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CHLORIDE IN THE SQUID GIANT AXON

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The classical experiments of Boyle & Conway (1941) first established that the distribution in frog muscle fibres of chloride as well as potassium ions depends partly on a double Donnan system, the majority of the internal anions and of the external cations being able to penetrate the membrane much less readily than Cl^- and K^+ ions respectively. Subsequent work on the chloride permeability of frog muscle has fully confirmed that the membrane is permeable to Cl^- ions, and in a resting fibre equilibrated with normal Ringer's solution the chloride conductance of the membrane is about twice as large as the potassium conductance (Hodgkin & Horowicz, 1959; Hutter & Noble, 1960). Moreover, Adrian (1960, 1961) has shown that in this tissue the size of the intracellular chloride concentration $[Cl]_i$ is consistent with a distribution of Cl^- ions determined by a purely passive mechanism. Thus the Nernst equilibrium potential for chloride, calculated as $E_{Cl} = RT/F \ln [Cl]_i/[Cl]_o$, is equal to the resting membrane potential E_m.

It has usually been assumed that in the squid giant axon chloride is passively distributed in a similar way. The evidence supporting this assumption is Steinbach's (1941) report that in a freshly dissected axon $[Cl]_i$ was about 36 m-mole/kg axoplasm; taking the water content of squid axoplasm as 880 g/kg (see p. 693) this corresponds to 41 m-mole Cl/kg H_2O. In squid plasma $[Cl]_o$ is 560 m-mole/kg H_2O (see Hodgkin, 1951), whence E_{Cl} would be -66 mV. This is close to the value of E_m recorded *in vivo* by Hodgkin & Keynes (see Hodgkin, 1958) and Moore & Cole (1960). Steinbach (1941) also found that during the first 30 min after isolating the axon $[Cl]_i$ rose to 75 m-mole/kg axoplasm, and thereafter remained steady for some time. Such a doubling of $[Cl]_i$ would correspond to a reduction of E_{Cl} to just under -50 mV, a figure not wholly incompatible with the lower resting potential normally observed in isolated axons. Against the view that chloride is passively distributed in squid axons should be set the arguments presented in a brief note by Mauro (1954) concerning the potential recorded internally with a silver/silver chloride electrode, and

the much higher values for $[Cl]_i$ in axoplasm from *Loligo pealii* found by Koechlin (1955) and Deffner (1961). However, it was not these facts that prompted the work to be described here, but a consideration of the rapidity of the net Cl movements described by Steinbach (1941). A chloride uptake of 39 m-mole/kg in 30 min corresponds to a net influx into a 500 μ squid axon of 284 pmole/cm^2.sec; and Steinbach also reported nearly as fast net movements of chloride outwards for axons soaked in low-[Cl] nitrate solutions. Fluxes of this order seemed difficult to reconcile with the tracer experiments of Caldwell & Keynes (1960) and Tasaki, Teorell & Spyropoulos (1961), which agreed in indicating that both influx and efflux of Cl are normally less than a tenth as great. A reinvestigation of the chloride content of squid axoplasm was therefore undertaken.

In December 1960 a number of analyses was made of the chloride content of extruded axoplasm from *Loligo forbesi*, and it soon became clear that Steinbach's low values for $[Cl]_i$ could not be confirmed, even in the freshest material the average value obtained being over 100 m-mole/kg. Since Steinbach's figures were consistent with a simple passive distribution of chloride, the new ones could not be, for the corresponding value for E_{Cl} was less than -40 mV. Hence it had to be supposed either that the internal chloride was partly bound, or that chloride was being transported inwards against the electrochemical gradient by some form of active transport mechanism. As will be seen, measurements of the chloride activity in extruded axoplasm ruled out the first explanation, and tracer experiments provided support for the second hypothesis, without, however, settling finally the question of the exact nature of the transport process. Two preliminary reports have been published (Keynes, 1962*a*, *b*), in which some of the average values quoted differ from those now given because they do not include the results of further experiments done at Plymouth during November and December 1962.

METHODS

Material. Most of the experiments involved the extrusion of axoplasm from giant axons from *Loligo forbesi*. Usually the hindmost stellar nerve trunks were dissected from refrigerated mantles, and a 10 mm length at the central end of each giant axon was quickly cleaned before the extrusion took place, in order to minimize contamination by extracellular chloride. As an additional precaution against contamination the nerves were sometimes rinsed for a few minutes in a chloride-free solution, either nitrate salts or dextrose being used to make the solution isotonic with sea water. Axons which were not fully excitable from end to end, or which had visibly damaged branches, were rejected. Occasionally living squid were available; the results obtained with their axons did not differ obviously from those with refrigerated mantles. A few samples of axoplasm were extruded directly from the central ends of undissected axons *in situ* in the mantle; this reduced the time between decapitation of the squid and extrusion of its axoplasm to 15–19 min.

Chloride analyses. The axoplasm samples were transferred to small flat-bottomed glass

tubes and quickly weighed. The axoplasm was dispersed in 1 ml. distilled water, and 2 ml. of 50 % acetic acid and 2 drops of octan-2-ol (to prevent foaming) were added. The chloride in the samples was then determined by electrometric titration against 0·01 N-$AgNO_3$ as described by Sanderson (1952). The standard $AgNO_3$ was delivered from an 'Agla' micrometer syringe which had a platinum wire sealed into its tip; in order to obtain steady potentials it proved important that the part of the tip below the wire should be drawn out fine over a length of at least 25 mm. A clean silver wire was dipped into the titration vessel, whose contents were vigorously stirred by a stream of air bubbles. The potential between the two wires was measured with a vibrating-reed electrometer (E.I.L. Vibron Model 33B). The potential was recorded after successive additions of 0·01 or 0·02 ml. of $AgNO_3$ solution until well past the end-point, which was determined graphically as the centre of the steep portion of the symmetrical S-shaped titration curve. Before beginning to add the $AgNO_3$ solution the potential was usually just above 300 mV; the end point was close to 180 mV. Titration of 1·0 ml. of 0·001 M-KCl on eight occasions always gave a small positive error, whose mean value was +0·03 μmole (the range of apparent Cl contents was 1·01–1·05 μmole). Supposedly Cl-free blanks gave starting potentials of 180 mV and apparent Cl contents less than 0·02 μmole. As a check on possible interference from the principal anions in squid axoplasm other than chloride, samples of L-glutamic and L-aspartic acids and of potassium isethionate were dissolved in 1 ml. water and titrated. There was no detectable chloride in 10 μmole of the two acids, while 100 μmole of the isethionate contained less than 0·3 μmole of chloride. When an appropriate quantity of Cl-free artificial axoplasm containing all three of these anions was added to 1·0 ml. of 0·001 M-KCl, the measured chloride content was still 1·03 μmole.

Determination of chloride activity. The samples of axoplasm were placed in a small glass pot whose capacity was about 100 μl., mounted close to a larger vessel containing the reference solution, which was 0·1 M-KCl for one series of measurements and artificial sea water for a second series. Into each pot silver wires were dipped, these being insulated with varnish except at the extreme ends, which were lightly coated with chloride; in order to avoid spurious potentials it was necessary to see that the chlorided tips of the wires were wholly immersed in the axoplasm or the reference solution. The potential between the wires was measured with a 'Vibron' electrometer. If with both wires immersed in the same pot the potential was greater than ±0·2 mV, the wires were cleaned and rechlorided. If with one wire in each pot, the reference solution in both pots, and a capillary bridge filled with saturated KCl solution or 0·6 M-KCl connecting the two pots together, the potential was greater than ±2 mV, the bridge was rejected. After extrusion the axoplasm was immediately transferred to the test pot and the wire was dipped into it; a capillary bridge was then held with one tip in the axoplasm and the other in the reference pot, and the potential was noted. Sometimes the measurement was repeated with a second capillary bridge. When the potential had been measured, which took a minute or two, the axoplasm was removed to a weighing bottle for determination of its chloride content. A similar procedure was followed for measuring chloride activity in certain other solutions, as described later.

Determination of chloride influxes. The method used was similar to that described by Hodgkin & Keynes (1957) for experiments with ^{45}Ca. Axons were dissected in pairs, 10 mm stretches were cleaned at the central ends, the ganglia were tied off and removed, and excitability was checked. One axon was then transferred to inactive artificial sea water containing an inhibitor (0·2 mM dinitrophenol, 2 mM cyanide, or 10^{-5} M ouabain) or to a modified test solution ([K] and [Na] were varied), while the other remained in normal artificial sea water at room temperature. When inhibition was judged to be complete (for times see Results) the axons were placed for exactly 20 min in stoppered tubes containing 4 ml. of similar solutions made up with ^{36}Cl instead of inactive chloride. After exposure to the radioactive solutions the axons were rinsed for 10–15 min in the inactive solutions, their excitability was checked again, and their diameters were measured. Axoplasm was

extruded from the cleaned ends on to glass slides and then transferred to planchettes on which it was spread out to occupy a roughly standard area (about 30 mm²), weighed and allowed to dry at room temperature. For 10 samples which were reweighed after drying the mean dry weight was 12·0 % of the wet weight, so that the water content of axoplasm may be taken as 880 g/kg. Counts were taken with an end-window counter in a Panax low-background assembly. The specific activities of the ^{36}Cl solutions were determined by diluting them by weight about 200 times, weighing out one drop of the diluted standard on to a planchette, and counting the dried drop. Errors from dilution of the ^{36}Cl sea water by inactive sea water transferred into the pots with the nerve trunks were avoided by frequent restandardization; the activity decreased by about 1·7 % for each nerve exposed. Another possible source of error was self-absorption of the 0·7 MeV β-radiation in the dried axoplasm; the counting rate for undried samples was 35 % below the count after drying the axoplasm, so that the self-absorption loss was not greater than 4 %. Owing to the long half-life of ^{36}Cl it was unnecessary to make any corrections for decay of the isotope.

Determination of chloride effluxes. Axons were cleaned, cannulated, and mounted in the cell described by Caldwell, Hodgkin, Keynes & Shaw (1960*a*). The internal chloride was labelled by injecting a 20–25 mm column of 1·0 M-K^{36}Cl from a microsyringe. The microsyringe worked on the same principle as that of Hodgkin & Keynes (1956), but was constructed more simply by using part of a Prior micromanipulator to operate the plunger of a Hamilton microlitre syringe (Model No. 7001 N) which had a length of glass capillary tubing 160 μ in diameter fixed to its needle. The cell was modified to reduce the collecting volume to 0·5 ml., so that the samples could be dried on planchettes for end-window counting. Samples were taken at 20–30 min intervals, and when sampling was complete the whole axon was dried on a planchette, in order to determine the amount of radioactivity remaining inside it.

Solutions. Axons were dissected in sea water, but all the experiments were done in the artificial sea water used by Hodgkin & Keynes (1955*a*), whose composition was (m-mole/l.): NaCl, 486; KCl, 10·4; $CaCl_2$, 10·7; $MgCl_2$, 26·0; $MgSO_4$, 29·2; Na phosphate, pH 7·6, 1·5. When [K] was changed, an equivalent quantity of Na was added or subtracted and the total [Cl] was kept constant at 568 mM. The radioactive solutions were made up from 1·0 and 0·1 M stock solutions and distilled water, added in appropriate quantities with an 'Agla' syringe, each addition being checked by weighing. The ^{36}Cl was obtained from the Radiochemical Centre, Amersham, as 1·0 M-Na^{36}Cl; its specific activity was about 14 μc/m-mole, which was high enough to permit a dilution of 2–4 times with inactive chloride. The pHs of the solutions were checked frequently with a glass electrode, a chlorided silver wire being used as reference electrode; the pH was always within the range 7·2–7·5.

RESULTS

Analyses of the chloride in axoplasm

Values obtained for the chloride contents of extruded axoplasm, expressed in m-mole/kg wet weight, are given in Table 1. The mean for 17 samples (excluding the two figures in brackets) was 108 m-mole/kg, with a standard error of ± 2 m-mole/kg. Taking the water content of the axoplasm as 880 g/kg, the mean value of $[Cl]_i$ was therefore 123 m-mole/kg H_2O. The first few samples were extruded from axons dissected from refrigerated mantles, the process of dissection taking about an hour. It seemed possible that the chloride contents were higher than those reported by Steinbach (1941) either because of a gain of chloride during dissection

or because some hours elapsed between decapitation of the squid on board ship and arrival of the mantle at the laboratory. However, the chloride was no lower in axoplasm extruded directly from refrigerated mantles without first dissecting out the nerve trunk, nor in axons dissected from a squid brought back alive to the laboratory. Even when the liveliest possible squid were used by doing the extrusions on board the collecting ship M.V. *Sarsia* immediately after the trawl had been brought in, and the time between decapitation and extrusion was reduced to about 17 min,

TABLE 1. Chloride contents of squid axoplasm

Date December 1960	[Cl] (m-mole/kg)	Conditions
12	109	Refrigerated mantle; axon dissected
13	104, 99	Refrigerated mantle; axons dissected
	121, 111	Refrigerated mantle; extruded *in situ*
14	122, 102	Living squid; axons dissected
	109, 106	Refrigerated mantle; extruded *in situ*
15	107, 113	Living squid; extruded *in situ*
	114, 109	Living squid; extruded *in situ*
	106, 105	Living squid; extruded *in situ*
16	89	Refrigerated mantle; axon dissected and left for 5 hr in sea water
	[95]	Same mantle; axon dissected and left for 5 hr in nitrate solution
	107	Refrigerated mantle; axon dissected and left for 5·8 hr in sea water
	[83]	Same mantle; axon dissected and left for 5·8 hr in nitrate solution
Mean and S.E.	108 ± 2	

the chloride content of the axoplasm was still uniformly more than 100 m-mole/kg. Another possible reason for the disagreement with Steinbach's (1941) values would be species variation, since he used *Loligo pealii* and not *L. forbesi*. However, recent analyses of axoplasm from *L. pealii* have given even higher chloride values than those in Table 1, for Koechlin (1955) found 140 m-mole/kg and Deffner (1961) found 151 m-mole/kg; Deffner also reported 150 m-mole/kg for axoplasm from squid of another family, *Dosidicus gigas*.

Two of the axoplasm samples listed in Table 1 were extruded from axons which had been soaked for several hours in a chloride-free medium made up by using nitrate instead of chloride salts in the artificial sea water (for composition see p. 693). Again in contradiction with Steinbach's observations, this treatment did not result in a rapid loss of chloride from

the axoplasm. Comparison of the two figures with the over-all mean for untreated axons suggests the occurrence of a slow chloride leakage of the order of 3 m-mole/kg.hr, which would not be incompatible with the effluxes of labelled chloride discussed on p. 701.

Methods for estimation of chloride based on the insolubility of AgCl might give misleading results if the axoplasm contained any other anion capable of forming an insoluble silver salt. As mentioned on p. 692, it was verified that glutamate, aspartate and isethionate did not interfere with the analytical technique, but the other constituents of axoplasm were not tested. However, the good agreement between the results of Koechlin (1955) and those of Deffner (1961) suggests that no other interfering substances are present in any quantity, since although the former estimated chloride gravimetrically as AgCl, the latter used Conway's (1935) micro-diffusion method, which depends on a wholly different principle. The only previously recorded chloride analyses for *L. forbesi* are those of Keynes & Lewis (1951), who found 72 m-mole/kg in unstimulated axons and 89 m-mole/kg in stimulated ones, from counts of the ^{35}S formed from ^{35}Cl in neutron-irradiated axoplasm samples. These values are somewhat lower than those now reported, but they were only obtained as a by-product of an investigation primarily concerned with the sodium and potassium in cephalopod axons, and were not claimed to be very reliable.

The activity of the chloride in axoplasm

One explanation for these unexpectedly high chloride figures would be that a substantial proportion of the chloride in axoplasm is not in free solution but is 'bound' in some sort of complex. This possibility was examined by making direct measurements of the activity coefficient of the Cl^- ions in extruded axoplasm with reversible Ag–AgCl electrodes. The experimental procedure has been described on p. 692, and the results are presented in Table 2. In the first set of measurements the reference solution was 0·1 M-KCl, and the capillary bridges were filled with saturated KCl solution in order to minimize liquid junction potentials. The figures obtained for γ_{Cl} in 1·0 and 0·15 M-KCl were not far from correct, while in axoplasm the activity coefficient was certainly not low as it would have been had much of the chloride been bound, but was actually somewhat higher than might have been expected for complete ionization of the chloride. In the second set of measurements artificial sea water was used as reference solution, so that the potentials could more easily be compared with those obtained by Mauro (1954) for intact axons, and the capillary bridges were filled with 0·6 M-KCl in order to reduce possible errors arising from diffusion of chloride into the small puddle of axoplasm. The disadvantage of this procedure was that the liquid junction potentials in the system could no

longer be neglected. Taking γ_{Cl} as 0·64 in artificial sea water (a.s.w.), the potential difference calculated for 0·1 M-KCl is +39·0 mV as compared with the observed +32·6 mV, which was the mean of several determinations. This suggests that $E_{0\cdot6\text{ M-KCl}} - E_{\text{a.s.w.}}$ was −6·4 mV, which agrees within the accuracy of the measurements with the value of −4 mV obtained by Baker, Hodgkin & Shaw (1962). However, errors from uncertainty as to the exact value of this junction potential could be avoided by subtracting the 32·6 mV from each of the potentials recorded for the

TABLE 2. Chloride activity of extruded axoplasm

Reference solution	Test solution	[Cl] (m-mole/kg H_2O)	Potential (mV)	γ_{Cl}
0·1 M-KCl	1·0 M-KCl	1032	−50·9	0·57
	0·15 M-KCl	151	− 8·0	0·70
	Axoplasm	142	−10·2	0·82
	Axoplasm	139	−11·2	0·87
	Axoplasm	153	−12·6	0·83
Artificial sea water	0·10 M-KCl	100	+32·6	(0·77)
	0·15 M-KCl	151	+22·9	0·75
	Axoplasm	133	+30·0	0·65
	Axoplasm	151	+23·0	0·75
	Axoplasm	132	+14·0	1·22
	Axoplasm	162	+25·0	0·64
	Axoplasm	172	+19·0	0·77

The values of γ_{Cl} in the test solutions were calculated from the potentials (in mV) relative to 0·1 M-KCl, in which the activity coefficient was taken to be 0·770 (Conway, 1952), by means of the relation

$$\frac{\text{Potential}}{58} = -\log_{10}\frac{[\text{Cl}]\times\gamma_{Cl}}{100{\cdot}4\times0{\cdot}770}.$$

In 1·0 M-KCl the correct value for γ_{Cl} at 20° C is 0·602; in 0·15 M-KCl it is 0·736.

axoplasm samples, so as to obtain, as before, the potential relative to 0·1 M-KCl. The resulting value for γ_{Cl} in 0·15 M-KCl was again almost correct, while the mean value for the five samples of axoplasm was 0·81. Some doubt remains as to the error introduced by the junction potential between the axoplasm and the 0·6 M-KCl in the bridge, but if $E_{0\cdot6\text{ M-KCl}} - E_{\text{axoplasm}}$ is taken as +4 mV, the mean value of γ_{Cl} is only reduced to 0·69. The second set of measurements thus agrees with the first in showing that there can be no appreciable binding of chloride in squid axoplasm.

As has already been mentioned, Mauro (1954) measured the potential between chlorided silver wires inside and outside intact squid axons, and reported that the internal potential was then about −35 mV. This represents the value of the resting membrane potential plus the difference in chloride activity between axoplasm and sea water, which from the last five lines in Table 2 averaged 22 mV plus a correction of the order of 8 mV for junction potentials. From these figures the size of the resting potential recorded with an internal electrode containing 0·6 M-KCl, which would be less than the true value by the same 8 mV junction potential correction,

would be expected to be -57 mV. This is reasonably close to what is normally seen in a freshly dissected axon, so that Mauro's (1954) observations on whole axons and those described here on extruded axoplasm are in tolerable agreement.

The influx of labelled chloride

Since the chloride in the axoplasm did not appear to be bound to any measurable extent, the next step was to use ^{36}Cl to see whether any evidence could be obtained for or against the occurrence of an 'active' uptake of chloride. The influx of chloride into the axons was measured by soaking them in solutions containing ^{36}Cl for exactly 20 min and then counting weighed samples of extruded axoplasm. This procedure avoided errors from extracellular radioactivity, but had the disadvantage that each axon provided only one value for the influx; the axons were therefore dissected in pairs from the same squid, and one was used as a control while the other was treated with an inhibitor. In order to make sure that inhibition was complete, the treated axons were exposed to the inhibitors in inactive solutions for ample periods (1·3–3 hr for DNP and CN, 0·4–1·2 hr for ouabain) before transferring them to the labelled solutions; during this pre-treatment the control axons were left in normal artificial sea water. Since whole nerve trunks were used, no attempt being made to clean the giant axon except over a few millimetres at the end from which the axoplasm was extruded, the measured influxes may have been slightly smaller than those that would have been obtained with cleaned axons. However, access of external ions to the surface of the giant axons does not seem to be greatly slowed by the presence of the small fibres, as is witnessed by the fact that removal of external sodium blocks conduction within 2 or 3 min, and in any case both control and treated axons would have been equally affected.

The first inhibitor to be tested was dinitrophenol, which at a concentration of 0·2 mM and pH around 7 effectively blocks both sodium efflux and potassium influx in squid axons within an hour of its application (Hodgkin & Keynes, 1955*a*; Caldwell, Hodgkin, Keynes & Shaw, 1960*b*). As the results in Table 3 show, treatment with 0·2 mM DNP consistently reduced the chloride influx by about half. Either comparison of the overall mean for the nine treated axons with that for the eight control axons, or examination of the mean influx ratio for eight pairs of axons, shows that the effect of DNP was highly significant ($P < 0{\cdot}01$). The action of DNP could either indicate some form of linkage between chloride influx and active transport of sodium and potassium, or could arise from the existence of separate transport mechanisms both dependent on metabolism. The effect of 0·01 mM ouabain, which rapidly blocks the sodium

efflux from squid axons (Caldwell & Keynes, 1959) without interfering with metabolism, was therefore investigated. As may be seen in Table 3(*c*), a clear negative answer was obtained, and ouabain did not significantly change the chloride influx. Thus the mean influx for ouabain-treated

TABLE 3. Effect of inhibitors on influx of labelled chloride

	Control axon		Treated axon		Influx ratio
	Diameter (μ)	Cl influx (pmole/cm².sec)	Diameter (μ)	Cl influx (pmole/cm².sec)	treated:control
		(*a*) Effect of 0·2 mM-dinitrophenol			
	745	12·7	762	7·2	0·57
	830	20·3	773	10·9	0·54
	—	—	682	9·1	—
	648	18·6	659	8·3	0·45
	564	33·3	534	19·0	0·57
	777	19·2	709	5·6	0·29
	750	25·2	670	5·9*	0·23
	733	26·3	728	9·0*	0·34
	738	19·4	732	7·8*	0·40
Mean	723	21·9 ± 2·2	694	9·2 ± 1·3	0·42 ± 0·05
		(*b*) Effect of 2 mM-CN			
	740	26·3	668	23·1	0·88
	793	41·5	740	55·0*	1·32
	745	21·4	690	18·3*	0·86
	650	24·4	650	6·9	0·28
			728	15·1	
			738	19·0*	
			733	9·1	
			733	8·6	
			800	17·7	
			792	7·9	
Mean	732	28·4 ± 4·5	730	17·4 ± 4·1	0·84 ± 0·21
		(*c*) Effect of 0·01 mM ouabain			
	693	27·6	670	15·6	0·56
	795	12·8	795	10·2	0·80
	727	12·0	761	13·3	1·11
	754	14·4	790	12·6	0·87
	733	26·4	723	16·4	0·62
	683	24·2	767	31·1	1·28
	820	30·1	960	28·7	0·95
	720	22·9	688	22·6	0·99
	835	20·3	838	30·3	1·49
Mean	751	21·2 ± 2·2	777	20·1 ± 2·9	0·96 ± 0·03

All the axons were still excitable over the whole of their length immediately before extruding the axoplasm, except those marked with an asterisk which only gave spikes over their central portions. The S.E. of some of the means is given. Temperature 17–22° C.

axons was close to the over-all mean for untreated axons, and the influx ratio for paired axons was close to unity. This finding suggests that Cl^- ions are not dragged inwards by the sodium pump, and that there is a separate channel for active inward transport of chloride.

In an attempt to distinguish between a special effect of DNP on chloride

influx and a general dependence on cellular metabolism, the effect of 2 mM-CN was also investigated, with results that were somewhat unsatisfactory because of their scatter. Both the over-all mean for CN-treated axons shown in Table 3(*b*) and the mean influx ratio for paired axons would suggest that cyanide did not have a significant effect, but this conclusion is greatly influenced by one treated axon which gave a particularly high chloride influx, and which ought possibly to be disregarded on the grounds that it was not excitable over the whole of its length. If the figures for this axon and two others in similar condition are rejected, the mean for CN-treated axons becomes $12{\cdot}6 \pm 2{\cdot}3$ pmole/cm^2.sec, which is significantly ($P < 0{\cdot}01$) less than the over-all mean for all untreated axons ($22{\cdot}8 \pm 1{\cdot}6$ pmole/cm^2.sec). However, the validity of this argument is uncertain, since three of the DNP-treated axons which were also not in perfect condition showed no sign of a raised influx, so that no firm conclusion can be reached on this point.

TABLE 4. Effect of changes in sodium and potassium concentration on influx of labelled chloride

	Axon 1		Axon 2	
	Diameter (μ)	Cl influx (pmole/cm^2.sec)	Diameter (μ)	Cl influx (pmole/cm^2.sec)
	(*a*) Axon 1 in K-free solution; axon 2 with 5 × normal [K]			
	738	47·5	760	51·3
	713	45·6	708	79·5
	950	39·8	943	78·8
Mean	800	44·3	804	69·9
	(*b*) Axon 1 with normal [Na]; axon 2 with $\frac{1}{4}$ × normal [Na]			
	564	25·9	615	25·6
	710	31·0	715	28·4
Mean	637	28·5	665	27·0

Another factor which might have been expected to alter the chloride influx had there been a linkage with the sodium pump was the external potassium concentration. Table 4(*a*) gives the influxes for three axons exposed to a K-free solution while paired axons were treated with 5 times normal [K]. Although more experiments would be desirable, it seems clear that the absence of external potassium raises rather than cuts down the influx, which confirms the separateness of the mechanism for active transport of chloride. In the K-rich solution the influx was evidently raised still further; the implications of this result are uncertain. Finally, Table 4(*b*) shows that when three quarters of the external sodium was replaced with choline, the chloride influx was unaltered. The object of this test was to examine the possibility, suggested by Shanes (1958), that an inward transport of Cl^- ions against their electrochemical gradient might

take place through the existence in the membrane of a mechanism which would permit the entry of neutral ion pairs of, say, Na^+ and Cl^-, thus allowing chloride to be moved uphill at the expense of the sodium concentration gradient. The insensitivity of the chloride influx to changes in external [Na] provides some evidence against this idea, but in the absence of any information as to the probable cation selectivity of an ion pair transport channel certainly does not rule it out conclusively.

The efflux of labelled chloride

The existence of a DNP-sensitive component of chloride influx will only account satisfactorily for the internal accumulation of chloride to a level higher than that corresponding to a passive electrochemical equilibrium if the action of DNP is shown to be asymmetrical and to have little or no effect on the chloride efflux. That this was indeed the case may be seen in Fig. 1, which illustrates measurement of the chloride efflux from an axon

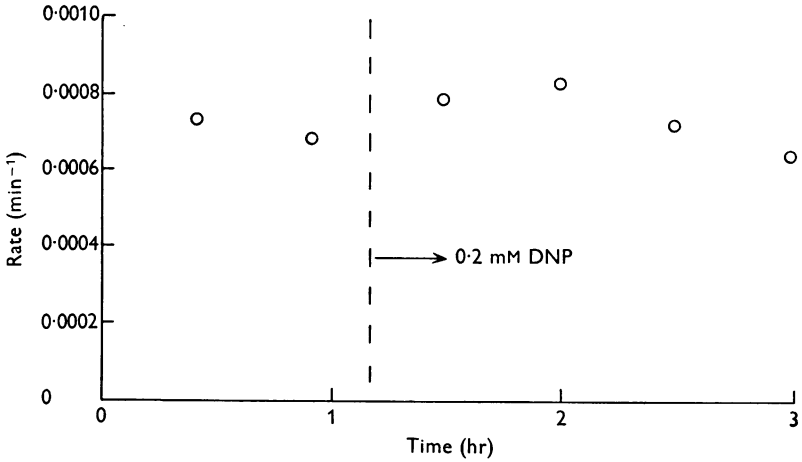

Fig. 1. Rate constant for loss of radioactivity from a squid axon loaded at zero time with a column of 1·0 M-K ^{36}Cl 23 mm in length; axon diameter 619 μ; temperature 17° C. At the end of the experiment the axon was still excitable over the whole of its length.

TABLE 5. Rate constants for efflux of labelled chloride from squid axons loaded by micro-injection

Axon diameter (μ)	Temperature (° C)	Rate constant (min^{-1})
742	20	0·00113
755	20	0·00037
716	18	0·00028
619	17	0·00071
Mean 708		0·00062

loaded with ^{36}Cl by micro-injection. There was no obvious change in efflux when the axon was treated with 0·2 mM DNP, either on this occasion or in another similar experiment. In two further experiments, treatment with 0·01 mM ouabain and 52 mM-K respectively had equally little effect. The mean rate constants for loss of ^{36}Cl in the four efflux experiments are listed in Table 5. Taking $[Cl]_i$ as 108 m-mole/kg axoplasm or 114 m-mole/l. axoplasm, the mean volume:surface ratio ($= \frac{1}{4} \times$ diameter) as 177 μ, and the rate constant as 0·00062 min^{-1} or $1{\cdot}03 \times 10^{-5}$ sec^{-1}, the mean chloride efflux would be 20·8 pmole/cm^2.sec. This estimate is close to that for chloride influx, as it should be if the dissected axons are nearly in a steady state as far as their internal chloride is concerned.

DISCUSSION

The experiments described here appear to establish clearly that the concentration of chloride ions in the axoplasm of the squid giant axon is two or three times greater than would be expected for a simple electrochemical equilibrium, and that this high value of $[Cl]_i$ results from the operation of a mechanism for uphill inward transport of chloride. It may be asked, first, whether the active uptake of chloride is purposeful, in the sense that a high $[Cl]_i$ can be shown to be functionally important, or whether it is merely accidental, being a secondary consequence of a linkage between chloride influx and the movements of some other ion. A possible advantage of a high intracellular chloride concentration would be the resulting increase in the specific conductance of the axoplasm and hence in the velocity of conduction of impulses. However, such an effect would only confer a marginal advantage on the axon, since the conductance increase on substituting 70 mM-Cl^- for an equivalent quantity of immobile anions would not be more than about 20%, so that the velocity, which is proportional to the square root of the conductance (Hodgkin, 1954), would rise by less than 10%. Another kind of way in which a difference between E_{Cl} and E_m could be advantageous would be through the existence somewhere on the axon or its cell body of synaptic junctions depending for their operation on changes in the chloride conductance of the post-synaptic membrane. The difficulty in accepting this idea is that, although synaptic mechanisms involving increases in chloride conductance have indeed been proposed (see Eccles, 1961), they have generally been invoked to account for the generation of inhibitory post-synaptic potentials, and there is no evidence that there are any inhibitory pathways in the stellate ganglion of the squid (see Bullock, 1948; Hagiwara & Tasaki, 1958). Moreover, if, as in the squid axon, E_{Cl} is substantially less than E_m, a local increase in chloride conductance will have a depolarizing and therefore excitatory effect rather than an inhibitory one. Nevertheless, there does

not seem to be any evidence that definitely rules out the possibility that in situations where $E_{Cl} < E_m$ there may be excitatory synapses operating through an increase in chloride conductance.

The lack of effect of ouabain seems clearly to exclude a coupling between chloride influx and the sodium pump, and the remaining alternatives are that Cl^- ions are pumped inwards by an independent mechanism or that they enter the axon through ion pair formation with Na^+. The effect of DNP would be compatible with the existence of a separate chloride pump dependent on a supply of phosphate-bond energy, but the rather inconclusive experiments with CN do not support this idea as well as one could wish. All the same, there are known to be mechanisms for active transport of chloride elsewhere, for example in the gastric mucosa, so that there is ample precedent for proposing the existence of a chloride pump in nerve membranes. Ion pair formation remains as an attractive possibility, subject to the objections that changes in $[Na]_o$ apparently had no effect on the chloride influx and that DNP would then have to be regarded as having a side effect on chloride transport not connected with its usual uncoupling action on oxidative phosphorylation.

The second question raised by these findings is that of their extension to other excitable tissues. For cardiac muscle fibres there is evidence (Lamb, 1961) that $[Cl]_i$ is somewhat higher than would be predicted from the value of E_m, but this conclusion might be altered if the chloride were apportioned between two compartments in addition to the extracellular space, as has been proposed for frog muscle (Harris, 1963), in a scheme which does not, however, suggest that chloride distribution is affected by forces other than straightforward electrochemical gradients. In considering the possible biological function of chloride movements it is clearly important to know the absolute value of E_{Cl} in relation to E_m, but the difficulty arises that after analysing any type of nerve or muscle fibre other than the squid giant axon it is necessary to make such large allowances for extracellular chloride that direct determination of the true value of $[Cl]_i$ becomes very uncertain, except when it has been raised by exposure to K-rich solutions. This is particularly true for myelinated nerve fibres, where lack of information as to the chloride content of the myelin sheath causes even greater uncertainty in arriving at a reliable value for $[Cl]_i$ in the axis cylinder. Until high-resolution chloride determinations can be made for frozen-dried tissue sections by techniques such as secondary X-ray microanalysis it will be hard to decide conclusively whether or not an intracellular chloride concentration high enough to make E_m substantially greater than E_{Cl} is a phenomenon peculiar to the squid giant axon or occurs more generally.

Finally, since many more values for chloride influx and efflux were

obtained in the course of these experiments than were reported by Caldwell & Keynes (1960), it seems worth while to repeat one or two of their calculations. In the first place, if the potassium efflux is taken from their paper as 38 pmole/cm².sec and $[K]_i$ as 370 m-mole/l. axoplasm or 400 m-mole/kg H_2O, and if the passive component of the chloride influx is (from Table 3) 9·2 pmole/cm².sec for $[Cl]_o$ = 568 mM, then from the constant-field relation (Hodgkin & Katz, 1949; Keynes, 1951) the ratio of P_K to P_{Cl} is 5·9. Taking the potassium influx into CN-treated uncleaned axons as 9·3 pmole/cm².sec (Caldwell *et al.* 1960*a*) for $[K]_o$ = 10·4 mM, and the chloride efflux as 20·8 pmole/cm².sec for $[Cl]_i$ = 123 m-mole/kg H_2O, the ratio of P_K to P_{Cl} is 5·3. These two estimates for the permeability ratio are in good agreement, and although the mean value of 5·6 is higher than the tentative figure of 2·2 used by Hodgkin & Katz (1949) it is probably in line with the recent studies of Baker *et al.* (1962) on perfused axons, which indicated that P_K is substantially greater than P_{Cl}. In order to account for an apparent fivefold discrepancy between the chloride conductance of the membrane calculated from the chloride fluxes and the electrical leak conductance as estimated by Hodgkin & Huxley (1952), Caldwell & Keynes (1960) suggested that the chloride fluxes might display some degree of non-independent behaviour of the 'single file' type (Hodgkin & Keynes, 1955*b*). However, this suggestion is not borne out by the figures now available, since the ratio of the chloride efflux to the passive influx is 2·3, which is below rather than above the flux ratio of 2·8 calculated from Ussing's (1949) independence relation for $E_m - E_{Cl}$ = 65 − 39 = 26 mV. It would therefore seem that some membrane current may be carried by anions other than chloride.

SUMMARY

1. Analyses of extruded squid axoplasm showed that even in the freshest material the chloride content was over 100 m-mole/kg wet weight. The over-all mean and its S.E. for 17 samples were 108 ± 2 m-mole/kg.

2. This intracellular concentration of chloride is well over twice that expected for a simple passive distribution between axoplasm and body fluids. The discrepancy could be explained either by binding of chloride in the axoplasm or by the occurrence of an 'active' uptake of chloride by the axon.

3. The activity coefficient for the chloride in axoplasm samples was determined with Ag–AgCl electrodes as being in the neighbourhood of 0·7. This seems close enough to the free solution value to rule out any substantial binding.

4. The existence of a mechanism for uphill inward transport of chloride

was supported by the demonstration that treatment with 0·2 mM dinitrophenol halved the influx of labelled chloride. The action of 2 mM cyanide was also tested, but the results were excessively scattered.

5. The influx of chloride does not seem to be linked to the transport of cations by the sodium pump, since it was not affected by 10^{-5} M ouabain and raised rather than lowered in a K-free solution.

6. The average rate constant for the efflux of labelled chloride was 0·00062 min^{-1}. The efflux was unaltered by treatment with dinitrophenol, cyanide or K-rich solutions.

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