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THE EFFECT OF DILUTING THE INTERNAL SOLUTION ON THE ELECTRICAL PROPERTIES OF A PERFUSED GIANT AXON

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The introduction of methods for perfusing giant axons has opened several interesting lines of investigation. The problem considered here arose from the observation that if artificial axoplasm is diluted with an isotonic sugar solution, action potentials can be obtained with relatively little potassium in the perfusion fluid (Baker, Hodgkin & Shaw, 1962*b*), or with none at all (Tasaki, Watanabe & Takenaka, 1962; Tasaki & Shimamura, 1962). It will be shown that, if a salt such as KCl is largely replaced by sucrose, the threshold for activating and inactivating the sodium-carrying system shifts towards zero or becomes positive; under these conditions action potentials can be obtained with practically no resting potential. Another interesting point is that if all the internal potassium is removed by perfusing with a solution containing a small amount of sodium or choline chloride, the axon gives an action potential with a plateau lasting 1–3 sec. These conclusions appear to be in general agreement with those based on experiments carried out during the preceding summer by Narahashi (1963*a*, *b*) and Moore, Narahashi & Ulbricht (1963).

METHODS

Material. Giant axons with diameters of 600–1000 μ were obtained from large specimens of *Loligo forbesi*. Live squid were used occasionally but as a rule we employed mantles which had been stored for a few hours in ice-cold sea water.

Experimental procedure. Axons were extruded and perfused in the way described by Baker, Hodgkin & Shaw (1962*a*). The cell in which the axons were mounted and the method of changing solutions need no description, since they were essentially the same as that given under method A in the paper just quoted. However, the following modifications were introduced in the method of recording membrane potentials: 1. The micro-electrode, which was filled with 0·6 M-KCl solution connected to an Ag-AgCl electrode, contained a 20 μ bright platinum wire instead of a bright silver wire of the same diameter (the function of this wire is to reduce the high-frequency impedance of the electrode; see Hodgkin & Katz, 1949: platinum is better than silver because it is less likely to be affected by changes in chloride concentration). 2. The shank of the micro-electrode was filled with an agar gel of 0·6 M-KCl, in order to ensure that there was no flow of KCl out of the electrode. 3. A cannula with a long terminal portion was sometimes used; after perfusion the sheath was

drawn up on the tip and retied; in this way the action potential in the cannula was increased so that it was often as large as in the rest of the axon. This modification was introduced because it was convenient to apply current to the cannula and to record potentials either in the cannula, or close to it. The experiments were done at room temperature, 18–23° C.

Junction potentials. Care must be exercised when determining the resting potential of an axon filled with a mixture of 99% isotonic sucrose and 1% salt. Figure 1 shows the experimental arrangement. In a few cases the resting potential was determined as the difference between the potentials recorded with the micro-electrode in positions 1 and 3. On the assumption that the saturated KCl bridge abolished the junction potential this should give the correct membrane potential. In the majority of instances the resting potential was measured as the difference between the potentials in position 1 and position 2, and a correction for 'junction potential' was applied by measuring the 2–3 potential for all

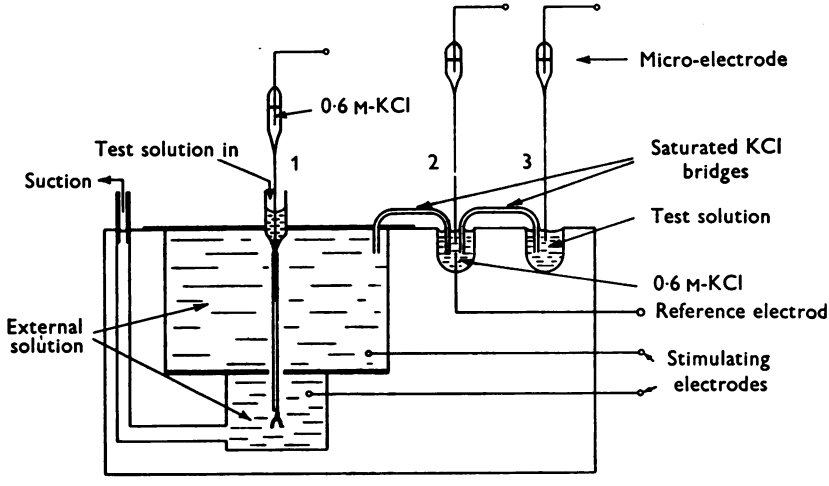

Fig. 1. Simplified diagram of recording arrangement showing method of eliminating junction potential (see text). The micro-electrode is shown in three different positions.

solutions at the beginning and end of each experiment. From the Henderson or Planck equations one expects a junction potential of less than 1 mV between 600 and 6 mM-KCl, and with a new micro-electrode the 2–3 potential difference was practically zero. However, after the electrode had been inside perfused fibres the 2–3 potential was 10–20 mV. The sign of the potential was such that the 600 mM-KCl in the capillary was negative to the solution of 6 mM-KCl; this suggests that the axoplasm, or whatever is stuck in the tip of the capillary, contains an excess of fixed negative charge and allows K^+ to diffuse more rapidly than Cl^-. Examples of the 'tip potentials' produced by different solutions are given in Table 1.

Solutions. The composition of the artificial sea water is given in Table 2. The internal solutions were similar to those described by Baker *et al.* (1962*a*), except that 1 molal sucrose instead of 0·98 molal glucose was used as isotonic diluting medium. The potassium contaminations of the isotonic sucrose and choline chloride solutions were measured by flame photometry and were found to be 0·013 and 0·005 mM respectively.

Single ion activities and activity coefficients, which were determined with sodium- and potassium-sensitive electrodes, are compared with values calculated from mean activity

coefficients in Table 3. In describing experiments it is convenient to refer to solution *A* as 600 mM-KCl, *B* as 300 and so on. Both concentrations and potassium activities are shown on graphs and tables, the former being expressed in m-mole/l. solution (mM) and the latter in m-equiv/kg H_2O. The activity coefficient of K, Na and Cl in sea water was taken as 0·68 (see Baker *et al.* 1962*a*); for the internal solution γ_{Cl} was taken as equal to γ_K.

TABLE 1. 'Tip potentials' produced by various test solutions in two different experiments

Test solution		Tip potential (mV)	
		Expt. 1	Expt. 2
0·6 M-KCl (isotonic)		0	0
Isotonic KCl: isotonic sucrose			
(parts)	(parts)		
1	1	0	0
1	5	− 1	− 1
1	24	− 3	− 7
1	99	− 5	− 17
Isotonic NaCl: isotonic sucrose			
(parts)	(parts)		
1	99	0	− 20
Isotonic choline-Cl: isotonic sucrose			
(parts)	(parts)		
1	99	− 6	0

The 'tip potential' gives the difference between the potential of the 0·6 M-KCl in the capillary and the test solution.

TABLE 2. Composition of external solutions (concentrations in m-mole/l. solution)

	K^+	Na^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-
Artificial sea water	10	463	11	55	602	2·5
K-free artificial sea water	—	473	11	55	602	2·5

RESULTS

Changes in the resting potential

Figure 2 illustrates the effect on the resting potential of varying the concentration of KCl inside axons immersed in K-free sea water; isotonicity of the internal solutions was maintained with sucrose. The points are readings taken at 5–20 min after introducing the test solution. This time allowed the main change in resting potential to occur, but the slow depolarization mentioned on p. 549 could have been missed in some cases. The full lines in Fig. 2 are for increasing concentrations and the interrupted lines for decreasing concentrations of KCl. The difference between the two sets of measurements may be partly due to incomplete equilibrium and partly to a decline in selectivity during the course of the experiment. Decreasing the KCl concentration from 600 or 300 to 100 mM sometimes increased the resting potential; this may be because a high internal chloride concentration prevents the potential reaching the level determined by the relative permeability of the membrane to Na and K.

TABLE 3. Activity coefficients of K and Na in various solutions

	1 Solution	2 K concentration (m-mole/l. solution)	3 K concentration (m-equiv/kg H_2O)	4 a_K (m-equiv/kg H_2O)	5 γ_K	6 γ_{KCl}
A	KCl	610	622	378	0·61	0·64
B	$\frac{1}{2}$KCl$\frac{1}{2}$S	305	338	224	0·66	0·68
C	$\frac{1}{6}$KCl$\frac{5}{6}$S	102	120	91	0·76	0·76
D	$\frac{1}{25}$KCl$\frac{24}{25}$S	24·4	29·4	25·3	0·86	0·85
E	$\frac{1}{100}$KCl$\frac{99}{100}$S	6·1	7·4	7·0	0·95	0·92
F	$\frac{1}{300}$KCl$\frac{299}{300}$S	2·0	2·5	2·5	1·00	0·95
		Na concentration		αNa	γNa	γNaCl
G	$\frac{1}{100}$NaCl$\frac{99}{100}$S	6·1	7·4	7·4	1·00	0·92

The isotonic KCl in row *A* contained 560 mM-KCl and 30 mM phosphate buffer at pH 7·7, and was identical with solution *E*, Table 1 of Baker *et al.* (1962*a*). In *B–F* this solution was diluted to varying extents with 1 molal sucrose (S). Columns 4 and 5 are based on measurements with a K- or Na-sensitive glass electrode standardized in 1·0, 0·1, 0·01 and 0·001 M-KCl or -NaCl. Column 6 is calculated from data in Robinson & Stokes (1959) on the assumption that the effects of sucrose and phosphate can be neglected.

The variability of the curves is thought to be at least partly due to differences in the relative permeability to Na and K and cannot be attributed solely to changes in the relative permeability to anions and

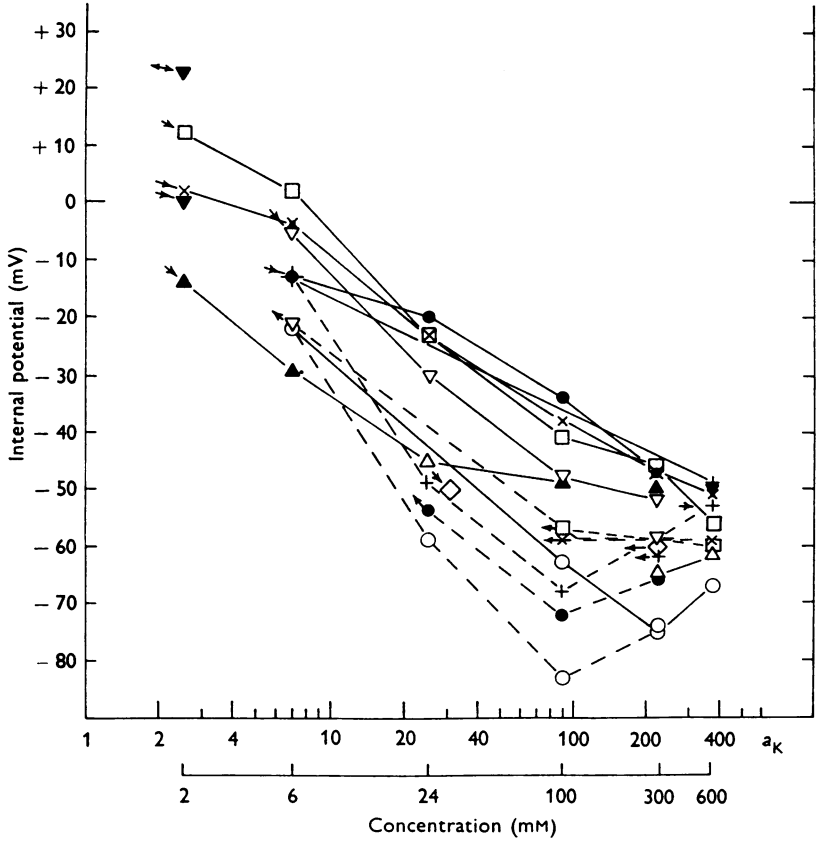

Fig. 2. Relation between the resting potential and the internal concentration of KCl for axons immersed in K-free artificial sea water. Abscissa, upper scale, potassium activity (m-equiv/kg H_2O); lower scale, concentration. Isotonicity of the internal solutions was maintained with sucrose. The interrupted lines are for decreasing concentrations of KCl and the full lines for increasing concentrations. Arrows indicate that the perfusion fluid was changed either to or from a K-free solution of 6 mM choline chloride + sucrose, or 6 mM-NaCl + sucrose. (The mean potential in these solutions was – 7 mV.) The results were obtained on 10 axons, each axon being shown by a different symbol.

cations. A fairly reliable estimate of P_{Na}/P_K can be made in the following way. When the internal KCl is diluted from 600 to 2 mM the chloride equilibrium potential changes from about – 2 mV to – 128 mV; under these conditions the resting potential changes from about – 60 mV to

about zero. At the potential, V', where the resting potential is equal to the chloride equilibrium potential, variations in chloride permeability should have no effect and V' should be given by

$$V' = \frac{RT}{F} \ln \frac{P_{Na} a_{Na.o}}{P_K a_{K.i}} \quad (1)$$

where R, T and F have their usual meaning; $a_{Na.o}$ and $a_{K.i}$ are the activities of external Na or internal K; and P_{Na} and P_K are permeabilities. This equation follows from the constant-field theory (Goldman, 1943; Hodgkin & Katz, 1949) or from the more general treatment of Patlak (1960). Table 4 summarizes the measurements and shows that P_{Na}/P_K varied between about 0·01 and 0·1; from experiments 1 and 2 it can be seen that the ratio tended to increase with time.

TABLE 4. Estimates of relative permeabilities to Na and K

Expt.	(m-equiv/kg H_2O) $a_{K.i}$	(mV) V'	P_{Na}/P_K
1*a*	32	−64	0·008
2*a*	36	−61	0·010
3*a*	41	−58	0·013
1*b*	50	−53	0·019
4*b*	60	−48	0·028
5*b*	70	−44	0·038
6*b*	90	−38	0·061
7*b*	95	−36·5	0·069
2*b*	98	−35·5	0·075

V' is the internal potential at which $V = V_{Cl} = 58 \log_{10} (a_{Cl.i}/a_{Cl.o})$ and was interpolated from curves drawn through points with 24 and 100 mM-KCl inside and K-free sea water outside (see Fig. 2). Experiments marked *a* are for decreasing concentrations, those marked *b* for increasing concentrations. P_{Na}/P_K was calculated from eqn (1) with $a_{Na.o} = 322$ m-equiv/kg H_2O; other activities are $a_{Cl.o} = 410$, $a_{Cl.i} \doteqdot a_{K.i}$, $a_{K.o} = a_{Na.i} = 0$.

The results in Table 5, which were obtained on a pair of axons, show that, with 6–100 mM-KCl inside the axon and 0–20 mM-K outside, the potential is fairly close to that given by

$$V_{K+0·01Na} = \frac{RT}{F} \ln \frac{a_{K.o} + 0·01 a_{Na.o}}{a_{K.i}} \quad (2)$$

Deviations at higher or lower internal concentrations are in the direction of the chloride equilibrium potential. The results in Table 6 (p. 554), which show that adding 270 mM choline chloride to 30 mM-KCl reduced the potential from −52 to −23 mV, again suggest an influence of chloride.

An attempt was made to calculate the relative permeability coefficients to K, Na and Cl by comparing the average results for increasing concentrations with the constant-field equation

$$V = \frac{RT}{F} \ln \frac{P_K a_{K.o} + P_{Na} a_{Na.o} + P_{Cl} a_{Cl.i}}{P_K a_{K.i} + P_{Na} a_{Na.i} + P_{Cl} a_{Cl.o}} \quad (3)$$

TABLE 5. Effect of varying $[K]_o$ and $[KCl]_i$ on the resting potentials of a pair of axons

	$[KCl]_i$	600	300	100	24	6·1	Axon
	$a_{K.i}$	378	224	91	25	7·0	
	V_{Cl}	− 2	− 15	− 38	−70	−102	
0 mM-K_o	V_1	—	− 74	− 83	−59	− 22	1
$a_{K.o} = 0$	V_2	− 67	− 75	− 63			
	$V_{K+0·01\ Na}$	−120	−107	− 84	−52	− 20	
10 mM-K_o	V_1	—	− 55	− 62	−34	− 3	2
$a_{K.o} = 6·8$	V_2	− 58	− 56	− 50	−24		
	$V_{K+0·01\ Na}$	− 91	− 78	− 55	−23	+ 9	
20 mM-K_o	V_1	—	− 58	− 40	−11	+ 23	2
$a_{K.o} = 13·6$	V_2	− 48	− 47	− 32	− 9		
	$V_{K+0·01\ Na}$	− 78	− 62	− 42	−10	+ 22	

V_1 is the resting potential with decreasing concentrations of K_i; V_2 with increasing concentrations. Potentials are in millivolts, concentrations in millimoles and activities in m-equiv/kg H_2O. The external medium was artificial sea water with $a_{K.o} + a_{Na.o} = 322$ m-equiv/kg H_2O.

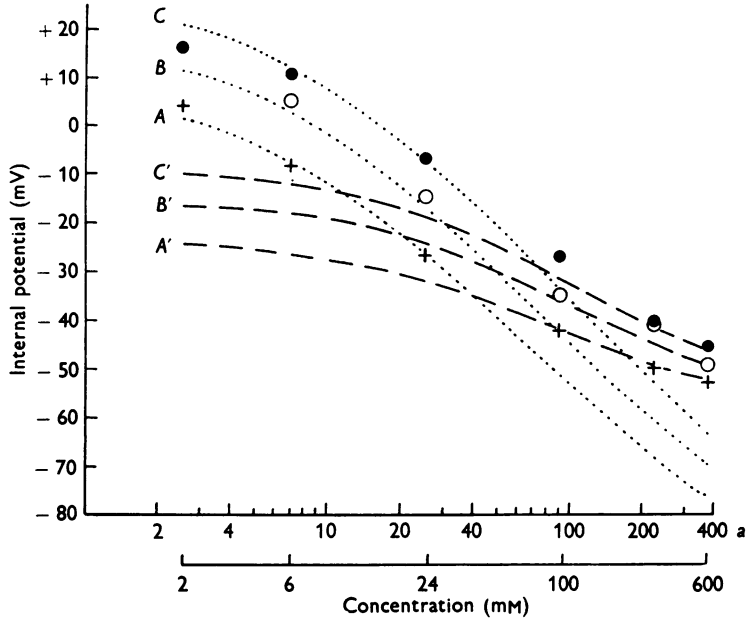

Fig. 3. Effect of increasing concentrations of internal KCl on average resting potential of axons immersed in artificial sea waters containing 0, 10 or 20 mM-K. Abscissa, upper scale, internal potassium activity (m-equiv/kg H_2O); lower scale, internal concentration of KCl. The points are averages of readings with increasing concentrations taken after at least 8 min equilibration; +, 0 mM-K_o, 17 axons, S.E. of mean 2–4 mV; ○, 10 mM-K_o, 5 axons, S.E. of mean 4–10 mV; ●, 20 mM-K_o, 3 axons, S.E. of mean 2–4 mV. The dotted curves, *A*, *B*, *C* were calculated from eqn. 3 for 0, 10, 20 mM-K_o with $P_K : P_{Na} : P_{Cl} = 1 : 0·035 : 0·02$, using an activity coefficient of 0·68 for sea water and $a_{Cl.i} = a_{K.i}$. The interrupted curves *A′*, *B′*, *C′* were calculated in a similar way but with $P_K : P_{Na} : P_{Cl} = 1 : 0·05 : 1$.

As can be seen from Fig. 3, the values observed with internal KCl concentrations of less than 50 mM were well fitted by $P_K : P_{Na} : P_{Cl} = 1 : 0{\cdot}035 : 0{\cdot}02$. With higher internal concentrations the resting potential was less than that calculated, indicating a poorer discrimination between potassium and other ions present. In this region permeability ratios such as $P_K : P_{Na} : P_{Cl} = 1 : 0{\cdot}05 : 0{\cdot}1$ gave a reasonable fit, but the analysis did not give clear information about the relative values of P_{Na} and P_{Cl} for the more concentrated internal solution.

Changes in the action potential: preliminary description

Figure 4 illustrates the effect of a wide range of internal KCl concentrations on the action potential; the external solution was K-free sea water and, except in record *D*, the internal solution was isotonic KCl diluted in

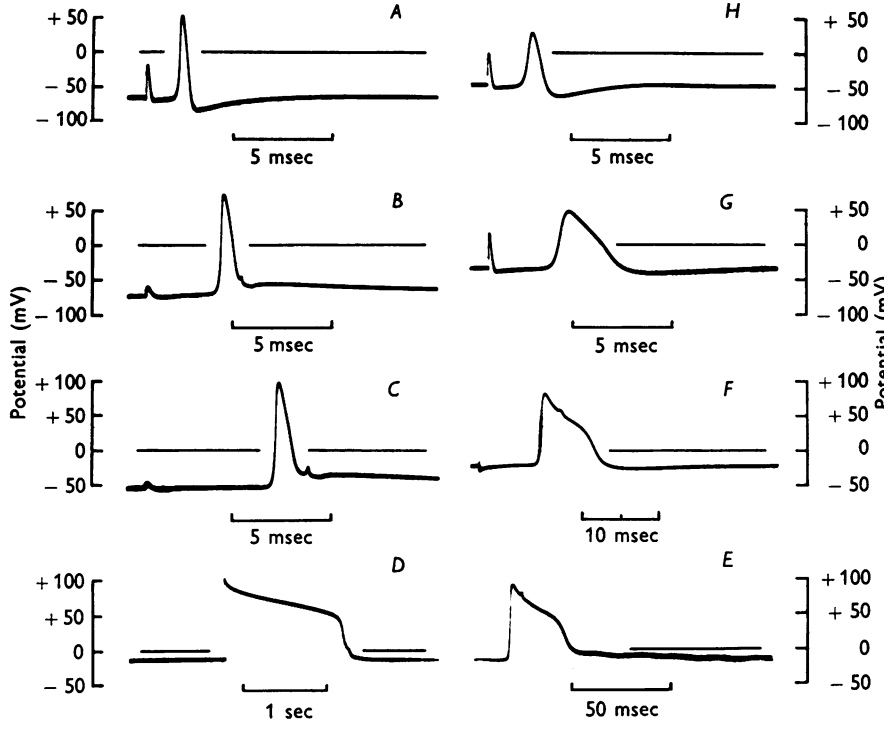

Fig. 4. Effect of diluting isotonic KCl with isotonic sucrose on action potential and resting potential. *A*, 300 mM-KCl; *B*, 100 mM-KCl; *C*, 24 mM-KCl; *D*, 0 mM-KCl; 6 mM choline Cl; *E*, 6 mM-KCl; *F*, 24 mM-KCl; *G*, 100 mM-KCl; *H*, 300 mM-KCl. Records *A–C* and *E–H* were taken 5–10 min and record *D* 24 min after changing internal solutions. External solution, K-free artificial sea water. Note change in time scale. Cannula artifacts in *B*, *C*, *E* and *F*. Electrode 5 mm from cannula. Temp. 23° C.

various proportions with isotonic sucrose. In record *D* the K-free internal solution was obtained by replacing the 24 mM-KCl sucrose used in record *C* with 6 mM choline Cl sucrose.

There are several interesting points about these records. Perhaps the most striking is that in the total absence of internal potassium, record *D*, the action potential lasted 1·3 sec and in form resembled the response of heart muscle. As can be seen from Fig. 5 such heart-like responses could be observed with 6 mM-NaCl instead of 6 mM choline Cl, so it cannot be argued that they are due to a specific effect of choline.

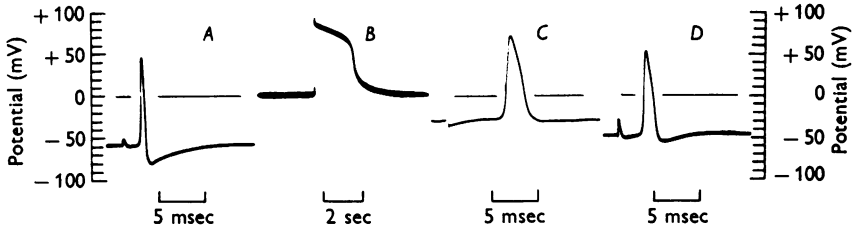

Fig. 5. Action potentials recorded internally from axon perfused with: *A*, 300 mM-KCl; *B*, 6 mM-NaCl; *C*, 24 mM-KCl; *D*, 100 mM-KCl; isotonicity was maintained with sucrose; records obtained 5–10 min after applying solutions; external solution K-free artificial sea water; temperature 21° C. Note change in time scale for *B*. From Baker, Hodgkin & Meves (1963).

Another surprising aspect illustrated by Figs. 4 and 5 is that action potentials can be obtained with so little resting potential. Apparently, if KCl is diluted with sugar the position of the sodium conductance and inactivation curves must be shifted along the voltage axis in the positive direction (see Baker *et al.* 1962*b*; Narahashi, 1963*a*, *b*). Further information about this aspect is given on p. 553.

The increase in the overshoot of the action potential as a_{K_i} is reduced agrees with previous observations and can be explained by assuming that when there is no sodium inside the axon the peak potential is determined by $\frac{RT}{F}\ln\frac{b\,a_{Na,o}}{a_{K,i}}$, where $b = P_{Na}/P_K$ for the active membrane.

Something should be said about the long time required for solutions diluted with sucrose to exert their full effect both on the resting potential and on the duration of the action potential. Although the solutions were applied for 5–10 min, it is clear from Fig. 4 that there is a marked difference between the records obtained with increasing and decreasing concentrations. The difference cannot be attributed to some irreversible action of the choline solution in record *D*; for the same effect was seen when 6 mM-NaCl was used to obtain a K-free solution. The most likely explanation is that

a very long time is required to reduce potassium to a low value by isotonic sucrose solutions. Thus in one experiment an axon perfused with 6 mM-KCl eventually gave a prolonged response similar to that in record *E* (Fig. 4), but only after the solution had been perfused for 40 min. When increasing concentrations of potassium are used, the fibre seems to equilibrate much more quickly ,and records *E–H* are therefore regarded as a more reliable indication of the effect of internal potassium concentration on the action potential and resting potential.

Repetitive responses during transitional stages

The sequence of events when a solution such as 600 mM-KCl is diluted 100 times with isotonic sucrose is often somewhat bewildering. Initially there may be an action potential of 160 mV and a resting potential of −60 to −70 mV. Subsequently the resting potential falls and the axon may fire repetitively for long periods. Sometimes the repetition occurs in bursts and on these occasions the resting potential usually alternates between two values, one at about −50 mV and the other at −30 mV; the period of the alternation is roughly 10 sec and the repetitive firing occurs when the resting potential is in its lower state (−30 mV). After 20–30 min these complicated effects disappear and the axon then gives a single response of the kind shown in Fig. 4*E*. We did not attempt to analyse this complex behaviour, which may arise partly from non-uniformity during the initial stages of perfusion. All that need be said about it is that since the variation of potassium permeability with potential can give a negative-slope conductance, it is not surprising to find that under appropriate conditions the resting potential can have two semi-stable states.

The cannula artifact

In the present experiments the cannula was filled with a column of perfusion fluid about 4 cm in length and was not drained before taking records. This exaggerates an artifact which has often been seen but has not previously been described. When the action potential reaches the cannula the potential of the fluid inside the cannula suddenly increases by 100 mV or more; this rapid change gets through the capacity of the glass insulation of the electrode and adds a small differentiated component to the action potential recorded from the tip of the electrode. The artifact is conspicuous in Fig. 4*C* and *E*, the long delay between rising phase and artifact occurring because the conduction velocity is reduced by the high resistivity of the sucrose solutions inside the axon.

Effect of concentration on threshold and inactivation

The effect on the threshold of changing the concentration of KCl inside the axon was determined, somewhat crudely, by applying a rectangular current pulse between the cannula and the external solution, and recording the electrical response with an internal electrode close to the tip of the cannula. The method was applied only if the action potential in the cannula was about the same size as in the rest of the axon. The distance between the recording electrode and the end of the cannula was about 1 mm, since records taken at shorter distances were complicated by an ohmic component caused by convergence of current into the tip of the cannula.

Figure 6 illustrates one of the experiments. The family of records in *A* gives the response to different currents with 6 mM-KCl inside the axon. The resting potential was −18 mV, the action potential 10 msec in duration and 127 mV in amplitude and the threshold potential about +20 mV.

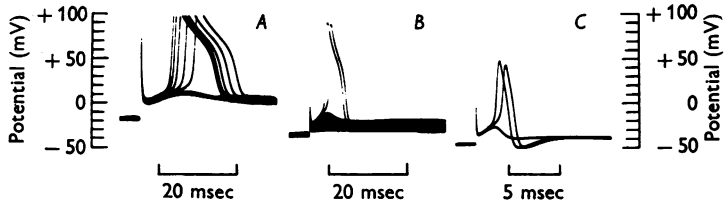

Fig. 6. Records showing critical potentials at three different internal KCl concentrations. Internal solution: *A*, 6 mM-KCl; *B*, 24 mM-KCl; *C*, 100 mM-KCl; isotonicity maintained with sucrose. External solution K-free artificial sea water. Stimulation by long-lasting rectangular current from cannula, with recording electrode 1 mm from cannula. Note change in time scale for *C*. Temp. 21° C. Other records gave the peak potential in *A* as +109 mV.

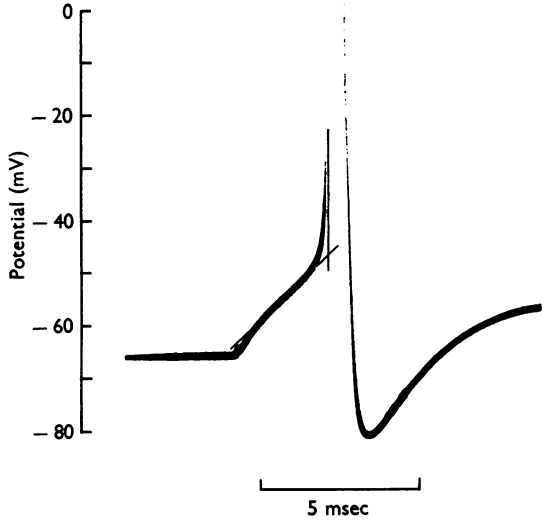

Fig. 7. Threshold potential with 300 mM-KCl inside axon, and K-free artificial sea water outside. The record shows the method of determining the critical potential, which is taken as the intersection of the two straight lines (see text). Experimental details as in Fig. 6, except that the recording electrode was 2 mm from the cannula. Temp. 20° C.

The records in *B* or *C* are similar families obtained with 24 mM-KCl (*B*) or 100 mM-KCl (*C*) inside the axon. They show that the effect of raising the internal KCl concentration to 100 mM is to shorten the action potential and to shift the threshold potential from about +20 to −20 mV.

From Fig. 7 it can be seen that the threshold potential with 300 mM-KCl

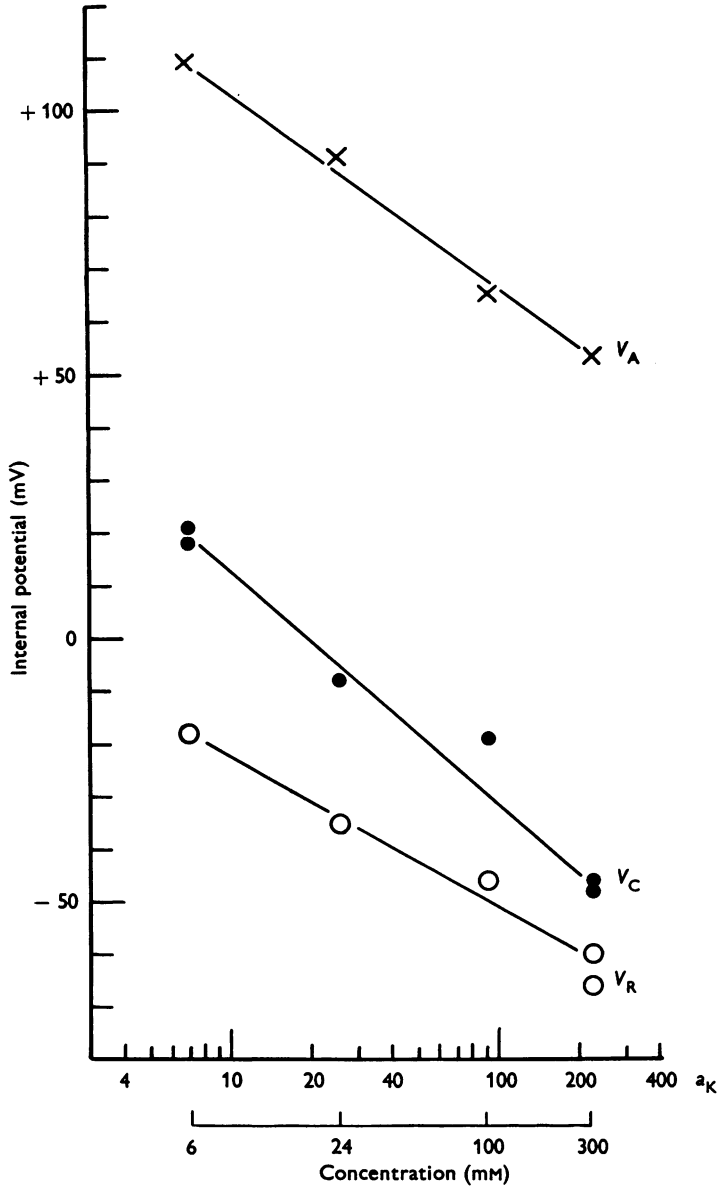

Fig. 8. Effect of varying the concentration of KCl inside the axon on resting potential, critical potential and overshoot. Abscissa, upper scale, internal potassium activity (m-equiv/kg H_2O); lower scale, internal concentration of KCl; ○, resting potential, V_R; ●, critical potential, V_C; ×, potential at crest of spike, V_A. The external solution was K-free sea water. Points at 6, 24 and 100 mM-KCl are from the axon of Fig. 6, those at 300 mM-KCl from two different axons.

inside the axon was about −47 mV. The difference between the threshold potentials in 300 and 100 mM-KCl may not be as great as 27 mV, for the 300 mM-KCl record was obtained at the beginning of an experiment and the 100 mM-KCl record in Fig. 6 at the end.

In order to express the variation of threshold in a more quantitative manner, a definition of critical potential is required. A convenient but arbitrary method is to equate the critical potential, V_c, with the potential defined by the intersection of lines drawn tangentially to the inflexions on the subthreshold and super-threshold parts of the response. Figure 7 illustrates the method. A different procedure which gave about the same result was to take the threshold potential from the maximum potential produced by a just-sub-threshold current.

The variations of the resting potential, critical potential and potential at the crest of the spike are given in Fig. 8. There are not enough points to establish the form of the curve relating V_c to $[KCl]_i$, but it seems clear that the critical potential must alter by 50 to 70 mV when the internal concentration of KCl is changed from 6 to 300 mM.

The inactivation curve was not measured directly, but from the variation of the maximum rate of rise of the spike with the level of membrane potential it appeared that dilution of isotonic KCl with isotonic sucrose shifted the inactivation curve along the voltage axis by about the same amount as the critical potential. This conclusion is strongly supported by the experiments of Narahashi (1963*a*, *b*). There was also evidence that the position of the inactivation curve was not altered to any large extent if K was replaced with Na, Li, choline or Cs. Thus in the experiments of Baker *et al.* (1962*b*), in which isotonic K_2SO_4 was replaced by Cs_2SO_4, the action potential declined as the resting potential fell from −50 to −40 mV, and failed shortly afterwards. A similar experiment in the present investigation showed that replacement of 300 mM-KCl with 30 mM-KCl and 270 mM choline Cl led to failure of the action potential at about the same level of resting potential. Before failure the rate of rise of the spikes was very greatly reduced, as would be expected if block were brought about by increasing inactivation.

Table 6 shows that a large action potential was present with 30 mM-KCl inside the axon, but that replacing this solution with 30 mM-KCl and 270 mM choline Cl made the axon reversibly inexcitable. It is true that the addition of choline chloride reduced the resting potential from −52 to −23 mV, but this cannot account for the difference, since depolarizing the axon filled with 30 mM-KCl to −22 mV left it excitable and reduced the maximum rate of rise of the spike by only 33%; from this it was estimated that the potential for half-inactivation, V_h, was −17 mV (see Hodgkin & Huxley, 1952); when filled with 30 mM-KCl and 270 mM

choline Cl, the axon gave a graded anode-break response after a hyperpolarization of 20 and 60 mV. From the relation between the height of the response and the preceding potential, the membrane potential for half-inactivation was estimated as -45 to -50 mV; this is about the same as in an axon filled with 300 mM-KCl, but markedly different from that in an axon filled with 30 mM-KCl. The conclusion is that the shift in the inactivation curve, and probably the threshold, are determined by the total salt concentration rather than by potassium concentration. Further experiments, preferably with the voltage-clamp method, are needed to put this result on a quantitative basis.

TABLE 6. Effect of adding 270 mM choline chloride to 30 mM-KCl on resting potential and action potential

Internal solution	$a_{K,i}$ (m-equiv/kg H_2O)	Resting potential (mV)	Action potential (mV)
300 mM-KCl	224	−59	112
30 mM choline Cl	0	− 2	Inexcitable
30 mM-KCl	31	−52	150
30 mM-KCl + 270 mM choline Cl	22	−23	Inexcitable
30 mM-KCl	31	−50	110

The external solution was K-free sea water. Isotonicity of the internal solutions was maintained with sucrose.

Properties of heart-like action potentials

Except in one rather poor experiment, heart-like action potentials were always observed when the axon had been thoroughly perfused with 6 mM-NaCl sucrose or 6 mM choline Cl sucrose. The external solution was K-free sea water in all cases, since we wished to avoid the possibility of K leaking into the internal solution. In the early stages spontaneous heart-like responses at a frequency of about 1 in 10 sec were sometimes observed, but after the solution had been applied for some time a shock was required to start the response. If the internal potential in the resting state was more positive than about -10 mV it was usually necessary to hyperpolarize the fibre for a few seconds before stimulation. These experiments showed that the duration and amplitude of the response increased with the duration and magnitude of the hyperpolarization (Fig. 9). This made it difficult to carry out quantitative experiments but several qualitative points have been established.

(1) The relative refractory period of the response was clearly very long. Thus when stimulating at 1 shock every 10 sec, alternate shocks often failed to evoke a response.

(2) As in Purkinje fibres (Weidmann, 1951) or in squid axons injected

with tetraethylammonium ions (Tasaki & Hagiwara, 1957), the plateau of the response could be terminated by applying a strong anodal current.

(3) Replacing 6 mM-NaCl sucrose by 2 mM-KCl + 4 mM-NaCl sucrose gave an action potential similar to that seen in Purkinje fibres (Draper & Weidmann, 1951). In this type of response, which is illustrated by Fig. 10, there was an initial spike and then a plateau. Such 'Purkinje-like'

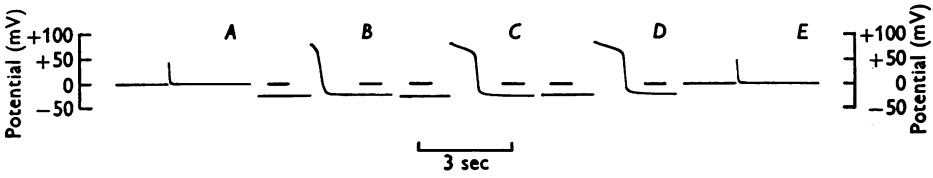

Fig. 9. Effect of hyperpolarization on duration and amplitude of the action potential. Internal solution, 6 mM-NaCl with sucrose; external solution, K-free artificial sea water. *A*, without hyperpolarization; *B*, *C*, *D*, approximately 1, 2, 3 sec after switching on the hyperpolarizing current; *E*, about 1 sec after break of hyperpolarizing current. Stimulation and polarization from cannula. Electrode 2 mm from cannula. Temp. 20° C.

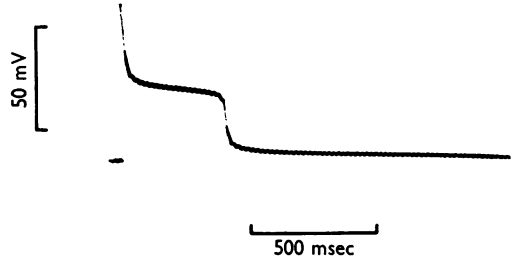

Fig. 10. 'Purkinje-like' action potential recorded from axon perfused with 2 mM-KCl + 4 mM-NaCl + sucrose; external solution, K-free artificial sea water. Electrode 4 mm from cannula. Temp. 19·5° C.

responses were often seen in the early stages of perfusion with 6 mM-NaCl or 6 mM choline Cl and probably occurred before all the internal potassium ions had been removed.

(4) The level of K at which heart or Purkinje-like responses appeared seemed rather variable. A Purkinje-type response with a plateau lasting 150 msec was seen in one axon filled with 6 mM-KCl, but this solution usually gave a normal type of action potential with a duration of 5–30 msec. It is possible that the production of responses with a long plateau depends on the ratio K:choline or K:Na rather than on the absolute level of K. Thus one axon filled with 2 mM-KCl gave a normal type of action potential (duration 10–60 msec), whereas two other axons filled with

2 mM-KCl + 4 mM-NaCl gave Purkinje responses with plateaus lasting 0·4–2·0 sec. A Purkinje-type response was also observed when an axon filled with 30 mM-KCl and 270 mM choline Cl was stimulated during a steady hyperpolarization to −100 mV.

(5) The heart-like action potentials were abolished and the axon became totally inexcitable if external sodium was replaced by choline; on restoring external sodium the heart-like response returned. This result indicates that the prolonged responses observed with K-free internal solutions resemble those produced by injecting TEA into squid axons in being sodium-dependent (Tasaki & Hagiwara, 1957), and differ from those of crab muscle, which occur in absence of external sodium and require divalent ions such as Ca, Sr or Ba (Fatt & Ginsborg, 1958). The maximum

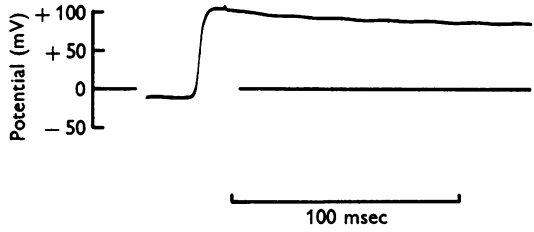

Fig. 11. Rising phase of a heart-like action potential on a fast time base. Internal solution, 6 mM choline Cl with sucrose; external solution, K-free artificial sea water. Cannula artifact after crest of spike. Electrode 5 mm from cannula. Temp. 23° C.

rate of rise of the heart-like action potentials was 20–100 V/sec and the maximum rate of fall, 0·1–1·0 V/sec. Figure 11 illustrates the rising phase of a heart-like response on a fast time base.

(7) From the effect of an added step of current there was evidence that the membrane conductance was fairly high during the plateau. This suggests that the resemblance of the response to a heart action potential is superficial, for the membrane conductance during the plateau of a Purkinje-fibre response is known to be less than in the resting state (Weidmann, 1951).

DISCUSSION

Although this series of experiments gave some unexpected results it does not conflict in any obvious way with the usually accepted form of the ionic theory. The dependence of the resting potential on the potassium concentration gradient is in qualitative agreement with conventional ideas and with the earlier experiments on perfused fibres (Baker *et al.*

1962*b*). The effect of diluting the internal potassium solution in increasing the overshoot has been described previously, and is to be expected if the active membrane is not perfectly selective to sodium ions (Baker *et al.* 1962*b*).

Further experiments should clearly be done in order to explain why the full effect of solutions consisting mainly of isotonic sucrose is exerted so slowly. Our impression is that the delay is too long for simple diffusion, and it may be that some potassium ions are retained in loose association with fixed negative charges in the residual layer of axoplasm. This would explain why isotonic solutions of Na, Cs, Li or choline salts seem to depolarize much more rapidly than isotonic sucrose. However, before accepting such an explanation it seems highly desirable to obtain direct information about the ease with which potassium and other ions can be removed from perfused axons.

Our experiments agree with those of Narahashi (1963*a*, *b*) and Moore *et al.* (1963) in showing that dilution of the internal potassium solution with a non-electrolyte causes both the inactivation curve and the relation between sodium conductance and membrane potential to shift in the direction of a more positive internal potential. Since this happened when the potassium solution was diluted with isotonic sugar, but not if K was replaced by Li, Na, Cs or choline, it is necessary to consider how a change in total salt concentration might alter the critical potential. A possible explanation is that fixed anions near the inside of the membrane might set up a potential difference between the inside of the membrane and the perfusion fluid, and that this p.d. increases when the salt concentration is reduced (cf. Tasaki *et al.* 1962). The anions might be carboxyl or phosphate groups at the inner edge of a lipid membrane, or they might be dispersed over a layer, extending perhaps throughout the residual protoplasm. In either case, the effect of the anions at low salt concentration would be to change the electric field in the membrane without altering the total p.d. between internal and external fluids. Thus if the membrane were permeable only to potassium ions and the activity of potassium in the perfusion fluid was equal to that in the external fluid, it is clear that at equilibrium there would be no potential difference between internal and external solutions. Under these conditions the effect of fixed anions in a layer immediately inside the membrane would be to concentrate cations in the region and to make it electrically negative to both internal and external solutions. From the ordinary Donnan principles it follows that the magnitude of the effect should increase as the total salt concentration in the perfusion fluid decreases. If the salt concentration were five times greater than that of the fixed anions, application of the Donnan equations indicates that the layer containing the anions should be 2·5 mV

negative to the perfusion fluid. On the other hand, if the salt concentration were reduced to one-tenth that of the fixed anions, the same type of calculation shows that the layer should be 58 mV negative to the perfusion fluid. Since excitability changes are likely to depend on the potential difference across the membrane rather than on the total potential difference between internal and external solutions, the hypothesis explains why reducing the internal KCl concentration from 300 to 6 mM leaves the membrane in a resting state even though the measured potential difference between internal and external solutions may be nearly zero.

Besides illustrating the effects of a low internal salt concentration on the relation between the internal potential and sodium permeability, the experiments showed that, with no K outside and 6 mM-NaCl or 6 mM choline Cl inside, there is a dramatic increase in the duration of the action potential and refractory period (cf. Narahashi, 1963*b*). These changes were clearly not due to an alteration of salt concentration or ionic strength, since replacing 6 mM-NaCl by 6 mM-KCl reduced the duration of the action potential from 1–5 sec to about 30 msec; a further reduction to about 1 msec occurred if the internal KCl concentration was increased to 300 mM. This last result agrees with previously developed concepts, for a shorter action potential is expected when the ion which normally repolarizes the membrane is restored. However, it is difficult to explain action potentials lasting 1–5 sec and relative refractory periods of 10–20 sec without postulating some change in the mechanism controlling sodium permeability. One possibility is that potassium ions are involved in making the sodium-carrying system refractory, and that with no potassium ions in the system both the rate at which inactivation develops, and the rate at which it is removed, are greatly retarded. Another is that in the absence of K, the potassium channel allows Na to pass, so that the normal distinction between the Na and K permeable phases of the action potential becomes somewhat blurred. In this connexion it should be kept in mind that, with 6 mM-NaCl inside and 460 mM-NaCl outside, an unselective increase in Na and K permeability could give an overshoot of about 100 mV. It is not known what ion is responsible for repolarizing the membrane in the absence of internal K, but since the rate of repolarization is exceedingly slow there is no difficulty in attributing repolarization to an inward movement of chloride ions.

SUMMARY

1. The effects of diluting isotonic KCl with isotonic sucrose inside giant axons were studied by the perfusion method; the external solution was usually K-free artificial sea water.

2. Reducing $[KCl]_i$ from 600 to 100 mM caused little change or occasionally an increase in resting potential. A further decrease to 6 mM-KCl reduced the resting potential by 30–60 mV in a reversible manner, but it might take 10–40 min for the potential to reach a steady value; equilibration was more rapid with increasing concentrations.

3. After prolonged perfusion with 6 mM-KCl the axon gave action potentials of 100 mV, although the resting potential was only about −10 mV.

4. The critical potentials for activating and inactivating the sodium mechanism varied with the internal concentration of salt, the threshold potential was about −45 mV with 300 mM-KCl and about +20 mV with 6 mM-KCl inside the axon.

5. There was some evidence that the shift in threshold and inactivation potential depended on the total salt concentration rather than on the potassium concentration of the internal fluid.

6. When all the internal potassium was replaced with 6 mM-NaCl or 6 mM choline-Cl the action potential was greatly prolonged and lasted 1–5 sec; replacing 6 mM-KCl reduced the duration to about 30 msec; a further shortening to 1 msec occurred when 300 mM-KCl was restored.

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