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EXPERIMENTS ON THE INJECTION OF SUBSTANCES INTO SQUID GIANT AXONS BY MEANS OF A MICROSYRINGE

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Many instructive experiments on cell physiology could be performed if it were possible to change the internal composition of cells as readily as their external environment. Studies like those of Flynn & Maizels (1949), on human erythrocytes, and of Desmedt (1953), on frog muscle, show that this is particularly true of problems connected with the ionic permeability of cell membranes, these authors having obtained interesting results by examining the behaviour of cells whose intracellular ionic concentrations had been altered by prolonged exposure to abnormal media. However, such a technique is only practicable in the case of substances which penetrate the membrane with relative ease. An alternative approach which does not suffer from this limitation is to use the method of microinjection. Two types of microinjector have been described—one depending on the ejection of small volumes of fluid from very fine micropipettes by applied pressure (e.g. Chambers & Pollack, 1927; Heilbrunn & Wiercinsky, 1947; Arvanitaki & Chalazonitis, 1951; Grundfest, Nachmansohn, Kao & Chambers, 1952); and the other on the discharge of ions from similar micropipettes by electrophoresis (Nastuk, 1953; del Castillo & Katz, 1955; Niedergerke, 1955; Coombs, Eccles & Fatt, 1955). Both these methods can be used for producing local concentration changes close to the tip of the microinjector, but they are unsuitable for introducing substances at a uniform concentration into large cells. The purpose of this paper is to describe some preliminary work with a new microinjection technique which enables accurately known volumes of fluid to be injected into a precisely defined region of a squid giant axon.

When a 100 μ capillary filled with fluid is pushed down the centre of a fresh 600 μ axon, pressures up to 200 mm Hg can be applied without discharging the fluid (Hodgkin & Katz, 1949*b*), because the axoplasm behaves as a fairly firm gel. The fluid can be forced into the axoplasm with sufficiently high

pressures, but this is liable to damage the axon, and the extent of the flow is somewhat indeterminate. Our new method is based on the principle of applying pressure while at the same time withdrawing the capillary from the axon; it is then relatively easy to fill with fluid the space being vacated by the tip of the capillary. As will be seen, this was done by constructing a type of microsyringe which automatically ejected a volume of fluid equal to its own external volume when it was being raised out of the axon.

The first experiments to be considered here were concerned with the injection of strong dye solutions into squid axons, and were primarily designed to test the performance of the microsyringe. After establishing that reproducible injections could be made, we spent a short time studying the effect on the resting and action potentials (recorded with a normal internal electrode) of injecting sodium, potassium, calcium, magnesium and tubocurarine chlorides, partly in order to check on the observations reported by Grundfest *et al.* (1952) and Grundfest, Altamirano-Orrego, Kao & Nachmansohn (1953). Finally, a number of experiments were done with injected ^{24}Na. These were preliminary in nature, but served to establish certain points about the behaviour of the sodium efflux which could not have been settled by other methods. Since the original experiments were done, the method has proved useful in a study of the calcium permeability of squid axons (Flückiger & Keynes, 1955), but most of the obvious lines of advance which it opens up still remain to be exploited.

METHOD

The construction of the microsyringe

A diagram illustrating the principle on which the microsyringe worked is shown in Fig. 1. A long (10–15 cm) uniform length of thin-walled glass tubing, the 'inner', 90–110 μ in external diameter, was fitted very closely into a shorter length of slightly larger tubing, the 'outer'. Both tubes were filled with water, and the inner was open at both ends, while the outer was sealed off at the top. The inner was held in a relatively wide glass tube filled with mercury, and the outer was fixed above it in such a position that the junction of the two capillaries was always beneath the surface of the mercury. The whole assembly was mounted on a Palmer stand so that the inner and outer could be raised and lowered together without changing their relative positions, while the wide tube holding the inner could also be moved up and down independently by means of a Palmer rackwork X-block, graduated in mm. The injector was filled by lowering the inner relative to the outer, and emptied by the reverse movement. Its satisfactory working depended on a really close fit between the capillaries; when the annular gap at their junction was only a few microns wide, surface tension forces enabled considerable pressures to be exerted. The mercury in the wide tube had the object of increasing these forces; at the same time it prevented evaporation at the junction and kept it free from dust.

In order to achieve a tight fit of inner and outer, and yet to allow them to slide relative to one another over a distance of about 35 mm without danger of jamming, it was essential for the diameter of the inner to be constant within rather narrow limits. The inners were therefore carefully selected from long lengths drawn down from 2 mm soft glass tubing (hard glass proved quite unsuitable for this purpose). The outers had to be accurately circular in cross-section, but did not need to be uniform in diameter. The operation of fitting inners and outers together was done under water, as follows: Using an outer with an appreciable taper, somewhat less than 90 μ in diameter

at the narrow end, an inner was inserted at the wide end, and pushed gently down until its tip stuck. The inner was then withdrawn a short distance, and the outer was scratched with a carborundum stone just above the place which the tip had reached, and broken off cleanly. The inner could now be pushed right through, and the wide end of the outer sealed off in a microflame (keeping the lower end of the inner under water). If, on removal of the matched capillaries from

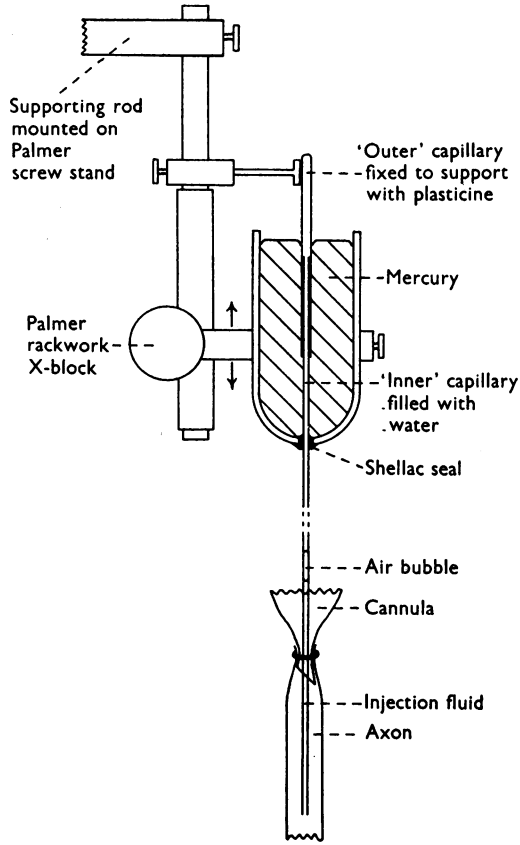

Fig. 1. Diagram (not to scale) of the microsyringe.

the dish of water, an air bubble could be sucked smoothly and in a dead-beat fashion up and down the inner, they were acceptable for final mounting in the rackwork assembly. The inner was sealed into the wide tube with shellac, this tube was mounted on its X-block, and the upper end of the outer was fixed with plasticine. After making a careful note of the limits between which it was safe to raise and lower the inner without either breaking the outer against the shellac seal or allowing the junction to emerge above the surface of the mercury, the wide tube was filled with mercury, and the microsyringe was ready for use.

Calibration of the microsyringe

In order to guard against back-lash arising from leakage of fluid at the junction between inner and outer (which did give some trouble in the first few microsyringes to be built), a small bubble of air was always sucked up before filling the inner with the test solution. By making certain that

this bubble returned exactly to the tip of the inner when the injection was complete, or (in a few early experiments) by making a proportional correction if it did not, satisfactorily reproducible results were obtained. In all the later experiments, the total distance through which the bubble moved was measured for each injection; it was about three times the relative movement of inner and outer. The exact volume delivered by the microsyringe was determined by filling it with ^{24}Na sea water of known specific activity, discharging its contents into sea water, and taking counts with a liquid counter in the usual way. For the two microsyringes used for the majority of the experiments, the average of duplicate measurements of this type agreed to within 1% with the volume calculated from the product of the distance travelled by the air bubble and the cross-sectional area of the inner, measured optically after breaking it off at several places. This agreement was, no doubt, fortuitously close, and the 'tracer' volume did not fit quite as well with the volume calculated from the product of the distance moved by the inner relative to the outer, and the external cross-sectional area of the top end of the inner. Thus the calculated volumes differed by −1, +8 and −23% from the tracer volumes, in three microsyringes for which the comparison was made. There did not appear to be a systematic discrepancy between the different methods of arriving at the volume delivered, and the tracer volume was therefore used for the three microsyringes for which it was available (covering by far the greater number of the experiments), while the latter method of working out the volume was used for a few early injectors.

Experimental procedure

Giant axons were dissected in cold sea water from the hindmost stellar nerve of *Loligo forbesi*, and carefully freed from small nerve fibres. They were cannulated (Hodgkin & Huxley, 1945) and transferred (for electrical experiments) to the recording cell described by Hodgkin & Katz (1949*a*). For the experiments with ^{24}Na a smaller cell was used, identical in principle, but scaled down so that the total volume of fluid surrounding the upper part of the axon was reduced to 6 ml. A 100 μ microelectrode (Hodgkin & Katz's Type *c*) was first inserted into the axon in the usual way, and the sizes of resting and action potentials determined at several positions of the tip, down to a depth of about 35 mm. The microelectrode was then removed, and the microsyringe, previously loaded with a test solution, was lowered into the cannula until the tip of the inner was just visible at the top of the axon. The shaft of the inner was now steered down the centre of the axon in the normal fashion, the cannula being raised steadily with the microsyringe at a fixed height, while the operation was watched with a binocular dissecting microscope and a 45° mirror. As there was already a track down the axon, this second insertion could be done relatively quickly. When the tip had reached the desired depth (usually about 5mm above the lowest point reached by the microelectrode), a column of fluid 3–20 mm in length was injected by smoothly raising the inner on its rackwork X-block, keeping the outer and the axon fixed. On completion of the injection, the tip of the inner was still 10–25 mm from the upper end of the axon; the final stage of removing the injector was therefore done by raising inner and outer together, using the Palmer stand. In the experiments with dyes, photographs of the injected column were taken as soon as the microsyringe had been removed. In the experiments concerned with the action of injected substances on the membrane potentials, a microelectrode was re-introduced into the axon, and lowered to the injected patch within 2 or 3 min of the time of injection. Sometimes several further injections were made into the same axon, the injector and the microelectrode being inserted alternately. In the tracer experiments, collection of the ^{24}Na appearing in the inactive solution outside the axon was begun as soon as the injector had been removed.

When recording action potentials, the axon was stimulated at about 40 per min between two platinum wires in the lowest, oil-filled, part of the cell. Recording methods were as described by Hodgkin & Katz (1949*a*). In many experiments no photographic records were taken, but a backing-off potential was applied from a voltage calibrator connected in series with the external Ag–AgCl electrode in the cell, and the voltage necessary to bring the steady potential or the peak of the spike back to zero level was measured directly.

Solutions

Dissection of the axons was done in fresh sea water, cooled to about 5° C. During the experiments, the recording cell was filled with an artificial sea water whose composition is given by Hodgkin & Keynes (1955). When dinitrophenol, cyanide or tubocurarine chloride were dissolved in the artificial sea water, the solution was carefully neutralized so that its pH (determined with bromthymol blue) was never far from 7. The solubility of the tubocurarine chloride was such that although a 1% solution could readily be made up in distilled water, the greatest amount which would dissolve in 0·57 M-KCl (as used for injection) corresponded to about 0·2%. For purposes of injection, the following solutions were taken to be isotonic with the artificial sea water: 0·57 M-NaCl, 0·57 M-KCl, 0·37 M-$CaCl_2$, 0·37 M-$MgCl_2$. The ^{24}Na samples were irradiated in the Harwell pile as 'Analar' sodium bicarbonate; they were then dissolved in a slight excess of 2 N-HCl, evaporated to dryness in a weighed silica crucible over calcium oxide at 100° C, and made up to a strength of 0·57 or 1·0 M in a 1·5 mM solution of sodium phosphate buffer, pH 7·6. Their final pH was always close to 7.

RESULTS

Injection of dyes

A microsyringe working on the principle just outlined should deliver a volume of fluid exactly equal to the volume vacated by the inner as it is raised out of the axon. In order to make certain that the apparatus was performing up to expectation, some tests were made with dye solutions, which enabled the precise location of the injected fluid to be checked visually. The high concentration of dye needed for this purpose made the fibres inexcitable, but the experiments served a useful function in showing that the contents of the syringe were introduced uniformly into the region of axoplasm which it was intended to inject. They also provided verification that there was no serious tendency, as there might have been had the inner not been properly uniform, for any of the test solution to be forced up the outside of the inner during its withdrawal from the axon in the act of injection.

Pl. 1 shows several series of photomicrographs taken in the course of experiments in which dyes were injected. Pictures A to E were taken with a 59 mm objective, while a 0·5% solution of methylene blue in isotonic KCl was injected. In A the shaft of the microsyringe is seen immediately before the injection, filled to the tip with dye solution. B was taken 15 sec after making the injection, and the demarcating air bubble can be seen close to the tip; the dye forms a fairly sharply defined patch in the centre of the axon. C was taken 57 sec after the injection; the microsyringe has now been removed, and the dye has begun to spread perceptibly towards the edges of the axon. In D (120 sec) the diffusion has made further progress, and at 430 sec the dye appeared to the naked eye to be distributed uniformly. The photograph taken at this time (E) still shows a faint dark streak along the axis. This almost certainly does not represent a concentration of methylene blue, since it is visible (though less conspicuous) in the part of the axon which had not been injected, but which had been penetrated by the microelectrode. The probable

cause of the streak is that the fluid left in the track of the microelectrode scatters less light than the undisturbed axoplasm.

The diffusion process is shown more clearly in the other photomicrographs of Pl. 1, which were taken with a 32 mm objective (overall magnification four times greater). It will be observed that whereas both methylene blue and eosin diffused readily through the axoplasm, the dye aniline blue did not, but formed an intense streak of colour which was virtually the same width after 10 min as it had been after 35 sec. It seems probable that the aniline blue somehow precipitated or combined with the axoplasm, so forming a barrier to further diffusion, but the precise reason for its different behaviour was not discovered. It cannot have been a question of the net charge on the dye molecule, since methylene blue and eosin both diffused fairly fast, despite their opposite charges, the former being basic like aniline blue, and the latter acidic. Possibly the effect was simply one of molecular size, the order of molecular weights of the three dyes being aniline blue > eosin > methylene blue. This order fits with the results of an experiment in which the rates of penetration of the same dyes into a 10% gelatine gel (in 0·5 M-KCl) were compared qualitatively; but in this test the aniline blue did diffuse some distance into the gelatine, although it certainly moved very much more slowly than either methylene blue or eosin. Another possibility is that there were impurities in the sample of aniline blue, which was a commercial product and may have contained traces of powerful protein precipitants such as zinc.

In order to make rough estimates of the numerical value of the diffusion coefficient, D, for methylene blue and eosin, the following procedure was adopted: The law governing the concentration, c, of dye in the axoplasm during the initial phase of the diffusion process after introducing a quantity Q per unit length as an instantaneous line source along the axis at time $t=0$ can readily be shown (see Carslaw & Jaeger, 1947, p. 218) to be

$$c=\frac{Q}{4\pi Dt}\mathrm{e}^{-(x^2+y^2)/4Dt}, \tag{1}$$

where x and y are the distances from the axis in Cartesian co-ordinates. The total amount of dye in a plane passing through the axon is

$$\int_{-\infty}^{+\infty} c\,\mathrm{d}y=\frac{Q}{2\sqrt{\pi Dt}}\mathrm{e}^{-x^2/4Dt}, \tag{2}$$

and this was taken to be linearly related to the optical density of the photomicrographs (an approximation which received some justification from the fact that the integrated density across the patch of dye did not change much while it broadened). This solution applies only to the period before the concentration at the cell boundary becomes appreciable, and it may be noted that it is the same as the solution for an instantaneous plane source because

the method of measurement involves summation along a series of planes parallel to the direction of observation. It follows that the distance ($x_{\frac{1}{2}}$), at which the density is half as great as it is on the axis, increases with time according to the relation

$$x_{\frac{1}{2}}^2 = 2{\cdot}77Dt. \tag{3}$$

Densitometer readings were made at a number of points across each of the first three photomicrographs taken after injecting the dyes, and $x_{\frac{1}{2}}$ was determined as 78, 119 and 164 μ respectively 3, 20 and 60 sec after injection of methylene blue, and as 87, 115 and 135 μ respectively 10, 35 and 60 sec after injection of eosin. These figures could not be substituted directly in equation (3), since it was necessary to make some allowance for the fact that the dyes were really introduced as cylindrical rather than as line sources. This was done by using pairs of figures and treating the results as if a line source had been introduced at an arbitrary time shortly before the injection. On this basis if the half widths at two times t' and t'' are $x'_{\frac{1}{2}}$ and $x''_{\frac{1}{2}}$ the diffusion coefficient is given by

$$2{\cdot}77\ D\ (t''-t') = (x''_{\frac{1}{2}})^2 - (x'_{\frac{1}{2}})^2. \tag{4}$$

For methylene blue the calculated values of D thus obtained were 1·7 and $1{\cdot}3 \times 10^{-6}$ cm^2/sec, while for eosin they were 0·83 and $0{\cdot}76 \times 10^{-6}$ cm^2/sec. According to Lison & Fautrez (1948) the diffusion constants in water at 18° C are around 4×10^{-6} cm^2/sec for both dyes, so that these admittedly very rough calculations indicate that in the axoplasm the dye molecules move two to five times more slowly than in free solution.

The effect of injected substances on the membrane potentials

Before discussing the main experiments on the behaviour of injected ^{24}Na, it is convenient to describe a few observations on the changes in resting and action potentials after injecting the chlorides of sodium, potassium, calcium, magnesium and tubocurarine. The effects on the membrane potentials were not investigated in great detail, but a limited number of measurements were made in order to verify that the electrical properties of the axons were not adversely affected by injection of the quantities of sodium used later in the tracer experiments. Some attention was also paid to the effect of injecting Mg^{2+} ions and curare, since Grundfest *et al.* (1952, 1953) had reported that conduction in squid axons could be blocked specifically by the introduction of surprisingly small quantities of these substances. Subsequently, Grundfest, Kao & Altamirano (1954) were unable to confirm the specific blocking effect of magnesium, and Grundfest (1953) mentioned that curare was not always as powerful an agent as had at first been supposed. Our results would indicate that neither substance has any specific blocking action.

Table 1 summarizes all the measurements made on membrane potentials

except those for the first two axons to be injected. These two earliest experiments gave results essentially similar to the later ones, but have been omitted because no accurate record was kept to enable an allowance to be made for some backlash in the injection, and because both axons were exceptionally small (one only 365 μ in diameter) and in poor condition. Determinations of this sort were only worth making with axons which were in reasonably good condition, and whose membrane potentials were not slowly declining even before any injections had been made. The relative constancy of the potentials in all the later experiments may be judged from the experiment illustrated in Fig. 2, which also gives some idea as to the time scale on which we worked.

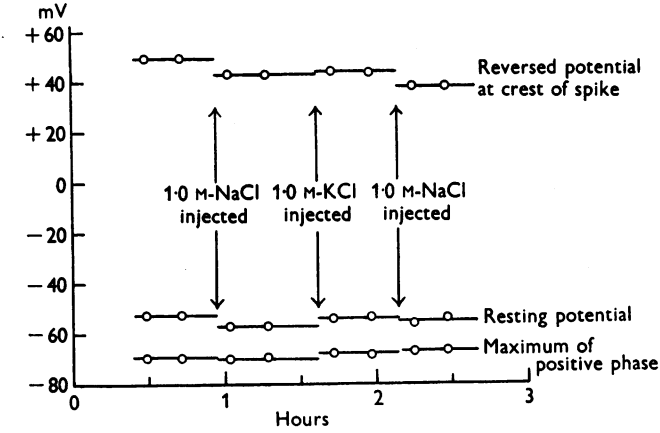

Fig. 2. The effect on the membrane potentials of injecting NaCl and KCl. The volume injected on each occasion was $7{\cdot}85 \times 10^{-6}$ ml./mm, and the length of the injected patch was 14 mm. Potentials were measured exactly in the centre of the injected region, and the axon diameter at this point, measured with the microelectrode in position, was 561 μ before making the first injection, and 579 μ after making the last one. Potentials are given as inside minus outside potential.

An important feature of the technique was that it made possible the injection of a fairly long column of the test solution, so that the membrane potentials could be measured near the centre of a uniformly treated portion of the axon. The space constant for the passive spread of potential in a squid axon in sea water is about 6 mm (Cole & Hodgkin, 1939), and for useful results it was desirable to make the potential measurements at a point separated by at least this distance from the untreated part of the axon. As Table 1 shows, this was not always achieved, but the majority of the determinations were made sufficiently far from the uninjected region to avoid gross errors from electrotonic current flow.

As reported by Grundfest *et al.* (1954), injections of KCl which raised the concentration inside the axon by about 30 mM had little effect on the resting potential, the spike or the positive phase (Expts. 1, 3, 4 and 9, Table 1). This is

TABLE 1. Effects on the membrane potentials of squid axons of making various injections

Expt.	Axon diam. (μ)	Solution injected	Volume injected (10^{-6} ml./mm)	Final concn. in axoplasm	Length injected (mm)	Distance from edge of injected patch (mm)	Resting potential		Reversed potential at crest of spike		Potential at maximum of positive phase	
							Before (mV)	After (mV)	Before (mV)	After (mV)	Before (mV)	After (mV)
1	550	0·57 M-KCl	8·8	22 mM	24	3	49·3	47·8	44·3	46·2	65·5	64·8
		1/1000 curare in 0·57 M-KCl	8·0	36×10^{-6}	24	3	47·8	47·5	46·2	42	64·8	63
		0·37 M-$MgCl_2$	8·2	13 mM	20	3	47·5	44·8	42	33·3	63	60·5
2	540	0·37 M-$MgCl_2$	7·8	13 mM	15	5	51·5	50	46·5	43·5	69·5	67·5
		0·37 M-$CaCl_2$	8·0	13 mM	15	5	50	32	43·5	Small spike	67·5	—
3	598	0·57 M-KCl	8·6	17 mM	20	8	52	52·3	50	47·3	70	69·5
		2/1000 curare in 0·57 M-KCl	6·7	60×10^{-6}	19	7	52·3	49·5	47·3	46	69·5	67·5
4	526	1·0 M-NaCl	10·7	49 mM	12	6	50·8	52	51·3	40·5	68·5	68
		1·0 M-KCl	10·7	47 mM	12	6	52	50·3	40·5	37·3	68	65·5
	550	1·0 M-NaCl	10·4	45 mM	12	6	50·3	49·3	37·3	27·3	65·5	64·5
5	498	0·074 M-$CaCl_2$ + 0·455 M-KCl	10·7	4 mM-Ca 25 mM-K	6	3	47	46	39	32	—	—
6	624	0·37 M-$CaCl_2$	10·7	13 mM	3	1·5	49	27	48	No spike	—	—
7	583	0·37 M-$MgCl_2$	7·85	11 mM	14	7	55	53	53	49	—	—
		2/1000 curare in 0·57 M-KCl	7·85	58×10^{-6}	14	7	54	51·5	48	50·5	—	—
		0·37 M-$CaCl_2$	7·85	10 mM	14	7	51·5	48	47·5	24	—	—
		0·37 M-$CaCl_2$	7·85	10 mM	14	7	48	36	26	Small spike	—	—
8	536	0·37 M-$MgCl_2$	7·85	13 mM	14	5	53·5	54	48·5	45	71	—
		1/1000 curare in 0·57 M-KCl	7·85	34×10^{-6}	14	6	52	51	46	45	—	—
		0·37 M-$CaCl_2$	7·85	12 mM	14	5	51	40–50	45	Small spike	—	—
9	561	1·0 M-NaCl	7·85	32 mM	14	7	52·5	57	49·5	43	69·5	69·8
		1·0 M-KCl	7·85	31 mM	14	7	57	53·8	43	43·8	69·8	68·3
	579	1·0 M-NaCl	7·85	30 mM	14	7	53·8	56	43·8	38	68·3	67·5
10	564	2·0 M-NaCl	7·85	64 mM	14	5	54	55	46	34	69	65

In Expts. 1 to 4 small corrections to the volume injected have been made to allow for back-lash; in the other experiments there was no back-lash. In Expts. 4 and 9 the axon diameter was measured at the beginning and end; in the other experiments it was measured after completing the injections. Potentials were measured to the nearest 0·5 mV and many of the figures are the averages of two or three determinations. In Expts. 2 and 7 the 'small spike' was only 7–8 mV in size, and could have arisen from electrotonic current spread from the uninjected region; in Expt. 8 the final spike amplitude was 26 mV. Temperature 17–23° C.

not surprising, because the potassium concentration would only have increased by about 10%, and the rise in potential from this cause might be counteracted by the fall expected from the increase in internal chloride concentration. If the membrane were perfectly selective to potassium, a 10% increase in internal potassium would raise the membrane potential by 2·4 mV. If, on the other hand, the permeability to Cl^- were 0·45 times that to K^+, as assumed by Hodgkin & Katz (1949*a*), the change predicted by the constant field equation (Goldman, 1943) would be a decrease of 3 or 4 mV. Differences between these values and those recorded experimentally might well arise from extraneous effects of the injection.

Injection of sodium chloride caused a marked drop in the reversed potential difference across the membrane at the crest of the spike and, on most occasions, a slight rise in the resting potential (Expts. 4, 9 and 10, Table 1). The effect on the action potential fitted well with the hypothesis that the overshoot depended on the sodium concentration ratio. The average reduction in the overshoot for four injections of 1·0 M-NaCl was 8·2 mV, the mean rise in internal sodium concentration for a single injection being 39 mM. If the initial internal sodium concentration is calculated from the size of the overshoot by the Nernst equation, an average value of 80 mM is obtained for the internal sodium before the injection. On this basis the change in overshoot resulting from the injection should be 10 mV. The rise in resting potential may occur because the increase in active sodium efflux caused by the greater internal sodium concentration makes a direct contribution to the resting potential which more than outweighs the effect of raising the internal chloride. However, further experiments are needed both to confirm the action of injected sodium on the resting potential and to establish a connexion with the activity of the sodium pump.

Fig. 3 shows a series of photographs of the action potential recorded internally in one of the experiments in which magnesium, curare and calcium were injected in turn. The first solution introduced was 0·37 M-$MgCl_2$; this caused an insignificant rise in the resting potential, and a small drop (3·5 mV) in the overshoot of the spike. The next solution was one of tubocurarine chloride (1 in 1000, dissolved in isotonic KCl); this reduced the resting potential by 1 mV, perhaps because of the KCl, and had even less effect on the action potential than the magnesium. Finally, 0·37 M-$CaCl_2$ was injected. This had a much greater blocking action, and 11 min after the injection the spike was reduced to only 26 mV.

The results of other experiments on the injection of magnesium and curare are summarized in Table 1. Injections of 0·37 M-$MgCl_2$, corresponding to 0·07 μg Mg^{2+}/mm over 14–20 mm, caused a slight reduction (averaging 5·1 mV) in the overshoot, but certainly did not block conduction. This result is not inconsistent with the more recent work of Grundfest *et al.* (1954), which gave

the blocking dose of magnesium as 1 μg, but not with the earlier report (Grundfest *et al.* 1953) from which it appeared that 0·0003 μg Mg^{2+} was sufficient to block conduction. Curare, also, seemed to have little effect. The strongest solution of tubocurarine chloride injected was 1 in 500 parts of isotonic KCl, which in two experiments (3 and 7, Table 1) gave a final concentration of curare in the axoplasm of about 60×10^{-6}, but caused no obvious reduction in the size of the action potential. This concentration was much greater than that at which tubocurarine from the same source had its normal blocking effect on a frog nerve-muscle preparation. The total quantity injected (about 15×10^{-3} μg curare/mm axon over 14–19 mm) was much greater than the blocking dose given by Grundfest *et al.* (1952)—a total injection of 10^{-5} μg.

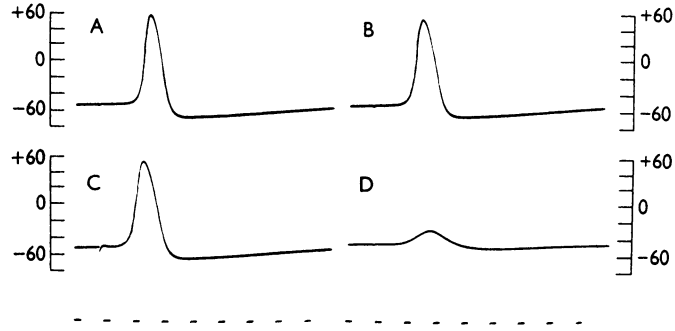

Fig. 3. Action potentials recorded with an internal electrode in a 536 μ squid axon. A, at the beginning of the experiment. B, 28 min after injecting 0·37 M-$MgCl_2$. C, 25 min after injecting 1 in 1000 tubocurarine chloride. D, 14 min after injecting 0·37 M-$CaCl_2$. Owing to a drift in the electrode potential there was doubt as to the correctness of the resting potential in record D. For further experimental details see Table 1, Expt. 8. Time marks 1250/sec. Temperature 17° C. Vertical scales give the internal potential in mV.

The one substance which was found to block conduction was calcium. Its effects were somewhat variable, since in the axon of Expt. 6 (which may have had a weak spot) an injection of only 3 mm length was sufficient to reduce the resting potential from 49 to 28 mV, and to abolish the spike altogether, whereas in Expt. 7 the first injection, of roughly the same amount of 0·37 M-$CaCl_2$ per mm axon over a total distance of 14 mm, only reduced the resting potential by 4 mV and the spike by 21 mV, and a second injection of the same quantity was needed before the spike was reduced to a small fraction of its original size. In addition to its effect on the membrane potentials, calcium caused two other interesting changes. In the first place, the axoplasm in the injected axons became fluid, as was observed by Chambers & Kao (1952), so that in the injected region the shaft of the micro-electrode could be moved from side to side without difficulty, in contrast to its relative immobility inside a normal axon. The average concentration of calcium in the axoplasm after

injection was about 10 mM, so that this observation is consistent with the experiments of Hodgkin & Katz (1949*b*) on the dispersal of extruded squid axoplasm by calcium ions, 10 mM-Ca^{2+} having caused complete dispersion in about 3 min. Secondly, the injected part of the axon became noticeably opaque, the upper and lower limits of opacity coinciding exactly with those of the injected column. This suggests that the opaque brown patches which appear at the bases of branches of the main axon which are damaged or cut off too short during dissection also arise from an entry of calcium into the axoplasm, as has been subsequently confirmed (see Flückiger & Keynes, 1955). Since calcium evidently acts on the axoplasm in a rather drastic manner, it seems likely that its effect on the membrane potentials is a general consequence of disrupting the internal structure of the axon, rather than a special action on the membrane itself.

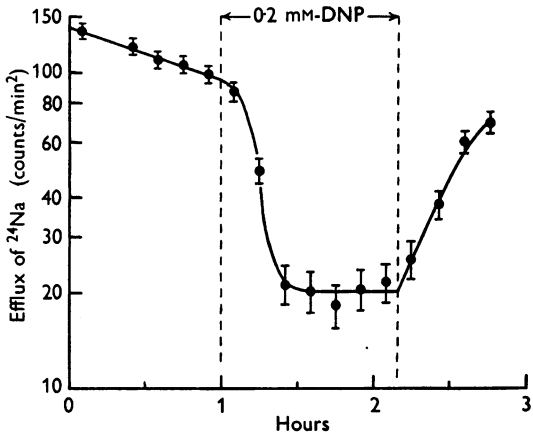

Fig. 4. The action of dinitrophenol on the sodium efflux from a squid axon after injecting ^{24}NaCl. Axon diameter 560 μ. Length injected 19 mm. Volume of injection $8{\cdot}25 \times 10^{-6}$ ml./mm. Temperature about 19° C. The axon initially gave a 96 mV spike over the whole of the injected region but in this particular experiment when tested at the end was inexcitable, although it still had about 35 mV resting potential. Vertical lines indicate $\pm 2 \times$ S.E. of the counts.

The injection of labelled sodium

The object of our first experiment on the injection of ^{24}NaCl was to check that labelled ions introduced into the axon by microinjection were extruded in the same way as those entering the axon during stimulation in ^{24}Na sea water, and that the efflux of radiosodium was then similarly affected by treatment with metabolic poisons like dinitrophenol. In Fig. 4 the rate of extrusion of ^{24}Na into the inactive sea water in the collecting chamber, expressed in counts/min per min and corrected for the decay of the isotope, is plotted semi-logarithmically against the time after injecting a 19 mm column of 0·57 M-NaCl containing ^{24}Na (0·57 M-Na*Cl) into a 560 μ squid axon. The sea

water was changed every 10 min after the injection, and the radioactivity of successive samples was determined with a liquid counter. On comparing this curve with that for a *Sepia* axon first stimulated in ^{24}Na sea water and then treated in the same manner (Hodgkin & Keynes, 1955) it will be seen that qualitatively there is no obvious difference between the behaviour of radiosodium injected into the axon and that entering the axon in a more physiological fashion during normal electrical activity. In each case the sodium efflux is reversibly reduced to a small fraction of its initial value by the action of 0·2 mM-DNP. In some other experiments, squid axons were treated with 2 mM-CN after injection of ^{24}NaCl, and again the effects were very like those described in our earlier paper.

During the initial part of this experiment, while the axon was still exposed only to normal artificial sea water, the efflux of ^{24}Na declined steadily. Knowing accurately how much radioactivity was injected into the axon, it is of some interest to see whether the rate of this decline fits well with that expected from the rate of dilution of intracellular ^{24}Na by inactive sodium entering the axon. In a system in which the total efflux of sodium, M_o, is directly proportional to the total internal [Na], we can write

$$M_o = p[\mathrm{Na}], \tag{5}$$

and the efflux of labelled sodium, M_o^*, will be given by

$$M_o^* = p[\mathrm{Na}^*]. \tag{6}$$

Taking p as constant, then $\dfrac{\mathrm{d}M_o^*}{\mathrm{d}t} = p\dfrac{\mathrm{d}[\mathrm{Na}^*]}{\mathrm{d}t}$,

so that

$$\frac{1}{M_o^*}\frac{\mathrm{d}M_o^*}{\mathrm{d}t} = \frac{1}{[\mathrm{Na}^*]}\frac{\mathrm{d}[\mathrm{Na}^*]}{\mathrm{d}t}. \tag{7}$$

Thus the rate constant for decline of the efflux should equal that for decline of the total radioactivity in the axon, which has been written as k in earlier papers. In the experiment of Fig. 4, the total radioactivity injected was 38,900 counts/min, while the initial value of $\mathrm{d}[\mathrm{Na}^*]/\mathrm{d}t$ was -140 counts/min^2. Hence

$$\frac{1}{[\mathrm{Na}^*]}\frac{\mathrm{d}[\mathrm{Na}^*]}{\mathrm{d}t} = -\frac{140\times 60}{38900} = -0{\cdot}22\ \mathrm{hr}^{-1}.$$

But the slope of the straight line drawn through the first five points, which corresponds to the value of $\dfrac{1}{M_o^*}\dfrac{\mathrm{d}M_o^*}{\mathrm{d}t}$, was appreciably greater, being $-0{\cdot}39\,\mathrm{hr}^{-1}$. It thus seems that the efflux of ^{24}Na declines faster than can be explained on the simple assumption made above.

This conclusion held good for every experiment in which the radiosodium efflux into sea water was followed for long enough to allow the rate constant for its decline to be calculated. It may be seen from Table 2, where the results

of eight such experiments are listed, that the average rate constant for loss of Na* was 0·20 hr^{-1}, while M_o^* declined just twice as fast. We therefore undertook a few experiments to examine the cause of the discrepancy.

TABLE 2. The rate of decline of the efflux of ^{24}Na from squid axons

Expt.	Axon diam. (μ)	Length injected (mm)	Volume injected (ml./mm)	[Na*] after injection (mM)	Initial M_o^* (pmole/ cm^2 sec)	Inital value of $\frac{1}{[Na^*]}\frac{d[Na^*]}{dt}$ (hr^{-1})	Value of $\frac{1}{M_o^*}\frac{dM_o^*}{dt}$ (hr^{-1})
1	560	19	$8{\cdot}25 \times 10^{-6}$	19·3	16·2	0·22	0·39
2	521	10	8·4	22·4	21·1	0·26	0·45
3	534	12	8·4	21·3	15·9	0·20	0·49
4	510	14	9·5	27·0	21·5	0·23	0·34
5	544	14	10·7	26·9	19·6	0·19	0·55
6	514	11	10·7	28·6	21·0	0·21	0·48
7	554	15	10·7	25·9	20·5	0·21	0·34
8	616	10	7·85	15·3	5·8	0·09	0·15
Mean	544	—	—	23·3	17·7	0·20	0·40

Axon diameters were measured at the end of the experiment, and where a second injection was made in the course of the experiment an allowance for the resultant swelling has been subtracted. Temperature 18–20° C.

The two most obvious explanations which might be put forward are these: In the first place, the total efflux may not be proportional to [Na], but may instead be constant, i.e. the sodium pump may operate at a fixed rate which is independent of the amount of sodium inside the axon. If the total internal sodium increases steadily this will result in the efflux of labelled sodium falling off faster than the concentration of labelled sodium. In this case, in the relation

$$\frac{M_o^*}{M_o} = \frac{[Na^*]}{[Na]}, \tag{8}$$

M_o is constant. Now in general

$$\frac{d[Na]}{dt} = (M_i - M_o)\frac{A}{V}, \tag{9}$$

and in an unlabelled solution

$$\frac{d[Na^*]}{dt} = -M_o^*\frac{A}{V}$$

where A and V are the cell surface and volume respectively, and M_i is the sodium influx. Substitution for [Na] from (8) in (9) and elimination of A/V leads to

$$\frac{1}{M_o^*}\frac{dM_o^*}{dt} = \frac{M_i}{M_o}\left\{\frac{1}{[Na^*]}\frac{d[Na^*]}{dt}\right\}. \tag{10}$$

This explanation of the discrepancy would require the sodium influx to be about twice as great as the efflux, which is not an impossible demand. A second hypothesis is that over short periods the sodium efflux is directly proportional to the internal sodium concentration, but that the proportionality

factor decreases exponetially with time; this might occur if the effectiveness of the sodium pump gradually declined Then instead of equation (5) we should have

$$M_o = p_o \, e^{-k_1 t}[\mathrm{Na}], \tag{11}$$

and instead of (6)

$$M_o^* = p_o \, e^{-k_1 t}[\mathrm{Na}^*]. \tag{12}$$

Differentiation of (12) leads to the relation

$$\frac{1}{M_o^*}\frac{dM_o^*}{dt} = -k_1 + \frac{1}{[\mathrm{Na}^*]}\frac{d[\mathrm{Na}^*]}{dt}. \tag{13}$$

This equation would also fit with the observations if the average value of the rate constant k_1 were about 0·20 hr^{-1}.

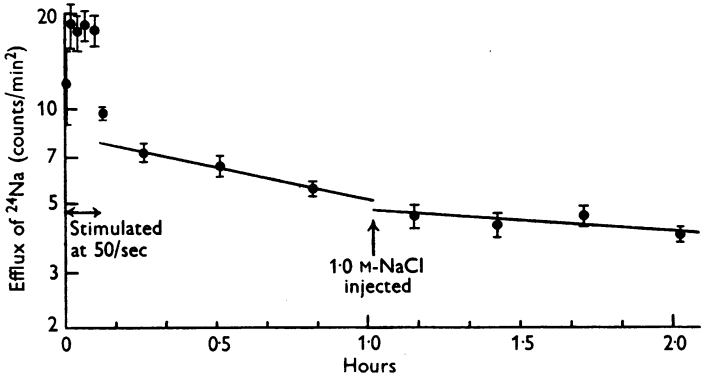

Fig. 5. The effect on the efflux of ^{24}Na of injecting inactive sodium chloride. An 11 mm length of the axon was injected with 0·57 M-Na*Cl at zero time, and 61 min later the same part of the axon was injected with 1·0M-NaCl. Axon diameter 514 μ. For a period of 8·5 min, ending 7 min after completing the injection of Na*Cl, the axon was stimulated at 50/sec. Vertical lines are $\pm 2 \times$ S.E.

The simplest way of testing the first hypothesis was to find whether or not the sodium efflux was changed by an injection of inactive sodium chloride. If the total sodium efflux were unalterable, then the sudden introduction of a large amount of inactive sodium would substantially reduce the efflux of radiosodium. The experiment illustrated in Fig. 5 shows that this was not the case. An 11 mm column of 0·57 M-Na*Cl was injected into a 514 μ squid axon, and after an initial short period of stimulation (see p. 611), the resting efflux of ^{24}Na was followed for about an hour. An 11 mm column of inactive 1·0M-NaCl was now injected over the same part of the axon, and the efflux was observed for a further hour. The volume of solution injected on each occasion was $10{\cdot}7 \times 10^{-6}$ ml./mm, so that the concentration of Na* in the axoplasm immediately after the first injection was 30 mM, while the extra amount of inactive sodium introduced in the second injection was about 50 mM. If the initial sodium content of the axon had been 50 mM, the constant efflux

hypothesis would predict a 40% drop in the efflux of ^{24}Na after the second injection, whereas it can be seen from Fig. 5 that although the efflux may have fallen slightly, the change was not statistically significant. Another experiment of the same sort, in which a 2·0 M solution of inactive sodium chloride was injected, also gave no appreciable reduction in the ^{24}Na efflux. These two experiments provide good evidence that over the range of internal sodium concentrations tested (up to about 130 mM) there is no obvious saturation of the sodium efflux, which rises proportionally when the total internal sodium is suddenly increased.

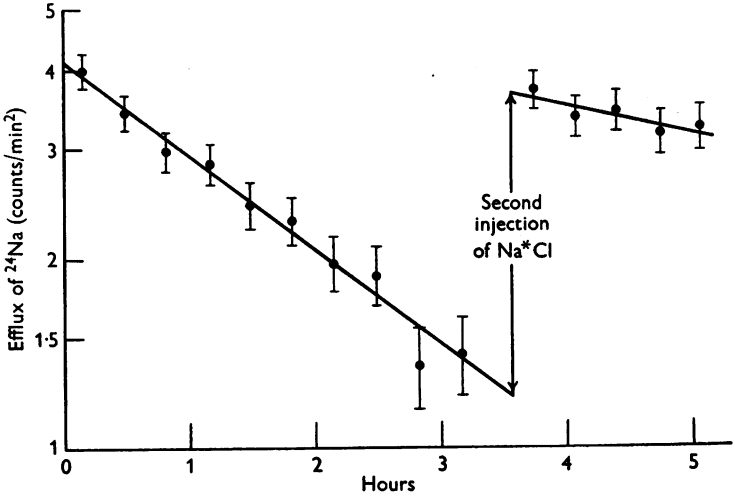

Fig. 6. The decline of the efflux of ^{24}Na with time. The first injection of a 14 mm column of 0·58 M-Na*Cl was made at zero time, and a second identical injection was made 3 hr 34 min later. The final axon diameter was 521 μ, and the axon diameter after the first injection only was taken as 510 μ. Vertical lines are $\pm 2 \times$ S.E.

The alternative hypothesis was examined in two experiments of the type illustrated in Fig. 6, involving duplicate injections of Na*Cl. In the case shown the mean axon diameter was 510 μ, and each injection consisted of a 14 mm column of 0·58 M-Na*Cl whose volume was $9{\cdot}5 \times 10^{-6}$ ml./mm. The specific activity of the ^{24}Na was 1 count/min equivalent to 70·6 pmoles Na*. The initial efflux of Na*, extrapolated back to zero time, was 4·11 counts/min^2; the total radioactivity injected was 1088 counts/min. The initial value of $\frac{1}{[Na^*]}\frac{d[Na^*]}{dt}$ was therefore $-0{\cdot}227$ hr^{-1}, which was, as usual, appreciably less than the value of $\frac{1}{M_o^*}\frac{dM_o^*}{dt}$, the slope of the straight line fitted to the counts taken over the first 3 hr being $-0{\cdot}344$ hr^{-1}. The value of k_1 in equation (13), which is the

difference between these two rate constants, was thus 0·117 hr^{-1}. Now the effect of the second injection of Na*Cl was to raise the efflux from 1·22 to 3·67 counts/min^2, an increase of only 2·45 counts/min^2. The rise in [Na*] was the same as before, so that the factor of proportionality between efflux and internal sodium concentration was not constant, but had decreased by 40% of its original value over the interval of 3·58 hr which elapsed between the two injections. Taking k_1 as 0·117 hr^{-1}, equation (12) predicts a fall of 34%, so that the results of the experiment fit fairly well with the assumptions on which equations (11) to (13) are based. The only respect in which agreement was not good was that the value of $\frac{1}{M_o^*}\frac{dM_o^*}{dt}$ after the second injection (0·098 hr^{-1}) was less than that expected $\left(\text{about } 0{\cdot}25 \text{ hr}^{-1}, \text{ from adding } k_1 \text{ to the new value of } \frac{1}{[Na^*]}\frac{d[Na^*]}{dt}\right)$; but since few counts were taken this discrepancy was of doubtful significance.

In the other experiment of this kind, the difference (k_1) between the rate constants for the decline of the efflux and for the loss of total radioactivity was 0·135 hr^{-1}. The two injections of Na*Cl were separated by 2·20 hr, whence the expected drop in the efflux proportionality factor was 26% of the value for the first injection. The observed fall was 24%, so that once more the results were closely consistent with the theoretical explanation. It should perhaps be added that no special significance should be attached to our choice of an exponential for the decline of p, since this form was taken merely for convenience in analysis. A linear decline would, no doubt, have fitted the results equally well, but would have involved a more complicated mathematical solution.

These two experiments also provide data for a further test of the relation between efflux and [Na] for a sudden rise in [Na]. Knowing how much radioactivity was lost from the axon to the external medium during the period between the two injections, the amount of Na* still remaining in the axon at the time of the second injection can be calculated. It is then possible to compare the effluxes per unit internal [Na*] immediately before and immediately after the second injection. In the experiment of Fig. 6, altogether 504 counts/min (after correcting for decay of the isotope) were collected in the first series of samples, so that 584 counts/min were left in the axon. The corresponding efflux was 1·22 counts/min^2. After the second injection, the axon contained 584 + 1088 = 1672 counts/min, and the efflux was 3·67 counts/min^2. The values of $M_o^*/[Na^*]$ were therefore 0·00209 min^{-1} before and 0·00219 min^{-1} after. A similar calculation for the other experiment gave figures of 0·00237 and 0·00251 min^{-1} respectively. Thus in both cases the efflux increased in strict proportion to the amount of sodium introduced into the axon.

The time lag in the operation of the sodium pump

At the beginning of experiments like those illustrated in Figs. 4 and 6, where the first samples were not collected until at least 10 min after injecting the ^{24}NaCl, there was no perceptible lag in the attainment of a steady sodium efflux. In some of the other experiments, the first samples were taken much earlier, in order to discover how quickly the injected ^{24}Na became completely mixed with the inactive sodium in the axoplasm. The counts taken in several of these experiments are plotted in Fig. 7, the results for the different axons being normalized by taking the extrapolated initial steady efflux as 1.

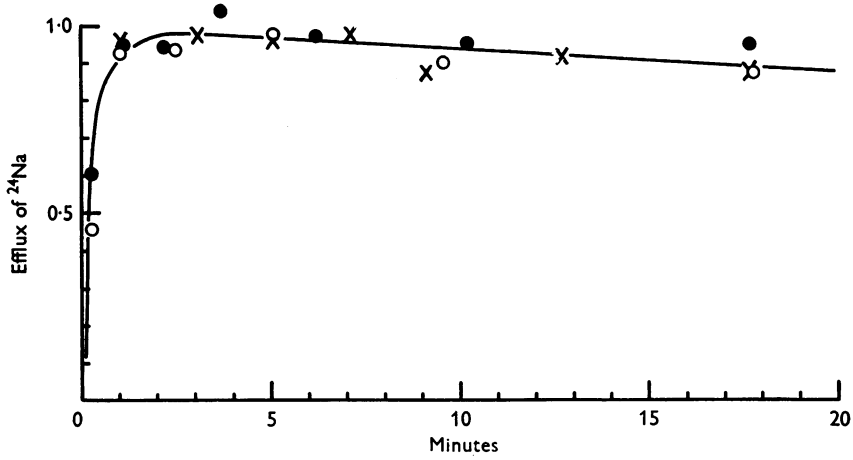

Fig. 7. Determinations of the resting sodium efflux immediately after injecting ^{24}Na. Abscissa: time after injection. Ordinate: efflux of ^{24}Na expressed as a fraction of the extrapolated efflux at zero time. ○, axon diameter 534 μ; ●, axon diameter 616 μ; ×, axon diameter 521 μ. Temperature 19–20° C.

The mixing process proved to be virtually complete about 1 min after the injection, and since the operations of removing the microsyringe and of changing the fluid in the collecting chamber each occupied several seconds, it was quite difficult to take a sample soon enough after the injection for the observed efflux to be much less than the final steady value. However, on three occasions the first sample was taken within 40 sec of making the injection, giving figures from which it is possible to derive information about the rate at which the ^{24}Na diffused through the axoplasm, and began to be extruded from the axon.

It is clear from Fig. 7 that the diffusion time is small compared with the time constant for decline of the efflux after reaching the steady state, so that it is legitimate to treat the problem as one of diffusion within an infinite cylinder whose surface is insulated. A general solution for the time course of such a diffusion process is given by Carslaw & Jaeger (1947, pp. 178, 306), from which it can be shown that the particular solution for the concentration, y, at

the surface of the cylinder $r=a$, after the introduction of an instantaneous line source at $r=0$ (i.e. on the axis of the cylinder) and at $t=0$ is

$$\frac{y}{y_\infty}=1+\sum_{\alpha_1,\,\alpha_2\ldots} e^{-\alpha^2 Dt/a^2}/J_0(\alpha), \tag{14}$$

where y_∞ is the final concentration after complete mixing, D is the diffusion coefficient, and α_1, α_2,..., are the positive roots of $J_1(\alpha)=0$. This expression is the same as that obtained by putting $r=0$ in Hill's (1948) equation (2) for the converse process of the concentration change on the axis of a cylinder after liberation of a sudden pulse at its surface. Hill plotted the expression as curve (i) in his Fig. 2, but this curve could not be used directly for the calculation of values of D from our results, because our observations of efflux were not instantaneous determinations, but were averages over a known period starting

TABLE 3. The rate of diffusion of ^{24}Na in squid axons

Expt.	Axon diam. (μ)	Mid-time of first collecting period (sec)	Efflux during first collecting period / Steady efflux	Theoretical value of Dt/a^2	D (cm^2/sec)
1	534	16	0·46	0·110	$5{\cdot}0\times10^{-6}$
2	514	21	0·65	0·181	5·7
3	616	17	0·61	0·157	8·8

In Expts. 1 and 3 the axons were resting. In Expt. 2 the axon was stimulated at 50/sec.

from $t=0$. It was therefore necessary to calculate the summated area under the curve given by equation (14) for various values of Dt/a^2, and thus to obtain a second curve relating the mean value of y/y_∞ to Dt/a^2, from which the figures listed in Table 3 were taken. It will be seen from this table that the average effective value of D, calculated as if the whole of the time lag in reaching the steady efflux arose from the slowness of diffusion through the axoplasm, was $6{\cdot}5\times10^{-6}$ cm^2/sec.

This calculation not only shows that Na^+ ions must be able to diffuse radially through the axoplasm at a rate not much smaller than in free solution (the self-diffusion coefficient of Na^+ ions at room temperature being about 10×10^{-6} cm^2/sec), but also sets a limit to any time delay which might occur in the operation of the extrusion mechanism. From equation (14) it follows that even if diffusion through the axoplasm had taken place at the full free solution value, the 0·5 level in Fig. 7 would not have been reached in less than about 10 sec for a 550 μ axon. Moreover, Hodgkin & Katz (1949*a*) noted that the layer of connective tissue surrounding the axon caused a lag in the blocking action of sodium-free solutions, and although our axons were more cleanly dissected than most of theirs, this connective tissue must have had some effect in delaying the appearance of labelled ions in the external medium. The fact that the ^{24}Na was really introduced as a 100μ column, rather than as the line source assumed in deriving equation (14), would operate

in the other direction, but over a radius of only 50 μ mixing would be complete in less than a second, so that this simplification does not introduce a serious error into the argument. The margin for possible delay in the working of the sodium pump is therefore reduced to no more than a few seconds. This is also true for the rather different mechanism involved in the accelerated passage of sodium through the membrane during electrical activity, since in one of the experiments (see Fig. 5 and Expt. 2, Table 3) the axon was stimulated at 50/sec before, during, and after the injection of ^{24}Na, and as one might expect the apparent value of D was close to the mean for the other two experiments. However, in this axon the effect of stimulation on the sodium efflux was unusually small (cf. Hodgkin & Keynes, 1955), so that no great weight can be attached to this observation.

The injection of ATP

One of the main incentives for developing the technique described in this paper was to see whether it was possible, by the injection of an energy-rich substance like ATP, to restore the sodium efflux in axons previously poisoned with dinitrophenol or cyanide. Several experiments were done on these lines, mostly using axons in which the sodium had been labelled by stimulation in ^{24}Na sea water, and in which the sodium efflux had then been inhibited by treatment with 2 mN-CN, this being followed by an injection of ATP and control injections of KCl. However, the long series of operations involved made the experiments difficult to complete satisfactorily, and the only conclusion we were able to reach was that ATP did not seem to cause a really dramatic recovery of the sodium efflux. There was also doubt about the purity of the ATP sample.

DISCUSSION

The electrical effects of injected ions

The conclusions from our very limited investigation of the electrical effects of injecting ions were largely negative. In confirmation of Grundfest *et al.* (1954) magnesium was found to have no specific blocking action in small concentrations. Tubocurarine chloride was also found to have no blocking effect in concentrations much greater than that required to interfere with neuromuscular transmission. Calcium caused block, but since this ion also tended to disperse the axoplasm (Hodgkin & Katz, 1949*b*), it may not have acted on the membrane, but may have simply destroyed the internal organization of the axon. The most definite result was that injection of sodium chloride reduced the overshoot of the spike, whereas similar quantities of potassium chloride had little effect. The change in overshoot agreed reasonably well with that calculated from the rise in internal sodium concentration. Grundfest *et al.* (1954) reported that injection of sodium not only reduced the spike, but also

changed its shape in a characteristic way. We did not notice any marked alterations in the shape of the spike, and it seems to us that the experiment illustrated in their fig. 10 is open to certain objections. The figure shows action potentials recorded at three microelectrodes separated by 2·9 and 4·9 mm. When 1·3 mm^3 of 0·51 M-sodium glutamate was injected through the central electrode, the centrally recorded spike changed appreciably, but the spikes at the two outer electrodes were less affected. The form of the central spike is about that which might be expected if the membrane in the central 5 mm had been made inexcitable but was still sufficiently intact for a change in potential to be produced by electrotonic spread. The increase in duration might then be partly due to the effect of averaging two spikes separated by a long conduction time, and to the delaying effect of the membrane capacity. If this deduction is correct, it follows either that the injected fluid did not spread uniformly along the axon or that the central part was damaged by the injection procedure, or both.

As the concentration changes brought about by the present method are limited in extent, our experimental evidence neither confirms nor contradicts the conclusion that the membrane potential is not dependent on the intracellular potassium concentration (Grundfest *et al.* 1954; Grundfest, 1955). We did not find much change in resting potential from injecting a small quantity of KCl, but little alteration was to be expected because K^+ and Cl^- would act in opposite directions, and the potassium concentration ratio was not much increased. Grundfest's (1955) main argument seems to be that, apart from the progressive depolarization and block which occur when a sufficient volume of any solution is introduced locally, large injections of potassium glutamate or aspartate do not have much effect on the resting potential, notwithstanding the prediction of the constant field equation that the resting potential should rise by about 30 mV if the internal potassium concentration is increased three- or four-fold, and if the accompanying anion is unable to cross the membrane. While it is true that equation (4) of Hodgkin & Katz (1949*a*) may not be exact—it has never been claimed to be more than a rough approximation—the change of 30 mV calculated from it would in any case be expected only if the following conditions were satisfied:

(1) That the potassium concentration were raised three-fold over a length of several space constants—ideally about 30 mm.

(2) That the anion injected with the potassium did not diffuse out through cut branches or through the membrane.

(3) That the fibre was not damaged by the injection.

In view of the quantities injected and of the conclusions of the previous paragraph, it seems that there is some doubt about these points.

The proportionality factor for sodium efflux

A direct proportionality between sodium efflux and intracellular sodium concentration has previously been assumed in theoretical treatments of ionic exchange in nerve and muscle by several authors (see, for example, Harris & Burn, 1949; Keynes, 1954), but has not been confirmed experimentally. It is therefore satisfactory to be able to provide evidence that, at least over short periods, the absolute size of the sodium efflux does change in proportion to the total internal concentration of sodium. This behaviour has the advantage of providing the cell with a simple means of automatically regulating its internal sodium level, since it ensures that after a bout of activity the sodium efflux at once rises, and that the excess sodium inside the cell is then pumped out at a gradually decreasing rate (as was concluded by Hodgkin & Keynes, 1955) until there is once more a balance between the resting sodium influx and the sodium efflux. Since the sodium concentration in freshly dissected squid axons seems to be about 40 mM (Hodgkin, 1951), and since, from Table 2, the mean efflux of sodium per internal m-mole/l. was 0·76 pmole/cm^2.sec, this resting sodium efflux in squid axons is probably in the neighbourhood of 30 pmole/cm^2.sec.

It should not be assumed that the tendency for the proportionality factor to decrease with time, which we have demonstrated here for squid axons, also applies to other cells. There is no evidence that it is applicable to muscle, nor do our figures for *Sepia* axons (Hodgkin & Keynes, 1955) reveal any comparable discrepancy between the rate constants for decline of the efflux and for decline of the total intracellular labelled sodium. Possible explanations of the effect, between which we cannot at present decide, are that it is due to progressive exhaustion of the metabolic reserves of the axon, or that the efficacy of the pump gradually falls off through escape of some component of the carrier mechanism, either from the outside of the membrane or via cut branches. It might also be related to an inward leakage of calcium through cut branches (Flückiger & Keynes, 1955).

The time lag in sodium extrusion

We have previously shown (Hodgkin & Keynes, 1953) that ^{42}K is able to diffuse longitudinally inside *Sepia* axons with a diffusion coefficient close to the value for free solution. If the intracellular proteins were arranged in a fibrillar fashion, they might provide an obstacle to radial movements, and diffusion in this direction might be appreciably slowed. However, our present experiments show that for ^{24}Na moving radially there can be very little obstruction of this sort, since diffusion must have occurred almost as fast as in free solution. For the much larger dye molecules of methylene blue and eosin, the protein skeleton of the axoplasm, or whatever other orderly internal

structure there is, evidently provided a more effective hindrance to free movement, but all the same did not slow diffusion by a very large factor. The molecules of aniline blue, which are larger still, were apparently unable to diffuse at all through the axoplasm, but factors other than simple size may have been involved.

The rapidity with which ^{24}Na reached the cell membrane and began to appear in the external medium is also of interest in that it proves that there cannot be a time lag of more than a second or two in the operation of the sodium pump. Furthermore, it appears to argue against the possibility that the sodium might have to be incorporated in some large intracellular molecule, or even in submicroscopic particles such as mitochondria, before being extruded from the cell—unless such molecules or particles are located permanently so close to the axon boundary as to constitute part of the membrane.

SUMMARY

1. A microsyringe is described which enables precisely determined volumes of fluid to be injected into squid giant axons uniformly over distances of 3–20 mm. The volume injected is about 1/25 of the axon volume per unit length.

2. The performance of the microsyringe was tested by injecting dye solutions. Methylene blue and eosin were observed to diffuse radially through the axoplasm, but more slowly than in free solution.

3. Injection of small quantities of KCl did not have any marked effect on the membrane potentials. Injection of similar amounts of NaCl reduced the reversed potential at the crest of the spike by an amount which fitted with that calculated from the change in sodium concentration ratio.

4. Injections of 0·37 M-$MgCl_2$ and 1 in 1000 tubocurarine chloride solution had no great effect on the axons. Injection of 0·37 M-$CaCl_2$ liquefied the axoplasm and tended to block conduction.

5. The efflux of ^{24}Na introduced into the axon by micro-injection was blocked in the usual way by metabolic inhibitors.

6. When $^{24}NaCl$ was injected, a steady rate of extrusion of labelled sodium was established within 1 min. The diffusion coefficient of sodium in the axoplasm can have been very little less than in free solution, and the sodium pump must operate with a lag of not more than a few seconds.

7. It was established that over short periods the sodium efflux was directly proportional to the internal sodium concentration, but that the proportionality factor declined with a time constant of about 5 hr.

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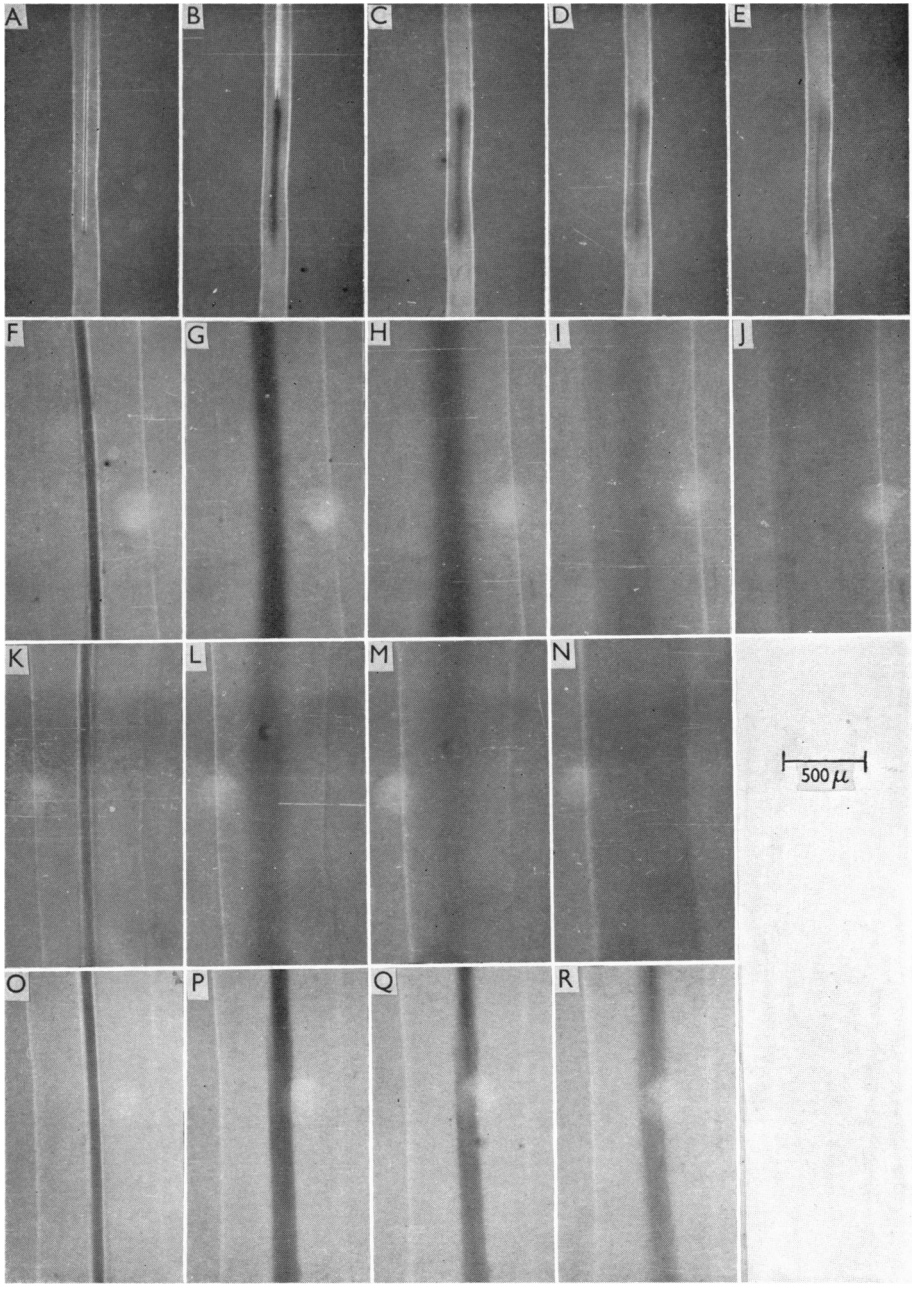
EXPLANATION OF PLATE

Photomicrographs of squid axons during injection of dyes. A, with shaft of microsyringe in position; B to E, respectively 15, 57, 120 and 430 sec after injecting a 3 mm column of methylene blue. Magnification quarter of that indicated on scale below. F to J, injection of methylene blue under higher power objective. F, before injection; G to J, respectively 3, 20, 120, and 600 sec after injection. K, before injecting eosin; L to N respectively 10, 35 and 600 sec after injecting eosin. O, before injecting aniline blue; P to R, respectively 5, 35 and 600 sec after injecting aniline blue. The dark streak down the centre of the axon in C, D and E may be seen to extend beyond the injected region, and was probably caused by a difference in the amount of light scattered by the disturbed axoplasm in the track of the microelectrode and microsyringe.



(*Facing p.* 616)