

Radiocalcium Release by Stimulated and Potassium-Treated Sartorius Muscles of the Frog

A. M. SHANES and C. P. BIANCHI

ABSTRACT Stimulation of frog (*Rana pipiens*) sartorius muscle accelerates release of Ca^{45} , but only during the period of stimulation. No appreciable difference is obtained in the calcium released per impulse whether stimulation is at a rate of 20/sec. or 0.5/sec. However, prior stimulation may appreciably increase the loss per impulse. In unfatigued muscles, the minimum amount of calcium liberated during an isotonic twitch is estimated to be about that previously calculated to enter, *viz.* $0.2 \mu\mu\text{mole/cm}^2$.

The time course of radiocalcium release during potassium depolarization depends on the nature of the contracture. When contracture is isometric, the rate of escape is doubled and declines only slowly; if isotonic, the rate is quadrupled but declines in a few minutes to a level maintained at about double that before potassium. The minimal calcium release during the first 10 minutes of potassium treatment is estimated to be about the same in both cases and about one-half to one-third the uptake. This, and especially the close equality of calcium entry and exit during electrical stimulation, are pointed out as not necessarily inconsistent with a transitory net entry of calcium, comparable to the influx, into restricted regions of the individual fibers.

INTRODUCTION

It has been demonstrated for frog striated muscle that the influx of calcium, as measured with radiocalcium, is increased by stimulation and early during potassium contracture; also, when the strength of single isotonic twitches is increased by replacing the chloride of the medium with nitrate, the entry of calcium per twitch increases proportionately (2). The present report is concerned with the increased release of Ca^{45} that we have found invariably accompanies electrical stimulation or exposure to potassium. A previous preliminary report (15) described an increase with stimulation. More recently, however, Harris (6) has failed to observe this effect. Our preliminary observations on Ca^{45} release were described earlier (1).

From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Department of Health, Education, and Welfare, Public Health Service, Bethesda. Dr. Bianchi is a Post-Doctoral Research Fellow of the United States Public Health Service. Received for publication, December 18, 1958.

Methods

The release of Ca^{45} from individual tissues, previously exposed for several hours to normal or modified Ringer's solution containing Ca^{45} , was followed as previously described (14), *viz.* by repeated exposure, usually for 5 minute intervals, to 2.5 ml. of stirred and oxygenated non-radioactive solutions. One-half ml. of the collected samples and of the acid extracts of the ashed residues, obtained after drying the tissues at the end of each experiment, was evaporated on planchets and counted; correction was made for self-absorption by having the same amount of salt present on all planchets. From these data "rate coefficient" curves were reconstructed. It will be recalled (*e.g.* reference 14) that these describe the time course of the average per cent *change* in radioactivity per minute that is estimated for each collection interval by dividing the activity appearing in the medium by the mean activity of the tissue during the collection and by the duration of the collection. The rate coefficient is particularly sensitive to experimental changes.

Stimulation or potassium was applied usually well after the rapid component of Ca^{45} escape is no longer evident, *i.e.* no earlier than 2 hours after washout, so that Ca^{45} was practically absent from the extracellular space. As previously described (2), the stimuli consisted of supramaximal rectangular pulses delivered by Tektronix or Grass square wave generators across the narrow column of solution recirculated past individual muscles. Stimulation was at a rate of 1 or $\frac{1}{2}$ per second for 5 minutes (or less if the isotonic contractions, which were always followed, became appreciably weaker) or at 20 per second delivered in 3 or 6 evenly spaced bursts such that the total time interval of stimulation and the total number of stimuli were about the same as at the lower rate. The number of stimuli delivered was registered with a radioisotope scaler. Cocaine was present in all Ringer's solutions, at a concentration of 2 mg. per cent in stimulation experiments and 14 mg. per cent in potassium runs, to prevent spontaneous activity or repetitive firing such as is particularly prone to occur when nitrate ion replaces the chloride of the medium.

All measurements were made at 25°C.

RESULTS

ELECTRICAL STIMULATION Fig. 1 demonstrates the increase in the rate coefficient observed in two muscles as a function of time during and following a 5 minute period of stimulation first at a low rate and then at a higher rate. The second release of Ca^{45} is not strikingly different from the first even though the contractile response was invariably weaker, as indicated by the tetanic contractions seldom exceeding the preceding twitches.

In Table I the data from these and additional muscles have been tabulated. The muscles in Fig. 1 can be identified in the table from the rates of stimulation employed. Thus, the upper graph was obtained with the lowermost

muscle in the table, and the lower graph with the sixth muscle from the top of the table.

The loss of Ca^{45} is expressed in Table I in several ways. Thus "Ca⁴⁵ change" is given in terms of the areas above the baseline; this is obtained by multiplying each increment of rate coefficient above the baseline by the time interval

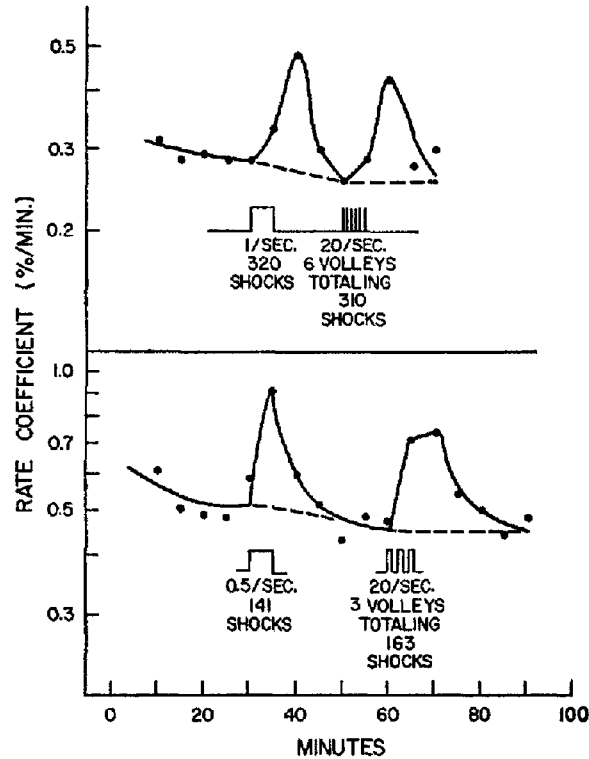


FIGURE 1. The effect of stimulation, first at a low rate then at a high rate for 5 minute intervals as shown, on the time course of the "rate coefficient" of Ca^{45} in a 98 mg. muscle (upper curve) and a 51 mg. muscle (lower curve).

of collection and by adding all the products so obtained for a given stimulation. This gives the *loss* of radioactivity of the muscle relative to (*i.e.*, divided by) the average in the fibers at the time of stimulation and is expressed in per cent. It will be recalled that we are dealing with Ca^{45} in the fibers only, for the extracellular space was cleared of Ca^{45} by a previous 2 hour washout in Ca^{45} -free Ringer's solution. The loss is uncorrected for the differences in the rate of fatigue among the muscles observable from the kymograph records. A correction for fatigue can be made on the assumption that the decline of contraction amplitude indicates a decrease in the number of active fibers, as

TABLE I
THE PERCENTAGE CHANGE OF Ca⁴⁵ IN MUSCLE FIBERS INDUCED BY STIMULATION

Also given are the Ca⁴⁵ spaces of the muscles during prior exposure to Ca⁴⁵-Ringer's solution for 3 hours; the per cent of this activity remaining (Ca⁴⁵ in fibers), chiefly in the fibers, 2 hours after clearing the extracellular space of Ca⁴⁵ by washing with non-radioactive Ringer's solution; and the effect of stimulation at this time and 40 minutes later. Other details in text.

Medium Ca Anion	Final weight mg.	Ca ⁴⁵ space mM	Stimulation rate	No. of stimuli	Ca ⁴⁵ in fibers	Ca ⁴⁵ change						Second stimulation period									
						First stimulation period		Corrected		Minimum		Second stimulation period		Minimum							
			sec. ⁻¹		per cent of initial	NO ₃	Cl	NO ₃	Cl	per cent	per cent	per cent	per cent	μmole/cm ²	μmole/cm ²	sec. ⁻¹	No. of stimuli	rate	loss/impulse	loss/impulse	
3 NO ₃	91.6	0.67	1	140	15.0	1.45	1.97	1.34	0.14	1.93	157	20	1.93								
3 Cl	80.8	1.00	1	140	23.7	1.28	1.34	1.34	0.22	2.98	157	20	2.98								
3 NO ₃	61.3	1.54	0.5	142	13.3	6.7	10.1	5.4	1.45	0.28*	150	20	0.28*								
3 Cl	63.3	1.42	0.5	112	24.1	4.6	5.4	5.4	1.31	0.11	150	20	0.11								
1 NO ₃	52.9	0.93	0.5	105	19.1	2.6	4.2	3.0	0.24	0.11*	163	20	0.11*								
1 Cl	51.1	1.14	0.5	141	26.0	2.05	3.0	3.0	0.21	0.23	163	20	0.23								
1 NO ₃	50.3	1.48	0.5	133	27.6	1.45	2.55	1.57	0.26	0.10*	157	20	0.10*								
1 Cl	43.0	1.33	0.5	133	35.6	1.20	1.57	1.57	0.19	0.47	161	20	0.47								
1 Cl	46.0	1.20	1	177	28.4	3.53	3.05	3.05	0.23	0.18	313	20	0.18								
1 Cl	41.5	1.27	20	257	39.3	3.05	3.05	3.05	0.19	1.1	176	0.5	1.1								
1 Cl	62.3	1.69	0.5	146	25.3	2.0	4.4	4.4	0.21	0.23	172	20	0.23								
1 Cl	64.3	1.51	20	156	34.3	4.4	4.4	4.4	0.49	0.19*	163	0.5	0.19*								
1 Cl	97.9	1.21	2 to 0.5	320	33.9	6.0	6.0	6.0	0.063‡	0.05‡	310	20	0.05‡								

* Negligible or exceptionally weak contraction.

‡ Contraction weak but sustained.

indicated in preliminary observations of spike amplitudes (*cf.* reference 2). The correction factor is obtained by dividing the area under the kymograph record into the area that would have been obtained had the amplitude of contraction been sustained. The corrected figures are given for those cases in which muscles stimulated in nitrate Ringer's solution were compared with their mates in normal Ringer's solution; this was necessary because failure is more marked in the former, as observed by Sandow (11) and subsequently confirmed (2). Such corrections were not feasible when comparisons were made between tetani and low rates of stimulation (*i.e.*, in most muscles for the second period of stimulation, and in the last 5 muscles for the first bout of contraction as well) since the tetanic response is so different from a twitch series.

The corrected Ca^{45} change is still not adequate as a basis for comparing muscles for it depends on what happens to be the Ca^{45} content in fibers at the time of stimulation and on the number of stimulations. Moreover, its significance in terms of the amount of calcium actually released depends on the amount of non-radioactive calcium represented by the Ca^{45} , in other words, the "specific activity"—the ratio of the radioactive calcium to the total calcium.

The Ca^{45} content of the fibers is accounted for by multiplying all Ca^{45} change figures by the " Ca^{45} in the fibers," which is expressed as per cent of the total activity taken up by the muscle fibers during the initial exposure to Ca^{45} -Ringer's solution. The number of stimulations can be allowed for by dividing the resultant product by the number of stimuli. And an estimate of the actual calcium transferred can be obtained on two assumptions: (*a*) that the calcium available for release is that which had entered previously with Ca^{45} in exchange with the fibers' store of calcium, so that the specific activity at the time washout begins is that of the medium and (*b*) that washout for 120 minutes does not appreciably change the specific activity. Assumption (*b*) is roughly true in view of the long (*ca.* 500 minute) time constant of washout (desaturation) curves (14). Assumption (*a*) may be the case even if equilibration is incomplete because the strong binding of calcium by intracellular components could lead to a peripheral cortical ring of myoplasm with radioactivity essentially at the specific activity of the medium. Observations on the poor diffusibility of Ca^{45} injected into giant axons (8) and on the limited spread of the contractile action of calcium injected into muscle fibers (10) are in accord with such a view. Studies of the kinetics of Ca^{45} emergence after different equilibration times are also in keeping with this (data to be published).

Even if not strictly correct, these assumptions are useful in providing a *lower* limit to the amount of calcium represented by the emerging Ca^{45} since the specific activity can be no higher than that of the original bathing medium. These assumptions are utilized when the above figure, obtained after division by the number of stimuli, is multiplied by the value of the Ca^{45} space, also given in Table I; this gives the mini-

imum loss per impulse in $\mu\mu\text{mole/gm}$. Multiplication by the Ca^{45} space gives the absolute calcium loss because this space, by definition, is the volume of radioactive medium equivalent in radioactivity to the radioactivity in 1 gm. of muscle at the end of the exposure to the Ca^{45} solution, and the numerical value of the space is the same as the micromoles of calcium which have exchanged because our media contained calcium at a concentration of 1 $\mu\text{mole/ml}$. These various manipulations of the data are equivalent to multiplying the corrected calcium loss per impulse by the specific activity of the Ringer's fluid.

The minimal losses were converted to $\mu\mu\text{mole/cm}^2$ by use of the conversion factor 300 cm^2/gm . (2), and these are the figures tabulated in Table I.

The minimum calcium released during the first bout of activity, when calculated as above, is in most cases about that obtained for the calcium uptake per impulse; *viz.*, 0.2 $\mu\mu\text{mole/cm}^2$ (2). This implies that no net change in the calcium content of whole muscle will be observed during activity, as actually noted some time ago (4). However, as pointed out in the Discussion, the magnitude of the calcium transfer is so small that more precise measurements of net transport are needed to establish the validity of our computations. It will also be noted later that the absence of a net change in muscle fibers does not necessarily imply the absence of net transfer in restricted regions of the fibers.

The second pair of muscles in Table I exhibits an exceptionally large release of calcium. Since both muscles were obtained from the same animal, a peculiarity of the frog rather than of the experimental conditions appears to be involved. It is noteworthy that equally large losses of calcium occur frequently but irregularly during the second period of stimulation. This can be seen to be unrelated to the stimulation rate, particularly in the last paired muscles, for their first responses as well as their second ones are quite comparable. Nor is the greater loss obviously related to calcium level, as shown by the first pair of muscles which, like the second pair, is in a Ringer's solution containing three times the usual concentration of calcium and yet releases the more usual amount of calcium.

From Table I it is evident that a relatively large, and frequently larger, release of calcium occurs during the second period of stimulation although, as already mentioned, the contractile responses during this period were invariably weaker. The return of action potentials to normal despite the weaker contractions might account for calcium losses identical with those obtained the first time but certainly not for the larger releases. Thus, it appears more likely that prior activity can, in some special way, augment the calcium liberated per impulse. How this is brought about remains to be resolved.

The available data in Table I comparing the calcium released by stimulation in NO_3^- and Cl^- -Ringer solutions do not indicate a larger loss in the former comparable to the larger gain observed in influx studies (2). However,

additional study appears desirable, preferably under conditions of reduced statistical error, such as may be obtainable with single fibers or with small muscle bundles.

Returning to Fig. 1, we may note another feature of the accelerated release of Ca^{45} during and following stimulation that was encountered; *viz.*, that the maximum loss of Ca^{45} occurs during the 5 minute collection interval *after* rather than *during* stimulation. However, this might easily be the consequence of the peculiarities of the kinetics when stimulation takes place in solution

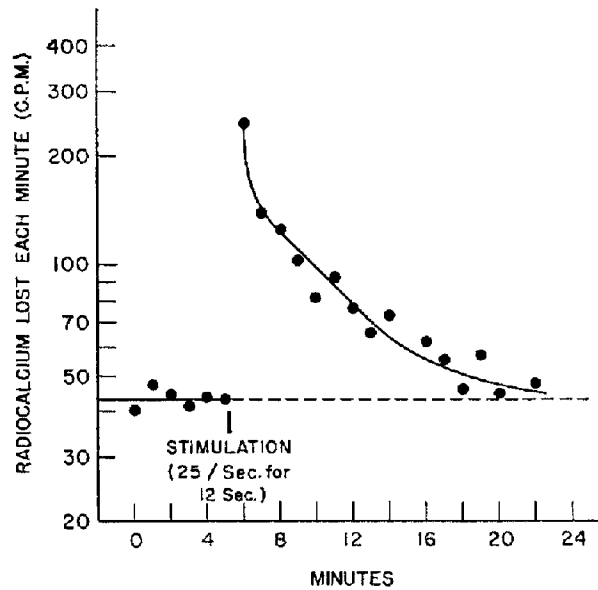


FIGURE 2. The effect of a single brief tetanic stimulus applied between sample changes on the Ca^{45} released by a 74 mg. muscle to samples of Ringer's solution replaced completely every minute.

and the first sampling collection associated with activity is taken immediately on cessation of stimulation. Note that the delay is absent or smaller in the smaller muscle.

A more critical test of the actual time course of Ca^{45} release was obtained by using very short collection intervals, intervals which, including the time required to change solutions, totaled only 1 minute. Stimulation, in the form of a brief tetanus, was introduced once during one of the changes of solution so that the muscle was in air at the time and the schedule was in no way altered. The typical result of one of two such experiments is shown in Fig. 2. It is immediately evident that there is no sustained accelerated loss of Ca^{45} such as would have occurred (a) in response to a rise in diffusible intracellular calcium or (b) as a result of stimulation of an active transport mechanism

such as is known for sodium and potassium. Rather, all of the Ca^{45} liberated as a consequence of activity has appeared in the extracellular space during stimulation, for the time course of decline of the increased Ca^{45} loss is about that for emptying of the extracellular space (14).

ELEVATION OF THE POTASSIUM CONCENTRATION OF THE MEDIUM It was previously demonstrated that a substantial increase in radiocalcium entry occurs only at the beginning of the brief isotonic contracture resulting from a large increase in the potassium level of the medium (2). The emergence of

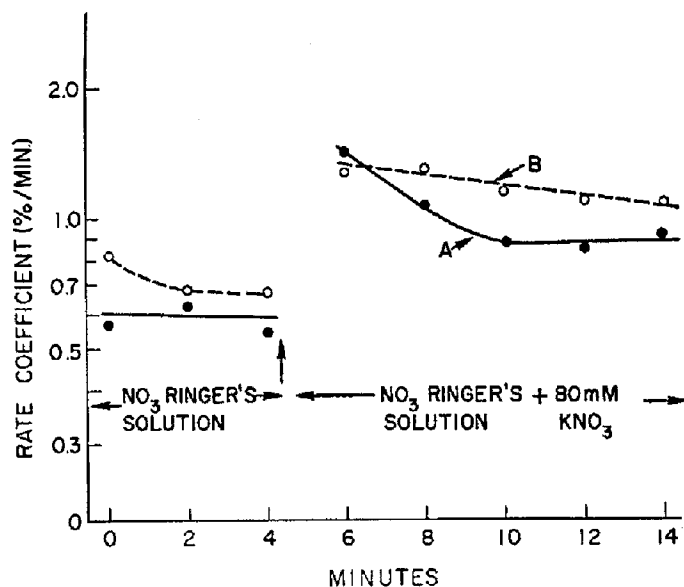


FIGURE 3. Comparison of the time course of the Ca^{45} rate coefficient, before and after the elevation of the potassium concentration, of 4 paired muscles mounted isotonicly (A) and isometrically (B).

Ca^{45} has now been examined during and following both isometric and isotonic contracture in elevated potassium and this is compared with uptake data obtained with both types of contracture.

In Fig. 3 are shown the averaged curves of Ca^{45} escape, expressed as rate coefficients, of paired muscles before and after subjection to high potassium media. One of each pair was mounted for isotonic, the other for isometric recording. It is immediately apparent that in both cases there is a rapid increase in the rate of Ca^{45} liberation by potassium which is maintained at about double the baseline rate, even after 10 minutes in the potassium. It will be recalled that maximum contracture is attained in about 15 seconds and relaxation is practically complete in approximately another 2 minutes despite the continued presence of excess potassium (2).

An important difference between the paired muscles is that, uniformly, those performing an isotonic contraction initially underwent twice the relative increase in rate coefficient (Table II), but this larger increment was transitory, disappearing in about the time required to wash out Ca^{45} from the extracellular space (Fig. 3). Since the extracellular space had been previously cleared of Ca^{45} by a 2 hour washout in radioisotope-free Ringer's solution, the additional Ca^{45} appearing with isotonic shortening would appear to be derived from the cells rather than from the extracellular spaces. The possibility remains to be explored that the elevated Ca^{45} in the extracellular space, resulting early from potassium treatment, was ejected more rapidly because of the shortening. In any case, these results suggest that Ca^{45} release from the fibers may be associated with at least two distinct processes: (a)

TABLE II
PERCENTAGE INCREMENT IN THE Ca^{45} RATE
COEFFICIENT OCCURRING IN ISOMETRICALLY AND ISOTONICALLY
MOUNTED MUSCLES DURING THE FIRST 2 MINUTES OF
EXPOSURE TO 80 mM POTASSIUM-RINGER'S SOLUTION

Isotonic	Isometric
131	63.5
332	114
144	57.7
204	170
203	101

shortening itself, (b) depolarization and/or potassium exchange. The two possibilities in (b) are presently inseparable, but studies of other depolarizing conditions (*e.g.*, with the veratrum alkaloids, that act by increasing sodium permeability (see reference 13)) may be expected to be helpful. It also remains to be seen whether the hypertonicity of the potassium solutions contributed to these results.¹

The minimum additional calcium liberated during 10 minutes in 80 mM potassium-Ringer's solution was estimated as for stimulation; these data are compared with the extra calcium uptake occurring during only 2 minutes of contracture in Table III. It should be kept in mind that the additional uptake actually occurs only very early during the contracture, and that the figures are undoubtedly less than half of the correct ones because the extracellular space in these experiments is less than half-saturated with Ca^{45} at the time

¹ Exploratory studies carried out since submission of this paper show that the increase in Ca^{45} liberation is obtained with potassium whether the ion replaces the sodium in the medium or whether it is present in excess.

the potassium concentration is raised (2). Nevertheless, unlike the situation during stimulation, the uptake figures are almost three times larger than the minimal release. It is also noteworthy that the estimated uptake or release is independent of the type of contraction or contracture; the initial difference in the rate of release during contracture is probably obscured by the large contribution of the later loss, which was not significantly different in the two types of contracture.

TABLE III
COMPARISON OF THE MINIMAL ADDITIONAL RELEASE OF
CALCIUM BY FOUR INDIVIDUAL PAIRS OF MUSCLES

One of each pair mounted isometrically, the other isotonically, subjected to 80 mM potassium for 10 minutes, and the average additional uptake of calcium by seven pairs of similarly mounted muscles subjected to the same solution for 2 minutes (\pm S.E.).

Minimal release $[(\mu\text{mole}/\text{gm.}) \times 10^3]$		
Isotonic (A)	Isometric (B)	B - A
9.4	11.4	+2.0
6.5	3.5	-3.0
9.1	8.0	-1.1
2.7	3.1	+0.4
Uptake $[(\mu\text{mole}/\text{gm.}) \times 10^3]$		
18.0 \pm 3	19.0 \pm 3	—

DISCUSSION

Our findings leave no doubt that muscle stimulation causes an increased release of Ca^{45} . The failure of Harris (6) to observe the increase in Ca^{45} liberation may have been caused by technical factors; *e.g.*, insufficient stimulation, excessively long collection intervals (his intervals appear to have been four times ours) so that "passive" outflux obscured the effect of stimulation, or the presence of irregular spontaneous activity, which also could have obscured the effect of stimulation. In our early experiments, carried out in the absence of cocaine, mild spontaneous activity was frequently encountered. This was successfully prevented by the regular use of at least 2 mg. per cent of the alkaloid in our solutions.

Many possibilities come to mind to account for the increased release of Ca^{45} during stimulation. Certain of the more conventional ones, such as an increase in permeability or the decline and reversal in membrane potential during the action potential, are less likely to play the part they do for mono-valent ions. That production of action potentials underlies the release at

least in part is suggested by (a) the frequently as large escape of Ca^{45} during a second period of stimulation, when the contractile response was weaker than during the first period of stimulation, and (b) the absence of a marked difference between muscles subject to several tetani or to individual twitches when the number of stimuli was the same. The maintained increase in Ca^{45} release by potassium appears in keeping with this since it may be due to the depolarization.

In view of the evidence that intracellular calcium is strongly bound (*e.g.*, 6, 8), and that even if it were not the free ion concentration would not appreciably exceed that in the medium (5, 14), special mechanisms appear to be required to reconcile this with our finding that the increase in release during activity at least equals and may appreciably exceed the entry. A brief release of the intracellularly bound calcium may be postulated; Heilbrunn (7, p. 606) has summarized evidence from a variety of cells suggestive of the freeing of calcium from the bound form. An increase in permeability to a soluble calcium complex also merits consideration in view of Zierler's finding that conditions that enhance passive ionic interchanges also favor the escape of large molecules such as aldolase (16).

These or other possibilities responsible for the increase in Ca^{45} release with activity may be peculiar to muscle, perhaps to its contractile mechanism (*e.g.*, the excitation-contraction coupling), for in the squid giant axon, although influx is increased with activity, outflux is unchanged. The 30-fold greater influx of calcium per impulse in muscle than in nerve despite the near equality of the resting influxes has already been noted as consistent with a special role for calcium in contraction (2). Further exploration of the differences among contractile and non-contractile tissues appears promising as a guide to new experiments.

It was noted before that the difference in the kinetics of Ca^{45} emergence under isometric and isotonic conditions of potassium contracture suggests that the shortening process itself is a contributor to increased calcium escape. This could well be the reduction in the (protein?) contents of the I band shown by the decline in density observed by Huxley and Taylor (9) to result from the constriction of the band during shortening. It remains to be seen whether, in keeping with this, electrical stimulation under isometric conditions causes a smaller release of Ca^{45} than under isotonic conditions and therefore is less efficient for maintenance of the intracellular calcium level in equilibrium with the environment.

The extent to which our minimal estimates of calcium release deviate from the actual figures requires determinations of influx concurrently with precise measurements of net changes. It has already been mentioned that previous reports of negligible changes in the calcium content of muscles stimulated *in situ* (4) cannot be regarded as strong support for our calculations. This is

because the unidirectional transfers are so very small. Thus, under the conditions of stimulation in Table I, the larger shifts of calcium for the total period of stimulation were chiefly under $0.1 \mu\text{mole/gm.}$, which is under 10 per cent of the calcium content of muscle (5, 6). A muscle weighing, say, 0.1 gm. placed in only 1 ml. of solution would raise the concentration of calcium by 0.01 mM, which certainly could not be detected, and this figure neglects the uptake of calcium that is now known to occur with activity (2). Perhaps other muscles capable of withstanding more and higher rates of activity would be useful for this purpose, especially if stimulated isometrically.

Although no net gain or even a net loss may ultimately be demonstrated to result from contraction or contracture in whole muscles or muscle fibers, the possibility that marked shifts occur in localized regions of individual fibers will remain to be explored. This is because influx and release may be separated temporally or spatially or merely reflect the same basic process such as the freeing of calcium bound near the fiber surface. For example, temporal separation of inward and outward components on a small time scale is a possibility in view of the strong evidence for such a phenomenon in respect of sodium and potassium (13). Or the liberation of calcium from cortical sites may well have components of inward and outward migration which are equal; with calcium normally present in the medium the outward movement may be self-exchange (*i.e.*, Ca^{46} for Ca^{40}) whereas in the absence of appreciable ionized calcium in the myoplasm, the entry is more likely to represent an actual net transport of calcium, possibly with chloride or nitrate (13), or by exchange with potassium. Spatial separation of calcium transport is an intriguing possibility in the light of the histological evidence for a non-uniform distribution of calcium in muscle fibers and particularly for a high content of calcium (or magnesium) in the vicinity of the Z line, from which it may be liberated during excitation (3, 11, 12; *cf.* 2); this possibility is of especial interest in view of the finding by Sten-Knudsen (personal communication) that the latent period of contraction is increased as the A band is separated from the Z line by stretch.

The following is regarded as the simplest sequence of events consistent with available data for frog sartorius fibers: (a) Depolarization (or agents that can act independently of depolarization) causes the release of calcium from the vicinity of the Z line into the I band; during ordinary stimulation this can be considered to occur during the true latent period. (b) Calcium migrates, possibly as a complex formed with soluble protein in the I band, to the A band; this would take place during, and perhaps be responsible for, latency relaxation. (c) Activation of contraction occurs when the calcium reaches the A band. (d) Calcium is "squeezed out," along with protein (9), during shortening of the I band in isotonic contractions; some release may also occur from the I and A bands during the preceding depolarization phase.

Autoradiographic studies of large, single muscle fibers subjected to suitable experimental conditions may be helpful in determining the correctness of these proposals. Histochemical methods may also prove useful.

REFERENCES

1. BIANCHI, C. P., and SHANES, A. M., *Anat. Rec.*, 1958, **132**, 413.
2. BIANCHI, C. P., and SHANES, A. M., *J. Gen. Physiol.*, 1959, **42**, 803.
3. CAREY, E. J., and ZEIT, W., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 31.
4. FENN, W. O., COBB, D. M., MANERY, J. F., and BLOOR, W. R., *Am. J. Physiol.*, 1938, **121**, 595.
5. GILBERT, D. L., and FENN, W. O., *J. Gen. Physiol.*, 1957, **40**, 393.
6. HARRIS, E. J., *Biochim. et Biophysica Acta*, 1957, **23**, 80.
7. HEILBRUNN, L. V., *An Outline of General Physiology*, Philadelphia, W. B. Saunders Company, 1952.
8. HODGKIN, A. L., and KEYNES, R. D., *J. Physiol.*, 1957, **138**, 253.
9. HUXLEY, A. F., and TAYLOR, R. E., *J. Physiol.*, 1958, **144**, 426.
10. NIEDERGERKE, R., *J. Physiol.*, 1955, **128**, 12P.
11. SANDOW, A., *Am. J. Physic. Med.*, 1955, **34**, 145.
12. SCOTT, G. H., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 349.
13. SHANES, A. M., *Pharmacol. Rev.*, 1958, **10**, 165.
14. SHANES, A. M., and BIANCHI, C. P., *J. Gen. Physiol.*, 1959, **42**, 1123.
15. WOODWARD, A. A., *Biol. Bull.*, 1949, **97**, 264.
16. ZIERLER, K. L., *Ann. New York Acad. Sc.*, 1958, **75**, 227.