Permeability of the squid axon membrane to several organic molecules[1]

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TASAKI, I, AND C. S. SPYROPOULOS. *Permeability of the squid axon membrane to several organic molecules.* Am. J. Physiol. 201(3): 413–419. 1961.—The permeability of the squid axon membrane to choline, guanidine, thiourea, urea, sucrose, starch, and cesium was investigated by use of radiotracers. In the resting state, the time constant for the loss of intracellular radioactive (free) choline, guanidine, and cesium was of the same order of magnitude as that for labeled sodium or potassium ions. Choline ion was found to bind rapidly with the constituents of the axon. In sea water containing labeled choline, there was accumulation of radioactivity by the axon. On repetitive stimulation, guanidine and cesium efflux was markedly increased; the choline efflux was slightly increased. Labeled thiourea and urea moved across the membrane far more readily than the univalent cations. Permeation of labeled sucrose and starch was very slow.

IN THE GIANT AXON OF THE SQUID, it is relatively easy to inject various isotopic tracers directly into the axoplasm and to follow their flux into the surrounding fluid medium. Using this method, movement of various radioactive inorganic ions across the squid axon membrane was investigated by Hodgkin and Keynes (1, 2) and by Tasaki, Teorell, and Spyropoulos (3).

In order to secure additional information as to the physicochemical nature of the excitable membrane, it seemed to us desirable to investigate the permeability of the axonal membrane to a variety of ions and neutral molecules which do not normally exist in the axon or in the sea water, or may exist at extremely low concentrations. In the present investigation, we are concerned with the movement of C^{14}-labeled choline, guanidine, thiourea, urea, sucrose, starch and Cs^{134} across the squid axon membrane under various experimental conditions.

METHODS

Material and technique. North Atlantic squid, *Loligo pealii*, available at the Marine Biological Laboratory in Woods Hole, were used. The technique of isolating and cleaning the axons was the same as that employed previously (3). The excised axons were 40–50 mm in length. Isotonic solutions containing radioactive tracers were uniformly injected into the 20 mm long cleaned portion of the axon. The technique of injecting tracers was the same as in the previous experiments. In most of the experiments, the axons were transferred after injection into a paraffin chamber in which the efflux of the radiotracer was determined.

The main compartment of the paraffin chamber was 20 mm long and had a capacity of approximately 0.5 cc. There was a narrow groove on each end of the main compartment, where the uninjected portion of the axon was introduced. A pair of platinum electrodes making contact with one of the terminal portions of the axon in the groove was used to stimulate the axon and another pair near the other end of the axon was used to record propagated impulses. The uninjected portions of the axons in the grooves were covered with Vaseline. A Grass stimulator (with a stimulus isolation unit) and a Tektronix oscilloscope were used for monitoring action potentials. The injected portion of the axon in the main compartment was immersed, as a rule, in normal sea water.

In the present series of experiments, tracer influxes were investigated only with radioactive choline ions; the technique employed will be described under RESULTS.

All experiments were carried out at room temperature (21–23 C).

Radioactive tracers. Choline-1,2-C^{14} chloride was obtained from Volk Radiochemical Co. A 0.5 M solution of this radioactive choline chloride had an activity of 0.55 mc/ml. This solution was stained with a small amount of chlorphenol red (to estimate the pH and monitor the injection). A volume of 0.5–1 mm^3 of the solution was injected into the axon.

C^{14}-labeled guanidine hydrochloride (6 mc/mM) was supplied by California Corporation for Biochemical Research. This chemical was dissolved in distilled water to make an isotonic solution. The injection fluid was prepared by mixing one part of this guanidine solution with two parts of 0.54 M KCl solution stained with a trace of chlorphenol red. The radioactivity of the final solution was approximately 1 mc/ml.

Radioactive thiourea and urea (C^{14}-labeled) were

Received for publication 20 February 1961.

[1] This work was done at the Marine Biological Laboratory, Woods Hole, Mass.

supplied by California Corporation for Biochemical Research. An isotonic solution of this chemical had an activity of approximately 8.3 mc/ml. The solution was diluted with a 0.54 M KCl solution (stained with chlorphenol red) by a factor of 4 to make the injection fluid (approx. 2.1 mc/ml).

Sucrose (C^{14}-labeled), supplied by California Corporation for Biochemical Research, had a specific activity of 1.65 mc/mM. An isotonic solution of this tracer stained with chlorphenol red (0.9 mc/ml) was used for injection.

Radioactive starch was obtained from Nuclear Chicago Corporation. The amount of 3.3 mg (0.05 mc) of this tracer was dissolved in 50 mm^3 of 0.54 M KCl solution stained with chlorophenol red, yielding a solution with an activity of 1 mc/ml.

Cs^{134} was purchased from Oak Ridge National Laboratories (10 mc of this isotope dissolved in 0.6 ml of 2.15 N HCl). The original solution was diluted with distilled water by a factor of 3 and the pH was adjusted with potassium bicarbonate. The solution was then diluted with an isotonic KCl solution (stained with chlorphenol red) by a factor of approximately 3, thus giving a final solution with a radioactivity of 1.7 mc/ml.

Measurement of radioactivity. Samples of approximately 1.2 cc of sea water containing C^{14}-labeled compounds were dried in planchets and the radiation from the samples was counted with a Nuclear Chicago automatic counter (models 181A and C-110B) in conjunction with a gas flow detector (model D-47 with a window) and a printing timer (model C-111B). Self-absorption of samples was kept constant by employing identical volumes of sea water in planchets. The radioactivity of the whole axon was measured at the end of each experiment. This was done by transferring the portion of the axon in the main measuring compartment into a planchet containing an identical volume of sea water as was employed with the extracellular samples; and after cutting the axon into small pieces in the planchet, the sample in the planchet was gently dried.

Method of analyzing effluxes of tracers which are not chemically inert. When a small amount of radioactive tracer is injected into a squid giant axon, the normal constituents of the axon may chemically bind the tracer, resulting in a gradual reduction of its freely diffusible form. Calcium, phosphate, and choline are examples of such chemically active ionized radicals. In such cases, the efflux of the tracer diminishes with time as a result of both the chemical binding and by transport across the membrane.

To treat such cases analytically, we make the following simplifying assumptions: *1*) the concentration of the free tracer is uniform within the axon, *2*) the transport of the free tracer across the membrane is proportional to the intracellular concentration of the unbound tracer, *3*) the rate of chemical binding is also proportional to the intracellular concentration of the free tracer, and *4*) the membrane is practically impermeable to the product of the chemical binding.

When the rate of chemical reaction is high, assumption *1* is not valid. Since the elimination of this assumption leads to a considerable mathematical difficulty, we adopt this even when it is only a poor approximation. Assumption *2* is probably valid as long as the external concentration of the tracer is practically zero. The squid axon membrane appears to have very low permeability to molecules of the size of sucrose; therefore, the efflux of the tracers in the bound form is likely to be very slow. The justification of assumption *3* derives from the situation that the reaction which depends on the square of the concentration of the tracer can not proceed because of the extremely low level of the intracellular tracer concentration. This assumption may, with some compounds, be only a poor approximation (e.g., choline).

Let C_f^* and C_b^* denote the concentration of the free and bound forms of the tracer in the axoplasm, respectively. The total concentration $(C_f^* + C_b^*)$ decreases with time as the result of transport across the membrane. According to assumption *2*, we have

$$-\frac{d(C_f^* + C_b^*)}{dt} = pC_f^* \qquad (1)$$

where p, the rate constant for transport, is equal to the "permeability coefficient" times the area-volume ratio of the portion of the axon under investigation (5). The rate of increase in C_b^* is, according to assumption *3*, given by

$$\frac{dC_b^*}{dt} = kC_f^* \qquad (2)$$

in which k is the rate constant for chemical reaction. Combining *equations 1* and *2*, we have

$$-\frac{dC_f^*}{dt} = (p + k)C_f^* \qquad (3)$$

the solution of which is obviously

$$C_f^* = C_o^* e^{-(p+k)t} \qquad (4)$$

where C_o^* is the intracellular concentration of the tracer immediately after injection. Introducing *equations 4* into *1*, we find that

$$-\frac{d(C_f^* + C_b^*)}{dt} = pC_o^* e^{-(p+k)t} \qquad (5)$$

We now multiply both sides of *equation 5* by v, the volume of the axoplasm under study, and convert the equation into a more convenient form. Denoting the amount of the radioactive material that came out of the axon in the ith collection period by Δn_i, the total amount

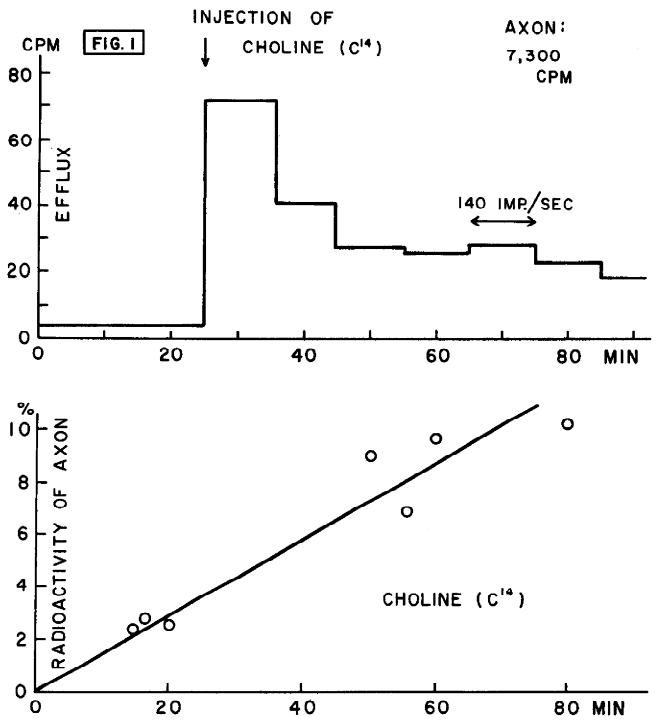

FIG. 1. *Upper:* appearance of radioactivity in sea water following injection of C^{14}-labeled choline chloride into squid giant axon; each period for collection of sea water was 10 min; injection pipette was introduced into axon approximately 30 min before injection. *Lower:* accumulation of radioactivity by squid giant axon immersed in sea water containing C^{14}-labeled choline chloride; the radioactivity per unit volume of the axon (expressed in percentage of radioactivity in medium) was plotted against time of immersion.

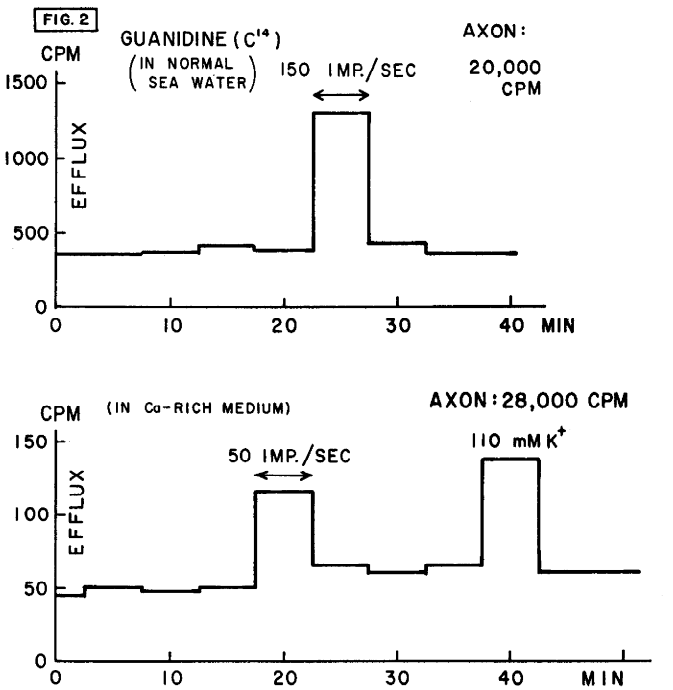

FIG. 2. *Upper:* efflux of C^{14}-labeled guanidine following uniform injection into squid giant axon (500μ diam.); axon was kept in normal sea water during experiment; each collection period was 5 min. *Lower:* comparison of effect of repetitive stimulation to that of potassium depolarization upon efflux of intracellular C^{14}-labeled guanidine; axon was kept in sea water, of which Ca content was raised to approximately 35 mM; axon diameter approximately 700μ.

of the radioactive material introduced into the axon n_o, which is equal to vC_o^*, is given by

$$n_o = \Delta n_1 + \Delta n_2 + \Delta n_3 + \cdots + n_a \qquad (6)$$

where n_a is the amount found in the axon at the end of the experiment. Insofar as one collection period, Δt, is much shorter than the over-all time constant, $1/(p + k)$, the time derivative of $v(C_f^* + C_b^*)$ can be replaced with $\Delta n_i/\Delta t$. Thus we have

$$\frac{\Delta n_i}{\Delta t} = p n_o e^{-(p+k)t} \qquad (7)$$

The determination of the two rate constants p and k from the measurement of Δn_i is straightforward. We plot Δn_i against time on a semilogarithmic scale; the slope of the straight line corresponds to $(p + k)$. The value of p can be determined from the efflux measurement at the beginning: $p = \Delta n_i/(n_o \Delta t)$. When the rate constant for chemical combination k is far smaller than p, the slope of the straight line should give the same value as that determined from the initial flux.

RESULTS

Choline ion. The measurable radioactivity of C^{14}-labeled cholin that could be introduced into a squid giant axon by the injection technique described under RESULTS was between 5,000 and 15,000 count/min. It is assumed that this radioactivity is due to 1,2-C^{14}-labeled choline and not by some unknown impurity. This amount of radioactive choline in an axon did not bring about any detectable change in the rate of propagation of impulses across the injected region. When such an axon was immersed in normal sea water, there was measurable radioactivity in the sample of sea water collected in a period of 5–10 min. The efflux of the radioactive compound was found to decrease to a low value in a period of 20–30 min after injection.

Because of this rapid decrease in the efflux, collections of the radioactive samples were made on the plate where the injection of the tracer was carried out. This technique was used previously in determining the rate of efflux of tritiated water and of Ca^{45} (3). An example of the results obtained by this method is presented in Fig. 1, *upper*. In this preparation the time constant of loss of intracellular radioactivity by transport across the membrane ($1/p$ in *equation 7* under METHODS) was found to be of the order of 15 hr. The time constant of

the diminution of the efflux, i.e., the value of $1/(p + k)$, described in METHODS, was found to be approximately 30 min in this case.

In six axons, between 400 and 550 μ in diameter, the time constant $1/p$ varied irregularly between 10 and 25 hr. This range is similar to that of the time constant for the loss of intracellular Na^{24} and K^{42} ions (3). The "time constant for chemical binding," $1/k$, varied between 20 and 40 min. The large variability in the value of $1/p$ can probably be attributed to the situation that, because of the rapidity of chemical binding, the initial amount of the tracer in the axon (n_o in METHODS) does not give a reliable value of the concentration of the unbound tracer molecule near the membrane.

The effect of repetitive stimulation upon the efflux of C^{14}-labeled choline was investigated. When the axon was stimulated at a frequency of 50–150 shocks/sec, there was an increase in the efflux of 10–20% above the level which would be expected if no stimuli were delivered (Fig. 1, *upper*). This finding does not necessarily indicate that the permeability to this ion species is altered only slightly during activity. Because of the great rapidity of chemical binding, it is possible that our measurement of the efflux is not determined by the membrane permeability. There is also a possibility that the rate of chemical binding is accelerated by repetitive stimulation. The possibility that the radioactive material found in the sea water is not choline itself cannot be excluded in the experiment above; however, the following (influx) experiment strongly supports the view that the axon membrane is permeable to choline ions.

It is a common practice among neurophysiologists to replace the sodium ion in the medium with choline when a reduction in the sodium concentration is desired. Therefore, it seemed interesting to investigate the process of uptake of C^{14}-labeled choline by the axon from the surrounding medium. The uptake of the radioactive choline was studied in the following manner.

Approximately 40 mm long axons were meticulously cleaned. About 20 min after cleaning, the axons were examined under dark-field illumination. The axons with clean, uniform appearance were then transferred into sea water containing radioactive choline. The radioactivity of the medium ranged from 6,000 to 40,000 count/min/mm^3 of sea water. After various incubation times, which varied between 15 and 80 min, the axons were transferred into a large volume of normal sea water and the diffusible radioactive choline outside the axon was removed by changing the sea water several times. After a period of 40–60 min in normal sea water, single electric shocks were delivered to the axons to test conduction. About 20 mm long middle portions of those axons which were capable of carrying impulses were then transferred into planchets and their radioactivity was determined by the procedure described under METHODS.

The results obtained are presented in the lower diagram of Fig. 1. The radioactivity of the axons (membrane inclusive) per unit volume is expressed in percentage of the radioactivity of the medium. Since the volume-to-area ratio of the axon varies with the diameter, axons of approximately the same diameter (400μ) were used in this experiment. It is seen in this diagram that the radioactivity of the axon increases approximately linearly with time of incubation. Within the range of radioactivity employed, the uptake of the labeled choline increases with an increase of the concentration of the isotope in the medium. The results in Fig. 1 indicate that the average concentration of the labeled choline in the axon rises at the rate of approximately 9% (of the external concentration) per hr, or 0.17%/min. The results obtained by exposing to radioactive choline only a 5 mm long portion of the cleaned axon (the remaining being immersed in mineral oil) were similar. (We have seen a similar phenomenon in *Nitella flexilis*; when these plant cells were immersed in the medium containing labeled choline there was a rapid accumulation of labeled compound by the cells.)

Based on these findings on the movement of labeled choline, one can explain a previous observation on the effects of substitution of the external sodium with choline (1, 3). It was shown previously that the efflux of Na* (which was 0.3–0.5%/min in axons immersed in normal sea water) was roughly doubled when the external sodium was replaced with choline. Such a replacement did not affect the movement of intracellular labeled potassium appreciably.

On account of the tendency of choline ions to combine with some normal constituents of the axon, the concentration of free choline ions in the axoplasm of a normal axon is expected to be very low. When the sodium ions in the normal sea water are replaced with choline, we expect a rapid uptake of choline ion by the axon. In the axons carrying no net current through the membrane, this movement of positively charged choline ions should be associated with either outward movement of some other cations or inward movement of anions. Since there is no sodium in the medium in this case, the movement of the intracellular sodium can be traced faithfully by the labeled sodium injected into the axon. The movement of chloride, sulfate, and other anions across the membrane was found to be much slower than that of cations. It appears to us therefore reasonable to conclude that the efflux of nonradioactive sodium ion (as revealed by the efflux of Na* in the absence of sodium in the medium) is a process required to satisfy the condition of electric neutrality. This in turn may imply that Na ions move across the membrane in the charged form.

Guanidinium ion. Guanidine, $(NH_2)_2C = NH$, is a very strong base, approaching the alkalies in strength. In aqueous solution, it is in an ionized form, $(NH_2)_2C = N^+H_2$, resonating among three equivalent structures (cf. p. 213 in [4]). Following injection of C^{14}-labeled guanidine into a squid giant axon, there was a readily detectable efflux of radioactive material into the surrounding fluid medium.

The time constant for the loss of intracellular radio-

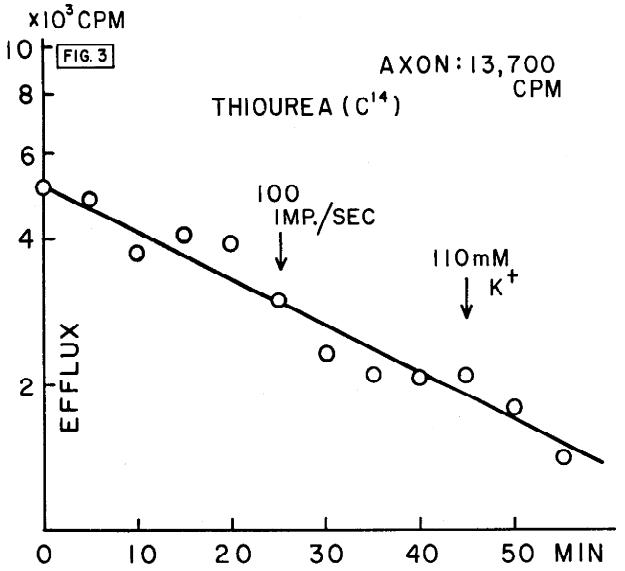

FIG. 3. Efflux of intracellular C^{14}-labeled thiourea plotted in semilogarithmic scale; each collection period 5 min; axon diameter 550μ.

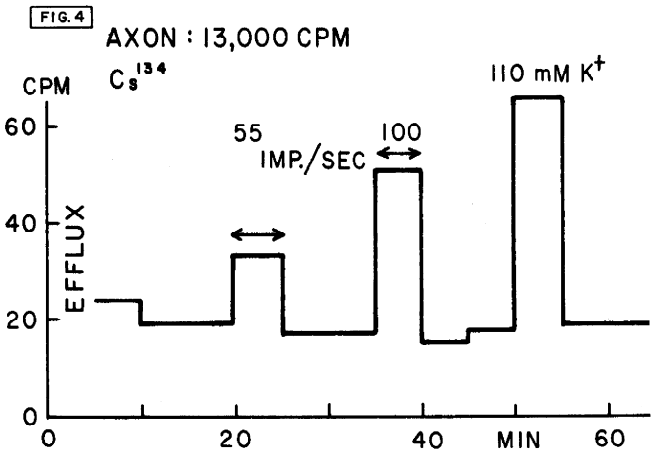

FIG. 4. Efflux of intracellular radioactive cesium during rest repetitive stimulation, and potassium depolarization; each collection period 5 min; axon diameter 220μ.

activity, as determined from the ratio of the radioactivity in the axon to the efflux, was found to be between 4 and 15 hr. This range coincides with that for the efflux of Na* and K*. The wide variation in the time constant appears to be at least partly attributable to the tendency of the axon to fire repetitively under the action of guanidine.

When the measurable radioactivity introduced into the axon was 20,000 to 30,000 count/min there was usually no spontaneous discharge of impulses as long as the axon was kept in normal sea water. Following high-frequency stimulation of such an axon, it was seen, on several occasions, that the axon kept on firing impulses repetitively after withdrawal of external stimulation. On a few occasions, spontaneous firing started immediately after injection of guanidine. Spontaneous discharge of impulses could be readily suppressed by a slight increase in the calcium content of the surrounding fluid medium.

The effect of repetitive stimulation was examined on five axons. As in the case of radioactive sodium and potassium ions, the efflux of radioactive guanidine was markedly increased by repetitive stimulation. At a frequency of 50 impulses/sec the efflux increased by a factor of 2 to 8 (Fig. 2). On account of the wide variation in the efflux at rest, no extensive effort was made to determine the frequency dependence of the efflux. Spontaneous discharges of impulses were also accompanied by a marked increase in the efflux.

An increase in the potassium content of the surrounding sea water from the normal value (9 mM) to 110 mM was found to increase the efflux by a factor of 2–3 (observation on two axons). An increase in the calcium content in the medium was found to decrease the resting efflux (three axons). Within 1 to 2 hr after injection, we could not find any evidence for chemical binding of radioactive guanidine with axonal constituents.

Thiourea and urea. The efflux of neutral tracers, $(NH_2)_2C^*S$ and $(NH_2)_2C^*O$, was measured by the standard technique described under METHODS. These two tracers, when injected intracellularly, came out of the axon at a time constant of 30–90 min. (The time constant for labeled thiourea was slightly shorter than that for labeled urea; but the difference observed may not be significant.)

Fig. 3 shows an example obtained with radioactive thiourea. The efflux is seen to obey a simple exponential law. The ratio of the efflux to the intracellular radioactivity (METHODS) remained approximately constant during the experiment, indicating that the bulk of intracellular tracer molecules remain freely diffusible during this period. The irregularity seen in the figure can partly be attributed to the experimental procedures designed to influence the fluxes.

The effect of high-frequency stimulation was investigated on six axons (three for thiourea and three for urea). In some instances repetitive stimulation seemed to have influenced the efflux slightly; but in general the effect was completely absent. The effect of potassium depolarization upon the efflux was equally dubious.

Sucrose and starch. By injection of C^{14}-labeled sucrose or starch, it was possible to obtain an intracellular radioactivity of the order of 10,000 count/min, or more. The efflux observed in a soaking period of 5 min was always close to the background activity of the counting machine (15–30 count/min). Because of this low radioactivity of the samples measured, there was considerable inaccuracy in our determination of the time constant for the loss of these tracers. The present es-

timation of the time constant is 50–150 hr for labeled sucrose and 150–200 hr for labeled starch. Because of the existence of cut branches on the surface of the axons, the value for the uniform intact portion of the membrane could be larger than the observed value. Such may also be the case for the determination of anionic effluxes (3). Neither repetitive stimulation nor potassium depolarization had any clear effect upon the efflux of labeled sucrose and starch.

Cesium-134. After injection of a small amount of KCl solution containing radioactive Cs, the measurable radioactivity of the axons was in the range between 10,000 and 30,000 count/min. The time constant for the loss of this intracellular radioactivity was between 5 and 15 hr. On stimulation of the axons at a frequency of about 50 impulses/sec the efflux was increased by a factor of 2–3. Depolarization of the axon membrane by immersion in a medium containing 110 mM KCl (prepared by mixing 550 mM KCl with artificial sea water) increased the efflux by a factor of 2.5–3. An example of the results obtained is presented in Fig. 4.

DISCUSSION

Based on a thermodynamic treatment of an open system (5), it was shown that the flux of a radioactive tracer per unit area of the squid axon membrane, J^*, can be expressed by

$$J^* = P(C_i^* - \xi C_o^*) \qquad (8)$$

where C_i^* and C_o^* are the concentrations of the chemically free radioactive species in- and outside the membrane, respectively, ξ is the Teorell factor (6, p. 364) which corresponds to the ratio C_i^*/C_o^* at the final stationary state and P is the permeability coefficient (7). When the nonradioactive analogue of the radioactive material normally exists in the axoplasm and in the medium, ξ is expected to coincide in the stationary (nonequilibrium) state with C_i/C_o, where C_i and C_o are the concentration of the nonradioactive analogue in- and outside the membrane, respectively. The coefficient P depends not only on the mobility of the tracer in the membrane but also on the factors that determine ξ (i.e., the interactions between various flows across the membrane). More recent thermodynamic studies indicate that *equation 8* is general enough to include the effect of possible (coupled but isoaffine) chemical reactions in the membrane.

In most of the experiments described in this paper, the external concentration of the tracer was close to zero. Under these circumstances, the intracellular concentration of the tracer is expected to fall exponentially toward zero with a time constant given by $v/(aP)$ in the absence of chemical binding, where v is the volume of the axoplasm and a is the area of the membrane of the axon under study. The coefficient p in *equation 1* in METHODS is equal to P in *equation 8* times the ratio a/v.

The experimental data described under RESULTS indicate that the value of P for small neutral molecules (thiourea and urea) is greater than the value for Na* or K*. The values of P for the univalent cationic tracers examined, namely choline, guanidine, and Cs*, were found to be of the same order of magnitude as the previously reported values for Na* and K*. The large neutral molecules, labeled sucrose and starch, were shown to move far more slowly than the cations mentioned above.

During repetitive stimulation of the axon, the efflux of the cationic tracers (remaining free in the axoplasm) was markedly accelerated. Depolarization of the axon membrane by an increase in the potassium content in the medium markedly increased the efflux of the cationic tracers; among the alkali metal ions examined, the effect of potassium depolarization seemed to increase progressively with increasing atomic numbers. Conversely, the effect of repetitive stimulation seemed to decrease progressively with increasing atomic numbers. We have studied also the movement of injected Rb^{86} and found that it is similar to that of labeled K or Cs. The effect of varying the calcium content in the medium appeared to be qualitatively the same for all of the univalent cationic tracers. The movement of the neutral molecules was not affected by repetitive stimulation nor by potassium depolarization.

These findings can be interpreted as providing additional support for the view that the squid axon membrane has a dense structure with negative fixed charge in the active state as well as in the resting state (3), as was suggested originally by Michaelis (8) and Teorell (9). The excitable membrane is, according to the present results, "permselective" in the sense that the cation permeability is much higher than the anion permeability both in the resting and active states. However, our experimental results do not give any support to the view that postulates the existence of high specificity with regard to chemical species among different alkali metal ions. The difference among different cations is, so far as our experimental results are concerned, quantitative and not qualitative. Recent experiments in our laboratory (10) on *Nitella* appear to be consistent with this conclusion. Effluxes of radioactive K, Cs, Rb, and guanidine were fractionated during the action potential. The time course of efflux of these isotopes roughly paralleled the time course of the impedance changes; whatever difference we could detect between these tracers appeared to be quantitative.

The implication of our finding, showing accumulation of choline ion by the axon, was discussed under RESULTS.

Finally, a brief discussion will be made of the relationship between the flux of a radiotracer and the flux of its nonradioactive analogue. When a biological system, such as a squid axon, is in a state not far from a stationary (but nonequilibrium) state, the flux of the nonradioactive component, J, can be described by $J = P(C_i - \xi C_o)$. Since P and ξ can be regarded as being common for the two isotopic species (cf. 5), it is possible to determine J if C_i, C_o, P, and ξ are known accurately

enough. If we assume that the chemical species under study is not a metabolite in the sense discussed elsewhere (5), and if the system is in a stationary state, then $J = 0$. In a special case where $\xi = 1$, the flux equation becomes $J = P(C_i - C_o)$ which is nothing more than Fick's law. Although the flux J can be expressed as the difference between $P\ C_i$ (which is sometimes called efflux) and $P\ C_o$ (influx), this division of J into two terms has to be regarded as a mere mathematical trick to calculate J. (Note that we distinguish J^* from J.)

When the chemical species under study exist mainly in the membrane or in the inclusions (mitochondria, etc.) near the membrane (as probably is the case with Ca^{++}), or when the amount of the species in the membrane varies with time (e.g., during excitation), the efflux equation discussed above becomes a very poor approximation or inapplicable.

The authors express their thanks to Dr. Toshihiko Oikawa who helped with the early phase of the experiments on the squid axon.

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