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POTASSIUM ACCUMULATION IN SMOOTH MUSCLE AND ASSOCIATED ULTRASTRUCTURAL CHANGES

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SUMMARY

1. The water content, extracellular (^{60}CoEDTA) space, ionic composition and ultrastructure of several mammalian smooth muscles were studied after incubation in solutions of varying ionic compositions and osmolarities.

2. Substitution of KCl for NaCl resulted in an increase in cell water, K and Cl, accompanied by little change in total wet weight. This was due to a reduction in the extracellular space.

3. Changes in extracellular osmolarity produced a wider range of cell volumes in high KCl solutions than in Krebs. The addition of 29–58 mM sucrose to high KCl prevented the swelling.

4. Electron microscopy of smooth muscle swollen in high KCl solution revealed light (less electron opaque than normal) fibres of increased diameter, reduction in extracellular space, and nuclear swelling. The normal thick filament lattice was destroyed in swollen, osmium-fixed smooth muscles.

5. The ultrastructural changes ascribed to swelling were absent in smooth muscles, (*a*) depolarized in high K_2SO_4 solutions, (*b*) in high KCl solutions with 29–58 mM sucrose, and (*c*) returned to normal Krebs solution for recovery from swelling.

6. Smooth muscles incubated in high KCl (swollen) and high K_2SO_4 (unswollen) exhibited similar contractile responses, suggesting the filament lattice was intact until fixation, and that the contractile mechanism can operate over a relatively wide range of actin to myosin separations.

7. Shrinkage of smooth muscles in high KCl solutions made hypertonic

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with the addition of 10% sucrose was accompanied by an aggregation of the thick filaments.

8. The cell water of fixed taenia coli was reduced (*a*) by incubation in hypertonic solution followed by fixation in normal glutaraldehyde, or (*b*) by fixation of normal tissue in hypertonic glutaraldehyde. Osmotic responses during aldehyde fixation may be a source of artifact in the visualization of the normal filament lattice.

INTRODUCTION

Boyle & Conway (1941) established that the replacement of Na by K in the bathing medium results in swelling of the frog sartorius muscle with the inward movement of KCl due to the much greater permeability of the fibre membrane to K than to Na. Substitution of 100 m-mole K/l. for Na resulted in a 102% increase in weight (Boyle & Conway, 1941). In contrast, equivalent substitution of K for Na produced no significant change in the wet weight of the guinea-pig taenia coli (Casteels & Kuriyama, 1966; Brading & Tomita, 1968). This finding has been ascribed to the exclusion of Cl from the fibres by active transport (Casteels & Kuriyama, 1966) or by a relative impermeability to Cl (Grundfest, 1968). Electrophysiological and ion flux observations, however, indicate that the mammalian smooth muscle membrane is more permeable to K than to Na and that it is also permeable to Cl (Brading, 1971; Burnstock & Straub, 1958; Casteels & Kuriyama, 1966; Casteels, 1969*a*, *b*; Bülbring & Tomita, 1969; Ohashi 1970).

The extracellular marker, ^{60}CoEDTA, allows the measurement of total water, ion contents, and extracellular space on the same muscle strip with greater accuracy than earlier methods (Brading & Jones, 1969). The present study was prompted by the availability of this technique and the chance observation during a study of Sr uptake, that the ultrastructure of vascular smooth muscles depolarized with high KCl solutions was distorted (Somlyo & Somlyo, 1971; A. V. Somlyo & A. P. Somlyo, unpublished observations). We studied two types of electrophysiologically (Somlyo & Somlyo, 1968; Somlyo, Vinall & Somlyo, 1969) and ultrastructurally (Devine, Somlyo & Somlyo, 1972) different vascular smooth muscles and the taenia coli.

Our investigations yielded information about the effect of cellular swelling on myofilaments. In view of the recent controversy regarding the organized form of myosin in vertebrate smooth muscle (see Discussion) we also determined the cell volumes of glutaraldehyde-fixed smooth muscle incubated under several osmotic conditions.

METHODS

Animals and tissue preparations

White male rabbits, 2·0–2·5 kg; and male guinea-pigs, 300–350 g were used. The animals were stunned, bled, and the following tissues dissected from rabbits: longitudinal muscle of intestine (taenia coli), portal-anterior mesenteric vein, upper thoracic aorta, and main pulmonary artery. Only the taenia coli was removed from the guinea-pigs. Longitudinal strips of the portal-anterior mesenteric vein and helical strips of the thoracic aorta and main pulmonary artery were cut. Warm Krebs solution was placed on the tissues during dissection and isolated strips were placed into Krebs at 37° C for trimming, and mounted on stainless-steel holders. Vascular strips were stretched to 1·5 times their excised length and the taenai coli to its *in vivo* length.

Solutions

The composition of the solutions used appears in Table 1. Solutions for extracellular space determination contained the Na salt of $CoEDTA^{2-}$, 2 mM, substituted for an equimolar amount of NaCl or the K salt of $CoEDTA^{2-}$ substituted for KCl in high K solutions. For electron microscopy $CoEDTA^{2-}$ was omitted. Hypertonic solutions were prepared by adding sucrose to the experimental solutions. Analytical reagent grade salts and double distilled well water were used.

Ion and water contents

Tissues were cut free from the holders (leaving the impaled ends behind), blotted on Whatman no. 40 filter paper and placed in tared polyethylene vials for weighing. Tissues were oven dried at 98° C for 20 hr to determine water contents and dry weights. Vials were routinely retared to correct for weight changes. Tissues were ashed overnight at 85° C in 1 ml. H_2O_2 (30 % w/v) containing sufficient $AgNO_3$ to over-titrate the Cl (Casteels & Kuriyama, 1965). Electrolytes were dissolved in 5 ml. 0·1 M-HNO_3, 0·01 M-H_3PO_4 and 10 mM-$LiNO_3$, and analysed by atomic absorption spectroscopy (Perkin–Elmer, model 303). Contents were expressed on the basis of wet weight and dry solid (d.s.). Analyses were routinely conducted on the experimental solutions, with chloride being measured on a Cotlove chloride titrator. The measured ion concentrations (usually within 2–3 % of the composition given above) were used in the corrections for the extracellular electrolyte. Osmolarities were determined on a Precision osmometer.

Extracellular space

The chelate of ^{60}Co to EDTA was employed as an extracellular marker (Brading & Jones, 1969; Frazer, Secunda & Mendels, 1972; Jones, 1970*a*; Jones & Swain, 1972). In high K solutions the K salt of EDTA was used. The $^{60}CoEDTA$ was prepared as before with about 5 % excess EDTA to insure almost complete chelation of ^{60}Co to EDTA. Tissues were equilibrated in $^{60}CoEDTA$ for 30 min (Brading & Jones 1969; Jones & Swain, 1972). The weight of water permeable to $^{60}CoEDTA$ was calculated on the basis of the ratio of counts per second in the strip and in a weighed amount of the experimental solution. The space was calculated on the basis of percentage wet weight and kg H_2O/kg d.s. Smooth muscle water was estimated by subtracting the $^{60}CoEDTA$ space from the total H_2O. Cell electrolytes were calculated by subtracting ($^{60}CoEDTA$ Space) × (ion concentrations in the experimental solution, $[\ ]_o$) from the total contents. This value divided by the smooth muscle water yielded the estimate for cell concentrations, $[\ ]_i$.

TABLE 1. Composition of the physiologic solutions

	Concentration mM								Gas (%)	
	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-	$H_2PO_4^-$	SO_4^{2-}	Glucose	O_2	CO_2
Krebs	143·8	5·9	1·2	1·2	128·4	24·9	1·2	—	5·6	95	5
High KCl	—	149·7	1·2	1·2	128·4	24·9	1·2	—	5·6	95	5
K_2SO_4	—	179·2	2·5	1·2	17·4	16·0	1·2	76	5·6	95	5
Locke	155·8	5·6	2·2	—	164·0	1·8	—	—	5·6	100	—
High KCl Locke	—	161·4	2·2	—	164·0	1·8	—	—	5·6	100	—

Tension

Tension was recorded, with a semiconductor strain gauge, Sanborn FTA10 or Grass FTO3 transducer, on a rectilinear recorder (Sanborn).

Electron microscopy

The muscle strips (less than 1 mm diameter) were stretched similarly to those used for the analytical studies, preincubated for 15 min and then incubated in either Krebs (control) or substituted solutions for half an hour prior to fixation. Tissues were fixed for 2 hr with 2% osmium tetroxide in 0·05 M cacodylate buffer (pH 7·4) with 1·2 mM-$CaCl_2$, rinsed in buffer, and block-stained for 1–2 hr with saturated aqueous uranyl acetate, dehydrated in a series of graded alcohols and embedded in Spurr's resin (Spurr, 1969). Thin sections were cut on a Porter-Blum MT2B ultramicrotome, stained with lead citrate and examined in a Hitachi HU 11E electron microscope with a 30 μm objective aperture. In preliminary studies, some preparations were also examined after prefixation with aldehydes followed by post-fixation with osmium. In KCl-swollen fibres this type of fixation resulted in a heterogeneous appearance with both light and dark (more electron opaque) cells in a given section (Somlyo & Somlyo, 1973). The difference in appearance of the cells probably represents the varying degrees of recovery (shrinkage) of swollen fibres during fixation by the aldehyde that does not abolish the osmotic behaviour (Somlyo, Devine & Somlyo, 1971; Results). For this reason, all the observations reported here were made after primary fixation with osmium, following the technique used for electron microscopic studies on osmotic behaviour of striated muscles (e.g. Huxley, Page & Wilkie, 1963).

The experimental protocol of several embeddings was unknown to the microscopist when he evaluated the degree of swelling or shrinkage of the tissue. A second 'blind' evaluation was carried out by another individual examining the representative electron micrographic prints.

Statistics

The results are expressed as mean plus and minus the S.E. of the mean. Student's t and linear regression were used, respectively, to test significance and correlation.

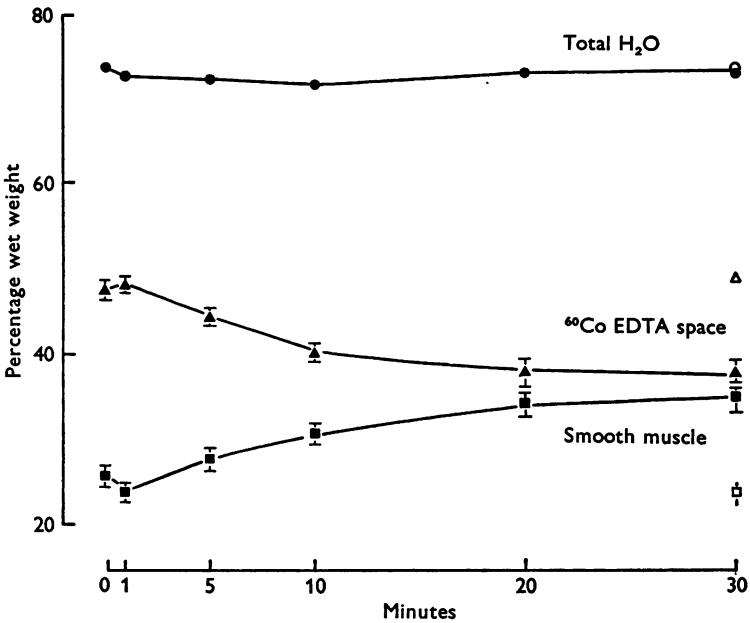
RESULTS

Redistribution of water and ions in high K solution

The time course of water and ionic redistribution after exposure to high KCl was determined on aortic strips equilibrated (90 min) in Krebs solution with ^{60}CoEDTA added for the last half hour. Five strips were transferred to the high KCl solutions, containing the same ^{60}CoEDTA concentration, for varying periods. The other two strips were removed directly from the Krebs solution for analyses initially (time = 0) and after 30 min. Changes in tissue water distribution are shown in Text-fig. 1. In other experiments total water decreased slightly during the first 10 min while peak tension was being developed, but returned to control levels by 20 min. Over this period, however, an important redistribution of water occurred: the extracellular space shrank and simultaneously, the smooth muscle swelled. Since the tissues had been pre-equilibrated in

^{60}CoEDTA before exposure to high KCl, isotope actually was lost from the aorta. This ruled out the possibility that contraction may have reduced the penetration of ^{60}CoEDTA and caused an erroneous underestimate of the extracellular volume. The water distribution was unchanged in the control tissues remaining in the Krebs solution for the additional 30 min, confirming that the 30 min equilibration period was adequate for determining the ^{60}CoEDTA space.

The changes in smooth muscle cell water are replotted in Text-fig. 2 together with the changes in intracellular electrolytes corrected for concentrations in the ^{60}CoEDTA space. K and Cl increased in parallel with

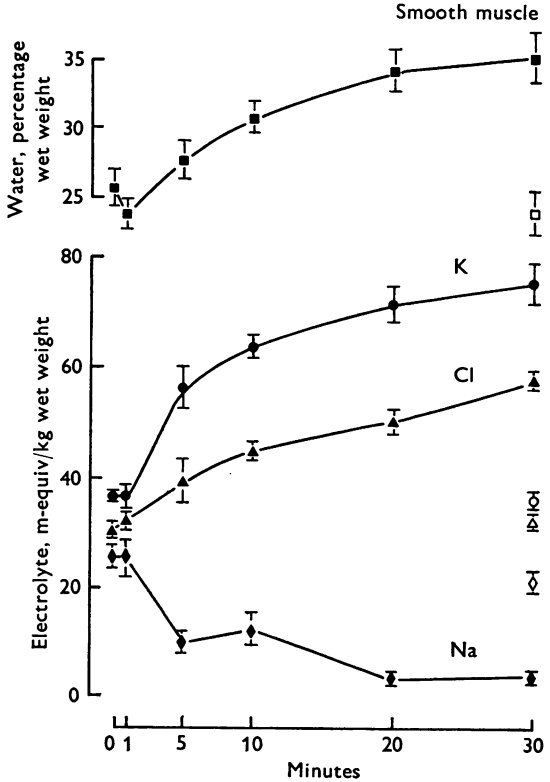


Text-fig. 1. Effects of high KCl solution on the distribution of water in rabbit aorta (eight animals). Abscissa: time in minutes after transfer to the high KCl solution. Ordinate: water components with total water (●), ^{60}CoEDTA space (▲), smooth muscle (■) in percent weight. Measurements of tissues remaining in normal Krebs are denoted by (○), (△) and (□) for the respective components. Standard error of mean represented by vertical bars except when less than the size of the symbol. Points are joined by straight lines.

smooth muscle H_2O, and Na contents decreased. The time course indicated that the redistribution of water and electrolytes in high KCl were essentially complete by 30 min. The electrolyte contents were stable in the control tissues that remained in Krebs solutions.

High KCl (30 min) caused cellular swelling and decreased extracellular

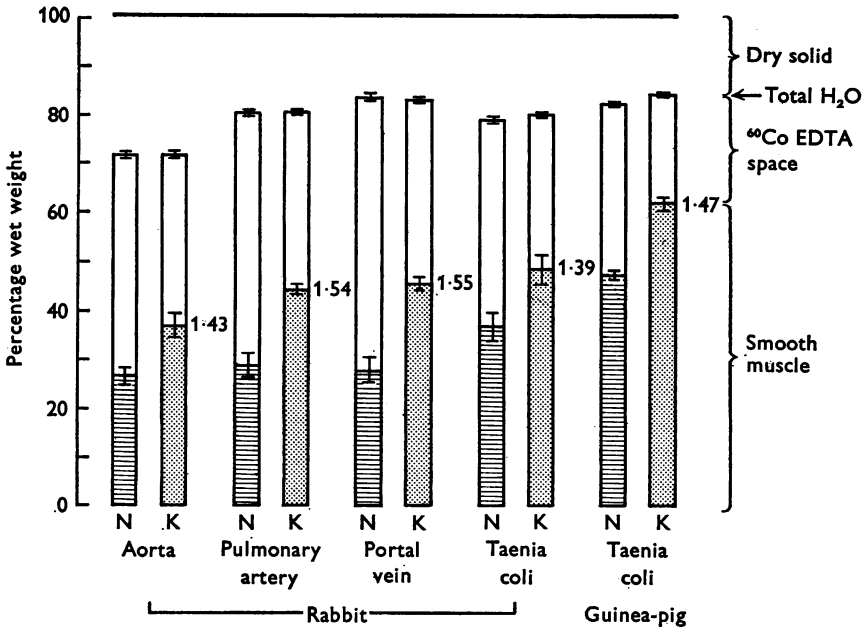
water in a variety of smooth muscles (Text-fig. 3). Total water increased only in the taenia coli of the guinea-pig ($P < 0{\cdot}001$), but to a smaller extent than the cellular swelling. The increases in cell water, 40–50 % of the control values, were highly significant ($P < 0{\cdot}001$ in all, but rabbit taenia coli with $P < 0{\cdot}01$).



Text-fig. 2. Effects of high KCl solution on cell water and electrolyte contents. Abscissa: same as in Text-fig. 1. Lower ordinate: electrolyte corrected for component dissolved in ^{60}CoEDTA space with K (●), Na (◆) and Cl (▲), upper ordinate: cell H_2O (■) as in Text-fig. 1. Tissues remaining in normal Krebs indicated by (○), (◇), (△), (□) for the respective components. Vertical bars represent ± S.E. of mean.

Shifts in electrolytes, on a dry weight basis, are given in Table 2. The dry solid representations can be converted into wet weight by dividing by 1·00 plus the total H_2O/kg d.s. Replacement of Na with K resulted in a net uptake of KCl. This was greater in those preparations having the larger initial cell volumes. A balance between the net changes ($\Delta Na + \Delta K - \Delta Cl$) indicated a slight excess of Cl uptake. Only in the taenia coli of the guinea-pig, however, was this suggestive ($P < 0{\cdot}025$). Estimates of intra-

cellular ions did not take into account ion adsorption to connective tissue elements (Manery, 1954; Keatinge, 1968; Brading & Jones, 1969). This may explain the apparently high $[Na]_i$ and $[Cl]_i$ calculated for rabbit aorta and the somewhat elevated values in main pulmonary artery (collagen plus elastin contents of these preparations were 50 and 30% dry weight respectively; A. W. Jones, unpublished). The increased $[K]_i$ of aorta and main pulmonary artery (but not of the other muscles) in high KCl solution may be due to K-Na exchange on connective tissue sites or to a basic difference between tonic and phasic smooth muscles.



Text-fig. 3. Water distribution in various smooth muscles. One muscle from each pair was equilibrated in normal Krebs solution (N) and one in high KCl solution (K). Cross-hatched portion of the columns represent smooth muscle water; open sections, ^{60}CoEDTA space; top of the column, total water; and the space above the column, dry solid. Vertical bars represent ± S.E. of mean for total water (upper) and smooth muscle water (lower). The ratio of smooth muscle water (normalized per kg dry solid, see text) in high KCl vs. normal Krebs are shown to the right of each group.

Buck & Goodford (1966) suggested that a differential form of the Boyle & Conway (1941) analysis would be more appropriate in the presence of ion adsorption. This was tested by relating changes in cell chloride, ΔCl, to changes in cell water, ΔH_2O, for the various smooth muscles (Table 2). The data fell along the regression: $\Delta Cl = 153\ H_2O + 14$ (S.E.

TABLE 2. Smooth muscle water and electrolyte composition in normal and high K solutions

Rabbit	No.	H_2O (kg/kg d.s.)			Total electrolyte (m-equiv/kg d.s.)			Total − ^{60}CoEDTA space (m-equiv/kg d.s.)				m-equiv/kg H_2O		
		Total	^{60}CoEDTA space	'Cell'	Na	K	Cl	Na	K	Cl	ΔK + ΔNa − ΔCl	$[Na]_i$	$[K]_i$	$[Cl]_i$
Aorta	8													
Krebs		2·50	1·58	0·92	327	134	326	103	124	130		112	135	141
		± 0·07	± 0·05	± 0·04	± 10	± 5	± 8	± 8	± 5	± 7				
High KCl		2·54	1·23	1·31	17	435	345	14	253	193	− 12 (7)	11	193	146
		± 0·08	± 0·04	± 0·08	± 7	± 16	± 12	± 7	± 17	± 11	± 14			
Main pulmonary artery	8													
Krebs		4·06	2·59	1·47	470	242	479	97	227	158		66	154	107
		± 0·11	± 0·04	± 0·09	± 9	± 12	± 14	± 8	± 11	± 15				
High KCl		4·13	1·86	2·27	24	703	527	20	430	296	− 5 (7)	9	189	130
		± 0·10	± 0·10	± 0·12	± 6	± 16	± 15	± 6	± 19	± 16	± 39			
Portal vein	9													
Krebs		5·16	3·41	1·75	536	343	531	41	320	88		23	183	50
		± 0·26	± 0·22	± 0·18	± 29	± 18	± 25	± 17	± 18	± 16				
High KCl		4·97	2·25	2·72	33	822	549	27	481	253	− 14 (9)	10	177	93
		± 0·15	± 0·08	± 0·12	± 5	± 29	± 12	± 5	± 23	± 9	± 30			
Taenia coli	7													
Krebs		3·79	2·01	1·78	338	361	404	49	349	151		28	196	85
		± 0·12	± 0·09	± 0·13	± 17	± 17	± 14	± 12	± 17	± 15				
High KCl		4·07	1·59	2·48	21	722	471	24	475	261	− 8 (7)	10	191	105
		± 0·12	± 0·06	± 0·15	± 6	± 16	± 7	± 8	± 24	± 11	± 37			
Guinea-pig														
Taenia coli	9													
Krebs		4·78	2·04	2·74	364	510	418	71	497	153		26	181	56
		± 0·06	± 0·06	± 0·06	± 8	± 12	± 8	± 5	± 12	± 6				
High KCl		5·49	1·46	4·03	36	929	570	33	714	385	− 51 (9)	8	177	96
		± 0·10	± 0·06	± 0·11	± 6	± 18	± 10	± 6	± 13	± 11	± 18			

$= \pm 10$; $r = 0{\cdot}929$). According to this relation the Cl concentration would be 153 mM or about half of the extracellular concentration (310 mM). This is consistent with an iso-osmotic cellular uptake of ions and water.

Effects of intermediate $[K]_o$

In order to compare experimental changes with the formulation derived by Boyle & Conway (eqn. (14), 1941), cell volumes were determined on guinea-pig taenia coli in intermediate $[K]_o$. The equation relating volume to changes in $[K]_o$ is:

$$V = \frac{\eta c}{c^2 - \Sigma 4[K]_o d} + \frac{\eta c}{c^2 - \Sigma 4[K]_o d}\sqrt{\left[1 - \left(1 - \frac{\epsilon^2}{\eta^2}\right)\left(1 - \frac{\Sigma 4[K]_o d}{c^2}\right)\right]}, \quad (1)$$

where

V = cell water, kg/kg d.s.;

c = total extracellular concentration, mM;

d = concentration of diffusible anion, mM;

η = content of non-diffusible solute, m-mole/kg d.s.;

ϵ = content of charged non-diffusible solute, m-equiv/kg d.s.)

The following estimates for these parameters were employed for normal Krebs solution: $c = 310$ mM; $d = [Cl]_o = 125$ mM; $(\eta/V) = c - [K]_i - [Cl]_i - (d)_i \simeq 60$ m-mole/l.: $(\epsilon/V) = [K]_i - [Cl]_i \simeq 125$ m-equiv/l. For cell water, $V = 2{\cdot}73$ kg/kg d.s. (Krebs Table 3), $\eta = 164$ m-mole/kg d.s. and $\epsilon = 341$ m-equiv/kg d.s. The experimental results and calculated smooth muscle water appear in Text-fig. 4. At $[K]_o = 50$ mM there was a small, 6%, but significant increase in experimental cell H_2O ($P < 0{\cdot}01$). With further increases in $[K]_o$ cell H_2O increased, but to a lesser extent than that predicted by eqn. (1). The experimental plot and the theoretically calculated curve intersected at $[K]_o$ of 120 mM.

There was a progressive increase in cell K and Cl (Table 3) with increasing $[K]_o$. Cell Na contents fell reaching a minimum at $[Na]_o = 27$ mM. Further decrease to 2 mM had little additional effect on cell Na. $[K]_i$ was stable over the range of $[K]_o$ whereas $[Cl]_i$ progressively increased.

Osmotic interactions

The above formulation of Boyle & Conway (1941) also predicts changes in cell volume with altered extracellular concentration, c. The dependence of guinea-pig taenia coli smooth muscle water on extracellular concentration, varied by dilution or addition of sucrose, appears in Text-fig. 5. The

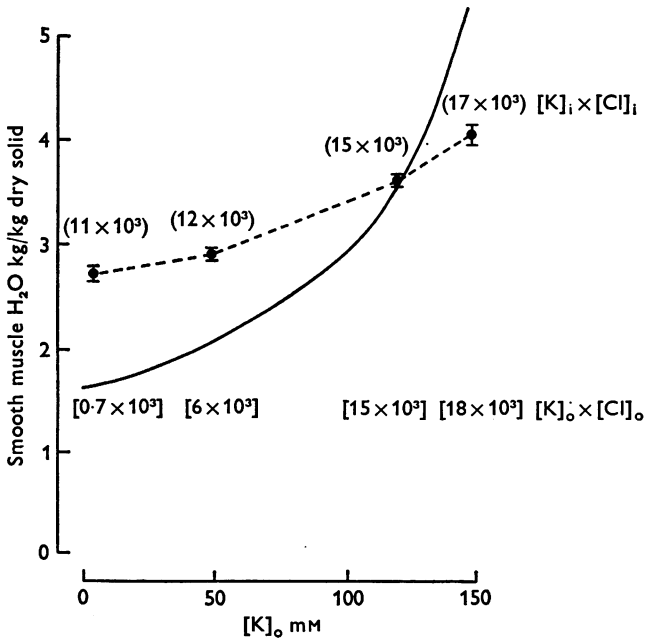
changes in cell water in normal Krebs solution closely followed the relation

$$V_{Os} = V_{288} \frac{288}{Os}. \qquad (2)$$

where:

V_{Os} = cell water, kg/kg d.s. in a solution of osmolarity, Os, m-osmole.

V_{288} = cell water kg/kg d.s. in normal Krebs of osmolarity = 288 m-osmole.



Text-fig. 4. Smooth muscle water at various $[K]_o$ Ordinate: experimental values for cell H_2O (●) in taenia coli (eight to nine guinea-pigs) ± S.E. of mean. Points are connected by straight lines. The curve was calculated from eqn. (1) (see text). The numbers in parentheses are products of $[K]_i \times [Cl]_i$ while those in square brackets are $[K]_o \times [Cl]_o$.

This behaviour was consistent with that of an osmometer with a constant content of solute (confirming, Brading & Setekleiv, 1968; Arvill, Johansson & Jonsson, 1969).

In contrast, high KCl solutions resulted in a much wider range of cell water contents. At low osmolarities smooth muscle water exceeded that in normal Krebs but approached similar values at high osmolarities. The cell water fell to within the normal range by the addition of 1–2 % sucrose (29–58 mM), consistent with the prediction of eqn. (1). Cell solute also

Table 3. Effects of varying $[K]_o$ on water and electrolyte distribution in guinea-pig taenia coli

$[K]_o$ (mM)	No.	H_2O (kg/kg d.s.)			Total electrolyte (m-equiv/kg d.s.)			Total − ^{60}CoEDTA space (m-equiv/kg d.s.)				m-equiv/kg H_2O		
		Total	^{60}CoEDTA space	'Cell'	Na	K	Cl	Na	K	Cl	ΔK + ΔNa − ΔCl	$[Na]_i$	$[K]_i$	$[Cl]_i$
5·9	8	4·78 ± 0·13	2·05 ± 0·12	2·73 ± 0·07	380 ± 26	506 ± 12	439 ± 21	84 ± 13	494 ± 12	169 ± 10	—	31	181	62
50	8	4·47 ± 0·09	1·56 ± 0·05	2·91 ± 0·05	215 ± 8	611 ± 9	401 ± 15	54 ± 4	534 ± 8	193 ± 13	− 13 ± 17	18	184	66
120	8	5·15 ± 0·12	1·55 ± 0·05	3·60 ± 0·08	75 ± 4	837 ± 10	501 ± 19	31 ± 4	650 ± 7	304 ± 15	− 31 ± 23	9	180	84
148	9	5·49 ± 0·10	1·46 ± 0·06	4·02 ± 0·11	36 ± 6	929 ± 18	570 ± 10	33 ± 6	714 ± 13	385 ± 11	− 51 ± 18	8	177	96

TABLE 4. Effects of varying osmolarity on water and electrolyte distribution in guinea-pig taenia coli

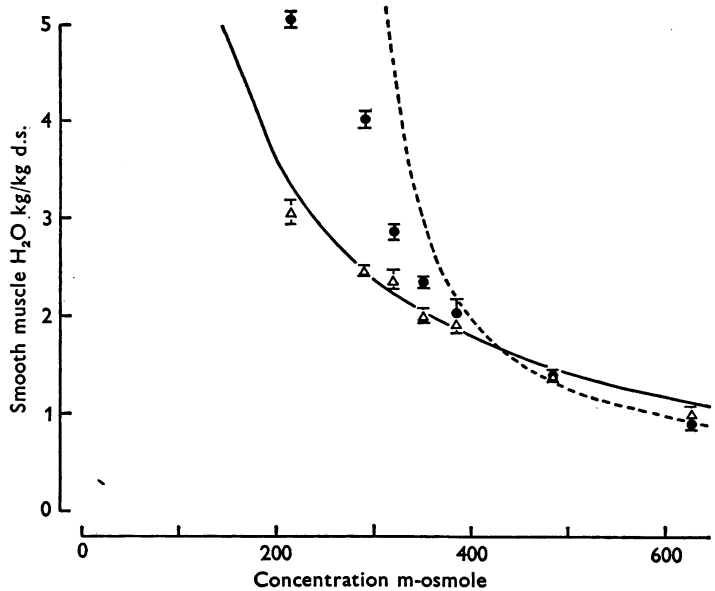
Osmolarity (m-osmole)	H_2O (kg/kg d.s.*) Total	^{60}CoEDTA space	'Cell'	Total electrolyte (m-equiv/kg d.s.)* Na	K	Total $-^{60}$CoEDTA space (m-equiv/kg d.s.)* Na	K	m-equiv/kg H_2O $[Na]_i$	$[K]_i$
Normal Krebs (seven animals)									
218	5·25	2·16	3·09	324	451	90	437	29	141
	± 0·20	± 0·12	± 0·12	± 21	± 15	± 32	± 14		
288	4·49	1·98	2·51	378	467	94	455	37	181
	± 0·12	± 0·09	± 0·04	± 13	± 11	± 18	± 11		
320	4·38	1·98	2·40	362	477	72	464	30	193
	± 0·14	± 0·04	± 0·11	± 14	± 10	± 11	± 10		
352	3·89	1·86	2·03	340	462	70	451	34	222
	± 0·08	± 0·12	± 0·06	± 16	± 4	± 5	± 4		
384	3·79	1·85	1·94	372	463	100	452	52	233
	± 0·10	± 0·07	± 0·07	± 9	± 8	± 10	± 8		
486	3·48	2·08	1·40	413	458	109	445	78	318
	± 0·12	± 0·07	± 0·08	± 13	± 13	± 10	± 12		
629	2·68	1·65	1·03	363	443	122	432	118	420
	± 0·08	± 0·05	± 0·08	± 17	± 9	± 15	± 9		
High KCl (seven animals)									
218	6·33	1·26	5·06	24	782	22	718	4	142
	± 0·10	± 0·02	± 0·08	± 6	± 18	± 6	± 18		
288	± 5·47	1·45	4·02	26	916	23	701	6	174
	± 0·11	± 0·07	± 0·08	± 2	± 16	± 2	± 16		
320	4·36	1·47	2·89	22	823	19	612	7	212
	± 0·04	± 0·07	± 0·08	± 4	± 12	± 4	± 12		
352	3·96	1·57	2·38	24	782	21	573	9	240
	± 0·08	± 0·06	± 0·05	± 6	± 18	± 6	± 17		
384	3·61	1·54	2·07	34	744	21	517	15	250
	± 0·16	± 0·05	± 0·14	± 9	± 12	± 9	± 11		
486	2·96	1·53	1·43	26	708	23	482	16	337
	± 0·06	± 0·08	± 0·05	± 7	± 11	± 7	± 11		
629	2·64	1·67	0·97	50	714	46	469	47	484
	± 0·04	± 0·05	± 0·04	± 4	± 11	± 4	± 11		

* Corrected for the weight of sucrose.

decreased (Table 4) as predicted by this formulation. High KCl solutions with 42 mM sucrose also maintained normal cell volume in rabbit portal vein.

Effects of bicarbonate

Substitution of K for Na in solutions of low HCO_3 (Locke solution) results in a marked increase of wet weight to fresh weight ratio in taenia coli of the guinea-pig (Brading, 1970; Brading & Tomita, 1972). The content and distribution of water and electrolyte in taenia coli equilibrated



Text-fig. 5. Smooth muscle water at various osmolarities in guinea-pig taenia coli. Ordinate: experimental values of cell H_2O in high KCl (●) and normal Krebs solution (△) ± S.E. of mean. Seven guinea-pigs were employed for each type of solution. Dry weights were corrected for the weight of the sucrose in the extracellular space. The solid curve was calculated from eqn. (2) (see text) while the curve represented by dashes was calculated from eqn. (1) (see text).

in Krebs and Locke solution appear in Table 5. Standard Locke solution resulted in decreased total water, cell water, K and Cl contents as compared to Krebs solution($P < 0{\cdot}05$). A significant decrease in the ^{60}CoEDTA space from $1{\cdot}83 \pm 0{\cdot}05$ in Krebs to $1{\cdot}66 \pm 0{\cdot}04$ kg/kg d.s. in Locke solution was also found in an extended series of thirteen animals ($P < 0{\cdot}01$, paired t test). High KCl Locke solution produced a marked increase in cell water with uptake of K and Cl, similar to that noted in high KCl Krebs

TABLE 5. Effects of Locke solution on water and electrolyte distribution in guinea-pig taenia coli (no. = 7)

Solutions	H_2O (kg/kg d.s.)			Total electrolyte (m-equiv/kg d.s.)			Total $-^{60}$CoEDTA space (m-equiv/kg d.s.)			m-equiv/kg H_2O		
	Total	^{60}CoEDTA space	'Cell'	Na	K	Cl	Na	K	Cl	$[Na]_i$	$[K]_i$	$[Cl]_i$
Krebs	4·51 ± 0·14	1·87 ± 0·08	2·64 ± 0·08	368 ± 13	510 ± 15	454 ± 18	94 ± 4	497 ± 15	216 ± 12	36	188	82
Locke	3·96 ± 0·08	1·70 ± 0·05	2·26 ± 0·04	354 ± 6	458 ± 13	462 ± 9	83 ± 6	448 ± 13	174 ± 11	37	198	77
High KCl Locke	4·78 ± 0·09	1·32 ± 0·04	3·46 ± 0·07	29 ± 9	909 ± 19	666 ± 15	27 ± 9	687 ± 16	439 ± 11	8	198	127
High KCl Locke + 2 % sucrose	3·58 ± 0·08	1·42 ± 0·06	2·16 ± 0·03	25 ± 6	765 ± 17	532 ± 16	23 ± 6	528 ± 16	291 ± 11	11	244	135

(Table 2). The decrease in the ^{60}CoEDTA space (Table 5), however, was not so great as that in high KCl Krebs (Table 2). The swelling in high KCl Locke solution could also be prevented by addition of 2% sucrose (58 mM).

Ultrastructure and cell volumes of fixed muscles

The normal ultrastructure of the smooth muscles examined in the present study, as observed after prefixation with an aldehyde, has been previously described (Devine *et al.* 1972; Somlyo, Devine, Somlyo & Rice, 1973; Somlyo & Somlyo, 1973). The present series confirmed the somewhat impaired preservation of the thin filaments and the swelling of the sarcoplasmic reticulum after primary fixation with osmium (without prefixation with an aldehyde; Somlyo, Devine & Somlyo, 1971; Somlyo, Devine, Somlyo & Rice, 1973; Somlyo & Somlyo, 1973). In control tissues, incubated in Krebs solution prior to fixation, the thick filament lattice was preserved, best in the portal anterior mesenteric vein (Pl. 1, fig. 1), adequately in the guinea-pig taenia coli (Pl. 4, fig. 1), and rather poorly in the rabbit pulmonary artery (Pl. 2). The latter finding is presumably the result of inadequate primary fixation of this tissue with osmium, perhaps due to poor diffusion of this fixative through the layers of elastin. In aldehyde prefixed pulmonary arteries, well organized rosettes of a central thick filament with surrounding thin filaments can be observed (Devine *et al.* 1972). The thick filament lattice was preserved after fixation at room temperature, although we agree with the conclusion (Shoenberg, 1973) that adequate fixation of guinea-pig taenia coli requires rather thin strips of tissue.

The ultrastructure of tissues incubated in KCl-substituted solutions showed characteristic changes indicative of swelling that were clearly recognizable by the electron microscopist without knowledge of the experimental protocol. Detectable at low magnification were an increase in cell diameter, a reduction in the cell to cell distances in muscle bundles (taenia coli and portal vein) and in adjacent smooth muscle fibres within a lamellar unit (main pulmonary artery) together with reduced electron opacity of the smooth muscle cytoplasm and the nuclei (Pl. 1, fig. 2; Pl. 3; Pl. 4, fig. 2). The swollen smooth muscle fibres in the main pulmonary artery, in transverse section, appeared cuboidal (Pl. 3) partially due to the 'smoothing out' of the normal cellular processes (Pl. 2). The nuclear profiles were round. Since changes in cell diameter could be due either to swelling or to contraction, micrographs of portal veins exposed to high KCl solution and to K_2SO_4 solution (see below) were compared. The number of complete or partial cell profiles per unit area (twelve micrographs) of K_2SO_4-treated tissue was 1·95 times that of tissues in

KCl (ten micrographs). This is an index of increased volume in KCl, but is not a quantitative measure. The size of the extracellular space, excluding surface vesicles (because of the low magnification), was estimated by integration by weight (Somlyo, Devine, Somlyo & North, 1971). The extracellular space in the K_2SO_4-treated smooth muscle was 19·7 % ± 2·2 S.E. (n = 8) compared with 5·6 % ± 0·7 S.E. (n = 7) in the KCl-swollen tissue (P < 0·001). Chemical analysis of a parallel group (Table 6) indicated that high KCl resulted in larger cell volumes and smaller extracellular spaces than K_2SO_4. The extracellular spaces in the electron micrographs were smaller than the ^{60}CoEDTA spaces, primarily because the micrographs selected showed muscle bundles and excluded the larger areas between fibre bundles and the adventitia.

TABLE 6. Water content and distribution in rabbit blood vessels

Main pulmonary artery	No.	Wet weight (%)		
		Total	^{60}CoEDTA space	'Cell'
High KCl	8	80·6 ± 0·5	36·2 ± 1·7	44·4 ± 1·4
K_2SO_4	6	77·1 ± 0·6	44·3 ± 0·9	33·8 ± 1·4
Portal vein				
High KCl	9	83·2 ± 0·6	37·7 ± 1·2	45·5 ± 1·0
K_2SO_4	6	77·2 ± 1·1	45·0 ± 1·8	32·2 ± 2·2

The light appearance of the smooth muscles swollen in high KCl solutions was due to dispersal of the (thin and thick) myofilaments and, most commonly, a complete disappearance of the thick filament lattice. In only a very few fibres (Pl. 1, fig. 2), which appeared more electron opaque at low magnification, was the thick filament lattice preserved. The intermediate (approximately 100 Å) filaments that are composed of neither actin nor myosin were preserved in KCl-treated and osmium fixed tissues as noted previously (Somlyo *et al.* 1973; Somlyo & Somlyo, 1973).

Mitochondria, unlike nuclei, did not appear swollen in even the most swollen smooth muscle fibres (Pl. 1, fig. 2; Pl. 3; Pl. 4, fig. 2). The sarcoplasmic reticulum was not well preserved after primary fixation with osmium and therefore could not be evaluated.

The above observations were consistent in every block of tissues (Table 7) exposed to high KCl. A guinea-pig taenia coli, examined after incubation in a solution in which 50 mM-KCl were substituted for NaCl,

was judged slightly (borderline) swollen by both the microscopist and the evaluator of the electron microscopic prints. The filament lattice of a portal vein incubated for 30 min in a normal Krebs solution, after the initial incubation in the high KCl solution, was normal.

If the destruction of the thick filament lattice was due to the swelling in high KCl, then the lattice should be preserved by preventing the swelling by substitution of an impermeant anion, sulphate (Table 6;

TABLE 7. Number of embeddings for electron microscopic examination after exposure to isotonic and anisotonic solutions

Incubation solution	Rabbit portal anterior mesenteric vein	Rabbit main pulmonary artery	Guinea-pig taenia coli
Krebs	5	3	2
150 mM-K (128 mM-Cl)	5	3	3
150 mM-K (128 mM-Cl) 1·43 % (42 mM) sucrose	3	2	—
150 mM-K (128 mM-Cl) 1·7 % (50 mM) sucrose	4	2	—
150 mM-K (128 mM-Cl) 10 % (292 mM) sucrose	3	1	—
179 mM-K (76 mM-SO_4)	2	1	—
50 mM-K (128 mM-Cl)	—	—	1
150 mM-K (128 mM-Cl) followed by Krebs	1	—	—

Adrian, 1956) or by the addition of sucrose (Fig. 5). Both of these pre-fixation schedules prevented the ultrastructural changes found in the high KCl solutions: the thick filament lattice was preserved in the K_2SO_4 (Pl. 5, fig. 1) and in the high KCl-1·43 % sucrose (Pl. 6) solution.

The hypertonic (with 10 % sucrose) high KCl solution produced changes in the thick filament lattice comparable to the effects of hypertonic (with sucrose) Krebs solution (Somlyo, Somlyo, Devine & Rice, 1971; Somlyo, Devine, Somlyo & Rice, 1973; Somlyo, Devine, Somlyo & North, 1971; Somlyo & Somlyo, 1973). The thick filament lattice was aggregated into ribbon-like structures in transverse section (Pl. 5, fig. 2) and the nuclear and cytoplasmic electron opacity were increased.

To determine whether the shrinkage induced by hypertonic solutions is detectable in tissues subsequently fixed with aldehydes, taenia coli from guinea-pig were incubated either in Krebs solution with 10 % sucrose or in normal Krebs solution before fixation for 2 hr in 2 % glutaraldehyde with

0·075 M cacodylate. Both groups lost water during fixation in comparison to unfixed tissues (Table 8). The extracellular space of control fixed tissues remained remarkably close to the normal (unfixed) values, indicating that the semipermeable properties of the membrane were not completely destroyed by glutaraldehyde. The cell volume was reduced to 53 % of control by the hypertonic incubation prior to fixation ($P < 0{\cdot}001$).

TABLE 8. Water content and distribution in taenia coli of the guinea-pig (no. = 6)

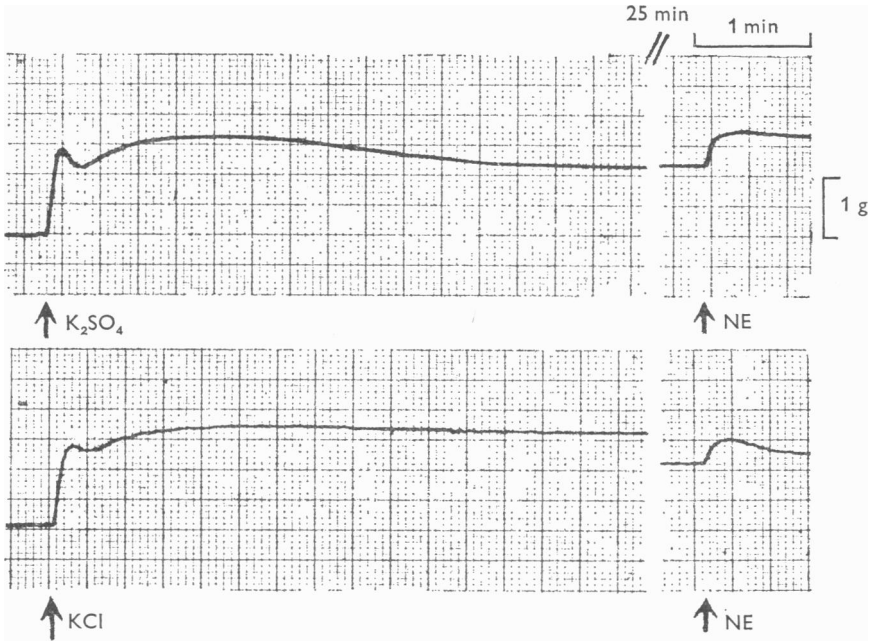
	Wet weight (%)			kg/kg d.s.		
	Total	^{60}CoEDTA space	'Cell'	Total	^{60}CoEDTA space	'Cell'
Krebs, fresh	80·4 ± 0·1	38·8 ± 2·0	41·6 ± 2·1	4·11 ± 0·04	1·98 ± 0·09	2·13 ± 0·12
Krebs, fixed	79·5 ± 0·5	44·1 ± 2·3	35·4 ± 1·9	3·90 ± 0·11	2·17 ± 0·16	1·73 ± 0·06
Krebs + 10 % sucrose, fixed	74·3 ± 0·5	50·7 ± 1·0	23·6 ± 1·0	2·90 ± 0·08	1·98 ± 0·07	0·92 ± 0·04

Fixation with glutaraldehyde made hypertonic with sucrose has been shown to reduce the wet weight (Somlyo, Devine & Somlyo, 1971). We found in the present study that the addition of 10 % sucrose to the glutaraldehyde fixative was sufficient to produce a reduction in cell water at the end of fixation from $1{\cdot}61 \pm 0{\cdot}04$ in the control to $0{\cdot}55 \pm 0{\cdot}04$ kg/kg d.s. (no. = 3) under hypertonic conditions. It should be emphasized that the tissues used for these experiments were in isotonic solutions up to the moment of fixation.

Contractile properties in high K solutions

The apparent destruction of the myofilament lattice in high KCl solution may have occurred either during incubation or as a result of increased susceptibility of swollen tissues to the extractive effects of osmium. The normalization of the ultrastructure by prevention of the swelling in high K solutions prior to fixation (see preceding section) suggested the latter to be the case. Comparison of the contractile properties under these conditions would also indicate to what extent high KCl damaged the myofilament lattice before fixation. We took advantage of the original observation of Evans, Schild & Thesleff (1958) that smooth muscles can be contracted by drugs even after depolarization. The contractile response to high KCl was compared to high K_2SO_4 as the latter did not produce swelling (Table 6). The responses to norepinephrine were tested after 30 min

depolarization, the time at which the analytical and electron microscopic observations were made. There was no significant difference in either the magnitude or the relative decline of the contractions produced by either solution in rabbit main pulmonary artery and rabbit portal vein. The response to norepinephrine was within the limits of variability for the two vascular strips (Text-fig. 6). In nineteen additional experiments (ten portal veins, nine main pulmonary artery strips) no significant differences were found.



Text-fig. 6. The effect of norepinephrine on the tension development of rabbit mesenteric vein after exposure to K_2SO_4 solution or high KCl solution. Addition of norepinephrine 10·0 μg/ml. (NE) caused comparable contractions in both muscle strips.

DISCUSSION

We found that iso-osmotic substitution of KCl for NaCl in the bathing medium increases the cell volume of smooth muscles. This swelling is associated with either no change in total tissue wet weight, as has been reported by previous workers (Casteels & Kuriyama, 1966; Brading & Tomita, 1968) or, when there is a statistically significant increase, it is relatively small. Our findings are consistent with the electrophysiological observations indicating significant permeability of the smooth muscle membrane to both K and Cl (Burnstock & Straub, 1958; Casteels & Kuriyama, 1966; Ohashi, 1970).

The absence of a major weight gain by tissues swollen in high KCl solutions can be explained by a redistribution of water from the extracellular into the intracellular space. It is interesting to note that Boyle & Conway had considered the possibility that intraspace water is progressively squeezed out as the muscle swells (page 18, Boyle & Conway, 1941). This effect, while of no major import in the closely packed frog sartorius muscle, can mask major degrees of swelling in smooth muscle cells embedded in a large extracellular matrix. Thus, limited expansion of smooth muscle fibres is initially compensated by the reduction of the extracellular space but, since this space is limited, a large cellular expansion must be associated with increases in tissue wet weight.

KCl was accumulated, during swelling, as an iso-osmotic solution compatible with the considerations of Donnan forces described by Boyle & Conway (1941) and showed no saturation behaviour, unlike the earlier observations of one of the authors based on experiments over a limited range (0–20 mM) of $[K]_o$ (Jones, 1970*b*; Jones & Karreman, 1969). Nor does it require for its explanation macromolecular interactions in the cytoplasm. The effect of small concentrations of an impermeant solute (sucrose) in preventing the swelling in high KCl cannot be readily explained by an effect on intracellular salt linkages as the primary mode of volume regulation. Instead, the behaviour of smooth muscles in high KCl resembles osmotic regulation across the capillary wall in the vascular system where, in the presence of large concentrations of permeant solutes on both sides of the separating membrane, osmotic control is due to relatively small amounts of impermeant solutes (Landis & Pappenheimer, 1963). The experimental value (between 29 and 58 mM) of sucrose required to maintain normal volumes in the high KCl solution is in good agreement with that calculated (50 mM) from the Boyle & Conway analysis (1941).

The calculated values of $[Cl]_i$ for smooth muscles bathed in solutions of up to a $[K]_o$ of 120 mM exceeded the values predicted by the Gibbs–Donnan equilibrium. This excess of internal chloride in smooth muscle has been noted by others (Buck & Goodford, 1966; Casteels, 1971; Daniel & Robinson, 1960) and ascribed to compartmentalization or to active transport.

The electron microscopic observations also give unequivocal evidence for swelling of smooth muscles incubated in high KCl solutions. The comparison of high KCl (swollen) and high K_2SO_4 (unswollen) contracted smooth muscles indicated that the swelling of the fibres is associated with the marked reduction in the extracellular space immediately surrounding the cells. The differences in the extracellular space as measured by ^{60}CoEDTA, while still highly significant, were less, presumably indicating

that the swollen smooth muscle fibres do not markedly encroach on the large islands of extracellular space between adjacent fibre bundles or on the vascular adventitia. Both of these regions would be included in the analytical, but not in the electron microscopic, estimates of the extracellular space.

The light (less electron opaque) appearance of KCl-swollen smooth muscles was associated with an absence of the thick filament lattice and considerable destruction of the thin filaments. This finding is consistent with previous suggestions (Somlyo, Devine & Somlyo, 1971) that swelling interferes with the preservation of the thick filaments in vertebrate smooth muscle. This probably represents an extraction artifact of fixation, since the contractility of swollen muscles was unimpaired and no ultrastructural damage was evident in the preparation returned to Krebs solution prior to fixation. The preservation of the thick filament lattice through the prevention of swelling (by sucrose or an impermeant anion) indicates that the removal of thick filaments in fixed KCl-swollen muscles is a consequence of the state of intracellular hydration, rather than of the K concentration. The ultrastructure of smooth muscle fibres incubated in 5 mM-EDTA-Krebs solution prior to fixation (Figs. 8–10 in Cooke & Faye, 1972) is indistinguishable from that of KCl-swollen fibres extracted during fixation (present study). The effects of EDTA (Cooke & Faye, 1972) probably also reflect the cellular swelling (gain in NaCl and H_2O) due to the chelation of extracellular divalent cations (Jones, 1970*b*; Jones, 1973; Gulati, 1973), followed by extraction of myofilaments during fixation (present study).

The similarity of the tensions maintained by the high KCl (swollen) and high K_2SO_4 (unswollen) depolarized muscles and their comparable contractile responses to norepinephrine suggest that the contractile mechanism of vertebrate smooth muscle can operate over a considerable range of separation of thin and thick filaments. The contractility of striated muscles over a variable range of actin to myosin separations (Huxley, 1953; Elliott, Lowy & Worthington, 1963; Rome, 1968) has been interpreted (Pepe, 1966; Huxley & Brown, 1967) as being compatible with a swinging cross-bridge mechanism of contraction in which the cross bridges can move laterally and exert force over a considerable range of thin-to-thick filament separation. A sliding filament mechanism of contraction also operates in mammalian smooth muscle (Somlyo *et al.* 1973), and a swinging cross-bridge mechanism probably also explains the normal contractile behaviour of vertebrate smooth muscles in various states of swelling.

The present discussion assumes that the thick (155 Å) filaments (Somlyo *et al.* 1973), represent the organized form of myosin in vertebrate smooth muscle, and that ribbons are thick filaments aggregated by osmotic

shrinkage. According to another view (Lowy & Small, 1970; Small & Squire, 1972), ribbons are the normal form of myosin in vertebrate smooth muscles, and their disaggregation gives rise to the thick filaments. Two of us have presented evidence elsewhere (Rice, McManus, Devine & Somlyo, 1971; Somlyo, Somlyo, Devine & Rice, 1971; Somlyo, Devine, Somlyo & Rice, 1973) suggesting that the ribbons are aggregates of the (normal) thick filaments produced by the preparatory techniques and that the regularity of the normal filament lattice gives rise to the narrowness of the 144 Å meridional X-ray reflexion (Lowy, Poulsen & Vibert, 1970) ascribed to the cross-bridges. A detailed discussion of these arguments is outside the scope of this presentation, but we note that the claim that our interpretation of the narrowing of the meridional reflexion is 'erroneous' (Small & Squire, 1972) has not been substantiated. In view of the regular distribution of thick filaments (Rice *et al.* 1971; Somlyo, Somlyo, Devine & Rice, 1971; present study), the narrowing of the meridional myosin reflexion in the equatorial direction can be due to an interference function generated by the not strictly random arrangement of the diffracting units (thick filaments) (Hosemann & Bagchi, 1962).

The preservation of the thick filament lattice requires isotonic conditions prior to and during fixation with aldehydes. The shrinkage of normal smooth muscle during fixation with hypertonic fixative, indicates that osmotic responses of smooth muscle can occur during glutaraldehyde fixation and are a potential source of electron microscopic artifact.

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EXPLANATION OF PLATES

All tissues were fixed in 2 % osmium tetroxide dissolved in 0·05 M cacodylate buffer (pH 7·4) with 1·2 mM-$CaCl_2$ added, and block stained with uranyl acetate. Sections were stained with alkaline lead citrate. Scale bar represents 0·5 μm.

COL	collagen	PAMV	portal-anterior mesenteric vein
EL	elastin		
MPA	main pulmonary artery		

PLATE 1

Fig. 1. Transverse section of smooth muscle cells from rabbit PAMV which was incubated in Krebs solution before fixation. Note the normal cell volume and extracellular space. Thick filaments (arrows) are in relatively regular arrays. × 13,500.

Fig. 2. Transverse sections of smooth muscle cells from rabbit PAMV fixed after incubation in high KCl solution. The cells are swollen, the extracellular space is reduced and the myofilaments are not well preserved. Only rarely were more electron opaque fibres (X) containing thick filaments observed in such blocks. × 13,500.

PLATE 2

Transverse section through rabbit MPA. The tissue was incubated in Krebs solution before fixation. × 13,500.

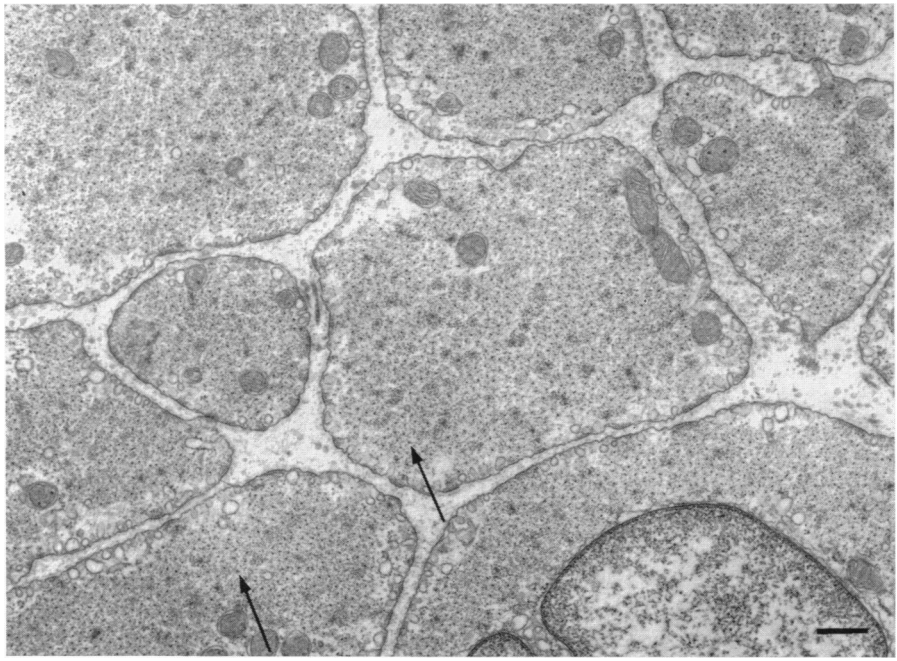


Fig. 1

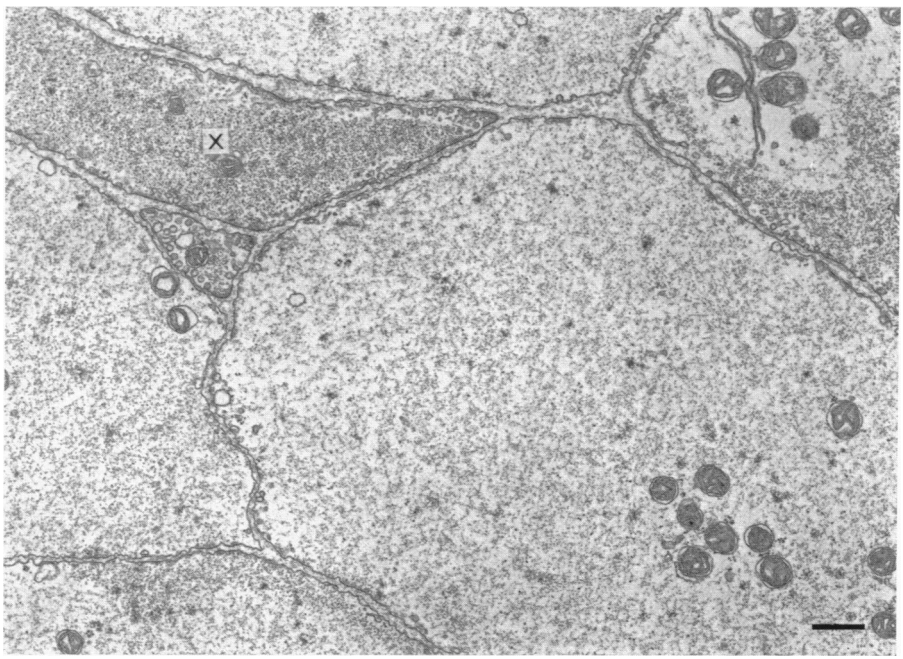
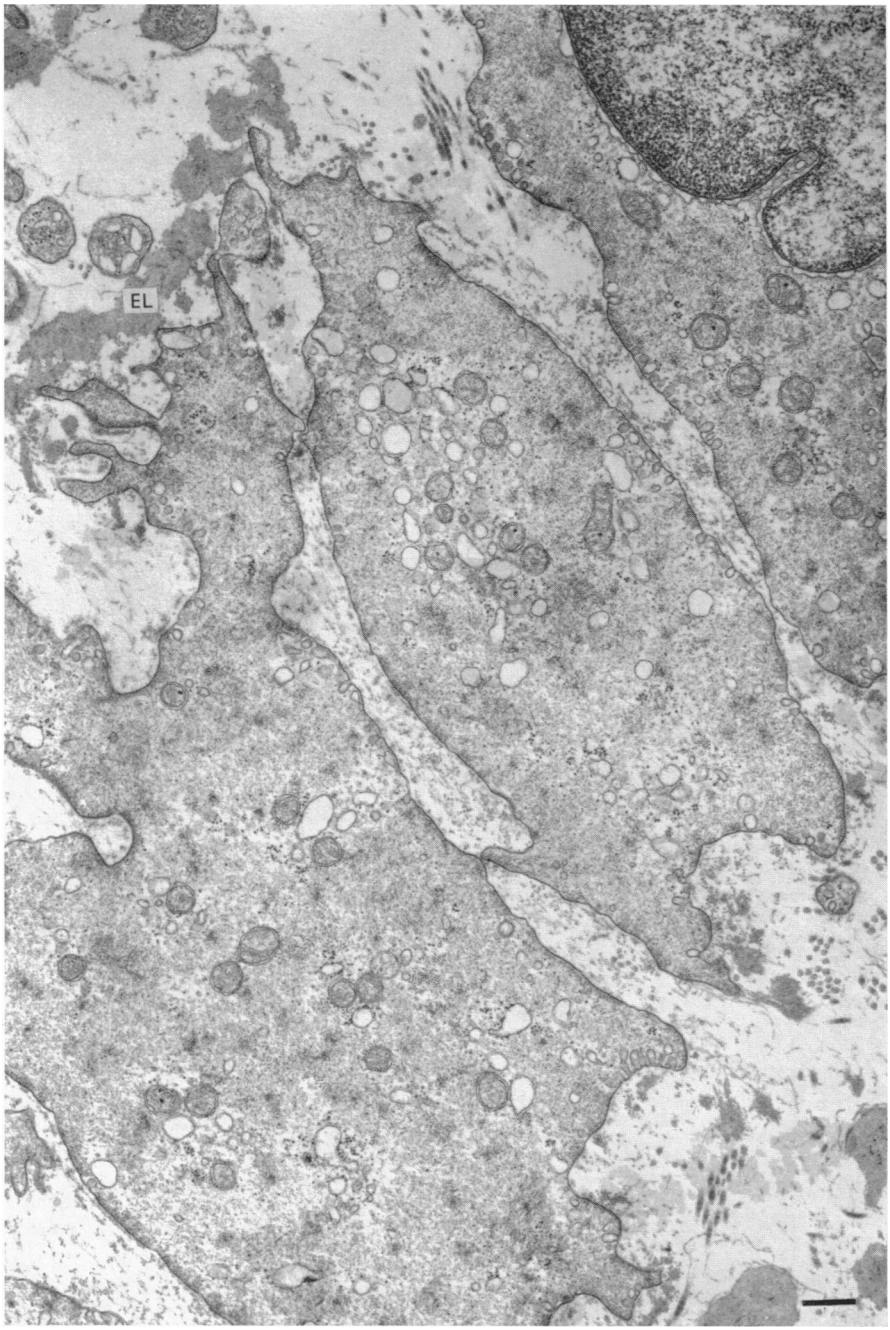
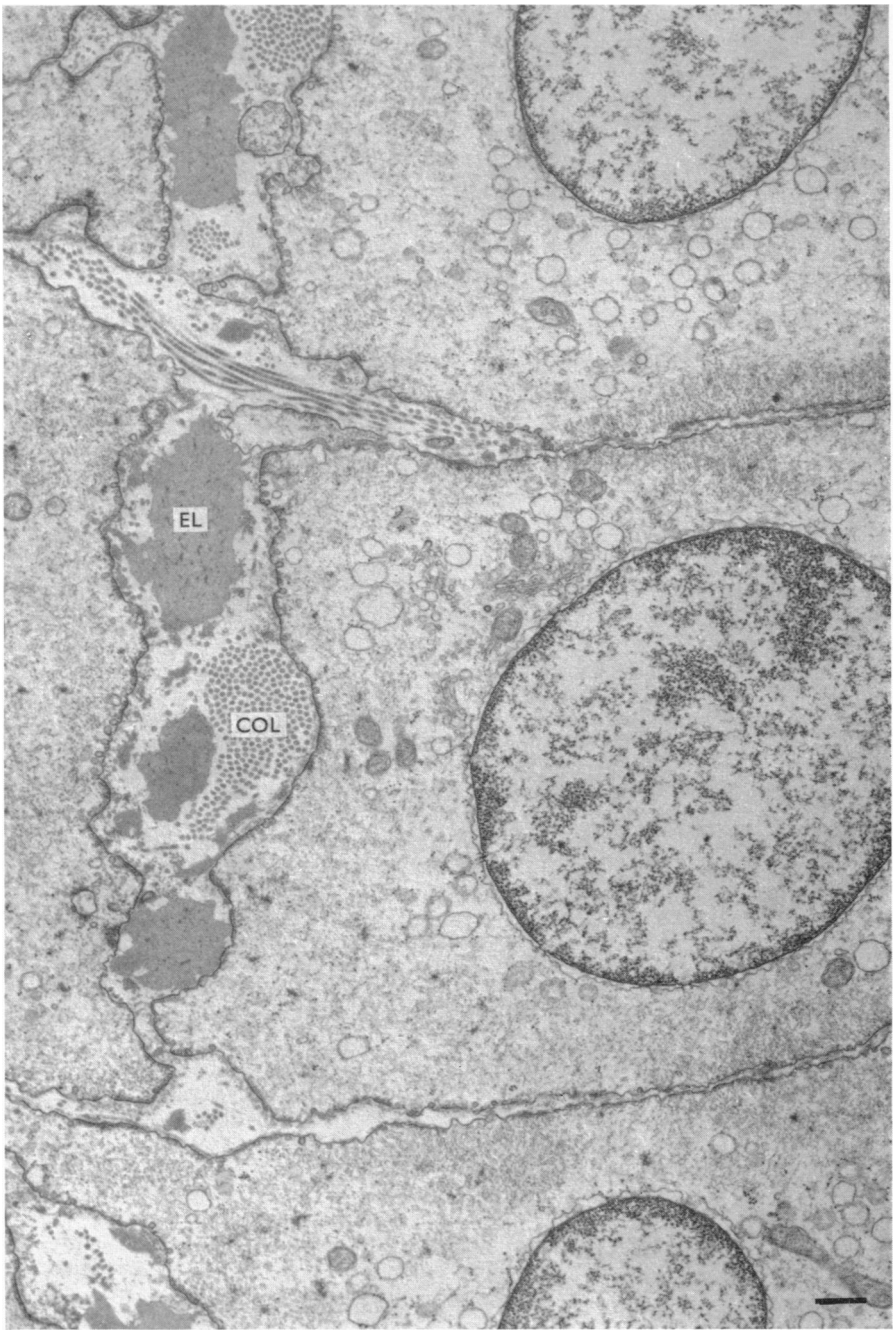


Fig. 2


EL



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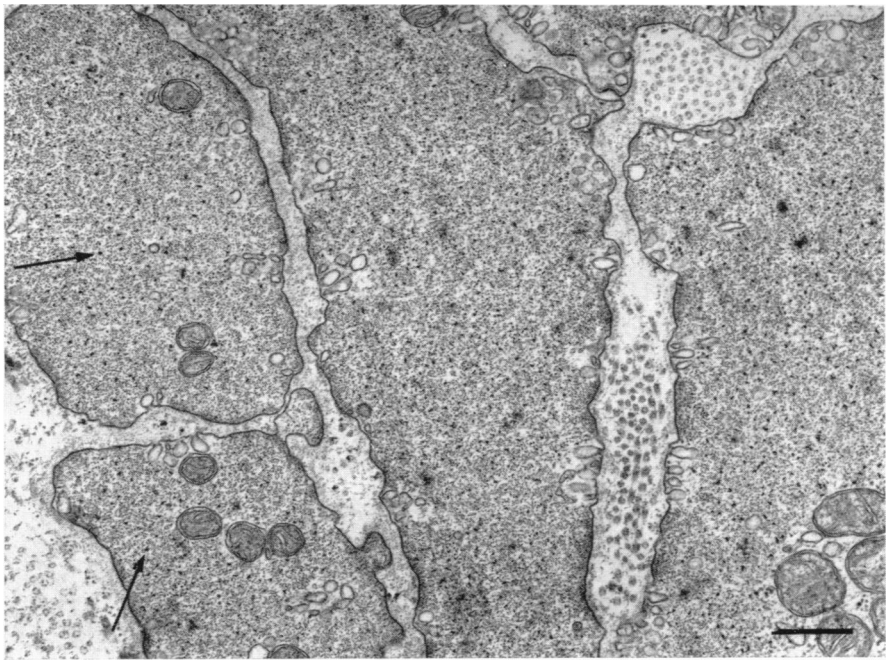

Fig. 1

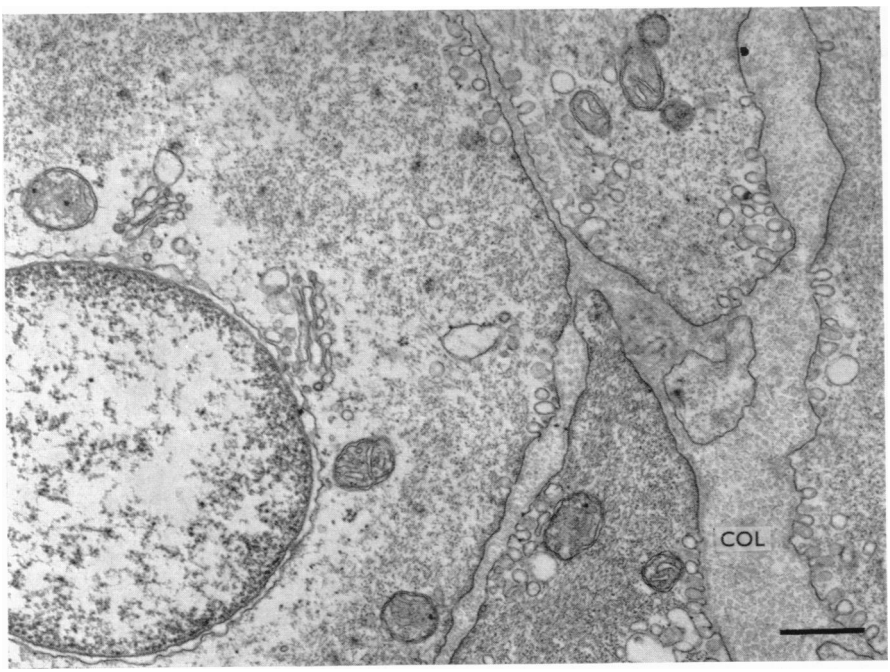

Fig. 2

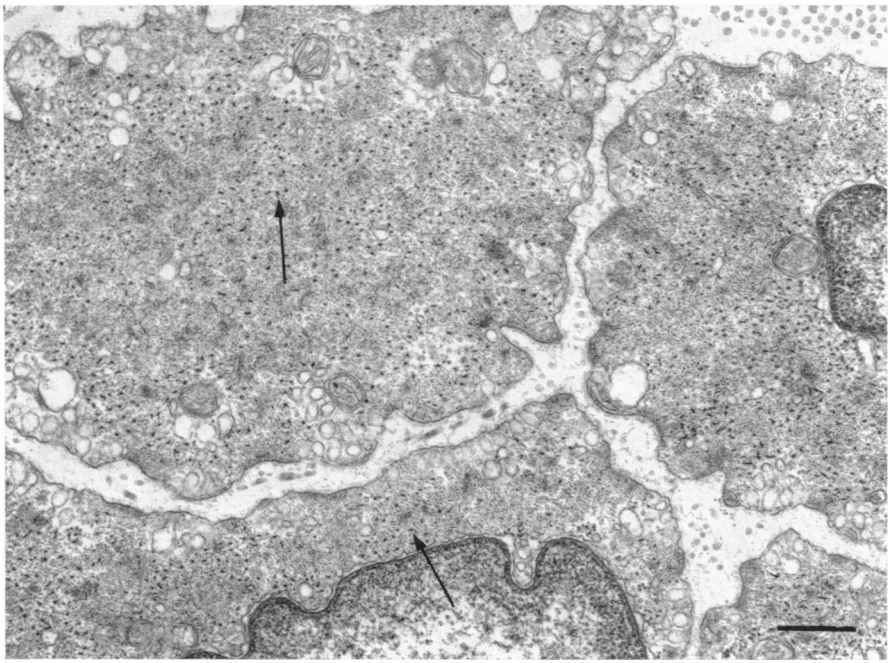

Fig. 1

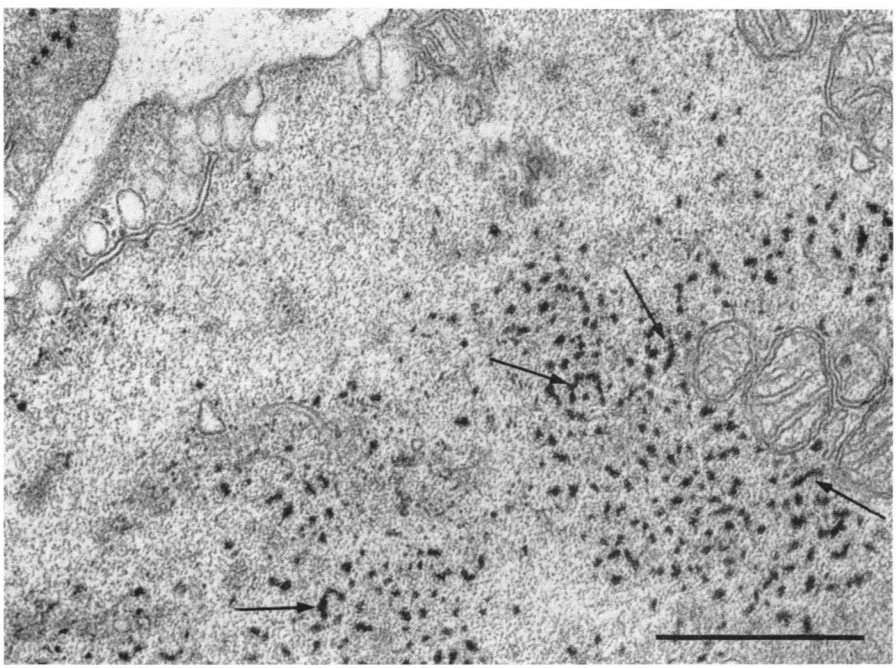

Fig. 2

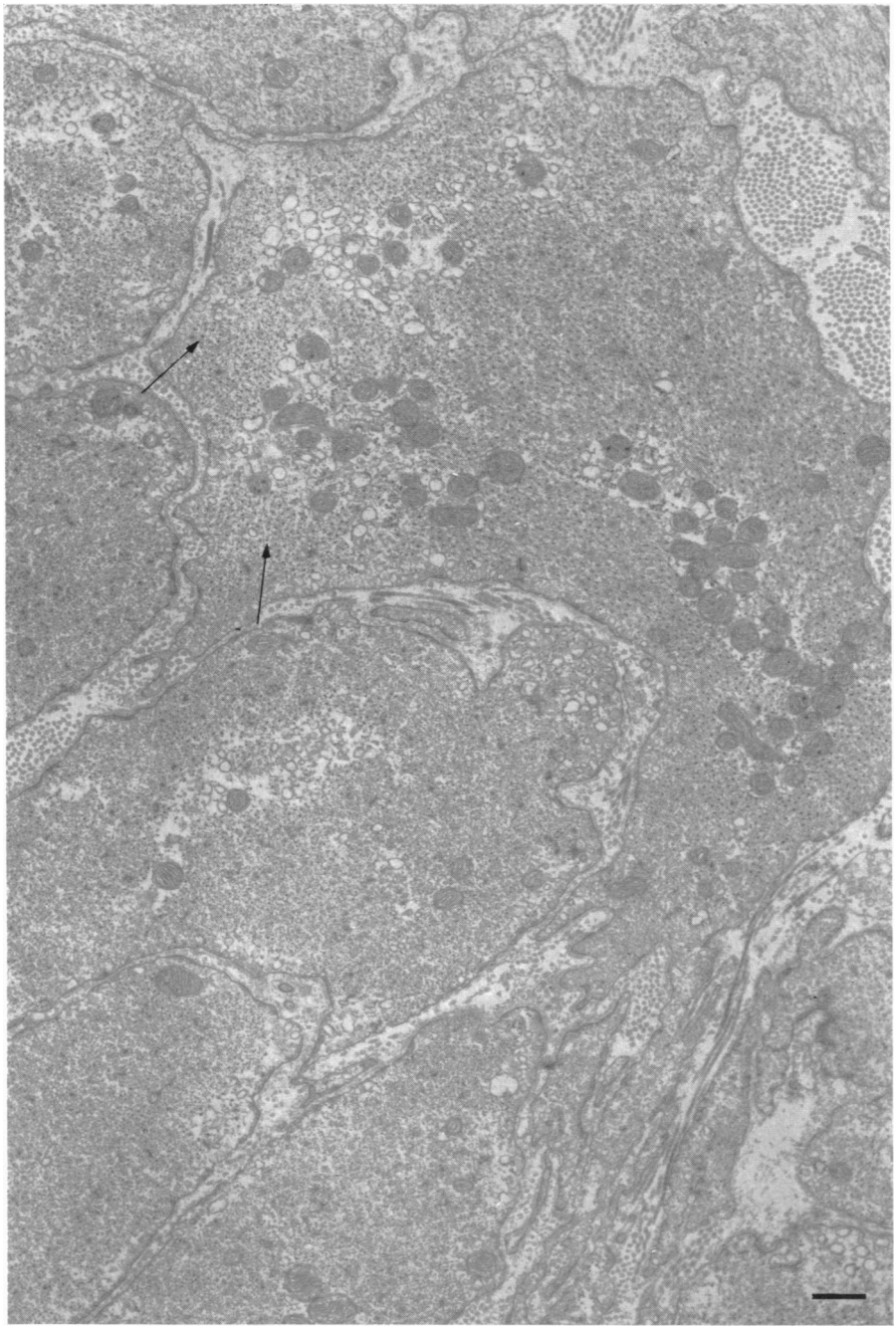

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PLATE 3

Transverse section through the rabbit MPA. The tissue was incubated in high KCl solution before fixation. Note the swollen cells and decreased extracellular space between adjacent fibres of the lamellar unit. Compare with the normal control tissue shown in Pl. 2. × 13,500.

PLATE 4

Fig. 1. Transverse sections of smooth muscle cells from the guinea-pig taenia coli. Thick filaments (arrows) are present. × 21,600.

Fig. 2. Swollen smooth muscle cells from the guinea-pig taenia coli. The tissue was incubated in high KCl solution before fixation. Myofilaments are poorly preserved. Compare the cytoplasmic density with Pl. 4, fig. 1. × 21,600.

PLATE 5

Fig. 1. Transverse section of smooth muscle cells from rabbit PAMV. The tissue was incubated in K_2SO_4 solution before fixation. Thick filaments (arrows) are present. × 21,600.

Fig. 2. Transverse section of parts of two shrunken smooth muscle cells from rabbit PAMV. The blood vessel was incubated in high KCl solution with 10% sucrose added. Thick filaments are aggregated into ribbon-like structures (arrows). × 54,800.

PLATE 6

Transverse section through several smooth muscle cells of the rabbit PAMV. The tissue was incubated in high KCl solution with 1·43% sucrose added. Cell diameter, extracellular space and myofilaments (arrows) appear normal. Compare with Pl. 1, fig. 1. × 13 500.