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CONTRACTURES IN A SUPERFUSED FROG'S VENTRICLE

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SUMMARY

1. A new perfused preparation of frog's ventricle is described, whose main advantage is that there are short diffusion distances between the cells and the washing fluid.

2. This preparation responds within a few seconds to alterations in sodium, potassium or calcium concentration, or alteration in the osmotic pressure of the bathing fluid. The speed of these alterations is consistent with the diffusional distances involved measured histologically.

3. On depolarizing the preparation with Ringer + excess potassium, tension development starts at about -25 mV and is still increasing at -8 mV (the lowest voltage studied). As in earlier experiments in frog ventricle (Niedergerke, 1956*b*) and in skeletal muscle (Hodgkin & Horowicz, 1960) the relation between potential and tension is very steep.

4. Sodium free contractures (Lüttgau & Niedergerke, 1958) are very rapid in onset with this preparation. To maintain the tension developed, a rapid rate of washing is necessary for 1–2 min. After this, slow washing is sufficient. It is proposed that this is due to the leaching out of cellular sodium.

5. In fresh preparations the twitch and maximum K contracture are of similar size when stimulated at 30/min in 1–2 mM-Ca. With the onset of hypodynamia the twitch tension falls, but the K contracture remains unaltered. In hypodynamic ventricles the sensitivity of the twitch to the ratio $[Ca]/[Na]^2$ declines, whereas that of the K contractures remains unaltered. Hypodynamia therefore does not affect the contractile elements themselves.

6. During the staircase phenomenon the K contracture and twitch size alter in a similar manner, as previously described by Niedergerke. The sodium free contracture, however, remains unaltered. This tends to favour the hypothesis that the staircase phenomenon is due to effects at the cell membrane rather than in the cell interior.

7. Maximum K contractures occur at a [Ca]/[Na]^2 ratio of about $0{\cdot}7 \times 10^{-4}$ mM^{-1}, a figure similar to that obtained in earlier experiments (Lüttgau & Niedergerke, 1958).

8. The length–tension curves of K contractures are similar to those previously described for heart muscle, using single twitches to generate tension.

9. Application of K free solutions produced no rapid contractures in this preparation.

INTRODUCTION

In single fibres from frog skeletal muscle potassium contractures are fully developed in less than 1 sec (Hodgkin & Horowicz, 1960), whereas in frog ventricle potassium contractures take some 20 sec to develop (Niedergerke, 1956*b*; Lüttgau & Niedergerke, 1958) and contractures due to sodium removal 1–2 min to reach maximum tension (Lüttgau & Niedergerke, 1958; Niedergerke, 1963).

It seemed likely to us that the difference between the rates of onset of these contractures was largely due to the differing conditions, for it would take longer for alterations in the bathing fluid to reach the membranes of the majority of the cells in strips of ventricle than to reach the membrane of a single muscle fibre. An opportunity to test this hypothesis occurred when we introduced a new preparation of frog ventricle for studying radioactive K exchanges (Gray, Lamb & McGuigan, 1964; Lamb & McGuigan, 1965*a*), whose main virtue was that the diffusion distances between most of the cells and the bathing fluid was very short. This paper reports results obtained with this new preparation. A preliminary account of some of these results has already been published (Anderson, Lamb & McGuigan, 1965; Lamb & McGuigan, 1965*b*).

METHODS

The superfusion technique. Frogs, unselected for species or length of storage, were decapitated and the hearts removed. The auricles and aortae were cut off by a single knife cut near the base of the ventricle. The ventricle was then placed upright on the cut edge and sectioned again by a vertical cut into anterior and posterior halves. Either half of the ventricle was then used for the experiment (occasionally with a small heart a $\frac{3}{4}$ ventricle was used). The half ventricle was washed clean of blood and mounted on a wax block (Fig. 1*a*) by two stout entomological pins through the base. A No. 6 braided silk thread was tied into the apex of the ventricle and led to an isometric transducer. The output was displayed on a Devices pen recorder (over-all frequency response 0–60 c/s $\pm$ 3 db). The ventricle could be driven at various rates by means of two large silver electrodes embedded in the wax and connected to a Digitimer. (The output stage of this stimulator is battery operated and hence has a low output impedance.) In each experiment the stimulus to give the maximum twitch tension was determined and then the voltage trebled to provide a safety margin. A typical threshold stimulus was 3 V at 2 msec. In preliminary experiments it was found that a

stimulation rate of 30/min gave the maximum twitch tension and this was adopted for most of the experiments.

The ventricle was irrigated by a jet of Ringer fluid falling on to it from a tube held some 2 cm above it. The excess fluid was sucked away from the trough surrounding the ventricle to a reservoir under the bench. The flow rate and the tube position were adjusted so that the ventricle swelled adequately with the minimum flow rate (usually 1 ml./sec). In a typical experiment the jet hit the surface of the ventricle at about 760 dynes/cm^2 (calculated from the radius of the jet) and finally escaped by spilling over the edge of the ventricle at a low pressure.

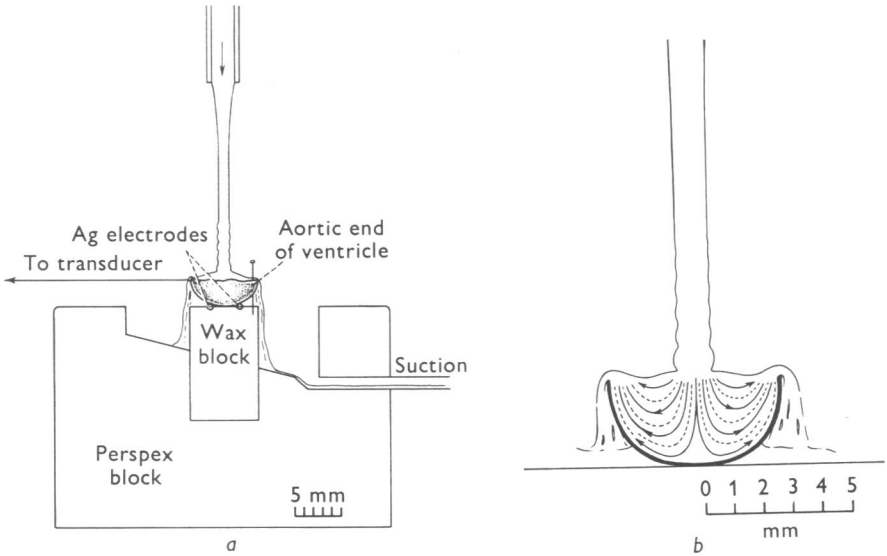

Fig. 1. (*a*) Diagram of experimental set-up. A half ventricle is pinned down to the wax block by two stout entomological pins through the base. The apex is then connected to the transducer by a silk thread. (*b*) Suggested route of fluid through the ventricle. Note the reflected waves on the perfusing fluid.

No detailed analysis was made of the physics of the preparation, but it seems likely that the following points are correct. (1) Between the point of impact of the jet and the exit over the edge of the ventricle potential energy is converted to kinetic energy and so the fluid must accelerate. This causes the jet to constrict just before it hits the ventricle (Fig. 1) and then gives the reflected waves up the jet. (2) The fluid probably takes a course through the ventricle as shown in Fig. 1*b*, moving at high velocity between the strands of cells but exerting little pressure on the ventricular walls. (3) The pressure across the ventricular walls at any point is simply the hydrostatic pressure at that point and so increases from above downwards in the ventricle. This means that the maximum hydrostatic pressure at the bottom of the ventricle is only about 0·5 cm of water. There are two pieces of evidence on this last point, (*a*) it was noticed that if the outside pool of Ringer was allowed to gather until it was equal to the height of the ventricle then the ventricle collapsed, and (*b*) histological sections of the ventricle fixed during superfusion (Fig. 2) showed that there is a greater separation of the strands of cells of the ventricle at the lower part of the ventricle than at the upper part and that the cell strands are thinner, a finding consistent with the pressure gradient. The general conclusion from these considerations is that the pressure across the ventricle is about normal but that the cells are subjected to much higher fluid velocities than usual in the intact animal.

Each test fluid was kept in a separate bottle on a shelf about 40 cm above the preparation and led through a rubber tube to a final glass delivery tube of 2 mm inside diameter. A Perspex block bored to be a close fit on these glass tubes was mounted above the preparation. To change fluids the tube in use was clipped off, removed from the Perspex block and replaced by a new tube. This operation usually took a few seconds and had the advantage that the first fluid drained away from the ventricle before the second fluid reached it and so little mixing of the fluids occurred. Usually a small artifact was produced on the record by the fluid changeover and this served to indicate the time of change. In order to produce the fastest access of the new fluid to the cells (as judged by the rate of rise of the tension to a potassium contracture fluid) it was found necessary to position the tube very accurately before re-starting the jet and to run the new fluid at a high rate after the changeover. This then meant that the new fluid suddenly hit the centre of the ventricle, distended it and so gained rapid access to most of the cells. With a little practice there was no difficulty in doing this with repeatable results.

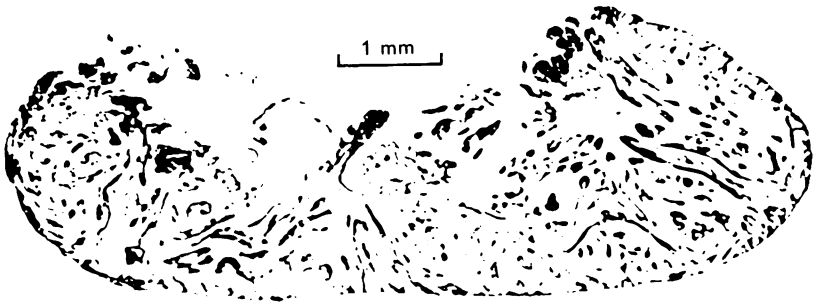

Fig. 2. Cross-section of superfused ventricle. Fixed with Bouin's fixative in a calcium free medium. Stained haematoxylin and eosin. Note that the cell strands at the lower part of the ventricle are thinner than those at the top.

Solutions. The composition of the Ringer used is shown in Table 1. This Ringer is based on that described by Adrian (1956).

(1) Ca^{2+} was added as molar $CaCl_2$ to the above solutions to make a final concentration in the range 0–10 mM.

(2) Intermediate values of potassium between 2·5 and 200 mM were obtained by mixing *C* and *D* or *E* and *F* in appropriate amounts.

(3) Solutions with reduced sodium concentration were made by mixing *A* and *E* for low potassium Ringer or *C* and *F* for high potassium Ringer.

Potential measurement. Electrodes were machine pulled and filled with 3 M-KCl. Usually their resistance varied between 20–40 MΩ. Pieces of ventricle were held in a bath made from Perspex and dental wax by light glass clips and impaled with the micro-electrodes through the epicardial surface. The irrigation of the cells was not as good as in the superfusion technique and therefore some minutes were allowed for diffusion to become complete before starting potential measurements in new fluids. No measurements of tip potential were made, but all the electrodes used were freshly made.

Histological measurements. A × 100 enlargement of Fig. 2 was made. The diameter of 398 strands of cells from various parts of the section was measured. The results were distributed in a slightly skew way due to the few thick strands near the top edge. The modal radius was 10 μ; the mean radius 14 μ with a standard error of 0·6 μ. Distended ventricles fixed *in vivo* have a mean strand size of 12 $\mu \pm 0{\cdot}3$ (J. F. Lamb & J. A. S. McGuigan, unpublished observations).

TABLE 1. Compositions of solutions used

Solution	Concentration mM						Sucrose g/l.	Relative tonicity
	Na^+	K^+	Cl^-	HPO_4^{-2}	$H_2PO_4^-$	Glucose		
A	120·0	2·5	121·1	2·15	0·85	5·55	—	1·00
B	5·0	117·5	121·1	2·15	0·85	5·55	—	1·00
C	120·0	200·0	317·6	2·15	0·85	5·55	—	2·53
D	120·0	2·5	121·1	2·15	0·85	5·55	123·0	2·53
E	5·0	2·5	5·1	2·15	0·85	5·55	73·1	1·00
F	5·0	200·0	202·6	2·15	0·85	5·55	73·1	2·53

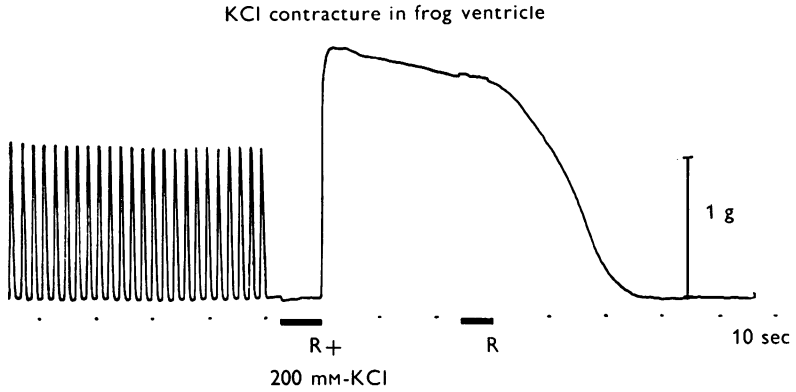

Fig. 3. Photograph of a record of a potassium contracture in a superfused frog's ventricle. Bars indicate changeover periods (also indicated by wavers on the tracing). Preparation driven at 30/min between contractures. $[Ca]_0$ = 1·8 mM. Recovery of tension at end slower than usual. Resting tension 0·3 g.

RESULTS

Potassium contractures

Figure 3 shows a typical potassium contracture obtained with this preparation and serves to indicate the general technique. Before the record was started the preparation was set at an initial tension of 0·3 g. The first part of the record shows the twitches obtained on stimulation at 30/min in 1·8 mM-Ca Ringer. The stimulation and then the normal jet of Ringer were stopped (indicated by the waver in the base line and the start of the bar). The jet of Ringer containing excess 200 mM-KCl (solution *C*) was then started (indicated by the end of the bar) and caused a large increase in tension. This reached a peak in 2–3 sec and started to decline. The changeover back to normal Ringer was made some 26 sec later and is indicated by the second bar. This caused an immediate relaxation in tension; considerably slower than the onset. After the record was taken

the resting tension was relaxed and stimulation restarted at 30/min. A rest of 5 min was then allowed before another contracture was elicited.

The fast rate of rise of the K contracture observed at this stimulation rate was not due to an initial action potential. This was shown in experiments in which the twitch was reduced in size by solutions of high osmotic pressure or ACh or abolished with raised potassium, with no change in the rate of development or size of the subsequent potassium contracture.

The osmotic pressure of the Ringer solution was increased 2·5 times by the addition of 123 g/l. sucrose. The ACh was used at a concentration of 10^{-5} g/ml. (6×10^{-5} M). Under these conditions the twitch was reduced to less than 4% of the K contracture. The K in the Ringer solution was raised to 10 or 20 mM to abolish the twitch. In each case the treatment was continued until maximum effect was obtained before a K contracture was elicited. For the raised osmotic pressure this took about 1 min, for ACh and raised K a much shorter time.

As originally observed by Niedergerke (1956*b*) the plateau of the contracture in heart is prolonged and declines slowly with time. In these experiments the plateau had a halftime of decline of about 60 sec.

Table 2 shows the individual results from seventeen such experiments during the second half of this series. These figures are taken from the first contracture recorded for each experiment with the associated twitch. With an external calcium concentration in the range of 1–2 mM the average tension developed was 75·4 g/g wet wt. of ventricle, the twitch/contracture ratio was 0·62 and the half-time ($T_{\frac{1}{2}}$) of development of tension 0·9 sec and relaxation of tension 6·6 sec. The following points may be made.

(1) The tension developed/wet wt. for an external [Ca] of 1·8–2 mM (78·1 g/g wet wt.) was similar to a [Ca] of 1 mM (74·0 g/g wet wt.) (see later).

(2) The twitch/contracture ratio of 0·62 is much larger than that of about 0·2 found by Hodgkin & Horowicz (1960) for frog skeletal muscle fibres. This is presumably a reflexion of the much longer action potential found in the heart, allowing the developed tension to go nearer completion. In the last seven experiments this ratio was 0·82, perhaps a reflexion of an improving technique. In general, throughout the experiments the contractures stayed relatively constant, whereas the twitches declined and therefore the ratios given here are the maximum recorded (see later).

(3) The relaxation times decreased in the last few experiments, e.g. the mean of the last seven is only 2·7 sec compared with 9·6 for the first nine. This was due to a more rapid rate of application of Ringer in these experiments. This recovery rate is still longer than the rate of onset, probably a result of the contracted state of the ventricle at this time increasing the diffusion distances for the normal Ringer. This may also account for the increasing slope of the initial part of recovery seen in this and some other records.

TABLE 2. Table showing data from last experiments in the series

Date	Wet wt. (mg)	Tensions g/g wet wt.				Twitch/ contracture ratio	$T\frac{1}{2}$ (sec)	
		Ca_0	Resting	Twitch	Contracture		On	Off
17.xii.64	—	1·8	—	—	—	0·84	0·50	8·0
21.xii.64	—	1·8	—	—	—	0·27	0·50	7·0
23.xii.64	32·1	1·8	7·2	20·9	68·5	0·31	1·00	5·5
29.xii.64	21·2	1·8	8·0	69·3	97·2	0·71	1·00	5·0
5.i.65	(1) 36·2	2	2·8	5·5	38·7	0·14	0·75	15·0
	(2) 16·0	2	12·5	21·9	68·8	0·32	0·50	15·0
7.i.65	24·0	2	10·8	62·5	116·7	0·54	0·50	18·0
8.i.65	(1) 19·0	1	17·4	23·7	57·9	0·41	0·75	6·0
	(2) 19·0	1	26·3	32·1	69·5	0·46	0·50	6·5
5.iii.65	(1) 11·0	1	22·7	112·7	143·6	0·79	2·00	3·0
	(2) —	1	—	—	—	1·00	0·50	1·0
9.iii.65	22·9	1	6·1	53·3	66·0	0·81	0·80	2·0
11.iii.65	(1) 22·6	1	11·9	44·2	53·5	0·83	2·00	3·0
	(2) 23·4	1	23·2	212·1	89·7	1·13	0·50	1·0
19.iii.65	29·4	1	12·6	30·6	39·5	0·78	1·00	6·0
15.iv.65	28·8	1	13·6	29·6	73·0	0·41	1·50	3·0
19.viii.65	20·5	1	9·3	61·0	73·2	0·83	0·50	—
		—						
Mean	23·3	—	12·4	47·8	75·4	0·62	0·87	6·6
S.E.	± 1·7		± 1·7	± 9·3	± 7·7	± 0·07	± 0·12	± 6·5

Fluid flow rates. Both the twitch and the potassium contractures were affected to some extent by the rate of fluid flow. The twitch tension was greatest when the ventricle was in air and least when high flow rates were used. Usually the difference was not very marked ($< 10\%$). At the usual rates of flow used to record twitches, the twitch tension was similar to that obtained when lying in a pool of Ringer. (If left in unstirred Ringer for any time the twitch tension declined when stimulated at 30/min, presumably due to anoxia or K accumulation, etc.) The rate of contracture tension development and size of tension produced by high K concentrations were very dependent on the initial rate of potassium application, but once a contracture was established variations in the flow rate had little effect. Figure 4 shows consecutive records taken from one preparation, showing the contractures produced (from above downwards) by maximum, intermediate and slow flows of Ringer + 200 mM-KCl. The fast flow ballooned the preparation, the intermediate flow stirred the strands of the ventricle and the slow flow caused a rapid change of the Ringer around the outside of the preparation. At the slower rates of fluid application the peak contracture tensions reached 79 and 76% of the corresponding tension on the declining part of the fastest contracture. In other experiments the relation between peak tensions reached on slow K application and the tension reached at a corresponding time after fast K application varied between this value down to 30%. Figure 4 also shows that slowing the fluid flow during the declining phase of the (upper) contracture has little effect.

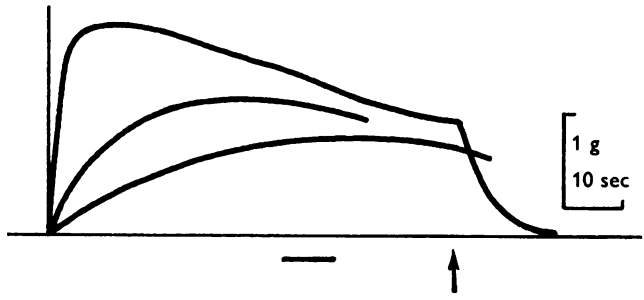

Fig. 4. Tracings of three KCl contractures. Contractures induced at vertical line by R + 200 mM-KCl. Upper record shows normal superfused record. In middle record jet of Ringer much slower and preparation not ballooned. In lower record Ringer dripping on to preparation at fast rate. Horizontal bar and arrow refer to upper record. For duration of the bar superfusion slowed, with little effect. At arrow R + 200 mM-KCl replaced with Ringer only. Three consecutive records from the same preparation. $[Ca]_0$ 1 mM.

With K concentrations near the threshold both the rate of onset and maintenance of the contracture were dependent on the flow rate, e.g. once a contracture was established with a high flow rate, maintenance of this

tension also required a high flow rate. This is probably due to K near the membrane being taken up by the cells or other stores and so reducing the concentration near the membrane unless the flow rate is fast. This would result in tension changes at near threshold concentrations of K but not at high K concentrations because of the relation between external K and tension development. A similar explanation would probably not account for the difference between the tension reached on slow K application and the corresponding tension after superfusion. The effect of the various rates of K application on the initial rate of tension development can probably be accounted for by the differing diffusion distances involved; thus the observed rate of tension development would be expected from strand sizes of 14 μ and the usual slow K diffusion in tissues when allowance is made for the non-linear relation which exists between external [K] and tension development. In Fig. 4 the $\frac{1}{2}$ ventricle was about 1 mm thick. The slowest application of fluid therefore corresponded to diffusion of K from one side into 1 mm of tissue. Judged by the time to peak tension of 30 sec the intermediate record of Fig. 4 probably corresponds roughly to that obtained with thin ventricular strips (Niedergerke, 1956*b*).

Decline of twitch tension with perfusion. In all the experiments described in this paper a contracture was generally elicited within a few minutes of killing the frog, and then repeated at about 5 min intervals for several hours. During this time the contractures usually remained constant, but might occasionally decline or increase slightly. The twitches invariably declined. Figure 5 shows a typical experiment. At the start of the experiment the twitch/contracture ratio was 95%, at the end of the experiment it was 32%. The initial contracture tension was 61 g/g wet wt., the final one 57 g/g wet wt. (Note that contractures were induced with 100 mM-KCl; 200 mM-KCl would probably have produced a greater tension.) The rate of rise of the first and last contracture were similar at a $T_{\frac{1}{4}}$ of 0·5 and 0·75 sec respectively. The decrease in twitch tension was associated with a decreased time to peak tension from 0·5 to 0·4 sec. This is the condition of hypodynamia of the frog's heart investigated by Clark (1913). The present results on the decline of the twitch tension are very similar to those of Clark (his Fig. 1) but show a faster decline. Clark showed that one factor in the production of this hypodynamic state was the rate of washing with Ringer, fast washing speeded up the process, lying in a pool of Ringer or recirculation of the Ringer slowed or even reversed the process. In the present experiments the washing was very vigorous and this probably accounts for the fast decline in twitch tension. In other experiments it was shown that the rate of stimulation also had an effect on the rate of formation of hypodynamia. At slow rates of stimulation the onset of hypodynamia was less rapid than at fast rates.

It seemed possible that the small twitch at the end of the experiment could have been due to poor conduction in the ventricle. This possibility was not entirely ruled out but it was observed (1) that the threshold for stimulation (about 3V at 2 msec width) was unchanged throughout the experiment, (2) that the response was still all-or-none and (3) that test stimulations at high voltages or long pulse lengths (up to 100 V or 20 msec), had no augmenting effect on the twitch tension. It therefore seems unlikely that poor conduction could have been occurring and probable that all the cells were being stimulated by the action potential.

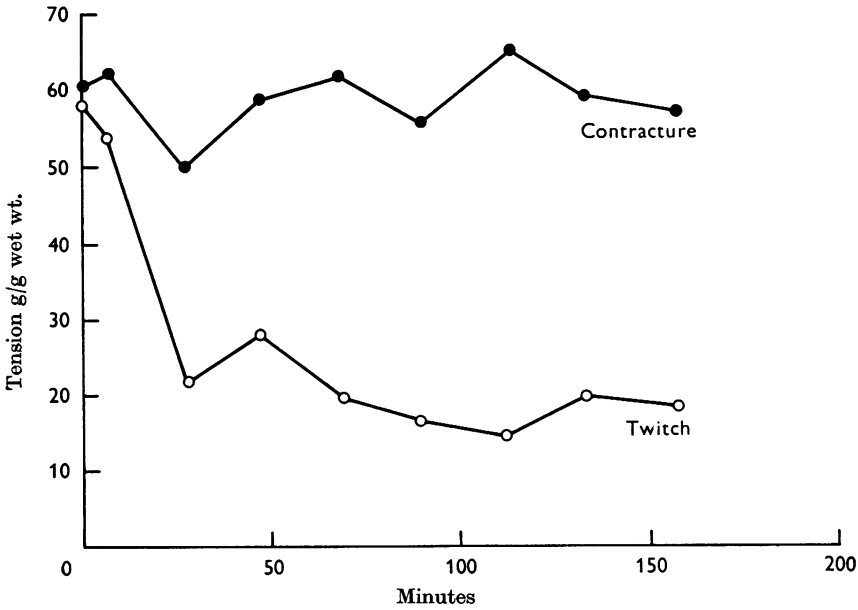

Fig. 5. Contracture and twitch tensions in a ventricle superfused for 3 hr and stimulated at 30/min. $[Ca]_0$ 1 mM. Contractures induced with R + 100 mM-KCl. Contracture tension remains relatively constant, twitch tension declines.

Length–tension curves. In some of the early experiments length–tension curves were measured, both for twitches and contractures, partly to check that the twitches were a good index of the steady-state tension, and partly to find out more about our technique. Figure 6 shows the results from part of one of these experiments. The whole experiment lasted 4 hr. During this time the contractures remained constant but the twitch/contracture ratio declined from 50 to 19%. Eight length–twitch tension curves and four length–contracture tension curves were done. The results illustrated in Fig. 6 are typical and were taken about the middle of the experiment.

The resting tension and the total and active tensions for the twitch and corresponding potassium contracture are plotted for each length. Also shown in Fig. 6 is the ratio twitch/contracture and the $T_{\frac{1}{2}}$ of the tension rise at each length. It can be seen that (1) at zero initial tension there is

no tension developed, (2) that the twitch/contracture ratio is constant over the range studied and that (3) the rate of rise of tension is not affected by the initial length. The general shape of these curves is very similar to

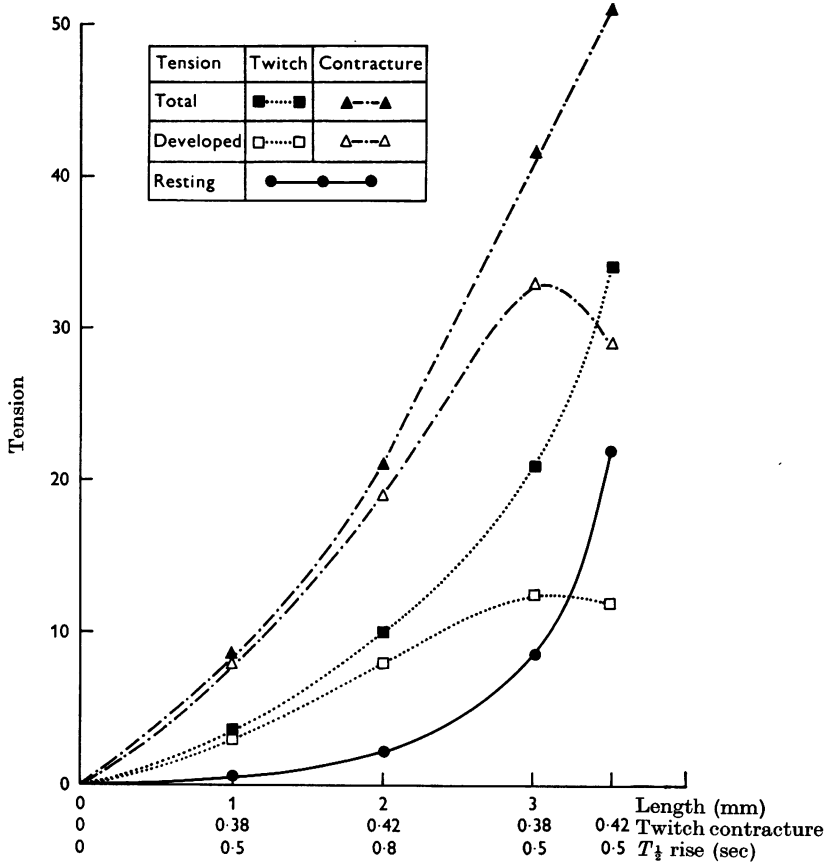

Fig. 6. Length–tension diagrams for a superfused frog's ventricle. Tension (in arbitrary units) plotted against initial length. Symbols are explained in the table. Also plotted are the ratio of twitch/contracture developed tensions and the $T_{\frac{1}{2}}$ of the rise in tension of the contractures. These curves were obtained during stretching. Some hysteresis was present so that the relaxation curves were displaced downwards. 20 in arbitrary units corresponds roughly to 80 g/g wet wt. in the other records. $[Ca]_0$ 1·8 mM.

those measured by Abbott & Mommaerts (1959) and Sonnenblick (1962) using single twitches to produce tension at various initial resting lengths. These results enable us to compare the rise times of contractures elicited in different positions on their respective length–tension curves.

Effect of different K concentrations on tension development. In order to depolarize the cell membranes $(K)_0$ must be increased. If this is done at the expense of $(Na)_0$ then, in heart cells, the sodium removal in itself sensitizes the contractile system to depolarization (Lüttgau & Niedergerke, 1958). If the increased $(K)_0$ is obtained by the addition of excess K, then the osmotic pressure of the Ringer is increased; and this affects the contractile system (Hajdu, 1953; Niedergerke, 1963).

Previous work on the relation between external potassium and tension development has been done by Niedergerke (1956*b*) and Lüttgau & Niedergerke (1958) using strips of frog ventricle. In the earlier paper K was substituted for Na isosmotically, while in the later paper excess K was added to normal Ringer containing 3 mM-Ca. As both sodium reduction and calcium increase augments the contracture tension at a constant potassium level (Lüttgau & Niedergerke, 1958; Niedergerke, 1956*b*), the present experiments were undertaken to measure the relation between external potassium and contracture tension at normal external calcium (1 mM, Boyle & Conway, 1941).

Figure 7 shows the results obtained with various concentrations of K but constant osmolarity (filled circles, solution *C* and *D* mixed). Underneath is plotted the mean membrane potential measured in five other experiments. It can be seen that on depolarization tension is produced between -39 and -30 mV (25–40 mM-K), is half maximum at -15 mV (100 mM-K) and is still increasing at -8 mV (200 mM-K). In a single experiment using 200, 300 and 400 mM-K there was little further increase in tension at the higher potassium concentrations. These higher potassium concentrations would be unlikely to depolarize the cells much further however. These experiments are still not ideal in that the osmolarity of the solutions is greatly increased. A few experiments were therefore carried out to measure the threshold for tension development using excess K only (and hence minimum osmotic change). In four experiments tension was just detectable ($>2\%$ of maximum) at 50, 50, 60 and 70 mM-K. Some of these results are plotted on Fig. 7 as open symbols. The mechanical threshold is therefore probably in the range of -27 to -21 mV under these conditions.

A notable feature of these osmotic experiments was that the effects of the increased osmotic pressure were apparent in a few seconds. This is shown in Fig. 8.

In Fig. 8*a* are shown the contractures produced by 50 mM excess K and 50 mM-K substituted for Na isosmotically. The contracture produced by these solutions + excess sucrose to bring the osmolarity up to that of Ringer + 200 mM-KCl are shown by the bar and the largest contracture. Figure 8*b* shows the contractures produced by 80 mM-K, 80 mM-K +

sucrose to make it isosmotic with +200 K and +200 K. It can be seen that the higher tension produced by the addition of sucrose is developed very rapidly. These results are interesting in that weight loss and membrane potential increase consequent on osmotic loss of water is slow in this preparation (E. Anderson & J. F. Lamb, unpublished).

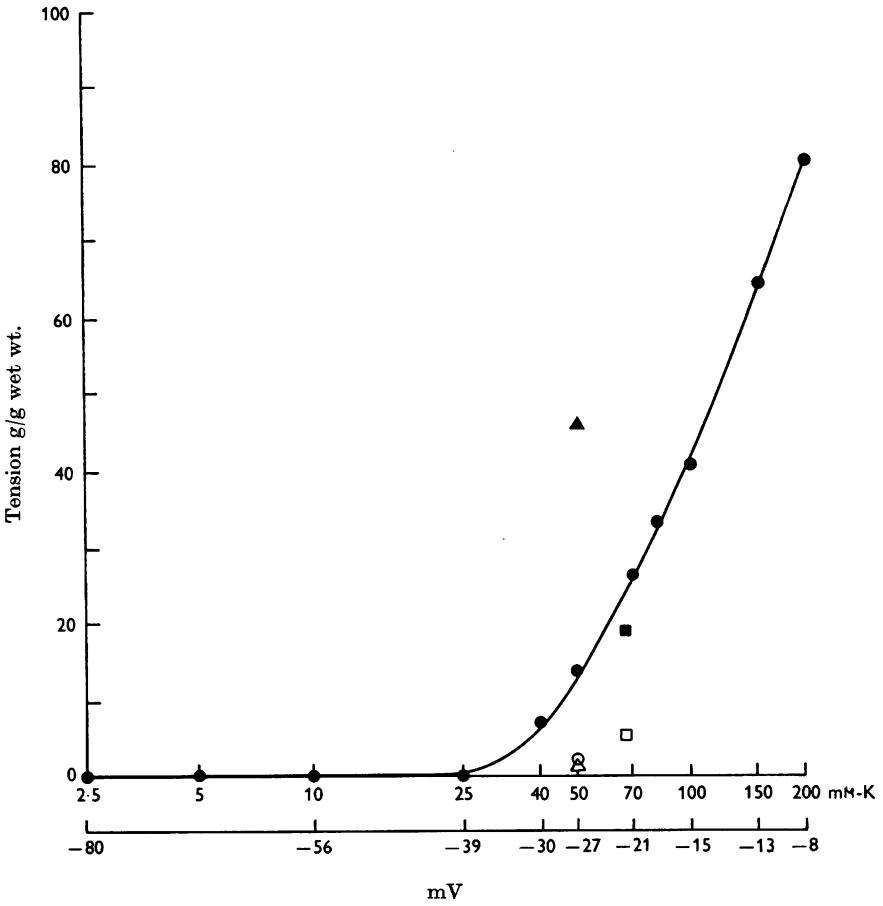

Fig. 7. Relation between external potassium concentration or membrane potential and contracture tension. Filled circles are mean values from six tension measuring experiments and five potential measuring experiments in which the K was varied at constant osmolarity (corresponding to R+200 mM KCl). Line drawn through them by eye. Open symbols are threshold values for tension development from three other experiments in which K was added as solid KCl to Ringer. Filled square is tension corresponding to open square but with added sucrose to increase the osmolarity. Filled triangle is tension corresponding to open triangle but with 50 mM-K for 50 mM-Na. $[Ca]_0$ 1 mM.

Effect of potassium-free fluids

It was first noticed by Ringer (1883) that K free solutions induced contractures in frog ventricle. As this method is still sometimes used for this purpose (e.g. see Thomas, 1960) it was thought of interest to try it. In two experiments K-free solutions (solution *A* with K omitted) were applied in the usual way. In neither of these experiments was any contracture tension developed, but in both after a few seconds a rapid series of large twitches occurred which persisted as long as the washing continued. Therefore it seems that the contractures produced by K-free solutions are of a different kind from that produced by K-rich solutions and may be the end result of quite complex changes in the cells.

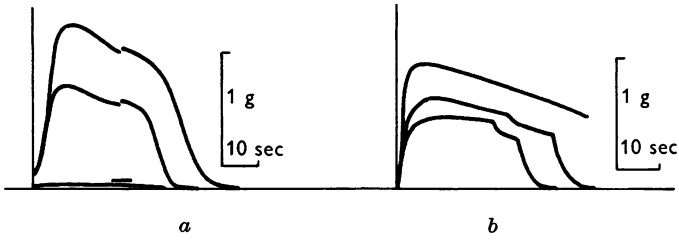

Fig. 8. Effect of increased osmolarity on contracture tensions at various values of external K. In (*a*) is shown (from below upwards) R (Ringer) + 50 mM-K, R + 50 mM-K + sucrose (bar), R with 50 mM-K for 50 mM-Na and R with 50 mM-K for 50 mM-Na + sucrose. In (*b*) is shown (from below upwards) R + 80 mM-K, R + 80 mM-K + sucrose, R + 200 mM-K. Sucrose added to make solutions isosmotic with R + 200 mM-KCl. *a* and *b* different preparations. [Ca] 1 mM.

Contractures to sodium removal

In 1913 Clark noticed that frog ventricle died in systole immediately upon placing it in a solution from which the NaCl had been omitted. Later Lüttgau & Niedergerke (1958) analysed this effect and showed that it was completely reversible and was due to the specific absence of Na from the Ringer. They suggested that the contracture produced by Na-free solutions was due to sodium–calcium antagonism at or near the cell membrane. In the absence of sodium, calcium could combine freely with the hypothetical molecule or site R so that enough CaR was produced to enter the cell despite the absence of depolarization. A feature of the sodium free contractures reported by Lüttgau & Niedergerke (1958) was that they were very slow in onset compared to the corresponding contracture produced by depolarization. In preliminary experiments with this new technique it was found that Na-free contractures developed at a similar rate to K contractures and therefore this was studied more fully.

Figure 9 (upper trace) shows a typical contracture produced by the sudden application of a Ringer in which the NaCl was all replaced by sucrose (solution *E*, containing 2·5 mM-K and 1·8 mM-Ca). The tension development occurs with a $T_{\frac{1}{2}}$ of 1·5 sec, whereas the previous and succeeding K contractures had a $T_{\frac{1}{2}}$ of onset of 1·3 and 1 sec respectively. The tension declined from the peak with a $T_{\frac{1}{2}}$ of 22 sec in this case. In other experiments (e.g. see Fig. 10 later) the plateau tension could be maintained by very fast Ringer flow. From these experiments it was concluded that in this preparation, the rate of tension development to Na-free

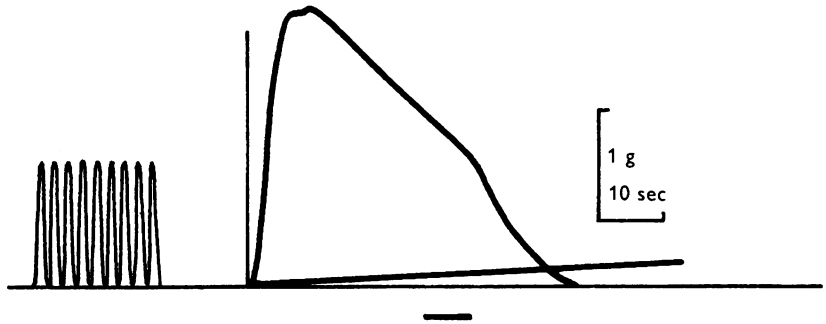

Fig. 9. Na free contractures. At vertical line sudden application of Na-free Ringer gave the large contracture; slow application gave the smaller trace. Bar indicates changeover back to normal Ringer. Sucrose replaced the sodium isosmotically. $[Ca]_0$ 1 mM.

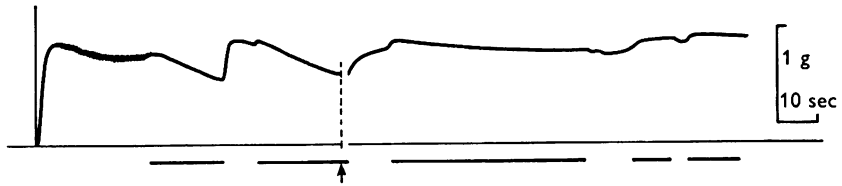

Fig. 10. Effect of varying rates of Na-free Ringer on tension development. Initially contracture induced and maintained by fast application. Each bar indicates a period of slow application of Na-free Ringer. The arrow and dotted line represents a gap in the record of 80 sec. Sucrose used to replace Na. $[Ca]_0$ 1 mM.

solutions was of the same order as that to high K solutions. Slow application of Na-free solutions (Fig. 9, flat trace; fluid rate same as +K Ringer in Fig. 4 middle trace and same experiment) gave much slower tension development than similar application of excess K solutions and so agreed with Lüttgau & Niedergerke's observations. The clue to the explanation for this was given by the observation that the plateaux of the Na-free contractures were very sensitive to the rate of fluid application. This effect is shown in Fig. 10, taken from another experiment. The record starts with

the induction and maintenance of a Na free contracture with a high rate of fluid flow (indicated by a wavering plateau); on slowing the fluid flow the plateau falls, on increasing the flow rate it rises again. This sequence could be repeated for 190 sec in this preparation before a slowing of the flow rate no longer affected the tension level. By this time the plateau had settled down to about its initial value. This record was taken 3 hr 20 min after setting up the preparation. Earlier, at 20 min from the start, the response to slowing the Na free fluid was abolished after 53 sec of superfusion. Similar results were obtained in another two preparations. A possible explanation of these results is to suppose that initially the rate of formation of the Na free contractures depends on a competition between Na removal by washing and its replenishment from tissue 'stores'; with adequate washing the Na around the cells is kept at a low level, with poorer washing the Na collects and desensitizes the membrane. Eventually all the Na is washed from these 'stores' and so the rate of washing is no longer important. With less efficient washing from the beginning of the contracture, full tension is not developed until all the Na has been leached from the tissue.

Effect of rate of stimulation on contractures. It is well known that the twitch tension of heart muscle varies with the rate of stimulation (the staircase or treppe phenomenon). Niedergerke (1956*a*) showed that the K contracture also varied with the stimulation rate. The present experiments were designed to study (*a*) the K contractures over a wider range of stimulation rates and (*b*) the Na free contractures at different stimulation rates. Figure 11 shows the results of two experiments on the effect of stimulation on the K contracture and Na free contractures at stimulation rates between 0 and 120/min. (The maximum rate chosen was 120/min, for at higher rates the ventricle would not follow regularity). In one experiment (open symbols) K contractures and Na free contractures (K = 2·5 mM) were elicited at 0, 2 and 30/min. In the other experiment (filled symbols) K contractures and Na and K free contractures were elicited at 120, 60, 30 and the spontaneous rate of 15/min. It can be seen that the K contractures vary with stimulation rate from a maximum at 120/min to a minimum at quiescence. (The minimum contracture is 20% of the maximum.) The rate of onset of the smallest contracture was 2–3 times slower than that of the fastest and was similar to that measured by Niedergerke (1956*a*). The upper part of the figure shows that the Na free contractures are independent of rate; their rate of onset also did not change with stimulation rate. In these experiments the maximum twitch tension occurred at 30/min; at rates of 120/min the twitches were barely detectable. Other less complete experiments gave similar results. In two experiments the effect of shortening the action potential with ACh (10^{-5} g/ml.) on the staircase

phenomenon was investigated. It was found that the phenomenon was still present even with the extremely short twitches produced by ACh.

Time course of calcium removal on the twitch. In order to calculate the diffusional distances involved in this preparation, an analysis similar to that described by Niedergerke (1957) was adopted. In two experiments the Ca concentration of the bathing fluid was varied over the range of 0·25–

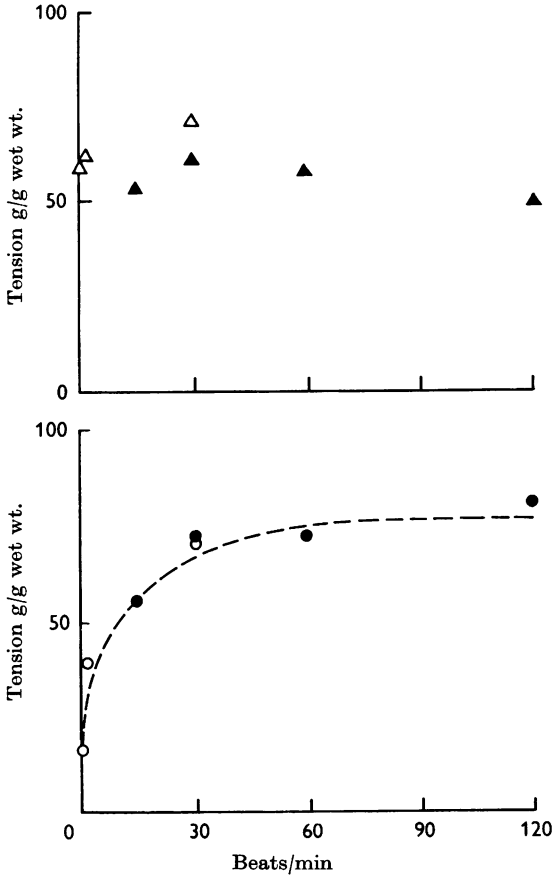

Fig. 11. Effect of various rates of stimulation on contractures. Upper graph shows Na free contractures, lower graph contractures to R + 200 mM-KCl. Line fitted by eye. Two different experiments shown by filled and open symbols. Sucrose used to replace Na. $[Ca]_0$ 1 mM.

3 mM and the twitch tension measured. The preparations were driven at 30/min. It was found that between 1 and 2 mM the tension was roughly proportional to the calcium concentration as also found by Niedergerke, In both preparations the tension changes consequent on changing from 2 mM to 1 mM-Ca were complete in less than 1 sec. It was difficult to be

more precise than this, so a $T_{\frac{1}{2}}$ of 0·33 sec was taken for the time course of the change.

The numerical solution of the diffusion equation for half saturation inside a cylinder given by Niedergerke (1957) was used to calculate the diffusion distances involved using this $T_{\frac{1}{2}}$ value and various values of diffusion coefficient for Ca. The equation is

$$R^2 = \frac{DT_{\frac{1}{2}}}{0{\cdot}063},$$

where R is the radius of the cylinder, $T_{\frac{1}{2}}$ is the time to half completion of the tension change consequent on changing from 2 mM to 1 mM-Ca in the Ringer and D the diffusion coefficient for calcium. Values of D used were (1) $7{\cdot}8 \times 10^{-6}$ cm^2/sec, the free solution value (Wang, 1953), (2) $2{\cdot}0 \times 10^{-6}$ cm^2/sec, that occurring in the extracellular space of various tissues (Harris & Burn, 1949; Creese, 1954; Keynes, 1954; Johnson, 1955) and (3) $0{\cdot}47 \times 10^{-6}$ cm^2/sec, the value for frog ventricle estimated by Niedergerke (1957) with ventricular strips. These values of diffusion coefficients give cylinder radii of 64, 33 and 15 μ respectively. The size of 15 μ is most similar to histological sections of this preparation (Fig. 2). Some caution is necessary in interpreting this result as the time course of extracellular diffusion in this preparation may be comparable to reversible uptake by the cell surfaces.

Effect of calcium variation on potassium contractures at two sodium concentrations. These experiments are essentially a repeat of those by Lüttgau & Niedergerke (1958). It was felt worth while to do these experiments in view of the differences in rates of onset of tension in these contractures compared to those carried out with ventricular strips. Figure 12 shows the results in three different preparations. Figures 12*a* and *b* are in the presence of normal sodium and 12*c* in Na free solution. These results show that above a [Ca]/[Na]^2 ratio of about $1{\cdot}39 \times 10^{-4}$ mM^{-1} (Ca of 2 mM and Na of 120 mM) maximum contracture tension is developed. The more extensive results in Table 2 show that at a ratio of $0{\cdot}7 \times 10^{-4}$ mM^{-1} (Ca = 1 mM) maximum tension is almost reached. This is rather similar to the $1{\cdot}5 \times 10^{-4}$ mM^{-1} obtained by Lüttgau & Niedergerke (1958) with ventricular strips and so there is no essential difference between the preparations in this respect.

These results on the potassium contractures were independent of the state of hypodynamia of the ventricle. The effect of calcium and sodium variation on the twitches were, however, dependent on the degree of hypodynamia. As already described by Clark (1913, Figs. 3 and 5) increased calcium concentration (above 1–2 mM) and decreased sodium concentration do not increase the twitch tension in fresh frog ventricles beating

spontaneously. This was verified on preparations driven at 30/min in the present experiments. On the other hand Clark showed that in hypodynamic ventricles Ca increase or Na decrease caused marked increases in the developed twitches. This has since been verified many times. We have shown that hypodynamia does not affect the sensitivity of the contractures to Ca and Na. These results can be summarized in another way by saying that in fresh ventricles maximum contracture and twitch tension is developed to similar values of the ratio $[Ca]/[Na]^2$ but that in hypodynamic ventricles maximum twitch tension occurs at a larger value of $[Ca]/[Na]^2$ than does maximum contracture tension.

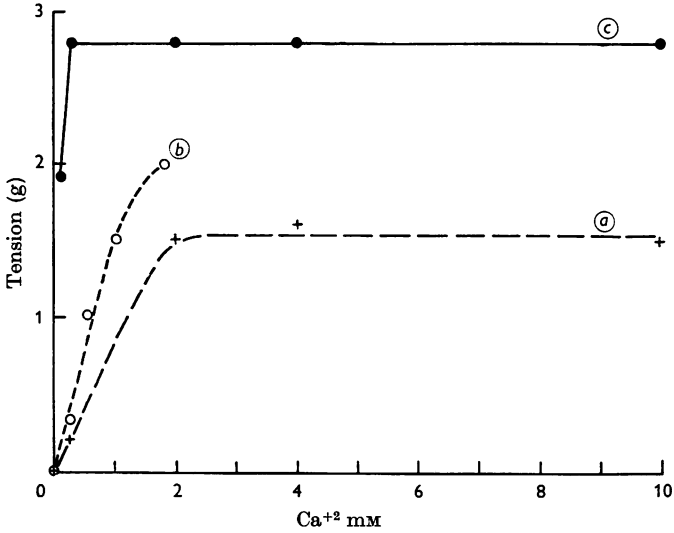

Fig. 12. Relation between calcium concentration and contracture tensions in typical experiments. (*a*) and (*b*) calcium varied at normal sodium. (*c*) calcium varied at zero sodium. Three separate preparations used.

DISCUSSION

The main reason for starting this work was to obtain a preparation of heart cells with rapid diffusion from the cells to the bathing fluid, so that experiments akin to those on single fibres of muscle and nerve could be done in the heart. The frog ventricle was an obvious starting point because of its sponge-like structure with the cells lying in the 'vascular space'. A drawback of the frog ventricle is that it normally has a low cardiac output and so it is difficult to control the composition of the fluid bathing the cells. The superfused technique described here has the virtue that it retains the advantages of the spongy structure of the ventricle, but allows a much faster washing rate than normal. This means that the composition of the

fluid between the cell strands can be better controlled. Histological sections (Fig. 2) show that, in the main, the diffusion distances from most of the cells to the bathing fluid are still kept short, but some thicker cell strands still remain near the cut surface. The time course of calcium effects on the twitch is in good agreement with these histological findings if the low diffusion constant of calcium found by Niedergerke (1957) is used. With the higher diffusion constants found in other tissues or in free solution much longer diffusion pathways would be expected. This may indicate 'unstirred layers' around these cell strands (Dainty & House, 1966).

With the normal stimulation rate of 30/min used here, the rate of onset of the K contractures is fast and comparable to those in skeletal muscle single fibres (Hodgkin & Horowicz, 1960). In the earlier work of Niedergerke and his associates with ventricular strips the K contractures were relatively quite slow in onset. The reason for this difference is mainly the slower stimulation rate used with the strips (for at 2/min the K contracture is much smaller and slower in onset than at 30/min), but partly due to the greater diffusion distance involved. In both ventricle preparations the K contractures last much longer than in skeletal muscle cells.

Two rather separate views on the staircase phenomenon appear to be currently discussed (Leonard & Hajdu, 1962). That due to Hajdu (1953) suggests that an optimum intracellular environment is required for maximum contraction. During rest the intracellular potassium concentration exceeds this; on resumption of activity this high potassium level falls gradually until the optimum value is reached again. The changes found experimentally are small, of the order of 3 m-mole/l. between the start and completion of the staircase phenomenon. An earlier view (Hofmann, 1926) suggested that the excitation contraction link became weaker during rest. Later Niedergerke (1956*a*) suggested that this was due to changes in the concentration of calcium in a superficial region of the cell (presumably as Ca complexed with a negatively charged complex, R). On depolarization this CaR saturates the contractile system in proportion to its concentration. The present experiments show that although the staircase phenomenon affects the K contractures it does not affect the Na free contractures. This result would be expected on Niedergerke's hypothesis, for the absence of Na would greatly increase the concentration of CaR whatever its starting value. The present results do not support Hajdu's hypothesis. To do so, the intracellular environment of the cells would need to alter in 2 sec. It must be pointed out however that if the sodium flux were as high as 100 p-mole cm^2 sec then in 2 sec the intracellular sodium could drop by 2 m-mole/l.

In fresh preparations we have shown that the twitches almost reach the same tension as the maximum K contractures, both at 30/min and at

lower stimulation rates. This is to be expected, for the ventricular action potential lasts for a long time and therefore allows the twitch tension to go near to maximum. With the onset of hypodynamia the situation changes for the twitch tension may drop to 1/5 of its previous value, whereas the contracture tension is unaltered, and so the action potential only releases a small proportion of the total available tension. The explanation for this change is not clear. There is certainly some shortening of the action potential, but this would seem to be too small to account for the change quantitatively. Clark (1913) argued that the semi-permeability of the heart cell membranes was altered in hypodynamia due to the loss of lipoid substances by perfusion. He showed that this process was reversible as the heart could be restored by serum, various soaps and other substances. The present evidence supports this view of a peripheral effect on the cell, by showing that the contractile elements are still capable of a normal contracture in a hypodynamic heart. From Clark's work it is apparent that the sensitivity of the twitch to $[Ca]/[Na]^2$ alters during hypodynamia so that a higher ratio is required for maximum contraction. We have shown that the contractures do not alter in a similar way, but retain a similar sensitivity to the ratio $[Ca]/[Na]^2$ throughout the development of hypodynamia.

The experiments on the relation between membrane depolarization (E_m) and tension development suggests that under normal conditions the tension threshold is between -20 and -30 mV. This figure is less than that of -55 mV for skeletal muscle (Hodgkin & Horowicz, 1960) and for frog ventricle of about -55 mV found by Niedergerke (1956) and Lüttgau & Niedergerke (1958). It is similar to the value of about -30 mV found by Lüttgau (1963) on frog muscle. The lower value found in the present experiments on frog ventricle is presumably due to the use of excess K with 1 mM-Ca rather than altering the Na or Ca level as done previously. If these results are correct it means that the contractile system only responds to rather a small part of the action potential, that below about -25 mV. In frog ventricle the 'plateau' starts at about $+20$ mV and persists to about -60 mV before the final rapid repolarization (Brady & Woodbury, 1960). This means that the contractile elements will be 'switched off' before the end of the action potential. This explains recent results we have obtained with perfused frog ventricles in which the peak of the mechanical twitch is about 100 msec before the T wave of the E.C.G.

In the present experiments the sodium free contractures have similar rates of onset to K contractures (at higher rates of stimulation) and to calcium alterations. In addition increased osmotic pressure has a very rapid effect on the contractures, much faster than alteration of the general

cell size consequent on bulk water withdrawal. Both these observations provide further support for the view that Ca and Na interact at or near the cell surface (Lüttgau & Niedergerke, 1958); water withdrawal from this region would increase the 'CaR' concentration.

In ventricular strips the Na free contractures are slow in onset (Lüttgau & Niedergerke, 1958), whereas in the superfused preparation they are very fast. It has been proposed that the difference is due to interaction between washing and leaching of Na from the tissues; in the poorly washed strips enough Na leaches from the tissue 'stores' to keep the amount of CaR low until all the Na has left these stores. Due to the non-linearity between $[Na]_0$ and tension development (Niedergerke, 1963, Fig. 4) small extracellular Na concentrations are sufficient for this purpose. If this explanation is correct then these tissues stores of Na are emptied in about 1–2 min, both in ventricular strips and in the superfused preparation. With ventricular strips the extracellular Na in sodium free solutions soon drops to a low level, therefore there should not be much back diffusion into the cells in either preparation.

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