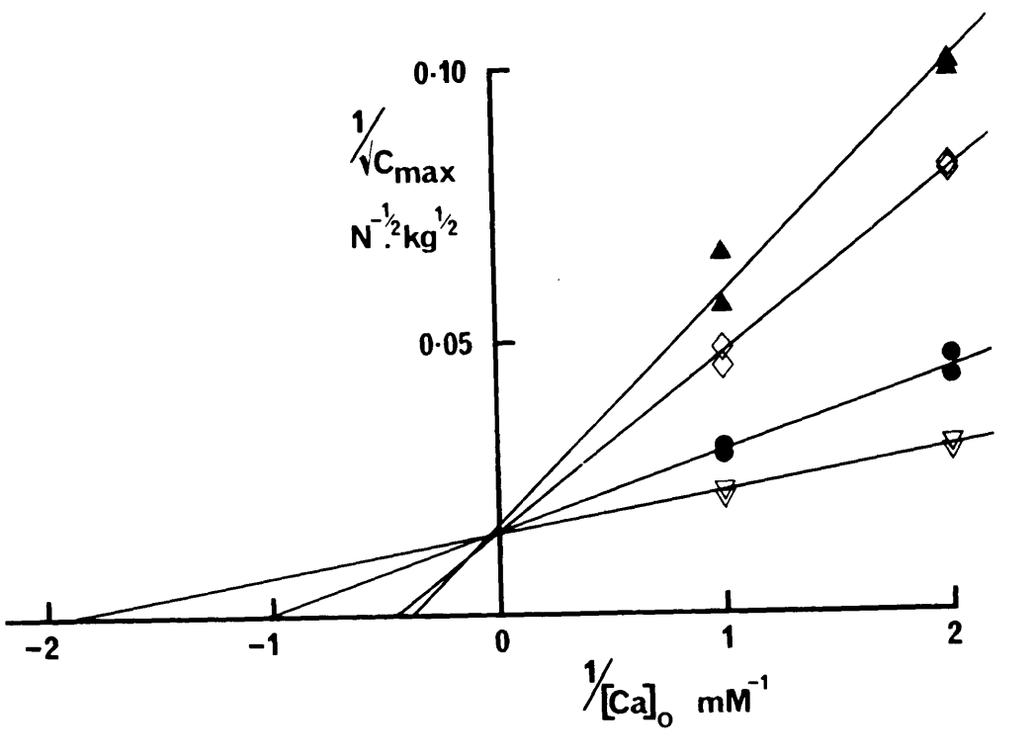

C_{max}
10^3 N.kg^{-1}
7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
$[Na]_o$ mM

Figure 21.

Left: Examples of the contracture responses to low-sodium solutions from the experiment illustrated in Figure 20 (left).

Right: A double reciprocal plot of the results illustrated in Figure 20 (right). The intercepts with the calcium concentration axis allow calculation of the value of K_1 and subsequently K_2, (equation 8). The common intercept with the y - axis was taken as the value for ω. These values of K_1, K_2 and were used to produce the curved li in Figure 20.


0 Mn
2 Mn
4 Mn
[Na]o mM
0
23·4
29·3
35·1
46·8
52·7
30 sec
2×10³ N.kg⁻¹


0·10
0·05
1/√Cmax
N⁻½kg½
−2
−1
0
1
2
1/[Ca]o mM⁻¹

reduces the maximum tension produced in zero -sodium solutions whereas a reduced calcium concentration merely shifts the tension curve to the left, the maximum contracture tension in zero-sodium being unchanged. The tension recordings of some of these responses are shown in Figure 21 (left).

Returning to the equation derived to describe the contracture responses (equation 8), then in the absence of manganese the term $\frac{Mn}{K_2}$ can be omitted. The value of K_1 for results of the type shown in Figure 20 (right) can be calculated by using the same method as was used previously to calculate the value of K_2 from Figure 19 (Page 51). It will be apparent that a plot of $\frac{1}{\sqrt[n]{C_{max}}}$ versus $\frac{1}{[Ca]}$ will have an intercept with the x-axis of $\frac{K_1}{[Na]^2}$. The results expressed in the form of a Lineweaver-Burk plot are shown in Figure 21 (right). If we let $\theta = \frac{Na^2}{K_1}$ and at any two sodium concentrations (Na_1 and Na_2) let the difference between any two intercepts with the x-axis $= \theta_1 - \theta_2 = \theta_3$, then

$$\theta_3 = \frac{[Na_1]^2}{K_1} - \frac{[Na_2]^2}{K_1}$$

$$\text{thus} \qquad K_1 = \frac{[Na_1]^2 - [Na_2]^2}{\theta_3}$$

From various combinations of pairs of intercepts in Figure 21 (right) the mean value of K_1 was found to be 0.80

(range 0.73 to 0.83). An alternative method of calculating K_1 is directly from the intercept at any given sodium concentration as the intercept $= \frac{K_1}{[Na]^2}$. The mean value of K_1 calculated by this method was 0.85.

The value of K_2 can be calculated if two sodium-manganese mixtures produce equal tensions while the calcium concentration is constant. If the sodium and manganese concentrations in the two mixtures are $[Na_1], [Mn_1]$ and $[Na_2], [Mn_2]$ respectively then,

$$\frac{\omega [Ca]}{[Ca] + \frac{[Na_1]^2}{K_1} + \frac{[Mn_1]}{K_2}} = \frac{\omega [Ca]}{[Ca] + \frac{[Na_2]^2}{K_1} + \frac{[Mn_2]}{K_2}}$$

$$\therefore \quad \frac{[Na_2]^2}{K_1} + \frac{[Mn_2]}{K_2} = \frac{[Na_1]^2}{K_1} + \frac{[Mn_1]}{K_2}$$

$$\therefore \quad K_2 = \frac{K_1([Mn_1] - [Mn_2])}{[Na_2]^2 - [Na_1]^2}$$

From sodium-manganese mixtures producing equal tension in this experiment the mean value of K_2 was calculated to be 2.84 (range 2.07 - 4.15) assuming $K_1 = 0.85$. Therefore using these values for K_1 and K_2 theoretical curves can be produced according to Equation 8 for various concentrations of sodium, calcium and manganese. The lines drawn through the points in Figure 20 are these calculated curves. The curves

derived from equation 8 reasonably fit the observed responses at low contracture tensions. However the predicted curves intercept the tension axis at about twice the observed maximum contracture tension in zero-sodium, zero-manganese solution. This is in accord with previous results (Chapman, 1974) where curves derived from equation 7 were found to intercept the y - axis at points 1.5 to 2 times greater than the observed maximum contracture tension. Possible reasons for this discrepancy have been suggested (Chapman,1974) and will be considered in more detail later. It is noticeable that in the presence of manganese the predicted curves show a much better fit with the observed responses even in zero-sodium solution.

(8) Sodium-induced tension in the presence of manganese

A small increase in tension can be observed before the relaxation when changing from a sodium-free solution (plus manganese) back to normal Ringer containing sodium (Chapman and Ochi,1971). This "off response" suggests that the re-addition of sodium may induce a small contracture. To investigate this further, trabeculae were exposed to sodium-free solutions (Tris replacement) containing manganese, thereby producing a small contracture. Various low concentrations of sodium were then reintroduced (Figure 22 left). This resulted in an increase in contracture tension with a maximum tension being induced by the addition of approximately 10 mM sodium (Figure 23 top).

Similar experiments were performed by the addition of lithium instead of sodium (Figure 22 right). However the

Figure 22.

Sodium and lithium induced contraction in the presence of manganese.

Left: Following exposure to sodium-free solution containing 8 mM manganese the addition of small amounts of sodium (concentrations are expressed in mM/litre) is able to produce a contracture.

Right: Similar contractures are induced by the addition of lithium (same preparation)but higher concentrations are required.

The bottom right trace shows the normal manganese inhibited sodium-free response without addition of lithium or sodium. The calcium concentration was 1 mM throughout.

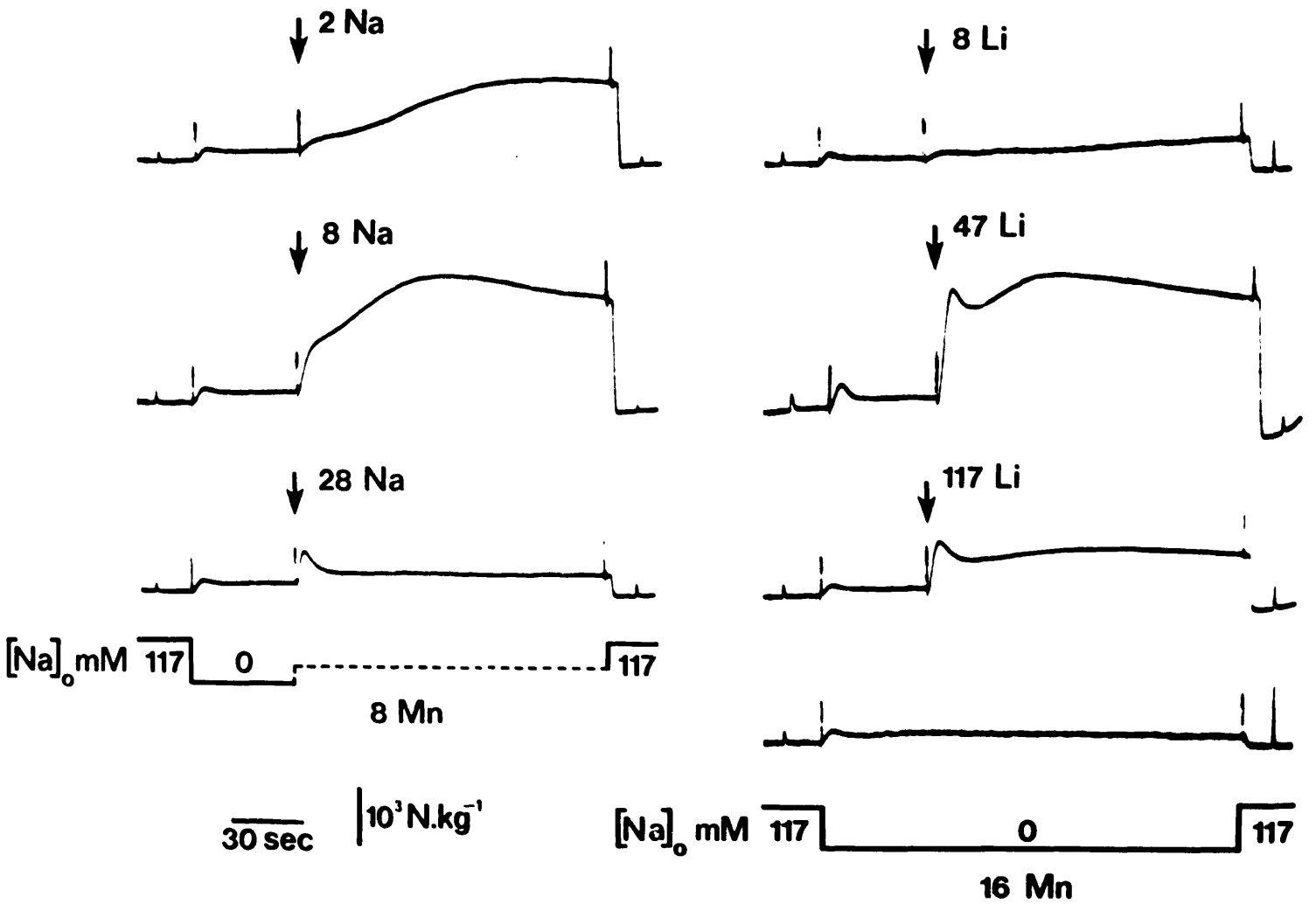
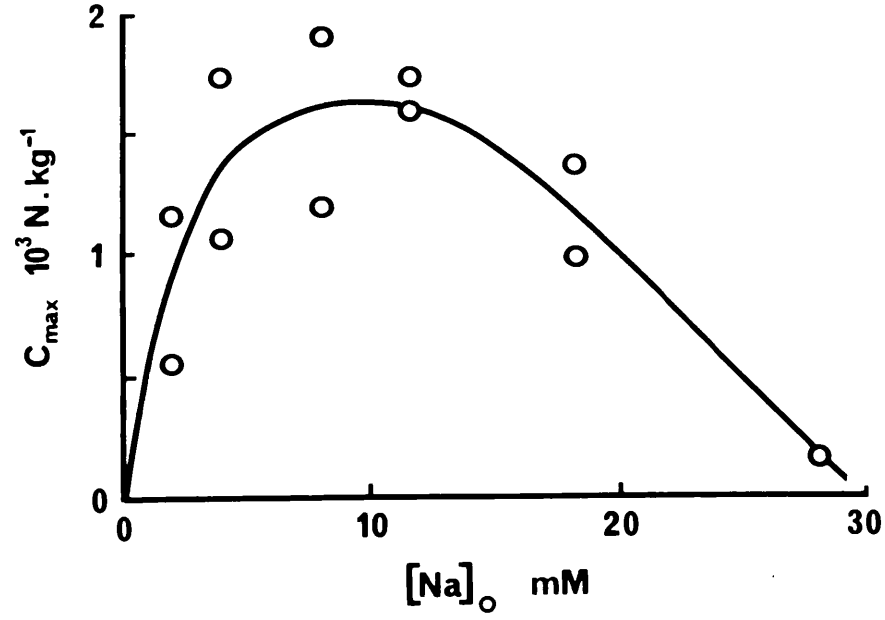
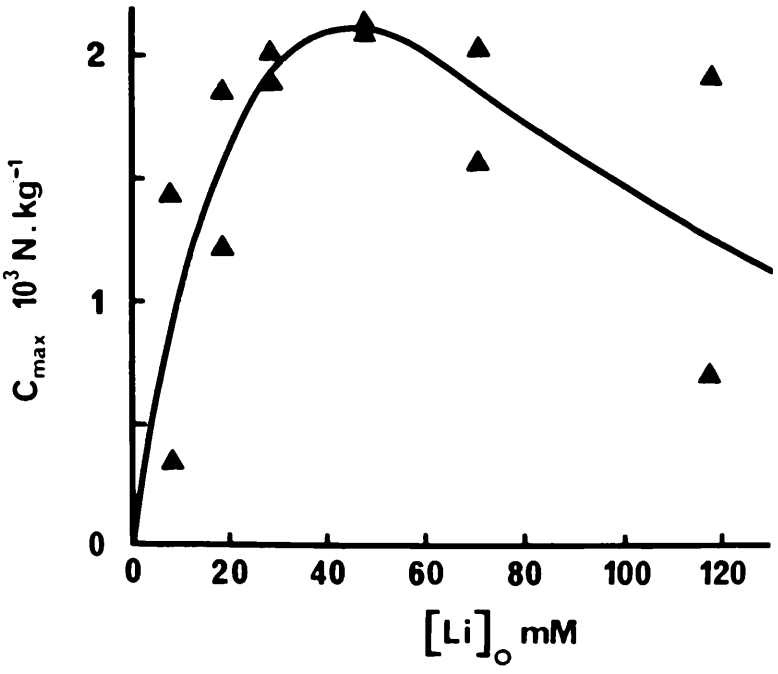

2 Na
8 Na
28 Na
[Na]o mM 117 0 117
8 Mn
30 sec
10³ N.kg⁻¹
8 Li
47 Li
117 Li
[Na]o mM 117 0 117
16 Mn

Figure 23.

Maximum contracture tensions induced by the addition of various sodium(top) and lithium concentrations (bottom) following exposure to sodium-free solution in the presence of manganese. Both sets of results were obtained from the same preparation, 8 mM manganese was present during the addition of sodium and 16 mM manganese during the addition of lithium. The experimental procedure was as described in connection with Figure 22. 1 mM calcium throughout .


2
1
0
C_{max} 10^3 N.kg^{-1}
0
10
20
30
$[Na]_o$ mM


2
1
0
C_{max} 10^3 N.kg^{-1}
0
20
40
60
80
100
120
$[Li]_o$ mM

optimum concentration for tension production is much higher with lithium (Figure 23 bottom). This could be due to the fact that lithium does not show the same antagonistic action to contracture production as sodium. Indeed as was shown previously (Figure 12) lithium produces no inhibition of the low-sodium contracture.

The contracture response to sodium or lithium addition is biphasic. With the addition of high concentrations of sodium or lithium only a short phasic response was observed followed by a small tonic tension (Figure 22). The phasic response may be the consequence of the gradual rise of the sodium concentration through an optimum for tension production and then on to the inhibitory range at higher concentrations. The mean optimum sodium and lithium concentrations for these experiments are given in Table 4.

Two other cations, hydrazine and hydroxylamine, are also capable of initiating contracture tension in the same way as sodium (Figure 24). Hydrazine initiated the largest contractures, a feature possibly related to its potentiating effects on contractile responses described previously.

An explanation advanced by Chapman and Ochi (1971) for the apparently paradoxical effect of sodium initiating contracture tension despite its known inhibitory effects is that sodium produces contractures by its action inside the cell. When exposed to sodium-free conditions the sodium concentration within the muscle cells should fall rapidly as sodium efflux in cardiac tissue is very rapid (Novotny and Bianchi,1973; Bosteels and Carmeliet,1972). It seems possible that under manganese inhibition of calcium influx the addition of sodium

TABLE 4

Optimum Sodium Concentration for Inducing Tension in Manganese Solutions

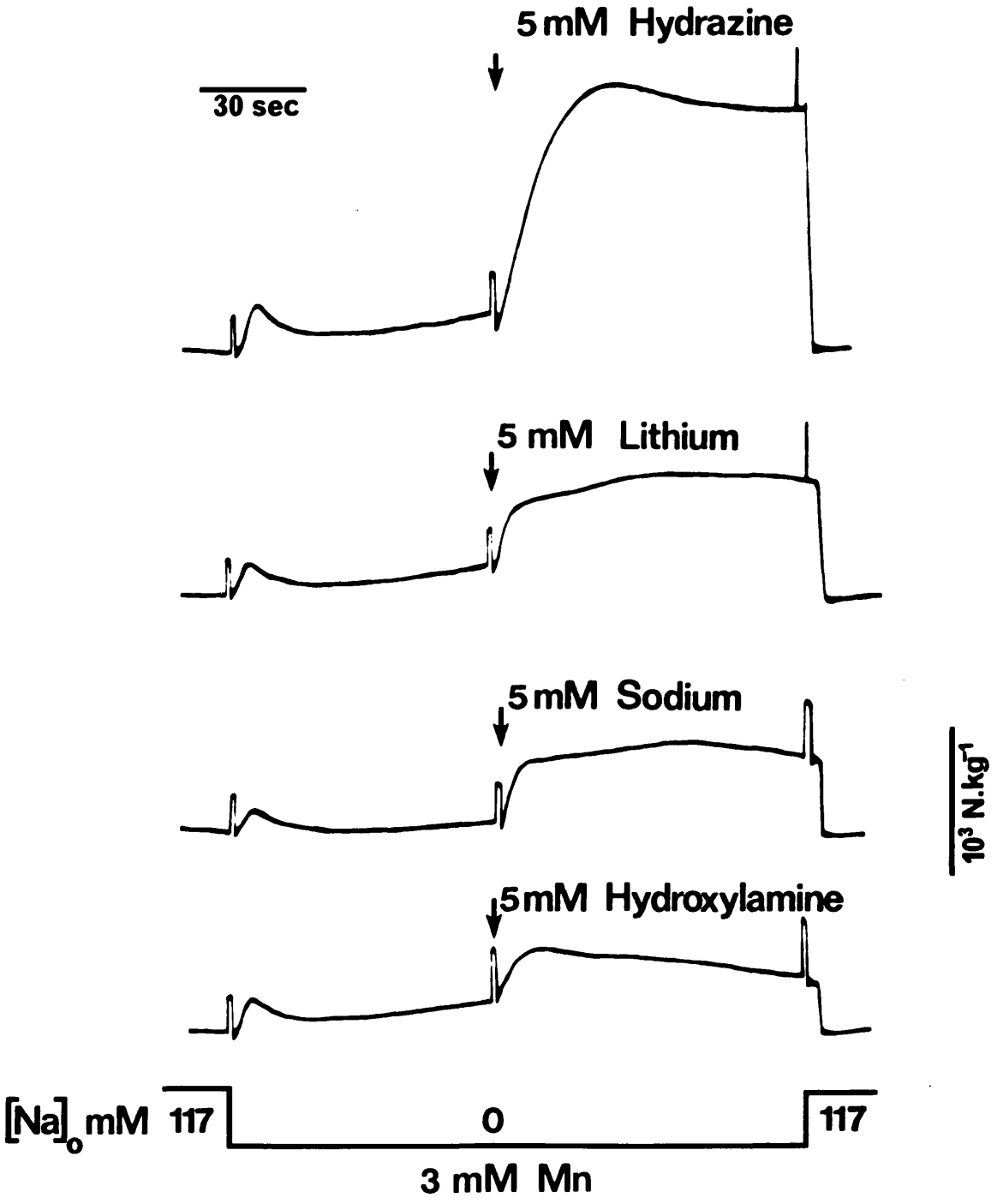
	Initial Phasic Tension	Tonic Tension
Mean Sodium Concentration (mM)	11.1	9.6
Standard Deviation (mM)	3.6	1.1
Number of experiments (n)	4	4

Optimum Lithium Concentration for Inducing Tension in Manganese Solutions

	Initial Phasic Tension	Tonic Tension
Mean Lithium Concentration(mM)	58.3	41.7
Standard Deviation (mM)	30.1	25.2
Number of experiments (n)	3	3

Figure 24.

Comparison of the contractures produced by hydrazine, lithium, sodium and hydroxylamine following exposure to sodium-free solution (Tris substitution) in the presence of 3 mM manganese. All recordings from the same experiment. 1 mM calcium throughout.


5 mM Hydrazine
30 sec
5 mM Lithium
5 mM Sodium
10³ N.kg⁻¹
5 mM Hydroxylamine
[Na]o mM
117
0
117
3 mM Mn

could increase calcium influx via a sodium-calcium exchange process similar to that suggested for squid axon (Baker et al,1969) and guinea pig heart muscle (Reuter and Seitz,1968). Thus in the presence of manganese the addition of small amounts of sodium could induce contracture rather than produce its normal inhibitory effect. In fact it has been observed that contracture tensions in high manganese concentrations are larger in the presence of small amounts of sodium than in its complete absence. This being the case it would be anticipated that increasing the internal sodium concentration would result in the potentiation of contracture tension. In the presence of strophanthidin (which would result in an increased internal sodium concentration) the contracture responses are in fact increased (Chapman,1974). It would appear that the tension induced by sodium and the other univalent cations in the present experiments is indeed due to calcium influx across the cell membrane because such contractures are prevented if calcium is absent from the bathing Ringer solution (Chapman R.A. - personal communication.

(9) Effects of increased internal sodium concentrations.

The internal sodium concentration was increased in these experiments by perfusion with low-potassium Ringer, 0.6 mM (Keenan and Niedergerke, 1967). If the tension induced by sodium addition to manganese inhibited sodium-free contractures was due to sodium acting internally then when the internal sodium concentration is raised, the low-sodium contracture should show less inhibition by a given manganese concentration. Figure 25 illustrates the effects of a reduced potassium

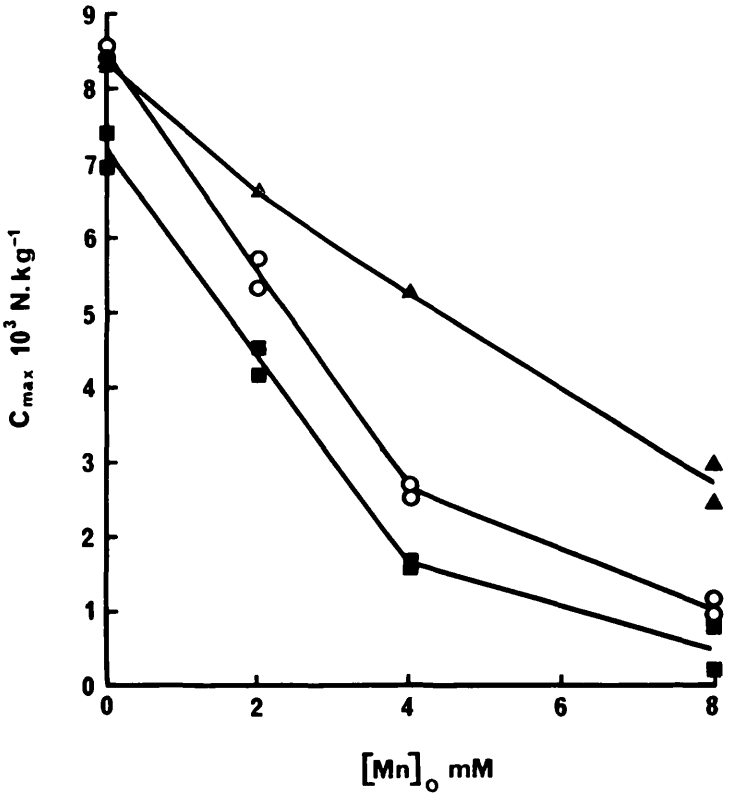
concentration on the manganese inhibition of the sodium-free contracture. The low potassium solutions produced a potentiation of the contractures. Figure 26 shows the effect of low-potassium solutions on the responses to various low-sodium solutions. The contractures in low-potassium solutions are clearly augmented as are the twitch responses (see tension recordings, bottom of Figures 25 and 26). This potentiation in low-potassium solutions could be the result of a direct influence of internal sodium on contracture tension, alternatively it could be due to a long term increase in the internal calcium concentration. This latter possibility was suggested by experiments where the muscle was maintained in 5 mM calcium Ringer (Figure 25 triangles). Thirty seconds before a sodium-free contracture was initiated the calcium concentration was reduced to 1 mM and maintained at this concentration throughout the subsequent contracture. This pretreatment would be sufficient to reduce the external calcium concentration during the contracture to approximately 1 mM i.e. to the same level as for the control responses (Figure 25, squares) as the exchange time of solutions in these preparations is about 3 seconds (Chapman and Tunstall, 1971). Thus it would appear that the concentration of calcium in an intracellular compartment was raised while the external calcium concentration had returned to the control level. The clear potentiation of the contractile response indicates that raising the calcium concentration in this compartment can increase the contracture tension under the conditions of these experiments. Therefore the potentiated contractures in solutions with a low potassium concentration could simply be

Figure 25.

Effects on the sodium-free contracture of a reduction in the potassium concentration of the bathing solution. The maximum sodium-free contracture tensions in normal (3 mM) potassium solutions are represented by the squares in the top graph. The contractures were inhibited by various concentrations of manganese. The muscle was then perfused with low-potassium (0.6 mM) Ringer. This potentiated the twitch contraction and the response to sodium-free solutions (also containing 0.6 mM potassium). After approximately 60 to 90 minutes these tensions remained stable at a new higher level. The sodium free contracture responses in the presence of 0.6 mM potassium were then tested over the same range of manganese concentrations (open circles).

The muscle was then re-equilibrated in normal Ringer and another set of measurements made in sodium free, 3 mM potassium solutions (squares).

Finally the muscle was equilibrated in a high calcium Ringer (5 mM) but with normal (3 mM) potassium concentration The responses to sodium-free solutions were measured over th same range of manganese concentrations (triangles). However in this case the muscle was returned to 1 mM calcium Ringer for 30 seconds prior to the addition of the contracture inducing solution which also contained 1 mM calcium. This procedure was intended to reduce the extracellular calcium concentration during these contractures to the same level as for those in the two previous parts of the experiment. Bottom: Some of the contracture recordings from the three parts of the experiment.


C_{max} 10^3 N.kg^{-1}
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
$[Mn]_o$ mM

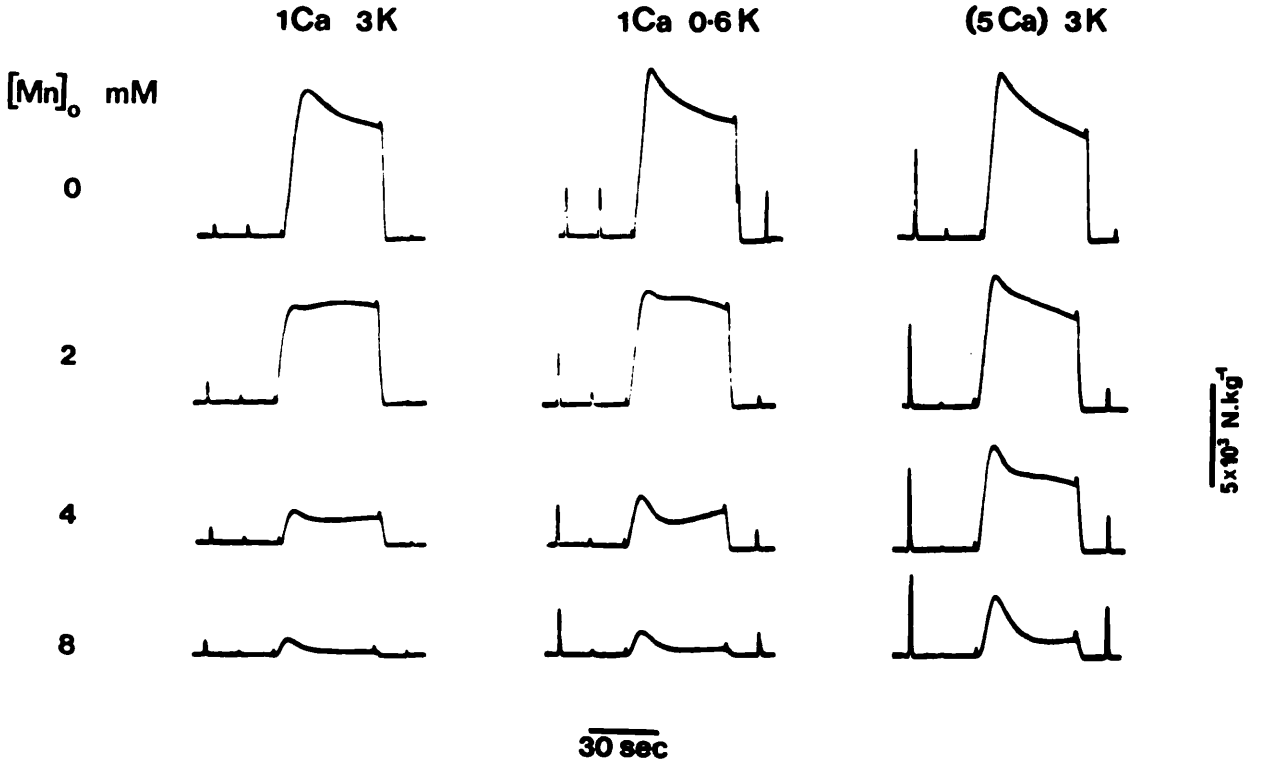

1Ca 3K
1Ca 0·6K
(5Ca) 3K
$[Mn]_o$ mM
0
2
4
8
5x10^3 N.kg^{-1}
30 sec

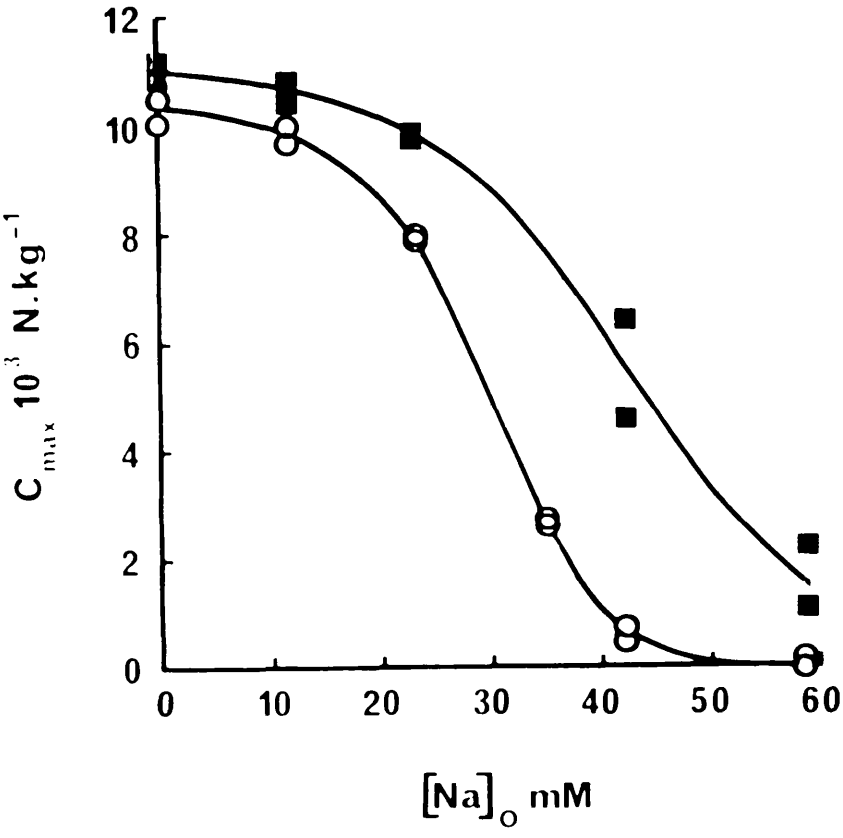
Figure 26.

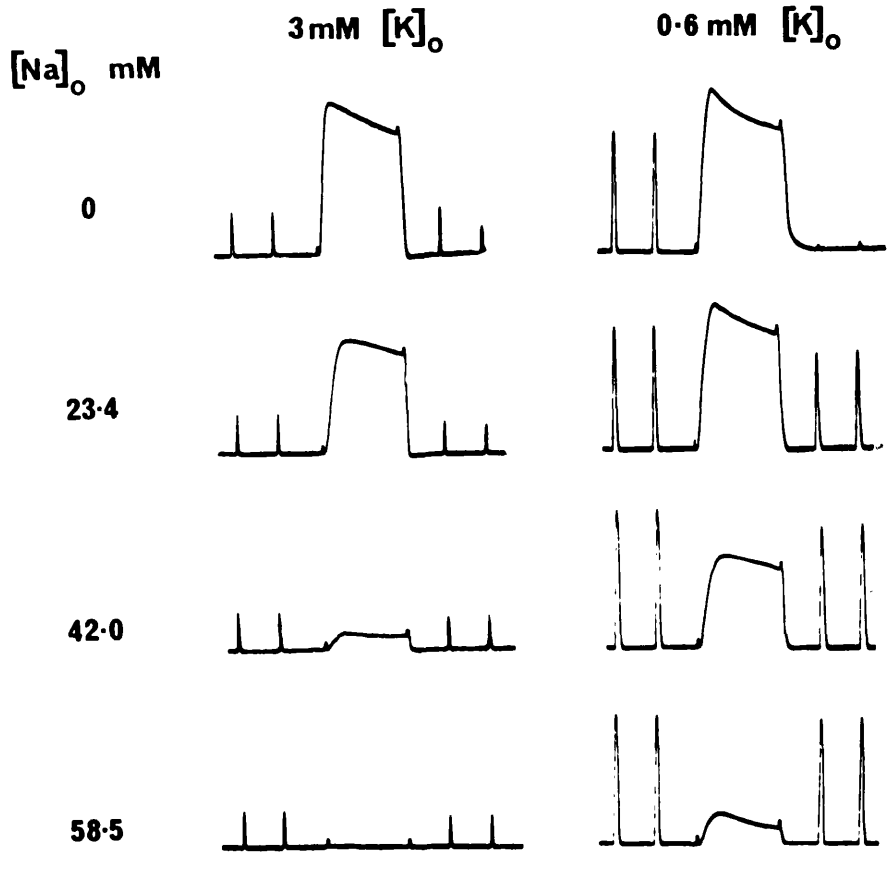
Comparison of the effects of normal (3 mM) and reduced (0.6 mM) potassium concentrations on low-sodium contractures. Experimental procedures similar to that described in connection with Figure 25. The muscle was initially perfused with normal 3 mM potassium Ringer and the maximum contracture tensions measured in various low-sodium solutions (open circles).

After equilibration (60 to 90 minutes) in 0.6 mM potassium Ringer the contracture response to low sodium solutions (0.6 mM potassium) were recorded (closed squares). The muscle was then returned to normal potassium Ringer befor a final set of measurements were made. 1 mM calcium throughout.

Top: Maximum contracture tensions in the various solutions.

Bottom: Some of the contracture recordings. Note the potentiation of the twitch contractions as well as the low-sodium contractures in solutions with a reduced potassium concentration.


12
10
8
6
4
2
0
0
10
20
30
40
50
60
$C_{max}\ 10^{-3}\ N.kg^{-1}$
$[Na]_o$ mM


3mM $[K]_o$
0·6 mM $[K]_o$
$[Na]_o$ mM
0
23·4
42·0
58·5

due to an increase of the internal calcium concentration. (Thomas, 1960, found a large calcium uptake by cells in potassium -free solution in frog heart.) This does not exclude the possibility that the increase of the internal calcium concentration is being produced as a result of an increased internal sodium concentration. Therefore under these conditions the degree of contracture response to low-sodium solutions would appear to be dependent, at least to some extent, on calcium other than that in the extracellular solution. This may only be the case in conditions that produce a very high internal calcium concentration. This could increase the response to contracture inducing solutions as a result of the high sarcoplasmic calcium concentration and the inability of calcium accumulating structures to take up more calcium. Although the 30 second pretreatment in the normal calcium concentration Ringer is sufficient to reduce the extracellular calcium concentration to control levels it is probably insufficient to allow equilibration of the calcium concentration elsewhere in the muscle (two compartments with slow exchange times have been recognised by Chapman ,1971 . One of these compartments had a time constant for calcium changes of about 30 seconds. Therefore as this calcium source has been suggested to be one of the two that produce the low sodium contracture it would be anticipated that the contracture responses would be potentiated if the calcium concentration at this site had not returned to the control level). Thus the problem clearly remains unresolved. Indeed it might well prove difficult to show categorically that the contracture potentiation in low-potassium solutions is due directly to an increased internal sodium, directly to an increased internal

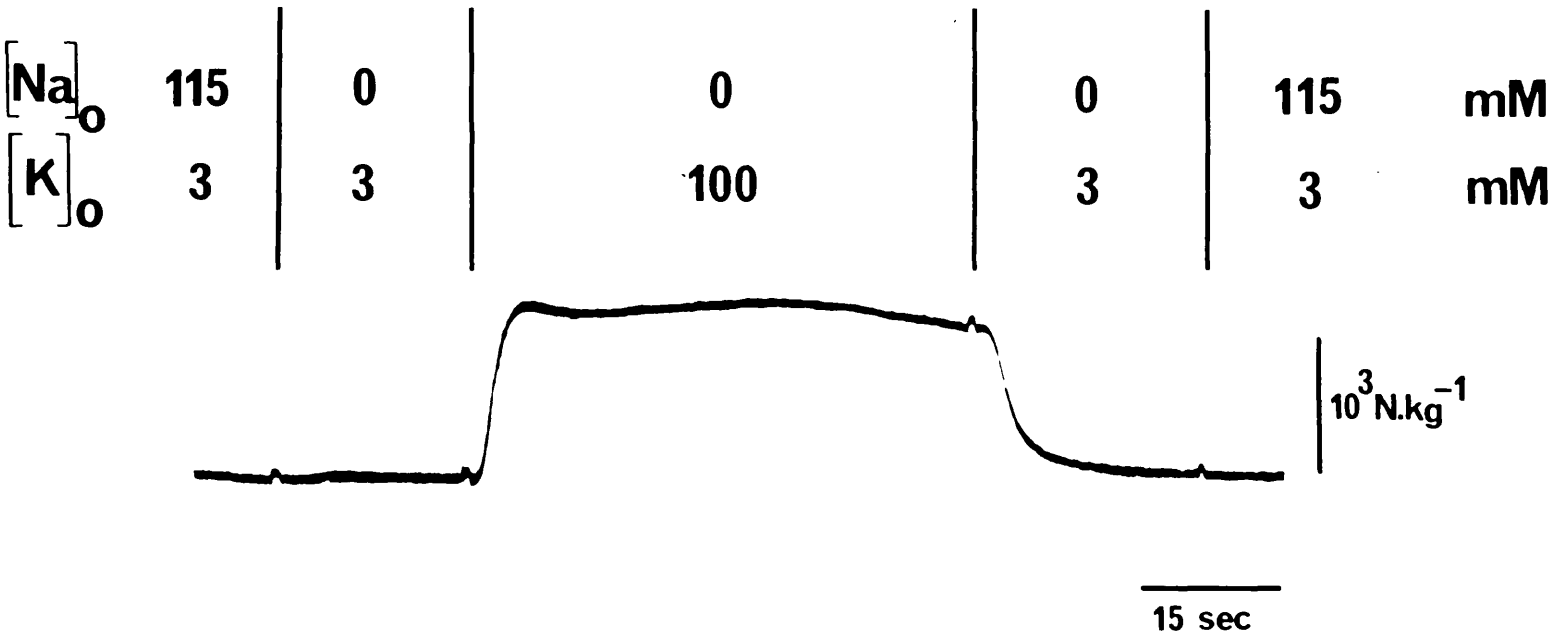
calcium, or indirectly to an increased internal calcium concentration via an increased internal sodium concentration. These changes may well be so closely interelated as to be inseparable.

(10) Depolarisation-induced tension and manganese.

Chapman and Tunstall (1971) found that the contracture response to potassium-rich depolarising solutions varied with the cube of the calcium concentration (i.e. $n = 3$ in equation 7). However the response to low-sodium solutions varied with the square of the calcium concentration (Chapman,1974). This suggested that an extra site for calcium release or binding was available in potassium-rich solutions. The calcium involved in the low-sodium response was suggested to originate from superficial sites in the cell e.g. the cell membrane. Therefore rapid responses to external solution changes are observed. The extra calcium source observed in the presence of potassium-rich solutions was supposed to correspond to the slowest compartment described by Chapman and Niedergerke (1970A), and this might correspond to an internal calcium store (e.g. the sarcoplasmic reticulum) which releases calcium upon depolarisation of the cell. If the two calcium sites involved in the low-sodium contracture are also involved in the potassium-rich contracture, then by eliminating the low-sodium response it should be possible to observe the tension produced by the extra calcium source in potassium-rich solutions. This in fact appeared to be possible (Chapman and Ochi,1971). In Figure 27 the preparation was maintained in 40 mM manganese, a concentration sufficient to almost completely inhibit the low-

Figure 27.

Contracture response produced by depolarisation with 100 mM potassium-rich solution in the presence of 40 mM manganese. When the low-sodium contracture is almost completely inhibited depolarisation with potassium-rich solution is still able to evoke a substantial contracture.


[Na]o
115
0
0
0
115
mM
[K]o
3
3
100
3
3
mM
10^3 N.kg^-1
15 sec

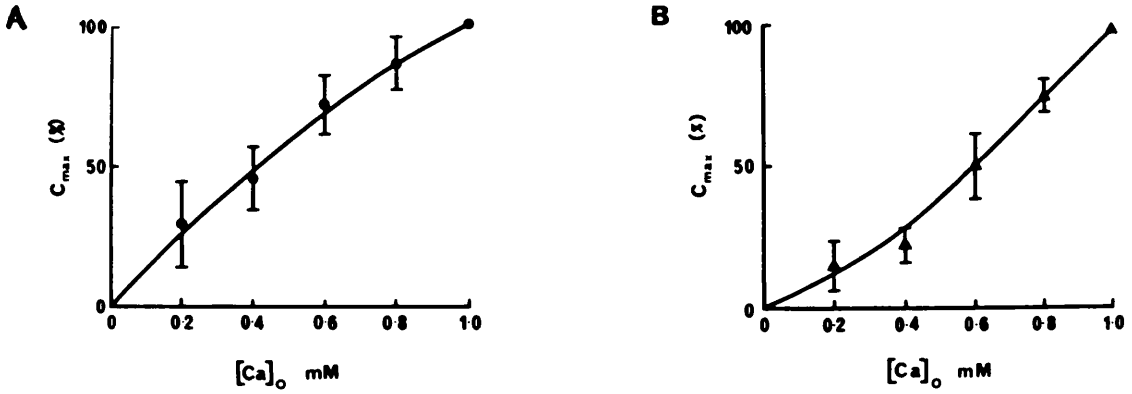
sodium contracture but when the potassium concentration was raised to 100 mM a large contracture developed. This contracture relaxed on return to normal potassium concentrations despite the absence of external sodium. However these depolarisation induced contractures are unlikely to be due solely to internal calcium release because it has been shown (R.A.Chapman unpublished) that these contractures are blocked in the absence of external calcium. It could be that a small amount of "trigger" calcium is essential in order to promote internal calcium release as has been suggested for skeletal muscle (e.g. Endo et al, 1970) or on the other hand calcium released during potassium depolarisation could originate from the external solution or from a membrane site. The depolarisation might increase the gradient for inward movement of CaR, the calcium complex responsible for contraction (Niedergerke,1963A), or release calcium from a membrane store which has a rapid turnover of calcium.

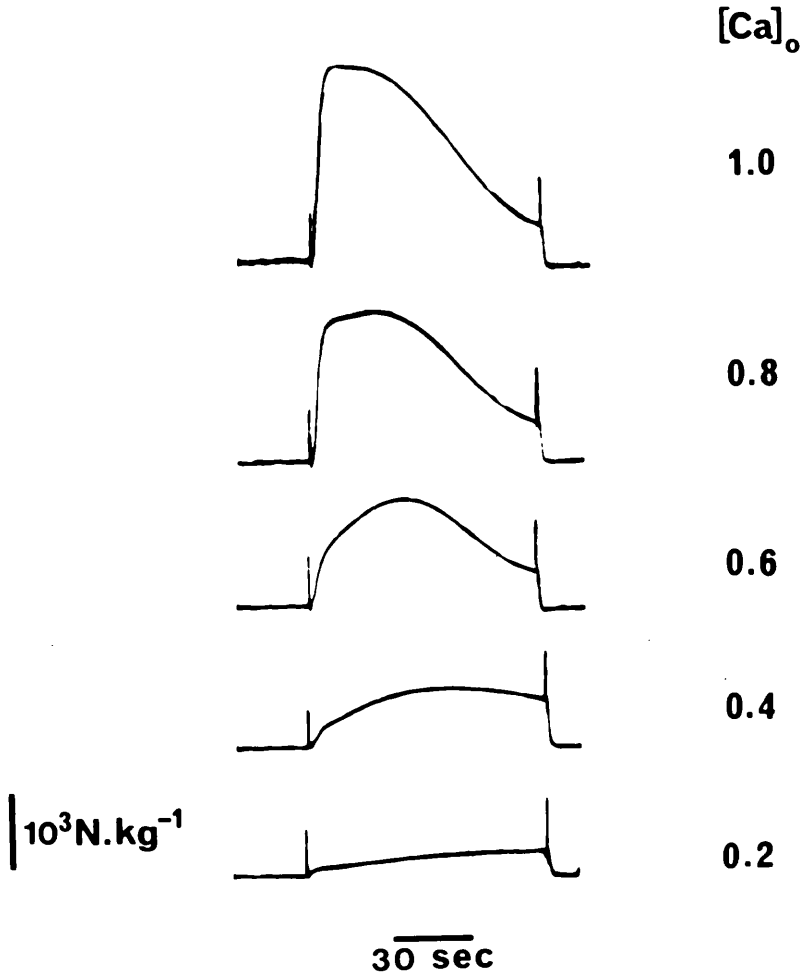
If depolarisation was simply increasing the gradient for CaR movement across the cell membrane it is unlikely that the relationship of tension production to variation of the external calcium concentration would be altered. This has been investigated as is illustrated in Figure 28. The tension recordings (bottom) are the responses evoked by 100 mM potassium solutions lacking sodium but containing 12 mM manganese. This manganese concentration was sufficient to inhibit completely the sodium-free contracture in the presence of 1 mM calcium. Graph A (Figure 28) gives the mean results from 4 similar experiments. The maximum contracture tension has been plotted as a percentage of that produced in 1 mM calcium.

Figure 28.

Contractures produced by potassium-rich solutions in the presence of 12 -20 mM manganese. The figure illustrates the results of 4 experiments where manganese was added at a concentration sufficient to inhibit almost completely the contracture response to sodium-free solutions in the presence of 1 mM calcium. The muscle was then equilibrated for 20 minutes at each of the various calcium concentrations before the readdition of manganese and a 100 mM potassium-rich, sodium-free solution with the calcium concentration indicated.

The maximum contracture tension in these solutions has been plotted as a percentage of the maximum contracture tension in 1 mM calcium (left). Points give the mean ($\pm$ S.D. of mean) of the 4 experiments. Some of the contracture recordings from one experiment are illustrated at the bottom. The contracture exhibited an initial rapid phasic response (not very clear in the experiment illustrated) followed by a slower rise to the maximum contracture tension. The graph on the right shows the mean contracture tensions of the initial phasic response, again expressed as a percentage of that in 1 mM calcium. The mean slope of plots of log contracture tension versus log calcium concentration was 0.94 $\pm$ 0.50 S.D. for the maximum contracture tension (left) and 1.39 $\pm$ 0.55 S.D. for the initial phasic response (right).


A
100
50
0
C_{max} (%)
0
0·2
0·4
0·6
0·8
1·0
$[Ca]_o$ mM
B
100
50
0
C_{max} (%)
0
0·2
0·4
0·6
0·8
1·0
$[Ca]_o$ mM


$[Ca]_o$
1.0
0.8
0.6
0.4
0.2
$10^3 N.kg^{-1}$
30 sec

(in these 4 experiments the manganese concentration required to block the sodium-free contracture varied between 12 and 20 mM). The mean slope of plots of log maximum contracture tension versus log calcium concentration was 0.94± 0.50 S.D. These results suggest that in potassium-rich solutions in which the low-sodium responses are blocked, the contracture tension varies linearly with the external calcium concentration. (The process of course shows saturation at high tension levels). It will be recalled that the relationship of tension to external calcium varied with the cube of the calcium concentration for potassium depolarisation contractures (Chapman,1971). The present results suggest that in the presence of high manganese concentrations there is a simple linear relationship with the external calcium concentration. This might indicate that depolarisation is not simply increasing the inward gradient for CaR.

The contractures were often more biphasic than those of the experiment illustrated in Figure 28 (bottom). Where, as in the case of the experiment illustrated, the two phases of the contracture were not easily discernable the tension has been measured after 10 seconds in the contracture solution. The initial phase of the contractures exhibits a slightly steeper relationship with the external calcium concentration. The mean shope of plots of log phasic contracture tension versus log calcium concentration was 1.39± 0.55 S.D.

(11) The caffeine contracture and manganese

Caffeine evokes contractures in skeletal muscle probably by releasing calcium from the sarcoplasmic reticulum (Weber and Hertz, 1968). At room temperature caffeine does not

produce contracture in frog heart when applied in normal Ringer (Chapman and Miller, 1974). However, following a contracture produced by potassium-rich and low-sodium solutions the addition of caffeine induces a large contracture. In sodium-free solutions the application of caffeine can induce a contracture even when the external calcium concentration is reduced to 10^{-8} M and therefore probably results from calcium release from an intercellular store (Chapman and Miller, 1973).

Figure 29 (bottom) illustrates the combined results of 3 experiments where caffeine contractures have been produced in sodium-free solutions in the presence (closed symbols) and absence (open symbols) of manganese. If manganese inhibits contracture tension by blocking calcium influx across the cell membrane, then the caffeine contracture would be expected to be unaffected by manganese. In these experiments there was little or no inhibition of the caffeine contracture by manganese except possibly at very low caffeine concentrations. The caffeine contracture recordings from one experiment are shown in Figure 29 (top). These results suggest that manganese does not produce its inhibitory effects directly on the contractile proteins nor on calcium release from intracellular calcium storage structures but that it probably acts at a more superficial site e.g. the cell membrane.

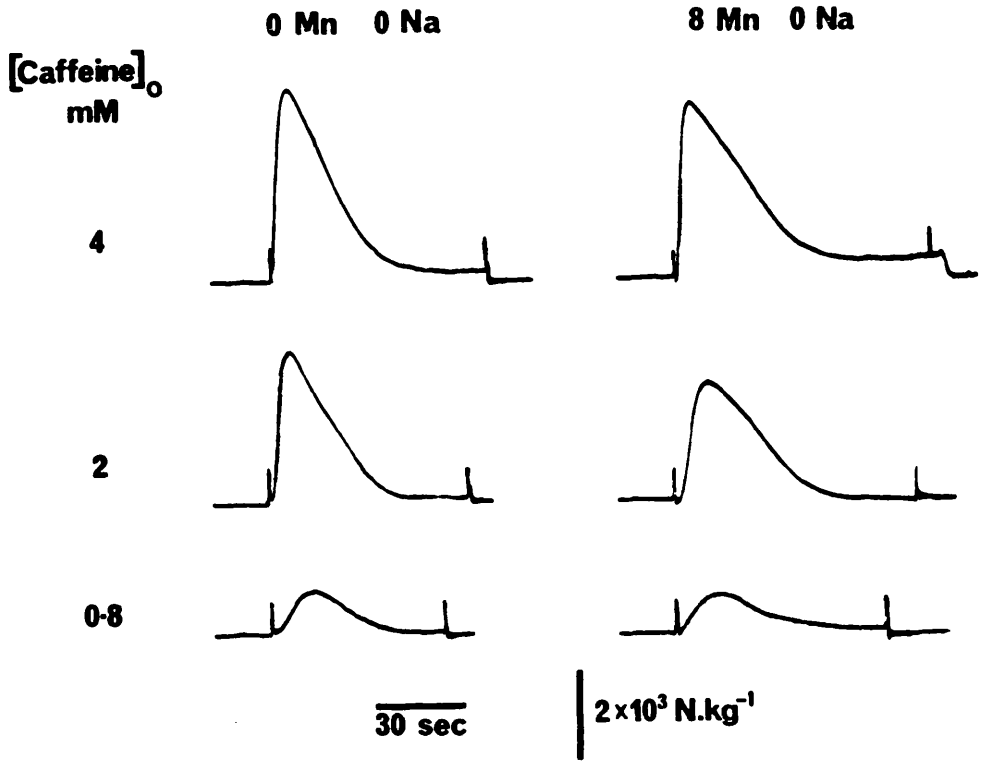
(12) The effects of D-600 on frog heart muscle

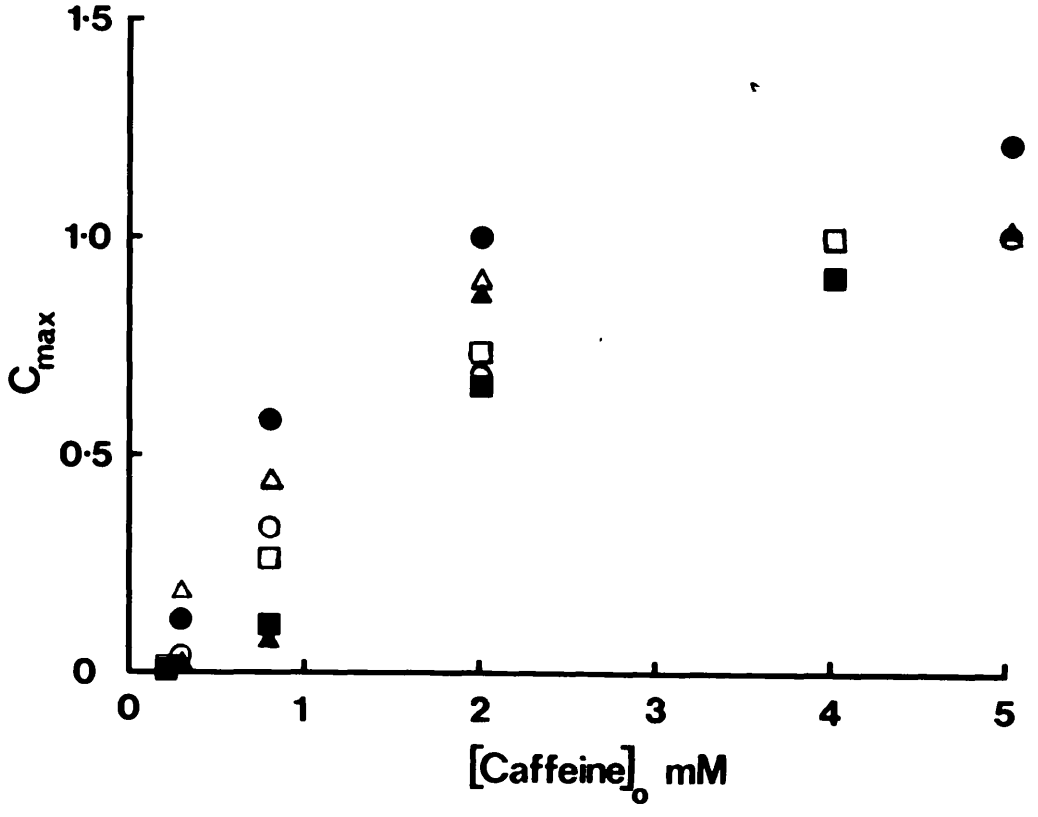
Verapamil and its methoxy-derivative, D-600 have been used as inhibitors of calcium influx in heart muscle

Figure 29.

Effect of manganese on the contracture induced by caffeine. Caffeine contractures were produced following relaxation of sodium-free contractures. The caffeine, also in sodium-free solution, produces a rapid increase in tension which subsequently relaxes spontaneously. When manganese was present it was added for a pretreatment of 30 seconds in sodium-free solution before the addition of the caffeine solution containing manganese. The combined results of 3 experiments are illustrated (bottom). The maximum contracture tension in the presence (closed symbols) and absence (open symbols) of manganese have been plotted as a fraction of the contracture tension produced by 4 or 5 mM caffeine in the absence of manganese (scaled to be equal to 1.0). 1 mM calcium throughout.

Top: Some of the contracture responses from one experiment.


0 Mn 0 Na
8 Mn 0 Na
[Caffeine]o
mM
4
2
0·8
30 sec
2×10³ N.kg⁻¹


1·5
1·0
0·5
0
Cmax
0
1
2
3
4
5
[Caffeine]o mM

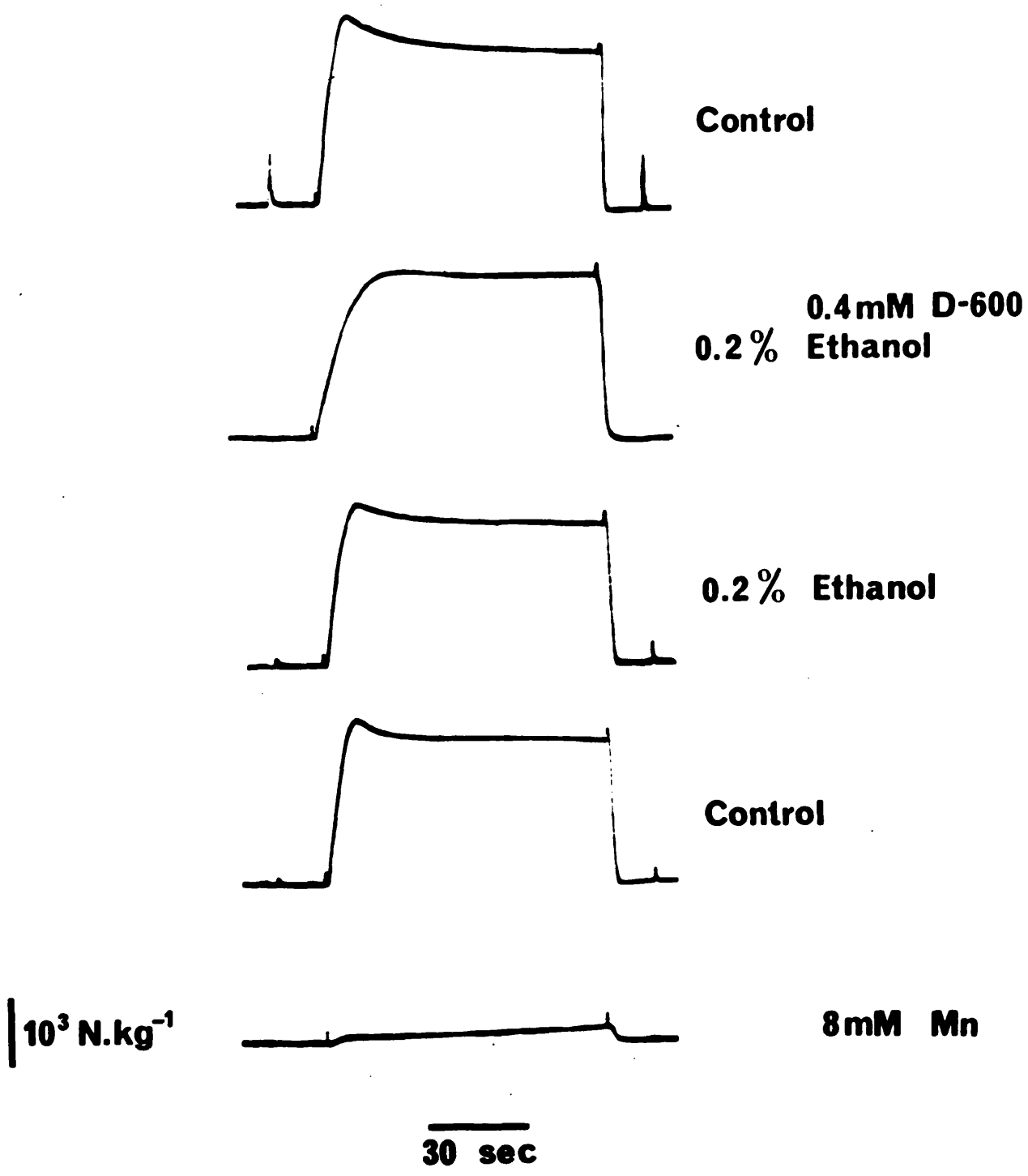
(e.g. Fleckenstein et al,1969) and in squid axon (Baker et al,1973). In the present experiments with frog atrial trabeculae the effect of D-600 has been compared with the inhibition produced by manganese.

The drug D-600 was required in very large doses (0.1 - 0.4 mM) in order to produce significant effects. In such concentrations D-600 produced an increase in twitch threshold and an inhibition of the twitch contraction. It inhibited to varying degrees the potassium-rich contracture but had little or no effect on the contracture produced by low-sodium solutions even after 8 minutes pretreatment (Figure 30). From the results with manganese, the low-sodium contracture would have been assumed to be the most sensitive to trans-membrane calcium block. However the results in Figure 30 show one of the largest inhibitory effects on the sodium-free contracture that has been observed in these experiments. Indeed in some experiments where the low-sodium response had become slow to reach its peak tension (e.g. at the end of a long experiment) the addition of D-600 actually potentiated the low-sodium contracture response. These results are clearly not compatible with the idea that the low-sodium contracture results from calcium influx across the cell membrane unless the mode of action of D-600 differs from that suggested by Fleckenstein et al, (1969). Alternatively the calcium influx that produces the low-sodium contracture may occur at a different site to the calcium influx of the action potential. The former site would be insensitive, and the latter sensitive to D-600 inhibition. Evidence for this suggestion comes from the results of Ashley et al, (1974).

Figure 30.

Effect of D-600 on the sodium-free contracture.

Top: Control contracture in sodium-free solution (Tris substitution). After 15 minutes the muscle was exposed to 0.4 mM D-600 (in ethanol solution) for 8 minutes. The contracture responses to sodium-free solution (also containing D-600) was then recorded (second from top). The middle trace shows the contracture responses in the presence of the same concentration of ethanol but without D-600 (again following 8 minutes pretreatment in 0.2% ethanol Ringer). After these procedures the control sodium-free contracture was little affected (second from bottom). Finally the large inhibitory effect of 8 mM manganese on the sodium free contracture was recorded (bottom trace). 1 mM calcium throughout.


Control
0.4 mM D-600
0.2 % Ethanol
0.2 % Ethanol
Control
10³ N.kg⁻¹
8 mM Mn
30 sec

In barnacle giant muscle fibre they found that the increased aqueorin light omission associated with a removal of external sodium(and presumed to be due to calcium influx across the cell membrane) was blocked by manganese but not by D-600. Thus in barnacle muscle the calcium influx following the removal of external sodium appears to be insensitive to D-600 inhibition.

It is possible that D-600 might act at a site other than the surface membrane. Indeed the long time required (10 - 20 minutes) for D-600 and verapamil to produce their inhibitory effects in heart muscle (Kohlardt et al,1972) could be evidence for this possibility. D-600 can produce irreversible contracture in crab leg muscle (R.C.Thomas and R.Vaughan-Jones - personal communication) so an effect on internal calcium storage structures might be indicated. In fact it has been demonstrated that D-600 can inhibit calcium uptake by the sarcoplasmic reticulum (Entman et al, 1972) but at higher concentrations than those effective on the excitation process. A similar action in frog heart muscle could decrease the calcium available for release during the twitch and potassium-rich contracture. This could account for the inhibition of these responses while the low-sodium contracture would be unaffected.

Some signs of irreversibility (or at least slow reversibility) on the twitch contraction were apparent in these experiments using D-600 on frog heart.

DISCUSSION (2)

(1) Manganese and the twitch contraction

A thorough investigation of the effects of manganese on the twitch contraction has not been possible because in manganese solutions: (a) the excitability of the tissue is affected, (b) there are complex changes in the duration of the action potential and (c) a contracture developes after longer periods in manganese containing Ringer. There is the possibility that useful information might be obtained on the source of the calcium involved in the twitch contraction by using short exposures to low manganese concentrations and employing an analysis similar to that of Chapman and Niedergerke (1971A) with calcium. However the simple experiments reported here show that manganese produces a rapid inhibition of twitch tension which could well indicate that it produces its inhibition by an action on the surface membrane of the cells.

(2) Intracellular effects of manganese

The contracture development after long periods in manganese Ringer suggests that the sarcoplasmic calcium concentration increases. This could occur by a decreased calcium efflux, an increased influx, a decreased uptake by an internal store or a release of calcium from internal storage sites. The results of Sabatini-Smith (1969) using rabbit heart and Keene et al (1972) with rabbit arterial muscle point to an inhibition of contraction by manganese due to a decreased calcium influx because radioactive calcium

uptake was reduced in manganese solutions. These findings appear to contradict the results of Conrad and Baxter (1963) who injected manganese into live rats then, after two hours, removed and fractionated the hearts. This treatment produced a doubling of the uptake of radioactive calcium by the cells mainly due to uptake in the microsome fraction. However, this increased uptake in vivo was probably the result of a positive inotropic effect of manganese caused by catecholamine release from the thyroid (Conrad, Trendley and Baxter, 1966).

R.A.Chapman and N.G.Rutherford (personal communication) have found that manganese is able to release calcium from rabbit skeletal muscle sarcoplasmic reticulum. An interference by manganese and other divalent cations with calcium uptake by the sarcoplasmic reticulum would be predicted because manganese can partially substitute for magnesium and calcium in the activation of the ATP-ase from rabbit skeletal muscle sarcoplasmic reticulum (Maclennan,1970). Carvalho (1968) reported that cadmium could exchange with calcium in the microsome fraction and that the calcium stimulated ATP-ase activity was inhibited by cadmium. Therefore calcium release or at least a decreased calcium uptake might be anticipated in the presence of cadmium. Therefore more than one effect of manganese might be responsible for the contracture that developes after long periods in manganese solutions.

A possible explanation for the contracture observed after prolonged exposure to manganese solutions is that the cation is able to substitute for calcium in activating the contractile

myofibrils. This possibility is rendered unlikely as the removal of manganese was found to produce a slow increase in contracture tension (Figure 9) at a time when the internal manganese concentration was decreasing (Part 3). However S. Ebashi (personal communication to R.A.Chapman) found that native actomyosin showed some superprecipitation in the presence of manganese but not in nickel or cadmium. Manganese appears to be able to bind to one of the calcium binding sites on the troponin molecule (Fuchs, 1971). He found that radioactive calcium bound to troponin was partially exchangeable with manganese, however the calcium binding at this site is not necessarily physiologically important. It would be of interest to know if this manganese-troponin complex inhibits activity. The only evidence presented by Fuchs along these lines was in the form of a personal communcation from D.J.Hartshorne who found that "cadmium but not manganese can activate the ATP-ase activity of natural actomyosin".

Barium and strontium produced contraction when injected into frog skeletal muscle fibres (Heilbrunn and Wiercinski, 1947) but magnesium was ineffective. The contraction produced by barium and strontium could have been due to calcium displacement from other sites in the muscle.

Seidel and Gergely (1963) found that strontium, cadmium, cobalt and manganese were partially able to reactivate the ATP-ase activity of calcium-free myofibrils but only at concentrations higher than the activating concentrations of calcium. Therefore the possibility of internal effects of manganese cannot be ignored in the analysis of these results, but they would seem to be likely to be small and possibly are not shared by all of the other transition elements.

The sustained contractions produced by depolarisation with potassium-rich solutions were much less sensitive to manganese inhibition than the twitch response. This may well endorse the suggestion that some of the twitch inhibition produced by manganese was due to incomplete excitation i.e. the effects of manganese on the action potential and threshold for stimulation.

(3) The potassium-rich contracture.

The production of substantial contractures by potassium-rich solutions despite the presence of a high concentration of manganese (Figure 27) would be evidence for an internal store of releasable calcium in frog heart if it is assumed that manganese can block calcium influx. However it has been found that external calcium is required for this contracture (R.A. Chapman-unpublished) so that an alternative explanation must be sought. There are various possibilities including (a) the calcium responsible for the contracture comes from the extracellular solution and the potassium depolarisation increases the gradient for the movement of the CaR complex across the cell membrane. (b) the calcium is released from a membrane store, this calcium being in rapid equilibration with extracellular calcium (c) calcium is released from an intracellular store by depolarisation but a small "trigger" calcium influx is required to initiate internal release.

It was suggested that the decrease of the potassium-rich contracture tension by low concentrations of manganese (i.e. up to 5 mM) could indicate what proportion of the response is

highly dependent upon calcium influx across the cell membrane (see Figure 10). The small further decrease in contracture tensions with concentrations of manganese greater than about 5 mM could be explained by hyperpolarising effects. Thus a portion of the potassium-rich contracture remains which is relatively insensitive to manganese inhibition.

An increased calcium uptake in potassium-rich solutions was found in frog heart (Niedergerke, 1959), rabbit aorta (Shibata, 1969) and frog skeletal muscle (Bianchi and Shanes, 1959). However the calcium concentration required to permit potassium contracture development in skeletal muscle appears to be much lower than that for smooth or cardiac muscle. Shibata (1969) found that in rabbit aorta a pretreatment with manganese (1 - 5 mM) prevented the increased calcium uptake associated with potassium-rich solutions.

In frog heart there appears to be an increased calcium influx throughout the whole duration of the potassium-rich contracture because the addition of manganese at any stage of the contracture produced a rapid decrease in tension ($t_{\frac{1}{2}}$ = 6 - 10 seconds with 8 mM manganese) to a new lower level. Therefore the response to potassium-rich solutions in frog heart (and in other muscles to varying degrees) appears to depend upon calcium released internally and calcium influx from the extracellular fluid.

The shape of the tension-depolarisation curve (Figure 11) in the presence of manganese is very similar to that produced by substitution of acetate for chloride in frog ventricle (Anderson and Foulks, 1974). Acetate substitution also

produced a decreased overshoot of the action potential, large increases in resting potential and action potential elongation i.e. similar changes to the early effects of manganese. They concluded that the effects of acetate were probably due to an increased potassium or a decreased sodium conductance. Acetate substitution decreased the potassium-rich contracture tension except at low potassium concentrations. A similar crossover of the tension-depolarisation curves appeared to occur at low potassium concentrations in these experiments with manganese (Page 38 and Figure 11) especially when the curves are corrected for the hyperpolarisation in manganese solutions. A plausible explanation for the reversal at low potassium concentrations of the normal inhibitory effects of manganese and acetate might be indicated by the results of Lindley et al (1973) on denervated frog skeletal muscle. They found that the potassium-rich contracture was approximately 7% inactivated when the muscle was maintained in 2.5 mM potassium control solutions compared to contractures produced after pre-treatment with 0.5 mM potassium Ringer. The hyperpolarisation in 0.5 mM potassium solution relieved the inactivation. Therefore the hyperpolarisation produced by pretreatment with manganese Ringer (or by acetate substitution) could decrease an inactivation of the potassium-rich contracture. This would effectively shift the tension-depolarisation curve to the left and allow tension production with potassium concentrations that were previously subthreshold.

In several tissues the addition of manganese (or other divalent cations) is able to restore the potassium-rich

contracture response after it has been abolished by complete removal of the extracellular calcium. Frank (1962) reported that cadmium, cobalt, nickel, magnesium and manganese were at least partially able to restore the contracture response to potassium-rich solutions in frog skeletal muscle when the contracture had been completely blocked by the removal of external calcium for 30 minutes. In smooth muscle manganese was also found to be capable of restoring the potassium contracture in calcium-free solution (Shibata,1969). The removal of calcium produces depolarisation in many excitable tissues (Shanes,1958) which could increase the inactivation of the potassium-rich contracture. The addition of manganese (or other divalent cations that can substitute for calcium in the maintenance of the resting potential)would therefore remove this inactivation and restore the potassium-rich contracture. These divalent cations could also act by displacing calcium from internal binding sites (Frank 1962) thereby temporarily restoring the potassium-rich response.

(4) The low-sodium contracture.

(a) Sodium substitutes.

The contracture induced by removal of sodium from the bathing solution was found to be very sensitive to manganese inhibition, (the I_{50} being approximately 0.6 mM manganese). The sodium-free contracture could be completely abolished by manganese concentrations (8 - 20 mM) that permitted large contractures in solutions with an elevated potassium concentration. It was therefore suggested that the low-sodium response is primarily dependent upon calcium influx

from the extracellular solution. Of the many sodium substitutes used, most gave large contractures with a fairly similar time course suggesting that it was the removal of sodium and not a positive effect of the substituted cation that produced the contracture. Only small contractures were obtained with magnesium substitution but magnesium would be expected to act as a calcium antagonist (del Castillo and Katz, 1954 A,B; Jenkinson, 1957). The other sodium substitutes (including potassium) produced approximately equal contracture tensions which were near to the maximum tension that the muscle could generate. Hydrazine substitution however resulted in up to 30% larger contracture tensions. This was probably related to the general potentiation of contractile responses in hydrazine solutions. Indeed a small contracture could be induced by the addition of only 12 mM hydrazine to normal Ringer.

(b) Membrane potential changes.

The resting potential measurements in low sodium solutions (Table 3) add further support to the suggestion that the hyperpolarisation in manganese solutions could be due to a decreased sodium conductance of the membrane. The increase in membrane potential in sodium-free solutions can be attributed to the decreased sodium conductance. Under these conditions manganese produced a hyperpolarisation equal to only 50% of that obtained in normal sodium Ringer. Chapman (1974) and Goto et al (1972) found even larger increases in resting potential in sodium-free solutions (approximately 10 mV).

(c) Is the low-sodium contracture dependent upon a calcium-sodium exchange?

The addition of manganese at any time during a sodium-free contracture produced a rapid decrease in tension (e.g. Figure 16). If manganese acts by decreasing calcium influx then the sodium-free contracture must be sustained by a continuous calcium entry. It was suggested (Chapman, 1974) that the spontaneous relaxation of the sodium-free response is due to a decreased sodium efflux as the internal sodium concentration decreases in the absence of external sodium. This decreased sodium efflux would slow the rate of calcium entry if a coupled sodium-calcium exchange mechanism exists. In the experiment illustrated in Figure 16 the return to a high contracture tension level even after long periods in manganese could suggest that the calcium influx block caused by manganese simultaneously results in a decreased sodium efflux i.e. tight coupling in an exchange process. Thus removal of manganese could have resulted in a return to a large contracture tension because the internal sodium concentration was still relatively high, thereby permitting a large sodium-calcium exchange. Some support for this theory can be obtained from the results of Baker et al (1969) where it was found that magnesium probably inhibits the calcium-dependent sodium efflux in squid giant axon. In the barnacle giant muscle fibre manganese inhibits calcium efflux (Russel and Blaustein,1974) into sodium seawater. Although this latter process is the reverse of that described previously it would not seem unreasonable that if manganese inhibits calcium binding it should also inhibit sodium binding to the

exchange system. This calcium efflux was sodium dependent as it decreased in lithium seawater (i.e. lithium was unable to substitute for external sodium) and there was no further decrease by the addition of manganese.

(d) Models of the calcium uptake system.

Two models have been assessed for their applicability to the contracture response to low-sodium solutions. The first model, where the calcium uptake process was assumed to occur via a carrier which could bind to sodium and calcium (or manganese if present) or could exist in a free state, indicated a tight binding of calcium to the carrier. This was not unexpected as Chapman (1974) reported that the sodium-free contracture could occur in calcium concentrations as low as 10^{-5}M and relaxation was only produced if the calcium concentration was reduced to 10^{-7} M. This would indicate a low dissociation constant for the binding of calcium. This suggested that the second model is more appropriate where a sodium-calcium exchange reaction on an anionic carrier is involved and where the carrier can only exist in combination with one of the various cations. Waud (1974) concluded that the responses predicted from both types of model should be similar and may be indistinguishable in some circumstances. He suggested that the high energy changes involved in the production of a free anionic receptor are too large to be feasible i.e. another cation in the bathing solution would automatically replace any cation leaving a carrier.

The second model has therefore been adopted and was found to reasonably predict the observed responses (Figures 19 and

20). However equation 8 (Page 50) predicts a sustained contracture and so cannot account for the spontaneous relaxation. The only other major disparity from the observed responses occurs in very low sodium concentrations in the absence of manganese i.e. an intercept with the tension axis was predicted at values up to twice the observed maximum contracture tension (Figure 20). This discrepancy could be due to a saturation of the contractile system before the calcium influx carrier is saturated. This possibility seems unlikely as up to 30% larger contracture tensions can be produced by e.g. the substitution of hydrazine for sodium. A more likely explanation for the discrepancy is that the low internal sodium concentration limits the calcium influx particularly when the external sodium concentration is reduced to near zero (Chapman,1974). He found that by increasing the internal sodium concentration with strophanthidin the maximum contracture tensions in low-sodium solutions were potentiated. Similarly in the present experiments increasing the internal sodium concentration with low-potassium solutions (Figures 25 and 26) increased the low-sodium contracture tension. Strophanthidin produces membrane depolarisation whereas low-potassium solutions cause hyper-polarisation. It is therefore reassuring that both procedures used to increase the internal sodium concentration produce the same effect on the contracture response suggesting that the potentiation of the low-sodium contracture is unlikely to be due to an effect on the membrane potential. It has also been found in these experiments that in high manganese concentrations contracture tensions were larger in the presence of small amounts of sodium rather than in its complete absence.

The value of n in equation 8 has been assumed to be 2 as the contracture tension is approximately proportional to the square of the calcium concentration, the square root of the manganese concentration and the fourth root of the sodium concentration. In some experiments the relationship of contracture tension to the manganese concentration was slightly steeper than would be anticipated assuming $n = 2$, however not steep enough to suggest that it might be 3. Many possible causes could contribute towards an apparent steeping of the relationship; e.g. (a) a slight calcium-induced calcium release from an internal store (Endo, Tanaka and Ogawa, 1970; Kerrick and Best, 1974). The low-sodium contracture has been assumed to originate from transmembrane calcium influx and not from internal calcium release. However under conditions of calcium loading (as has been suggested to occur in manganese solutions) the possibility of calcium induced release of calcium from an intracellular store is made more likely; (b) the slight hyperpolarisation produced in manganese solutions; (c) an effect by manganese on the potential distribution within the cell membrane. The analysis of the results has been carried out by assuming that the antagonism between manganese and calcium occurs by competitive binding to a receptor site. However in some tissues it has been suggested that a charge shielding effect, where no binding occurs, is completely able to account for the antagonism by divalent cations e.g. D'Arrigo (1973). In Part 1 the adsorption of divalent cations to negatively charged membrane sites was advanced as a possible explanation for some of the effects produced by manganese on the resting and action potential. The

influence of the various divalent cations on the contracture responses described here indicate that competitive binding is likely to be mainly responsible for the observed inhibition. The order of magnitude difference between the extent of the inhibition produced by some of the divalent cations $Cd > Mn \doteq Co \doteq Ni > Mg$ indicates a binding rather than a shielding phenominum. Shielding effects involve no binding energy so approximately equal inhibitory effects of the divalent cations would be anticipated. The low calcium concentration required for the sodium-free contracture suggested a tight binding to a carrier and the low I_{50} for manganese inhibition (approximately 0.6 mM) would be more appropriate to a competitive binding rather than to a charge shielding influence. Charge shielding effects alone could predict most of the inhibitory effects observed in the presence of divalent cations in earthworm longitudinal somatic muscle (Ito et al, 1970), cockroach giant axon (Narashashi, 1966) and crayfish axons (D'Arrigo, 1973). It would therefore seem likely that similar effects occur in other tissues. In fact D'Arrigo (1974) has suggested that acidification can increase the negative charge on cell membranes so that all tissues become charge-shielding sensitive. Thus the contracture responses may well be subject to two inhibitory influences, a competitive binding by manganese effective at low concentrations, and a charge-shielding effect which only has a significant influence in relatively high manganese concentrations. This could explain why the effect of manganese on the maximum upstroke velocity of the action potential was only noticeable in manganese

concentrations above about 8 mM (Hagiwara and Nakajima, 1966). Therefore a small additional inhibitory influence via a charge shielding effect could produce larger inhibitions than those predicted simply from a model involving the competitive binding of inhibitor to a receptor, thereby creating a steeper relationship than anticipated. An actual binding of divalent cations to negatively charged membrane sites could have similar effects on the potential distribution within the membrane (see e.g. Frankenhauser and Hodgkin, 1957). Here, binding energies would be important so an inhibition by the various divalent cations by this method cannot be excluded.

It is interesting that the low-sodium contracture becomes biphasic in the presence of manganese. The biphasic nature of the inhibited contracture might simply be an indication that the contracture in the absence of manganese is also composed of two components. These might not be separable because they are so large and succeed each other so closely. The initial phasic response normally appears slightly less sensitive to manganese inhibition than the tonic contraction. The higher the manganese concentration the slower is the rate of rise of the tonic tension and the longer is the time to peak tension. The initial phasic response appears to have a short time course relatively independent of the manganese concentration. This phasic contraction is unlikely to be due to a non-equilibration of manganese throughout the tissue because pretreatment periods in manganese Ringer from between 10 and 120 seconds produce

similar biphasic responses on removal of sodium, an almost maximal inhibition being observed after 15-30 seconds pre-treatment. Manganese might produce its inhibition by combining with the carrier molecule-calcium complex CaR in the terminology used here) as calcium dissociates inside the cell. This could produce a delay in the inhibition and thus a biphasic contracture. An alternative possibility is that the phasic tension might be an indication that there is a very slow turnover of calcium bound to the carrier so that the phasic response is due to the release into the cell of calcium that was bound prior to the application of manganese. The tonic tension might then be produced as a result of a saturation of internal calcium uptake systems and the continued influx of calcium. If the carrier molecule is able to bind two calciums, or one calcium plus one manganese ion (instead of sodium) then a biphasic contracture could be produced if the R Mn Ca complex moved more slowly across the membrane than the RCa_2 complex.

When the internal sodium concentration is reduced to such a low level that calcium influx is slowed and lower than the rate of calcium uptake by internal calcium accumulating structures then relaxation of the low-sodium contracture could occur.

(5) Induction of tension by sodium and other univalent cations.

The tension induced by the addition of small amounts of sodium to sodium-free solutions containing manganese

could be interpreted as an effect on an internal calcium store. Palmer and Posey (1967) found a sodium-induced release of calcium from rabbit skeletal muscle sarcoplasmic reticulum. The half maximal effect for sodium-induced release being achieved with approximately 1 mM sodium. Lithium was also capable of releasing calcium but to a lesser degree. In mechanically disrupted rat heart (where the surface membrane appears to have been removed) Kerrick and Best (1974) observed sodium induced tension similar to that produced by the addition of caffeine. They therefore concluded that sodium was able to induce calcium release from the sarcoplasmic reticulum in heart muscle. The observation that the sodium induced contracture is blocked by the absence of calcium from the bathing Ringer would suggest that the calcium which activates tension crosses the cell membrane rather than being released from an internal store.

An alternative suggestion to account for sodium induced contraction involves a sodium-dependent increase of calcium influx across the surface membrane. A sodium-calcium exchange has been implicated in the development and subsequent relaxation of the low-sodium contracture. In order to produce a sodium induced tension the added sodium would have to enter the cells. The return of that sodium to the extracellular solution could then promote the influx of calcium. Clearly the sodium entry cannot occur coupled with calcium efflux and still lead to the production of tension. Thus no sodium-induced tension could be observed

following the relaxation of a normal sodium-free contracture (although a maintained increase in tension by the addition of 14 mM sodium was found in goldfish heart by Busselen et al, 1973). In the presence of manganese however the addition of sodium did produce tension (figure 25). Under these conditions it is assumed that some manganese would also be bound to the carrier so that sodium entry could occur coupled to manganese exit. The sodium that had entered could now exchange with external calcium thereby resulting in a net calcium influx and contracture development. A sodium-manganese exchange would not be observable in terms of the tension response of the muscle as neither cation appears to produce tension directly. This type of antagonism has therefore not been included in the derivation of equation 8. R.C.Thomas and R. Vaughan-Jones (personal communcation) using sodium sensitive micro-electrodes have found that the addition of manganese produces a rapid decrease in the internal sodium concentration in crab leg muscle which could indicate a coupled sodium-manganese exchange in this tissue.

Baker and Crawford (1971) using squid axon, and Ashley and Ellory (1972) working with barnacle and crab muscle fibres, have described a magnesium efflux which appears to be dependent upon external sodium and is inhibited by internal calcium. The latter authors postulated a magnesium-sodium exchange carrier which is common to the calcium efflux system i.e. calcium and magnesium could compete internally for binding to the carrier (calcium having a higher affinity than manganese) to exchange with external sodium.

A calcium-manganese exchange (if present) might be expected to produce tension by a manganese efflux. Conditions that were likely to produce an efflux of manganese i.e. manganese removal after some time in high manganese concentrations often produced contracture potentiation e.g. Figure 9 where a larger contracture developed on return to manganese-free Ringer or Figure 16 where a rapid increase to a large contracture tension was produced on manganese removal from the sodium-free solution. However alternative explanations could be advanced to. account for these responses e.g. the removal of an inhibitory effect of external manganese in the case of the former or the maintenance of a high internal sodium concentration in manganese solutions for the latter.

It could be suggested that the rate at which tension is produced on addition of sodium in the present experiments is rather fast if sodium is acting inside the cell membrane. However the rate of tension generation is no faster than that produced by e.g. the addition of caffeine (Figure 28) which only appears to be able to act by releasing intracellular calcium. Therefore it would seem reasonable to suppose that the univalent cations are able to induce tension by an effect on the inside of the cell membrane.

The contracture induced by lithium, hydrazine and hydroxylamine (Figure 24) indicates that the sodium required to produce the sodium induced tension should enter the cell by a route other than via the sodium-calcium exchange carrier if the sodium induced tension results from an internal action

of the sodium. Lithium, hydrazine and hydroxylamine would be required to enter by a different route or these cations would inhibit the low-sodium contracture and no such inhibition was found.

A quite different explanation was advanced by Baker et al (1969) to account for the activation by external sodium and lithium of the sodium-calcium exchange in the squid giant axon. They postulated an extra external site which can accept univalent alkali metal ions to promote sodium and calcium movements through the exchange carrier. If a similar situation exists in frog heart then this "activator" site should also accept the hydrazine and hydroxylamine cations. One drawback to this model is that no sodium or lithium induced contraction was observed in the absence of manganese in these experiments. Hydrazine was however shown to produce a small contracture when added to normal Ringer. The inability of sodium and lithium to induce tension following the relaxation of a sodium-free contracture was possibly due to the low internal sodium concentration (following exposure to sodium-free solutions) being unable to support sodium-calcium exchange. Therefore the results are not inconsistent with the presence of such an external activator site for univalent cations.

Hydrazine, hydroxylamine and lithium have long been known to be capable of substituting for sodium in the maintenance of the action potential in nerve and muscle cells (Lorente de Nó et al, 1957; Koketsu and Nishi 1959; Tasaki, Singer and Watanabe, 1965). Hille (1971, 1973) reported that all

these cations could pass through the sodium channel of the sciatic nerve of the frog with a relatively high permeability ($\geqslant 50\%\ P_{Na}$). Therefore hydrazine, hydroxylamine and lithium would appear to act like sodium at the sodium channel site of the action potential and at the activator site of the sodium-calcium exchange system (be this intracellular at the exchange site itself or extracellular at a separate site), but do not act like sodium at the external site of the exchange system.

(6) Use of low-potassium solutions to increase the intracellular sodium concentration.

In experiments where an attempt was made to increase the internal sodium concentration there was a clear potentiation of contracture responses (e.g. Figure 25 and 26) consistent with the internal sodium being an important factor in regulating contractile activity (Langer,1973; Chapman, 1974). Brindly (1968) produced contractures in barnacle muscle after injecting sodium to increase the internal sodium concentration while substituting lithium for external sodium. Increased internal sodium concentrations also increased calcium influx in squid giant axon (Baker et al, 1969). Novotny and Bianchi (1973) found that both internal sodium and calcium concentrations increase in low potassium solutions in frog heart. In the present experiments it was not possible to say whether the contracture potentiation in low potassium solutions was due to a direct effect of an increased internal sodium concentration or to the general increase in the internal calcium concentration following the increase in internal sodium.

(7) Various effects of D-600

The effects of D-600 are difficult to interpret. The threshold for the twitch increased and the contraction was completely inhibited within a few minutes in 0.4 mM D-600. However the sodium free contracture was unaffected.

D-600 has been reported to inhibit twitch contractions in cat heart (Fleckenstein et al, 1969) toad skeletal muscle (Chiarandini and Bently,1973), guinea-pig atrium (Thyrum, 1974) and to decrease the delayed calcium influx in squid giant axon Baker et al (1973). However it seems unlikely that D-600 only produces its effects by a simple calcium blocking action (Kohlhardt, 1972). The slow onset of the inhibitory effects (Thyrum, 1972) and the inability to wash out its effects even after two hours (Singh and Vaughan-Williams, 1972) are incompatible with such a simple blocking effect.

Verapamil and D-600 have been found to decrease calcium uptake by the sarcoplasmic reticulum from dog heart (Entman et al, 1972) and rabbit skeletal muscle (Balzer,1972) but in much higher concentrations ($I_{50} \doteq 10^{-3}$M) than those required to inhibit twitch contractions ($I_{50} = 10^{-6}$M). In toad skeletal muscle verapamil at 10^{-6} M blocked the Solandt effect (the increase in respiratory rate when the potassium concentration is increased) but had no inhibitory effect on the contracture produced by potassium-rich solutions even at a concentration of 10^{-4} M. The inhibitory effects produced on the acetyl choline contracture could not be reversed by raising the calcium concentration.

Shigenobu et al, (1974) suggested that verapamil is not a specific calcium blocker. The slow sodium channel of embryonic chick heart was blocked by verapamil but was unaffected by manganese. Thus at least in this tissue verapamil appeared to inhibit both sodium and calcium entry during the action potential. In crab leg muscle verapamil and D-600 can produce irreversible contracture (Thomas R.C. and Vaughan-Jones, R., personal communication). Therefore these drugs appear to be able to produce more than one effect on muscle.

Assuming that D-600 can block a late calcium entry in cardiac muscle as it does in squid axon (Baker et al,1973), then this could account for the inhibition of cardiac twitch contractions which are influenced by calcium influx during the depolarisation of the action potential (Weidmann,1959) The lack of an effect on the sodium-free contracture would suggest that D-600 is unable to block the sodium-calcium exchange site and that this calcium entry route differs from the entry route associated with the action potential. Further evidence to support this suggestion comes from the finding by Ashley et al (1974) that D-600 and verapamil were unable to inhibit the increased calcium influx associated with a replacement of extracellular sodium in barnacle muscle fibres. The results of sodium substitution on the contracture responses also suggest that the sodium site of the sodium-calcium exchange carrier differs from the sodium entry channel of the action potential (because lithium, hydrazine etc. do not inhibit the sodium-free

contracture). However in the absence of an acceptable method of voltage clamping frog heart muscle (Johnson and Libermann, 1971; Tarr and Trank, 1974) conclusions involving calcium or sodium currents associated with the action potential must remain very speculative.

The results therefore suggest a sodium-calcium exchange process exists in frog heart muscle. If this occurs via a carrier molecule, then one with properties similar to that suggested by Reuter and Seitz (1968) in guinea-pig heart would be appropriate. They concluded that a carrier with four sites exists capable of binding two calcium, two sodium plus one calcium or four sodium ions. Sodium and calcium appear to compete for binding to these sites as does manganese (and certain other divalent cations) when present. If the exchanges are coupled then two sites could exist on the inside of the membrane and two on the outside. Lithium (or hydrazine or hydroxylamine) might be able to substitute for sodium at the internal sites but not at the external ones. Alternatively these univalent cations act at a separate external activator site. It seems reasonable that the carrier sites should not be identical on both sides of the membrane as the internal calcium and sodium concentrations are much lower than those externally. Garrahan and Glynn (1967) similarly suggested differences in the ion affinities, on internal and external sites of the carrier involved in sodium-sodium exchange in red blood cells.

The results indicate that internal sodium probably has

an important influence on the contractility of the muscle and that calcium has to compete with sodium and manganese for binding sites in order to cross the cell membrane. The model used to produce equation 8 reasonably predicts the observed responses of the muscle. However the long term effects of manganese could suggest that an increase of the internal manganese concentration could have a significant influence on the contracture responses. An attempt has therefore been made to assess the possible uptake of manganese by the tissue.

PART 3. TISSUE ANALYSIS TO DETERMINE MOVEMENTS OF MANGANESE IN VENTRICULAR MUSCLE.

METHODS (3)

(1) Canulation of ventricles for manganese flux measurements.

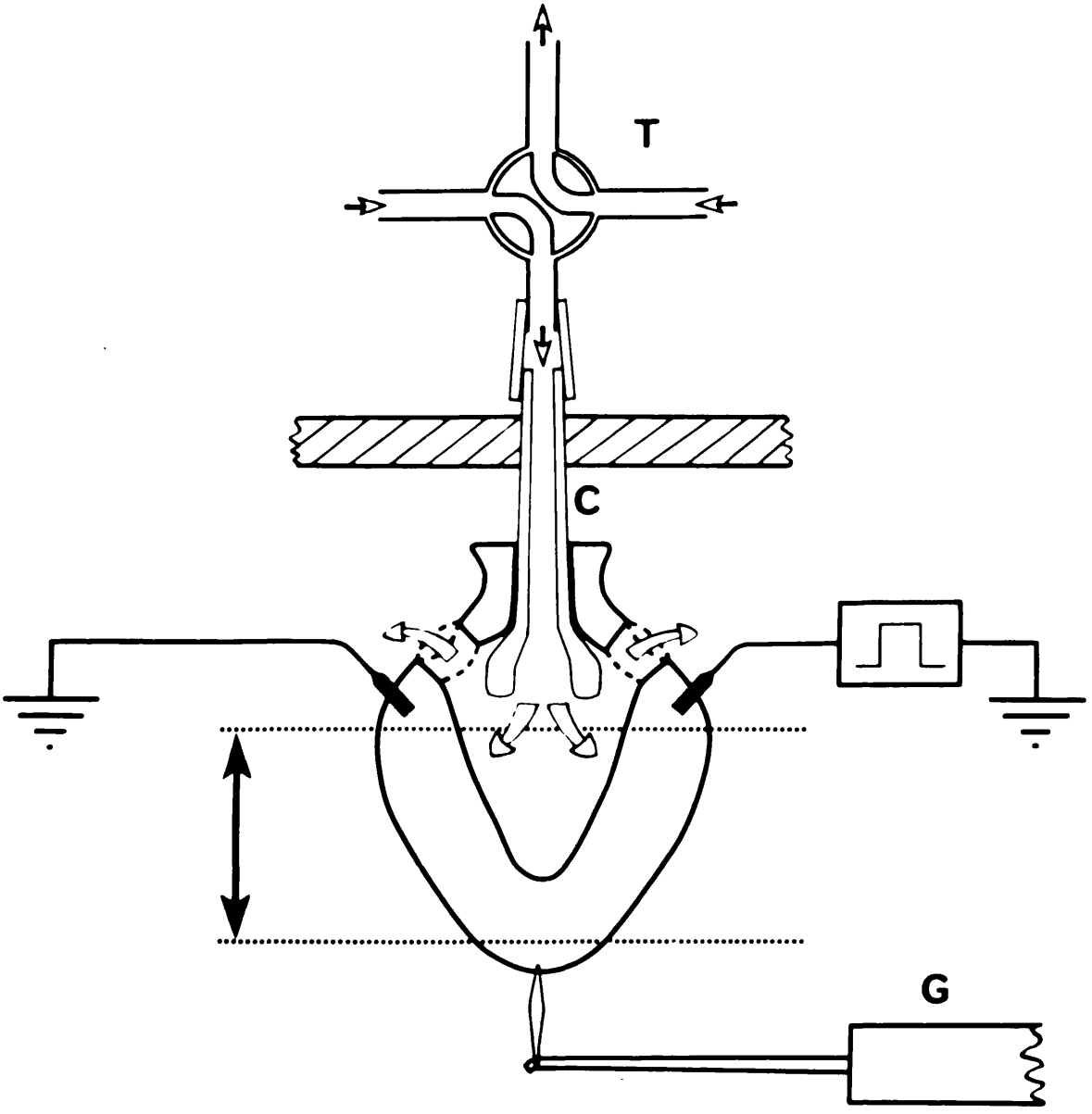
The manganese uptake and its rate of efflux were measured by exposure of whole ventricles to manganese containing Ringer for various times. The manganese content of the ventricle was then determined by atomic absorption spectroscopy of the digested ventricle after various periods of washout in Ringer lacking manganese.

Whole ventricles were used because of the need to use a large amount of tissue to permit accurate analysis. Niedergerke (1963) showed that frog ventricular strips took up more ^{45}Ca for a given tissue weight than did whole ventricles and suggested strips contained more injured tissue which then absorbed more ^{45}Ca. It seems reasonable to assume that injured tissue might absorb more manganese in a similar fashion. Whole ventricles were therefore used with care being taken to try to ensure that damaged tissues were not included in the material used for analysis of manganese content.

In order to obtain a reasonable estimate of the time course of manganese fluxes a fairly rapid perfusion technique is necessary. Whole ventricles were cannulated (Figure 31). Fine nylon thread (100 μ) was used to suture the top of the ventricles to a glass cannula with a bulbous end. Two holes were cut in the top of the ventricular walls for efflux of the perfusate. This ensured that the outer

Figure 31.

A cannulated ventricle for measurement of the manganese content of the tissue. Isolated ventricles were sutured to the cannula (C), the apex being tied to a force transducer (G). The perfusion solutions were changed by the tap(T). After flowing into the ventricle the perfusion solution leaves via two holes cut into the top. The ventricles were stimulated at a rate of 4 min^{-1} through two Ag:AgCl wires positioned near to the cannulated end. The section of tissue between the arrow-heads was analysed for its manganese content.


T
C
G

surface of the heart was also quickly perfused with new solution after a perfusate change and in addition prevented the outside of the heart drying up. Solutions were stored in Marriot bottles to maintain a constant flow rate through the heart of 4 mls per minute. This was sufficient to maintain the ventricle in a slightly distended state approximately equivalent to diastole, except during stimulation when the ventricle showed normal complete contraction. The perfusing solutions were maintained at 22 - 24°C by passing the tubes carrying them to the heart through a water bath. Care was taken to remove all traces of pacemaker tissue (identified by its slightly lighter colouration near the top of the ventricle) since spontaneous contractions, if not prevented, occur at a rate of approximately 20 $minute^{-1}$ at room temperature, which could affect the flux rates.

Stimulation was produced via two $Ag:AgCl_2$ electrodes on opposite sides of the ventricle. Stimulation rate was 4 $minute^{-1}$ at approximately 5 X threshold. In order to maintain complete contraction throughout the ventricle the stimulus was increased in manganese containing Ringer to compensate for the threshold increase.

A piezo-resistive strain gauge (Pye Dynamics TS1) was tied to the apex of the ventricle with fine nylon suture thread. The strain gauge output was monitored by one of the channels of a Devices Pen recorder driven by a Devices D.C.2. amplifier.

(2) Tissue Analysis

At the end of the experiment a section of ventricle (that between the two arrow heads in Figure 31) was removed with two scissor cuts. This ensured that none of the injured tissue, at the top or at the apex of the ventricle was used in the analysis. A single cut was then made along the length of the ventricle. It was opened up and gently blotted for 15 seconds on Whatman No.1 filter paper. The tissue was weighed and added to 3 mls of 2M - nitric acid in a test-tube. After leaching for 24 hours, the nitric acid was evaporated on a sand bath for a further 24 hours. The residue was dissolved in 2.5 mls of distilled water and subsequently analysed for manganese content using an atomic absorption spectrophotometer (Unicam S.P.90 sensitivity 0.1 part per million).

RESULTS. (3)

(1) Estimation of the rate of extracellular solution exchange.

The contracture development observed after long periods in manganese containing Ringer could not be accounted for by a simple competitive inhibition of calcium entry into the cells. Therefore the possibility that manganese itself could enter the cells was investigated. It was found that manganese was taken up by the tissue and an estimate of the rate of uptake and the rate of efflux of manganese has been attempted.

Measurements of these rates are limited to some extent by the rate of change of solution in the extracellular space i.e. by the adequacy of the perfusion procedure. An estimate of the rate of solution exchange in the ventricle can be made because the contraction of frog heart is dependent upon calcium in the perfusion solution (Ringer,1883). The removal of calcium produces a decline in twitch tension and the rate of decline can give an indication of the rate of decrease of the extracellular calcium concentration and thus the rate of exchange of the extracellular solution. As part of the experimental procedure each ventricle was exposed to calcium-free Ringer as is illustrated in Figure 32A and the extracellular clearance time estimated. A rapid decrease in tension was observed in zero-calcium solution. The rate of change of tension with time of perfusion can be shown by plotting twitch tension against

Figure 32.

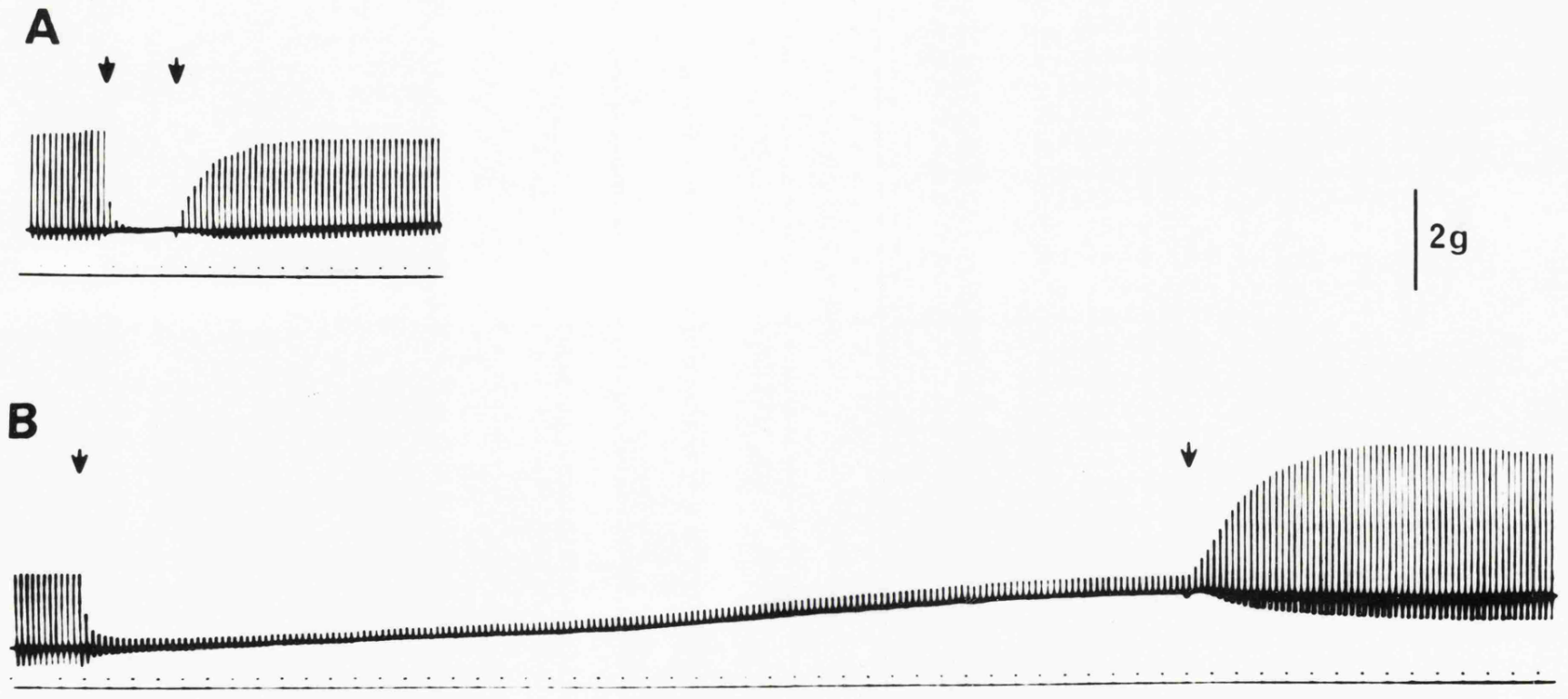
Illustration of the experimental procedure used in the cannulated ventricle experiments. The tension recordings are the ventricle contractions produced in response to electrical stimulation at a rate of 4 min^{-1}.

A. Tension changes produced by removal of calcium from the perfusion solution. Between the arrows the muscle was exposed to calcium-free Ringer.

B. Tension changes produced by the addition of 8 mM manganese to the normal Ringer. This ventricle was perfused (between the arrows) with 8 mM manganese followed by a 15 minute "washout" perfusion period in manganese free Ringer. The ventricle was then removed from the canula and its manganese content assayed.

The time trace below each record shows one minute intervals. The deviation of the relaxed muscle tension away from the time trace illustrates the slowly developing contracture produced in manganese Ringer.

The calcium concentration was 1mM in the normal and in the manganese containing Ringer.


A
2g
B

time as illustrated in Figure 33. The removal of calcium produced a decline of tension (Figure 33, left) which normally demonstrated two approximately exponential phases. There was a slow phase with a mean half-time of change ($t_{\frac{1}{2}}$) of 39.7, S.E. 5.4 sec n = 36) and superimposed on this was a faster component with mean $t_{\frac{1}{2}}$ = 6.7, S.E. 0.7 sec(n = 36). The readdition of calcium produced a return of twitch tension with a single exponential time course (Figure 33, right).

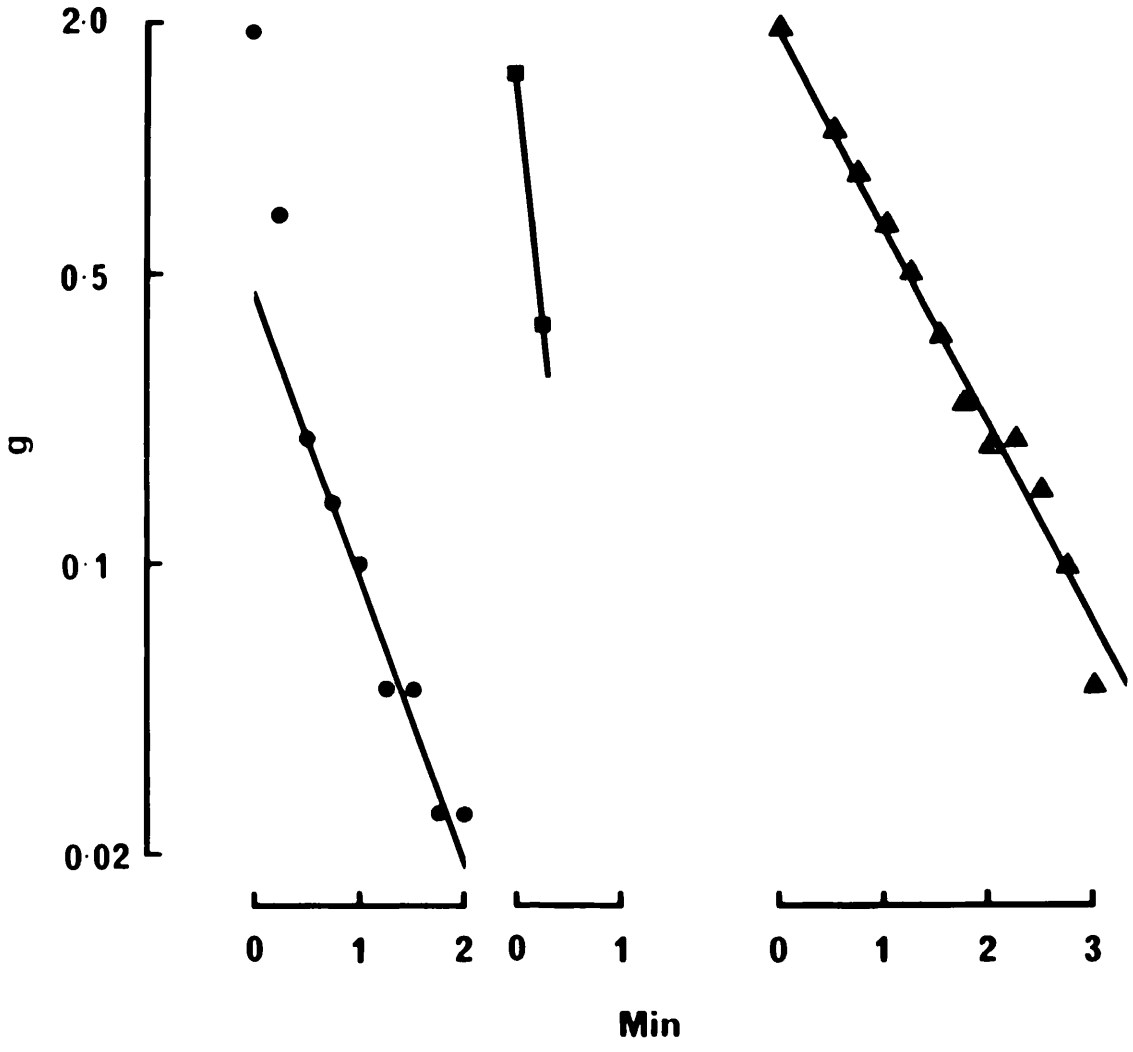
The composite time course of changes of twitch tension produced by an altered extracellular calcium concentration has been previously described for frog ventricle (Chapman and Niedergerke,1970A). The decline of twitch tension with three exponential components was interpreted as an alteration of the calcium concentration that activates contraction in three compartments in the muscle (Chapman, 1971). The fastest component to change was suggested to be that directly in equilibrium with the extracellular solution (Ca_I in the terminology of Chapman, 1971). It therefore seems likely that the fastest component measured in these whole ventricle experiments corresponds to Ca_I . The results therefore indicate that the mean half-time for solution exchange in these whole ventricles could be less than 7 seconds. This would seem to be a reasonable estimate as the perfusion rate was 4 mls minute and the ventricle weight rarely exceeded 100 mg. so that the ventricles were perfused with approximately 40 times their volume of Ringer per minute.

Figure 33

Twitch tension changes produced by the removal and readdition of calcium. The experimental procedure was that illustrated in Figure 32A.

The decline in twitch tension in calcium-free Ringer is shown on the left on semi-logarithmic coordinates. The decline was normally exponential but with a faster component, only observable in the early stages of the decline. This faster component was separated by a curve peeling technique (middle graph). The readdition of calcium produced a return of twitch tension with a simple, single exponential time course (right).

Regression lines were calculated for the points, the half-time for the fast component of tension decline (middle) being taken as the half-time for extracellular solution exchange.


2·0
0·5
0·1
0·02
g
0
1
2
0
1
0
1
2
3
Min

Following the exposure to calcium-free solutions the ventricles were perfused with 1 mM calcium Ringer for at least 15 minutes prior to the addition of manganese.

Two series of experiments were performed to investigate the transmembrane flux of manganese. In one group the ventricles were exposed to 8 mM manganese for 60 minutes followed by a washout period in the absence of manganese for times ranging from 0 to 150 minutes. In the other series the ventricles were perfused with 8 mM manganese Ringer for various periods ranging from $3\frac{1}{2}$ to 120 minutes followed by a fixed washout time of 15 minutes in the absence of manganese.

(2) Manganese efflux from ventricles

The rate of manganese efflux from the cells can be estimated from the results shown in Figure 34. The ventricles were loaded with manganese for 60 minutes. The manganese content of the ventricles after various washout times in manganese-free Ringer has been measured. The efflux shows a slow approximately exponential time course ($t_{\frac{1}{2}}$ = 67.5 minutes) but with a faster component (replotted Figure 34 top right) with a $t_{\frac{1}{2}}$ of 2.0 minutes. The perfusion and analysis technique is inadequate to separate rapidly changing manganese flux components at the start of the efflux period so this phase might itself be composed of more than one component. The results show that the efflux of some of the manganese taken up by the cells is very slow. The presence of a compartment from which manganese is only slowly

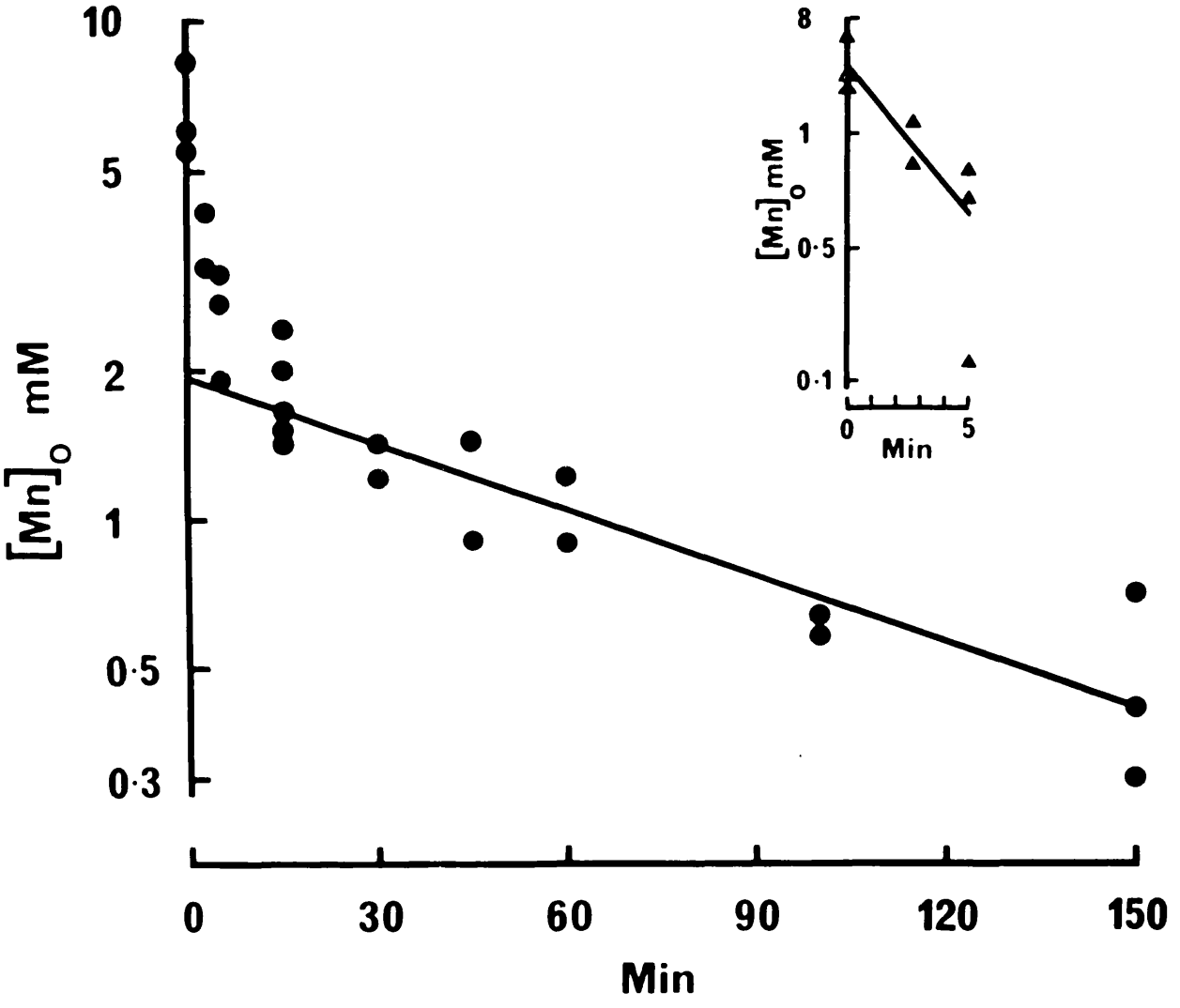
Figure 34.

Manganese content of cannulated ventricles after various periods of "washout" in manganese-free Ringer. The ventricles were perfused with 8 mM manganese for 60 minutes. The perfusion solution was then changed to normal Ringer for the times indicated before the ventricles were removed from the canula and assayed for their manganese content. Each point represents one ventricle. The line has been drawn according to the equation,

$$[Mn] = b\, e^{-kt}$$

with $b = 1.93$, $k = 0.0103\ min^{-1}$ and t=the time in manganese-free Ringer. This is the regression line for the points between 15 and 150 minutes so that a half-time of approximately 67.5 minutes is indicated for the rate of manganese efflux during this period.

The inset shows the faster component of manganese efflux at the beginning of perfusion in manganese-free Ringer. This was separated from the slow component by curve peeling. The regression line for these points gives a half time of 2.0 minutes. This technique is inadequate to separate very rapid changing components e.g. the half-time for extracellular solution exchange has been estimated as approximately 7 secon (Figure 33). Thus more than one component of the efflux is likely to be present during these early stages. The manganes content of five control ventricles i.e. without exposure to manganese was 0.084 ± 0.037 S.D. of mean. Calcium concentrati was 1 mM in all perfusion solutions.


10
5
2
1
0·5
0·3
[Mn]o mM
0
30
60
90
120
150
Min
8
1
0·5
0·1
[Mn]o mM
0
5
Min

released provides a basis for the suggestion that the long term potentiation of contracture responses observed after manganese treatment is due to the presence of manganese within the cells.

(3) Manganese uptake by ventricles.

In these experiments the manganese loading periods ranged from 3½ to 120 minutes. An estimate of the uptake of manganese by the muscle is difficult due to contamination by extracellular solution (the extracellular space has been estimated to be approximately 20% of tissue volume by Niedergerke,1963A). In order to overcome this problem the ventricles were perfused with manganese-free Ringer for 15 minutes after exposure to manganese solutions. This procedure ensured that the extracellular manganese content of the tissue could be neglected. The 15 minute washout procedure means that the rate of manganese uptake by the slow compartment (described in the manganese efflux experiments) was measured. The results are shown in Figure 35 (top). A lot of variability in the uptake is noticeable. One possible reason for this variation was thought to be the contracture development in manganese solution (e.g. Figure 32B). A ventricle that had been exposed to manganese Ringer for 120 minutes was observed to be in strong contracture even after 15 minutes perfusion with manganese-free Ringer. This ventricle had the highest manganese content of all the ventricles analysed. Strong contracture impedes the effectiveness of the perfusion system and could therefore produce a slowed manganese efflux

Figure 35.

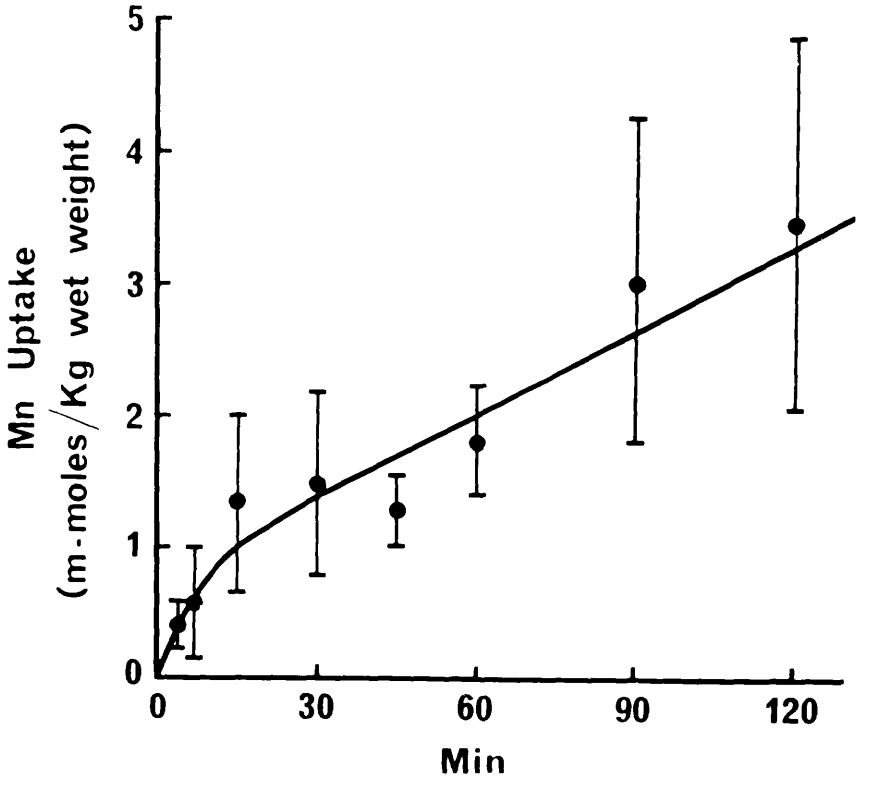
The rate of uptake of manganese by whole canulated ventricles. The ventricles were perfused with 8 mM manganese for the times indicated followed by a 15 minute 'washout' period in manganese-free Ringer to remove extracellular manganese. This washout time means that the uptake of manganese into the slow compartment illustrated in Figure 34 was being analysed. The points are the mean $\pm$ one S.D. of the mean of 5 ventricles at each sampling time. The line through the points (top) has been drawn according to the equation

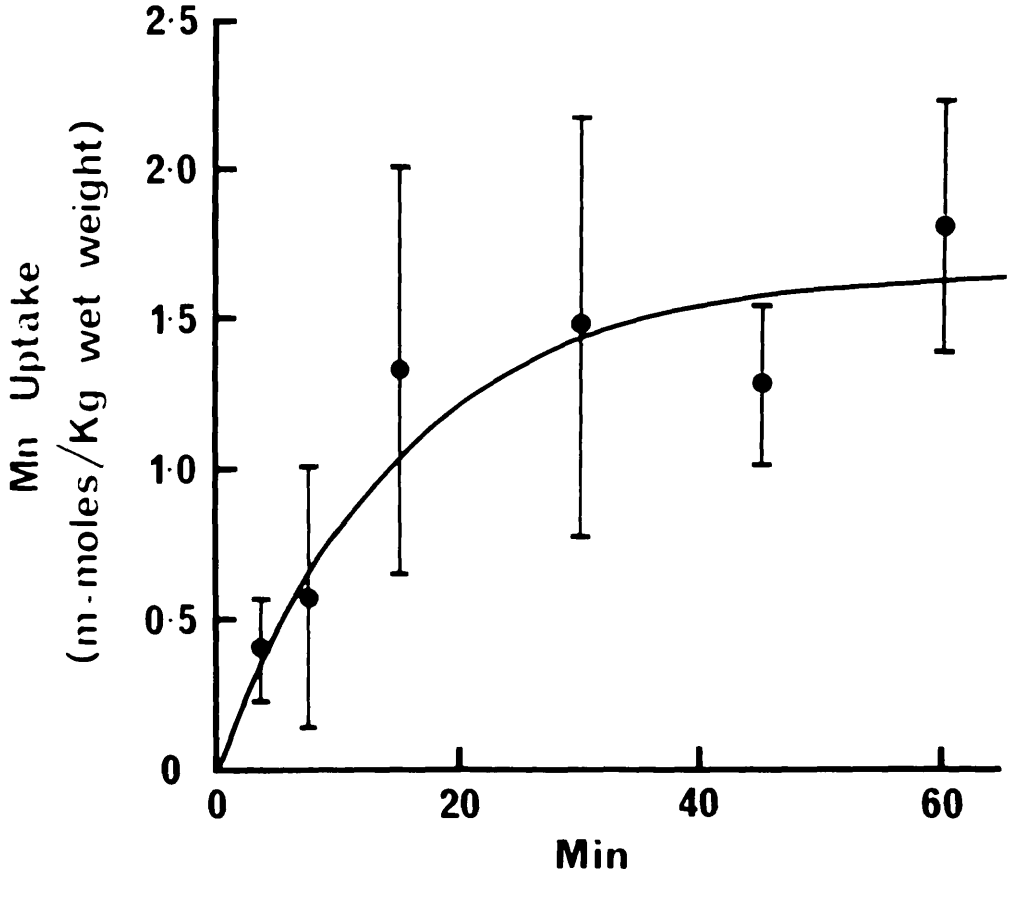
$$[Mn] = a\,t + b(1 - e^{-kt})$$

with $a = 0.21$, $b = 0.75$, $k = 0.15\ min^{-1}$ and t = the time (minutes) of exposure to manganese solution. The results would therefore be accord with an uptake process having a saturable and a non-saturable component. However the strong contracture after very long periods in manganese Ringer suggested that the washout of manganese might be hindered. The results of points up to 60 minutes have therefore been replotted(bottom). The line through the points has been drawn according to the equation

$$[Mn] = b\,(1 - e^{-kt})$$

with $b = 1.65$ and $k = 0.067\ min^{-1}$


Mn Uptake
(m-moles/Kg wet weight)
0
1
2
3
4
5
0
30
60
90
120
Min


Mn Uptake
(m-moles/Kg wet weight)
0
0·5
1·0
1·5
2·0
2·5
0
20
40
60
Min

pattern. Therefore an accurate estimate of the manganese uptake by the muscle might only be possible with short exposures to manganese solutions. With this in mind the results for exposures of manganese only up to 60 minutes have been replotted (Figure 35 bottom) and are shown to reasonably fit the simple equation,

$$\text{Mn content} = b(1 - e^{-kT})$$

where b and k are constants and T is the time of exposure to the manganese containing solution.

The contracture development of whole ventricles in manganese Ringer (Figure 32B) is similar to that observed in auricular trabeculae (Figure 9). Other similarities include the rapid decline of twitch tension on addition of manganese, the slow recovery of the twitch response after long periods in manganese solutions and the potentiated responses on return to manganese-free Ringer.

DISCUSSION (3)

(1) Consequences of manganese accumulation by the muscle.

The results of this final section show that relatively large concentrations of manganese can be taken up by frog heart muscle and that the loss of a significant part of this manganese is very slow although the contractile response recovers relatively quickly. This slow efflux provides an explanation for the long term potentiation of contractures produced in low sodium solutions and the slowly developing contractures observed in normal Ringer containing manganese (Figure 32). Intracellular manganese could produce these effects if it increased the sarcoplasmic calcium concentration. This might occur by several mechanisms:-

(a) Inhibition of calcium efflux. Although an inhibition of calcium efflux by intracellular free manganese seems likely, it is doubtful whether a net increase of the internal calcium concentration could occur by this method because manganese also reduces the calcium influx.

(b) An exchange of intracellular manganese for extracellular calcium in much the same way as sodium has been suggested to exchange could lead to an increased sarcoplasmic calcium concentration.

(c) There will probably be a displacement by manganese of calcium at internal binding sites e.g. many proteins bind calcium but show a greater affinity for manganese (Gurd and Wilcox, 1956).

(d) The numerous mitochondria which store calcium might be expected to release some calcium in exchange for manganese. Mitochondria appear to store calcium as hydroxyapetite, $[Ca_3(PO_4)_2]_3 \cdot Ca[OH]_2$ (Lehninger and Rossi, 1963). They have been shown to be capable of accumulating large quantities of manganese probably as manganese phosphate (Chappel et al, 1963). Lehninger and Rossi (1963) suggest that it is likely that calcium can be replaced by manganese to some extent in mitochondria but Chappel, J.B. (personal communication) suggests that it is doubtful whether manganese can directly displace intramitochondrial calcium. However both groups appear to agree that manganese can compete with calcium for uptake. Therefore an inhibition of the reuptake of calcium released e.g. during stimulation could produce an increased sarcoplasmic calcium concentration.

(e) Manganese has been shown to produce ^{45}Ca release from the sarcoplasmic reticulum of rabbit skeletal muscle (Chapman R.A. and Rutherford N.G., personal communcation) and probably also inhibits uptake.

Manganese can partially exchange with calcium bound to troponin (Fuchs, 1971). Therefore the contracture potentiation observed in manganese solutions could be due to an activation of the contractile proteins by manganese instead of calcium. The low affinity of troponin for manganese might be compensated for by a high internal manganese concentration if a substantial amount of the internal manganese is free in the cytoplasm as the calcium concentration released to produce

maximal activation of contraction is only $1 - 3 \times 10^{-5}$M (Ebashi et al., 1969). However the replacement of calcium by manganese on the troponin molecule does not necessarily indicate that the manganese-troponin complex can permit tension generation as does the calcium-troponin moiety.

The manganese uptake experiments indicated a fairly rapid influx of manganese. However the results do not exclude an even faster uptake as a 15 minute washout period in manganese-free Ringer was used prior to the analysis of the manganese content of the ventricles. A very rapid uptake might therefore be hidden if the efflux was correspondingly fast. The value of the rate constant, K (see subscripts of Figures 34 and 35), of manganese uptake and efflux by the slowly changing compartment was at least six times faster for uptake than for efflux. Sarri and Johnson (1971,1972) found a similar assymetry in the rate of calcium uptake and efflux in rabbit heart, the former being three times faster. They interpreted this as an effect of internal calcium on its own permeability. Regardless of the cause of the assymetry the results show a fairly rapid uptake of manganese and only a slow efflux. This would produce a build-up of the total intracellular manganese concentration with successive exposures to manganese containing solutions.

CONCLUDING DISCUSSION

(1) Manganese entry into muscle cells during the action potential.

Recent work on cardiac muscle has suggested that calcium influx during the depolarisation of the action potential is an important regulator of contractility. In general, exposure of cardiac muscle to manganese produces a negative inotropic effect. If this decrease in contractility is due to a decreased calcium influx during the action potential then manganese would appear to inhibit this influx. There is some evidence e.g. Ochi (1971) that manganese can substitute for calcium in this process because a slow inward current is observed and action potentials are produced in manganese solutions free of sodium and calcium in guinea-pig heart muscle. Other workers have also suggested that this calcium entry route is not specific for calcium e.g. strontium and barium can substitute (Masher,1973; Pappano and Sperelakis 1969; Vereecke and Carmeliet 1971) and an entry of ^{28}Mg during the action potential has been observed in squid axon (Baker and Crawford 1971). The present results give evidence for an entry of manganese during the action potential in frog heart. A 5 mV decrease in overshoot was produced with 2 mM manganese but higher concentrations produced little; or no further decrease. The small reduction in overshoot with high manganese concentrations (e.g. approximately 6.5 mV with 16 mM manganese) was much less than would be expected from the work of Niedergerke and Orkand (1966) who

found that the overshoot decreased by 18.3 mV for a ten-fold reduction in the calcium concentration (the present experiments have demonstrated an approximate equivalence of the effects of calcium and manganese on the threshold for stimulation, resting membrane potential and action potential duration). Therefore it is suggested that manganese contributes to the inward current during the action potential. This would counteract the decrease in overshoot due to inhibition of calcium entry by manganese. This manganese entry might be the major cause of the large manganese accumulations described in Part 3. Such accumulations would reduce the manganese equilibrium potential and tend to decrease the overshoot if a manganese inward current was operative at this time in much the same way as occurs in high calcium solutions (Niedergerke and Orkand, 1966B). However such a reduction would be prevented if the sarcoplasmic manganese concentration was decreased by internal binding or compartmentalisation.

(2) Surface membrane effects of manganese.

Experiments described in Part 1 have indicated that the large threshold increases in manganese and the decrease in the rate of rise of the action potential (Hagiwara and Nakajima 1966) are due to a decrease in the peak sodium conductance of the membrane. The hyperpolarisation observed in manganese solutions seems unlikely to be due to an effect on the potassium conductance (Orkand,1962; Chiarandini and Stefani,1973) and would be produced by a decrease in the

resting sodium conductance. This increase in membrane potential in the presence of manganese has been shown to be incapable of producing the large increases in the stimulation threshold observed (even when any possible release of endogenous catecholamines or acetyl choline is blocked). Therefore a charge shielding or absorption effect of manganese at negatively charged membrane sites probably occurs. These effects would alter the electric field within the membrane thereby reducing the sodium(and possibly calcium) conductance.

Graded action potentials were produced in manganese solutions with stimuli near to the threshold. This is evidence for the theory that the plateau component of the action potential is sustained by neighbouring cell interaction e.g. the syncytial nature and slow conduction velocity of the tissue could permit re-enterant excitation and thus prolongation of the plateau. This would mean that the long action potential of frog heart (much longer than that of mammalian cardiac tissue) is primarily a property of the slow conduction velocity and spongy structure of the tissue.

(3) Inhibition of Contraction by manganese.

The results of Part 1 have also shown that quantitative studies on the effect of manganese on the normal twitch contraction are rendered difficult and probably inaccurate due to four major complicating factors:

(a) Biphasic changes in the action potential duration and thus the duration of the active state.

(b) Variation in the action potential duration with stimulus intensity in the presence of manganese.

(c) Stimulation threshold increases.

(d) Contracture development with longer exposures to manganese.

Therefore analysis was confined to the contractures produced by depolarisation with high potassium solutions and those produced (without large changes in membrane potential) by low sodium solutions. Manganese produced large inhibitions of the contracture responses elicited by a reduction of the external sodium concentration (I_{50} approximately 0.6 mM). For these contractures the tension produced was proportional to ratio $[Ca]_o / [Mn]_o$. The similarity of the inhibitory effects of sodium and manganese on contracture tension led to the derivation of equation 8 i.e.

$$C_{max} = \propto \left[\frac{\omega\ Ca}{Ca + \frac{Na^2}{K_1} + \frac{Mn}{K_2}} \right]^n$$

This assumes that sodium and manganese show a simple competitive antagonism towards calcium for external binding sites which permit the entry of calcium into the cell and subsequently the generation of tension. The results indicate a tight binding of the cations to an anionic receptor site. In this process the cations can be envisaged as undergoing ion exchange reactions, the relative affinities for binding being in the order $Ca > Mn > Na$. The other divalent

cations used in this work show inhibitory effects similar to that of manganese on the low sodium contracture, the degree of inhibition being Cd > Co ≏ Mn ≏ Ni > Mg.

Manganese caused much less inhibition of the contractures induced by depolarisation of the cell membrane with high potassium solutions than those produced by low external sodium. This appears to be due to the fact that the effect of sodium is very similar to manganese and sodium was present in much higher concentrations than manganese in the potassium-rich solutions. Therefore the effects of manganese were being observed against the background of the large inhibitor concentration already present. Previous work has suggested that the potassium depolarisation contracture is largely dependent upon the release of internal calcium. The lack of a large inhibitory effect of manganese on the potassium-rich contracture could thus also be an indication of this type of internal calcium release.

The resistance of the potassium-rich contracture to inhibition by manganese is less when the external calcium concentration is reduced (Figure 10). This might indicate that calcium entry is required in order to trigger internal calcium release. From experiments like the one illustrated in Figure 10 it appears that a proportion of the contracture is sensitive to manganese inhibition (large initial decline in contracture tension with increasing concentrations of manganese) while another part is relatively insensitive (the small decrease in tension between 4 and 16 mM manganese could be due to the hyperpolarising effect of manganese

combating the extent of the depolarisation). The manganese dose-dependence of the initial decline is similar to that of the inhibition of the low sodium contracture.

(4) Tension induced by univalent cations.

It has been shown that following exposure to low sodium solutions, in the presence of manganese, the addition of sodium, lithium, hydrazine and hydroxylamine induces contraction. This led to the proposition that sodium might have two actions, one inside and one outside the cell membrane (Chapman and Ochi, 1971). Sodium inhibits calcium entry at the external surface of the membrane but could potentiate the entry of calcium by its presence at an internal membrane site. The receptor sensitivities at the internal and external sites should differ because of the vastly different sodium and calcium concentrations in the internal and external environments (but of course it is the localised sodium and calcium activities adjacent to the membrane sites which are important. These are difficult to estimate and may differ greatly from the values calculated on the basis of tissue analysis).

An alternative interpretation of these results is that sodium, lithium, hydrazine and hydroxylamine can act at an external activator site to increase the rate of the sodium-calcium exchange system.

(5) The potentiation of contraction by manganese.

During the course of these experiments it became apparent that manganese could induce an activation of contractile

activity contrary to its normal inhibitory effects. This exhibited itself in the slow contracture development with prolonged exposure to manganese solutions (Figures 9 and 32b), the slow partial recovery of twitch tension following the initial rapid decline and the potentiation of successive low-sodium contractures throughout an experiment. This opened the possibility that manganese was taken up by the cells during exposure to manganese containing solutions and produced its activating effects internally. In experiments designed to test this suggestion, it was found that there was a rapid uptake and a relatively slow loss of part of the manganese absorbed which led to its accumulation by the muscle.

Despite the large accumulations of manganese found in these experiments the possibility cannot be excluded that compartmentalisation in the cell reduces the sarcoplasmic free manganese to very low levels. The mitochondria, which are known to be capable of absorbing large quantities of manganese and are very numerous in frog heart, might play a major rôle in this respect. The slow time course of some of the efflux of manganese from the cells (Figure 34) could indicate such a compartmentalisation. The faster component of manganese efflux (Figure 34, top right) was much slower than the extracellular solution exchange time and could therefore correspond to the rate of efflux of manganese from the sarcoplasm. If manganese carries current during the action potential then on return to manganese-free solutions this current will be outward as the equilibrium

potential for manganese is now very negative. This fast component might therefore be the result of manganese efflux via this route.

Apart from the mitochondria the sarcoplasmic reticulum might also help decrease the sarcoplasmic manganese concentration. Any such manganese uptake has been shown to have little effect on the caffeine contracture (Figure 29) i.e. on the calcium released by the addition of caffeine, this calcium being presumed to originate from the sarcoplasmic reticulum. This indicates that, at least in the short term, any manganese uptake by the sarcoplasmic reticulum is not accompanied by a substantial loss of the calcium releasable by caffeine.

The binding of sarcoplasmic manganese to proteins and glycoproteins is likely to be an important feature of the reduction of the activity of free manganese in the sarcoplasm. The affinity of manganese binding is greater than that of calcium in many cases (Gurd and Wilcox 1956) so the entry of manganese could produce internal calcium release. This would contribute to the potentiating effects observed in manganese solutions.

The possibility that manganese can activate contraction by substitution for calcium on the troponin molecule has not been satisfactorily resolved. More experiments are required on extracted contractile proteins and the ions required for their activation. If the long term potentiating effects of manganese are due to a direct activation of

contraction then the ability of manganese in this respect must be small because of the relatively small effects observed (although this could conceivably be due simply to a very active reduction of the free sarcoplasmic manganese concentration to very low levels).

(6) Comparison of manganese with other calcium inhibitors.

One of the primary objectives of this work was to try to estimate the usefulness of manganese as a typical competitive inhibitor of calcium entry in order to investigate the calcium entry system. Various other ions and compounds have been used in the past to block calcium entry. Some of these have been tested in the present experiments for comparison with manganese.

The drugs D-600 and verapamil appear to have been used successfully previously to inhibit calcium entry e.g. in cardiac muscle (Fleckenstein et al. 1969) and squid axon (Baker et al. 1973). In the present experiments D-600 increased the threshold for stimulation, inhibited the potassium rich contracture to a small extent but did not inhibit the contracture produced by removal of sodium. This means that if D-600 inhibits the slow inward current of voltage clamp experiments (which has been presumed to be the calcium entry required for the twitch contraction) then the low-sodium contracture is not produced by calcium entry via this route. Therefore does D-600 block calcium entry via the sodium-calcium exchange system? In barnacle muscle fibres the answer appears to be no (Ashley et al. 1974). If

the low sodium contracture in frog heart is due to sodium-calcium exchange then D-600 does not block this type of calcium movement. It would be of interest to know if D-600 blocks the better characterised sodium-calcium exchange observed in the squid giant axon.

Of the other divalent cations employed magnesium appears similar to manganese but only at approximately ten-fold higher concentrations. Cobalt and nickel produced similar effects to manganese but cadmium was approximately ten times more potent an inhibitor.

In conclusion, manganese appears to be a useful tool as an antagonist of calcium entry, although any ion which itself enters cannot be considered completely satisfactory especially as internal effects of manganese have been alluded to. However these effects are relatively small in magnitude. Measurements of the uptake of magnesium, nickel, cobalt and cadmium have not been attempted but might show that they are more suitable in this respect. The usefulness of manganese in the estimation and elimination of calcium fluxes in frog heart might only be possible because of a very efficient internal binding thereby reducing the free sarcoplasmic manganese concentration to near control levels.

SUMMARY

1. The effects of manganese (and some other transition elements) on the electrical and contractile properties of frog heart muscle have been investigated.

2. The action potential became graded in the presence of manganese i.e. both the overshoot and the action potential duration were dependent upon the intensity of stimulation.

3. Manganese produced only small decreases in the overshoot and duration of the action potential with relatively high stimulus intensities. The results suggest that manganese inward current may replace that normally produced by calcium movements.

4. The cells were hyperpolarised by approximately 10 mV for a ten-fold increase in the manganese (or calcium concentration). This hyperpolarisation could not completely account for the large increases in the threshold stimulus for contraction produced in manganese and high calcium solutions. These effects produced by manganese (and similar changes produced by the other divalent cations employed) are consistent with a charge shielding or absorption effect of these ions on the cell membrane.

5. Many of the changes in the action potential, resting potential and excitability of the tissue could be explained by a decreased sodium conductance of the cell membrane together with an inhibition of calcium fluxes.

6. The twitch contraction was rapidly and reversibly inhibited by manganese but the complicated changes produced on the excitation process make quantitative assessment of this inhibition difficult.

7. The contracture responses produced by low-sodium solutions were greatly inhibited by manganese (I_{50} approximately 0.6 mM) and are largely accounted for by assuming a competitive inhibition of calcium binding by manganese. This type of inhibition appeared similar to that produced by sodium.

8. The drugs D-600 and verapamil produced little or no inhibition of the low-sodium contracture.

9. Sodium, lithium, hydrazine and hydroxylamine were able to induce contraction in the presence of manganese following exposure to sodium-free conditions.

10. Increasing the intracellular sodium concentration by exposure to low potassium solutions potentiated both the low-sodium contracture and the twitch contraction. The results add further support to the suggestion that intracellular sodium is important in the regulation of calcium influx.

11. Manganese produced less inhibition of the potassium-rich contracture than of that produced by low-sodium solutions. This suggests that either another site is available for calcium release during depolarisation which is relatively insensitive to manganese inhibition or that depolarisation increases the gradient for the inward movement of the

calcium complex responsible for contraction.

12. The hyperpolarisation in manganese solutions is insufficient to account for the inhibition of the contractures produced by low-sodium or potassium-rich solutions.

13. Contractures produced by the addition of caffeine were not inhibited by manganese. This is consistent with the idea that the inhibitory effect of manganese is due to an action at the cell membrane.

14. Measurements of the uptake of manganese by whole canulated ventricles indicated a large accumulation and a slow loss of part of the accumulated manganese on return to manganese-free solutions.

15. Although manganese appears to be a useful tool in the study of calcium fluxes and contraction of frog heart muscle the effects of the manganese that accumulates inside the cells and the effects of manganese on the excitation process often produce complications.

REFERENCES

ADRIAN, R.H. (1956). The effect of internal and external potassium concentration on the membrane potential of frog muscle. J.Physiol. 133, 631-658.

ADRIAN, R.H. and FREYGANG, W.H. (1962). The potassium and chloride conductance of frog muscle fibres. J.Physiol. 163, 61-103.

ANDERSON, E.R. and FOULKS, J.G. (1974). Mechanism of the effect of acetate on frog ventricular muscle. Can. J.Physiol. Pharmac. 52, 404-423.

ASHLEY, C.C. and ELLORY, J.C. (1972). The efflux of magnesium from single crustacean muscle fibres. J.Physiol. 226, 653-674.

ASHLEY, C.C., ELLORY, J.C. and HAINAUT, K. (1974). Calcium movements in single crustacean muscle fibres. J.Physiol. 242, 255-272.

BABSKII, E.B. and DONSKIKH, E.A (1972). Opposite nature of the effect of manganese and nickel ions on action potentials of myocardial fibres. Doklady Akademii Nauk S.S.S.R. 209, 699-702.

BAKER, P.F. (1972). Transport and metabolism of calcium ions in nerve. Progr. Biophys. Mol. Biol. 24, 177-223.

BAKER, P.F., BLAUSTEIN, M.P., HODGKIN, A.L. and STEINHARDT, R.A. (1969). The influence of calcium on sodium efflux in squid axons. J.Physiol. 200, 431-458.

BAKER, P.F. and CRAWFORD, A.C.(1971). Sodium dependent transport of magnesium ions in giant axons of Loligo forbesi. J.Physiol. 216, 38P-39P.

BAKER, P.F., MEVES, H. and RIDGEWAY, E.B. (1973). Effects of manganese and other agents on the calcium uptake that follows depolarisation of squid axons. J.Physiol. 231, 511-526.

BAKER, P.F., MEVES, H. and RIDGEWAY, E.B. (1973). Calcium entry in response to maintained depolarisation of squid axons. J.Physiol. 231, 527-548.

BALZER, H. (1972). The effects of quinidine and drugs with quinidine-like action (propranolol, verapamil and tetracaine) on the calcium transport system in isolated sarcoplasmic reticulum vesicles of rabbit skeletal muscle. Naunyn Schmied. Archs. Pharmac. 274, 256-272

BEELER, G.W. Jnr. and REUTER, H.(1970A). Membrane calcium current in ventricular myocardial fibres. J.Physiol. 207, 191-209.

BEELER, G.W. Jnr. and REUTER, H.(1970B). The relation between membrane potential, membrane currents, and activation of contraction in ventricular myocardial fibres. J.Physiol. 207, 211-229.

BENDALL, J.R. (1953). Further observations on a factor (the "Marsh" factor) effecting relaxation of ATP-shortened muscle fibre models, and the effect of Ca and Mg ions upon it. J.Physiol. 121, 232-254.

BIANCHI, C.P. and SHANES, A.M. (1959). Calcium influx in skeletal muscle at rest, during activity and during potassium contracture. J.Gen.Physiol. 42, 803-815.

de BOER, S. (1918). Le liquide de perfusion des coeurs de grenouilles d'ete. Arch néerl. Physiol. 2, 352-357,

BOSTEELS, S., and CARMELIET, E. (1972). Estimation of intracellular Na concentration and transmembrane Na flux in cardiac Purkinje fibres. Pflugers. Archs. 336, 35-47.

BRADY, A.J. and WOODBURY, J.W. (1960). The sodium-potassium hypothesis as the basis of electrical activity in the frog ventricle. J.Physiol. 154, 358-407.

BRINDLY, F.J. and MULLINS L.J. (1968). Sodium fluxes in internally dialysed squid axons. J.Gen.Physiol. 52, 181-211.

BUSSELEN, P. and CARMELIET, E.(1973). Protagonistic effects of Na and Ca on tension development in cardiac muscle at low extracellular Na concentrations. Nature N.B. 243, 57-58.

CALDWELL, P.C. and WALSTER, G. (1963). Studies on the micro-injection of various substances into crab muscle fibres. J.Physiol. 169, 353-372.

CARAFOLI, E., TIOZZO, R., LUGLI, G., CROVETTI, F., KRATZING, C. (1974). The release of calcium from heart mitochondria by sodium. J. Mol. Cell. Cardiol. 6, 361-371.

CARSTEN, M.E. (1964). The cardiac calcium pump. P.N.A.S. 52, 1456.

CARVALHO, A.P. (1968). Effects of potentiators of muscular contraction on binding of cations by sarcoplasmic reticulum. J.Gen.Physiol. 51, 427-442.

del CASTILLO, J. and KATZ, B. (1954A). The effect of magnesium on the activity of motor nerve endings. J.Physiol. 124, 553-559

del CASTILLO, J. and KATZ, B.(1954B). Changes in the end-plate activity produced by pre-synaptic polarisation. J.Physiol. 124, 586-604.

CHAPMAN, R.A.(1971). Is there a T-system in frog cardiac muscle cells? J.Physiol. 215, 48P-49P.

CHAPMAN, R.A.(1971). Experimental alteration of the relationship between the external calcium concentration and the contractile force generated by auricular trabeculae isolated from the heart of the frog Rana pipiens. J. Physiol. 218, 147-161.

CHAPMAN, R.A.(1974). A study of the contractures induced in frog atrial trabeculae by a reduction of the bathing sodium concentration. J.Physiol. 237, 295-313.

CHAPMAN, R.A. and MILLER, D.J. (1972). Caffeine contractures induced in frog auricular trabeculae in the absence of external calcium. J.Physiol. 225, 52P-54P.

CHAPMAN, R.A. and MILLER, D.J. (1974). The effects of caffeine on the contraction of the frog heart. J.Physiol. 242, 589-613.

CHAPMAN, R.A. and NIEDERGERKE, R.(1970A). Effect of calcium on the hypodynamic frog heart. J.Physiol. 211, 389-421.

CHAPMAN, R.A. and NIEDERGERKE, R. (1970B). Interaction between heart rate and calcium concentration in the control of contractile strength of the frog heart. J.Physiol. 211, 423-443.

CHAPMAN, R.A. and OCHI, R.(1971). The effects of manganese ions on the contractile responses of isolated frog atrial trabeculae. J.Physiol. 222, 56P-58P.

CHAPMAN, R.A. and TUNSTALL, J. (1971). The dependence of the contractile force generated by frog auricular trabeculae upon the external calcium concentration. J.Physiol. 215, 139-162.

CHAPPEL, J.B., COHN, M. and GRANVILLE. (1963). "Energy linked functions of Mitochondria". Page 219. Edit.B. Chance. Acad. Press. New York.

CHIARANDINI, D.J. and BENTLY, P.J. (1972). The effects of verapamil on metabolism and contractility of the toad skeletal muscle. J.Pharmac. Exp. Therap. 186, 52-59.

CHIARANDINI, D.J. and STEFANI, E.(1973). Effects of manganese on the electrical and mechanical properties of frog skeletal muscle. J.Physiol. 232, 129-147.

CONRAD, L.L. and BAXTER, D. J.(1963). Effects of manganese on Q-T interval and distribution of calcium in rat heart. Am. J.Physiol. 205, 1209-1212.

CONRAD, L.L., TRENDLEY, R.L. and BAXTER, D.J. (1966). Positive inotropic effect of manganese on dog myocardium. Am. J. Physiol. 210, 357-359.

CORABOEUF, E. and VASSORT, G. (1967). Effects de la tétrodotoxine du tétréthylammonium et du manganèse sur l'activité du myocarde de Rat et de Cobaye.

CORABOEUF, E. and VASSORT, G.(1968). Effect of some inhibitors of ionic permeabilities on ventricular action potential and contraction of rat and guinea-pig heart. J. Electrocard. 1, 19-30.

COSMOS, E. and HARRIS, E.J. (1961). In vitro studies of the gain and exchange of calcium in frog skeletal muscle. J. Gen.Phys. 44, 1121-1130.

COSTANTIN, L.L. (1968). The effect of calcium on the contraction and conductance threshold in frog skeletal muscle. J.Physiol. 195, 119-132.

COSTANTIN, L.L., FRANZINI-ARMSTRONG G. and Podolsky, R.J. (1965). Localisation of calcium-accumulating structures in atrial muscle fibres. Science, 147, 158-159.

D'ARRIGO, J.S. (1973). Possible screening of surface charges on crayfish axons by polyvalent metal ions. J.Physiol. 231, 117-128.

D'ARRIGO, J.S. (1974). Axonal surface charges: Binding or screening by divalent cations governed by external pH. J.Physiol. 243, 757-764.

DALTON, J.C. (1958). Effects of external ions on membrane potentials of lobster giant axon. J.Gen. Physiol. 41, 529-542.

DODGE, F.A. and RAHIMIMOFF, R. (1967). Co-operative action of calcium ions in transmitter release at the neuro-muscular junction. J.Physiol. 193, 419-432.

EBASHI, S., EBASHI, F. and KODAMA, A. (1967). Troponin as the Ca^{++} receptive protein in the contractile system. J.Biochem. 62, 137-138.

EBASHI, S., ENDO,M., OHTSUKI, I. (1969). Control of muscle contraction. Quart. Rev.Biophy. 2, 351-384.

EBASHI, S. and KODAMA, A. (1966). Interaction of troponin with F-actin in the presence of tropomyosin. J.Biochem. 59, 425-426.

EBASHI, S. and LIPMANN, F. (1962). Adenosine triphosphate linked concentration of calcium ions in a particulate fraction of rabbit muscle, J.Cell. Biol. 14, 389-400.

EDWARDS, C. and LORKOVIĆ, H. (1967). The rôles of calcium in the excitation-contraction coupling in various muscles of the frog, mouse and barnacle. Am. Zool. 7, 615-622.

ENTMAN, M.L., ALLAN, J.C., BARNET, E.P., GILLETTE, P.C., WALLICK, E.T. and SCHWARTZ, A. (1972). Mechanisms of calcium accumulation and transport in cardiac relaxing system (Sarcoplasmic reticulum membranes): Effects of verapamil, D-600, X537A, and A23187. J.Mol. Cell Cardiol. 4, 681-687.

ENDO, M., TANAKA, M. and OGAWA Y. (1970). Calcium induced release of calcium from the sarcoplasmic reticulum of skinned skeletal muscle fibres. Nature. 228, 34-36.

FATT, P. and GINSBORG, B.L. (1958). The ionic requirements for the production of action potentials in crustacean muscle fibres. J.Physiol. 142, 516-543.

FLECKENSTEIN, A., TRITTHART, H., FLECKENSTEIN, B., HERBST, A., GRÜN, G.(1969). Eine neve Gruppe kompetitiver Ca^{++} Antagonisten (Iproperatril, D-600, Prenylamin) mit storken Hemmelffekten auf die elektromechanische Koppling im Warmbluter - Myokard. Pflug. Arch. 307, R25.

FORD, L.E. and PODOLSKY, R.J. (1970). Regenerative calcium release within muscle cells. Science. 167, 58-59.

FRANK, G.B. (1962). Utilisation of bound calcium in the action of caffeine and certain multivalent cations on skeletal muscle. J.Physiol. 163, 254-268.

FRANKENHAEUSER, B. and HODGKIN, A.L. (1957). The action of calcium on the electrical properties of squid axons. J.Physiol. 137, 218-224.

FUCHS, F. (1971). Ion-exchange properties of the calcium receptor site of troponin. B.B.A. 245, 221-229.

FUKUDA, Y. (1972). Mechanism of calcium induced fibrillation in the toads heart. Jap. J.Physiol. 22, 25-37.

GARRAHAN, P.J. and GLYNN, I.M. (1967). The behaviour of the sodium pump in red cells in the absence of external potassium. J.Physiol. 192, 159-174.

GILBERT, D.L. and EHRENSTEIN, G. (1969). Effect of divalent cations on potassium conductance of squid axons: Determination of surface charge. Biophysical Jnl. 9, 447-463.

GOTO, M., ABE, Y. and KAWATA, H. (1961). An analysis of the intracellular action potential of the cardiac muscle. Kyushu J. Med. Sci. 12, 187-195.

GOTO, M., and BROOKS, C. McC. (1969). Separable spike and plateau action potentials and their rôles in contraction of frog ventricle. Proc. Soc. Exp. Biol. and Med. 131, 1427-1431.

GOSSWEILER, N., KIPFER, K., PORETTI, G., RUMMEL, W. (1954). Der Einflub von Callum auf den Kaliumaustritt aus Muskelgewebe. Pflugers Archiv. 260, 154-160.

GRAHAM, G.D., BENNET, R.B. andWARE, F. (1969). Potassium effects on transmembrane potentials in frog ventricle. Am. J. Physiol. 216, 1360-1366.

GURD, F.R.N. and WILCOX, P.E. (1956). Complex formation between metallic cations and proteins, peptides, and amino acids. Adv. Prot. Chem. 11, 311-427.

HAGIWARA, S. and NAKAJIMA, S. (1965). Tetrodotoxin and manganese ion : Effects on action potential of the frog heart. Science 149, 1254.

HAGIWARA, S. and NAKAJIMA, S. (1966). Differences in Na and Ca spikes as examined by application of tetrodotoxin, procaine and manganese ions. J.Gen.Physiol. 49, 793-806.

HAGIWARA, S. and TAKAHASHI, K. (1967). Surface density of calcium ions and calcium spikes in the barnacle muscle fibre membrane. J.Gen.Physiol. 50, 583-601.

HASSELBACH, W. and MAKINOSE, M. (1961). Die calcium-pump der "erschlaffungsrana" des muskels und ihre abhorgigheit von der ATP-spaltung. Biochem. Z. 333, 518-528.

HEILBRUN L.V. and WIERCINSKI, F.J. (1947). The action of various cations on muscle protoplasm. J.Cell Comp.Physiol. 29, 15-32.

HILLE, B. (1968). Charges and potentials at the nerve surface : divalent ions and pH. J. Gen. Physiol. 51, 221-236.

HILLE, B. (1970). Ion channels in nerve membranes. Prog. Biophys. Mol. Biol. 21, 1-32.

HILLE, B. (1971). The permeability of the sodium channel to organic cations in myelinated nerve. J.Gen.Physiol. 58, 599-619.

HILLE, B. (1972). The permeability of the sodium channel to metal cations in myelinated nerve. J.Gen. Physiol. 59, 637-658.

HODGKIN, A.L. and HUXLEY, A.F. (1952A). Currents carried by sodium and potassium ions through the membrane of the giant axon of Loligo. J.Physiol. 116, 449-472.

HODGKIN, A.L. and HUXLEY, A.F.(1952B). The components of membrane conductance in the giant axon of Loligo. J.Physiol. 116, 473-496.

HODGKIN, A.L. and HUXLEY, A.F. (1952C). The dual effect of membrane potential on sodium conductance in the giant axon of Loligo. J.Physiol. 116, 497-506.

HOFFMAN, B.F. and SUCKLING, E.F. (1956). Effect of several cations on transmembrane potentials of cardiac muscle. Am. J.Physiol. 186, 317-324.

HUXLEY, A.F. (1959). Local activation in muscle. Ann. N.Y. Acad. Sci. 81, 446-452.

HUXLEY, A.F. and TAYLOR, R.E. (1958). Local activation of striated muscle fibres, J.Physiol. 144, 426-441.

ITO, Y., KURIYAMA, H. and TASHIRO, N. (1970). Effects of divalent cations on spike generation in the longitudinal somatic muscle of the earthworm. J.Exptal. Biol. 52, 79-94.

JENERICK, H.P. and GERARD, R.W. (1953). Membrane potential and threshold of single muscle fibres. J.Cell. Comp. Physiol. 42, 79-102.

JENKINSON, D.H. (1957). The nature of the antagonism between calcium and magnesium ions at the neuromuscular junction. J.Physiol. 193, 419-432.

JOHNSON, E.A. and LIEBERMAN M. (1971). Heart: Excitation and Contraction. Ann. Rev. Physiol. 33, 479-532.

KAVALER, F. (1959). Membrane depolarisation as a cause of of tension development in mammalian ventricular muscle. Am. J.Physiol. 197, 968-970.

KEENE, J.J., SEIDEL, C.L. and BOHR, D.F. (1972). Manganese on calcium flux and norepinephrine inducued tension in arterial smooth muscle. Proc. Soc. Expt. Biol. and Med. 139, 1803-1805.

KEENAN, M.J. and NIEDERGERKE, R.(1967). Intracellular sodium concentration and resting sodium fluxes of the frog heart ventricle. J.Physiol. 188, 235-260.

KERRICK, W.G.L. and BEST, P.M. (1974). Calcium ion release in mechanically disrupted heart cells. Science 183, 435-437.

KEYNES, R.D. and SWAN, R.C. (1959). The effect of external sodium concentration on the sodium fluxes in frog skeletal muscle. J.Physiol. 147, 591-625.

KLEINFELD, M. and STEIN, E.(1968). Action of divalent cations on membrane potentials and contractility in rat atrium. Am. J.Physiol. 215, 593-599,

KLEINFELD, M., STEIN, E. and AGUILLARDO, D. (1966). Divalent cations on action potentials of dog heart. Am.J.Physiol. 211, 1438-1442.

KOHLARDT, M., BAUER, B., KRAUSE, H., and FLECKENSTEIN, A. (1972). Differentiation of the transmembrane Na and Ca channels in mammalian cardiac fibres by the use of specific inhibitors. Pflugers Arch. 335, 309-322.

KOKETSU, K. and NISHI, S.(1959). Restoration of neuro-muscular transmission in sodium-free hydrazinium solution. J.Physiol. 147, 239-252.

KUMAGAI, H., EBASHI, S. and TAKEDA, F.(1955). Essential relaxing factor in muscle other than myokinase and creative phosphokinase. Nature. 176, 166.

LAMB, J.F. and McGUIGAN, J.A.S. (1966). Contracture in superfused frogs ventricle. J.Physiol. 186, 261-283.

LANGER, G.A. (1973). Heart: Excitation-contraction coupling. Ann. Rev. Physiol. 35, 55-86.

LINDLEY, B.D. KIRBY, A.C., STUESSE, S.C., PICKEN, J.R.(1973). Mechanical threshold and inactivation in denervated frog muscle. Am. J.Physiol. 225, 171-176.

LINEWEAVER, H. and BURK, D.(1934). The determination of enzyme dissociation constants. J.Am. Chem. Soc. 56, 658-666.

LORENTE DE NÓ, R., VIDAL, F. and LARRAMENDI, L.M.H. (1957). Restoration of sodium deficient frog nerve fibres by onium ions. Nature 179, 737-738.

LUTTGAU, H.C. (1963). The action of calcium on potassium contractures of single muscle fibres. J.Physiol. 168, 679-697.

LUTTGAU, H.C. and NIEDERGERKE, R.(1958). The antagonism between Ca and Na ions on the frogs heart. J.Physiol. 143, 486-505.

MACLENNAN, D.H. (1970). Purification and properties of an adenosine triphosphatase from sarcoplasmic reticulum. J.Biol.Chem. 245, 4508-418,

MAHLER, H.R. (1961). The use of amine buffers in studies with enzymes. Ann. N.Y. Acad. Sci. 92, 426-439,

MARCEAU, F. (1904). Recherches sur la structure et de développement camparés des fibres cardiaques dans la série des vertebrés. Ann. Sci. Nat. 8^{e} serie, Zoologie 19, 191-365.

MARSH, B.B. (1951). A factor modifying muscle fibre syneresis. Nature 167, 1065-1066.

MASHER, D. (1973). Electrical and mechanical responses in ventricular muscle fibres during barium perfusion. Pflugers. Arch. 342, 325-346.

MASHER, D. and PEPER, K. (1969). Two components of inward current in myocardial muscle fibres. Pflugers Arch. 307, 190-203.

MATOBA, T. (1973). Mechanism of positive inotropic actions of hydrazinium ions on bullfrog heart muscles. Jap. Heart Jnl. 14, 257-266.

MEIRI, U. and RAHAMIMOFF, R. (1972). Neuromuscular transmission : Inhibition by manganese ions. Science 176, 308-309.

MILLER, D.J. (1974). Caffeine and the contraction of frog heart muscle. Ph.D.thesis, University of Leicester,

MINES, G.R. (1913). On functional analysis by the action of electrolytes. J.Physiol. 46, 188-235.

MORAD, M. and ORKAND, R.K. (1971). Excitation - contraction coupling in frog ventricle : Evidence from voltage clamp studies. J.Physiol. 219, 167-189.

NARAHASHI, T. (1966). Dependence of excitibility of cockroach giant axons on external divalent cations. Comp. Biochem. Physiol. 19, 759-774.

NARAHASHI, T. and YAMASKI, T. (1960). Mechanism of the after-potential production in the giant axon of the cockroach. J.Physiol. 151, 75-88.

NATORI, R.(1954). The property and contraction process of isolated myofibrils. Jikeikai Med.Jnl. 1, 119-126.

NIEDERGERKE, R. (1955). Local muscular shortening by intracelluarly applied calcium. J.Physiol. 128, 12P.

NIEDERGERKE, R.(1956). The potassium chloride contracture of the heart and its modification by calcium. J.Physiol. 134, 584-599.

NIEDERGERKE, R.(1959). Calcium and the activation of contraction. Experentia 15, 128.

NIEDERGERKE, R.(1963). Movements of Ca in frog heart ventricles at rest and during contractures. J.Physiol. 167, 515-550.

NIEDERGERKE, R. and LUTTGAU, H.C. (1957). Antagonism between calcium and sodium ions. Nature 179, 1066-1067.

NIEDERGERKE, R. and ORKAND, R.K. (1966). Thedual effect of calcium on the action potential of the frogs heart. J.Physiol. 184, 291-311.

NOBLE, D. and TSIEN, R.W. (1969). Reconstruction of the repolarisation process in cardiac Purkinje fibres based on voltage clamp measurements of membrane current. J.Physiol. 200, 233-254.

NONOMURA,Y., HOTTA, Y. and OHASHI, H. (1966). Tetrodotoxin and manganese ions : Effects of electrical activity and tension in taenia coli of guinea-pig. Science 152, 97-98.

NOVOTNY, I. and BIANCHI, C.P. (1973). Distribution and efflux of sodium from frog heart ventricles perfused with normal and sodium-free Ringer's solution, Pflugers Arch. 339, 113-124.

OCHI, R. (1970). The slow inward current and the action of manganese ions in guinea-pigs myocardium. Pflugers. Arch. 316, 81-94.

OHSHIMA, H. (1969). Reversible plateau Vs. spike action potential of amphibian cardiac muscle fibre. Jap. J.Physiol. 19, 569-598.

OOTA, I., TAKAUJI, M. and NAGAI T. (1972). Effect of manganese ions on excitation - contraction coupling in frog sartorius muscle. Jap.J.Physiol. 22, 379-392.

ORKAND, R.K. (1962). Chemical inhibition of contraction in directly stimulated crayfish muscle fibres. J.Physiol. 164, 103-115.

PAGE, S.G., and NIEDERGERKE, R. (1972). Structures of physiological interest in the frog heart ventricle. J. Cell. Sci. 11, 179-203.

PALMER, R.F. and POSEY, V. (1967). Ion effects on calcium accumulation by cardiac sarcoplasmic reticulum. J. Gen. Physiol. 50, 2085-2095.

PAPPANO, A.J. and SPERELAKIS, N. (1969). Spike electrogenesis in cultured heart cells. J.Physiol. 217, 615-624.

REUTER, H. and SEITZ, N. (1968). The dependence of calcium efflux from cardiac muscle on temperature and external ion composition. J.Physiol. 195, 451-470.

RINGER, S. (1883). A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. J.Physiol. 4, 29-47.

ROSSI, C.S. and LEHNINGER, A.L. (1963). Stoichiometric relationships between accumulation of ions by mitochondria and the energy-coupling sites in the respiratory chain. Biochem. Z. 338, 698-713.

ROUGIER, O., VASSORT, G., GARNIER, G., GARGOUIL, Y.M. and CORABOEUF, E. (1969). Existence and rôle of a slow inward current during the frog atrial action potential. Pflugers. Arch. 308, 91-110.

RUSSEL, J.M. and BLAUSTEIN, M.P.(1974). Calcium efflux from barnacle muscle fibres. Dependence on external cations. J.Gen.Physiol. 63, 144-167.

SAARI, J.T. and JOHNSON, J.A. (1971). Decay of calcium content and contractile force in the rabbit heart. Am.J.Physiol. 221, 1572-1575.

SAARI, J.T. and JOHNSON, J.A. (1972). Build-up and decay of calcium content and contractile force in the rabbit heart. Can.J.Physiol. Pharmac. 50, 1206-1210.

SANDOW, A.(1965). Excitation-contraction coupling in skeletal muscle. Pharmacol. Rev. 17, 265-320.

SABATINI-SMITH, S. and HOLLAND, W.C. (1969). Influence of manganese and ouabain on the rate of action of calcium on atrial contractions. Am.J.Physiol. 216, 244-248.

SCARPA, A. and GRAZZIOTTI, P. (1973). Mechanism for intracellular calcium regulation in heart. 1. Stopped-flow measurements of Ca^{++} uptake by cardiac mitochondria. J.Gen.Physiol. 62, 756-772.

SCHMIDT, H. (1960). Die wirkung von calcium-ionen auf das membranpotential markhaltiger nervenfasern. Pflugers Arch. 271, 634-654.

SEIDEL, J.C. and GERGELY, J. (1963). Studies on myofibrillar adenosine triphosphatase with calcium free adenosine triphosphate. J.Biol. Chem. 238 III, 3648-3653.

SHANES, A.M. (1958). Electrochemical aspects of physiological and pharmacological action in excitable cells. Pharmac. Rev. 10, 59-165.

SHIBATA, S. (1969). Effects of Mn^{++} on ^{45}Ca content and potassium induced contraction of the aortic strip. Can. J.Physiol. Pharmac. 47, 827-829.

SHIGENOBU, K., SCHNEIDER, J.A. and SPERELAKIS, N. (1974). Verapamil blockade of slow Na and Ca responses in myocardial cells. J.Pharmac. Exp. Therap. 190, 280-288.

SINGH, B.N. and VAUGHAN-WILLIAMS, E.M. (1972). A fourth class of antidysrhythmic action? Effect of verapamil on ouabain toxicity on atrial and ventricular intracellular potentials and on other features of cardiac function. Cardiovas. Res. 6, 109-119.

STALEY, N.A. and BENSON, E.S. (1968). The ultrastructure of frog ventricular cardiac muscle and its relationship to mechanisms of excitation-contraction coupling. J.Cell Biol. 38, 99-114.

STANLEY, E.J. and REITER, M. (1965). The antagonistic effects of sodium and calcium on the action potential of guinea-pig papillary muscle. Naunyn. Schmied. Arch. Pharmack. 252, 159-172.

TAKAHASHI, Y. and HOLLAND, W.C. (1969). Effect of manganese on transmembrane potential and contractility of atrial muscle. Am. J.Physiol. 217, 1280-1286.

TAKAYA, K. and REITER, M. (1972). Effect of divalent manganese ions on action potentials and contractility of cardiac muscle. Naunyn. Schmied. Arch. Pharmak 275, 213-226.

TARR, M. (1971). Two inward currents in frog atrial muscle. J.Gen.Physiol. 58, 523-543.

TARR, M. and TRANK, J.W. (1974). An assessment of the double sucrose-gap voltage clamp technique as applied to frog atrial muscle. Biophys. Jnl. 14, 627-644.

TASAKI, I., SINGER, I. and WATANABE, A.(1966). Excitation of squid giant axons in sodium-free external media. Am. J. Physiol. 211, 746-754.

THOMAS, L.J. Jnr. (1960). Increase of labelled calcium uptake in heart muscle during potassium lack contracture. J. Gen. Physiol. 43, 1193-1206.

THYMRUM, P.T. (1974). Inotropic stimuli and systolic transmembrane calcium flow in depolarised guinea-pig atria. J.Pharmac. Exp. Therap. 188, 166-179.

TRITTHART, H., VOLKMANN, R., WEIBS, R., and FLECKENSTEIN, A. (1973). Calcium-mediated action potentials in mammalian myocardium. Naunyn Schmied. Arch. Pharmac. 280, 239-252.

VEREECKE, J. and CARMELIET, E. (1971). Sr action potentials in cardiac Purkinje fibres. 1. Evidence for a regenerative increase in Sr conductance. Pfluger, Arch. 322, 60-72.

WALKER, J.L. and LADLE, R.O. (1973). Frog heart intracellular potassium activities measured with potassium micro-electrodes. Am. J. Physiol. 255, 263-267.

WARE, F. (1961). Effects of Ca deficiency and excess on transmembrane potentials in frog heart. Am. J. Physiol. 201, 1113-1119.

WAUD, D.R. (1974). Absorption isotherm Vs ion-exchange models for the drug-receptor reaction. J. Pharmac. Exp. Therap. 188, 520-528.

WEBER, A. and HERTZ, R.(1968). The relationship between caffeine contracture of intact muscle and the effect of caffeine on reticulum. J.Gen. Physiol. 52, 750-759,

WEIDMANN, S. (1957). The effect on the cardiac membrane potential of the rapid availability of the sodium carrying system. J.Physiol. 127, 213-224.

WEIDMANN, S.(1959). Effect of increasing the calcium concentration during a heart beat. Experentia. 15, 128.

WEISS, G.B. and BIANCHI, C.P. (1965). The effect of potassium concentration on ^{45}Ca uptake in frog sartorius muscles. J. Cell. Comp. Physiol.65, 385-392.

WILBRANDT, W. von and KOLLER, H. (1948). Die calciumwiring am froschlarzen als funktion des ionengleichgewichts zwischen und umgebung. Helv. physiol. pharmac. Acta. 6, 208-221.

WINEGRAD, S. (1971). Studies of cardiac muscle with a high permeability to calcium produced by treatment with ethylenediaminetetracetic acid. J. Gen. Physiol. 58, 71-93.

WINEGRAD, S. and SHANES, A.M. (1962). Calcium flux and contractility of guinea-pig atria. J. Gen. Physiol. 45, 371-394.

THE EFFECTS OF MANGANESE ON FROG HEART MUSCLE

by D. ELLIS
(January 1976)

ABSTRACT

1. The effects of manganese (and some other transition elements) on the electrical and contractile properties of frog heart muscle have been investigated.

2. The action potential duration and overshoot were dependent upon the intensity of stimulation in the presence of manganese.

3. The cells were hyperpolarised by approximately 10 mV for a ten-fold increase in the manganese (or calcium concentration). This hyperpolarisation could not completely account for the large increases in the threshold stimulus for contraction produced in manganese and high calcium solutions.

4. The twitch contraction was rapidly and reversibly inhibited by manganese.

5. The contracture responses produced by low-sodium solutions were greatly inhibited by manganese (I_{50} approximately 0.6 mM) and are largely accounted for by assuming a competitive inhibition of calcium binding by manganese.

6. The drugs D-600 and verapamil produced little or no inhibition of the low-sodium contracture.

7. Sodium, lithium, hydrazine and hydroxylamine were able to induce contraction in the presence of manganese following exposure to sodium-free conditions.

8. Increasing the intracellular sodium concentration by exposure to low potassium solutions potentiated both the low-sodium contracture and the twitch contraction suggesting that intracellular sodium is important in the regulation of calcium influx.

9. Manganese produced less inhibition of the potassium-rich contracture than of that produced by low-sodium solutions.

10. The hyperpolarisation in manganese solutions is insufficient to account for the inhibition of the contractures produced by low-sodium or potassium-rich solutions.

11. Contractures produced by the addition of caffeine were not inhibited by manganese.

12. Measurements of the uptake of manganese by whole canulated ventricles indicated a large accumulation and a slow loss of part of the accumulated manganese on return to manganese-free solutions.