J. Physiol. (1965), **176**, *pp.* 180–190
With 8 text-figures
Printed in Great Britain

THE LOSS OF RADIOACTIVE ^{45}CALCIUM FROM THE SMOOTH MUSCLE OF THE GUINEA-PIG TAENIA COLI

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(*Received* 18 *December* 1963)

In the previous paper (Bauer, Goodford & Hüter, 1964) it has been shown that the calcium content of intestinal smooth muscle rises on stretching, on exposure to sodium-free solution, on cooling, during anoxia and after dissection. The calcium content also rises when the potassium concentration of the bathing solution is raised (Urakawa & Holland, personal communication). Under all these conditions there is, at least, an initial contraction, and these observations therefore show that under different experimental conditions the calcium content of the taenia is higher after a period of contraction.

This increase in tissue calcium could occur in two ways. Either the uptake of calcium might be faster without sufficient increase in the rate of loss of calcium to maintain a steady state, or the uptake of calcium might be steady, while the rate of loss diminished. In this paper an attempt has been made to distinguish between these possibilities by measuring the loss of tracer calcium from the tissue.

METHODS

Solutions

The 'normal' solution was a modified Krebs's saline solution prepared from isotonic stock solutions (Goodford, 1962). It contained (mM): Na^+ 137, K^+ 5·9, Ca^{2+} 2·5, Mg^{2+} 1, Cl^- 144, HCO_3^- 5·9, D(+)-glucose 11·5, and was equilibrated with a gas mixture of 99 % O_2/1 % CO_2. 'Glucose-free' and 'sodium-free' solutions were prepared as described by Bauer *et al.* (1964). High potassium solutions (40 to 100 mM) were either prepared by using isotonic KCl instead of isotonic NaCl solution, or by adding solid KCl. Radioactive ^{45}Ca was supplied as a 3·8 mg/ml. $CaCl_2$ solution (specific activity 20 mc/ml.) by Oak Ridge National Laboratory, U.S.A.

Preparation of the taenia

In each experiment one guinea-pig weighing 300–600 g was stunned and bled. The abdomen was opened and up to four pieces of taenia 25 mm long were rapidly dissected. The average muscle weight was 7 mg so that diffusion delays would not be appreciable unless the rate of diffusion of calcium in the interspaces were much less than in aqueous

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solution (cf. Freeman-Narrod & Goodford, 1962, for the case of Na, which has an almost identical diffusion coefficient).

Each muscle was mounted on a long-armed stainless-steel spring by piercing both ends of the taenia with one arm, and hooking the loop of muscle so formed on to the other arm (Fig. 1). The spring applied a tension of 2 g to the taenia, and was lowered by a thread into a small jar containing 4 ml. oxygenated Krebs's solution at 35° C. Muscles mounted in this way were viable for many hours, contracting when the potassium concentration was

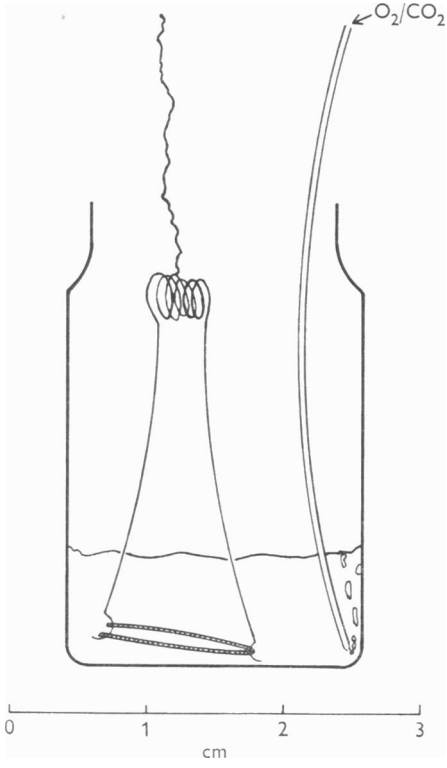

Fig. 1. Each muscle was mounted on a long-armed stainless steel spring and immersed in oxygenated ^{45}Ca Krebs's solution in a small glass jar. During tracer wash-out the muscle and spring were rapidly transferred down a series of such jars containing inactive Krebs's solution.

raised and relaxing in normal solution when 2×10^{-8} adrenaline was added. They could be transferred from one jar to another in about 1 sec, and were loaded with tracer ^{45}Ca by placing them in a jar with 2 ml. radioactive solution (specific activity 250 μc/ml.) of the same chemical composition as the inactive Krebs's solution.

Experimental procedure

A series of identical jars was prepared, each containing 4·0 ml. inactive Krebs's solution oxygenated through a thin polyethylene tube, but the first jar contained 12·0 ml. inactive solution in order to wash away any radioactivity which had splashed on to the spring

during the loading period. The radioactive muscle was then transferred from jar to jar leaving some tracer in each. At the end of the experiment the muscle was soaked in three further jars of inactive solution for 72 hr altogether. The amount of radioactivity left in the final jar was always less than the amount in the last jar of the actual experiment, and no further attempt was made to extract tracer calcium from the tissue.

Determination of radioactive calcium

To 4 ml. of solution in each wash-out jar 9 ml. isopropyl alcohol and 2 ml. of phosphor solution (5 g/l. diphenyloxazole in toluene) were added. The liquids were stirred thoroughly, kept in a dark-room overnight, and scintillations were counted the following morning in the same jars using a pulse analyser.

Control experiments

Chujyo & Holland (1963) and Niedergerke (1963*a*) have observed that glass or metal surfaces can adsorb tracer calcium and release it again during a subsequent wash-out period. If the metal spring or the glass jars in the present experiments adsorbed ^{45}Ca in this way the observations could be seriously distorted. However, only 5% of the radioactivity in the first wash-out jar came from the spring in control experiments, and this fell to less than 1% in each of the succeeding jars. In order to check if any radioactivity remained in the jars from previous experiments a large number of blanks were always measured. If the taenia liberated any chemiluminescent substance into the Krebs's solution this could cause spurious counts, but no such counts were measured when inactive muscles were used as controls. A more serious error, however, would arise if the ratio of Krebs's solution to counting solution was not constant, as an extra 0·1 ml. of inactive Krebs's solution would diminish the count rate by 2%.

RESULTS

^{45}Ca *loss at* 35° *C*

Figure 2 illustrates the longest wash-out experiment in the present series. The rate of ^{45}Ca efflux (counts min^{-2}) was calculated from the radioactivity in each jar, and was plotted as a fraction of the initial rate. The amount of radioactivity in the muscle (counts min^{-1}) at any time was equal to the sum of the counts in all subsequent jars, and was plotted as a fraction of the amount at the start of the experiment. Both series of points are shown in Fig. 2 starting from a common origin on the same semi-logarithmic axes, and the decline of both is approximately linear after 100 min. However, the lines never become parallel as they would if ^{45}Ca were being released at a single exponential rate from a single compartment, and so tracer calcium in this experiment did not behave homogeneously (Persoff, 1960). A similar conclusion was reached from each of six further experiments in which muscles were transferred to a fresh jar every 10 min between 120 and 220 min of efflux. The effluent counts had an average half-time of 77 min (Table 1), corresponding to a rate constant of 0·009 min^{-1} which agreed with Chujyo & Holland's (1963) value of 0·01 min^{-1} under similar conditions. The muscle counts in each experiment

had a significantly longer $T_{\frac{1}{2}}$ (average 151 min) than the effluent counts, showing that the slow exchange of ^{45}Ca was heterogeneous.

Bauer *et al.* (1964) could not detect the slow exchange of calcium in their uptake experiments because the experimental scatter prevented them from measuring the last stages of exchange. They therefore interpreted

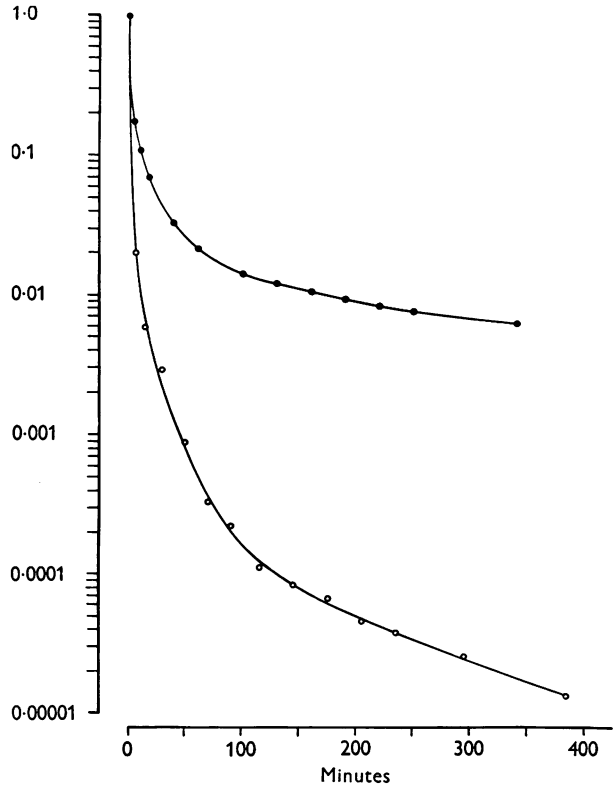

Fig. 2. Observations in normal solution at 35° C after 210 min immersion in radioactive ^{45}Ca Krebs's solution. Abscissa, time in min after transfer of the taenia coli to the first inactive jar for tracer wash-out. Logarithmic ordinate scale along which both curves have been shifted so that they start from a common origin. Open circles, the rate of ^{45}Ca efflux (counts min^{-2}) as a fraction of the initial rate. Closed circles, the amount of radioactivity in the muscle (counts min^{-1}) at each time of observation, as a fraction of the amount at the start of the wash-out. Note that the two curves never become parallel. See text.

the uptake on the basis of two rapidly exchanging components, and the first minutes of ^{45}Ca loss have been treated in the same way in Fig. 3. A half-time of 2·7 min was then obtained which agreed with the value of 2–3 min for the corresponding period of uptake (Table 2). In both uptake and wash-out experiments there was an initial faster exchange

TABLE 1. Loss of ^{45}Ca after 120–220 min of wash-out

Expt.	$T_{\frac{1}{2}}$ of effluent counts (min)	$T_{\frac{1}{2}}$ of muscle counts (min)
1	81 ± 12	129 ± 5
2	72 ± 12	97 ± 3
3	79 ± 21	156 ± 8
4	100 ± 31	174 ± 5
5	90 ± 54	219 ± 14
6	56 ± 11	150 ± 7
7	59 ± 24	129 ± 5
Mean	77	151

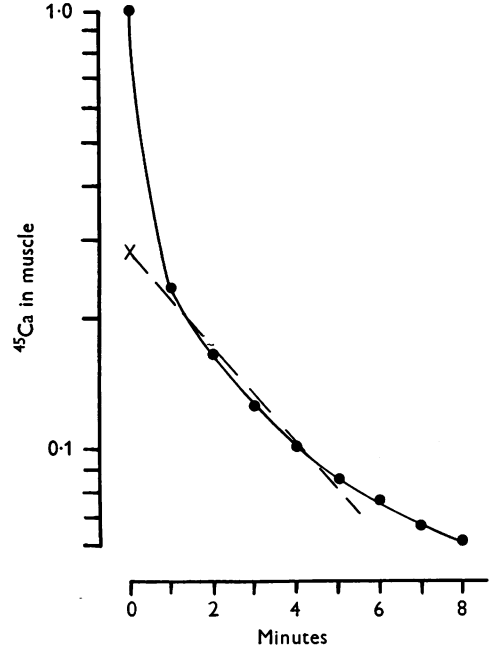

Fig. 3. Muscle counts in normal solution at 35° C during the first minutes of wash-out. Abscissa, time in min after transfer of the taenia to the first inactive jar. Logarithmic ordinate, the amount of radioactivity in the muscle (counts min^{-1}) as a fraction of the initial amount. See text.

which was larger (71 % as opposed to 42 %) during wash-out because radioactive Krebs's solution was carried with the muscle into the first wash-out jar. Control experiments showed that this superficial Krebs's solution nearly doubled the weight of a piece of taenia (average increase in weight + 82 ± 7 (20) %), and the efflux corrected accordingly is given in the last line of Table 2. It then shows almost identical characteristics with the first minutes of uptake (middle line).

TABLE 2. A comparison of the first phases of ^{45}Ca uptake and loss (see text)

Reference	Method	Phase I		Phase II	
		$T_{\frac{1}{2}}$ (min)	%	$T_{\frac{1}{2}}$ (min)	%
Present observations	Loss	0·5	71	2·7	29
Bauer *et al.* (1964)	Uptake	0·5	42	2–3	58
Present observations corrected	Loss	0·5	48	2·7	52

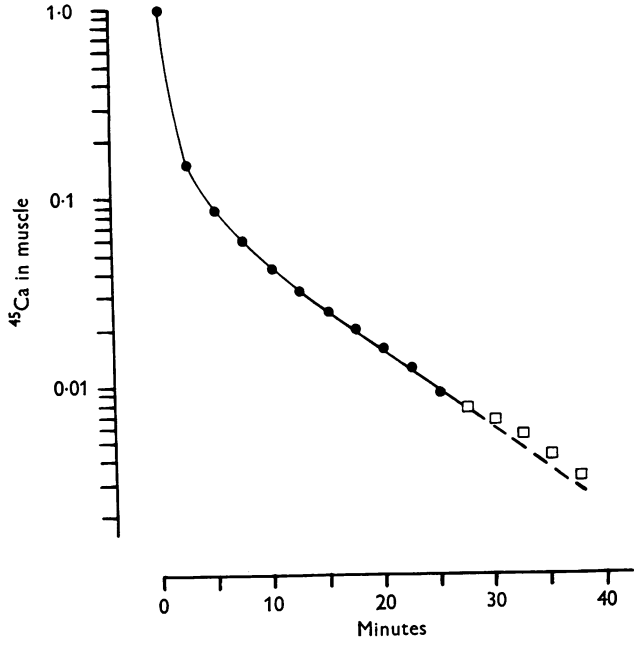

Fig. 4. Average muscle counts in five wash-out experiments at 35° C. Abscissa and ordinate as in Fig. 3. Solid circles, in normal solution. Open squares, in 100 mM potassium solution. Note the slight transient slowing of ^{45}Ca loss after high-potassium immersion.

High-K, Na-free, and oxygen-free glucose-free solutions

When taeniae were transferred to sodium-free solution, high-potassium solution, or oxygen-free glucose-free solution at 35° C there was a sudden contraction and a simultaneous increase in calcium content (Bauer *et al.* 1964; Urakawa & Holland, personal communication). Figure 4 shows the loss of ^{45}Ca, first into a normal solution and then into a series of jars containing 100 mM-K solution. The rate of loss of ^{45}Ca diminished slightly for a few minutes at this time, but the effect was very small and was not always noticeable. Figure 5 shows the effect of 55 mM potassium solution, of sodium-free solution and of oxygen-free glucose-free solution at a later

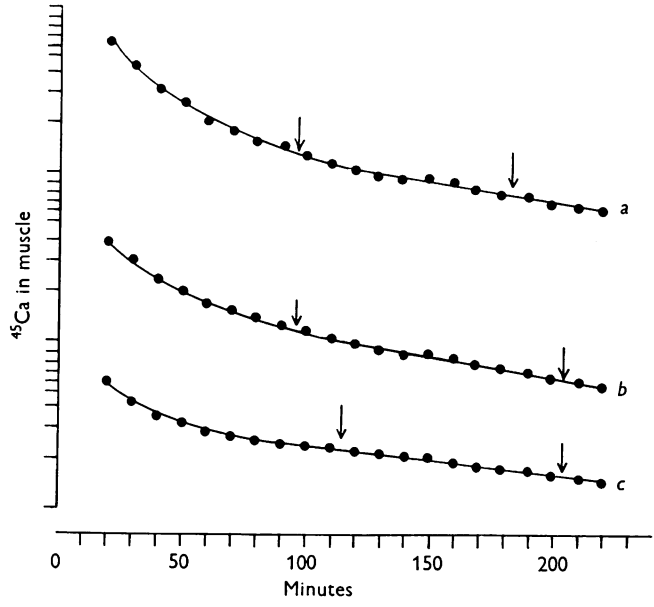

Fig. 5. Muscle counts in three experiments at 35° C. Abscissa and ordinate as in Fig. 3. The middle and bottom lines were moved one and two decades down the logarithmic ordinate scale to avoid overlap. The chemical composition of the inactive solution was changed in each experiment between the arrows to: (*a*) 55 mM potassium, (*b*) sodium-free, (*c*) oxygen-free glucose-free. These changes produced no significant effects.

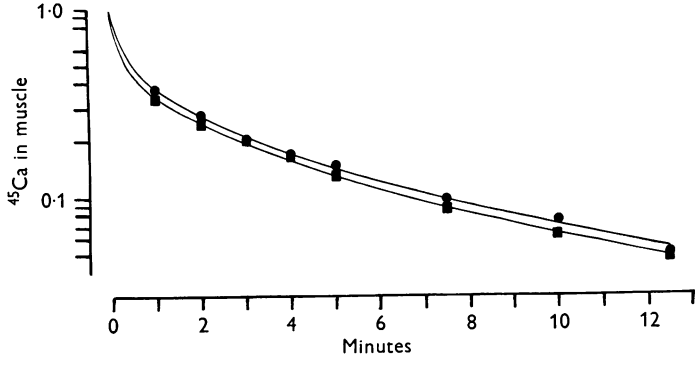

Fig. 6. Muscle counts in one experiment at 35° C. Abscissa and ordinate as in Fig. 3. Solid circles, in normal solution. Solid squares, with 2×10^{-4} M-DNP added. Slightly more ^{45}Ca was lost in the presence of DNP, but the small effect was attributed to a difference in the apparent extracellular space.

stage of wash-out, after an initial 200 min loading period. The results were reproducible, and in no case was any significant effect observed.

The action of 2,4-dinitrophenol (DNP)

Figure 6 shows a single experiment in which two similar muscles were loaded with ^{45}Ca for 15 min, and then transferred to inactive solution with or without 2×10^{-4} M-DNP. This was a high concentration (Bülbring &

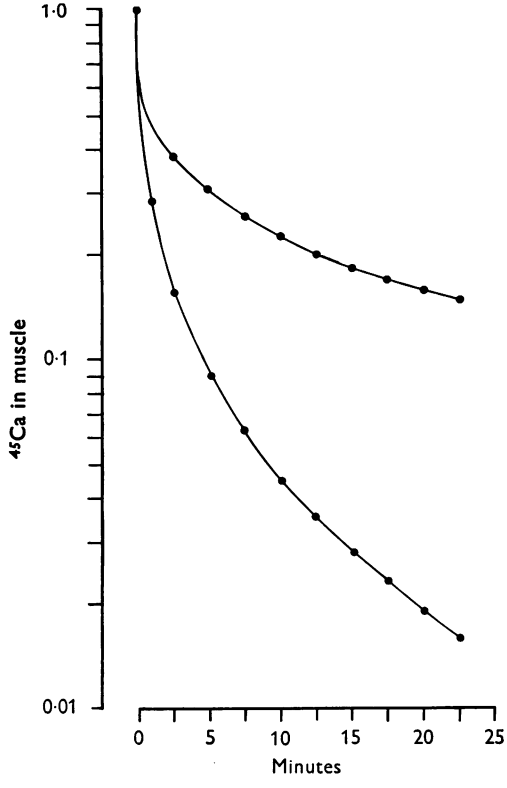

Fig. 7. Average muscle counts in five experiments in normal solution. Abscissa and ordinate as in Fig. 3. Upper curve at 4° C. Lower curve at 35° C.

Lüllmann, 1957) which caused the muscle to relax, but it did not change the rate of loss of ^{45}Ca appreciably. The small constant difference between the muscles was already observed at the first minute, and was attributed to a difference in the apparent extracellular spaces.

The effect of temperature change

Figure 7 shows the average results of five experiments in which the loss of tracer at 35° C was compared with that at 4° C. A greater proportion

of the radioactivity remained in the muscle at the lower temperature, confirming the observation of Bauer *et al.* (1964) that the amount of slowly exchanging calcium increased in the cold. In further experiments muscles were loaded with ^{45}Ca at 4° C and wash-out began at that temperature. After half an hour this was increased to 22 or 35° C, and the rate of loss of tracer at once rose, measured by the gradient of the curve immediately before and after warming. In the experiment shown in Fig. 8, for example, the half-time fell from 41 min (corresponding to a rate constant K of

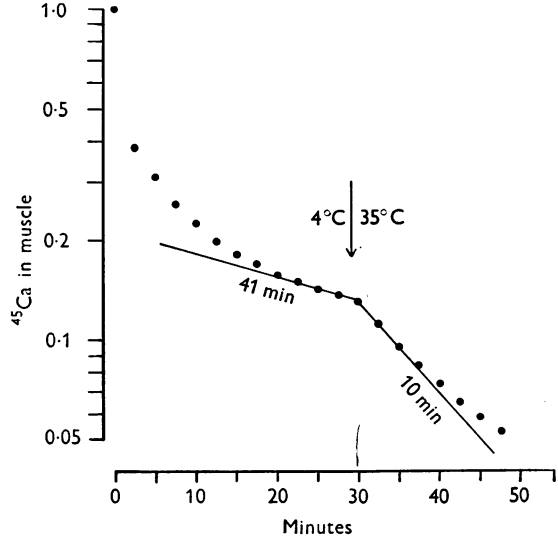

Fig. 8. Muscle counts in one experiment in normal solution. Abscissa and ordinate as in Fig. 3. Wash-out began at 4° C and the temperature was suddenly raised to 35° C during the experiment. See text.

0·017 min^{-1}) to 10 min (0·069 min^{-1}) when the temperature was increased by 31° C. On the basis of the Arrhenius equation

$$\ln K = -\frac{A}{RT} + \text{constant} \quad (R = \text{gas constant})$$

a straight line should be obtained when the logarithm of the rate constant is plotted against the reciprocal of the absolute temperature (T), and the results did not deviate significantly ($0{\cdot}1 > P > 0{\cdot}05$) from linearity when plotted in this way. The parameter A had a value of 6400 cal/mole, corresponding to a Q_{10} of 1·5 which may not be too high for a purely physical process. The same Q_{10} was measured in three further experiments when DNP was added to the wash-out solutions at concentrations between 6×10^{-5} and 6×10^{-4}M. The Q_{10} of calcium loss from a muscle which had first been killed by boiling in isotonic saline solution was 1·4.

DISCUSSION

The present experiments, and those of Bauer *et al.* (1964), showed that the uptake and loss of ^{45}Ca followed a similar time course during the first minutes of exchange. Slower components were just detected in the previous paper, and were observed for periods up to 6 hr during the wash-out of tracer. Even at this time, however, the loss of ^{45}Ca from the taenia did not follow a single exponential course, suggesting either that exchange was heterogeneous (Creese, Neil & Stephenson, 1956; Van Liew, 1962; Niedergerke, 1963*b*) or that the rate was declining as the tissue aged during the course of an experiment (Bauer, Goodford & Hüter, 1963).

Sodium-free, high-potassium, and oxygen-free glucose-free solutions made the taenia contract for a time at 35° C, and caused the total muscle calcium to increase simultaneously. But no changes in ^{45}Ca loss were observed under these conditions except for a slight transient slowing in some of the high potassium experiments, and even this effect cannot be regarded as established since Schatzmann (1961) observed a slight acceleration when the potassium concentration was raised. At 4° C the rate of tracer loss was reduced significantly, and a Q_{10} of 1·5 was measured between 4 and 35° C. Muscles which had been pre-treated with DNP or pre-heated to 100° C gave similar low Q_{10} values.

A possible interpretation for the present observations is that the rate of loss of calcium from the taenia coli *in vitro* is largely controlled by a physical process, unaffected by changes in the composition of the bathing solution. This does not exclude the possibility of a calcium-extrusion mechanism analogous to the 'sodium pump', but it implies that such a 'calcium pump' is not the rate-limiting stage in calcium extrusion. For this reason it cannot be detected when the muscle is poisoned with DNP. The observations suggest that there is a faster inflow of calcium in the sodium-free, high-potassium and oxygen-free glucose-free solutions while the taenia contracts. The over-all loss of calcium from the muscle is limited by the postulated physical process and the total muscle calcium therefore increases. When the taenia eventually relaxes its total calcium content remains high, but the extra calcium is relatively inexchangeable (Bauer *et al.* 1964). This extra calcium may not be associated with the contractile mechanism, and is stored in the tissue until it leaks back into the extracellular pool by the slower rate-limited process.

SUMMARY

1. The smooth muscle of the guinea-pig taenia coli was loaded with tracer ^{45}Ca in radioactive Krebs's solution *in vitro*, and the loss of ^{45}Ca into inactive Krebs's solution was measured for periods of up to 6 hr.

2. At no time did the loss follow a single exponential course, suggesting either that the exchange was heterogeneous or that the rate was declining as the tissue aged during the course of an experiment.

3. No changes in the rate of ^{45}Ca loss were observed in the absence of sodium, in oxygen-free glucose-free solution or in the presence of 2,4-dinitrophenol 2×10^{-4}M at 35° C. In some experiments there was a slight transient slowing in high-potassium solution. At 4° C the rate of loss was slowed, with a Q_{10} of 1·5 between 4 and 35° C.

4. It is suggested that the rate of loss of calcium from the taenia coli *in vitro* may be largely controlled by a physical process, unaffected by changes in the composition of the solution bathing the muscle.

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