J. Physiol. (1963), **167**, *pp.* 551–580
With 9 text-figures
Printed in Great Britain

MOVEMENTS OF Ca IN BEATING VENTRICLES OF THE FROG HEART

By R. NIEDERGERKE

From the Department of Biophysics, University College London

(*Received* 5 *October* 1962)

The hypothesis that entry of calcium into cardiac muscle fibres is responsible for the initiation of contraction is supported by results described in the preceding paper (Niedergerke, 1963), where an increased uptake of labelled calcium by frog ventricles was shown to be associated with contractures initiated by suitable changes in the sodium and potassium concentrations of the external fluid. In the present work further support for this hypothesis has been obtained under more physiological conditions by examining the calcium fluxes occurring during a series of propagated twitches.

Since twitch tension is known to become greater with increasing [Ca] in a suitable range of concentrations, it seems reasonable to expect a corresponding increase in influx. However, such a result would not by itself be a decisive test of the hypothesis, for it is known that the additional calcium influx associated with propagation of the nerve action potential also increases with the external calcium concentration (Hodgkin & Keynes, 1957), and it is likely that the same effect will apply to other excitable tissues, including cardiac muscle. More convincing evidence would be obtained if it could be shown that the increased twitch tension resulting from a reduction of the external sodium concentration is also associated with an increased influx of calcium. A further possibility, also based on the known features of the antagonism between calcium and sodium, is to compare the influxes of calcium occurring at two levels of calcium concentration, but with the concentration of sodium so adjusted that the twitch heights are similar.

A series of such experiments is described in the first part of this paper. In the second part corresponding measurements of the effect of stimulation on the efflux of labelled calcium are presented. It was especially interesting to observe that the increased uptake during stimulation was matched by a quantitatively similar change in the magnitude of efflux. This provides direct support for the subsidiary hypothesis that the cells possess a mechanism whereby they can maintain the internal calcium concentration at a relatively constant level.

METHODS

Animals. To remove a likely source of variability hearts from frogs of a single species, Dutch *Rana temporaria*, were used throughout. Care was taken to keep the animals in good condition by avoiding overcrowding, and by early segregation of the sick. Nevertheless, with the first batch of frogs (used from August to January) a progressive deterioration occurred in the response of the ventricles to continuous stimulation, as described in the first part of the 'Results' section. Although it is not clear whether this was in fact related to a deterioration in the condition of the animals or a result of hibernation, additional precautions were observed with the second batch (used from April to August). These animals were provided with 'dry land' and continuously renewed water, and were fed on a diet consisting alternately of raw liver dipped in cod-liver oil (forced feeding) and meal worms (taken voluntarily). With this treatment they remained in good condition throughout the time of use.

Material and procedure of analysis. These were essentially similar to those employed in the previous paper (Niedergerke, 1963). Ventricles (wet weight from 40 to 70 mg) were isolated as before and after carefully removing the superficial connective tissue were attached to the polyfluoroethylene cannula illustrated in Fig. 1*b* in that paper. The cannula was inserted through the atrioventricular hole and held with a thin strand of nylon thread tied round a small ring of auricle, which after careful dissection always weighed substantially less than 1 mg, so that the total quantity of injured tissue present should be minimal. Perfusion was carried out as previously described but at a faster rate of 2–4 ml./min. Ventricles were stimulated by the passage of short electric current pulses (of twice threshold strength) from two Pt-disk electrodes attached to opposite surfaces of the perfusion vessel. To avoid polarization the direction of successive shocks was alternated by using the currents of a charging and discharging condenser.

Perfusion fluids. The composition of 'normal' 1 mM-Ca-Ringer was that described in the previous paper. The Ca concentration was altered by adding or subtracting weighed amounts of solid $CaCl_2$, the Na concentration by replacing NaCl with osmotically equivalent choline Cl. To these Na-depleted fluids 2×10^{-5} M atropine sulphate was added in order to antagonize the inhibitory effect of the choline.

Oxygenation and CO_2-narcosis of the ventricles. Fluids were oxygenated by means of a polythene tube introduced into the perfusion chamber. Oxygen which was normally used could quickly be replaced by a CO_2-rich mixture (30 % CO_2 + 70 % O_2) which was found to cause an almost instantaneous and, if applied for relatively short times, reversible inhibition of the spontaneous beating of the ventricle. No attempt has been made to determine whether the effect is merely due to a lowering of the pH in the perfusion fluid (from about 7·6 to about 5·6), or in addition, to a 'specific' action of the CO_2 on the cardiac cells.

Temperature. The first experiments were made at room temperature (varying between 18 and 20° C) and the remainder at about 7° (5–8) C (see Results).

Determination of the cell dimension. In order to determine the circumference and cross-sectional area of ventricle fibres a number of electron micrographs of the frog ventricle were made in collaboration with Dr H. E. Huxley. A strip was fixed in a 1 % solution of osmium tetroxide, buffered with veronal acetate at pH 7·5. After dehydration the tissue was embedded with araldite and stained with 1 % phosphotungstic acid. Cross-sections, about 300 Å thick, were photographed to have a final magnification of about 10,000 times. The areas of 200 different fibres were measured with a planimeter, and their circumferences with a map reader. Oblique sections, recognizable by the characteristic appearance of the myofilaments, were rejected.

RESULTS

Effects of modified Ringer's fluid on the strength of the heart beat

The aim of the first experiments to be described was to determine how rapidly the twitch height of the ventricle reached its new level following application of the modified Ringer's fluids used under comparable conditions in the main series of experiments with labelled calcium. Ventricles were attached to a conventional Straub cannula, and the excursions of their tips recorded with a light isotonic lever writing on a smoked drum. The cannula was supplied with fluid at 6–8° C from storage vessels placed some distance above it, the fluid leaving the ventricle through the aortic hole. A constant perfusion pressure difference of 30–40 mm water (depending on the size of the ventricle) was maintained by placing a suction tube at the appropriate height within the cannula. Ventricles were stimulated at a rate of 10 shocks/min by the application of short electric shocks between two stainless-steel wires, one placed within the cannula, and the other at the external surface of the ventricle. With this arrangement it was found that the twitches increased in a roughly linear fashion with increasing Ca concentration over the range 0·5–1·5 mM, maximal responses being obtained with between 2 and 3 mM. A typical experiment is illustrated in Fig. 1, which shows the changes in twitch peaks when 0·5 mM-Ca-Ringer was alternated with fluid containing either 1 or 2 mM-Ca, or with 50 % of the sodium replaced by choline. As had been expected from the antagonism between calcium and sodium, twitches are of similar height after either a fourfold increase in calcium, or a reduction by half in the concentration of sodium. It should be noted that, before changing the fluid, the ventricles were emptied to a residual volume of about 0·1 ml., as determined by weighing controls treated in the same way and then blotted. With this procedure, and an average rate of perfusion of 3 ml./min, the new concentration should have been established within the ventricular cavity 5–10 sec after the change of fluid. The half-times for build-up and decay of the responses illustrated in (1), (2) and (3) of Fig. 1 were 20, 15 and 12·5 sec, respectively, and these values lie within the range of half-times of from 10 to 20 sec observed in 4 other such experiments.

These results show that under such conditions the extracellular equilibration of calcium and sodium, which probably limits the rate of change of tension (Niedergerke, 1957), is fairly rapid, being practically complete within 1 min. Therefore, in all later experiments with tracer Ca, an equilibration period of 1 min in the radioactive fluid before stimulation was considered adequate.

Appreciably lower rates of equilibration of the tension responses were observed, particularly toward the end of prolonged experiments, when

changing from high to medium Ca concentrations (e.g. from 4 to 1 mM) and also from low to medium Ca concentrations (e.g. from 0·25 to 1 mM). This phenomenon has not been further investigated, but it is likely that under these special conditions the rate-determining process is no longer extracellular.

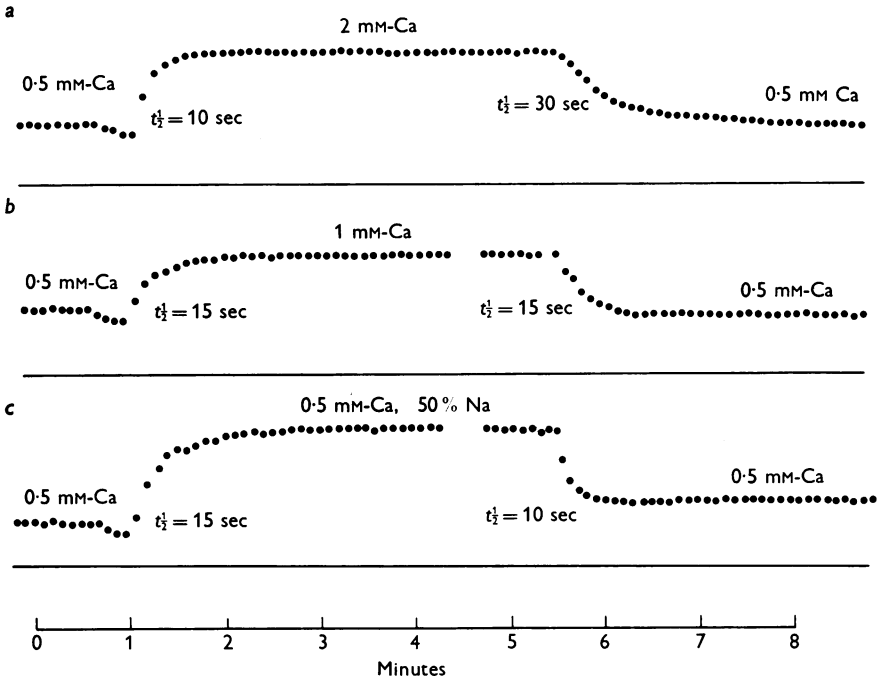

Fig. 1. Time course of the effect of changing Ca or Na concentrations on the amplitude of the heart beat. Tracings of maximal excursions of a heart lever. Ringer's fluid changed from 0·5 mM-Ca + 100 % Na to (*a*) 2 mM-Ca, (*b*) 1 mM-Ca and (*c*) 0·5 mM-Ca + 50 % Na 50 % choline, and vice versa. Initial dip before increase of the beats is a mechanical artifact due to emptying of the cannula. Atropine sulphate 2×10^{-5} M present throughout.

Procedure and preliminary experiments with ^{45}Ca

The procedure was essentially similar to one described in the preceding paper (Niedergerke, 1963; see discussion in connexion with Figs. 12 and 13). As before, a ventricle which had been resting in normal Ringer's fluid for about 2 hr was exposed to ^{45}Ca during a period of activity (in the present experiments a series of twitches). It was then perfused with successive volumes of inactive fluid, the perfusate being kept for counting. Finally, the ventricle itself was prepared for counting. In this way the tracer content of the tissue at various times after exposure to ^{45}Ca could be determined. A proportion of this tracer was, however, 'extra-fibre' and to

allow for this it was necessary to make a series of control experiments with unstimulated ventricles, other conditions being identical. By subtracting the 'washing-out' curves of these controls, a measure of the effect of stimulation could be obtained. A standard period of 11 min was chosen for perfusion with active solutions, since by the end of this time 'extra-fibre' uptake was largely complete and the resting uptake still relatively small (Niedergerke, 1963). It should also be noted that control ventricles

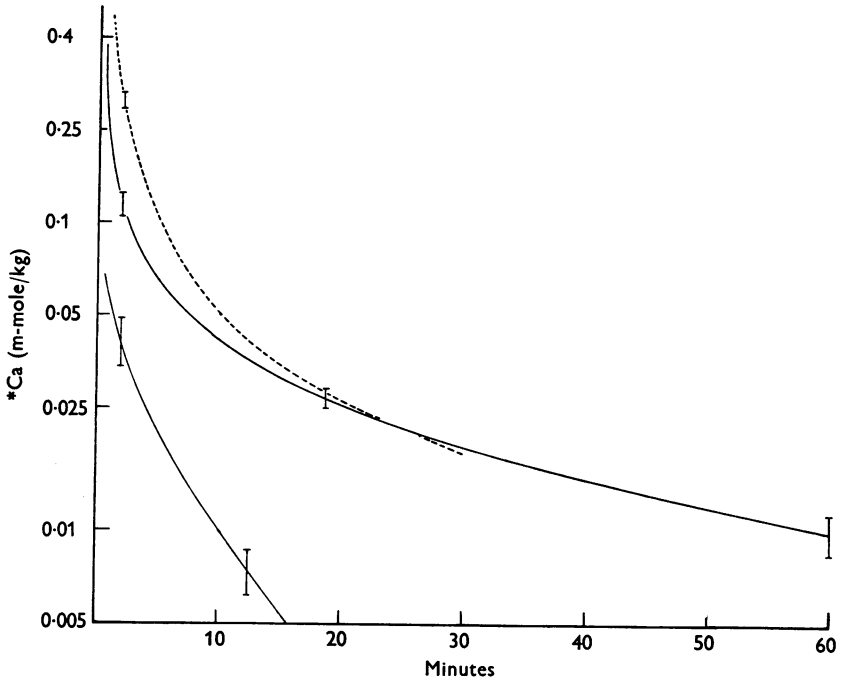

Fig. 2. Washing-out curves of unstimulated ventricles. Ordinate, tracer Ca retained by the ventricle (m-mole/kg) on logarithmic scale. Abscissa, time after removing ventricles from the radioactive fluids. Upper two curves, combined results from 4 resting ventricles which had been perfused for 11 min with 1 mM-*Ca-7Ringer (—) or with 4 mM-*Ca-Ringer (-----). Vertical bars show 2 × S.E. The lower curve gives the mean result of two experiments in which the tracer was washed off from the cannula and some residual auricle (vertical bars indicate range).

not naturally quiescent were made so by the addition of 5×10^{-4} M-ACh to both the load and washing-out fluids. In agreement with results described in the preceding paper no effect of acetylcholine on either uptake or efflux was observable under these conditions.

The upper two curves in Fig. 2 have been drawn through the mean values of two series of such control experiments with perfusion fluids which contained 1 and 4 mM-Ca, respectively. (For these, as in other such deter-

minations, at least four experiments were made in establishing each 'control' curve.) The variability of these controls, indicated by the length of the vertical bars (= ± S.E. of the mean), is probably mainly due to the presence of varying amounts of connective tissue and other 'extra-fibre' material which can absorb Ca. In support of this it was observed that tracer release was large from ventricles possessing relatively large amounts of connective tissue. Such ventricles were discarded in later experiments.

The observation that the washing-out curves obtained after loading with 1 and 4 mM-Ca are significantly different only during the first 10 min of washing out can best be explained by supposing that most of the preceding uptake was into superficial regions from which the tracer is easily lost again. That the two curves cross after 20 min is probably fortuitous; different batches of frogs had been used in the two series of experiments. The lowest curve in Fig. 2 was obtained in a further series of control experiments made to assess the contribution to the upper curves from tracer adhering to the cannula and to a typical ring of auricle, attached to the cannula by a nylon thread, as in all the present experiments. It can be seen that the contribution to the washing-out curves from these sources, though substantial at first, diminished greatly with time.

The procedure for the determination of the additional uptake resulting from stimulation is illustrated in Fig. 3, which shows the results of two experiments in which ventricles had been stimulated to give 100 beats in a 1 mM-*Ca-, 50 % Na-Ringer's fluid. Beating which would usually have continued spontaneously after cessation of stimulation was arrested in the first five volumes of washing fluid which had been equilibrated with the CO_2-rich gas mixture. Some ventricles remained quiescent after this period of narcosis, as for example, in the experiment of Fig. 3*a*, but more commonly spontaneous beating appeared after some 10–20 min, as in the experiment 3*b*, in which this continued at a constant frequency of about 17 beats/min up to the end. Subtraction of the controls gives the lower pair of the smooth curves (open circles), which, except for an initial period of about 5 min, can be approximated by straight lines. The extrapolation of these lines to zero time gives a measure of the additional uptake of Ca associated with activity. The results obtained in such experiments are discussed in detail in a later section.

The main uncertainty in this method arises from the fact that the 'stimulated' and 'control' curves were determined with different ventricles. An estimate of the error which may have been introduced in this way was obtained by 'correcting' the washing-out curves of stimulated ventricles by subtracting the control curves which showed the largest and smallest 'extra-fibre' uptake, respectively. The results so obtained are also plotted in Fig. 3 as the two interrupted lines. It was found that the extreme

estimates of the uptake differed in 9 experiments on the average by about 10 % from the values obtained from the 'average' controls. This error, determined with ventricles which had gained about 0·1 m-mole/kg additional *Ca during stimulation, increases of course with smaller and

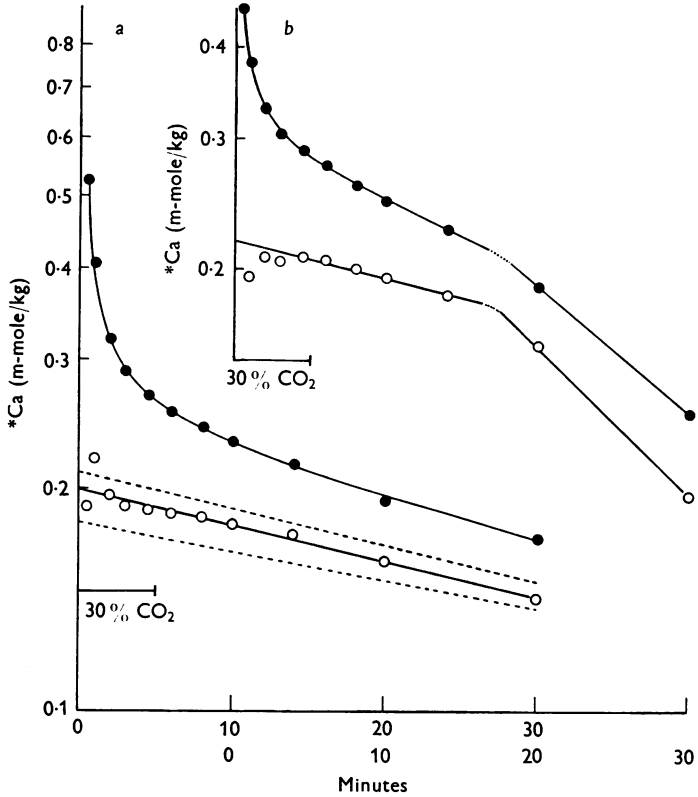

Fig. 3. Illustration of method to correct for 'extra-fibre' *Ca. Both ventricles had been stimulated with 100 shocks at the rate of 20 shocks/min in 1 mM-*Ca + 50 % Na + 50 % choline-Ringer. The first 5 volumes of the washing-out fluids had been equilibrated with 30 % CO_2 to inhibit spontaneous beating. The ventricle of Fig. 3*a* was quiescent throughout washing-out, that of Fig. 3*b* started beating spontaneously after about 17 min. Subtraction of the average washing-out results of 4 resting ventricles subjected to the same soaking and washing-out procedure as those stimulated gave the results shown by open circles to which straight lines have been drawn by eye. For explanation of the interrupted lines, see text.

decreases with larger *Ca gains. It will be seen in Fig. 3*b* that the negative slope of the washing-out curves increases greatly with the onset of spontaneous beating, indicating that Ca efflux becomes larger as a result of ventricular activity. This finding will be examined further in a later section.

In the experiment of Fig. 3*b* the increase in the rate of Ca loss was approximately fourfold, corresponding to the ratio τ_1/τ_2 (= 85 min/22 min) of the time constants obtained from the

slopes of the straight lines used to approximate the Ca loss. However, the correction procedure discussed in the preceding paragraph also limits the accuracy of the estimates of the two time constants, particularly those describing Ca release at rest, for, as can be seen in Fig. 3*a*, the two interrupted lines differ in slope as well as in the intercept they make on the ordinate. In this experiment the time constants estimated from the slope of the two lines were different by a factor of 1·3, but in other experiments factors of up to 2 have been obtained. Owing to this uncertainty, which arises mainly because the lines are short as well as rather flat, only rough estimates of the resting time constants could be obtained. (Prolonging the pre-stimulation period would have tended to reduce these errors, but at the cost of uncertainty as to the effects of the extended period of narcosis with CO_2-rich fluids.) However, time constants during periods of activity could be determined with considerably greater accuracy, i.e. with error of up to about 20 %, because the slopes are then steeper and the correction for 'extra-fibre' Ca had become substantially smaller.

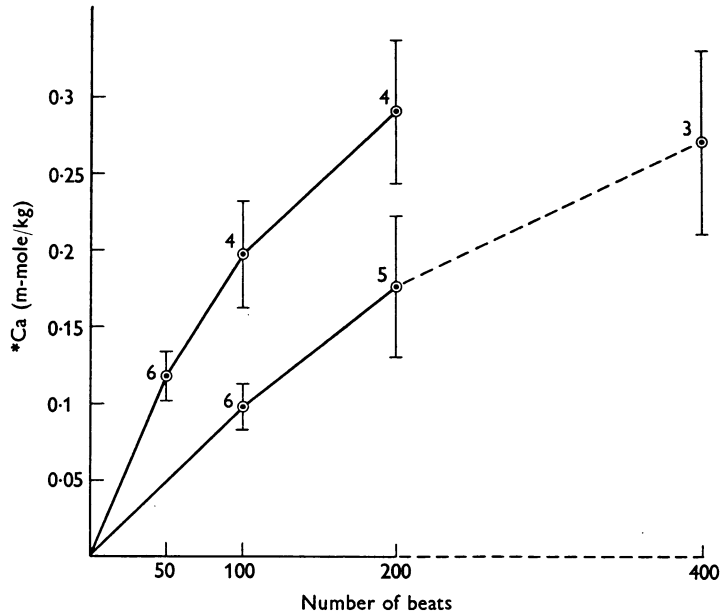

Fig. 4. Extra *Ca uptake associated with a series of beats in the presence of either 1 mM-*Ca-Ringer (lower curve) or 1 mM-*Ca + 50 % Na + 50 % choline-Ringer (upper curve). Rate of stimulation was about 20 shocks/min (varying between 19 and 25), except in the three experiments with a total of 400 beats during which the frequency was 40 shocks/min. Numbers are ventricles used in obtaining a given *Ca concentration. Vertical bars 2 × S.D. Temperature, 18–22° C.

Figure 4 summarizes the results of experiments in which the uptake of ventricles had been determined after 50–400 beats in the presence of either normal Ringer's solution or 50 % Na + 50 % choline-Ringer, both fluids containing 1 mM-*Ca. Although in some experiments satisfactory results were obtained at a stimulus frequency of 40/min (see results after 400 beats), in others the twitch height gradually declined with continued stimulation ('negative staircase', Niedergerke, 1956). Subsequently fre-

quencies of about 20/min, just above the natural frequency of the ventricular pace-maker, were used with more satisfactory results. It should be noted that ventricles stimulated with less than 200 pulses were placed in the tracer solution for an initial period without stimulation so that the time of exposure to *Ca should be constant, 11 min in all experiments.

The results plotted in Fig. 4 show clearly that the uptake of tracer is increased by stimulation and that the magnitude of the increase is, as was expected, greater when the bathing fluid contained less Na. If it can be assumed that the uptake of *Ca per twitch in any one experiment is constant, as would appear reasonable, the non-linearity of the build-up of fibre *Ca indicates that there is a simultaneous loss of tracer. This makes it difficult to calculate the absolute magnitude of the influx from these results.

As a next step the *Ca uptake was determined in the presence of 4 mM-*Ca-Ringer's fluid in which the ratio $[Ca]/[Na]^2$, and thus the strength of the heart beat, is increased to the same extent as with 50 % Na-Ringer. Since at the time at which these experiments were made the ventricles failed to maintain beats of constant size in these fluids, even with a rate of stimulation of only 20/min, the rate was further reduced to 10 shocks/min. Because this was below the natural frequency of the ventricular pace-maker it also became necesary to lower the temperature of the bathing fluid to about 7° C.

Table 1 summarizes the results obtained under these conditions. It is seen that the uptake of *Ca in the two contrasted groups is rather similar; thus the average content of *Ca in the ventricles after 100 beats in the presence of 4 mM-*Ca-Ringer was 0·286 m-mole/l. as compared with 0·266 in the presence of 50 % Na-Ringer. However the small number of determinations and the fact that even under these conditions four

TABLE 1. Uptake of *Ca after 100 beats (10 beats/min) in the presence of

Ventricle reference	(*a*) 4 mM-*Ca-Ringer (m-mole/kg)	(m-mole/l.)	Ventricle reference	(*b*) 1 mM-*Ca + 50% Na + 50% choline-Ringer (m-mole/kg)	(m-mole/l.)
a 1	0·2	—	*b* 1	0·17	—
a 2	0·185	—	*b* 2	0·2	—
a 3	0·19	—	*b* 3	0·15	—
a 4	0·22	—	*b* 4	0·22	—
a 5	[0·07]	—	*b* 5	[0·115]	—
a 6	[0·085]	—	—	—	—
a 7	[0·11]	—	—	—	—
Mean	0·199	0·286	—	0·185	0·266
S.D.	0·016	0·023	—	0·029	0·041

The *Ca-uptake in units of the volume of myocardium was obtained by dividing the experimental values of *Ca/(ventricle weight) by the factor 0·695 which is the ratio (vol. of myocardium)/(ventricle wt.) (Niedergerke, 1963). Values in brackets have been omitted for the calculation of the means because these ventricles showed signs of the 'negative staircase' (see text).

ventricles showed a 'negative staircase' accompanied by a greatly reduced *Ca uptake (values in brackets) makes the result rather tentative.

Rather surprisingly tracer uptake at the reduced temperature and rate of stimulation seems little different from that at room temperature. This may be seen from a comparison of the *Ca uptake after 100 beats in the presence of 1 mM-*Ca-Ringer at room temperature, of 0·141 m-mole/l. (corresponding to a value of 0·098 m-mole/kg of Fig. 3) with the corresponding value at 7° C of 0·135 m-mole/l. (Table 2, I*a*). A tentative explanation of this result is to suppose that the expected reduction of the *Ca influx due to low temperature is matched by a corresponding lengthening of the action potential. That cooling in fact prolongs the action potential of the heart is well known (Woodbury, Hecht & Christopherson, 1951) and under conditions similar to those of the present experiments the duration of the potential was found to increase from about 0·8–1 sec at 20° C to 2–2·5 sec at 8° C (Niedergerke & Orkand, unpublished).

To avoid the shortcomings described in the preceding paragraph a further series of experiments was made at the lower temperature with Ca concentrations of up to only 2 mM.

Later experiments

In these, the main series of experiments, both uptake and release were examined by using ventricles which were first stimulated in the *Ca-containing fluids either 100 or 600 times, corresponding to loading times of 11 and 61 min, respectively. The detailed procedure during the subsequent washing-out varied somewhat, depending on the aim of the experiment. For the determination of the uptake as in Fig. 3 the ventricles after transfer from the loading solutions were first perfused for a fixed period of 19 min, during which they remained quiescent owing to the CO_2-narcosis and its after-effects. They were then stimulated for 20–40 min to permit the effect of activity on release of Ca to be examined. Finally, they were again made quiescent for a time by perfusion with CO_2-rich washing fluids. The results of a number of such experiments are illustrated in Fig. 5. The bathing fluids used contained 1 mM-Ca or either 2 mM-Ca or 0·5 mM-Ca + 50 % Na, corresponding to a ratio of $[Ca]/[Na]^2$ of twice normal. It is seen that as in Fig. 3*b* the slope of the lines increased greatly during activity. Although in some experiments this region of increased efflux could be approximated by a single straight line (slope $= -(\tau_{s(I)})^{-1}$), in others (e.g. of the curves III*l* and III*i*), the rate of loss usually declined towards the end of long periods of stimulation, so that a second straight line (slope $= -(\tau_{s(II)})^{-1}$) was needed to fit the later points. The results of all experiments of this kind are summarized in Table 2, where values are listed for the uptake of *Ca in the presence of the three test fluids and also for the exponential time constants of release at rest, τ_r, (column 3) and during stimulation $\tau_{s(I)}$ and $\tau_{s(II)}$ (columns 4 and 5), respectively; together with the lowest level of the fibre *Ca, as a percentage of the previous gain, to which this had declined with a particular time constant. The most striking feature of these

results is the magnitude of the tracer which can be released from the cells during activity. Thus in the experiments in which the washing-out was preceded by 100 twitches in the labelled fluids the amount of tracer remaining after a period of activity was only 10–15 % of the previous gain in ventricles, I*e*, II*g*, II*d* and III*f*. Even after the long-loading period (during which 600 stimuli were given) a decline in *[Ca_i] to 22·5 and 17 % (in ventricles II*l* and III*i*) occurred, and there is no reason to suppose that more prolonged activity would not have reduced this further. This marked exchangeability of Ca and the fact that the exchange kinetics are approximately exponential are most easily explained by supposing that during activity the tracer enters and leaves some cellular compartment, perhaps the cell itself, in which it mixes readily with unlabelled Ca. In considering this possibility further it is necessary to discuss the time course of exchange in rather greater detail.

The kinetics of such single-compartment systems are well understood (cf. Hodgkin, 1951). The finding that fibre *Ca falls with time according to the expression:

$$[{}^*Ca_i] = [{}^*Ca_i]_{(t=t')} \exp -\left(\frac{t-t'}{\tau_s}\right), \quad \text{for} \quad t > t', \tag{1}$$

where t' is the time at the onset of stimulation in the washing-out fluid, suggests that the release of the tracer bears the following relation to m_{out}, defined as the efflux/fibre volume,

$$-\frac{d\,[{}^*Ca_i]}{dt} = m_{out}\frac{[{}^*Ca_i]}{[Ca_i]}, \tag{2}$$

where $[Ca_i]$ is the concentration of exchangeable Ca inside the fibre. Hence

$$\frac{1}{\tau_s} = \frac{m_{out}}{[Ca_i]} = \frac{s}{v}\frac{M_{out}}{[Ca_i]}, \tag{3}$$

where M_{out} is the efflux/fibre surface and s/v the ratio of the surface/volume of the fibre.

The relationship between M_{out} and $[Ca_i]$ is not known with certainty but will be assumed in interpreting the present results to be linear, as is the case for sodium efflux and $[Na_i]$ in the cephalopod axon (Hodgkin & Keynes, 1956). If this is in fact correct τ_s will remain constant and tracer release strictly exponential. This may at first sight seem to be in conflict with previous evidence that the time constant becomes smaller with increasing $[Ca_i]$ (Niedergerke, 1963). However, $[Ca_i]$ appears to change much less in the present than in the former experiments. Thus the largest tracer uptake during the present experiments was observed in the presence of 2 mM-Ca-Ringer after 600 twitches, when a steady state had been approached. This value of 0·57 m-mole/l. (Table 2, II*b*),

TABLE 2. ^{45}Ca inward movement in frog ventricles during series of beats (Stimulation 10 shocks/min; temperature 6–8° C)

Ventricle reference 1	*Ca uptake (m-mole/l.) 2	τ_r (min) 3	$\tau_{s(I)}$ (min) 4	$\tau_{s(II)}$ (min) 5	$\frac{\tau_{s(I)}}{\tau_{s(II)}}$ 6	$\frac{\bar{\tau}_{s(I)}}{\bar{\tau}_r}$ 7	F $(min)^{-1}$ 8	Influx/beat (μ-mole/l.) 9	Influx/beat (p-mole/cm^2) 10
I in the presence of 1 mM-Ca-Ringer									
(*a*) 100 twitches									
I*a*	0·121	85	25 (55 %)	—	—	—	0·121	1·46	—
I*b*	0·135	150	24 (66 %)	—	—	—	0·123	1·66	—
I*c*	0·145	115	14 (20 %)	—	—	—	0·14	2·03	—
I*d*	0·141	120	20 (32 %)	24·5 (16 %)	0·82	—	0·127	1·79	—
I*e*	0·108	140	17 (23 %)	24·5 (13·3 %)	0·69	—	0·132	1·43	—
I*f*	0·158	75	—	—	—	—	—	—	—
Mean	0·135	110	19·6	—	0·75	0·18	—	1·67	0·15 [0·14]
S.D.	0·02								
(*b*) 600 twitches									
I*g*	0·453	134	30 (37 %)	—	—	—	$\frac{\bar{\tau}_{s(I)}\text{ (after 100 beats)}}{\bar{\tau}_{s(I)}\text{ (after 600 beats)}}$		
I*h*	0·49	125	51 (53 %)	—	—	—			
I*i*	0·475	200	23 (33 %)	—	—	—			
I*j*	0·518	264	43 (47 %)	—	—	—			
I*k*	0·396	150	31 (32 %)	—	—	—			
Mean	0·466	168	34·1	—	—	0·2		0·58	
S.D.	0·046								
II in the presence of 2 mM-Ca-Ringer									
(*a*) 100 twitches									
II*a*	0·266	280	32 (50 %)	—	—	—	0·117	3·11	—
II*b*	0·187	300	26 (51 %)	—	—	—	0·12	2·24	—
II*c*	0·23	125	—	—	—	—	—	—	—
II*d*	0·216	120	—	—	—	—	—	—	—
II*e*	0·259	170	23 (39 %)	—	—	—	0·123	3·18	—
II*f*	0·223	290	28·4 (44·5%)	—	—	—	0·119	2·66	—
II*g*	0·158	120	19·6 (22 %)	25·6 (12·4 %)	0·77	—	0·128	2·02	—
Mean	0·22	185	25·4	—	0·77	0·14	—	2·64	0·23 [0·22]
S.D.	0·038								

TABLE 2 (*continued*)

Ventricle reference 1	*Ca uptake (m-mole/l.) 2	τ_r (min) 3	$\tau_{s(I)}$ (min) 4	$\tau_{s(II)}$ (min) 5	$\frac{\tau_{s(I)}}{\tau_{s(II)}}$ 6	$\frac{\bar{\tau}_{s(I)}}{\bar{\tau}_r}$ 7	F $(\text{min})^{-1}$ 8	Influx/beat (μ-mole/l.) 9	Influx/beat (p-mole/cm²) 10
(*b*) 600 twitches									
II*h*	0·59	270	32 (55 %)	—	—	—		$\frac{\bar{\tau}_{s(I)}\text{ (after 100 beats)}}{\bar{\tau}_{s(I)}\text{ (after 600 beats)}}$	
II*i*	0·648	250	43 (55 %)	—	—	—			
II*j*	0·576	190	61·5 (62·5 %)	—	—	—			
II*k*	0·619	230	41·6 (44·4 %)	55 (34·5 %)	0·76	—			
II*l*	0·396	105	27·4 (40 %)	32 (22·5 %)	0·86	—			
Mean	0·566	199	39·6	—	0·82	0·2		0·64	
S.D.	0·1								
III in the presence of 0·5 mM-Ca + 50 % Na + 50 % choline-Ringer									
(*a*) 100 twitches									
III*a*	0·18	80	—	—	—	—	—	—	—
III*b*	0·194	100	—	—	—	—	—	—	—
III*c*	0·216	130	—	—	—	—	—	—	—
III*d*	0·151	64	7 (24 %)	14 (10 %)	0·5	—	0·188	2·84	—
III*e*	0·266	138	11·5 (23 %)	—	—	—	0·149	3·96	—
III*f*	0·165	72	7·8 (26 %)	9·8 (11 %)	0·8	—	0·178	2·94	—
III*g*	0·223	145	15·9 (49 %)	18 (26 %)	0·88	—	0·135	3·01	—
III*h*	0·19	140	14 (43 %)	17·6 (24 %)	0·8	—	0·14	2·66	—
Mean	0·198	104	10·7	—	0·75	0·1	—	3·08	0·27 [0·22]
S.D.	0·036								
(*b*) 600 twitches									
III*i*	0·302	90	14·1 (37 %)	21 (17 %)	0·67	—		$\frac{\bar{\tau}_{s(I)}\text{ (after 100 beats)}}{\bar{\tau}_{s(I)}\text{ (after 600 beats)}}$	
III*j*	0·322	128	16·5 (32 %)	—	—	—			
III*k*	0·41	155	26 (30 %)	—	—	—			
III*l*	0·266	153	15 (50 %)	25·7 (32 %)	0·58	—			
III*m*	0·381	156	24 (55 %)	—	—	—			
Mean	0·332	134	(18·5	—	0·63	0·14		0·58	
S.D.	0·06								

Figures for *Ca uptake column (2) (m-mole/l. myocardium), calculated from experimental values (m-mole/kg ventricle) as described in Table 1. τ_r, $\tau_{s(I)}$, $\tau_{s(II)}$ time constants of *Ca-release at rest and at the beginning and during subsequent period of stimulation, respectively. Mean values of time constants, $\bar{\tau}$, are geometric means. Figures in brackets are levels of *[Ca] (% of the initial) to which the tracer in the tissue declined during series of twitches with a given exponential time constant. F (column 8), $= \{\tau_{s(I)}[1-\exp(-t/\tau_{s(I)})]\}^{-1}$, the factor used for the calculation of the influx according to equation (5). Figures in column (9) = (product of numbers in columns (2) and (8)/stimulus frequency, 10 shocks/min). Figures in square brackets are estimates of the 'average' influx (see text p. 567).

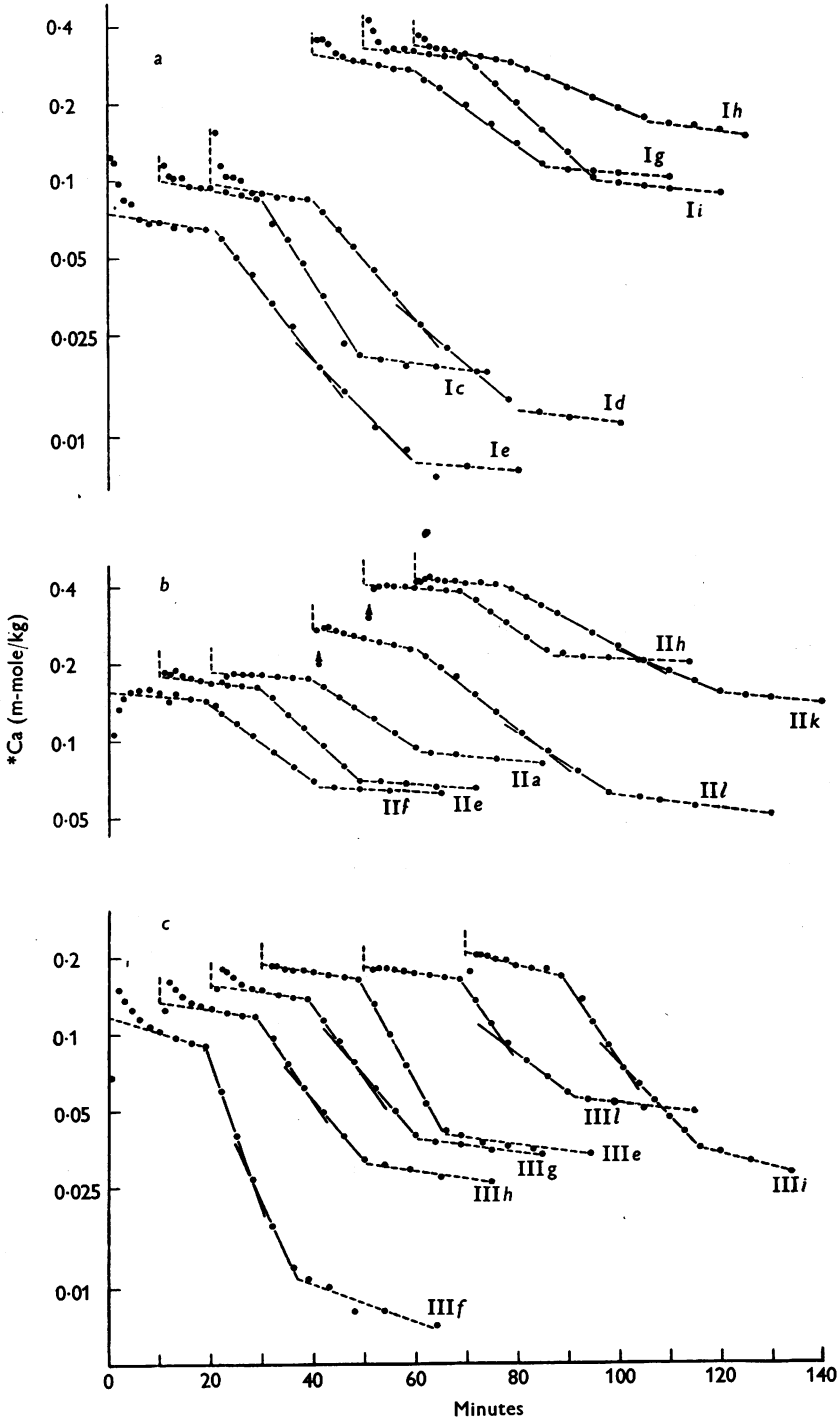

Fig. 5. For legend see opposite page.

corresponding to 0·39 m-mole/kg, is below the range of concentrations over which a reduced time constant had been observed in the previous experiments (e.g. discussion in connexion with Fig. 12*a*, Niedergerke, 1963). Also, such changes in $[Ca_i]$ as appear to occur cannot account for the observed deviation from the exponential law, e.g. the eventual flattening of the washing-out curves. For this was invariably observed, even with curves obtained with ventricles perfused with 1 mM-Ca-Ringer, when $[Ca_i]$ remained practically unaltered. (The approximate constancy of $[Ca_i]$ during activity in 1 mM-Ca-Ringer is suggested by the comparison of the magnitude of exchangeable Ca, of 0·49 m-mole/l. determined in the presence of this fluid (Niedergerke, 1963) with that of 0·466 m-mole/l. (Table 2, I*b*) after 600 beats when a steady state of exchange has almost been attained (cf. Fig. 8). Making this assumption, *Ca uptake should be given by

$$\frac{d\,[*Ca_i]}{dt} = \frac{s}{v} M_{in} - \frac{s}{v} M_{out} \frac{[*Ca_i]}{[Ca_i]} = m_{in} - m_{out} \frac{[*Ca_i]}{[Ca_i]}, \qquad (4a)$$

where m_{in} and M_{in} are influxes/(fibre volume) and (fibre surface), respectively. Hence by substituting equation (3) and integrating with the present initial conditions

$$[*Ca_i] = m_{in} \tau_s \{1 - \exp - (t/\tau_s)\}, \qquad (4b)$$

and

$$m_{in} = \frac{[*Ca_i]}{\tau_s \{1 - \exp - (t/\tau_s)\}}. \qquad (5)$$

Equation (5) allows the influx during the short series of beats to be calculated from the experimentally observed values for $[*Ca_i]$ (the gain of tracer) and τ_s (the time constant of release). This is permissible because the rate of release, and probably also that of the uptake, remain approximately constant during these 10 min periods, as is suggested by the results of Fig. 5. However, since it was not possible to determine these pairs of values simultaneously the assumption will be made that the time constant

Legend to Fig. 5.

Fig. 5. Release of *Ca from individual ventricles at rest and during stimulation. Washing-out curves corrected for 'extra-fibre' *Ca and approximated by straight lines, as illustrated in Fig. 3. Interrupted lines, periods of rest initiated by narcosis with 30 % CO_2-saturated fluids. Continuous lines, periods of activity during which ventricles were driven at the rate of 10 shocks/min. (*a*) 1 mM-Ca-Ringer as loading and washing-out fluid. Tracer uptake with ventricles I*c*, I*d*, I*e* during 100 beats, with ventricles I*g*, I*h*, I*i* during 600 beats. (*b*) 2 mM-Ca-Ringer as loading and washing-out fluid. Tracer uptake in ventricles II*a*, II*e*, II*f* with 100 beats, in ventricles II*b*, II*h*, II*k* with 600 beats. (*c*) 0·5 mM-Ca + 50 % choline-Ringer (in presence of $2 \cdot 10^{-5}$ M atropine sulphate). Tracer uptake in ventricles III*e*, III*f*, III*g*, III*h* with 100 beats, in ventricles III*l* and III*i* with 600 beats. Temperature, 6–8° C.

of release during stimulation in the tracer fluid is equal to $\tau_{s(I)}$, the time constant obtained about 20 min later during the initial period of stimulation in the washing-out fluids. The results of such calculations, using the data for [*Ca_i] and $\tau_{s(I)}$ after 100 beats, are listed in Table 2 (col. 9). Thus with 1 mM-Ca-Ringer as perfusion fluid, the influx/twitch comes to $1{\cdot}67 \times 10^{-3}$ m-mole/l. To convert these figures into fluxes per square centimetre of fibre surface they have been divided by $1{\cdot}14 \times 10^4$/cm, this being the average ratio (surface/volume) of the myocardial fibres as determined from the data illustrated in Fig. 6. Thus in the present example a value of 0·15 p-mole/cm² is obtained (column 10 of Table 2).

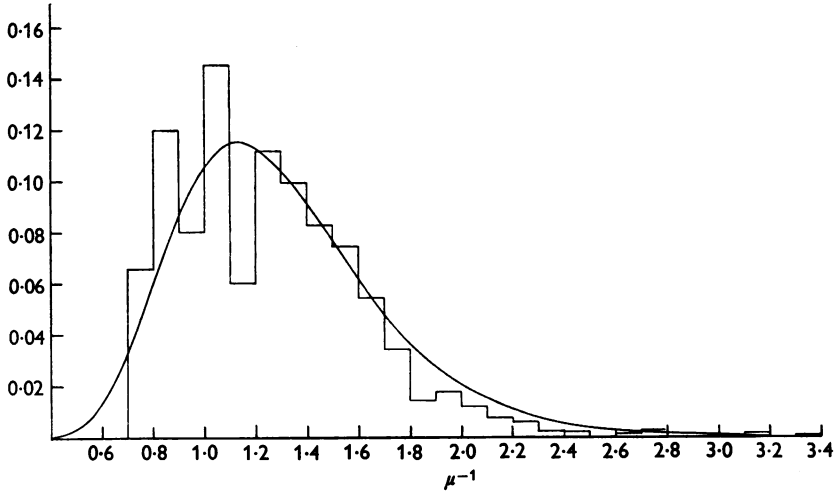

Fig. 6. Histogram showing distribution of perimeter/area as index of the ratio s/v from 200 ventricle fibres. Ordinate, relative area occupied by fibre cross-sections in any one class of this ratio (class division 0·1 μ^{-1}). Continuous curve, log-normal curve with geometric mean of 1·14 and S.D., λ of 0·3. No allowance has been made for possible shrinkage of the histological material during the procedures of fixation and embedding. Linear shrinkage of up to about 10 % may be assumed to have occurred (H. Huxley, personal communication). Accordingly the maximum of the distribution for fibres *in vivo* may lie to the left of the present maximum by about 0·1–0·2 units (μ^{-1}), depending on the extent to which the membrane participates in the shrinkage.

From a comparison of the calculated values it is clear that the influx per twitch is approximately the same in 2 mM-Ca as in 0·5 mM-Ca + 50 % Na-Ringer's fluid (0·23 p-mole/cm² as compared with 0·27 p-mole/cm²). Both values are more than 50 % larger than that obtained with 1 mM-Ca-Ringer's fluid. This result supports a hypothesis of the previous paper according to which the magnitude of Ca influx is related to the ratio $[Ca]/[Na]^2$ in the external fluid rather than to the external [Ca] as such.

An alternative assumption for the calculation of the influx by means of

equation (5) is to suppose that Ca uptake is accompanied by release of this ion at the rate which was observed after the 1 hr periods of exposure to the tracer fluids (see following paragraph). Accordingly values of [*Ca_i] determined after 100 beats were substituted into eqn. (3), together with those of $\bar{\tau}_{s(I)}$ after 600 beats, [*Ca_i] and $\bar{\tau}_{s(I)}$ being the means listed in the corresponding columns of Table 2. It is seen that the values for the influx so obtained (square brackets, col. 10, Table 2) are, with the exception of that with 0·5 mM-Ca + 50 % Na-Ringer as bathing fluid, only slightly smaller than those of the first calculation.

It should be noted here that these alternative calculations are based on two hypotheses outlined in a later section to explain the observed deviations from the exponential kinetics. The first is consistent with the hypothesis that the efflux gradually declines with time of stimulation (cf. discussion in connexion with Fig. 7*b*). Since after 10 min of stimulation followed by 19 min of rest little, if any, decline will be expected the time constant of release during 100 beats should be, as assumed, close to that found at the beginning of stimulation in the washing-out fluid. According to the other hypothesis the individual fibres within a ventricle have different rate constants, τ^{-1}, of release. If, as appears reasonable (cf. discussion in connexion with Fig. 7*a*), this variation of τ^{-1} associated with different fibre volumes can be assumed to follow a log-normal distribution, the initial time constant determined after 600 beats, i.e. when a steady state has been approached, should correspond to the 'average' time constant of the muscle (cf. discussion in connexion with eqn. (8)), Creese, Neil & Stephenson (1956). Values of the influx calculated on this basis (square brackets, col. 10, Table 2) would thus be a measure of the 'average' influxes of the ventricle.

Considering now the magnitude of the efflux during activity, this can be calculated by substituting corresponding pairs of values for [Ca_i] and τ_s in eqn. (3). These values may be obtained from the experimental data for ventricles at the beginning of the 10 min period of stimulation in the Ca-labelled fluids: (1) Because up to this time ventricles had been resting in 1 mM-Ca-Ringer for a period of about 2 hr, after which a steady state with respect to the Ca fluxes had probably been established. In the preceding paper the steady-state concentration [Ca_i] in the presence of 1 mM-Ca-Ringer was determined and a value of 0·49 m-mole/l. obtained, which may thus be used for the present calculation. (2) As in the calculation of the influx the rate of Ca release may be assumed to be approximately the same at this time as during the initial phase of the second period of stimulation, i.e. in the washing-out fluid. Thus the required time constants would correspond to the values of $\tau_{s(I)}$ obtained after 100 twitches in the load solution (Table 2, col. 4). To obtain the extra efflux directly associated with activity the resting efflux during a period of activity must be subtracted. Although this cannot be determined with any great precision, owing to the inherent inaccuracy in the determination of resting time constants (cf. page 567), the final error will not be large, since the resting influx is only 20 % or less of the total during a period of activity (cf. the ratio $\bar{\tau}_{s(I)}/\bar{\tau}_r$ of col. 7, Table 2 which is a measure of this fraction).

Table 3 lists the values for the efflux calculated in this way, by using the following expression derived from eqn. (3)

$$\text{efflux/twitch} = \frac{v}{s}\,\frac{\bar{\tau}_r - \bar{\tau}_{s(I)}}{\bar{\tau}_r \cdot \bar{\tau}_{s(I)}}\,\frac{[Ca_I]}{f}, \qquad (6)$$

where $\bar{\tau}_r$ and $\bar{\tau}_{s(I)}$ are the means of the time constants given in Table 2 and f is the stimulus frequency. Comparing these results with the corresponding figures of the influx in Table 2 it is seen that influx and efflux in the presence of 1 mM-Ca-Ringer are similar in magnitude (0·15 and 0·18 p-mole/cm², respectively). The difference between these figures, rather

TABLE 3. Ca efflux during heart beats (stimulation 10 shocks/min, temperature between 6 and 8° C)

Composition of perfusion fluid	$\frac{\bar{\tau}_r - \bar{\tau}_s}{\bar{\tau}_r \cdot \bar{\tau}_s}$ (min)$^{-1}$	Efflux/beat (m-mole × 10^{-3}/l. of myocardium)	Efflux/beat (p-mole/cm² fibre surface)
1 mM-Ca, 100 % Na	0·042	2·05	0·18
2 mM-Ca, 100 % Na	0·034	1·67	0·15
0·5 mM-Ca, 50 % Na + 50 % choline	0·084	4·1	0·36

than indicating a true difference in influx and efflux, is more probably a consequence of taking too large a value for $[Ca_I]$; a likely reason suggesting 0·49 m-mole/l. to represent an over-estimate of $[Ca_I]$ has been discussed in the legend of Table 5 of the preceding paper. However, the value for the efflux in the presence of 0·5 mM-Ca + 50 % Na-Ringer does seem to be larger, and that with 2 mM-Ca-Ringer smaller than the corresponding influxes. This is consistent with the observation that although the magnitude of the influx is similar in the two cases, yet the final gain of tracer after 600 beats is considerably smaller in the presence of 0·5 mM-Ca + 50 % Na-Ringer than with 2 mM-Ca-Ringer (cf. the corresponding figures of 0·33 m-mole/l. and of 0·57 m-mole/l. in Table 2).

The results of the experiments with 1 mM-Ca-Ringer as bathing fluid may be used to obtain an estimate of the relative increase in Ca influx during activity in relation to the resting influx. Since the fibres are close to a steady state in this fluid the approximate resting influx is given by

$$M_{in} = M_{out} = \frac{v}{s}\,\frac{[Ca_I]}{\bar{\tau}_r}.$$

Values calculated by means of this expression, taking the average figure for $\bar{\tau}_r$ of either 110 or 168 min (Table 2I, *a* and *b*, column 3) and the values for $[Ca_I]$ and s/v as above, ranged from 0·0065 to 0·0043 p-mole/cm².sec. If the extra influx/twitch is taken as occurring over the whole duration of the action potential (from 2 to 2·5 sec at 7° C), the extra influx lies between

0·15/2·0 and 0·15/2·5 = 0·075 and 0·060 p-mole/cm² sec. Thus the influx would increase between ten and twentyfold during activity. It is interesting to note that this rise in influx, which is associated with a fairly weak twitch, is similar to that obtained during 'threshold' contractures, where a factor of 12 was observed (Niedergerke, 1963).

Further analysis of the time course of exchange

Since the interpretation of the preceding results is based on the assumption that uptake and release of Ca from the cells follow an exponential law, the observed deviation from this law must now be discussed. The two features of this deviation are that the washing-out curves eventually flatten and that *Ca release during activity is slower, if determined after the ventricle had been stimulated 600 rather than 100 times during the initial perfusion with labelled fluids (cf. the larger time constants $\bar{\tau}_{s(1)}$ in sections (*a*) as compared with (*b*) of Table 2). A possible explanation of these features can be found in the variation in the size in the myocardial fibres and so of their surface/volume ratios. Thus fibres which have large values of s/v will be expected to exchange calcium more rapidly than thick fibres. The decline of the tracer in the ventricles should therefore be faster at the beginning than at the end of a washing-out period, and should also be faster after 100 as compared to 600 beats in the labelled fluids, because after the short series of beats the small fibres will contain a relatively larger proportion of the total tracer content of the ventricle.

To test this hypothesis the ratio perimeter/area of cross-sections from 200 fibres was determined from electron micrographs, to provide an index of the surface/volume ratio of the fibres. These ratios have been plotted in Fig. 6 (on the abscissa) against the relative area occupied by the fibre cross-sections (ordinate), so that a column in the histogram represents the volume fraction of myocardium which, according to the hypothesis, exchanges Ca with a given time constant. The resulting 'positive-skew' distribution can satisfactorily be approximated by a log-normal distribution, using a geometric mean of s/v equal to $1{\cdot}14/\mu$ and standard deviation (of the natural logarithm), λ, of 0·3, both values being obtained by probit analysis (Finney, 1952). If it is assumed that the reciprocal time constants are in fact distributed log-normally, the *Ca gain, $\Delta[{}^*Ca_i]$, of a small volume element of myocardium having the time constant τ_s should be given by eqn. (7).

$$d[{}^*Ca_i] = \frac{m_{in}}{m_{out}}[Ca_i]\left(1-\exp\frac{t}{\tau_s}\right)(2\pi\lambda^2)^{-\frac{1}{2}}\exp-\frac{[\log(1/\tau_s)-\log(1/\tau_m)]^2}{2\lambda^2}\,d\log\frac{1}{\tau_s}. \quad (7)$$

Assuming as before that M_{in} remains constant during a series of beats and $M_{out} \propto [Ca_i]$, then

$$\frac{m_{in}}{m_{out}} [Ca_i] = \frac{s}{v} M_{in} \tau_s$$

is a constant for all fibres and, for example, equal to $m_{in(m)} \tau_m$, where τ_m is the geometric mean time constant and $m_{in(m)}$ the corresponding average Ca influx/volume fibre. The total tracer content of a ventricle, [*Ca_i], can then be obtained by integration [*Ca] =

$$m_{in(m)} \cdot \tau_m \left\{ 1 - (2\pi\lambda^2)^{-\frac{1}{2}} \int_{-\infty}^{+\infty} \exp\left(-\frac{t}{\tau_s} - \frac{[\log(1/\tau_s) - \log(1/\tau_m)]^2}{2\lambda^2} \right) d \log \frac{1}{\tau_s} \right\}, \tag{8}$$

where

$$(2\pi\lambda^2)^{-\frac{1}{2}} \int_{-\infty}^{+\infty} \exp\left(-\frac{1}{\tau_s} - \frac{[\log(1/\tau_s) - \log(1/\tau_m)]^2}{2\lambda^2} \right) d \log \frac{1}{\tau_s} = \phi(t) \tag{8a}$$

is identical with the eqn. (8) of Creese *et al.* (1956), describing the decline in radioactivity of the fully loaded muscle during washing-out. The function $\phi(t)$ has been numerically evaluated by these authors.

Considering first the decline in radioactivity during washing-out, Fig. 7*a* shows two of the numerical solutions of eqn. (8*a*) for λ of either 0·3, i.e. corresponding to that of the distribution in Fig. 6, or of the larger value of 0·5. The curves which gradually flatten have, nevertheless, been approximated by two straight lines as when considering experimental washing-out curves (cf. Fig. 5). The initial line was drawn through the points corresponding to $t = 0$ and $t = \tau_m$, its slope being close to $(-\tau_m)^{-1}$ (Creese *et al.* 1956). The line (slope $= (-\tau_2)^{-1}$) through the remaining points was fitted by eye. Taking the ratio τ_m/τ_2 as a measure of the flattening of these curves values of 0·88 and 0·74 were obtained for this ratio with λ equal to 0·3 and 0·5, respectively. By comparison, the corresponding average ratio $\tau_{s(I)}/\tau_{s(II)}$ of the experimental washing-out curves came to 0·74 (average of 11 determinations) and has thus the same value as obtained from the theoretical distribution with standard deviation $\lambda = 0{\cdot}5$ rather than 0·3, the value suggested by the results of Fig. 6. Thus the variation in fibre diameter alone cannot account for all the deviation from the exponential law. Possibly some other source of variation for τ_s exists. Tentatively assuming this to be the case, the exchange of Ca should then be determined by eqn. (8) and (8*a*) with $\lambda = 0{\cdot}5$ as parameter. To test whether the experimentally observed gain of *Ca (Table 2, col. 2) after 100 and 600 beats, respectively, can satisfactorily be described by eqn. (8*b*) the smooth curves in Fig. 8 have been drawn according to this equation after substituting for τ_m the average values of $\tau_{s(I)}$ obtained after long loading (which are, as discussed above, approximately equal to τ_m) and for $m_{in(m)}$ the

corresponding values of the average influxes (in square brackets, col. 10, Table 2). It is seen that of the values for [*Ca_i] after a period of 600 stimuli, i.e. of the points not 'used up' by this curve fitting, the value for *Ca gain in the presence of 1 mM-Ca-Ringer (Fig. 8*a*) lies close to the curve.

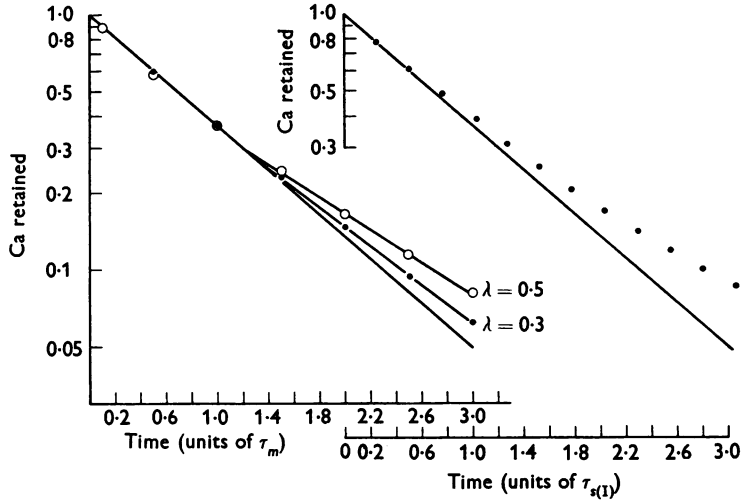

Fig. 7. Theoretical washing-out curves to illustrate two possible explanations of the deviation from the exponential decline. Ordinate = proportion of Ca retained by the tissue. (*a*) Decline of tracer in a ventricle made up of volume elements with which are associated the reciprocal time constants, τ_s^{-1}, which are distributed log-normally; the standard deviation, λ, being either 0·3 or 0·5. Initial portion of both curves is approximately exponential with time constant τ_m (the geometric time constant). The later parts of the curves have been approximated by a second straight line with slope of − 0·88 (λ = 0·3) and − 0·74 (λ = 0·5). (*b*) Decline of [*Ca_i] on the assumption that the efflux declines linearly with time at rate α, where α = 1/150 min^{-1}. A value of 19·6 min has been chosen for $\tau_{s(I)}$, the initial time constant, this being the one observed during ventricular activity in 1 mM-Ca-Ringer. Straight line, slope of the curve at zero time.

However, the predicted values for the *Ca gain in the other two cases, although correct in their magnitude relative to another, i.e. in the ratio ([*Ca_i] in 2 mM-Ca-Ringer/[*Ca_i] in 0·5 mM-Ca + 50 % Na-Ringer), are both too high.

A second factor which may also have contributed to the departure from the exponential law is a gradual decline in both efflux and influx during a series of twitches. Obviously a decline in efflux would account for the observed increase in the time constant describing tracer release; on the other hand the assumption of a decreased influx would provide an explanation for the two values of [*Ca_i] (in Figs. 8*b* and *c*) to be lower than that predicted by the hypothesis discussed in the preceding paragraph. Such

a change in both influx and efflux might be related to the shortening of ventricular action potentials which has been shown to occur during prolonged stimulation (Niedergerke, 1956). This effect, if very pronounced, gives rise to a 'negative staircase' which has already been observed to be associated with a reduction in *Ca uptake (cf. discussion in connexion with Table 1).

To show quantitatively that a simultaneous reduction in both influx and efflux could explain the observed deviation from exponentiality, the magnitude of the effect to be expected has been estimated by assuming

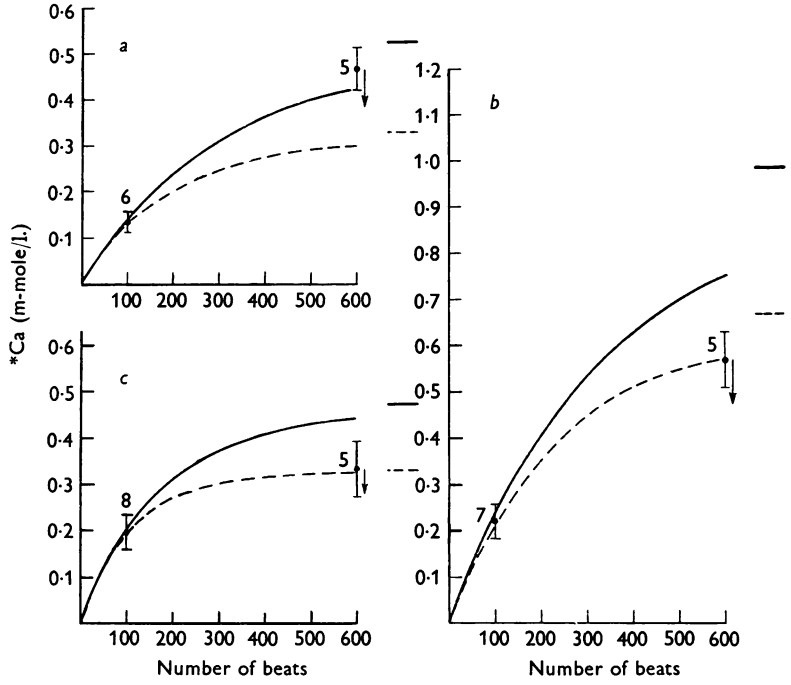

Fig. 8. Further analysis of the two explanations for the deviation from exponential kinetics (see also Fig. 7). The points show the extra gain of *Ca associated with a series of beats in the presence of (*a*) 1 mM-*Ca-Ringer, (*b*) 2 mM-*Ca-Ringer and (*c*) 0·5 mM-*Ca + 50 % Na + 50 % choline-Ringer (values taken from Table 2). Abscissa, number of beats. The lines have been drawn according to eqn. (8) (—) and (9) (-----). The arrows at the points of [*Ca_i] after 600 beats indicate a correction to allow for the fact that after 61 min of exposure to the tracer the resting influx also contributes to the total uptake, since this had been determined by means of subtracting controls obtained after only 11 min of soaking. The approximate magnitude of this correction, which corresponds to the length of the arrow, was estimated on the assumption that the resting influx forms a constant fraction of the extra influx in the different fluids. Using the data obtained with 1 mM-Ca-Ringer and assuming a steady state, this fraction, which is given by $\bar{\tau}_{s(I)}/(\bar{\tau}_r + \bar{\tau}_{s(I)})$ comes to 0·15 and it seemed reasonable to apply this figure also to the other values.

that the fluxes decrease linearly in the course of an hour to 3/5 of the initial value. This value was chosen to account for the observed differences between the slopes of the washing-out curves after 100 and 600 beats (cf. the ratios $[\bar{\tau}_{s(I)}$(after 100 beats)$]/[\bar{\tau}_{s(I)}$(after 600 beats)$]$ in Table 2) and also for the change in slope of a single washing-out curve (cf. the theoretical curve of Fig. 7*b*), thus ignoring, for simplicity, the variation in fibre size. The appropriate solution of eqn. (4*a*) then becomes

$$[^*Ca_i] = \bar{m}_{in} \cdot \bar{\tau}_{s(I)} \left\{1 - \exp - \left(\frac{t - \frac{1}{2}\alpha t^2}{\bar{\tau}_{s\,(I)}}\right)\right\}, \qquad (9)$$

where $\alpha = (150 \text{ min})^{-1}$ and $\bar{m}_{in}$ and $\bar{\tau}_{s(I)}$ are the means of the influxes and time constants after short loading.

The interrupted curves in Fig. 8 have been drawn with eqn. (9) and it is seen that the fit to the results obtained with 2 mM-Ca-Ringer and 0·5 mM-Ca + 50 % Na-Ringer is rather better than with the smooth curves, whereas the reverse is true for the result with 1 mM-Ca-Ringer. From this it appears that a combination of both factors, i.e. a variation in the time constant of different fibres and a shortening of the action potential, would account satisfactorily for the observed deviation from the simple exponential kinetics. However, yet another possibility which cannot entirely be excluded is that Ca may be contained in two separate compartments within individual fibres and correspondingly may exchange with two different rates. The exchange kinetics of such a system is described by the sum of two exponentials (cf. Harris, 1950) and may show a similar departure from the exponential law as described above.

DISCUSSION

The present experiments show that beating of the heart is accompanied by an additional influx of Ca ions into the muscle fibres and that this influx increases both when the external [Ca] is increased and when [Na] is reduced, two ionic changes which cause the beat to become stronger. Further, although the range is limited in which these ion concentrations could be changed without apparently altering the size of the action potential, it was found that effects on Ca influx are about the same when the ratio $[Ca]/[Na]^2$ is altered to a given extent by adjusting either [Ca] or [Na]. These results support the hypothesis that Ca entry during an action potential is responsible for the initiation of the twitch, and are also consistent with the idea that Ca crosses the cellular membrane after combination with specific molecules or sites for which Ca and Na ions compete (Lüttgau & Niedergerke, 1958).

If ionic Ca is in fact released inside the cell, the observed influx per beat

of 0·15–0·25 p-mole/cm² (col. 10, Table 2) corresponds to a transfer of positive charge equivalent to 0·3–0·5 p-mole of monovalent cations. This is only about 10% of the minimum ion transfer occurring during the action potential, i.e. 3·6–6 p-mole monovalent cation/cm², calculated as the charge required to depolarize a membrane of capacity 3–5 μF/cm² by 120 mV. It is possible that this relatively small fraction of current flows in specific areas of the surface membrane containing the sites which initiate the contraction.

The quantitative analysis set out on page 561 was made on the assumption that the major proportion of the exchangeable Ca is contained in a single compartment of the cell or alternatively is uniformly distributed within the cell itself. The relatively small deviations from the simple kinetics expected on such a system could be satisfactorily explained as due to variation in the time constants of exchange in different fibres and, possibly in addition, to a reduction in the efflux with continued stimulation. It should, however, be stressed that this simple picture may nevertheless be the end result of a much more complicated chain of events. In relation to this, it may be recalled that the results of the previous paper (Niedergerke, 1963) were interpreted by postulating the presence of two different forms of cellular Ca, corresponding to 'activator' and to an inactive form, respectively. These, although able to interact, could conceivably be located in different cellular compartments. However, this should not result in appreciable divergence from the simple exponential kinetics if, as there is reason to believe, the inactive Ca normally constitutes the larger fraction of the total. The experimentally observed uptake and loss of tracer would then be determined by the exchange of this inactive component.

That the exchangeable Ca is present in two different forms within the cell may also be inferred from the present results by comparing the Ca entry as expressed in terms of concentrations, during a single beat (1·6–3·1 μ-mole/l., cf. column 9 of Table 2) with the quantity (0·33–0·57 m-mole/l., cf. column 2, Table 2) present in the cell when a steady state has been approached, i.e. after 600 beats in the radioactive fluids. It is difficult to imagine that such small concentration changes could by themselves be responsible for the activation of contraction. It therefore seems more reasonable to assume that during the initial process of muscular activation there is a drastic rise of the concentration of 'activator'-Ca which may possibly correspond to ionic Ca, and this is followed by a rapid inactivation or binding.

It is of interest to consider to what extent the rapid rise and fall in tension during a twitch can be accounted for in these terms. According to the simple model proposed in connexion with Fig. 14 of the previous paper the concentration of activator-Ca, $[Ca_I]$, should be related to the

influx, m_{in}, and the rate constant β of the deactivating reaction by the equation

$$\frac{d[Ca_I]}{dt} = m_{iu} - \beta[Ca_I]. \quad (10)$$

Figure 9*b* illustrates the rise and fall of $[Ca_I]$, predicted by this model, in response to the extra influx of Ca during individual action potentials. It is assumed that the time course of the influx follows the general shape of the action potential of the heart, in that it rapidly rises to a constant level

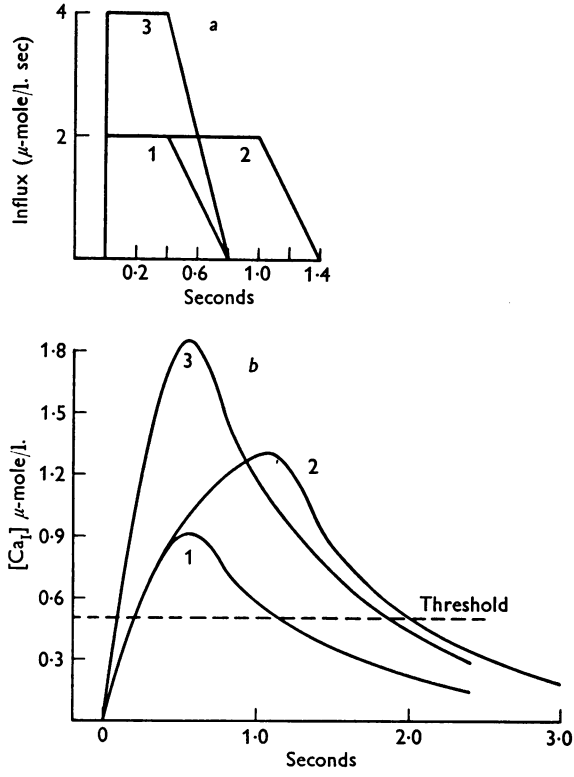

Fig. 9. Rise and fall of hypothetical activator in response to individual action potentials. (*a*) Assumed time course of the extra Ca influx, m_{in}, during three different action potentials, (1) of short, (2) of long duration and (3) of short duration but with twice normal influx. Ordinate, Ca influx (μ-mole/sec/l. myocardium). (The hypothetical Ca uptake due to the influx of curve (1) is 1·2 μ-mole/l., i.e. somewhat less than the influx/twitch, of 1·67 μ-mole/l. in the presence of 1 mM-Ca-Ringer (Table 2, I, col. 9)). (*b*) Change in Ca_I associated with the corresponding change of influx in (*a*). Curves obtained by plotting the integral of equation (3) against time. Ordinate, concentration of Ca_I (μ-mole/l.) (The threshold concentration of Ca_I has been assumed to be 0·5 μ-mole/l., and is thus similar though somewhat below that of 0·75 μ-mole/l. of the previous illustration of this model (fig. 15, Niedergerke, 1963)).

which is maintained during the plateau after which it declines somewhat more slowly corresponding to repolarization. For β a value of 2 sec^{-1} was chosen so as to make the rate with which the concentration $[Ca_I]$ rises and falls, respectively, similar to that of the rise and fall in tension during an isometric twitch. The curves in Fig. 9*b* were obtained by plotting the integral of equation (10) against time, m_{In} for each being taken from the corresponding function plotted in Fig. 9*a*. Curve (1) illustrates the change in $[Ca_I]$ associated with an action potential of normal duration. Curve (2) shows the effect of an increased duration at the same level of influx as in (1), corresponding to a prolonged action potential, and curve (3) the effect of an increase in influx at a constant duration, corresponding to the effects on twitch tension of increasing the ratio $[Ca]/[Na]^2$. The shapes of these curves resemble those of heart twitches in several respects: (1) The maximum is attained shortly after termination of the plateau of the action potential (cf. Figs. 4 and 5, Lüttgau & Niedergerke, 1958). Associated with the prolongation of the action potential the peak becomes larger and the time to the peak is prolonged, whereas the initial rate of rise is unaltered, features shown by the time course of tension development under these conditions (cf. Fig. 4, Niedergerke, (1956), and Fig. 5, Lüttgau & Niedergerke, 1958). (3) The curve associated with an influx of enhanced magnitude is analogous to that of a twitch response to an increased ratio $[Ca]/[Na]^2$ in that the initial rate of rise and the peak are increased, the time to the peak remaining unaltered (cf. Fig. 4, Lüttgau & Niedergerke, 1958).

Although this simple model evidently interprets the present findings in a qualitatively satisfactory way, it probably requires refinement to take into account two additional processes which have been neglected for simplicity. (*a*) Diffusion of 'active' Ca to the site of its action within the cell must be expected to cause an initial delay in the rise of the activator concentration in the fibre interior and thus of the twitch. As may be recalled the magnitude of this delay as calculated for twitch fibres of 100 μ diameter has been the reason for rejecting the hypothesis that activation may be due to a substance which diffuses from the external membrane into the fibre (Hill, 1949). However in cardiac fibres of the frog with an average fibre diameter of somewhat less than 5 μ this objection does not hold, because the diffusion time is shorter by a factor of more than $(100/5)^2 = 400$ as compared to skeletal muscle fibres. Thus taking Hill's (1948) example of a substance which after being suddenly released by the action potential at the fibre membrane diffuses inwards, and using his symbols and quantities, the relation kt/a^2 for frog heart fibres 40 msec after the release should be equal to 3·2 (k, the diffusion constant, being equal to 5×10^{-6} cm^2/sec and a, the radius of the fibre, assumed to be cylindrical, 0·00025 cm). Substituting this figure in the relevant solution of the diffusion equation (Hill,

1948, Fig. 2) for this case (which is related to, though somewhat different from, the present situation of a short but maintained influx through the membrane), shows that by that time the substance would have attained its final value in the fibre centre while being practically zero in the centre of skeletal muscle fibres of 50 μ radius (Hill, 1949). It appears possible that this initial delay together with the consideration that 'active' Ca has probably to exceed a threshold concentration before initiating contraction, may contribute to the rise in tension being S-shaped rather than initially linear as predicted by the model. (The main factor responsible for the initial delay is probably the series-elastic element of the muscle (cf. Hill, 1951).)

(*b*) The result that skeletal muscles resist quick stretches with their full isometric tetanus tension some 20–40 msec after stimulation suggests that full activation of contraction occurs long before the twitch attains a maximal value (Hill, 1949). Some such discrepancy between the rates of activation and tension development is likely to exist also in cardiac muscle, although it should be mentioned that Abbott & Mommaerts (1959) failed to obtain such a result. In this case tension rise and decay would thus be partly, or perhaps even largely, determined by the contractile process, e.g. by the speed of internal muscular shortening, rather than reflect the rise and fall of the activator. In terms of the model of Fig. 9 this would mean that the rate of deactivation is faster (the rate constant β larger) than at present assumed.

Although these qualifications must be borne in mind the possibility remains that the time course of a twitch is to an extent determined by the level of 'activator' Ca throughout the cell. The experiments summarized in Table 2 showed that the additional Ca uptake per twitch when expressed as a concentration came to only up to 3 μ-mole/l. fibre. It is interesting, therefore, to note that recent experiments have shown (Podolsky & Hubert, 1961; and Podolsky, personal communication) that contractures of myofibrils can under suitable conditions be induced by fluids containing as little as 10^{-5} mole $CaCl_2$/l. It is also known that myofibrillar ATPase can be activated by ionic Ca concentrations of less than 10^{-5} M (illustration 6, Weber, 1959). Probably related to this effect is the finding that 'super-precipitation' of actomyosin in the presence of ATP is accelerated by Ca concentrations down to 10^{-6} M (Ebashi, 1961). Further relevant observations have been made with certain isolated muscle granules which inhibit both the ATP splitting and the contraction of isolated myofibrils (Portzehl, 1957). Nagai, Makinose & Hasselbach (1960) were able to show that this inhibition may be suppressed with Ca concentration of 10^{-5} M (e.g. illustration 5 in their paper). It is conceivable that the importance of calcium for contraction of living heart fibres, as suggested by the present

work, will find an explanation in terms of these mechanisms. However, further speculation on this point must await a fuller understanding of the underlying biochemical processes.

It is of particular interest that Ca efflux also increased during activity, and to an extent which, at least in 1 mM-Ca-Ringer's fluid, matched that in influx. Thus the cells are able to maintain the internal Ca concentration at a relatively constant level during activity, clearly an essential requirement for the continuously beating heart. The mechanism of the increase in efflux is uncertain but must be largely by some 'active' process because it occurs against the electrochemical gradient for this ion (cf. Niedergerke, 1963). Unfortunately the time resolution of the present experimental method does not allow the efflux to be determined in the course of a single beat. It might be mentioned, however, that no measurable delay could be detected between the onset of beating and the increase in efflux.

An earlier observation that the rate of uptake and loss of Ca was hardly altered during stimulation should be mentioned in this context (Niedergerke, 1957). This is readily understood on recalling the procedure of the former experiments which were mainly concerned with the rate of extracellular equilibration in heart strips. With these strips a fraction of rapidly exchanging and presumably extra-fibre Ca was found comprising about 5 times the quantity contained in the extracellular fluid spaces. Movements of this substantial amount of Ca, which was moreover much delayed by extracellular diffusion, must be expected to have masked in these experiments the relatively small cellular Ca movements associated with stimulation.

It should finally be mentioned that the present findings are in agreement with previous results on *Ca movements which were examined with cardiac tissue other than that of the frog. Thus Henrotte, Cosmos & Fenn (1960) observed increased exchangeability of Ca in beating turtle ventricles during the 'Treppe', i.e. under conditions of increased contractility. In the mammalian heart Sekul & Holland (1959) and Winegrad & Shanes (1962) showed that an extra uptake of tracer Ca was associated with stimulation and the latter authors also found this uptake to become greater on raising the external Ca concentration.

SUMMARY

1. The methods described in the preceding paper have been used to study the uptake and release of Ca in cardiac cells of frog ventricles during series of beats, in the presence of Ringer's fluid of varying composition.

2. Both Ca uptake and release were increased during activity. The cellular Ca thereby exchanging with extracellular Ca was about up to 0·6 m-mole/l. fibre.

3. Ca release from the ventricle followed an approximately exponential time course. Such deviations from exponentiality as were observed could be explained by allowing for probable variations in the time constant associated with the release in individual heart fibres.

4. The extra Ca influx per beat through the surface of a heart fibre of average size (at 7° C) was about 0·15 p-mole/cm^2 in the presence of 1 mM-Ca-Ringer, 0·23 p-mole/cm^2 in 2 mM-Ca-Ringer and 0·25 p-mole in 50 % Na- + 50 % choline-Ringer. The extra influx in the latter two cases was thus increased to about the same extent, with respect to that in 1 mM-Ca-Ringer, while twitch tensions had risen to a similar level in response to a twofold increase of the external ratio [Ca]/[Na]^2.

5. The extra influx associated with activity in 1 mM-Ca-Ringer was approximately 10–20 times larger than the resting influx.

6. The rise in efflux during activity was of similar magnitude to that of the influx; in the presence of 1 mM-Ca-Ringer extra influx and efflux were almost exactly equal, in agreement with the observation that the concentration of exchanging cellular Ca remained approximately constant (0·4–0·5 m-mole/l.) in this fluid.

7. Activation of contraction during a twitch has been explained in terms of these findings and the model proposed in the previous paper by assuming that the action potential causes a sudden increase in Ca influx and so raises the concentration of 'activator-Ca' in the cell, thereby initiating contraction, and that fairly rapid inactivation of this 'activator' is responsible for relaxation after cessation of the action potential.

I wish to express my indebtedness to Professor Katz for constant encouragement and to Dr H. E. Huxley for allowing me to use unpublished material. Dr D. H. Jenkinson's help during the preparation of the manuscript is gratefully acknowledged.

REFERENCES

ABBOTT, B. C. & MOMMAERTS, W. F. H. M. (1959). A study of inotropic mechanisms in the papillary muscle preparation. *J. gen. Physiol.* **42**, 533–551.

CREESE, R., NEIL, M. W. & STEPHENSON, G. (1956). Effect of cell variation on potassium exchange of muscle. *Trans. Faraday Soc.* **403**, 1022–1032.

EBASHI, S. (1961). Calcium binding of vesicular relaxing factor. *J. Biochem., Tokyo*, **50**, 236–244.

FINNEY, J. (1952). *Probit Analysis*, 2nd ed., pp. 236–245. Cambridge University Press.

HARRIS, E. J. (1950). The transfer of sodium and potassium between muscle and the surrounding medium. Part II. The sodium flux. *Trans. Faraday Soc.* **46**, 872–882.

HENROTTE, J. C., COSMOS, E. & FENN, W. O. (1960). Ca exchange in isolated turtle ventricle. *Amer. J. Physiol.* **199**, 779–782.

HILL, A. V. (1948). On the time required for diffusion and its relation to processes in muscle. *Proc. Roy. Soc.* B, **135**, 446–453.

HILL, A. V. (1949). The abrupt transition from rest to activity in muscle. *Proc. Roy. Soc.* B, **136**, 399–420.

HILL, A. V. (1951). The effect of series compliance on the tension developed in a muscle twitch. *Proc. Roy. Soc.* B, **138**, 325–329.

HODGKIN, A. L. (1951). The ionic basis of electrical activity in nerve and muscle. *Biol. Rev.* **26**, 339–409.

HODGKIN, A. L. & KEYNES, R. D. (1956). Experiments on the injection of substances into squid giant axon by means of a microsyringe. *J. Physiol.* **131**, 592–616.

HODGKIN, A. L. & KEYNES, R. D. (1957). Movements of labelled calcium in squid giant axons. *J. Physiol.* **138**, 153–282.

LÜTTGAU, H. C. & NIEDERGERKE, R. (1958). The antagonism between Ca and Na ions on the frog's heart. *J. Physiol.* **143**, 486–505.

NAGAI, T., MAKINOSE, M. & HASSELBACH, W. (1960). Der physiologische Erschlaffungsfaktor und die Muskelgrana. *Biochem. biophys. acta*, **43**, 223–238.

NIEDERGERKE, R. (1956). The 'staircase' phenomenon and the action of calcium on the heart. *J. Physiol.* **134**, 569–583.

NIEDERGERKE, R. (1957). The rate of action of calcium ions on the contraction of the heart. *J. Physiol.* **138**, 506–515.

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

PODOLSKY, R. J. & HUBERT, C. E. (1961). Activation of the contractile mechanism in isolated myofibrils. *Fed. Proc.* **20**, I, 301*b*.

PORTZEHL, H. (1957). Die Bindung des Erschlaffungsfaktors von Marsh an die Muskelgrana. *Biochem. biophys. acta*, **26**, 373–377.

SEKUL, A. A. & HOLLAND, W. C. (1959). Cl^{36} and Ca^{45} exchange in atrial fibrillation. *Amer. J. Physiol.* **197**, 752–756.

WEBER, A. (1959). On the role of calcium in the activity of adenosine 5′-triphosphate hydrolysis by actomyosin. *J. biol. Chem.* **234**, 2764–2769.

WINEGRAD, S. & SHANES, A. M. (1962). Calcium flux and contractility in guinea pig atria. *J. gen. Physiol.* **45**, 371–394.

WOODBURY, L. A., HECHT, H. H. & CHRISTOPHERSON, A. R. (1951). Membrane resting and action potentials of single muscle fibres of the frog ventricle. *J. Amer. Physiol.* **164**, 307–318.