Permeability of Squid Axon Membrane to Various Ions

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ABSTRACT The permeability of the squid axon membrane was determined by the use of radioisotopes of Na, K, Ca, Cs, and Br. Effluxes of these isotopes were measured mainly by the method of intracellular injection. Measurements of influxes were carried out under continuous intracellular perfusion with an isotonic solution of potassium sulfate. The Na permeability of the resting (excitable) axonal membrane was found to be roughly equal to the K permeability. The permeability to anion was far smaller than that to cations. It is emphasized that the axonal membrane has properties of a cation exchanger. The physicochemical nature of the "two stable states" of the excitable membrane is discussed on the basis of ion exchange isotherms.

INTRODUCTION

The permeability of the squid axon membrane to various ions can be determined either by the use of isotopic tracers or by measuring the effects of the ions in question upon the membrane potential. In the tracer method of permeability measurement, the concentrations of the radioisotopes needed are as a rule far smaller than those of the normally existing ions. One can therefore safely assume that the physicochemical state of the membrane is not altered by the experimental procedure. In the potential method of permeability measurement, however, this is not usually the case.

In order to evaluate the membrane permeability by measuring the concentration effect of an ion upon the membrane potential, it is necessary to vary the ion concentration on one side of the membrane to a much greater extent. A change in the membrane potential is usually accompanied by an alteration in the membrane resistance and very often by a reduction or loss of excitability. In such an altered state the electrochemical properties of the membrane (mobilities of various ions, ion selectivities, etc.) can be very different from those in the original, unaltered state. Thus it is difficult to estimate the membrane permeability to various ions in the normal, resting (and excitable)

state by this method (*cf.* Krogh, 1946, p. 181). In this respect, the tracer method is more ideal than the potential method.

In determining the membrane permeability by the tracer method, it is highly desirable to measure both influxes and effluxes of tracers across the membrane. In the squid giant axon, it is relatively easy to measure tracer effluxes. By measuring the radioactivity in the sea water surrounding an axon into which a tracer has been injected, the time course of tracer efflux can be followed accurately (Hodgkin and Keynes, 1956; Tasaki, Teorell, and Spyropoulos, 1961). In the past it was extremely difficult to measure tracer influxes with reasonable accuracy because the intracellular tracer concentration could only be determined by extruding the axoplasm. When intracellular perfusion of the squid axon was found to be feasible (Oikawa *et al.*, 1961; Baker *et al.*, 1961), therefore, it seemed worth while to reinvestigate the permeability of the axon membrane to various ions by the use of this new technique.

The present paper deals mainly with the results of measurements of tracer fluxes across the squid axon membrane under the condition of continuous intracellular perfusion. The electrophysiological properties of the axons intracellularly perfused by the technique developed in this laboratory are reported elsewhere (Tasaki, Watanabe, and Takenaka, 1962; Tasaki and Shimamura, 1962). The results of the present measurements support the view that there is negative fixed charge in the axonal membrane. Based on the concept of "bistability" of the excitable membrane, the electrochemical nature of the resting and action potential is discussed.

METHODS

North Atlantic squid, *Loligo pealii*, available at the Marine Biological Laboratory, Woods Hole, were used. Giant axons, 450 to 600 μ in diameter and 40 to 50 mm in length, were dissected out, and the middle portion of the preparation was cleaned under a dissecting microscope with the use of darkfield illumination. The preparation was then mounted on the horizontal platform of a lucite chamber for the subsequent manipulation, namely, either for intracellular injection of radioactive tracers or for cannulation and perfusion.

The method of injecting tracers into the axons is described elsewhere (Tasaki, Teorell, and Spyropoulos, 1961). The tracers for injection were dissolved in isotonic (0.53 M) KCl solution, neutralized with $KHCO_3$, and this solution was introduced uniformly over the entire cleaned portion (15 to 20 mm in length) of the axon. The injected portion of the axon was kept in normal (non-radioactive) sea water which was replenished at 2 to 2.5 min. intervals. The uncleaned portion was imbedded in vaseline. Two pairs of platinum electrodes, one pair near each end of the axon, were used to stimulate and record propagated nerve impulses.

The technique of perfusing squid giant axons devised in this laboratory has been reported elsewhere (Oikawa, Spyropoulos, Tasaki, and Teorell, 1961). A slightly

different technique was developed by Baker, Hodgkin, and Shaw (1961) for larger squid axons. In most of the present investigation, the method of double cannulation was used.

Two large cannulae, the larger one approximately 350 μ and the smaller about 160 μ in diameter, were inserted into the axon at its two ends. The smaller cannula was used to introduce perfusing fluid into the axon and the larger one for drainage (see the insets of Figs. 3 and 4). The distance between the tips of the two cannulae was 7 to 9 mm in most experiments. In order to prevent clogging of the drainage cannulae, it was necessary to remove the major portion of the axoplasm in the cleaned part of the axon before starting perfusion. This was accomplished by sucking the axoplasm into the large cannula. The axoplasm in the large cannula could be removed by inserting the small cannula into the large one. Manipulation of these cannulae was done by the use of micromanipulators of the Peterfi type.

The small cannula was connected to one end of a polystyrene tube filled with perfusion fluid. The other end of the tubing was connected to a large (50 ml) syringe filled with air, the pressure of which was gauged with a mercury manometer. In the range between 4 and 12 mm Hg in pressure, a flow rate of 0.6 to 3 ml/hr. (10 to 50 mm^3/min.) was obtained. This flow rate appeared to be necessary to make accurate measurements of the radioactivity in the perfusing fluid at intervals of 2 to 5 min. The axon was usually covered with vaseline except for the 8 mm long perfused portion in the middle.

Propagated nerve impulses were induced by electric shocks applied near one end of the axon by means of a pair of platinum electrodes and were recorded with another pair near the other end. When spatially uniform stimulation was required, a pair of silver-wire electrodes was inserted into the middle portion of the axon (see Fig. 2). One of the silver wires (50 μ in diameter) had a bare surface of 6 mm length and was used for stimulation; the other wire had an uninsulated surface of about 1 mm in the middle of the bare region of the first wire and was used for recording. All the experiments were done at room temperature (23°C).

Various kinds of perfusing fluids were used during the course of development of the technique. In most of the experiments described in the present paper, the following solution was used: K_2SO_4, 38.5 gm and sucrose 140 gm dissolved in 1 liter of distilled water. With a view to removing heavy metal ions, the solution was treated with approximately 10 gm of cation exchange resin (amberlite 50 in hydrogen form) and then neutralized with K_2HPO_4. The pH of the solution was 7.25.

Radioactive tracers were obtained from various sources. Long half-life radioactive sodium, Na^{22} (2.6 years half-life), was obtained from Abbott Laboratories, Oak Ridge, Tennessee, in the chloride form (0.03 mc/ml); approximately 0.3 ml (10 μc) of the original solution was mixed with 3.5 ml of normal sea water and was used to measure tracer influxes. Short half-life (15 hrs.) radioactive sodium, Na^{24}, was purchased from Iso-serve, Inc., Cambridge, Massachusetts; it was supplied as 80 mM sodium chloride solution, its radioactivity being higher than 2 mc/ml at the time of arrival at the laboratory. As a rule, 10 to 20 μc of this original solution was mixed with 2 ml of sea water and was used to study influxes. When a study of tracer effluxes was desired, approximately 15 μc of Na^{24} was introduced into 2 ml of perfusing fluid.

Radioactive potassium, K^{42} (12.7 hrs. half-life), was supplied by Iso-serve, Inc., in the chloride form. The radioactivity of the original (0.3 M) solution was greater than 1 mc/ml at the time of arrival at the laboratory. Radioactive rubidium, Rb^{86} (19 days half-life), was obtained from Oak Ridge National Laboratory; the original solution (RbCl in 1.5 M HCl with a concentration of 7.5 mc/ml) was diluted with distilled water by a factor of 3 and was neutralized with $NaHCO_3$ for influx measurements and with $KHCO_3$ when efflux determinations were required. Radioactive cesium, Cs^{134} (2.3 years half-life), supplied by Oak Ridge National Laboratory was in a 2.1 M HCl solution (13.0 mc/ml); the original solution was diluted with distilled water by a factor of 4 and was neutralized in the same manner as in Rb^{86}. On several occasions, Cs^{137} (30 years half-life), was used; the chloride form of this tracer was diluted with distilled water and was neutralized with $NaHCO_3$ to obtain a neutral isotonic solution of approximately 0.5 mc/ml. The level of radioactivity of these univalent cationic tracers in the perfusing fluid or in sea water was, as a rule, 10 to 20 μc/ml.

A univalent anionic tracer, Br^{82} (36 hour half-life), was supplied by Oak Ridge National Laboratory as potassium salt solution (approximately 35 mc/ml at the time of arrival at the laboratory). Radioactive calcium, Ca^{45} (164 days half-life), was obtained from Oak Ridge National Laboratory and was in the chloride form dissolved in 0.92 M HCl; it was diluted with distilled water by a factor of 1.6 and was neutralized with $NaHCO_3$.

Samples containing radioactive tracers were dried in planchets, and their radioactivity was determined with a Nuclear Chicago Corp. low background counter (model C112 and C110B) in conjunction with a gas flow detector (model D-47 with a window) and a printing timer (model C111B). The background radioactivity of this machine was roughly 1.5 CPM. A Tektronix oscilloscope (type 502), a Grass stimulator with a stimulus isolation unit, and a Grass kymograph camera were used to examine and record electric responses of the axon.

RESULTS

1. *Effluxes of Injected Univalent Tracers*

Comparisons of the fluxes of various radioactive tracers injected in squid giant axons were made in previous papers (Tasaki, Teorell, and Spyropoulos (1961); Tasaki and Spyropoulos (1961)). In order to show a slight difference in behavior between perfused and unperfused axons, injection experiments using Na^{24}, K^{42}, and Rb^{86} were repeated in the present investigation.

The tracers were introduced uniformly over the entire cleaned portion (15 to 20 mm long) of the axon. The sea water surrounding the "hot" portion was replenished at intervals of approximately 2.5 min. The fluid was collected during every 5 min. period and was transferred into a planchet; its radioactivity was determined by the usual method. The procedure was continued for a period of approximately 1 hr. At the end of this period, the radioactivity

remaining in the axon was measured. Typical examples of the results obtained are presented in Fig. 1.

It is seen in this figure that the levels of the effluxes expressed in counts per minute are approximately the same for the three examples. The radioactivity in the axon at the end of the experiments is the lowest for Na^{24} and the highest for Rb^{86}; this indicates that the efflux expressed in percentage of the intracellular radioactivity decreases with increasing atomic numbers. The existence

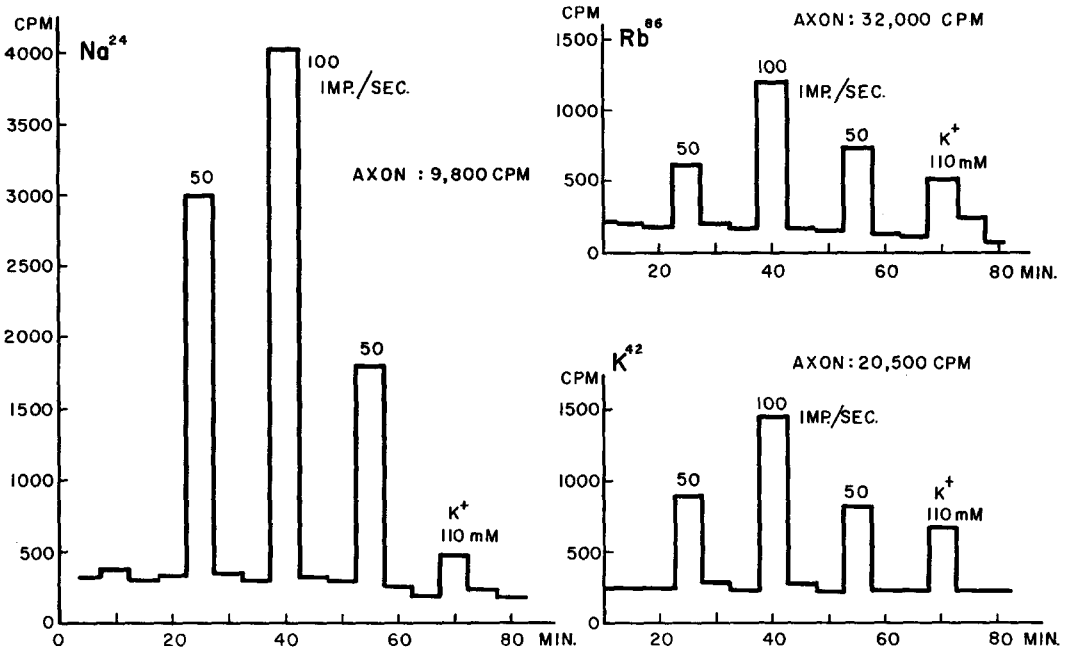

FIGURE 1. Effluxes of radioisotopes of three different alkali metal ions injected into squid giant axons. Ordinate, radioactivity in counts per minute in sea water collected in each collection period of 5 min. Abscissa, time after intracellular injection of radiotracers. Radioactivity remaining in axons at end of experiment is given. The effects of repetitive stimulation (at the rate of 50, 100, and 50 impulses per sec.) and of potassium depolarization are shown. 23°C.

of this trend was pointed out in a previous study (Tasaki *et al.*, 1961). The Na^{24} efflux in the absence of electric stimulation of the axon was approximately 0.4 per cent of the intracellular radioactivity per minute. The corresponding value for K^{42} was roughly 0.2 per cent per min. The efflux of Rb^{86} was about 0.1 per cent per min.

It is seen in Fig. 1 also that the effect of repetitive electrical stimulation shows a tendency to decrease with the atomic number. With radioisotopes of sodium, repetitive stimulation at a frequency of 50 impulses/sec. was found to increase the level of efflux (averaged in a period of 5 min.) by a factor of 5 to 9.

In the case of labeled potassium and rubidium, the efflux during repetitive stimulation at the same frequency was around three times the resting level. With radioisotopes of cesium (Tasaki *et al.*, 1961), the effect of repetitive stimulation was less marked than with those mentioned above.

The effect of depolarization with an approximately 110 mM KCl (prepared by mixing an isotonic KCl solution with normal sea water) upon the resting efflux showed a tendency to increase in the order from Na^{24} toward Cs^{134}.

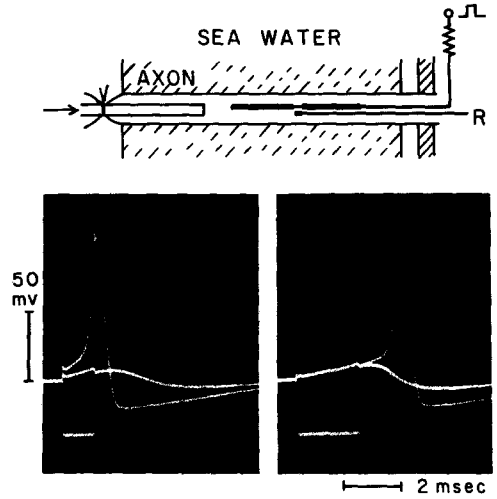

FIGURE 2. Action potentials recorded from an intracellularly perfused squid giant axon. Approximately 2 ml of perfusing fluid (K_2SO_4 solution) flowed before these records were taken. In each record two sweeps of oscilloscope beam were superposed. The uninsulated portions of the recording (*R*) and the stimulating electrodes are indicated by the thick lines in the axon (not to scale).

Regular, monotonic changes in behavior with the atomic number indicate that the difference among various alkali metal ions is quantitative, and not qualitative.

2. *Properties of Perfused Axons*

Electric properties of the axons perfused with isotonic KCl or K_2SO_4 solution were investigated in this laboratory mainly during the summer of 1960. Calculations of isotonicity were based on Formulae and Methods IV issued by the Marine Biological Laboratory, Woods Hole. Inserting a longitudinal glass pipette electrode into the perfusing fluid, the resting and action potentials were measured across the surface membrane. The potentials remained normal for a considerable period of time (20 min. to 3 hr.) and then deteriorated rapidly. The effects of altering the composition of the perfusing fluid were dis-

cussed in recent articles by Tasaki *et al.* (1962). Baker *et al.* (1961) also made a systematic study of this point.

Fig. 2 shows action potentials recorded with long intracellular metal wire electrodes inserted into the perfused portion of a cleaned axon. In order to increase the length of the perfused region, the drainage cannula was removed before insertion of the electrode set. The stimulating electrode covered the major portion of the cleaned region (7 mm); the recording electrode had a bare spot in the middle of the stimulating electrode. Stimulating currents, supplied by a voltage pulse generator through a high resistance, were adjusted

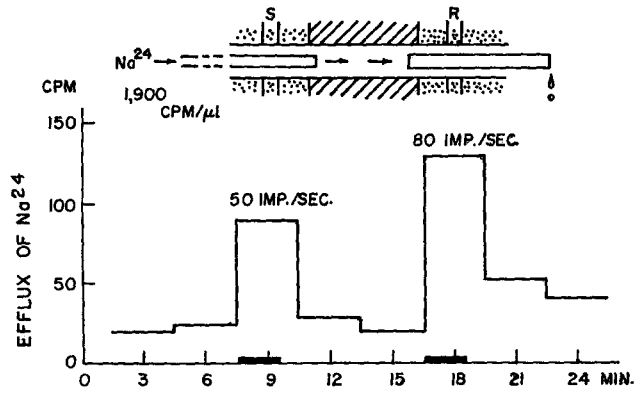

FIGURE 3. Efflux of radioactive sodium in a perfused squid giant axon. The radioactivity of the surrounding sea water collected in every 3 min. period is plotted against time after the start of perfusion. The radioactivity in the perfusing fluid is indicated. The periods during which repetitive stimuli were delivered are indicated by the heavy line on the abscissa.

to threshold. The interior of the axon was perfused, before these records were taken, with approximately 2 ml of isotonic potassium (sulfate) solution; this volume corresponds roughly to 800 times the total axoplasm of the portion under study. (While these recordings were being taken, the flow of the fluid was temporarily arrested.) It is seen that the amplitude and the configuration of the action potential are very similar to those obtained from unperfused axons.

Although the main purpose of the present investigation was to compare the *influx* of Na^{24} with that of K^{42} in perfused axons, effluxes of these radioisotopes were examined on several occasions. Fig. 3 shows an example of the efflux measurements using Na^{24}. The radioisotope was dissolved in the perfusing fluid (K_2SO_4 solution); the radioactivity was 1900 CPM for a volume of 1 μl of the perfusing fluid. The volume of the perfused portion of the axon was approximately 1.3 μl (0.5 mm diameter and 6.5 mm length). This means that the total radioactivity inside the axon was maintained at a constant level of approximately 2500 CPM during the entire period of perfusion. The rate of the

resting efflux of Na^{24} was then 0.3 per cent per min.; this value is slightly *smaller* than the corresponding figure in the unperfused axon. The results of measurements on two other axons agreed with the value just mentioned within 25 per cent accuracy.

When the action potential of a perfused axon started to deteriorate, the resting efflux of labeled sodium was found to start increasing rapidly. In spite of the fact that these perfused axons are under drastic, unnatural experimental conditions, there was always a distinct increase in the efflux of Na^{24} in response to repetitive stimulation. (In the example of Fig. 3, repetitive stimuli were delivered to the axon for the first 2 min. period during one collection time of 3 min.) It is possible that a part of the observed radioactivity may have derived from the efflux taking place in the unperfused portions near the orifices of the two cannulae. It was my impression that high frequency stimulation accelerated the deterioration of the potentials.

3. *Influxes of Labeled Na and K in Perfused Axons*

Influxes of radiotracers were examined on axons with 7 mm long cleaned portions. The uncleaned portions of the axons were imbedded in vaseline (see Methods). The perfusing fluid ran through the interior of the cleaned portion and flowed out of the drainage cannula (see Fig. 4, top). The radioisotopes were dissolved in filtered natural sea water and were introduced into the chamber (of approximately 0.5 ml capacity) in which the perfused portion lay. The samples for measuring the influxes were collected from the distal end of the drainage cannula. In order to avoid contamination of the samples by the radioactive material outside the axon, a wide partition made of parafilm was placed between the outlet of the drainage cannula and the perfusion chamber. The fluid collected during a period of either 3 or 5 min. was assembled in one planchet and its radioactivity was determined by the standard method.

When sea water containing either Na^{24} or K^{42} was introduced into the chamber, the fluid collected from the drainage cannula became radioactive immediately. When the sea water containing radioisotopes was replaced with normal sea water, there was an immediate fall in the radioactivity of the collected fluid. Since such rises and falls in the radioactivity could be clearly demonstrated at intervals of 3 min. or less, there was little doubt that the major part of the observed radioactivity was a reflection of the tracer influxes across the perfused portion of the axon membrane. When the action potential started to deteriorate during perfusion, there was a marked increase in the influx.

In Fig. 4, an example of the results is presented in which the influx of K^{42} was compared with that of Na^{24}. When sea water containing K^{42} was introduced into the chamber, there was a measurable transport of the radioisotope

into the perfusing fluid. Approximately 30 sec. before the end of the 5 min. collection period, the radioactive sea water was removed and the chamber was washed with normal sea water twice. During the next collection period, the radioactivity of the sample fell roughly to a quarter of the preceding level. When, during the following collection period, sea water containing Na^{24} was introduced into the chamber, there was another rise in the radioactivity. At intervals of 5 min. K^{42} and Na^{24} were applied to the axon alternately until the electric responses of the axon started to deteriorate about 80 min. after the start of perfusion. To avoid the effect of stimulation upon influxes, precaution was taken to apply single test shocks mainly during the period in which non-radioactive sea water filled the chamber.

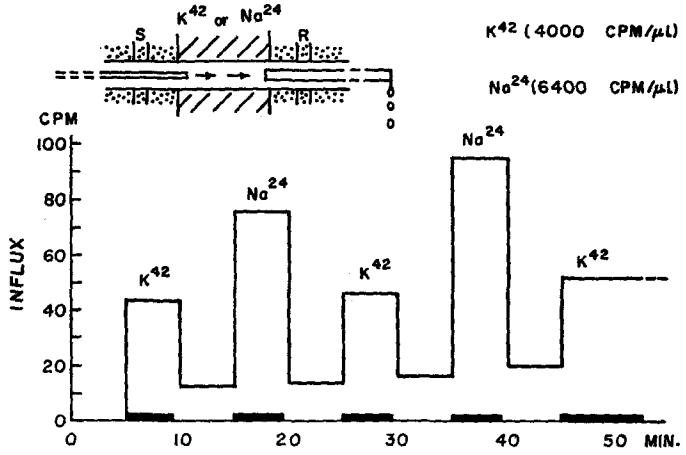

FIGURE 4. Comparison of fluxes of radioactive potassium and sodium into perfused squid giant axon. The radioactivity in perfusing fluid collected in every 5 min. period was plotted against time after start of perfusion. The bars on the base line indicate the periods during which sea water containing either K^{42} or Na^{24} was applied.

In the example furnished in Fig. 4, the level of radioactivity of the Na^{24}–sea water was 1.6 times as high as that of the K^{42}–sea water. It is seen in the figure that the radioactivity brought about by application of Na^{24} is higher than that caused by K^{42}; the radioactivity ratio in the two cases (measured as it rises above the slowly rising background activity) was found to be very close to the radioactivity ratio in the medium.

Influx of radioactive sodium was determined on ten different axons (five axons with Na^{22} and five axons with Na^{24}). The radioactivity of the medium varied widely between 1000 CPM for a volume of 1 μl of the medium to 8000 CPM/μl. There was a direct proportionality between the radioactivity in the medium and the radioactivity in the collected samples. There was no recog-

nizable difference between the results obtained with two different kinds of radioisotopes of sodium.

The results from five measurements in which collection periods of 5 min. were adopted are as follows: (1) tracer Na^{24} (influx 77 to 95 CPM)/(medium 6,400 CPM/μl); (2) Na^{22} (33 to 35)/(1,700); (3) Na^{24} (46 to 59)/(3,650); (4) Na^{24} (approximately 16)/(1,350); (5) Na^{22} (approximately 27)/(1,630). The numerals in the first parentheses represent the influxes of radiotracers (determined with a low background counter); the numerals in the second parentheses are the radioactivity in 1 μl of the surrounding medium. In all these cases, the ratio of the first figure to the second gives a value of 12 to 20 CPM in 5 min. for a level of radioactivity of 1,000 CPM/μl in the medium. The results from the remaining five axons using 3 min. collection periods gave values approximately three-fifths times as large as those mentioned above. As long as the perfused axons were capable of carrying nerve impulses, the sodium influx remained at a roughly constant level. The variation among different axons was reasonably small.

It is possible to convert the results of these influx measurements into a form which can be readily compared with the results of efflux measurements. We denote the flux of tracer α per unit area of the membrane by J_α, the total membrane area under study by A, the volume of the portion of the axon under study by v, and intra- and extracellular concentrations of the tracer by C'_α and C''_α, respectively. Then, under the conditions that $C''_\alpha = 0$, the following equalities exist:

$$-v\frac{dC'_\alpha}{dt} = -AJ_\alpha = AP'_\alpha C'_\alpha,$$

where P'_α is the "permeability coefficient" for efflux (see Discussion). We can express the total flux AJ_α in counts per minute per unit collection period and the total amount of the tracer in the axon vC'_α in counts per minute. The quantity employed in the discussion of Fig. 1 for comparison of different tracers is the ratio $(AJ_\alpha)/(vC'_\alpha)$, which corresponds to $(A/v)P'_\alpha$. (Note that the last quantity has the dimension of the reciprocal of time and also that $A/v = 4/D$, where D is the diameter of the axon.)

Under the conditions that C'_α is vanishingly small, the permeability coefficient for influx P_α can be defined by

$$J_\alpha = P_\alpha C''_\alpha.$$

In discussing the results of Fig. 4, we have expressed C''_α in CPM/μl and AJ_α in CPM per 5 min. collection period. We note that $(A/v)P_\alpha = AJ_\alpha/(vC''_\alpha)$ and also that v is approximately 1.3 μl in these experiments. Hence, by dividing the observed value of AJ_α for labeled sodium (12 to 20 CPM per 5 min.) by the product vC''_α (1300 CPM), it is found that the value of $(A/v)P_\alpha$ for sodium is 0.009 to 0.016 per 5 min. or roughly 0.2 to 0.3 per cent per min. This value is of the same order of magnitude as the

value of $(A/v)P'_\alpha$ determined by Na^{24} efflux measurements. It should be remembered in this connection that both P_α and P'_α vary with the experimental conditions (namely, with the concentrations and fluxes of all non-radioactive ions and neutral molecules) and that in general $P_\alpha \neq P'_\alpha$.

Under the present experimental conditions, the perfusing fluid does not contain sodium ion, radioactive or non-radioactive. In a stationary state, the specific radioactivity of sodium is considered to be constant throughout the system. The flux of the non-radioactive sodium can therefore be followed by the measurement of the flux of the radiotracer. The result of the present influx measurements indicates that the intracellular sodium concentration would rise from zero to 0.2 to 0.3 per cent of the external sodium concentration in 1 min. after cessation of the flow of the perfusing fluid. This rate is close to (or slightly smaller than) the rate of sodium-potassium exchange in the excised axon immersed in potassium-free medium. Steinbach and Spiegelman (1943) reported that 24 to 54 per cent of the intracellular potassium exchanged with sodium in approximately 3 hrs.; the initial rate of this exchange is then approximately 0.3 per cent per min.

The Na content in sea water is approximately 0.53 equivalent/liter. From this it follows that the influx of sodium into perfused axons is of the order of $(2 \text{ to } 3) \cdot 10^{-10}$ equivalent $sec.^{-1}$ cm^{-2}. This influx would carry a charge across the membrane at the rate of approximately 20 to 30 μa cm^{-2}; obviously there is no such membrane current because there is a simultaneous K flux of approximately the same magnitude in the reverse direction. The K efflux evaluated by the same principle on two intracellularly perfused axons was slightly smaller than the value estimated by Shanes and Berman (1955) and was close to the value of Na influx mentioned above. Elimination of K in sea water had little effect upon the K efflux.

Similar influx measurements with K^{42} were made on nine axons, of which five axons were used for comparing the K^{42} influx with influxes of other tracers. Again, denoting influxes during 5 min. collection periods in the first parenthesis and the radioactivity of the medium in the second parenthesis, the results are as follows: (1) (31 to 40 CPM)/(medium 4,000 CPM); (2) (41 to 57)/(3,650); (3) (approximately 42)/(2,940). These results indicate that the K^{42} influx in 5 min. would be 8 to 15 CPM when the level of radioactivity in the medium was 1,000 CPM/μl. In the remaining six measurements in which a collection period of 3 min. was adopted, the radioactivity of the medium was varied between 1,000 and 8,000 CPM/μl. There was a clear proportionality between the level of radioactivity of the medium and the influx. When these data were converted into figures representing K^{42} influxes in 5 min. for radioactivity of 1,000 CPM/μl in the medium, values between 7 and 20 CPM were obtained. This wider range of variability among different axons could at least partly be ascribed to the uncertainty about the residual level of influx observed following removal of the hot sea water. The value of K^{42} influx observed, 8 to 15 CPM (or 7 to 20 CPM) for 1,000 CPM/μl in the medium, is not significantly different from the corresponding value for Na^{24}, 12 to 20 CPM.

4. *Influxes of Radioactive Cesium, Rubidium, Bromine, and Calcium*

Because of the difficulty of obtaining large squid in Woods Hole during the summer of 1961, the following measurements were made only on a limited number of axons. In many cases, influxes of different ions were compared on the same axons, as in the experiment of Fig. 4.

Influx of radioactive cesium was determined on five axons (two axons with Cs^{134} and three axons with Cs^{137}). The radioactivity of the surrounding sea water was varied between 1,800 and 8,000 CPM/μl; the influx was found to vary directly with the level of radioactivity of the medium. There was no significant difference between the two radioisotopes of cesium ion. The influx in 5 min. was 6 to 10 CPM for a level of 1,000 CPM/μl in the medium. This value is approximately one-half of the corresponding value for labeled sodium. Comparison of Na^{24} and Cs^{137} on the same axon indicated that the difference was significant.

Determination of Rb^{86} influx was carried out only on two axons. The radioactivity of the sea water was 3,500 CPM/μl. The influx in 5 min. was 15 to 18 CPM for 1,000 CPM/μl in the medium. This value is not significantly different from the values for radioactive sodium and potassium.

An attempt was made to measure influxes of Cl^{36} in perfused axons without success. The main difficulty arose from the low level of radioactivity in the medium containing Cl^{36}. The influx of Br^{82} was determined on four different axons. In the first axon, a comparison was made of K^{42} and Br^{82} influxes with radioactivity in the medium chosen at about the same level for these two radioisotopes (approximately 3,000 CPM/μl). The influx of Br^{82} was roughly one-twentieth of the value for K^{42}. In the second axon, comparison was made with Na^{24}; again the Br^{82} influx was close to one-twentieth of that of Na^{24}. In the remaining two axons, slightly higher values (1 to 3 CPM in 5 min. for 1,000 CPM/μl in the medium) were obtained; the influx of this labeled anion was not influenced by repetitive stimulation of the axon to any significant degree.

The influx of Ca^{45} was measured on only three different axons under intracellular perfusion. The influx was found to be 3 to 6 CPM in 5 min. for 1,000 CPM/μl in the medium. This value is significantly smaller than the corresponding values for univalent cationic tracers. It is known that Ca ion undergoes chemical binding with the normal constituent of the axon and is partially immobilized (*cf.* Frankenhaeuser and Hodgkin (1957); Tasaki *et al.* (1961)). Probably this binding accounts for the small value observed in these experiments. The observed influx of the radioactive calcium is a reflection of a continuous influx of non-radioactive calcium under the present experimental conditions.

DISCUSSION

Under conditions of continuous intracellular perfusion, the influxes of radioactive sodium and potassium were of comparable magnitude for equal concentrations in the medium (Fig. 4). Most of the axons used had no visible branch in the portion measured. The membrane resistance of perfused axons was not significantly different from that of unperfused axons. There is hardly any doubt, therefore, that the labeled cations in these influx measurements were transported through the excitable portion of the axonal membrane.

In measuring influxes under these conditions, the radioactivity of the outflowing perfusion fluid was of the order of 1/10,000 of the level in the surrounding medium. The tracer flux, J_α, was proportional to the tracer concentration, C_α'', in the external medium, namely, $J_\alpha = P_\alpha C_\alpha''$ where P_α is the permeability coefficient for influx of species α. (P_α, defined in this manner, is almost equivalent to the permeability constant of Collander and Krogh (see Krogh, 1946, p. 154).) From a physicochemical point of view, the permeability coefficient is determined primarily by the mobility and the concentration of the ion in question within the major diffusion barrier in the membrane (*cf.* Tasaki, 1960). The selectivity (see *e.g.* Helfferich, 1962, p. 151) of the membrane matrix and the concentrations of other ions and neutral molecules on both sides of the membrane are the factors which determine the distribution of the radioisotope in the membrane.

The experimental findings described under Results show that the permeability coefficient for Na ion is roughly equal to that for K ion. The permeability coefficients for these ions determined from efflux measurements were not significantly different from those evaluated from influx measurements under these experimental conditions.

The statement made in the last paragraph is obviously inconsistent with the widely accepted view that the resting axonal membrane is far more permeable to K ion than to other ions (Bernstein, 1912; Hodgkin, 1951). This view is supported by a variety of experiments. We now wish to resolve the inconsistency between the previous results and the findings described in this paper.

The difficulty of determining membrane permeability by potential measurements was discussed in the introduction to this paper. In the experiments in which the external Na and K ion concentrations are altered, it is customary to assume that the membrane potential is determined by the concentrations of these two cations and the permeability ratio, P_K/P_{Na}, and that the permeability ratio remains unaltered by concentration changes. Actually, however, P_K/P_{Na} changes when the external concentrations are altered (see Fig. 1). Since different univalent cations are expected to compete with the intramembrane divalent cations with different strength, a change in the external concentration of a univalent cation can alter the membrane potential by changing the fraction of the negative sites in the membrane occupied by divalent

ions (see below). Except in the case of axons immersed in K-rich media, in which the normal excitability is lost and P_K is larger than P_{Na} (see *e.g.* Fig. 1), there is no simple relationship between the K ion concentration ratio across the membrane and the membrane potential. In other words, the results of these potential measurements do not conflict with the findings described in this paper.

The permeability of the axonal membrane to various ions is often assessed by the method of voltage clamp. This method offers extremely valuable means of evaluating various electric components of the equivalent circuit proposed by Hodgkin and Huxley (1952). It should be noted, however, that deduction of the electrochemical properties of an ionic membrane from the components of an equivalent electric circuit is not compelling. During transient stages, the transport numbers of various ions should be functions of the space coordinate across the membrane. Under ordinary experimental conditions, electric current should be carried mainly by Na ion near the outer membrane surface and, at the same moment, mainly by K ion near the inner surface. Even in time-independent stages, the general mathematical solution of the Nernst-Planck equations for ion fluxes through a uniform membrane (Teorell, 1951; Schlögl, 1954) is very different from the equations describing the properties of the equivalent circuit.

If one assumes that individual alkali metal ions are transported through separate specific channels or pores in the axonal membrane, the use of an electric circuit model may be helpful. However, the existence of such separate channels in the membrane is questionable, because the difference in behavior among different inorganic and organic cations is known to be only quantitative and not qualitative (Fig. 1, *cf.* also Tasaki and Spyropoulos, 1961; Tasaki and Shimamura, 1962). Furthermore, the potential difference across such a channel cannot be expressed by the Nernst equation because of the effect of fixed charges on the wall of the channel (*cf. e.g.* Kobatake's equation for porous membrane (Kobatake, 1958)). The application of the concept of equivalent circuit to the axonal membrane is, in some respects, analogous to a description of the behavior of a vacuum tube oscillator in terms of an AC generator; its usefulness is limited.

It is generally recognized that a high K ion concentration in the interior of various cells cannot be taken as an indication of low Na permeability of the membrane (see *e.g.* Krogh, 1946, pp. 181–184).

The radiotracer technique was often used in previous investigations for determination of the permeability of the squid axon membrane. The difference between P_K and P_{Na} determined by this technique has always been rather small (Rothenberg, 1950; Shanes and Berman, 1955; Tasaki *et al.*, 1961). In unperfused axons immersed in normal sea water, the ratio P_K/P_{Na} determined with injected radioisotopes is between 1:3 and 1:2. A prolonged (30 min. or more) treatment of such axons with cyanide (2 mM) is known to change this ratio to a value around 3:1 to 1:1 (Hodgkin and Keynes, 1955; unpublished experiments in this laboratory). This cyanide experiment shows that a change in the flow of metabolites across the membrane and/or production of some abnormal metabolites during cyanide poisoning alters P_{Na} more strongly than it does P_K.

It is important to note that all the values of P_K and P_{Na} determined by influx and

efflux measurements are not completely independent. In axons internally perfused with Na-free fluid and immersed in K-free artificial sea water, P_K for efflux was found to be roughly equal to P_{Na} for influx. This approximate equality is expected from the condition of electroneutrality on the assumption that the charges carried by anions and divalent cations are negligible. Since the internal K concentration is roughly equal to the external Na concentration in these experiments, the conservation of electric charge leads to the approximate relation $J_K + J_{Na} = 0$. When the mobilities of these two cations are very different, interdiffusion of this type creates an electric field within the membrane, tending to accelerate the slower species and to retard the faster species (see *e.g.* Helfferich, 1962, p. 358). Under these circumstances, the difference between P_{Na} and P_K determined by influx measurements should not be *reduced* by the electric field. The direct measurement of P_K/P_{Na} for influxes shows that the ratio is not very different from unity and, consequently, that the diffusion potential associated with the interdiffusion of Na and K is not very large.

The permeability of the axonal membrane for a univalent anion Br^{82} was very low, being of the order of one-twentieth of that for Na^{24} and for K^{42}. There was no significant increase in the flux of this and other anionic tracers during repetitive stimulation (Tasaki *et al.*, 1961). It is also known that complete substitution of intra- and/or extracellular chloride with sulfate or glutamate alters neither the resting nor action potential to any significant degree (Tasaki *et al.*, 1962). These findings strongly support the view that the axonal membrane has fixed negative charges, as originally suggested by Michaelis (1933) and by Teorell (1936).

Now in the light of the newly accumulated experimental data, a question may be asked: What determines the potential difference across the squid axon membrane in the resting and active states? This question has been discussed to some extent in two recent articles from this laboratory (Tasaki *et al.*, 1962). Our idea of the nature of the resting and action potential is based on the concept of *two stable states* (Tasaki and Hagiwara, 1957) in the axonal membrane with negative fixed charges. The qualitative aspects of the idea are illustrated by the diagram in Fig. 5, in which the equivalent fraction of the univalent cation in the critical layer of the axonal membrane is plotted against the corresponding value in the medium.

The medium is considered to contain in general a mixture of univalent and divalent cations. The abscissa of the diagram represents the fraction $C_1/(C_1 + 2C_2)$, where C_1 and C_2 are the concentrations of the univalent and divalent cations, respectively, in the medium. The ordinate represents the corresponding value in the critical layer in the membrane. When the fraction in the medium is zero, namely, when the medium contains only divalent cations, the membrane also contains only divalent cations. As the fraction in the medium increases (portion AB in the diagram), the fraction in the membrane increases also. When the fractions are equal to unity (upper right-hand

corner), there are only univalent cations in the system. A continuous change in the ionic fraction in the membrane resulting from a continuous change of the medium is expected to produce a continuous change in the membrane conductance and potential.

It is known that a continuous rise in the univalent cation concentration in the external fluid medium can give rise to a sudden, discontinuous change of the membrane conductance and potential in various excitable tissues (see Hill

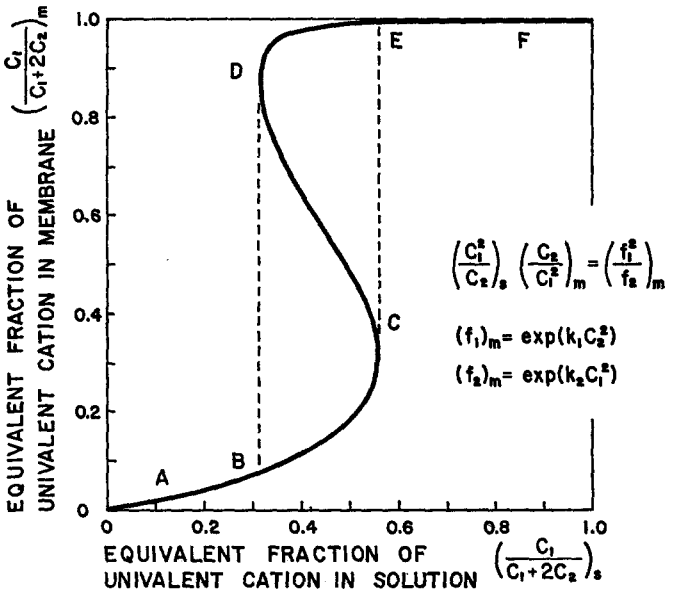

FIGURE 5. Theoretical ion exchange isotherm calculated for a cation exchange membrane immersed in a mixture of univalent and divalent cations. C_1, f_1, C_2, and f_2 represent concentrations and activity coefficients of the univalent and divalent cations, respectively. Subscripts m and s stand for membrane and solution phases, respectively. In calculation it is assumed that $k_1 = k_2 = 6(C_1 + 2C_2)_m^{-2}$ and $(C_1 + 2C_2)_m = 2(C_1 + 2C_2)_s$. The two stable states of the axonal membrane are explained in terms of this S-shaped ion exchange isotherm.

and Osterhout, 1938; Tasaki, 1959). Such discontinuities cannot be produced by a system with a simple, monotonic ion exchange isotherm. Thus, we arrive at an S-shaped isotherm which can be derived theoretically by introducing in the mass action law a special type of concentration dependence of the activity coefficients (given in the figure). Working on various kinds of crystalline cation exchangers, Barrer and Falconer (1956) have actually obtained this type of isotherm and clarified the thermodynamical and statistical mechanical basis of the phenomenon. The configuration of the isotherm in Fig. 5 suggests that occupancy of two neighboring charge sites by two cations of different valencies is energetically unfavorable.

Portion ABC in the diagram represents one stable state (resting) and portion DEF the other stable state (active) of the membrane. The discontinuous change in the properties of the membrane at a certain value of $C_1/(C_1 + 2C_2)$ is expressed by the vertical line CE. Abolition of a prolonged action potential by an increase in the Ca ion concentration in the external medium (Spyropoulos, 1961) is expressed by the pathway EDB. The portion of the curve labeled CD is *unstable*; like the intermediate portion of the isotherm given by van der Waals' equation of state (see *e.g.* Guggenheim, 1957, p. 156), the state expressed by this portion can never be realized.

We regard electric stimulation as a means of altering the ionic fraction in the membrane by forcing the intracellular univalent cation into the membrane. A brief stimulating pulse is expected to bring about transition CE and subsequently EDBA. When the membrane current is adjusted so as to bring the ionic fraction of the membrane to the unstable portion CD, there appears a "mixed state" in which the membrane is represented by a mixture of portions in two distinct states. The concept of mixed state was first introduced in order to explain the origin of a subthreshold response and of the intermediate portion (with a negative conductance) of the voltage-current relationship. A gradual depolarization of the squid axon membrane by a K-rich medium is also attributed to intervention of mixed states.

The two dimensional diagram of Fig. 5 explains only the process of generation of prolonged, "square-top" action potentials. For a description of an ordinary action potential, additional dimensions representing the proportion of different univalent cations in the membrane are required. A sudden change in the divalent cation concentration in the membrane is expected to alter the selectivity toward various univalent cations (see *e.g.* Helfferich, 1962, p. 103). Tobias *et al.* (1962) suggest that a large change in the water content of the membrane may result from such a change. Following a transition from one stable state to the other, therefore, a gradual shift in the predominant univalent cation species (hence, in general, a gradual change in the membrane potential) is expected to take place. The detail of the rate processes determining the configuration of the action potential is not yet well understood.

The importance of Ca ion in excitation processes is well known in classical physiology (see *e.g.* Heilbrunn, 1952) and has been reemphasized by several recent investigators (Brink, 1954; Koketsu and Nishi, 1960; Liberman *et al.*, 1961; Oomura *et al.*, 1961; Spyropoulos, 1961; Tobias *et al.*, 1962; and others). It should be pointed out, however, that two stable states of the type discussed above can, in principle, arise in a membrane immersed in a mixture of two kinds of univalent ions with very different radii (*cf.* Helfferich, 1962, p. 193).

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