Movement of radioactive tracers across squid axon membrane[1]

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TASAKI, I., T. TEORELL AND C. S. SPYROPOULOS. *Movement of radioactive tracers across squid axon membrane.* Am. J. Physiol. 200(1): 11–22. 1961. – Radioactive tracers of potassium, sodium, water, chlorine, sulfate, phosphate and calcium were injected into squid giant axons and the movement of these tracers across the surface membrane was traced either by the standard isotope technique or by the method of gamma-ray spectrometry. The time constants of loss of these tracers through the axon at rest were found to be approximately 8 hours for K^{42}, approximately 3 hours for Na^{24}, 0.7–2 minutes for tritiated water, 25–55 hours for Cl^{36}, 50–85 hours for radioactive sulfate, approximately 50 hours (at the onset) for radioactive phosphate and 20–30 minutes (at the onset) for Ca^{45}. The time constants for the last two tracers were found to increase during experiments, suggesting chemical binding of these tracers with the substance of the membrane and the axoplasm. The effects of repetitive stimulation, potassium depolarization, calcium and magnesium deficiency, etc., upon the movement of the tracers were investigated. Expressed in terms of Krogh's permeability, the ratio of the sodium permeability to the potassium permeability varied under different experimental conditions within the range of 2:1–1:5. The existence of negative fixed charges in the squid axon excitable membrane complex was suggested.

THE PRESENT REPORT deals with the movement of radioactive tracers through the surface membrane of the squid giant axon under various experimental conditions. Radioactive potassium, sodium, water, chlorine, sulfate, phosphate and calcium were used as tracers. These elements constitute the major inorganic components existing normally across the membrane and are thought to play important roles in the maintenance of the normal function of the axon.

Previously, the problem of isotope movement across the squid axon membrane was investigated by Keynes, Hodgkin and co-workers (1–4), by Shanes and Berman (5), Rothenberg (6), Nevis (7) and others. The major portion of the work is a repetition and expansion of this previous work, using what we regard as somewhat improved techniques. The standard method adopted in the present investigation was to inject a solution containing one or two of the tracers into the axon and to measure the radioactivity that appeared in the surrounding sea water. This method which had been used in some of the experiments by Hodgkin and Keynes (1, 2) is more sensitive and reliable than the conventional method of soaking an axon in a bathing fluid containing tracers and detecting the radioactivity of the whole axon.

In all the previous investigations, the experimental data obtained by the use of tracers were analyzed with the intention of gaining information as to the movement of the nonradioactive species. In the present investigation, however, tracers were used simply to determine the relative mobilities, or permeabilities, of various ions through the membrane complex. From the thermodynamical point of view, a tracer injected into a squid giant axon is driven into the surrounding sea water primarily by the gradient of the electrochemical potential of this radioactive species across the membrane. The flux of the tracer is given by the product of this 'driving force' (with due consideration of the membrane potential and the flow of metabolites) and the mobility of the tracer.

The movement of isotopic tracers across various membranes was discussed on the basis of thermodynamics of irreversible processes recently by Kedem and Katchalsky (8), Nims (9) and by Meares (10). Using a cation-exchange resin membrane as a model of the nerve membrane, U. F. Franck and I. Tasaki (unpublished) investigated the basis of the thermodynamical treatment of the tracer experiments on the nerve. The relationship between the permeability as measured by the isotope method and the mobility of the particle species under investigation was discussed by Tasaki (11). In the present article, however, we are concerned mainly with the results of the measurements and not with any elaborate analysis of the data.

METHODS

Microinjection. Giant axons, 0.5–0.7 mm in diameter and about 45 mm in length, were isolated from the squid, *Loligo pealii*. Axons were then cleaned under dark-

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field illumination using a technique employed for isolation of frog nerve fibers. Care was taken to avoid severing small branches close to the surface of the membrane. Axons with large branches (about 40 μ or larger) were discarded.

The axon was mounted horizontally on a glass platform (approximately 40 mm in width) and was stretched by means of threads, one tied on each end. A glass pipette of approximately 70 μ in outside diameter was used to inject radioactive material into the axon. The tip of the glass pipette was covered with a smooth layer of sticky wax; this wax covering at the tip reduced the chance of inflicting injury to the axon during insertion. The technique of microinjection was described previously (12). As a rule, the radioactive material was injected uniformly over a 20 30-mm-long portion of the axon.

Radioisotopes. K^{42} (designated hereafter by K*) and Na^{24} (Na*) were obtained from Brookhaven National Laboratory by air shipment. Carbonate salts of these isotopes were neutralized with HCl and diluted with a 0.55 M KCl solution, the resulting radioactivity being approximately 1 (sometimes 0.3) mc/cc. In order to estimate the pH of the injected fluid and the distribution of this fluid in the axon (following injection), the 'hot' solution was stained with chlorphenol red (approximately 0.05%). In the experiments in which Na* and K* were counted simultaneously by γ-spectrometry, a solution containing approximately 1 mc of K* and approximately 0.5 mc of Na* in 1 cc was prepared. The volume injected into the axon was of the order of 0.3–1.0 cu mm.

Tagged water, H^3_2O, was obtained from New England Nuclear Corporation (10 mc/gm). The injected fluid was prepared by adding 40 mg of KCl to 1 gm of tritiated water and a trace of chlorphenol red.

Radioactive chlorine, Cl^{36}, was obtained from Oak Ridge National Laboratory in the form of hydrochloric acid. The original solution (2.58 M) was diluted with distilled water and was neutralized with KOH, resulting in a 1 M solution of KCl* whose radioactivity was approximately 0.012 mc/cc. (The asterisk designates the tagged species throughout this paper.)

Sulfate containing S^{35} was prepared from the sodium salt solution (50 mc/cc) supplied by Oak Ridge National Laboratory. The solution was diluted, neutralized and, by adding KCl, made isotonic. The radioactivity of the final solution was approximately 5 mc/cc.

The isotopic tracer of phosphate, containing P^{32}, was prepared from the sodium salt supplied by Oak Ridge National Laboratory. The original solution (3.3 mc/cc) was first diluted with water and neutralized and then was made isotonic by adding KCl. The final solution had a radioactivity of approximately 0.7 mc/cc.

The isotope of calcium, Ca^{45}, was obtained from the Oak Ridge National Laboratory. The solution for intracellular injection contained 0.09 M Ca^*Cl_2 and 0.5 M KCl. Care was taken to inject only a limited amount (0.1–0.3 cu mm) of this mixture along the axis of the axoplasm.

Radiation counters. For measuring the radioactivity of Na*, K* and P*, a manually operated counter, model 165 of Nuclear Instrument & Chemical Corporation, with a detector D-32, was used. To measure the radioactivity of Cl*, S* and Ca* (and occasionally for Na* and K*), an automatic sample changer and counter, a combination of model 181A, C-110B, C-111B and a gas flow counter D-47 (Nuclear Chicago) was used.

The following procedure was used for measuring tritiated H_2O. A 50λ aliquot of the fluid bathing the axon (0.5 cc) was mixed in a mixture of three parts [[0.4% 2.5-diphenyl-oxazole, 0.01% *p*-bis[2-(5-phenyl-oxazolyl)] benzene in toluene]] and one part methanol. The total volume was either 10 cc or 15 cc. In a few experiments, the 50λ sample was placed in a mixture containing 1 cc hyamine and 10 cc of toluene containing 0.4% 2,5-diphenyloxazole and 0.01% *p*-bis[2-(5-phenyl-oxazolyl)]-benzene. The mixture was counted with the manually or automatically operated Tri-Carb liquid scintillation spectrometer (model 314-DC) of Packard Instrument Co. The counting efficiency was approximately 4–6%.

Radiation discriminator. The method of discriminating the radiation of Na* from that of K* was similar to that described originally by Öbrink and Ulfendahl (13). The detector used was a scintillation counter, RCA model-DS5. A radiation counter, model 186 of Nuclear Chicago, and a radiation analyzer, model 1810 of Nuclear Chicago, were used in conjunction with a Sorensen a. c. regulator, type 1001.

The effective concentration of Na* and of K* was determined in the following manner: the reading of 'channel I' of the discriminator gave primarily the amount of gamma radiation in the energy range of 1.48–1.62 mev, responding mainly to K* which has a sharp energy maximum at 1.51 mev. 'Channel II' gave primarily the amount of gamma radiation with energy greater than 2.42 mev, responding mainly to Na*. When a solution contained only K*Cl, 'channel I' of the discriminator gave a reading which was 6.0 times as great as that of 'channel II'. When a solution containing only Na*Cl was introduced, the ratio of the reading of 'channel I' to that of 'channel II' was found to be 0.233. These ratios were independent of the concentrations.

The problem of the contribution of beta radiation from K*, and to a lesser extent from Na*, was obviated by employing identical plastic tubes (Nuclear Chicago, model TT-1) and identical volumes of samples (2 cc). The latter was accomplished by diluting the collected fluid with sea water.

The concentrations of Na* and K* in a mixture were determined by the formulae

$$[Na^*] = \frac{R_2 - R_1/6.0}{1 - 0.233/6.0} \qquad (1a)$$

$$[K^*] = \frac{R_1 - 0.233\,R_2}{1 - 0.233/6.0} \qquad (1b)$$

where R_1 and R_2 represent the reading of 'channel I' and 'channel II' respectively with due regard for the activity decay.

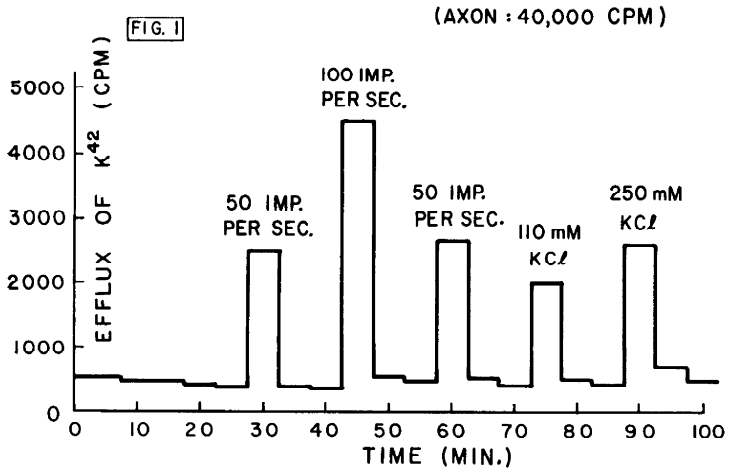


FIG. 1. Efflux of radioactive potassium ions injected into a 20-mm-long portion of a squid giant axon (approximately 500 μ in diameter) plotted as a function of time after injection. *Ordinate* represents radioactivity (in c.p.m.) in total volume of 1.2 cc of sea water collected in each period of 5 min. Stimulus frequencies and concentration of KCl used for K depolarization are given, 23°C.

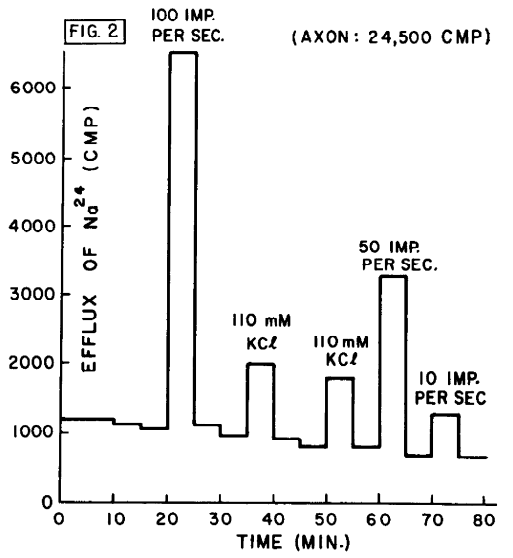


FIG. 2. Similar to fig. 1, except that radioactive sodium was used instead of potassium.

Nerve chamber. In most of the experiments, the axon was transferred immediately after injection of a tracer into a paraffin chamber in which the movement of the tracer was measured. The chamber consisted of a smooth cavity (0.4 0.5 cc capacity and 15–20 mm diameter) and of two grooves, one on either side of the middle cavity. The fluid surrounding the axon in the middle cavity was nonradioactive and was as a rule, but not always, sea water. In each of the two grooves, the axon was making contact with a pair of platinum electrodes, a pair on one end of the axon being used for stimulation and the other pair on the opposite end for recording action potentials extracellularly. The portions of the axon in the grooves were completely covered with Vaseline. The distance between the edge of the main cavity and the nearest platinum electrode was about 5 mm.

A Dumont oscilloscope and a Grass stimulator in conjunction with an isolation unit were used to monitor propagated action potentials. The radioactivity of the axon was measured by transferring the portion of the axon in the middle cavity into a planchet together with the usual amount of sea water and drying the sample. This was done to compensate for the self-absorption of the sample.

RESULTS

PART I. MOVEMENT OF K* AND NA*

Separate determination of the efflux of K and Na*.* The technique employed in this series of experiments was to inject one of these tracers into a squid giant axon and then determine their flux into the surrounding sea water. An attempt was always made to inject the radioactive solutiog uniformly along the entire portion of the axon under study. The axon was transferred immediately after injection into a paraffin chamber. The chamber contained 0.4 cc of normal sea water. Within every 5-minute interval, the sea water bathing the axon was replaced twice. All three collections were combined. Samples of fluid, each 1.2 cc in volume, containing radioisotopes of potassium or sodium were dried and their radioactivity was measured by the standard technique.

A typical example of the results obtained with K* is presented in figure 1. In this example, the surrounding sea water collected at 5-minute intervals, shows radioactivity of 500–400 counts/min. (c.p.m.). When the axon was stimulated at a rate of 50 impulses/sec. for a period of 4.5 minutes, the radioactivity of the sample (collected in a 5-min. interval) suddenly increased to about 2500 c.p.m. On cessation of stimulation, the efflux of K* immediately returned approximately to the resting value.

In all six experiments with K*, the effect of stimulation was highly reproducible. There was a close proportionality between the frequency of stimulation and the loss of radioactivity from the axon (up to 100 impulses/sec.). The loss of radioactivity at rest varied roughly with the amount of radioactivity in the axon (determined at the end of each experiment). Every 5 minutes, approximately 1 % of the radioactivity of the axon was found in the surrounding normal sea water. Conditions in which bathing fluid was not sea water are described later. Stimulation at a rate of 50 impulses/sec. increased this rate of loss by a factor of 3–6. (In the early stages of the investigation, we encountered three axons in which the increase was only twofold at 50 impulses/sec.; these axons were capable of carrying 100 impulses/sec. A nonuniform intra-axonal distribution of K* and/or a partial injury may account for the difference in results.)

Using the same technique, the loss of Na* was investigated. Qualitatively speaking, the behavior of Na* was very similar to that of K* (fig. 2). When a small quantity of isotonic KCl solution containing Na* (whose activity

was 0.1–1 μc) was injected into a 15–20-mm-long stretch of an axon, there was a measurable amount of radioactivity in the surrounding sea water collected over a period of 5 minutes. This efflux at rest was increased by a factor of 2–7 by stimulation at a rate of 50 impulses/sec. There was an approximate proportionality between the frequency of stimulation (up to 100 impulses/sec.); but the results with Na* were less reproducible than those with K*. The loss of Na* in a resting period of 5 minutes varied between 2.5 and 3.3% of the radioactivity in the axon (5 axons at about 22°C).

In order to compare the rate of loss of various tracers, the expression 'time constant' of tracer loss can be used instead of the expression percentage loss of radioactivity per unit time. Under the conditions of the present experiments, the amount of the radioactive ions C* in the axoplasm falls exponentially with time; the asymptote is obviously C* = 0. Therefore, $dC^*/dt = -C^*/\tau$, where τ is the time constant. When Δt is small enough as compared with τ, the time constant can be determined by the formula $\tau = -\Delta t \cdot (C^*/\Delta C^*)$, where $-\Delta C^*$ is the amount of radioactivity lost from the axon in time Δt and C* the total radioactivity of the axoplasm at that moment.

The time constant for the loss of K* was in most cases between 7 and 9 hours at about 22°C; in the later stages of the present investigation (in which the discrimination technique was used), time constants of about 15 hours were frequently obtained. The corresponding figure obtained previously by Shanes and Berman (5) using the soaking technique, is 3.3 hours, which is less than half of the value obtained in the present study. The figure obtained by Keynes (3) for Sepia axons (about 200 μ in diameter) also using the soaking technique, averaged 6.7 hours.

The time constant for the loss of Na* at 22°C was between 2.5 and 3.5 hours. The value obtained by Shanes and Berman (5) was 4.8 hours. Hodgkin and Keynes (1) do not explicitly mention the time constants, but their results do not seem to be very different from those stated above.

Simultaneous determination of efflux of Na and K**. In this series of experiments, a mixture of K* and Na* in the form of chloride and bicarbonate was injected into the axoplasm. The amounts of these tracers appearing in the surrounding medium were determined by the gamma-ray spectrometry technique described by Öbrink and Ulfendahl (13). This technique turned out to be extremely useful for a precise comparison of the movements of K* and of Na*. Such precision would have been impossible without going into tedious statistics on a great number of axons.

The procedure for collecting the fluid surrounding the axon was the same as was used in the preceding experiments. At the end of every experiment, which lasted 1–2 hours, the portion of the axon in the paraffin chamber was transferred to a plastic test tube (Nuclear Chicago, model TT-1) for determining the radioactivity of K* and of Na*. In order to get the total amount of each tracer at the initial moment of an experiment, we added up all the activity of the fractions analyzed to the amount remaining in the axon after the end of the experiment. The ratio of the amount of a tracer in the bathing fluid to the amount present in the axoplasm was divided by 5 (min.) to obtain the 'loss of activity per minute' (fig. 3).

In all the experiments (on 7 axons) made at room temperature (22°–23°C), it was found that the loss of Na* was greater than that of K*, confirming the results obtained by separate measurements of these two tracers. The ratio of loss of Na* to that of K* ranged between 1.3 and 2.2, the average being 1.63.

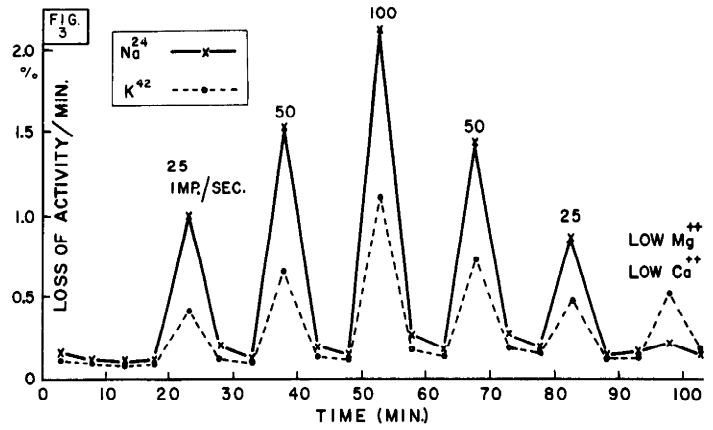
The time constant for loss of K*, calculated as in the preceding section, varied between 8 and 15 hours. This value is slightly greater than that obtained by the method of injecting K* alone; the difference may be attributed to a slight difference in the technique of manipulation or microinjection of the axon. The time constant for the loss of Na* varied between 4 and 15 hours.

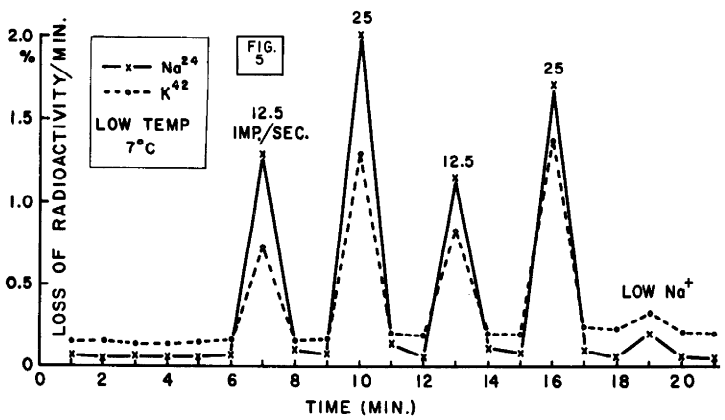
On stimulation of the axon, the loss of the intracellular tracers was greatly accelerated. In all the experiments the loss of Na* was approximately twice as large as that of K*, the ratio ranging between 2 and 2.5 at room temperature (22°–23°C).

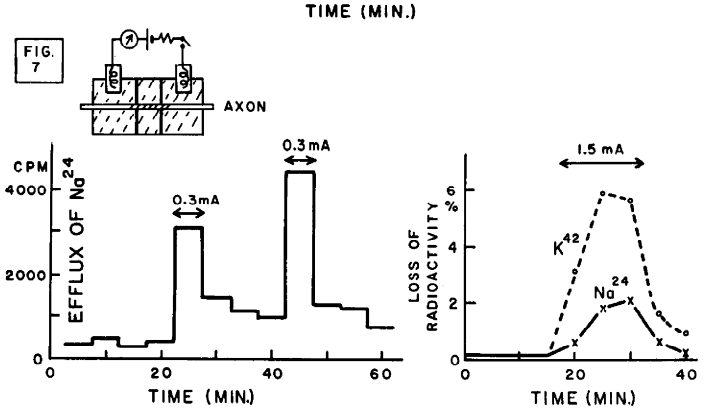
It seems worth emphasizing at this moment that the experimental results showing a great loss of radioactive sodium during activity do not necessarily reflect a great loss of nonradioactive sodium from the axon. It is not possible as a rule to follow the movement of the bulk sodium by tracing the movement of Na*, because the 'driving force' (viz. the gradient of the electrochemical potential) acting upon Na* is very different from that upon nonradioactive Na ions. The same statement can be made of the relationship between the movement of the bulk potassium and the loss of K*.

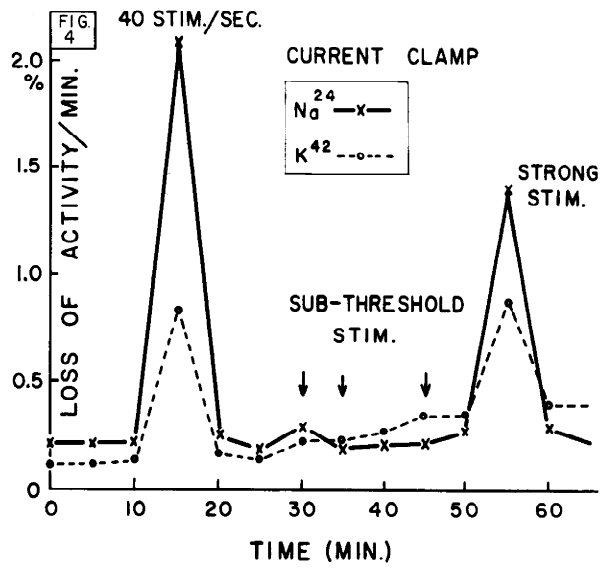
In the experiments described up to this point, the axons were excited by a pair of external electrodes making contact with the portion of the axon embedded in Vaseline. In a few instances, we attempted to measure the movements of Na* and K* in axons stimulated with an intracellular electrode. These observations were carried out in the following manner:

A mixture of Na* and K* was injected into a 20-mm-long portion of an axon. Upon withdrawal of the injection pipette, an internal electrode set made with two silver wires was introduced into the injected portion of the axon. The electrode set consisted of a current wire 50 μ in diameter, with 17-mm-long uninsulated portion, and a recording wire with a small uninsulated portion in the middle of the current electrode. The axon carrying these internal electrodes was mounted on a chamber with two Lucite partitions (sealed with Vaseline). The three pools of sea water separated by these two partitions were grounded with large Ag-AgCl (agar) electrodes. With this arrangement, a strong pulse of current flowing outwardly through the axon membrane is expected to activate the entire portion of the axon in the middle pool simultaneously. The fluid collected from the middle pool was analyzed with the gamma-ray spectrometer.









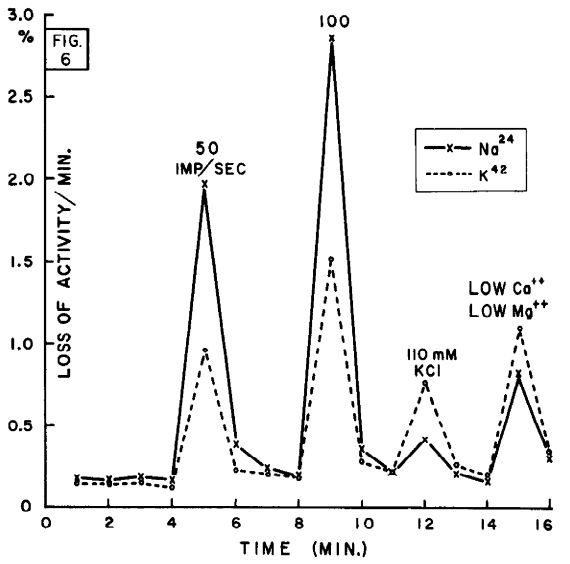


FIG. 3. Simultaneous determinations of effluxes of radioactive Na and K injected into a squid giant axon (approx. 400 μ in diameter). *Ordinate* represents loss of radioactivity/min. expressed in percentage of radioactivity present in the axon; *abscissa* is time after injection of a mixture of Na* and K*. Stimulus frequencies are given. In period between 95 and 100 min., the axon was immersed in artificial sea water from which $MgCl_2$ and $CaCl_2$ were eliminated; to several test stimuli delivered during this period, the axon responded with multiple conducted responses, 23°C.

FIG. 4. Similar to fig. 3 except that axon was stimulated with a longitudinal current electrode in the axon. Stimulating currents were supplied by a square pulse generator (30–50 v. and 0.25 msec.) connected to current electrode through a 1-megohm resistor and a 0.1 μf condenser. Stimulus frequency was 40/sec. throughout. Axon diameter varied from 600 μ at the proximal end to 400 μ at the other end. Decrease in efflux of Na* near the end of experiment is not necessarily related to the stimulus intensity.

FIG. 5. Similar to fig. 3 except that axon was transferred to a nerve chamber kept at 7°C immediately after intracellular injection of a mixture of radioactive Na and K. Axon diameter was approximately 400 μ.

FIG. 6. Effects of repetitive stimulation, depolarization by sea water containing 110 mM KCl, and of sea water containing no divalent ions upon the efflux of radioactive Na and K ions through squid axon membrane. Fiber diameter was approximately 500 μ.

FIG. 7. Effect of outward-directed membrane current upon rate of loss of Na* (*left*) or Na* and K* (*right*) from squid giant axons. *Ordinate* represents radioactivity in fluid collected at intervals of 5 min. from the pool of sea water in which the cathode was immersed; *abscissa*, time after injection of Na* or a mixture of Na* and K*. Duration and intensity of the current are indicated. *Blackened portion* of axon in diagram (not to scale) indicates the zone carrying the radioactive tracer.

Figure 4 shows an example of the results obtained by the use of an internal stimulating electrode. It is seen in this figure that subthreshold stimulation of the axon did not bring about any significant increase in the movement of the tracers; a slight increase seen in the figure may be attributed, at least partly, to occasional firing of impulses while attempts were being made to keep the stimulus intensity at a barely subthreshold level. The ratio of the loss of Na* to that of K* did not seem different from that obtained with external electrodes.

Effect of cooling on efflux of K and Na**. Axons were cleaned and injection of a mixture of K* and Na* along the axis of the axons was carried out at room temperature. The axons were then transferred to a paraffin chamber kept at low temperature. For stimulation of the axon, a pair of external electrodes (making contact with the portion of the axon embedded in Vaseline) was used. The conduction velocity of the axon was monitored with another pair of electrodes on the other end of the axon.

In the axons suddenly transferred to cold sea water, the efflux of Na* at rest was found to become smaller than that of K*. One example of the three consistent experiments performed at low temperature is presented in figure 5. Between 7° and 8°C, the loss of K* from the axon per 5-minute interval was 0.7–0.9% of the total amount of K* in the axon; this figure is only slightly smaller than the value obtained at room temperature. The loss of Na* during the same period of time was 0.3–0.4% which corresponds to ½ to ½.5 times the loss of K*. These findings are consistent with the results obtained by Hodgkin and Keynes (1) who found that the Na* efflux depends more markedly on temperature than the K* efflux.

On repetitive stimulation of cooled axons, the rate of Na* loss was markedly enhanced. At a frequency of 12.5 impulses/sec., the loss in a period of 5 minutes was found to be 6–7% of the internal Na*, corresponding roughly to a 20-fold increase in the rate of loss. There was a definite increase in the rate of loss of K* during activity, but the increase was less marked than that of Na*.

Effect of abnormal ionic environment on efflux of Na and K**. The effect of increasing the external potassium concentration upon the movement of K* and of Na* was examined on 12 axons, all yielding consistent results. The potassium content was increased usually by mixing natural sea water with a 500 mM KCl solution; in two experiments, potassium-rich media were prepared by replacing NaCl in artificial sea water with the proper amount of KCl. When the potassium content in the medium was raised from the normal value (9 mM) to 110 mM, the rate of loss of K* from the axoplasm was accelerated by a factor of 3–4 at room temperature (figs. 1, 2 and 6). The rate of loss of Na* was also increased; the loss in sea water containing 110 mM KCl was 1.7–2 times as great as in normal sea water. In sea water containing 250 mM KCl there was a more rapid loss of the intracellular K* and Na*. When these potassium-rich media were replaced with normal sea water, there was a restoration of nerve conduction.

Removal of both calcium and magnesium ions in artificial sea water induced a pronounced increase in the rate of loss of K* and Na* from the axoplasm (fig. 6). Replacement of natural sea water around an axon with artificial sea water containing 423 mM NaCl, 9.0 mM KCl, 9.3 mM $CaCl_2$, 22.9 mM $MgCl_2$, 25.5 mM $MgSO_4$ and 2.2 mM $NaHCO_3$ did not affect the rate of loss of the monovalent tracers. When this medium was replaced with a Ca^{++}-free Mg^{++}-free sea water (containing only 423 mM NaCl, 9 mM KCl and 2.2 mM $NaHCO_3$), the axon responded, as is well known, (e.g. Arvanitaki (14)), to a single shock with multiple responses and sometimes the axon fired spontaneously. Under these conditions, the loss of K* from the axoplasm was accelerated by a factor of 5–10, while the efflux of Na* was increased by a factor of 1.5–5. Since repetitive stimulation in normal sea water affects the Na* efflux more than the K* efflux, the effect of low calcium can not be regarded simply as the result of spontaneous firing. Furthermore, the same results were obtained in the axon which showed no spontaneous firing of conducted responses. The importance of calcium ions in the process of excitation has been repeatedly stressed [see reviews of Heilbrunn (15) and Brink (16)].

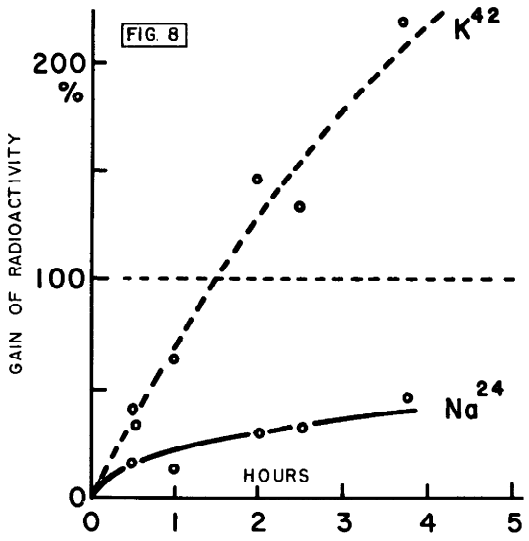
Replacement of NaCl in artificial sea water with choline chloride was found to increase the rate of loss of intracellular Na* ions by a factor of approximately 2. This finding is consistent with the results obtained by Hodgkin and Keynes (1) who reported a 20–50% increase of the tagged sodium in the Sepia axon in Na-free choline sea water. The effect of removal of sodium upon the movement of K* was slight at room temperature.

Effect of electric current on efflux of Na and K**. A maintained outwardly directed current through the membrane was found to markedly increase the efflux of intracellularly injected Na* and/or K*.

The arrangement employed in four of the six experiments of this series is illustrated by the diagram in figure 7, top. The axon was mounted on a chamber consisting of three compartments separated by two Lucite partitions. The small middle pool, approximately 5 mm wide, was filled with sea water and the fluid was replaced at 3-minute intervals. In each of the two lateral pools, a large electrode of the Ag-AgCl-sea water (agar) type was immersed. A battery (6 v.) was used as a source of electric current. The current intensity was varied by means of a variable resistor (max. 60 KΩ). The current was longitudinal up to the lateral pool where the current flowed through the membrane. The radioactivity was measured in the lateral pool.

Two examples of the results of the experiments of this type are furnished in figure 7. In these experiments, the major portion of the 'hot' region of the axon was situated in the middle pool. The fluid in one of the lateral pools was replaced twice every collection period of 5 minutes. The radioactivity of the collected fluid was counted as in the previous experiments.

Before the battery circuit was closed, there was a small flux of tracers into the fluid bathing the axon. When a cathodal (depolarizing) current of 0.09–1.5 ma was sent through the axon, there was a marked increase in the



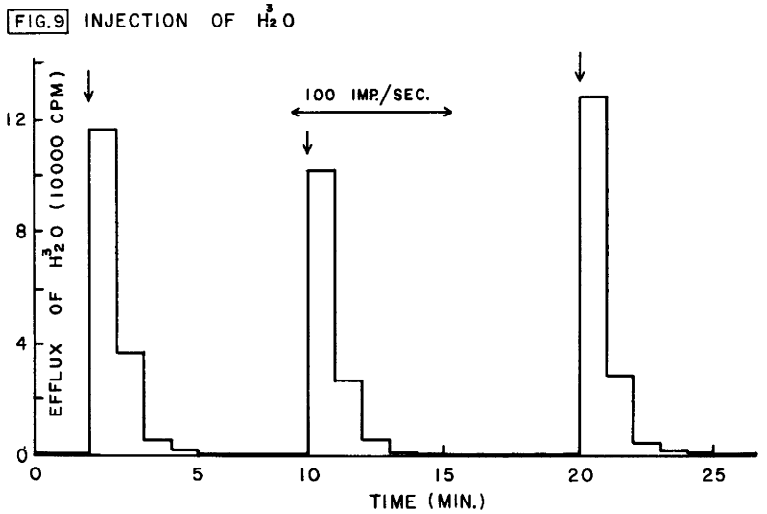


FIG. 8. Accumulation of K* and Na* in axoplasm of the axons immersed in sea water containing both K* and Na*. *Ordinate* represents concentration of radioactive ions expressed in percentage of the concentration in the bathing sea water; *abscissa* is time of immersion, 23°C.

FIG. 9. Loss of tritiated water from a squid giant axon following intracellular injection. The bathing sea water was collected every 1 min. *Arrows* indicate time at which tracer was injected into axon. Second injection was made while the axon was being stimulated at 100 shocks/sec., 23°C. No correction was made for the efficiency of counting arrangement (see METHODS).

flux of both K* and Na*. In those cases where a mixture of K* and Na* was used, the flux of K* in the stationary state was found to be 1.5–3 times as great as that of Na*. When a slightly different electrode arrangement was used (in which the two lateral pools were connected to the anode and the middle pool was connected to the cathode), the flux of K* during maintained cathodal depolarization was found to be 3–6 times the flux of Na* (observation on 2 axons).

The interpretation of the experiments just alluded to is complicated by the situation that the flux is affected not only by the mobilities of the tracers in the membrane but also by the mobilities in the axoplasm. Nevertheless, the results may be taken as indicating a similarity in the effects of depolarization by cathodal current and those caused by high potassium or by low calcium.

From the results of voltage clamp experiments, Hodgkin and Huxley (17) suggested that the sodium carrier mechanism is completely 'inactivated' by maintained depolarization. If this is so, no increase in the movement of Na* should be observed in this experiment. Our experiments show that the mobility of sodium ions remains at a high level during maintained depolarization. Our finding is also consistent with the conclusion drawn from the electrohydraulic nerve analog which predicts an over-all increase in the efflux of salt during the early part of prolonged voltage clamp (18).

Simultaneous determination of influx of K and Na**. When a clean axon was immersed in 'hot' sea water containing K* and Na*, both the axoplasm and the sheath (axonal membrane, Schwann cell layer and adhering small amount of connective tissue) became radioactive. We made a concentrated though unsuccessful attempt to determine the relative amount of Na* and K* in the sheath by the use of gamma-ray spectrometry. The results obtained suggested that an appreciable amount of Na* may be retained in the sheath. However, in part because of the difficulty of removing the axoplasm from the sheath completely, we could not obtain any quantitative information as to the ratio of Na* and K* in the sheath.

It is relatively easy to obtain more-or-less clean samples of axoplasm from axons soaked in 'hot' sea water. Previously, Rothenberg (6) and Hodgkin and Keynes (2) studied the radioactivity of the axoplasm by this method. The method of measuring the radioactivity of a whole axon is very simple, but this simple method does not give reliable results when the concentration of the tracer in the axoplasm is low.

Our experiments to measure the influx of K* and Na* were carried out in the following manner: approximately 10 cleaned axons were kept in normal sea water for a period of 0.5–1.5 hours. Then, all the axons were transferred into a large Petri dish containing 'hot' sea water. After various incubation periods, the axon was removed from the bathing fluid and its ability to conduct impulses was tested by delivering a single shock to the axon. Subsequently, the excess sea water adhering to the axon was removed by resting the axon on a 'Kimwipe' tissue. After discarding about 1-cm terminal portions of the axon, the axoplasm was extruded by a technique described elsewhere (19). The volume of the axoplasm was measured by sucking the extruded mass into a hemocytometer pipette. The axoplasm was then transferred into a plastic test tube for determination of the radioactivity of K* and Na* by the scintillation counter. The concentration of Na* and of K* in the bathing sea water was determined by measuring the radioactivity of a known volume of fluid.

In figure 8, the radioactivity of the axoplasm was

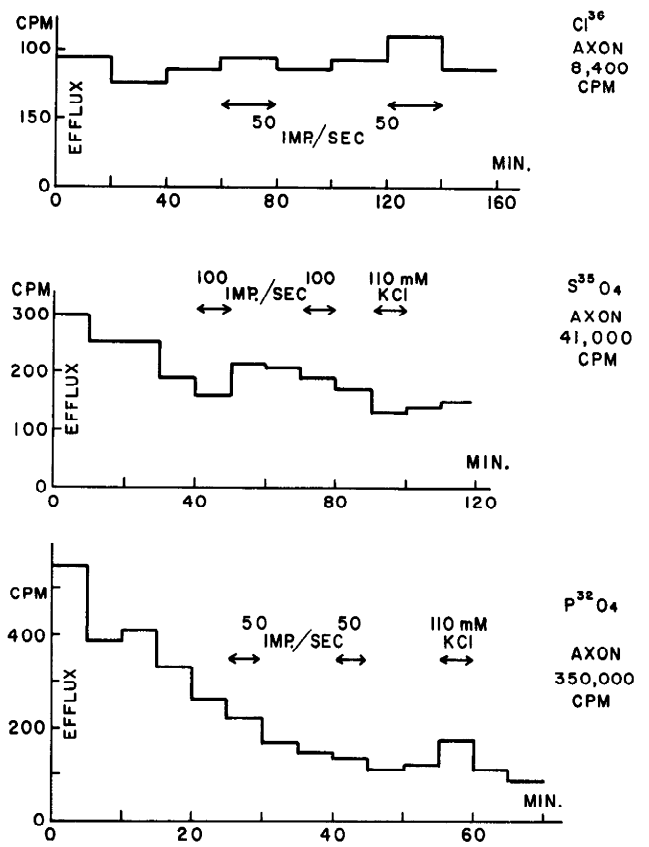


FIG. 10. Efflux of 3 different radioactive anions, chloride (*top*), sulfate (*middle*) and phosphate (*bottom*), injected into squid giant axons. Collection period was 20 min. for Cl*, 10 min. for S^*O_4 and 5 min. for P^*O_4. Intra-axonal injection of tracers was completed approximately 5 min. before the time marked zero. Axon diameter was 600 μ (*top*), 450 μ (*middle*) and about 500 μ (*bottom*), 22°–23°C.

plotted against the time of immersion of the axons in sea water containing K* and Na*. The concentration of the tracers in the bathing fluid was taken as 100% in this figure. It is seen that the concentration of K* in the axoplasm increased well above the level in the bathing fluid. The concentration of Na* remained at a low level; it is possible, however, that there was some contamination of the axoplasm by the sea water or by the material in the sheath, tending to raise the Na* level above the true value.

As will be discussed later, the concentration ratio for Na* across the membrane is expected to reach, in the final stationary state, that for nonradioactive Na; namely, the asymptote of the concentration of Na* in the axoplasm is given by $[Na^*]_i/[Na^*]_o = [Na]_i/[Na]_o$, where $[Na]_i$ and $[Na]_o$ are the concentrations of 'cold' sodium ion in- and outside the axon membrane, respectively. In an analogous manner, the asymptote of the concentration of K* is $[K^*]_i/[K^*]_o = [K]_i/[K]_o$. This qualitatively explains why the concentration of K* in figure 8 rises well above 100% while that of Na* ceases to rise at a low level. Because of the technical difficulties in this experiment, it is doubtful whether the data can be used for further quantitative analysis.

Nevertheless, our data, as well as the previous data obtained by Rothenberg (6), suggested that the time constant for potassium exchange is much longer than that for sodium exchange.

PART II. MOVEMENTS OF OTHER TRACERS

Efflux of tritiated water. In a preliminary experiment, it was found that the movement of tritiated water across the squid axon membrane was extremely rapid. The bathing sea water collected in a period of 5 minutes following injection of $H^3{}_2O$ was found to contain 80–90% of the radioactive water injected. It became necessary on this account to employ a special nerve chamber in which the injection pipette could remain in the axon while collections were made of the bathing sea water. The portion of the axon bathed in sea water (approximately 0.5 cc) was 15–17-mm long; the remaining portions were embedded in Vaseline. Stimulating and recording electrodes (platinum wires) made contact with the portion of the axon in Vaseline. The time required for injection was 5–10 sec. We could follow the loss of radioactive water from the axon at an interval of ½ minute by this method.

The time required for loss of 63% of the injected tritiated water was found to vary between 0.7 and 2 minutes at room temperature. These values are in agreement with the data obtained by Nevis (7) who employed the soaking method. At low temperatures (5°–8°C), there was a slight, statistically significant delay in the loss of radioactivity.

We spent a considerable amount of time in attempting to find out the means of influencing the rate of loss of tritiated water from the axon. In very few instances, a high frequency stimulation seemed to have accelerated the loss; but in the majority of the cases, the effect was not clear at all (fig. 9). The effects of electric current, sodium deficiency, potassium depolarization, calcium- and magnesium-free solutions upon the movement of water were also investigated extensively; we could not obtain any clear-cut results.

It must be emphasized that the time constant given above may very well not reflect only the time required to cross the surface structures of the axon; it may simply reflect the time expended in reaching the surface from the site of injection. If the axoplasm is a serious bottleneck, the variability in the results may partly be accounted for by the degrees of departure of the injection tract from the center of the axon.

*Efflux of Cl**. On account of the extremely low activity, the amount of radioactivity which could be introduced into a squid axon by massive injection of KCl* was only 7000–9000 c.p.m. After injection, the axon was transferred to a nerve chamber of the type used in the experiments of figures 1 and 2. A sample of sea water, bathing such an axon for a period of 20 or 30 minutes, gave radioactivity well above the background level of the counting machine. In four of five determinations, only 0.03–0.05% of the intracellular Cl*, came out of the axon in a period of 1 minute; in one axon, the loss was approximately 0.07%/min. This means that the time

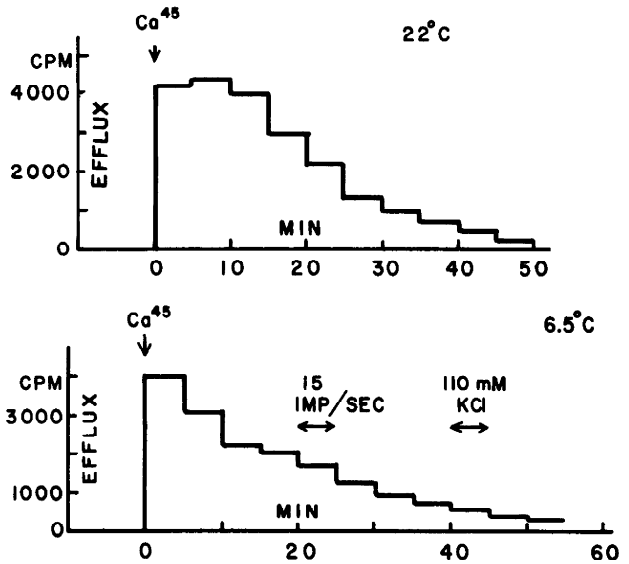


FIG. 11. Efflux of radioactive Ca ions following intra-axonal injection at room temperature (22°C) and at low temperature (6.5°C). *Arrows* mark time of injection. Collection period was 5 min., and bathing fluid was renewed 3 times in each collection period. Axon diameter 590 μ (*upper*) and 500 μ (*lower*).

constant (time required for the amount of intracellular tracer to reach 63% of the initial value) was 25–55 hours, roughly 10 times the values for the monovalent cationic tracers.

The following consideration suggests that the difference between the cationic tracers and Cl* could be more pronounced in intact axons than in excised ones. As is well known, there are on the surface of squid giant axons a number of small branches which are inevitably cut on excision and cleaning. These 'holes' in the membrane may be less 'permselective' than the intact portion of the membrane. Undoubtedly there is a continuous flow of inward current through these holes, tending to carry anions outward and cations inward. Any injury inflicted upon the axon is expected to reduce the 'permselectivity' of the membrane. It is possible, therefore, that the movement of Cl* is even slower in intact axons than in excised ones.

Previously, Shanes and Berman (5) reported that the permeability of the squid axon membrane to sodium, potassium and chloride ions is of the same order of magnitude. They measured the fall in the radioactivity of the whole axon following immersion in sea water containing Cl*. According to Steinbach (20), the concentration of chloride in the squid axon is only about 40 mM. The discrepancy between the earlier results on Cl* efflux and ours can then be accounted for as due to the difference in the technique employed. When the amount of Cl* in the sheath is appreciable as compared with the amount in the axoplasm, the rate of loss of Cl* from the whole axon is expected to be faster than the value determined by the method of intracellular injection of Cl*.

The effect of repetitive stimulation upon the efflux of intracellular Cl* was investigated on five axons. In two axons, there was no increase in the efflux; in the remaining three axons, there was an increase of 5–10% at a stimulus frequency of 50 impulses/sec. The results from the axon which gave the most conspicuous increase was shown in figure 10, top. In the case of K* or Na*, the effect of repetitive stimulation was always conspicuous even when the concentration of the tracer in the axoplasm was very low. The absence of a clear effect of repetitive stimulation was common to all the anionic tracers examined.

Because of the long immersion period required for detecting the movement of Cl*, the effects of various agents have not been investigated thoroughly. The effect of depolarization by a 110 mM KCl sea water in one axon showed a slight increase in the rate of loss, but in another axon the effect was dubious.

Efflux of S^*O_4. With sulfate ions containing S*, the radioactivity in the axoplasm could be raised to a level of 50,000 c.p.m. by intracellular injection. The loss of radioactive sulfate from such an axon was found to be 0.02–0.035% of the total amount in the axoplasm in 1 minute (measurement on 2 axons). This indicates that the time constant for the loss of radioactive sulfate is slightly longer than that of Cl*. Neither repetitive stimulation nor depolarization by potassium-enriched (110 mM KCl) sea water showed any detectable effect upon the movement of this tracer (fig. 10, middle).

Efflux of P^*O_4. The behavior of phosphate ions containing P* was slightly different from that of radioactive sulfate. Following a massive injection of this tracer, there was a loss of radioactivity from the axon of the order of 0.03%/min. This value, which is comparable to that of the radioactive sulfate, was found to decrease gradually to 0.008–0.015%/min. in the course of about 1 hour. This rate of loss of the intracellular radioactive phosphate was so low (the time constant being of the order of 150 hr.) that the total radioactivity of the axon remained practically unchanged for many hours.

The gradual decrease in the rate of loss was observed in all the experiments with P^*O_4 (5 axons). This indicates that the loss of P^*O_4 from the axoplasm does not obey a simple exponential law. We believe that this phenomenon is related to the participation of phosphate in the metabolic activity of the axoplasm and the sheath. Such participation by sulfate would be expected to be of a lower order of magnitude. Essentially the same result was obtained by Keynes and his collaborators in *Loligo forbesi* (personal communication).

*Efflux of Ca**. The loss of radioactive calcium from the axoplasm did not follow a simple exponential time course. In a preliminary experiment, it was found that sea water collected within 5–10 minutes following injection of Ca^*Cl_2 showed a high level of radioactivity. In the later experiments, therefore, the process of injection was carried out in the nerve chamber where collection of the bathing fluid was made; the procedure was similar to that used in the observation of the movement of tritiated water.

Care was taken to inject the radioactive solution uni-

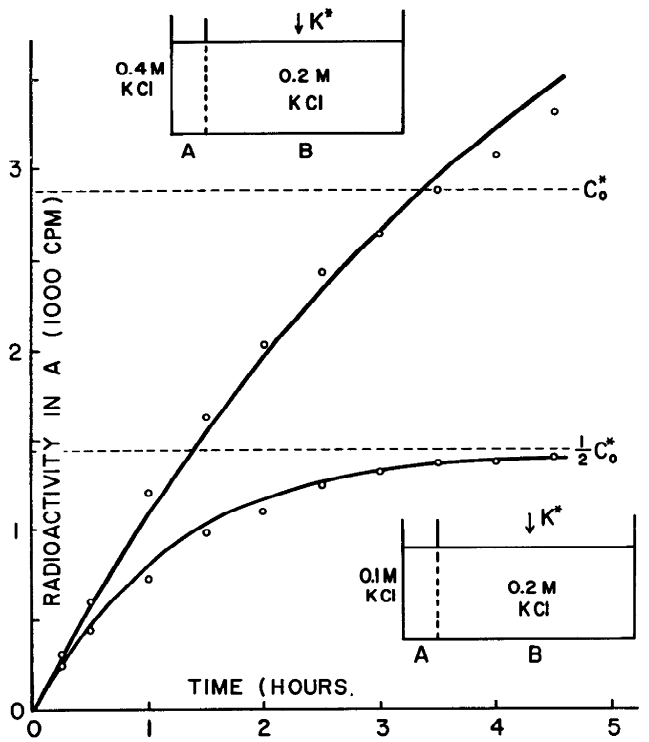


FIG. 12. Movement of K* through a cation-exchange membrane. Experimental conditions are illustrated by the two diagrams in figure. Note that rate of accumulation of K* in the small compartment (A) is the same at $t = 0$ under 2 different experimental conditions.

formly along the axis of the axon; when the procedure was satisfactory, a long column of the red injection fluid was formed in the center of the axon. Higher final concentrations of injected Ca* resulted as a rule in a block of conduction. The experimental data obtained from those axons incapable of carrying 100 impulses/sec. or more after a period of about 1 hour of injection, were discarded.

In the first several collection periods (each 5 min.), there was always a surprisingly large efflux of radioactive calcium (fig. 11). The loss of radioactivity was estimated to be 3–5 %/min. in this period; if this rate of loss were maintained, the radioactivity in the axoplasm would decrease at a time constant of 20–30 minutes. In the following collection periods, the rate of loss of the radioactivity decreased more rapidly than was expected from a simple exponential time course.

The movement of tagged calcium was investigated previously by Hodgkin and Keynes (2). Insofar as the slow movement of Ca* in the later stage is concerned, our results appear to be essentially the same as theirs. The rapid phase was present in all of our experiments (8 axons).

Hodgkin and Keynes (2) maintain that the tagged calcium remaining in the axoplasm was not free; they applied an electric field to the axon and found no displacement of the radioactive zone of the whole axon. We did not make corresponding observations. The departure of the time course of appearance of Ca* in the outside medium from the exponential may reasonably be related to the ability of Ca* to bind to cellular components (the surface inclusive).

We examined the effect of repetitive stimulation on the movement of tagged calcium. We could not detect any change in the rate of loss of radioactivity even in the stage during which the rate was rapid (fig. 11, right). The effect of depolarization by potassium was not clear either.

It must again be pointed out that, as in the case of tritiated water, the axoplasm may provide an appreciable barrier to the movement of Ca*. The measured time constant then does not necessarily reflect the mobility of calcium ions through the excitable membrane complex.

DISCUSSION

We have described in the preceding section the experimental results with a minimum of interpretation. We have seen that the squid axon membrane is more permeable to the cations than to the anions examined. The time constant of loss of intracellular Cl* was found to be an order of magnitude larger than that of intracellularly injected monovalent cations. This fact strongly suggests that the nerve membrane has properties of a 'charged membrane' in which negatively charged radicals are more-or-less immobile. A long time ago, Michaelis (21) pointed out the possibility that cell membranes are charged. This idea was put in a quantitiative form by one of us (Teorell, 22–24) and by Meyer and Sievers (25). Now it is apparent that there is a similarity between the squid axon membrane at rest and the cation-exchange membrane.

Besides the fixed charge of the membrane, the metabolism of the axon has to be taken into consideration in the discussion of the movement of the tracers through the axon membrane. Since the pioneer work of Osterhout and Stanley (26), it is well known that the characteristic distribution of ions across the cell membrane is the result of metabolism. Our theoretical treatments of the metabolism determining the resting membrane potential and the distribution of ions were presented elsewhere Teorell (24), Tasaki and Spyropoulos (11, and manuscript in preparation). The coupling between the continuously flowing metabolites and other ions in the membrane is considered to determine the distribution of ions across the resting membrane. When a tracer amount of a radioisotope is introduced on one side of the membrane, the gradient of the electrochemical potential driving the radioisotope is very different from that acting upon the nonradioactive species of the same element. In the final stationary state, the two isotopic species are expected to be distributed across the membrane at the same concentration ratio.

Under these circumstances, the flux of the radioisotope (J^*) can be described by the following equation:

$$J^* = P(C_i^* - \xi C_o^*), \qquad (2)$$

where C^*_i and C^*_o are the concentrations of the radioisotope in- and outside the membrane, respectively, ξ

the concentration ratio C_i/C_o of the nonradioactive species of the same element and P the 'permeability constant' of Collander and Bärland (27), Krogh (28, 29) and Teorell (24). This equation states simply that the flux J^* is proportional to the deviation from the distribution of the radioisotope in the final, stationary state. The flux J^* is related to the time derivative of intracellular concentration of the radioisotope by the equation

$$aJ^* = -v\frac{dC_i^*}{dt} \qquad (3)$$

where a is the area of the membrane and v the volume of the axon. In the appendix of this paper, several observations on artificial membranes are described to illustrate the significance of P and ξ.

When the extracellular concentration of the radioisotope is close to zero as it was in most of the present experiments, the term containing ξ in the flux equation (2) is eliminated and P can be determined by measuring the ratio J^*/C^*_i. Under RESULTS, we have compared the permeabilities of various ions in terms of the loss of the intracellular radioactivity in unit time. We have seen that the 'permeability' of the squid axon membrane to sodium ions is slightly greater than (or roughly comparable to) that of the potassium ions. The membrane appears to be highly permeable to the unbound form of calcium ions.

Unfortunately, the relationship between the permeability P and the ionic conductance is not simple. The relationship between the ionic conductances and the membrane potential is also very complicated in the biological systems. Our point of view on these problems is presented elsewhere (24 and manuscript in preparation).

It is important to point out that *equations 2* and *3* are valid only when the system under study is in a stationary state with respect to all the nonradioactive ion species. Strictly speaking, a resting axon may not be in a stationary state. When an axon is suddenly placed in an abnormal ionic environment, the axon at that moment is not in a stationary state. However, if a new roughly stationary state is soon reached, the observed time constant for the loss of radioactivity can be taken as a measure of the membrane permeability to the ion under study. An axon suddenly transferred to a bath of cold sea water is in all probability in a nonstationary state. When an axon is subjected to repetitive stimulation, the system is obviously in a nonstationary state, and therefore, interpretation of the results is more difficult.

The experiments of figures 1–7 revealed that the potassium permeability was increased more strikingly by potassium depolarization than the sodium permeability. Application of Ca-free, Mg-free sea water and maintained cathodal polarization by electric current were also shown to increase markedly the potassium permeability. It appears to us that the effects of these agents are closely related, because, in a membrane with negative fixed charge, enrichment of potassium in the membrane (either by cathodal polarization or by immersion of the axon in a potassium-rich medium) is expected to lower the amount of calcium in the membrane. In a cation-exchange membrane, it is well known that the flux of monovalent ions can be reduced by the addition of divalent ions to the system.

Repetitive stimulation increased the outward movement of the monovalent cationic tracers, particularly sodium. This can be interpreted as the result of: *a*) an increase in permeability to cations, *b*) a change in the forces driving ions through the membrane, *c*) a change in the amount of cations held by the membrane during activity, and *d*) transport of cations by membrane currents flowing between the resting and active areas of the membrane.

A pronounced decrease in the squid axon membrane resistance during activity is well known (30). Since the flux of anions is not increased during activity (fig. 10), the membrane appears to retain its negative fixed charge. Any change in the density of the negative charge is expected to alter the amount of cations held in the membrane. A rhythmical change in this chemical capacity should increase the movement of tracers through the membrane.

The force that drives an ion through the membrane consists of two terms; one is the deviation of the membrane potential from the thermostatic equilibrium level of the ion and the second is the resultant force arising from the movement of other ions and of water. A rise of the intracellular potential (or a decrease in negativity) during activity increases the force driving cations outwardly. The net result of the interaction with other ions is expected to change during activity; but it is difficult to estimate the magnitude of this change. The experiment of figure 7 suggests that the effect of the local current associated with a propagated impulse is not a predominant factor in the movement of cationic tracers. For the movement of nonradioactive sodium and potassium, the local current and the current flow due to nonuniform excitation at the wave-front of a propagated impulse appears to play a significant role.

It is desirable to know in what phase during activity the enhanced movement of Na^* or K^* takes place. The duration of the action potential of a squid axon injected with tetraethylammonium chloride and subjected to low temperature is often as long as 0.2–1 second. We could increase the time resolution of detection of the radioactivity to the extent that the efflux of tracers during various phases of this prolonged action potential could be determined. Our investigation along this line is still in progress and the results will be presented in a subsequent paper.

APPENDIX

The purpose of this appendix is to illustrate the significance of the *equations 2* and *3* under simple experimental conditions. Two identically designed Lucite chambers, each consisting of two compartments, were constructed to simulate the situation in which the cell interior and the surrounding fluid medium constitute two compartments. The partition between the two compartments was

made with a cation-exchange resin membrane (Ionics, Inc., CR-61) which, as is assumed for the squid axon membrane, is more permeable to cations than to anions. The small compartment was approximately 22.5 cc in volume and the large one had a capacity of 2500 cc. The area of the cation-exchange membrane was 22.5 cm^2, the volume-to-area ratio being 1 cm.

In the first observation, the two compartments were filled with a 0.2 M KCl solution. Then, approximately 3 μc of K*Cl was introduced into the small compartment. The loss of radioactivity through the permselective membrane was followed by taking samples of 0.1 cc from the small compartments at intervals of 30 minutes. The fluid in both the compartments was stirred vigorously during the entire course of the observation. The loss of radioactivity was found to follow an exponential time course and the time constant determined was between 2.4 and 2.6 hours. This time constant remained unaltered when the KCl concentration in the large compartment was varied between 0.1 and 1.0 M.

We denote the concentration of K* in the small compartment by C_i* and that in the large compartment by C_o*. *Equations 2* and *3* applied to the case above is

$$-\frac{dC_i^*}{dt} = P(C_i^* - \xi C_o^*), \qquad (4)$$

where P is the 'permeability constant' and ξ the concentration ratio of nonradioactive KCl. (Note that v/a is equal to 1 cm.) The observation mentioned above indicates that P is given by the reciprocal of 2.4–2.6 hours. Since $C_o^* \div$ 0 under the conditions of this observation, the term containing does not affect the time course of the 'efflux' of K*.

In the second observation, the 'influx' of K* was determined using the same experimental setup (fig. 12). The concentration of nonradioactive KCl in the large compartment was 0.2 M and that of K*Cl was 0.08 μc/cc in both chambers. The concentration of KCl in the small compartment was, in one experiment (fig. 12, bottom) 0.1 M, and in the other (top) 0.4 M. Since the volume of the large compartment was so large, there was no change in the radioactivity during the course of the observation (except for the effect of the decay of K* which was automatically taken care of by measuring the radioactivity of all the samples at one time). The concentration of K* in the small compartment was maintained at zero for a period of approximately 30 minutes by circulating nonradioactive KCl solution (0.1 M in 1 experiment and 0.4 M in the other): this was done to establish a stationary state (satisfying the condition $C_i^* = 0$) at the onset of the experiments. The abscissa in figure 12 is the time after K* was allowed to accumulate in the small compartment. The ordinate represents the radioactivities in the small compartments as a function of time.

Equation 4 applied to the observations of figure 12 has the following forms:

$$\frac{dC_i^*}{dt} = P'(C_o^* - \tfrac{1}{2}C_i^*) \qquad (5)$$

for the case $C_i:C_o = 2:1$ and

$$\frac{dC_i^*}{dt} = P'(C_o^* - 2C_i^*) \qquad (6)$$

when $C_i:C_o = 1:2$.

At $t = 0$, $C_i^* = 0$ in both cases. At this moment the flux of K* should be the same as that in the first observation; therefore, the value of P' for the second observation should be equal to P in the first observation. By integrating *equations 5* and *6*, with respect to t we have

$$C_i^* = 2C_o^*(1 - e^{-0.5Pt}), \qquad (5')$$

and

$$C_i^* = 0.5\, C_o^*(1 - e^{-2Pt}). \qquad (6')$$

The continuous curves in figure 12 were obtained by introducing into these equations the observed radioactivity in the large compartments (C_o^* = 1880 c.p.m.) and the permeability constant determined in the first observation ($P = 1/2.4$ hr.). The agreement between the observed and calculated values attest to the approximate validity of *equations 2* and *3* in the DISCUSSION under the conditions of the present experiments.

In an experiment where the fixed charge density would be comparable or less than the bulk total ion concentrations the exact significance of ξ, as employed here, would be r_2/r_1 (r's are the Donnan ratios at the membrane boundaries) as can be inferred from Teorell's basic equations applied to KCl (where the interior diffusion potential vanishes; expecially *eq. 12* in (24)). However, when the membrane charge is relatively higher, as is the case in the present experiments, it can be shown that r_2/r_1 approaches C_i/C_o. Only in such a case would the change in C_i be sufficiently small during the period of the experiment. The permeability constant P is in general a function of the charge-bulk concentration relation.

REFERENCES

1. HODGKIN, A. L. AND R. D. KEYNES. *J. Physiol.* 128: 61, 1955.
2. HODGKIN, A. L. AND R. D. KEYNES. *J. Physiol.* 138: 253, 1957.
3. KEYNES, R. D. *J. Physiol.* 114: 119, 1951.
4. KEYNES, R. D. AND P. R. LEWIS. *J. Physiol.* 113: 73, 1951.
5. SHANES, A. AND M. D. BERMAN. *J. Gen. Physiol.* 39: 279, 1955.
6. ROTHENBERG, M. A. *Biochim. et biophys. acta* 4: 96, 1950.
7. NEVIS, A. H. *J. Gen. Physiol.* 41: 927, 1958.
8. KEDEM, O. AND A. KATCHALSKY. *Biochim. et biophys. acta* 27: 229, 1958.
9. NIMS, L. F. *Yale J. Biol. & Med.* 31: 373, 1959.
10. MEARES, P. *Tr. Faraday Soc.* 55: 1970, 1959.
11. TASAKI, I. *Science* 132: 1661, 1960.
12. BRADY, R. O., C. S. SPYROPOULOS AND I. TASAKI. *Am. J. Physiol.* 194: 207, 1958.
13. ÖBRINK, K. J. AND H. R. ULFENDAHL. *J. Appl. Radiation & Isotopes* 5: 99, 1959.
14. ARVANITAKI, A. *J. Neurophysiol.* 5: 89, 1942.
15. HEILBRUNN, L. V. *An Outline of General Physiology* (3rd ed.). Philadelphia: Saunders, 1952, 818 pp.
16. BRINK, F. *Pharmacol. Rev.* 6: 243, 1954.
17. HODGKIN, A. L. AND A. F. HUXLEY. *J. Physiol.* 116: 497, 1952.
18. TEORELL, T. *Acta Soc. med. Upsalien.* 65: 4, 1960.
19. SPYROPOULOS, C. S. *J. Neurochem.* 5: 185, 1960.
20. STEINBACH, H. B. *J. Cell. & Comp. Physiol.* 17: 57, 1941.
21. MICHAELIS, L. *Kolloid Ztschr.* 62: 2, 1933.
22. TEORELL, T. *Proc. Soc. Exper. Biol. & Med.* 33: 282, 1935.
23. TEORELL, T. *Ztschr. Elektrochem.* 55: 460, 1951.
24. TEORELL, T. *Progr. in Biophysics* 3: 305, 1953.
25. MEYER, K. H. AND J. F. SIEVER. *Helvet. chim. acta* 19: 649, 1936.
26. OSTERHOUT, W. J. V. AND W. M. STANLEY. *J. Gen. Physiol.* 15: 667, 1932.
27. COLLANDER, R. AND H. BÄRLUND. *Acta Botanica Fennica* 11: 5, 1933.
28. KROGH, A. *Proc. Roy. Soc., London, ser. B* 133: 140, 1946.
29. HOLM-JENSEN, I., A. KROGH AND V. WARTIOVAARA. *Acta Botanica Fennica* 36: 1, 1944.
30. COLE, K. S. AND H. CURTIS. *J. Gen. Physiol.* 22: 649, 1939.