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THE SODIUM CONTENT OF THE SMOOTH MUSCLE OF THE GUINEA-PIG TAENIA COLI

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In vitro preparations of the guinea-pig taenia coli bathed with normal perfusion fluid have an average resting potential of −51 mV (Holman, 1958). The average height of the action potential is 59 mV, and the distribution of ions is such that these action potentials could arise from changes in the selective permeability of the membrane to sodium and potassium ions (Goodford & Hermansen, 1961). However, the spike height and the reversal of polarity during an action potential are maintained for 30–60 min when the sodium ions in the bathing solution are completely replaced by another cation (Holman, 1957; Axelsson, 1961; Bülbring, Kuriyama & Twarog, 1962) even if the substituent ion has very different properties from sodium (e.g. lithium, hydrazine, choline or *tris*-hydroxymethylaminomethane (*tris*)). In order to aid the interpretation of these results the sodium content of normal and sodium-depleted muscles has now been determined.

Over 90 % of the muscle sodium is lost during the first 3 min immersion in lithium solution, and it has been suggested (Goodford & Hermansen, 1961) that some of this rapidly exchanging sodium might be bound at an extracellular site. Such a hypothetical site might concentrate sodium, so that it would no longer be possible to calculate the extracellular sodium from a knowledge of the extracellular space and the sodium concentration in the bathing solution. To investigate this possibility the sodium content was determined after immersion of the taenia in solutions of low sodium concentration (1–7 mM-Na) but no evidence was obtained that sodium was being concentrated at such a binding site. Some evidence will, however, be presented that the muscle sodium was not simply distributed between the intracellular and extracellular spaces.

METHODS

The methods used were essentially those of Freeman-Narrod & Goodford (1962). Physiological saline solutions were prepared (Table 1) from isotonic stock solutions which had been made with Analar grade materials and glass-distilled water. The stock solution of *tris*

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chloride was prepared from the free base (Sigma grade 7–9) by partial neutralization with hydrochloric acid to pH 7·3. The sodium content of the low-sodium solutions was checked on the flame photometer at the end of experiments. It was not difficult to prepare a solution containing less than 10^{-6} parts of sodium so long as glass-ware which had never been exposed to sodium solutions was used, but on a few occasions much higher concentrations were

TABLE 1. The proportions of isotonic stock solutions used to prepare physiological saline solutions (see Krebs, 1950). Choline and *tris* solutions were prepared analogously to the lithium solutions. All solutions were gassed with 99 % O_2 + 1 % CO_2

Stock solution (g/l.)	Sodium solution	Lithium solution	Lithium (x part Na)
NaCl (9)	116	—	x
LiCl (6·5)	—	116	116−x
$KHCO_3$ (15·3)	5	5	5
D(+)-glucose (54)	5	5	5
$MgCl_2.6H_2O$ (21)	1·5	1·5	1·5
$CaCl_2$ (12)	3	3	3

observed and the results of these experiments were rejected. The sodium concentration of 'sodium-free' solutions rose towards 10^{-5} when a piece of taenia was introduced, so that they could not be used a second time, but this error was small enough to be neglected in 'low-sodium' experiments when the concentration already exceeded 1 mM-Na. The composition of the control physiological saline ('sodium solution') was (mM): Na^+ 137, K^+ 5·9, Ca^{2+} 2·5, Mg^{2+} 1, Cl^- 144, HCO_3^- 5·9, and D(+)-glucose 11·5, gassed with 99 % O_2 + 1 % CO_2, and differed from the solution used by Freeman-Narrod & Goodford (1962) in having a higher chloride and lower bicarbonate concentration. The CO_2 concentration was reduced in order to keep a pH of 7·3.

Thinner pieces of taenia were dissected than those used previously in order to minimize diffusion delays (Goodford & Hermansen, 1961), the mean weight being 9·6 ± 0·2 (289) mg for a 25 mm length of muscle.

RESULTS

Lithium solution at 35° C

Lithium was the first cation to be substituted for sodium, because the lithium ion could be readily detected by flame photometry, and because the effects of this substitution upon skeletal muscle were already well established (Keynes & Swan, 1959*a*, *b*). Pieces of taenia were therefore transferred to a solution in which all the sodium ions had been replaced by lithium, when there was a rapid fall of muscle sodium and gain of lithium. These were equal and opposite during the 3 min immediately following the transfer (Fig. 1), but thereafter the sodium was lost less rapidly. However, very little sodium was left in the tissue at 15 min, and after 60 min only 1·5 ± 0·6 (11) m-mole Na/kg wet wt. remained. 33 m-mole Li/kg wet wt. was gained during this period, but there was no change in the wet weight of the muscle and a slow loss of potassium now compensated for the lithium gain.

Goodford & Hermansen (1961) found that ^{24}Na exchange appeared to be complete after 15 min, but the present result suggests that some of the muscle sodium was lost at a slower rate in sodium-free solutions, although

it might be more freely exchangeable under normal conditions. The 1·5 m-mole Na/kg wet wt. which remained so firmly after 60 min in sodium-free solution might in some way account for the continuing electrophysiological activity of the taenia. The loss of radioactive ^{24}Na was therefore studied in order to confirm whether more ^{24}Na was really retained by a muscle bathed in lithium solution.

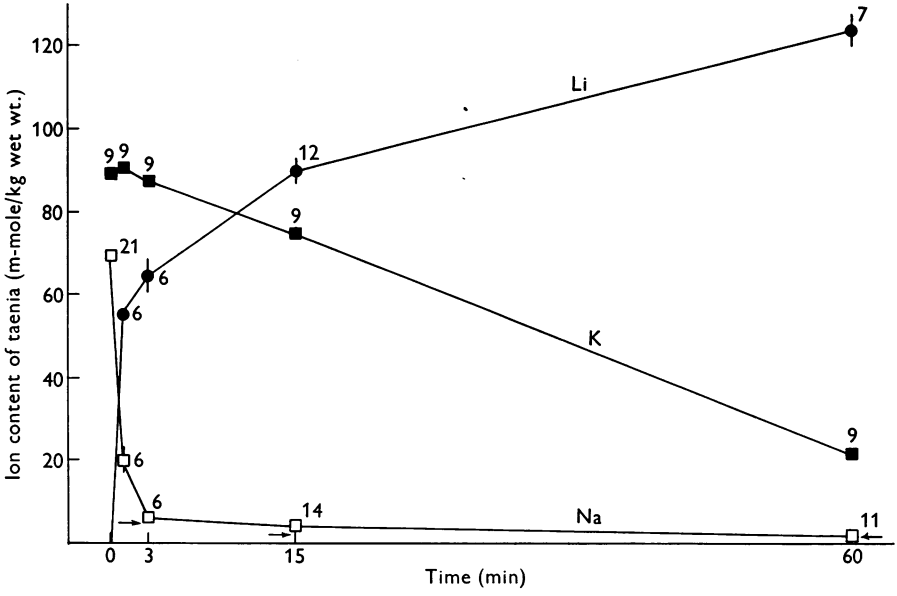

Fig. 1. Observations at 35° C. Abscissa, time after immersion of the taenia in a sodium-free solution prepared with lithium salts. Ordinate, the sodium, potassium and lithium contents of the taenia, determined by flame photometry, ±1 S.E. (for those observations in which the S.E. exceeded the size of the symbol on the figure), and the number of observations printed next to each point. Arrows indicate corresponding observations made using ^{24}Na. The taeniae had previously been allowed 1 hr to equilibrate in sodium solution at 35° C.

Pieces of taenia coli were placed in ^{24}Na sodium solution for 15 min, and were then transferred to inactive solutions prepared either with lithium or with sodium salts. They were removed in pairs after a further 3, 15, and 60 min, weighed, ashed and counted to determine the residual ^{24}Na; in every case more tracer remained in the muscle washed with the lithium solution, and although the difference was small (corresponding to 0·56 ± 0·076 (6) m-mole Na/kg wet wt. at 15 min) it was highly significant ($P < 0·001$).

Lithium solution at 4° C

It has been shown (Freeman-Narrod & Goodford, 1962) that the ionic content of the taenia coli was still changing after 1 hr equilibration at 4° C,

so a further hour was allowed for the muscle to settle down in sodium solution at this temperature. The sodium content was then 122 ± 3 (9) m-mole/kg wet wt. and the potassium content was low (Fig. 2).

The behaviour on transfer to lithium solution (Fig. 2) was quite different from that observed at 35° C. The sodium content fell rapidly at first, but came to a new steady value of 50 m-mole/kg wet wt., which remained

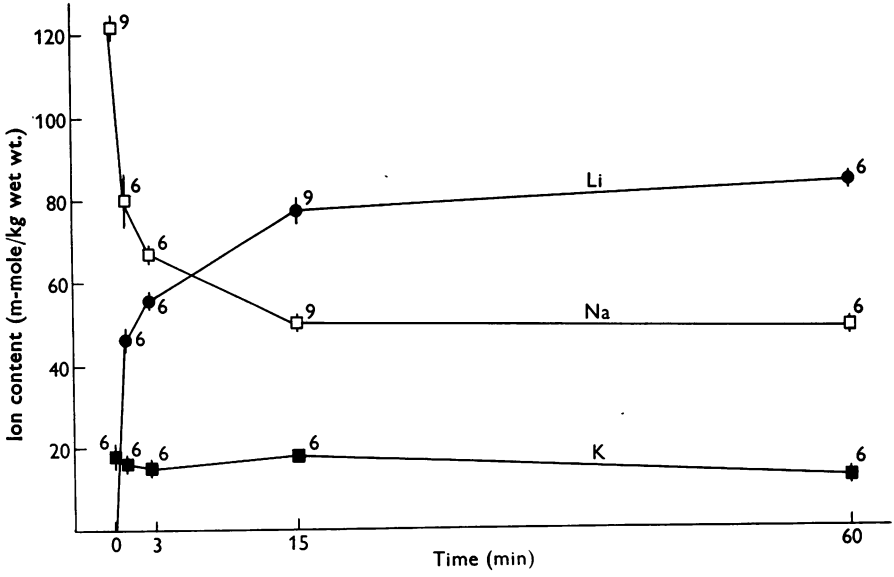

Fig. 2. Observations at 4° C. Abscissa, time after immersion of the taenia in a sodium-free solution prepared with lithium salts. Ordinate, the sodium, potassium and lithium contents of the taenia determined by flame photometry ± 1 S.E. (for those observations in which it exceeded the size of the symbol on the figure). The experiment started after taenia had been 2 hr in sodium solution at 4° C.

firmly in the tissue throughout the period of immersion in sodium-free solution. The lithium entry was rapid at first, but the slower phase of uptake was less marked at 4° C and only 6-mole Li/kg wet wt. was gained in the period 15–60 min. The potassium content also showed no significant alteration throughout the period of the experiment, and the total cation content (K + Na + Li) did not change. The amount of sodium remaining in the muscle after 1 hr at 4° C may be compared with the amount found by Freeman-Narrod & Goodford (1962) in normal solution when 49 m-mole Na/kg wet wt. did not exchange with tracer sodium at 4° C.

Another series of experiments confirmed that the loss of tracer sodium at 4° C was substantially the same into sodium or lithium solutions. Pieces of taenia were set up for 1 hr at 35° C, were transferred to ^{24}Na solution at the same temperature and after another hour were cooled to

4° C while still immersed in the radioactive solution. They were left for 3 hr to equilibrate, this procedure being designed to allow all the muscle sodium to exchange with tracer. They were then transferred to inactive solutions prepared either with lithium or with sodium salts. After 15 min they were removed in pairs but again no difference was detectable between the lithium-treated and the sodium-treated muscles.

An exponential analysis of the curves in Figs. 1 and 2 might give an impression of accuracy which the results do not justify, but one may compare the rapid sodium losses at 4° C with those at 35° C. In order to increase the accuracy of these comparisons additional experiments were carried out and the final results are shown in Table 2. Sodium loss was very rapid during the first 3 min at 35° C, but the additional loss of 5 m-mole/kg wet wt. (from $63 \pm 3{\cdot}0$ (*23*) to $68 \pm 1{\cdot}3$ (*6*)) between 3 and 15 min was not significant. On the other hand, although the initial rate was slower at 4° C, the process continued between the third and the fifteenth minute, during which an additional 23 m-mole/kg wet wt. was lost (from $55 \pm 4{\cdot}0$ (*12*) to $78 \pm 1{\cdot}3$ (*6*)).

TABLE 2. The loss of sodium (m-mole/kg wet wt.) from the taenia after immersion in lithium solution

Time in lithium (min)	Sodium lost at 35° C	Sodium lost at 4° C
1	$49 \pm 2{\cdot}7$ (*25*)	$42 \pm 5{\cdot}9$ (*12*)
3	$63 \pm 3{\cdot}0$ (*23*)	$55 \pm 4{\cdot}0$ (*12*)
15	$68 \pm 1{\cdot}3$ (*6*)	$78 \pm 1{\cdot}3$ (*6*)

The greater portion of the loss during the first minute was presumably from the extracellular space, since Goodford & Hermansen (1961) estimated the extracellular sodium to be 48 m-mole/kg wet wt. at 35° C. The rate at which this sodium diffused out of the muscle would not be expected to be greatly influenced by temperature, so that at 4° C the smaller rapid loss observed during the first minute most probably reflected the smaller extracellular space (0·22 ml/g; Born, 1962) in the cold. On the other hand by 15 min a new steady state had been set up, and thereafter no more sodium was lost at either temperature. The greater loss at 4° C by that time may have reflected the increased intracellular sodium which the tissue originally contained in the cold.

Choline and tris solutions at 35 and 4° C

The lithium ion was a convenient substitute for sodium because it could be determined in the ashed taenia by flame photometry. However, the effects described might have been due not to removal of sodium from the solution, but to some specific property of the high concentration of lithium.

The experiments illustrated in Figs. 1 and 2 were therefore repeated with the choline ion as a sodium substitute. The muscles contracted in the choline solution at 35° C, but at both temperatures the losses of sodium and potassium from the tissue were identical to those described for lithium.

The *tris*$^+$ ion (Lüttgau & Neidergerke, 1958), was also used as a sodium substitute. Pieces were analysed after a 15 min period in *tris*, and their sodium content was 4·3 ± 1·1 (9) m-mole/kg wet wt. (compare with Fig. 1).

Electrophysiological differences have been observed when taeniae coli were immersed in sodium-free solutions prepared with lithium, choline or *tris* (Bülbring & Kuriyama, 1961). The similarities of the ionic movements here described do not help to interpret these differences.

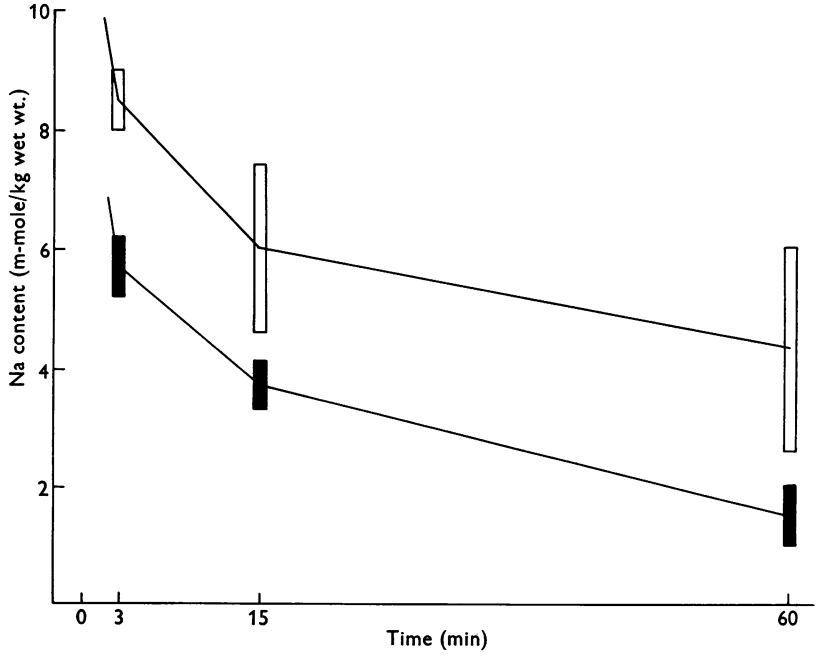

Fig. 3. Observations at 35° C. Abscissa, time after immersion of the taenia in low-sodium solutions prepared with lithium salts. Ordinate, the sodium content of the taenia determined by flame photometry ±1 S.E. Upper curve, 7 mM sodium solution; lower curve, sodium-free. Experiment started after 1 hr equilibration in sodium solution at 35° C.

Low sodium concentrations

The taenia coli might adsorb sodium from a solution of very low sodium concentration if there were sodium binding sites on the tissue. In this case the sodium content, and perhaps the electrophysiological properties, might be critically dependent upon the complete removal of sodium from the

solution. Pieces of taenia were therefore transferred to a lithium solution which contained 6 parts (7 mM) of sodium at 35° C, and the sodium content fell similarly to the earlier results. Figure 3 illustrates the later stages of this loss on an enlarged scale, and shows that the muscles immersed in lithium (6 parts sodium) solution retained about 2·5 m-mole Na/kg wet wt. more sodium than those immersed in pure lithium. This agrees satisfactorily with the difference of 2·45 m-mole Na/kg wet wt. which would be predicted from the extracellular space (about 350 ml./kg wet wt. at 35° C; see Discussion) and the solution concentration (7 mM), so that no indication was obtained that sodium was being selectively taken up and bound by the tissue.

However, the excess of lithium ions might compete with sodium for binding sites on the muscle: the formation of a lithium complex would be favoured, relative to sodium, because the lithium ion is the smaller (Fajans, 1923). No such effect would be expected when sodium-free solutions were prepared from choline, but the mean difference of sodium contents between muscles immersed in lithium (3 parts Na) and choline (3 parts Na) solutions was in fact only $1{\cdot}2 \pm 3{\cdot}0$ (8) m-mole/kg wet wt. In further experiments to detect sodium binding taeniae were placed at 4° C in low-sodium solutions prepared from choline instead of lithium. The residual sodium after 15 min immersion in a solution containing 1 part (1·2 mM) sodium was $55{\cdot}5 \pm 2{\cdot}2$ (4) m-mole/kg wet wt.; in 3 parts (3·5 mM) sodium $57{\cdot}5 \pm 2{\cdot}2$ (4); and in 6 parts (7 mM) was $56{\cdot}5 \pm 2{\cdot}7$ (4), so that there was no significant difference between these means. In other experiments at 35° C pieces of taenia were transferred for 1 min to a sodium-free choline solution, and then for 14 min more to fresh sodium-free choline, so that they had every opportunity to lose sodium. Half the taeniae were transferred for a further 15 min to a solution containing 3·5 mM-Na, and both groups were then analysed. There was again no significant effect, the taeniae in sodium-free solution containing $5{\cdot}1 \pm 0{\cdot}5$ (9) m-mole Na/kg wet wt. and in low-sodium $6{\cdot}3 \pm 0{\cdot}7$ (9).

50 % *Li solution*

Taeniae were set up in a normal solution at 35° C for 1 hr, and half the pieces were then transferred to a solution in which 50 % of the sodium had been replaced by lithium (68 mM-Na^+; 68 mM-Li^+). After another 15 min all the samples were removed for analysis, and the sodium content of the muscles in the half-sodium solution was then half the content of normal pieces ($51{\cdot}9\,\% \pm 2{\cdot}5\,\%$ (16)). There was a small potassium loss and a larger gain of lithium, so that the total cation (K + Na + Li) content changed by an insignificant amount ($3{\cdot}2 \pm 2{\cdot}3$ (15) m-mole/kg wet wt.).

Control experiments

In the experiments in which ^{24}Na was used to measure the sodium content of the taenia the determination was repeated by flame photometry on the same muscles in order to make a direct comparison between the methods. At 4° C the results differed by only $0{\cdot}02 \pm 0{\cdot}9$ (5) m-mole Na/kg wet wt., but at 35° C it was possible to observe a real difference, because the total amount of sodium to be determined was smaller. In every case the sodium content, calculated by counting ^{24}Na and assuming that complete exchange had taken place, was less (arrows in Fig. 1) than the content by flame photometry, and the effect was statistically significant. For example, in one experiment, after 15 min in ^{24}Na followed by 15 min in lithium, the counting method indicated $1{\cdot}23 \pm 0{\cdot}14$ (6) and flame photometry $3{\cdot}00 \pm 0{\cdot}37$ (6) m-mole Na/kg wet wt., so that the difference of $1{\cdot}77 \pm 0{\cdot}49$ (6) was significant at the 0·02

TABLE 3. Analysis of variance of the sodium content of pieces of taenia coli from different guinea-pigs after immersion in normal solution for different times

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio	P
Between guinea-pigs	9	9,523	1,060	6·8	< 0·001
Between times	6	430	71	0·46	> 0·2
Remainder	29	4,521	156	—	—
Total	44	14,474	—	—	—

level of probability. The small difference was to be expected, because the 15 min loading time in ^{24}Na solution was probably insufficient for complete exchange, and suggested that a correction factor should be applied to the results with tracer sodium before they could be compared directly with the flame photometry observations. Thus it was also observed that taeniae which had been loaded with ^{24}Na for 15 min and washed with inactive sodium solution for another 15 min still contained $0{\cdot}69 \pm 0{\cdot}06$ (8) m-mole ^{24}Na/kg wet wt., declining to $0{\cdot}52 \pm 0{\cdot}08$ (4) m-mole ^{24}Na/kg wet wt. after 60 min washing, again because a small portion of the muscle sodium exchanged with a very long half-time. To examine this possibility further, pieces of taenia were divided into two groups. One group was transferred to ^{24}Na solution for 24 hr, the other for 15 min. Both were then compared after a 15 min washing in inactive sodium solution, when the muscles soaked in tracer sodium for 24 hr contained ^{24}Na corresponding to $0{\cdot}72 \pm 0{\cdot}09$ (16) m-mole Na/kg wet wt. more than the controls.

At 35° C the taeniae were usually equilibrated for 1 hr before being transferred to sodium-free solution, but in some of the last experiments a period of 24 hr was allowed to ensure complete isotopic exchange. After such a period *in vitro* the properties of the tissue might have changed, and a separate comparison was therefore made by flame photometry of the effects of sodium-free solutions upon the taenia after 1 and 24 hr equilibrations. No differences in the ionic contents were observed.

Estimates of the sodium content of the taenia coli always showed greater variation than the potassium content and there were frequently marked variations from one guinea-pig to the next. The sodium content of the taenia from ten animals was therefore determined after different periods of immersion *in vitro*, and the analysis of variance (Table 3) showed a highly significant variation from animal to animal. Many of the previous comparisons were therefore repeated, pairs of muscles from the same guinea-pig being used; for example, the difference between the sodium contents at 4 and 35° C was confirmed in separate experiments in which eight muscles were dissected from each animal in pairs situated in adjacent positions *in vivo*. One muscle from each pair was studied at 4° C and the other at 35° C; two pieces at each temperature were analysed after equilibration in sodium solution and two after a further 15 min in lithium. The results of these experiments agreed closely with the observations in Figs. 1 and 2, confirming that variation between guinea-pigs was not an important source of error under the conditions of these experiments.

DISCUSSION

It has already been observed that the electrical activity of crustacean muscle (Fatt & Katz, 1953), frog neuromuscular junction (Koketsu & Nishi, 1959), frog skeletal muscle (Koketsu & Nishi, 1960) and mammalian smooth muscle (Daniel & Singh, 1958) can be maintained in a sodium-free solution, and it has been concluded either that sodium is not essential for the depolarizing mechanism in these tissues, or that some sodium is still present. In the experiments here described two independent methods, flame photometry and counting tracer sodium, were used to measure the amount of sodium remaining in smooth muscle after immersion in a sodium-free solution. The results were in satisfactory agreement, and showed that after 60 min in sodium-free solution at 35° C the residual sodium was only $1{\cdot}5 \pm 0{\cdot}6$ m-mole/kg wet wt. The electrophysiological behaviour of the tissue in similar solutions has already been described (Axelsson, 1961; Bülbring & Kuriyama, 1961; Bülbring & Kuriyama, personal communication), and showed two distinct transitions: an initial inhibition of spontaneous discharge which lasted for 2–3 min was followed by a long period during which frequent large action potentials were observed. The membrane polarity was reversed during these action potentials, but the rates of rise and fall were reduced.

The observations of Freeman-Narrod & Goodford (1962) and the present results both show that the sodium of the taenia coli was not simply distributed between an intracellular and an extracellular space in a two-compartment system. Up to 4 m-mole Na/kg wet wt. remained in the tissue after a 15 min immersion in sodium-free solution at 35° C, showing that some of the muscle sodium was slow to exchange. On the other hand, there was a loss of $68 \pm 1{\cdot}3$ m-mole Na/kg wet wt. during the first 15 min immersion. This rapid loss significantly exceeded the extracellular sodium dissolved in free solution as calculated from the extracellular inulin space (39 ± 7 %, Durbin & Monson, 1961; 333 ± 8 ml./kg wet wt., Goodford & Hermansen, 1961; 0·30 ml./g, Born, 1962; 337 ± 9 ml./kg wet wt. Goodford, unpublished observations) and the concentration of sodium in the bathing solution (137 mM). One may therefore describe the sodium in the taenia coli as distributed in at least three different ways: (1) a very rapidly exchanging component which was presumably extracellular; (2) another rapidly exchanging component difficult to distinguish from the first, but quantitatively in excess of the sodium in the extracellular inulin space; (3) a component which exchanged more slowly. Similar conclusions may be reached from the observations at 4° C, where still more sodium was lost rapidly in the first 15 min (72 ± 4 m-mole Na/kg wet wt. in Fig. 2 or $78 \pm 1{\cdot}3$ in Table 2), although the extracellular space was actually lower (0·22 ml./g at 0° C; Born, 1962).

Some of the present experiments were designed to test Goodford & Hermansen's (1961) suggestion that the rapidly exchanging sodium might be in part bound to the muscle, but no supporting evidence was obtained. The sodium movements in the taenia therefore seem to consist of two components besides extracellular exchange. Like the potassium exchange observed by Goodford & Hermansen (1961) these could correspond to the cytoplasmic and nuclear compartments of the cell.

Under normal conditions the intracellular sodium concentration, calculated on the conventional two-compartment model, was always lower than concentration in the solution. The sodium equilibrium potential was therefore opposite in sign to the resting membrane potential, so that the rising phase of the action potential and the reversal of polarity could be caused by an increase in the permeability of the cell membrane to sodium. When the sodium in the solution was half replaced by lithium the total sodium content of the taenia was halved, which indicated that there was a diminished intracellular concentration. If, as the results suggested, the intracellular concentration was also halved there would be no change in the transmembrane sodium concentration gradient, and no change in the value predicted by the Nernst equation for the sodium equilibrium potential. Bülbring & Kuriyama (personal communication) have observed that during an action potential under these conditions the membrane potential was still reversed, which could be an approach to the unchanged sodium potential.

A similar interpretation is more difficult after prolonged exposure to completely sodium-free conditions at 35° C, for two reasons. First, one would expect at least some of the residual sodium to be within the cells, although if this were bound to intracellular components, as seems probable, the intracellular activity of sodium ions might still be low. Secondly, it would be necessary to postulate that a proportion of the sodium was so distributed that it could carry depolarizing charge across the membrane, but that its loss into the bathing solution was prevented in some way. For example, the large number of vesicles recently found in smooth-muscle cell membranes (Shoenberg, personal communication) might conceivably be concerned.

Alternatively, some quite different process might be responsible for the rising phase of the action potential in sodium-free solution. Such a process could be an increased membrane permeability to another ion besides sodium, so long as the concentration gradient of the relevant ion was of the appropriate sign. An increased permeability to the cation substituting for sodium, or to Ca^{2+} (Schatzmann, 1961) or to any intracellular anion except Cl^- would offer a satisfactory explanation. This effect would not have to operate during the first minutes in sodium-free solution: there was then an

inhibition of electrical activity which coincided with, and might be caused by, the rapid loss of sodium from the muscle. The eventual abolition of electrical activity observed after a prolonged immersion in sodium-free solution (Bülbring and Kuriyama, personal communication) might be due to an accumulation of the sodium-substitute cation inside the cell, to the loss of intracellular anion, or to the loss of intracellular potassium.

SUMMARY

1. The sodium, potassium and lithium contents of the smooth muscle of the guinea-pig taenia coli have been determined after immersion in sodium-free solutions prepared with lithium chloride, choline chloride or *tris*-hydroxymethylaminomethane hydrochloride.

2. More sodium was lost rapidly from the muscle than could be accounted for by the material in the extracellular space. But 1·5 m-moles/kg wet wt. at 35° C and 50 m-moles/kg wet wt. at 4° C remained firmly in the tissue during immersions in sodium-free solution lasting 1 hr.

3. The observations indicated that the muscle sodium was not simply distributed between the intracellular and extracellular spaces.

4. The results are discussed with reference to the known ability of smooth muscle to generate action potentials in sodium-free solutions.

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