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REPLACEMENT OF THE AXOPLASM OF GIANT NERVE FIBRES WITH ARTIFICIAL SOLUTIONS

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In 1937 Bear, Schmitt & Young showed that substantial quantities of axoplasm could be squeezed out of the cut end of a giant nerve fibre of *Loligo*. This technique has been widely used for obtaining samples of axoplasm, but little attention has been paid to the electrical properties of the thin sheath which remains after the contents of the nerve fibre have been extruded. Since extrusion involves flattening the axon with a glass rod or roller it is natural to suppose that the membrane would be badly damaged by such a drastic method of removing axoplasm. However, in the autumn of 1960 impulses were recorded from extruded sheaths which had been refilled with isotonic solutions of potassium salts (Baker & Shaw, 1961) and on further investigation it turned out that such preparations gave action potentials of the usual magnitude for several hours (Baker, Hodgkin & Shaw, 1961). Tasaki and his colleagues at Woods Hole have also been successful in perfusing the giant axons of *Loligo pealii* and several methods, some evidently developed before ours, were described by Oikawa, Spyropoulos, Tasaki & Teorell (1961). We have made no serious attempt to compare different methods and all the experiments described here were carried out by perfusing sheaths from which the bulk of the axoplasm had been removed by extrusion. The first paper deals with technique and with some general properties of perfused fibres, including histology and electron microscopy. The second is concerned with the electrical effects of changing the internal solution and ends with a discussion of both sets of results.

METHODS

Material

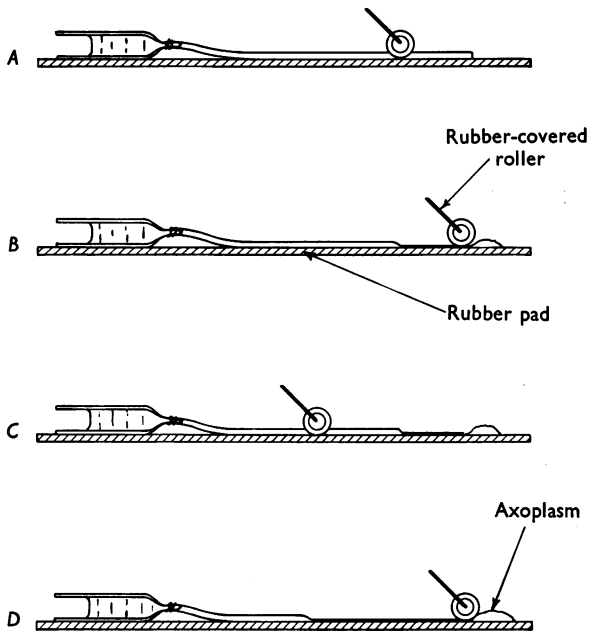
Giant axons from large specimens of *Loligo forbesi* were used throughout the investigation. The mantle lengths of the squid were usually 30–50 cm, and the diameters of the axon 700–900 μ. Living squid were used occasionally but as a rule we employed refrigerated mantles. In this technique live squid are decapitated as soon as the trawl is brought up and

the central 30 cm of mantle is dissected and placed in a large Thermos flask filled with ice-cold sea water. The dissection of the nerves in the laboratory is started about 2 hr later.

Giant axons were left in the nerve trunk and were not cleaned.

Experimental procedures

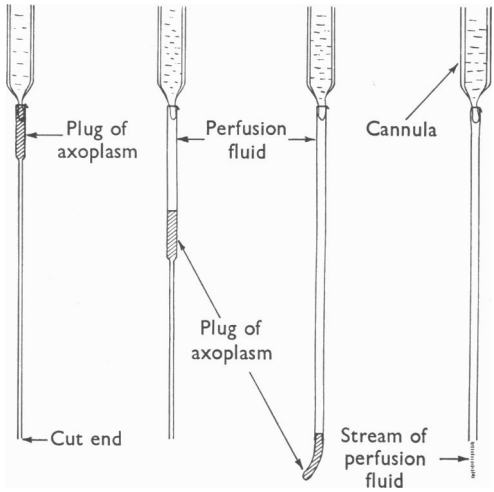
Extrusion and perfusion. A cannula filled with perfusion fluid was tied into the distal end of a giant axon of length 6–8 cm. The axon was placed on a rubber pad and axoplasm was extruded by passing a rubber-covered roller over it in a series of sweeps. The first sweep started at about 1·5 cm from the cut end, the second at 3 cm and so on (Text-fig. 1). At the end of this operation all the axoplasm had been removed except for a column 5–10 mm in length near the cannula. The axon was then suspended vertically in a large beaker of sea water. The cannula was connected to a mechanically driven 'Agla' syringe and perfusion fluid was forced into the axon at about 6 μl./min. This moved the plug of axoplasm along the axon as shown in Text-fig. 2 and after about 4 min a stream of perfusion fluid (which was denser than sea water) could be seen flowing out of the cut end of the axon. Occasionally, when the axon narrowed in the middle, the plug of axoplasm stuck and in these instances the experiment usually had to be abandoned.



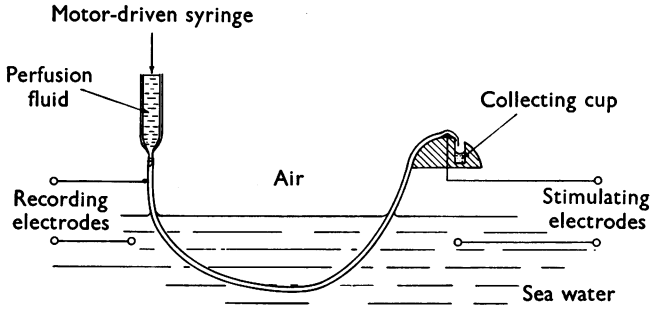
Text-fig. 1. Extrusion of axoplasm.

After establishing a flow through the axon, its excitability was tested by using the apparatus shown in Text-fig. 3. Out of a total of 207 attempts 147 axons were excitable after extrusion and inflation. By excitable we mean that there was an all-or-nothing action potential over at least 2 cm of axon. This is not a critical test since it is well known that the action potential can propagate through damaged regions. However, about 60 % of the excitable axons continued to give action potentials for 1–5 hr and in a number of instances

the failure of the axon could reasonably be attributed to use of a faulty solution or excessive perfusion pressure. Our general impression is that with squid of mantle length greater than 30 cm the method gave a reliable preparation in about 50 % of trials.



Text-fig. 2. Re-inflation of extruded axon with perfusion fluid. The fluid was driven into the cannula by a motor driven 'Agla' syringe at a rate of about 6 μl./min.



Text-fig. 3. Standard arrangement for recording external action potentials from perfused axons.

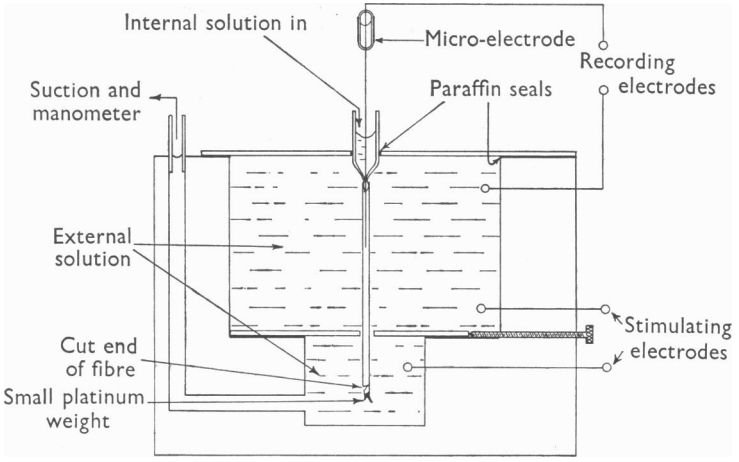
Measurement of membrane potentials in perfused fibres without change of solution

After an axon had been extruded and perfused it was tied at the lower end, filled with perfusion fluid and impaled with an internal electrode by almost exactly the same method as that used with an intact axon. The apparatus was similar to that described by Hodgkin & Katz (1949*a*) and the only variation in technique was in the method of lowering the microelectrode. The axoplasm of an intact axon is solid and a sideways movement of the bottom of the fibre bends both internal electrode and axon but does not move one relative to the other. An electrode is steered down an intact axon by bending the fibre below the electrode in such a way that, when moved down, the electrode remains near the centre of the fibre. As would be expected, perfused fibres behaved like sheaths filled with fluid and there was no evidence of any internal structure. Steering, therefore, involved relatively small movements of the

lower part of the axon and unless the fibre was both straight and fully inflated the electrode often touched the surface. One might suppose that this would be disastrous and axons were occasionally damaged by the internal electrode. However, in many instances we recorded action potentials of 100–110 mV for several hours after lowering an electrode into a shrunk and slightly curved axon, with virtually no steering. Since the micro-electrode appeared to touch the surface at several points it would seem that the membrane is protected, perhaps by a thin film of axoplasm. Another indication of the robustness of the inner surface is provided by the observation that although passing air bubbles through axons reduced the action potential it did not make them inexcitable (see p. 352).

Measurement of membrane potentials with change of solution

Method A. A relatively simple way of changing solutions with the internal electrode in position is shown diagrammatically in Text-fig. 4. In this system the fibre was open at the bottom and was not kept fully inflated. To introduce a new solution the standard solution was first removed from the cannula, which was then refilled with the test solution. Application of a negative pressure of a few centimetres H_2O to the cell caused fluid to enter the fibre and, after about 30 sec, to flow out of the cut end.

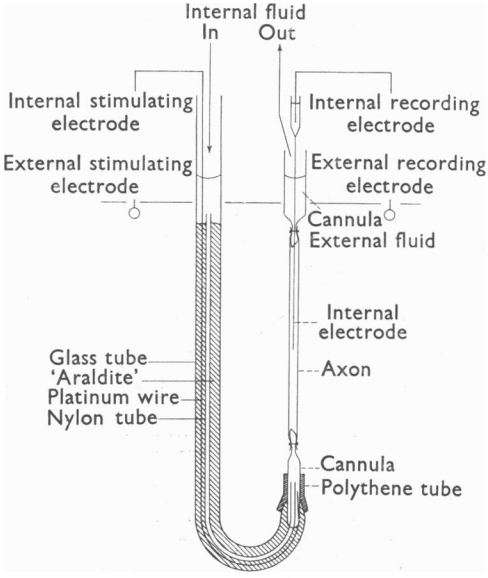


Text-fig. 4. Diagram of apparatus for recording with an internal electrode from perfused axon (method *A*). Application of negative pressure to the cell caused fluid to flow through the axon. Negative pressure was produced by pulling gently on the piston of a large, greased, air-filled syringe connected to the cell and to a manometer by polythene tubing.

The method had the advantage that the dead space was small and that setting up the fibre was a relatively simple operation. Its disadvantages were that the volume of the fibre was not constant and that the internal electrode might touch the membrane.

Method B. Text-figure 5 is a diagram of the fibre in position. Fluid was introduced at the left by a syringe and was withdrawn at the right by a nylon tube attached to a suction device. In order to change the internal solution, fluid was removed from the left-hand reservoir and immediately replaced with the new solution. Fluid then drained through the axon until the system came to rest with fluid in both cannula and reservoir at the same level; this height was 1–3 cm and was sufficient to keep the axon inflated. Removing fluid from the left-hand reservoir did not cause a back-flow because the axon collapsed on to the lower cannula and stopped the flow.

The procedure for setting up the axon was as follows: The axon was cannulated, extruded and perfused in the usual way. After tying off the open end and inflating with solution, the axon was cut close to the tie and a second cannula introduced and tied in. The doubly cannulated axon was mounted by pushing the polythene tube, which was permanently attached to the small cannula, over the inverted end of the U-tube. The polythene tube was then tied in position and the whole assembly lowered into a large cell filled with sea water. When introducing the internal electrode an attempt was made to keep it in the centre of the axon by adjusting the position, in the horizontal plane, of the lower cannula relative to the fixed upper cannula.



Text-fig. 5. Diagram of apparatus for recording with internal electrode during perfusion of doubly cannulated axon (method *B*). The drawing is not to scale.

Setting up an axon in the apparatus was a hazardous procedure and there was always a danger that one or other cannula would be blocked by pieces of axoplasm. It was important to make sure that there was a free flow through the axon before introducing the second cannula. Formation of an air bubble at the final stage of mounting the axon was avoided by allowing fluid to drain both from the lower cannula and from the end of the U-tube at the moment when the union between them was made.

Solutions

Table 1 gives the compositions of some typical solutions. The tonicity of the internal solutions was adjusted to be the same as that of sea water by the vapour-pressure method of Hill (1930), modified as described by Krogh (1939). Analar or 'specpure' salts were used if available; potassium methylsulphate was supplied by Hopkin & Williams; potassium isethionate (twice recrystallized) was normally prepared from sodium isethionate (British Drug Houses) by ion exchange with Amberlite IR 120.

Activity measurements

A solution of K_2SO_4 which is isotonic with Plymouth sea water contains 1·05 equiv K/l. and has an ionic strength which is so high that the activity coefficient of K^+ (γ_K) cannot be

computed reliably from published values of the mean activity coefficient. Approximate measurements of the activity coefficients of K and Na in the main solutions employed were therefore made with glass electrodes selective to K or Na (Table 2). The electrodes were supplied by Electronic Industries Ltd. and potentials were read with a Vibron electrometer, the general procedure being very similar to that used when determining pH with a glass electrode; junction potentials were 'abolished' with a saturated KCl bridge. Since the solutions contained either K or Na but not both ions the results did not depend on the

TABLE 1. Composition of solutions. Concentrations in mg ions/l. solution

	External solutions K^+	Na^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	HCO_3^-
A Sea water (S.W.)	10	470	10	54	550	29	—
B Artificial sea water (A.S.W.)	10	526	50	—	633	—	2·5
C High-K A.S.W.	538	—	50	—	635	—	2·5

	Internal solutions K^+	Main anion	Phosphate* as $H_2PO_4^-$
D K_2SO_4	1050	500	30
E KCl	610	560	30
F K isethionate†	*ca.* 610	*ca.* 560	30
G K methylsulphate	720	670	30
H Isotonic glucose	0·98 mole/l. H_2O		

* pH adjusted to about 7·7 adding by KOH to KH_2PO_4 in final solution.

† Early isethionate solutions contained 5 mM-Mg and some HCO_3, Cl and Na.

Internal solutions containing sodium were made by replacing K by Na on a mole-for-mole basis.

TABLE 2. Activity coefficients of Na and K in various solutions

Row	1 Solution X = K or Na G = glucose	2 Concentration K or Na (equiv/l.)	3 K-electrode ΔV (mV)	4 Na-electrode ΔV (mV)	5 From ΔV γ_K	6 From ΔV γ_{Na}	7 From $\gamma\pm$ γ_K	8 From $\gamma\pm$ γ_{Na}
A	0·5 M-XCl	0·50	0	0	(0·64)	(0·68)	0·64	0·68
B	0·1 M-XCl	0·10	−37	−38	0·74	0·75	0·76	0·78
C	1·0 M-XCl	1·00	+16·5	+19	0·62	0·70	0·60	0·66
D	Sea water	0·47 (Na) 0·01 (K)	—	− 1·6	(0·68)	0·68	—	—
E	NaCl	0·61	—	+ 4·5	—	0·66	—	—
F	X_2SO_4	1·05	+ 6·9	+ 7·0	0·40	0·43	0·48	0·51
G	$\frac{1}{2}X_2SO_4\frac{1}{2}G$	0·525	− 5	− 5·6	0·50	0·52	0·55	0·57
H	$\frac{1}{3}X_2SO_4\frac{2}{3}G$	0·350	−14	−15	0·53	0·53	0·58	0·61
I	$\frac{1}{6}X_2SO_4\frac{5}{6}G$	0·175	−27	—	0·63	—	0·66	0·67
J	$\frac{1}{12}X_2SO_4\frac{11}{12}G$	0·088	−42	—	0·69	—	0·72	0·73
K	X methylsulphate	0·72	+ 3·7	+ 8·5	0·52	0·66	—	—

The electrode potential was read to about 0·5 mV with 0·5 M-KCl or NaCl as the reference solution; the temperature was about 20° C. Solutions *E*–*K* were isotonic with sea water and contained phosphate buffer (see Table 1). Values enclosed in brackets were assumed. In columns 7 and 8, γ_+ was calculated from values of $\gamma_\pm$ in Taylor (1931) by the formula $\gamma_+ = \gamma_\pm^{z_+/z_-}$ where z_+ is the valency of the cation and z_- of the anion. This formula cannot be expected to apply to concentrated solutions of an asymmetrical electrolyte such as K_2SO_4. The difference between γ_K and γ_{Na} in row *K* has not been checked and might be due to water in the salts used. In calculating a_K and a_{Na} in mixtures of isotonic K_2SO_4 and isotonic Na_2SO_4 the approximation $\gamma_K = \gamma_{Na} = 0{\cdot}42$ will be used.

ability of the electrodes to discriminate between Na and K. We are indebted to Dr R. D. Keynes for assistance with these measurements, some of which were made at the A.R.C. Institute of Physiology, Babraham.

Recording electrodes

The external electrode was a silver wire, coated electrolytically with chloride, which made contact either with sea water or, in some cases, with a 0·6 M-KCl solution connected to the external fluid by a 3 M-KCl-agar bridge.

The internal electrode consisted of a 100 μ glass capillary filled with 0·6 M-KCl and containing a 20 μ silver wire, to reduce the high-frequency impedance (see Hodgkin & Katz, 1949*a*, Fig. 2*c*). A silver–silver-chloride electrode made contact with the KCl solution in the shank of the electrode but did not touch the 20 μ silver wire. Tests showed that the presence of the 20 μ silver wire in the capillary did not make the electrode sensitive to the concentration of chloride in the fluid in which the electrode was immersed. However, it was necessary to allow for junction potentials at the tip of the electrode.

Table 3. Corrections for junction potential between 0·6 M-KCl and test solution

Test solution		$E_{0\cdot6\text{ M-KCl}} - E_{\text{test solution}}$ (mV)
0·6 M-KCl		0
0·6 M-NaCl		−4
Sea water		−4
Isotonic solution:	K_2SO_4	+7
	Rb_2SO_4	+7
	Cs_2SO_4	+7
	Na_2SO_4	+4
	Li_2SO_4	+4
Isotonic K_2SO_4 : isotonic glucose		
(parts)	(parts)	
1	1	+5
1	2	+4·5
1	5	+4
1	11	+2
Isotonic K isethionate		+4
Isotonic K methylsulphate		+3

3 M-KCl bridges and an internal electrode filled with 0·6 M-KCl were used in making these measurements.

Junction potentials

In a few experiments the resting potential was determined as the difference between the potential of the micro-electrode (1) in solution *X* inside the axon and (2) outside the axon in contact with a small volume of solution *X* which was connected to the external solution by a 3 M-KCl bridge. On the assumption that 3 M-KCl abolished the junction potential this should give the resting potential directly. An equivalent procedure was to measure the potential difference between the internal and external solutions and to apply a correction for the junction potential between the 0·6 M-KCl in the electrode and the internal or external solution. The necessary corrections, which were determined with 3 M-KCl bridges, are given in Table 3. As an example of the method of applying the correction, suppose that the potential of the 0·6 M-KCl in the micro-electrode changes from 0 to −60 mV on transferring it from sea water in the recording cell to the inside of an axon containing isotonic potassium sulphate. From Table 3 it follows that the corrected potential of the sea water is +4 mV and that the K_2SO_4 solution inside the axon is at −67 mV. The corrected potential difference is therefore taken as −71 mV.

Electron microscopy

At least 2 hr elapsed between the death of the squid and the fixation of the giant fibre. During this period, the mantle or nerve was kept in refrigerated sea water. Nerves kept in this way showed little change in structure even after 24 hr.

All the axons studied were electrically excitable before fixation. For a general comparison of intact, extruded and perfused nerves, 1½ cm lengths of fibre were removed from the peripheral end of the nerve trunk, loosely tied to glass supports and fixed by immersion for 1–2 hr in ice-cold 1 % osmium tetroxide solution, buffered with acetate-veronal at pH 8·0 and made isotonic by addition of a suitable volume of three-times-isotonic artificial sea water (Palade, 1952; Villegas & Villegas, 1960*b*). In the case of perfused nerves it was possible to replace the artificial axoplasm with an ice-cold perfusion fluid containing osmium tetroxide buffered with acetate-veronal at pH 7·8 and made isotonic by addition of KCl. This method proved very satisfactory and a series of nerves was fixed in this way, in some instances osmium also being included in the external solution. Ten axons, all of which had been perfused initially with isotonic K_2SO_4 (solution *D*, Table 1), were examined after OsO_4 fixation.

A few perfused nerves were also fixed by the following four methods: 1, Vapour fixation by suspending the axon over buffered (pH 8·0) isotonic 2 % osmium tetroxide at 4° C for 4 hr. 2, Vapour fixation by suspending the axon over citrate-buffered (pH 6·8), isotonic formalin solution for 4–6 hr at 4° C, usually followed by a short treatment in 1 % osmium tetroxide solution. 3, Unbuffered, isotonic 3 %, potassium permanganate at room temperature (Zebrun & Mollenhauer, 1960), 4, Buffered, isotonic 1 % potassium permanganate at 4° C (Luft, 1956).

Vapour fixation was used in an attempt to minimize leaching out of any residual protoplasm from perfused nerve fibres. A check on the efficacy of fixation, which is largely independent of the experimental procedures applied to the giant fibre, is obtained by studying the small nerve fibres which are always present in the preparation. Both osmium tetroxide solution and vapour and formaldehyde vapour gave essentially similar results. Potassium permanganate gave very poor fixation. Even in the best instances it caused appreciable swelling of the Schwann cell layer of the small fibres and, in these same preparations, markedly damaged the Schwann cell layer of the giant axon.

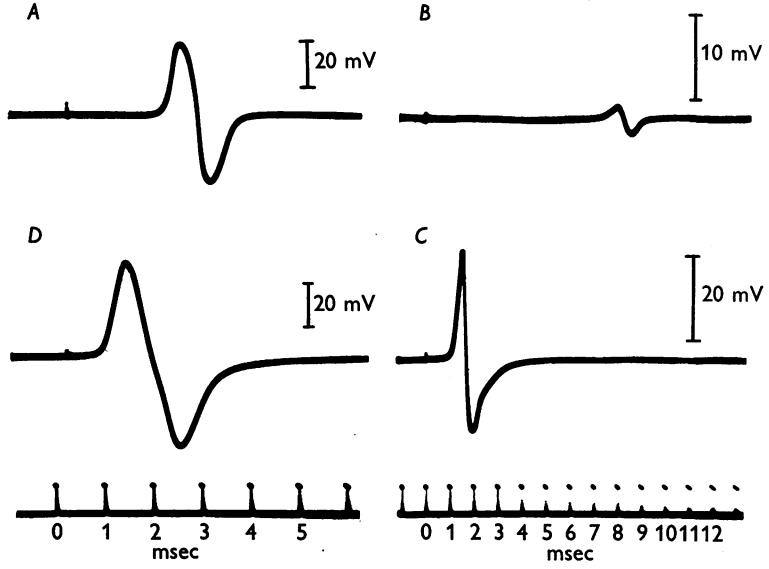
After osmium fixation, nerves were dehydrated in successive changes of ethanol and, in some instances, tissue blocks were stained by substituting a 1 % solution of uranyl nitrate in absolute ethanol for the final alcohol treatment in the dehydration procedure. Material was embedded in araldite (Glauert & Glauert, 1958) and transverse sections were cut on an A. F. Huxley microtome. Those sections showing silver interference colours were selected and either picked up directly on to a bare grid or, if further staining was expected, were mounted on a 'formvar' film. Contrast was frequently increased, in sections which had not previously been stained, by floating the grid section-side downwards for periods up to 30 min on a warm 1 % solution of uranyl nitrate in 75 % ethanol. After staining, the grids were thoroughly washed by agitation in clean 75 % ethanol. The sections were examined in a Siemens Elmiskop 1 electron microscope at 60 kV.

RESULTS

Moving on axoplasm

A simple way of showing that the membrane is not destroyed by extrusion is to roll out axoplasm from the central half of an axon and then to refill the flattened sheath by moving in axoplasm from the distal half. (Axons normally taper slightly in the direction away from the ganglion,

so there is less danger of axoplasm sticking if it is moved towards the ganglion.) Such experiments showed that the part reinflated with axoplasm was electrically excitable and that the action potential which propagated through it had about the same amplitude as before extrusion (for records see Baker *et al.* 1961).



Text-fig. 6. Effect of extrusion and filling with isotonic potassium sulphate on externally recorded action potential. *A*, intact axon; conduction distance, 40 mm; recording distance 13 mm, time scale below *D*; *B*, extruded axon; conduction distance 28 mm; recording distance 3 mm, time scale below *C*; note, gain is four times that in *A*; *C*, after filling 'sheath' with isotonic K_2SO_4, same conduction and recording distance as in *B*; *D*, as in *C* conduction distance 25 mm, recording distance 13 mm (as in *A*). Axon 155, temperature 17·4° C. The mean diameter of the intact axon was 830 μ and of the filled axon 810 μ. The conduction distance was in sea water and the recording distance in air.

Replacement with artificial solutions

Preliminary description. Text-figure 6, record *A*, shows the action potential of an intact axon recorded with external electrodes. Between *A* and *B* axoplasm was extruded; the action potential was still present but owing to the high internal resistance of the flattened axon it propagated more slowly and the component recorded with external electrodes was greatly reduced. On filling the axon with isotonic potassium sulphate solution the external action potential and conduction velocity increased to values somewhat greater than those in the intact axon (records *C* and *D*). Since isotonic potassium sulphate has a higher electrical conductivity

than axoplasm it is reasonable that both the external action potential and the conduction velocity should be larger after filling with this solution than in the intact axon.

Evidence that the small, slowly propagating action potential in record *B* was carried in the flattened giant axon and not by small fibres is provided by the following points:

(1) It could be elicited by a shock applied between an external electrode in a large volume of sea water and an external electrode connected to the inside of the giant axon through the cannula; since the whole of the cannulated end of the nerve was immersed in a large volume of sea water there was virtually no possibility of stimulating small fibres.

(2) The action potential was all-or-nothing in contrast to the compound wave produced by small fibre activity.

(3) In other experiments it was shown that the response gradually increased in size and the conduction time gradually shortened as the fibre was inflated.

The small size of the external action potential in extruded axons is of interest because it indicates that most of the axoplasm has been squeezed out. The externally recorded spike, V_o, is related to the spike at the membrane, V_m, by equation (1) (see Hodgkin & Rushton, 1946).

$$\frac{V_o}{V_m} = \frac{r_o}{r_o + r_i}, \tag{1}$$

where r_o and r_i are the external and internal resistances per unit length, respectively. In Text-fig. 6 the amplitude of the action potential in the extruded axon was about 1·5 mV as against 34 mV in the intact axon or about 100 mV at the membrane. With these figures it follows from equation 1 that r_o/r_i was 0·5 in the intact axon and 0·015 in the extruded axon. This indicates that extrusion reduced the amount of axoplasm to about 3 % of that in the intact axon. Values obtained in other experiments varied between 2 and 10 %.

The increase in internal resistance accounts satisfactorily for the low conduction velocity of the extruded axon. If the total area of the membrane remains constant—which implies folding in the shrunken condition —and the local properties of the membrane are unchanged by extrusion, it follows from the local circuit theory that the velocity θ, should vary as

$$\theta = k(r_o + r_i)^{-\frac{1}{2}}, \tag{2}$$

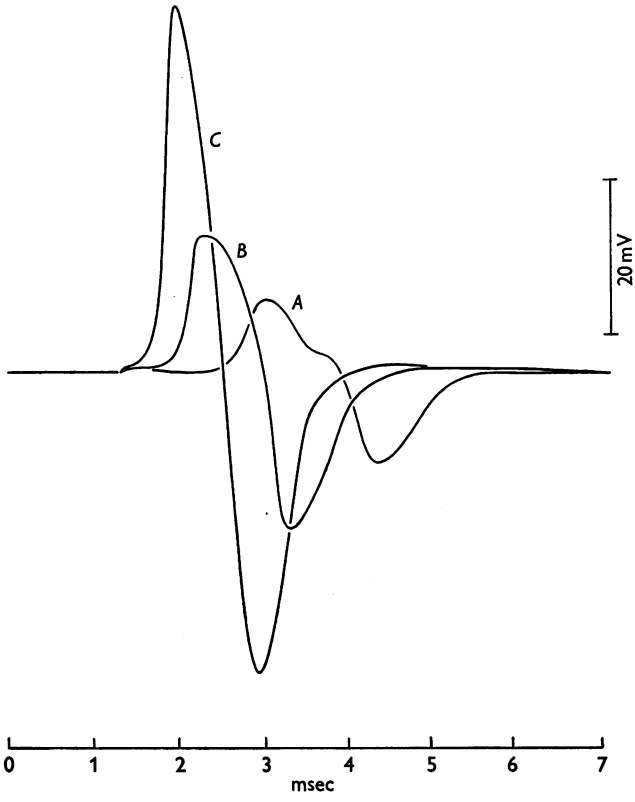
where k is a constant (see Hodgkin, 1954). On comparing this with equation (1), it can be seen that if a change in internal resistance alters the externally recorded action potential by a factor α the conduction velocity

should alter by $\alpha^{\frac{1}{2}}$. Applying this result to the experiment of Text-fig. 6, it follows that the velocities in extruded and intact axons should be in the ratio of $\sqrt{(1{\cdot}5/34)} = 0{\cdot}21$ which agrees with the observed values of 4·1 m/sec for the extruded axon and 20 m/sec for the intact axon. In other instances the velocity in the extruded axon was sometimes much less than that calculated. For example, in one experiment extrusion reduced the external action potential to $\frac{1}{25}$ and the velocity to $\frac{1}{14}$ instead of $\frac{1}{5}$ as calculated. Possible explanations are that flattening is irregular or that minor injury is more important in flattened than in inflated axons. These factors might also explain why extruded axons sometimes failed to give action potentials until filled with artificial axoplasm. Evidence for uneven flattening was provided by the exceedingly irregular action potentials observed in extruded sheaths if the recording distance exceeded the wavelength of the spike.

The effects of a change in volume on conduction velocity are best illustrated by experiments in which the fibre contained the same solution throughout the whole test (Text-fig. 7). A fibre which had been filled with isotonic potassium sulphate was drained of most of its internal fluid by raising it into air until only the open end at the bottom was left in sea water. With such an arrangement the tendency of the elastic sheath to force fluid out of the fibre was assisted by gravity and the fibre shrank to a greatly reduced volume. After fluid had drained from the fibre it was tied at the lower end and tested electrically. The small diphasic action potential from the shrunken fibre is shown in trace *A* of Text-fig. 7. On running fluid into the fibre, the spike increased and when fully inflated (*C*), it was about six times greater than in *A*. The conduction velocity also increased on inflating the fibre, as may be seen from the reduction in conduction time and the approximation of the first and second phase of the diphasic action potential. In Table 4 the observed values of conduction velocity are compared with values calculated from equations (1) and (2). Further information about the effects of inflation and hydrostatic pressure on the action potential is given on p. 344.

Preliminary tests of various perfusion fluids. Text-figure 8 provides evidence that the perfusion fluid had rapid access to the surface membrane. Record *A* was obtained with isotonic potassium sulphate flowing through the axon; 4 sec before record *B* the solution was replaced by isotonic sodium sulphate; records *B–E* show the rapid block associated with the change from K to Na and *F–J* the rapid recovery when isotonic potassium sulphate was replaced.

Experiments of this type showed that axons remained excitable during perfusion with the following solutions: isotonic potassium sulphate, methylsulphate, ethanesulphonate, chloride and isethionate. These solu-



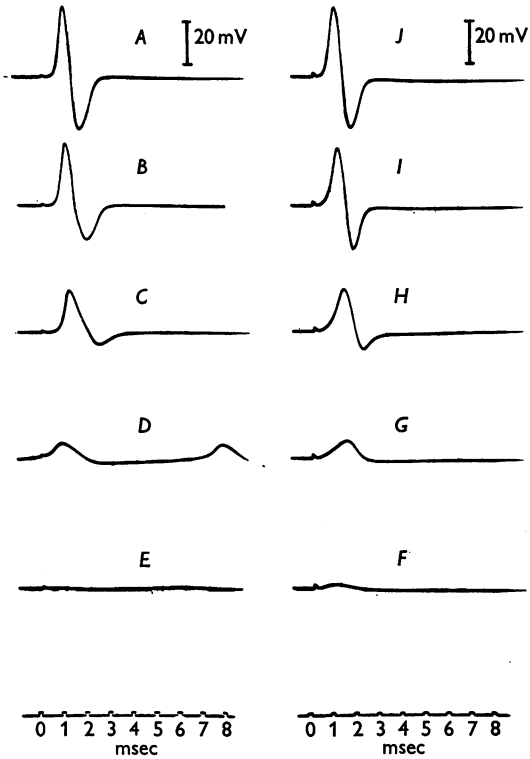
Text-fig. 7. Effect of varying the degree of inflation on the external action potential and conduction velocity of a fibre filled with isotonic K_2SO_4. *A*, shrunk fibre. *B*, partly inflated. *C*, fully inflated. The conduction distance (in a large volume of sea water) was 14·2 mm and the interelectrode distance (in air) was also 14·2 mm. Axon 73, 14·6° C.

TABLE 4. Effect of inflating fibre on external spike and conduction velocity

Row	I Condition of fibre	II External spike (mV)	III $\frac{r_o}{r_i}$	IV Conduction velocity (m/sec) observed	V Conduction velocity (m/sec) calculated
1	Fully inflated in large volume of sea water	—	0	35	—
2	Fully inflated in air (record *C*, Fig. 7)	47	0·925	—	25·2
3	Partly inflated in air (record *B*, Fig. 7)	17·3	0·215	14·2	15·3
4	Shrunk in air (record *A*, Fig. 7)	9·2	0·103	10·7	11·2

Action potential across membrane determined with internal electrode = 98 mV. In column III r_o/r_i was calculated by eqn. (1), taking V_m = 98 mV. In column V velocities were calculated by eqn. (2), assuming r_i was the same in rows 1 and 2 and that r_o was the same in rows 2, 3 and 4

tions contained 30 mM phosphate buffer, usually at pH 7·7, and were isotonic to within a few per cent. Short-term experiments indicated that the action potential was little changed over a range in tonicity of ± 10 % and a range in pH of 7–8·5 (pH 6·1 blocked conduction reversibly). On one occasion the concentration of phosphate buffer was reduced to 3 mM



Text-fig. 8. Effect on external action potential of replacing isotonic K_2SO_4 by isotonic Na_2SO_4. *A*, with K_2SO_4 in axon. *B* to *E*, 3·5, 7, 10·5, 14 sec after starting perfusion of isotonic Na_2SO_4; 30 sec interval. *F–J*: 7, 10·5, 14, 21 and 80 sec after starting perfusion of isotonic K_2SO_4. Axon 61, 16·5° C. Note that the fibre was firing repetitively in record *D*. Same gain and time scale throughout.

without ill effect. In solutions containing sulphate, phosphate buffer could be replaced by histidine or borate buffers, but with methylsulphate instead of sulphate as the main anion solutions made with these buffers appeared mildly toxic. A possible explanation is that sulphate or phosphate reduced the level of ionized calcium inside the axon. Magnesium and bicarbonate ions were included in the internal solutions used in the initial experiments but were later found to be unnecessary.

The solutions were nominally calcium-free and the effect of adding calcium has not yet been determined in a reliable manner. A single experiment showed that addition of 10 mM-Ca to isotonic potassium methylsulphate solution buffered with borate blocked the action potential rapidly and there was a partial recovery when the axon was perfused with isotonic K_2SO_4 buffered with phosphate.

Isotonic K_2SO_4 and potassium isethionate solutions were used in most of the experiments and there was satisfactory evidence that those media preserved the electrical properties of the membrane for considerable periods of time. A volume of 3–4 ml., which was more than a hundred times that of the fibre could be perfused without making the axon inexcitable and axons remained excitable for 3–5 hr. The action potential and resting potential were of the usual size (p. 346) and, as will be shown in the next section, inflated axons were capable of carrying a large number of impulses.

TABLE 5. Number of impulses carried by inflated and intact axons

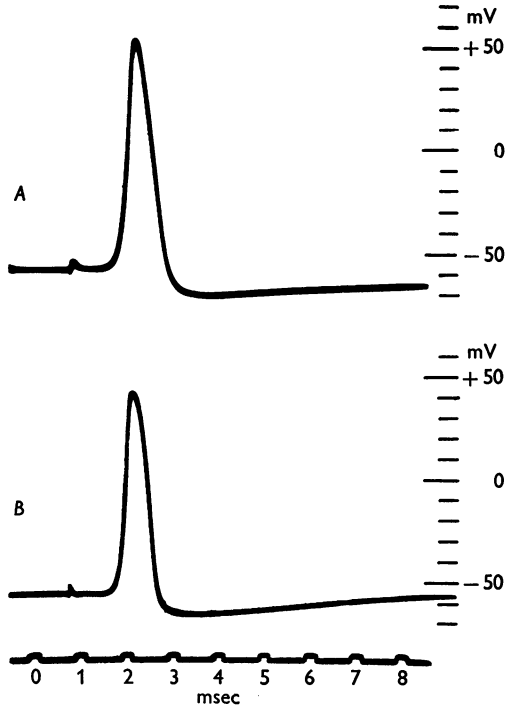
Row	Axon	Diameter (μ)	Temperature (° C)	Condition	Internal solution	Period of stimulation (min)	Main stimulation frequency (shocks/sec)	Number of impulses
1	59	770	15	Fully inflated	K- isethionate	120	50	$3{\cdot}6 \times 10^5$
2	101	720	21	40 % inflated	K_2SO_4	80	50	$2{\cdot}3 \times 10^5$
3	114	880	18	60 % inflated	K_2SO_4	120	50	$4{\cdot}1 \times 10^5$
4	115	810	18	Intact	Axoplasm	107	50	$3{\cdot}9 \times 10^5$
5	118	750	19·5	Intact	Axoplasm	186	125	$1{\cdot}1 \times 10^6$

Row 1; further perfusion at end of stimulation increased external spike but conduction soon failed. Row 2; attempt at further perfusion wrecked fibre. Row 3; further perfusion after $2{\cdot}1 \times 10^5$ impulses gave substantial improvement; further perfusion after $4{\cdot}1 \times 10^5$ impulses gave no real improvement. Row 4; pair to axon in row 3. Row 5; central half of long axon blocked with pressure in middle; at the end of the experiment an attempt to resuscitate stimulated half by moving in axoplasm from distal unstimulated part wrecked fibre.

Massive stimulation

Table 5 illustrates a series of tests in which intact and inflated axons were stimulated until the action potential failed. The inflated axons were closed by a ligature at the end away from the cannula and there was no steady flow during the period of stimulation. In some cases the fluid inside the fibre was renewed when conduction started to fail. The frequency of stimulation was usually 50/sec and the axon was considered to fail when the action potential ceased to be all-or-nothing. This is an unsatisfactory criterion and the results in Table 5 cannot be regarded as fully quantitative. However, it is clear that axons which have been filled with K_2SO_4 or potassium isethionate can carry $3–5 \times 10^5$ impulses and that this

is of the same order as the maximum number carried by isolated axons that have not been extruded. If the entry of Na and loss of K is assumed to remain at 4 pmole/cm^2. impulse (Keynes & Lewis, 1951) throughout the period of stimulation, 5×10^5 impulses should raise the internal sodium and lower the internal potassium in an 800 μ axon by 100 mM. One might therefore expect that an inflated axon which had been stimulated to exhaustion could be rejuvenated by renewing the internal fluid. Some improvement was obtained in this way but it was difficult to decide whether to attribute the improvement to an alteration in internal concentration or to a change in the degree of inflation. Our tentative conclusion is that accumulation of sodium and loss of potassium are not the only factors limiting the number of impulses carried by inflated axons.



Text-fig. 9. *A*, action potential recorded with internal electrode from extruded axon filled with isotonic potassium sulphate (16° C). *B*, action potential of an intact axon, with same amplification and time scale (18° C). The voltage scale gives the potential of the internal electrode relative to its potential in the external solution (no correction for junction potential). In record *A* correction for junction potentials would shift the scale 11 mV upwards.

TABLE 6. Action potentials (mV) and resting potentials (mV) in perfused fibres

I Internal solution	II Number of axons	III Mean or range	IV Action potential	V Under-swing	VI Resting potential	VII Potential at crest of spike	VIII Potential at bottom of underswing
K_2SO_4	33	Mean ± S.D.	107 ± 7	15 ± 6	−68 ± 5	+39 ± 5	−83 ± 4
		Range	91 to 120	0 to 22	−59 to −81	+24 to +51	−73 to −91
K isethionate	7	Mean ± S.D.	97 ± 9	12 ± 7	−59 ± 5	+38 ± 7	−70 ± 6
		Range	86 to 108	0 to 17	−55 to −70	+27 to +48	−58 to −77
KCl	4	Mean	97	21	−58	+39	−79
		Range	90 to 106	19 to 22	−54 to −60	+36 to +46	−74 to −82
K methylsulphate	2	Mean	115	9	−67	+48	−75
		Range	114 to 115	9	−65 to −68	+47 to +49	−74 to −77
$\frac{1}{3}$ K_2SO_4 $\frac{2}{3}$ glucose	2	Mean	122	< 0	−75	+46	−69
		Range	110 to 134	< 0	−72 to −79	+40 to +52	−63 to −76

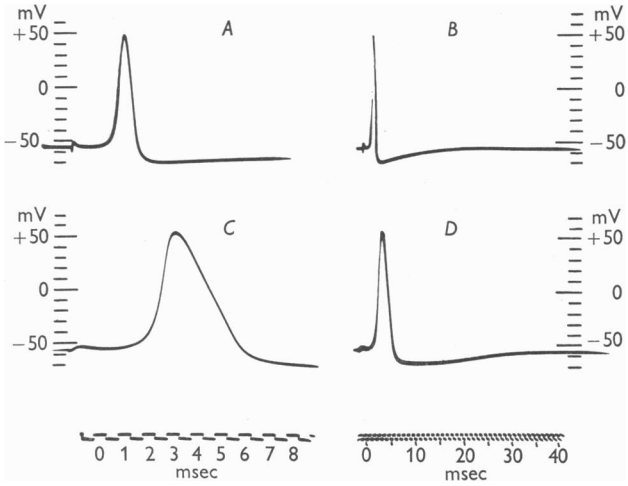
For the composition of the solutions see Table 1. Potentials have been corrected for junctional potentials in columns VI–VIII. The experiments with K isethionate were carried out at an early stage and the isethionate solutions contained some Na, Cl, Mg and HCO_3. ± is the standard deviation of individual observations about the mean. Mean potentials are based on values at the beginning of each experiment (first two groups) or when solutions were first applied (last three groups). Temperature 16–20° C.

Experiments with internal electrodes

Magnitude of action potential in fibres filled with isotonic potassium solutions. Text-figure 9*A* illustrates the action potential of an axon which had been extruded and filled with isotonic potassium sulphate. For comparison a record from an intact axon taken at the same gain and time-base speed is given below. The two action potentials are similar and the minor differences between them might be attributed to variation between axons.

Table 6 summarizes the main results obtained with internal electrodes and establishes that fibres filled with isotonic solutions of potassium salts can give action potentials of approximately the normal size. The rates of change of potential were also within the normal range, typical values at 18° C being 750 V/sec for the maximum rate of rise and 300 V/sec for the maximum rate of fall.

An interesting point is that in axons filled with isotonic potassium sulphate the potential at the bottom of the underswing (V_U) was more constant than either the resting potential or the underswing. Thus the fibre with the largest resting pontential (−81 mV) had an underswing of 1 mV and V_U was about the same as in a fibre with a relatively low resting potential (−62 mV) and an underswing of 22 mV. Further consideration



Text-fig. 10. Effect of temperature on action potential of axon filled with isotonic potassium sulphate. *A*, 16·8° C fast time scale. *B*, 16·8° C slow time scale. *C*, 4·9° C fast time scale (same as *A*). *D*, 4·9° C slow time scale (same as *B*). The zero is the potential recorded when the micro-electrode was in sea water outside the axon; correction for junction potential would shift the zero 11 mV upwards. Axon 94.

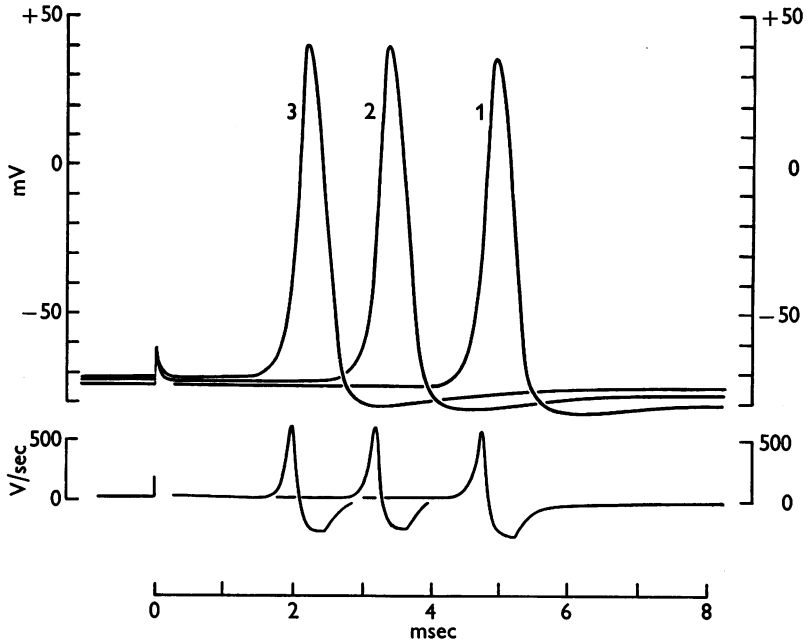
of this point and of other questions raised by Table 6 will be deferred to the following paper.

Effect of temperature. One of the more striking properties of nerve fibres is that the duration of the action potential is greatly increased by a fall in temperature. Text-figure 10, which shows the action potential of an axon filled with isotonic K_2SO_4 at 16·8° C and at 4·9° C, indicates that the temperature-dependence of the spike in a perfused axon is similar to that observed by Hodgkin & Katz (1949*b*) in intact axons. The result supports the view that temperature has a direct influence on the membrane and that its action in prolonging the spike does not depend on some change in the bulk of the axoplasm.

Effects of hydrostatic pressure difference on the action potential. A hydrostatic pressure difference of 2–3 cm of water between the internal and external solutions was required to keep the fibre inflated to its normal size. When using axons closed by a ligature at the lower end it was found that reducing the hydrostatic pressure below about 1 cm often caused the axon to shrink and to move fluid into the cannula. The extent to which this occurred was variable, perhaps because of differences in the mechanical properties of the sheath and of other elastic tissue in the nerve trunk. However, the volume of fluid inside the fibre could always be reduced by allowing the fibre to drain from a cut at the bottom end. In the experiment of Text-fig. 11 the axon had first been drained in this way and was then tied at the bottom. Record 1, which was obtained with an internal electrode, shows the action potential with no hydrostatic pressure difference between cannula and external fluid. The spike was of normal amplitude but propagated at only about 10 m/sec. On raising the hydrostatic pressure difference to 4 cm the fibre volume increased to its normal size with the result that the conduction velocity rose to about 24 m/sec but, in contrast to records with external electrodes, there was no immediate change in the amplitude or shape of the action potential. This is expected on the local-circuit theory, for a dimensional argument indicates that the form of the propagated spike depends only on the local properties of the membrane and that it should not be altered by a change in external or internal resistances (Hodgkin, 1954). No significance should be attached to the small increase in amplitude seen in Text-fig. 11; small changes in the opposite direction were observed in other tests.

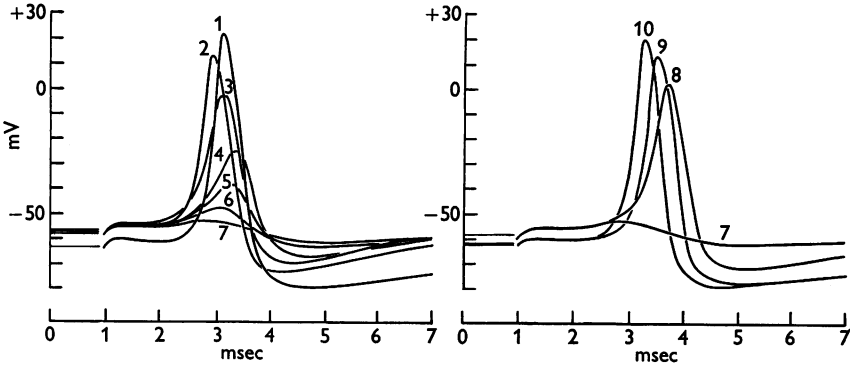
From experiments such as these we concluded that inflating the axon by pressures of 2–4 cm water had no immediate effect on the shape or size of the action potential. However, if a pressure of 3–6 cm water was maintained for several minutes the action potential underwent a gradual decline. In the experiment of Text-fig. 11 after maintaining the pressure of 4 cm for 5 min the action potential was reduced from 112 to 80 mV and

the resting potential from −72 to −60 mV; little further change occurred during the next 5 min. On releasing the pressure there was a partial recovery, the action potential after 4 min being 85 mV and the resting potential −61 mV. Later on, raising the pressure to 12 cm abolished the action potential and reduced the resting potential to −50 mV. On releasing the pressure the resting potential returned to −60 mV but there was no recovery of the action potential. In this case the effect of pressure was irreversible. However, in other axons its action was much more reversible. Text-figure 12 illustrates such an experiment. Records 1–7 show that applying a pressure of 12 cm of water for 2·5 min lowered the



Text-fig. 11. Effect of inflating axon on action potential recorded with an internal electrode. The upper trace shows the internal potential and the lower trace the rate of change of potential. 1, shrunk axon under no hydrostatic pressure difference. 2, partly inflated axon with a hydrostatic pressure difference of about 1 cm H_2O. 3, fully inflated axon a few seconds after raising hydrostatic pressure difference to about 4 cm H_2O. With 4 cm H_2O, the axon was inflated to about its normal diameter of 600 μ; in 1 it was shrunk down, the diameter was difficult to measure but was estimated as 250 μ in one plane and 400 μ in the other. Axon 191; temperature 18° C; filled with isotonic K_2SO_4; corrected for junction potential; conduction distance about 40 mm; distance of electrode from cannula = 5 mm. The increase of 1 cm hydrostatic pressure was produced by adding fluid to the cannula, that from 1 to 4 cm by applying 3 cm negative pressure to the external fluid. The experimental arrangement was similar to that in Text-fig. 4 except that the axon was tied at the bottom.

resting potential by 5–7 mV and reduced the action potential from 85 mV to a non-propagating response of a few millivolts. Records 7–10 show the relatively slow recovery which took place when the pressure was reduced. The upper part of the axon evidently recovered before the lower part and the first indication of recovery was the appearance of a 60 mV action potential 2 min after reducing the pressure; during the subsequent 20 min period there was a further slow recovery of the spike to 82 mV.



Text-fig. 12. Effect of raising the hydrostatic pressure difference between cannula and external solution on the action potential of an axon filled with isotonic K_2SO_4. Record 1, pressure difference approximately zero; records 2–7 taken at following times after raising pressure difference to 12 cm H_2O; 2, 11 sec; 3, 30 sec; 4, 60 sec; 5, 75 sec; 6, 90 sec; 7, 150 sec. After 3 min the pressure difference was reduced to zero and records 8, 9, 10 were obtained 2, 6 and 21 min later. The pressure difference was raised by applying suction to the external fluid (see Text-fig. 11). Axon 94; 16° C; corrected for junction potential.

It seems rather unlikely that the small decrease of resting potential in the experiment of Text-fig. 12 could account for the complete abolition of the spike. Alternative explanations are that excessive stretch of the membrane impairs the mechanism responsible for the increase in sodium permeability or that it produces a general increase in ionic permeability without greatly reducing the discrimination between K and Na. The second idea is supported by the observation that the initial effect of pressure is to shorten the underswing.

Light microscopy

Plate 1, fig. 1 is a low-power view of a transverse section of an intact axon in a nerve trunk; fig. 2 was prepared by a similar method but with an axon from which the axoplasm had been extruded; fig. 3 shows an axon which was extruded and then fully inflated with isotonic KCl; fig. 4 is similar to fig. 3 but the hydrostatic pressure on the cannula was reduced

and the axon has shrunk down to some extent. Figure 5 is a high-power view of the intact axon, and fig. 6 of the fully inflated axons. These photographs show that extrusion and subsequent perfusion remove most of the axoplasm. It is hard to say exactly how much axoplasm is left but a tentative estimate of 3 % was obtained from photographs such as these.

Electron microscopy

Intact axons (*Pl.* 2. *fig.* 1 *and Pl.* 3). The structure of the intact giant axon was similar to that described by Geren & Schmitt (1954) in *Loligo pealii* and by Villegas & Villegas (1960*a*) in a tropical squid *Doryteuthis plei*. The axoplasm contained mitochondria and small vesicles or possibly tubules. The mitochondria were evenly distributed throughout the axon but the vesicles, which varied in diameter from 200 to 2000 Å, were most numerous within a few microns of the surface. The axoplasm of intact axons was surrounded by an unbroken axolemma, which from its folded appearance did not seem to be under tension. Both axon and Schwann cell membrane could occasionally be resolved into two lines separated by 30 Å, but a clear-cut unit membrane of the kind described by Robertson (1960) was not often seen in material fixed with OsO_4. Plate 3 shows one of the many processes of the Schwann cell layer, which project into the axon for a distance of up to 2 μ.

Extruded axons. Apart from reducing the amount of axoplasm and throwing the Schwann cell layer into a series of folds, extrusion did not have any obvious effects on the structures seen in the electron microscope.

Perfused axons (*Pl.* 2, *figs.* 2 *and* 3; *Pl.* 4). Electron micrographs taken at low magnification showed that the small amount of protoplasm remaining in perfused axons was distributed round the periphery of the fibre. In most specimens the layer of residual axoplasm was of rather indeterminate thickness, but when a clearly defined edge was visible the thickness usually varied from 1 to 12 μ. In the fibre shown in Pl. 2, fig. 2, which had a diameter of 700 μ, the average thickness of the layer was 7 μ; this amounted to 4 % of the axoplasm in the intact axons. This is in rough agreement with the estimate of 2–10 % obtained from the magnitude of the external action potential in extruded axons. The layer of residual axoplasm was markedly less electron-dense than the axoplasm of intact fibres, perhaps because osmiophilic material was lost into the perfusion fluid. The fibrous elements and vesicles in the layer were more conspicuous than in intact axons. Some of the vesicles have two membranes and may be swollen mitochondria with few cristae visible. The larger vesicles (0·4–1·6 μ) with a single membrane might be derived from damaged mitochondria, and the smaller ones (200–4000 Å) by swelling of the small, membrane-bounded structures seen in intact axons.

The axolemma was similar to that in intact axons and was largely unbroken. The few breaks that did occur amounted to much less than 0·5 % of the total area of the membrane and it is not known whether they are anything more than fixation artifacts. The folds in the axolemma and the projections from the Schwann cell layer were less conspicuous (compare Pls. 3 and 4); this might be because they were flattened by the pressure exerted in the initial stages of perfusion.

There were no obvious differences between the Schwann cell layers of perfused and intact axons. In both cases the layer was traversed by double osmiophilic lines similar to those described by Geren & Schmitt (1954). Frankenhaeuser & Hodgkin (1956) regarded these structures as cracks between Schwann cell processes and suggested that the space between the two osmiophilic lines was the path through which ions move to and from the axolemma. They assumed that the after-effects of impulses arose from accumulation of potassium ions in the space immediately outside the axolemma and that the rate at which the after-effects decline was determined by the speed with which the potassium ions escaped through the cracks between the Schwann cells. Since extrusion or perfusion might alter the layer of Schwann cells, it was worth while to see whether the after-effects were modified by these procedures. A single test with an axon filled with isotonic K_2SO_4 solution showed that there was in fact little change, for the underswing during a 50/sec tetanus declined in much the same way as in an intact axon. This fits with the absence of any marked change in the Schwann cell layer but does not help to decide whether or not Frankenhaeuser & Hodgkin's suggestion is correct.

Structural alterations in perfused fibres. Plate 5, fig. 1, illustrates an effect seen in some perfused fibres and also occasionally in intact fibres which had been kept for 24 hr. It shows one of the large spaces (S) which sometimes develop in the Schwann cell layer without causing any obvious change in the axolemma. In other instances, the axolemma may separate from the Schwann cell membrane, forming a space such as G. The mechanism by which this occurs is not clear but since similar changes were seen in extruded fibres the cause of the separation is more likely to be mechanical than chemical. Both structural alterations seem stable to normal perfusion fluids, but passing an air bubble which filled a few millimetres of axon tended to strip off the axolemma, perhaps from regions where it had already begun to separate from the Schwann cells. Plate 5, fig. 2 is from an axon through which an air bubble had been passed. It shows one of the small regions from which the axolemma had been removed, leaving the inside of the axon in contact with a Schwann cell and also with one of the pairs of osmiophilic lines which cross the Schwann cell layer. If these lines are the boundaries of the aqueous channels

through which ions move, such damage would short-circuit the internal and external solutions. However, even after passage of an air bubble the damaged regions did not occupy more than 0·5 % of the total membrane area and this seems compatible with the observations that the action potential was reduced but not abolished by perfusing an air bubble.

The conclusion from the studies with the electron microscope is that although perfused axons showed occasional signs of damage, or perhaps of poor fixation, the axolemma and Schwann cell layer were in essentially the same state as in an intact axon.

SUMMARY

1. Giant nerve fibres of *Loligo*, from which the axoplasm had been extruded, conducted impulses if the 'sheath' was refilled either with natural axoplasm or with isotonic solutions containing salts of potassium.

2. The extruded 'sheath' could also conduct impulses, but in that case the conduction velocity was low and the externally recorded action potential was small.

3. Axons filled with isotonic solutions of potassium isethionate or sulphate remained excitable for up to 5 hr and could conduct 3×10^5 impulses.

4. Experiments with internal electrodes showed that axons filled with isotonic potassium solutions such as sulphate, methylsulphate, isethionate or chloride or with isotonic mixtures of K_2SO_4 and glucose, gave action potentials of amplitude 90–130 mV and resting potentials of 50–80 mV.

5. The effect of temperature on the action potential of a perfused axon was similar to that found previously with intact axons.

6. Inflating a collapsed 'sheath' by raising the pressure from zero to 1–5 cm H_2O increased the conduction velocity without changing the internally recorded spike; if a pressure of more than about 5 cm was maintained, the action potential declined and eventually failed; the block was often reversible and was associated with some decrease of resting potential.

7. Electron micrographs indicate that the axon membrane and Schwann cell layer were still present in extruded and perfused axons. They also show that about 95 % of the axoplasm was removed by extrusion and subsequent perfusion; the size of the spike recorded externally from extruded axons was consistent with this estimate.

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EXPLANATION OF PLATES

The following abbreviations are used in labelling the plates: A, axolemma; C, connective tissue sheath; F, fibrous elements in axoplasm; M, mitochondrion; O, osmiophilic lines crossing the Schwann cell layer; P, protoplasm of giant axon; S.C., Schwann cell; SCM, Schwann cell membrane; SCN Schwann cell nucleus; S.F., small nerve fibre; V, vesicle.

All the plates are from transverse sections. All the electron micrographs are of specimens fixed with OsO_4. The axons in Pl. 2, fig. 3 and Pl. 4 were fixed by perfusion and subsequent immersion, others by immersion.

PLATE 1

Light micrographs of sections of intact, extruded and perfused axons, all left in nerve trunk.

Fig. 1. Intact axon.

Fig. 2. Extruded axon.

Fig. 3. Perfused axon which had been fully inflated with isotonic KCl.

Fig. 4. Similar to Fig. 3 but with axon not fully inflated.

Fig. 5. Surface of intact axon at higher magnification.

Fig. 6. Surface of perfused axon with same magnification as in Fig. 5. Note that the residual axoplasm reaches a maximum thickness of 10 μ near a Schwann cell nucleus.

All sections were stained with Azan. Fixatives were: Figs. 1, 2 and 5, Bouin's Fluid; Figs. 3, 4 and 6, methanol, acetic acid and formic acid mixed in ratio 8:1:1.

PLATE 2

Low magnification electron micrographs.

Fig. 1. Intact axon.

Fig. 2. Perfused axon; tissue block stained with uranyl nitrate.

Fig. 3. Perfused axon at higher magnification. Arrows mark the ends of a double osmiophilic line crossing the Schwann cell layer. Note numerous vesicles in axoplasm.

PLATE 3

High magnification electron micrograph of an intact axon; section stained with uranyl nitrate. Arrows mark regions in which the membrane can be resolved into two lines. Note: the projection from the Schwann cell layer; also that the axolemma is more folded than the Schwann cell membrane.

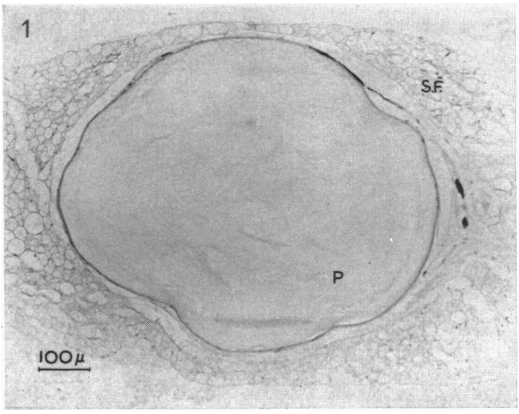
PLATE 4

High magnification electron micrograph of a perfused axon showing a region comparable to that in Pl. 3. Note that the axolemma is less folded than in Pl. 3 but is otherwise similar.

PLATE 5

Electron micrographs showing alterations in the Schwann cell layer of perfused fibres. Fig. 1 shows a space (S) within the Schwann cell layer and the separation (G) of the axolemma and Schwann cell membranes. Note that the axolemma is still intact. Fig. 2 is from an axon through which an air bubble had been passed; it shows one of the small regions from which the axolemma had been removed.

(*Facing p.* 354)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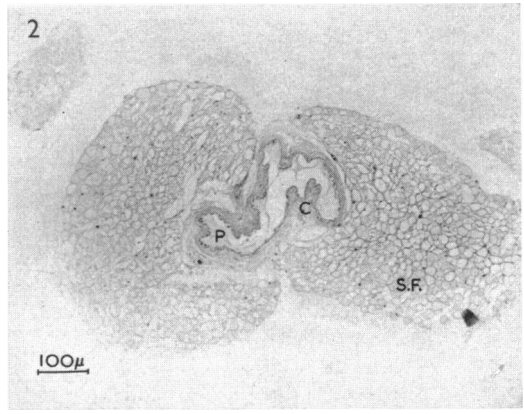
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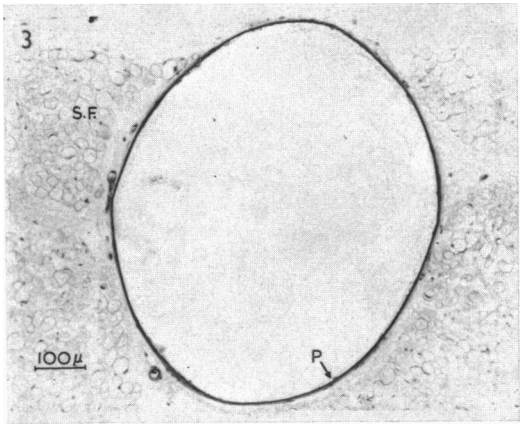
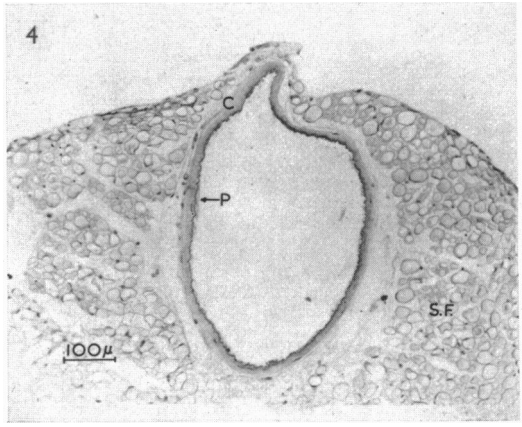
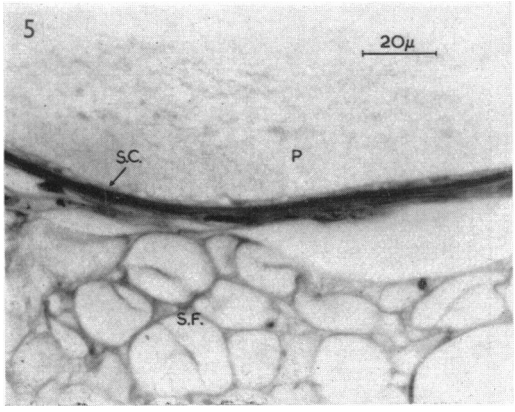
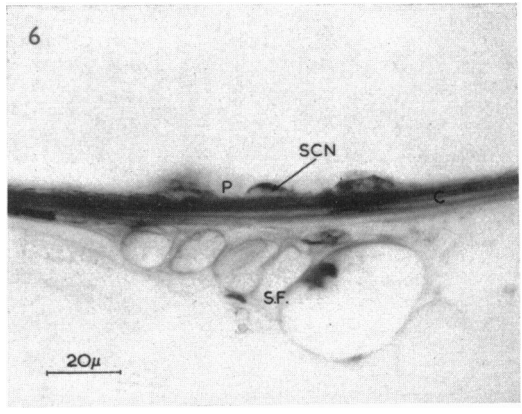
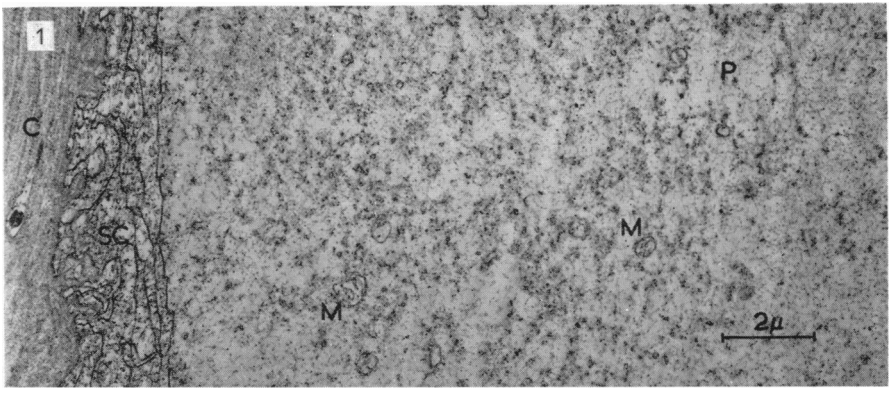
S.F.
100μ

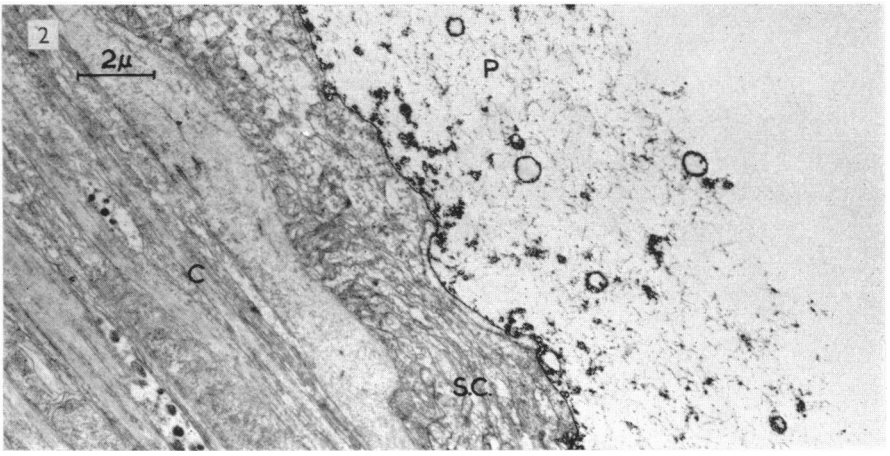

3
S.F.
100μ
P

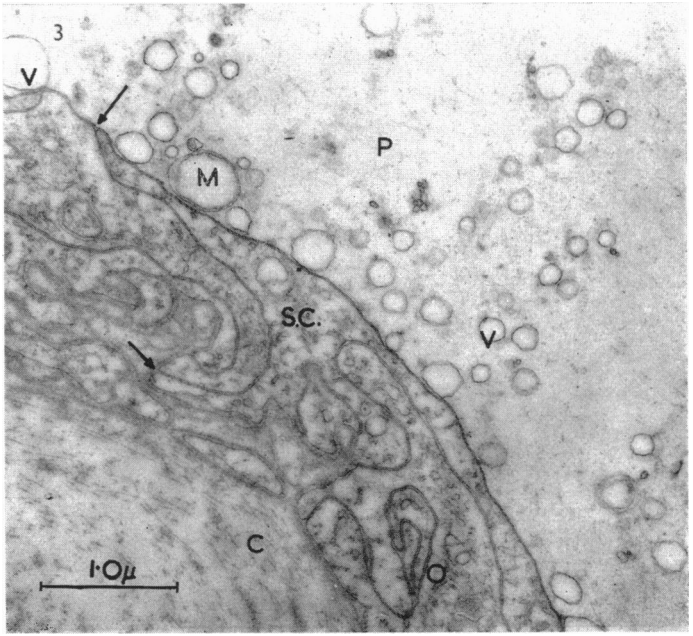

4
C
P
S.F.
100μ

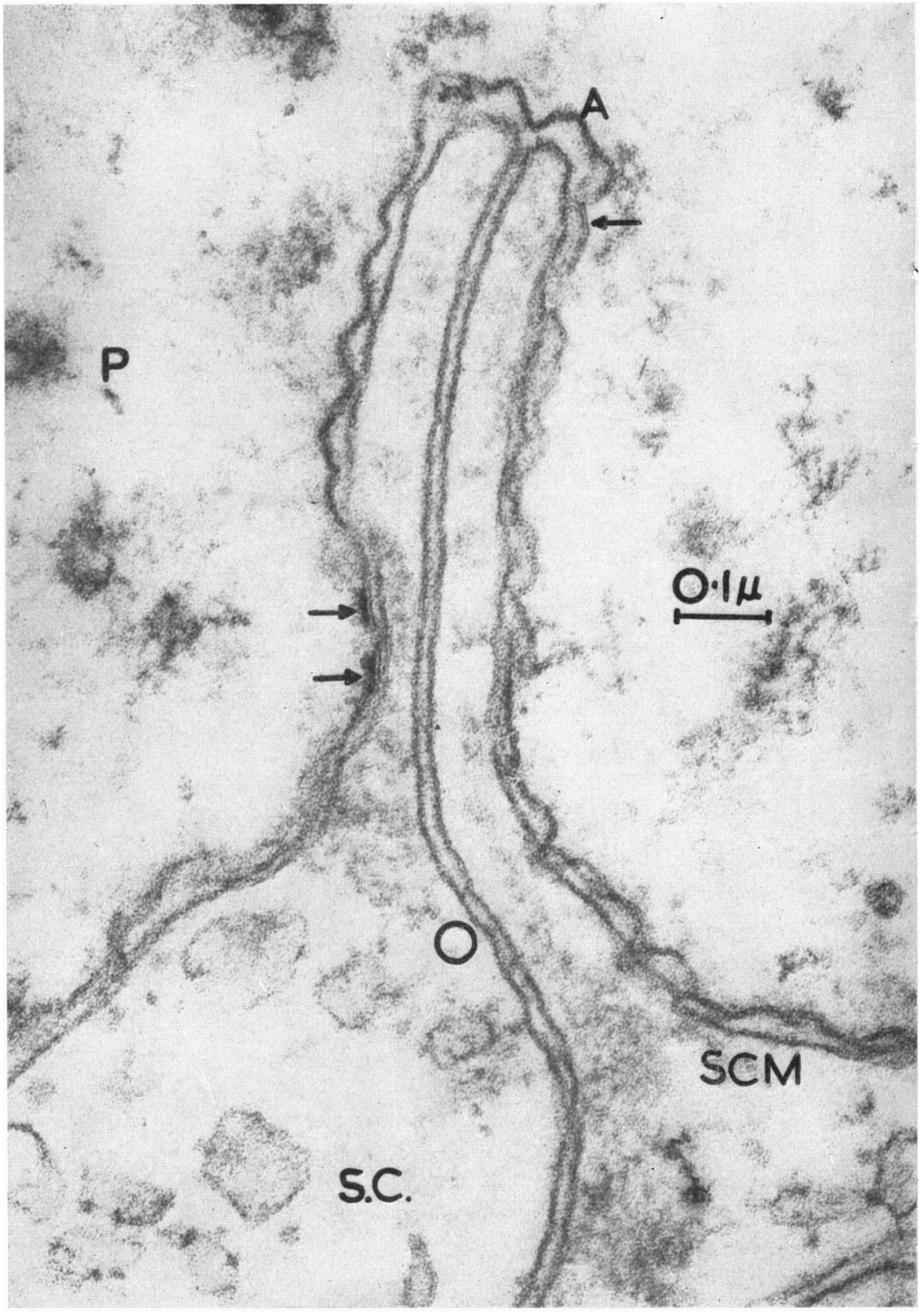

5
20μ
S.C.
P
S.F.


6
SCN
P
C
S.F.
20μ


1
C
SC
M
M
P
2μ


2
2μ
C
P
SC


3
V
M
P
SC
V
C
1·0μ



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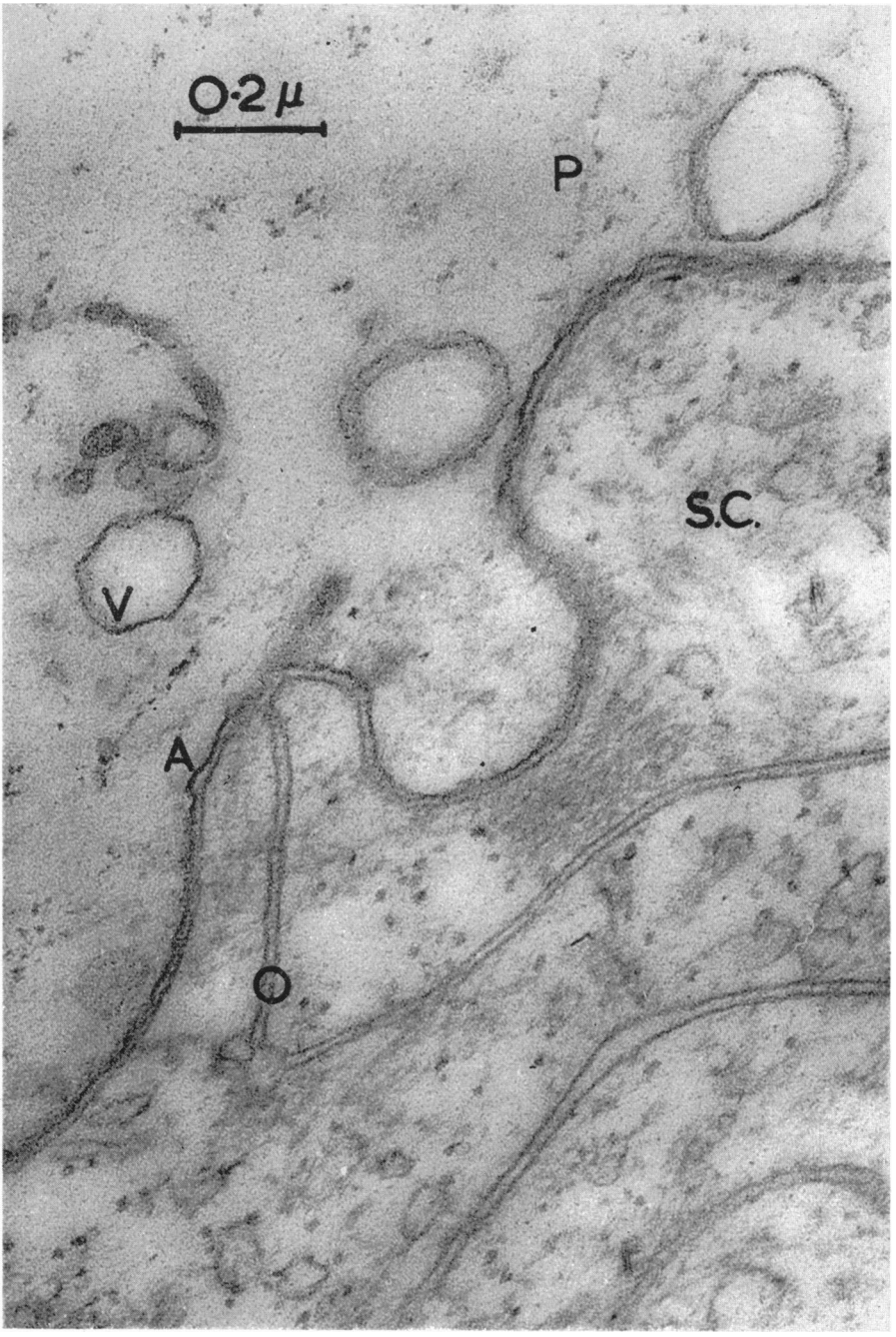

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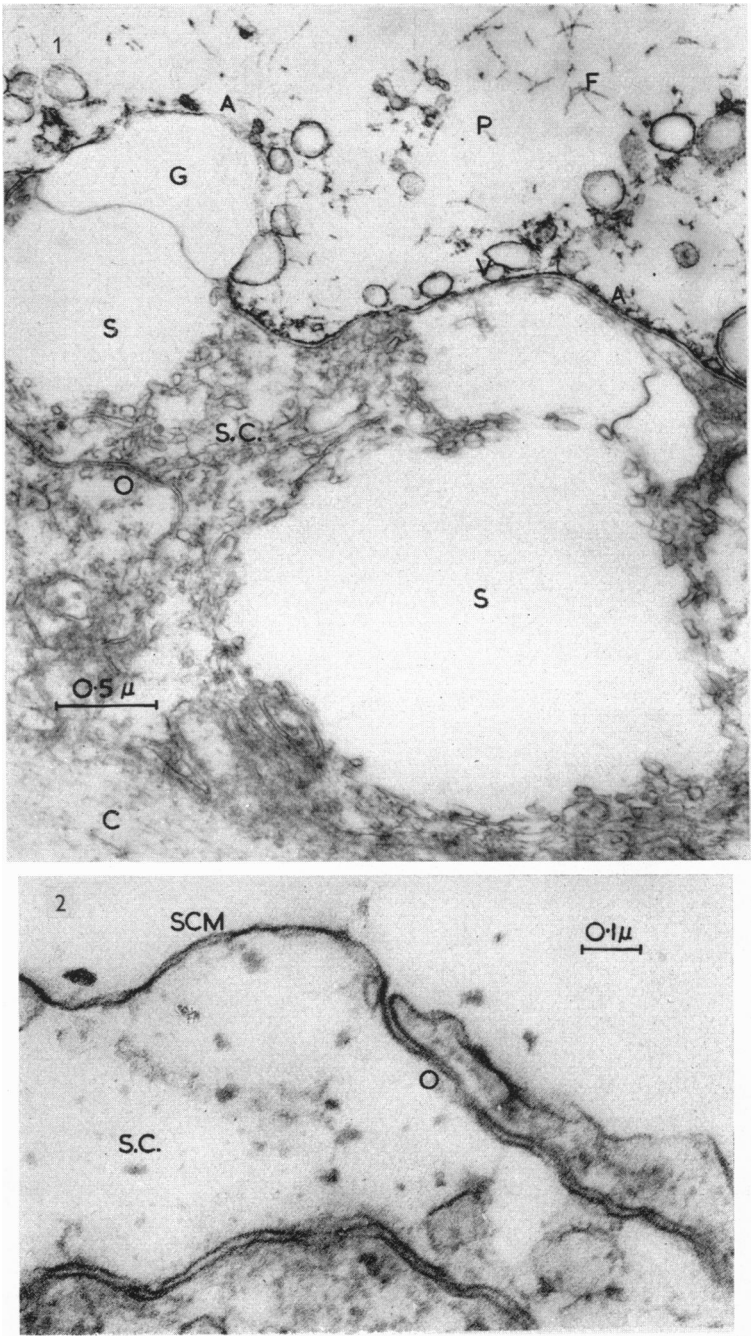

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