# Permeability of the Giant Axon of *Dosidicus gigas* to Calcium Ions

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It is a commonly accepted fact that calcium ions, apart from regulating the permeability of cell membranes to various ionic species, determine the level of membrane electrical potential at which nerve fibers fire impulses. Since these properties ascribed to $Ca^{++}$ seem to be of great importance, a great deal could be gained by having detailed information regarding the permeability of nerve fibers to $Ca^{++}$, the physical state of intracellular calcium, or, in general, any chemical reaction in which nerve fiber constituents and calcium ions participate.

The techniques of microinjection and internal perfusion used in this work have been shown previously to work successfully in the giant axon of *Dosidicus* (1–3). Only results obtained in nerve fibers whose electrical properties were undamaged through the whole experiment were considered. In the microinjection technique, action potentials were recorded only with external electrodes, whereas in the internally perfused fibers the additional control of internal recording was also effected. Stimulating electrodes were externally applied in both techniques. The details of the methods have been (4) and will be published elsewhere.

If a tracer amount of $^{45}Ca^{++}$ is microinjected into a giant axon kept in sea water and the kinetics of outflow is immediately followed, it is found that the efflux of $^{45}Ca^{++}$ as a function of time is described by two exponentials, a very fast one and a very slow one. This behavior of calcium ions seems to correspond to an inherent property of nerve fibers and not to an artifact, due to faulty technique in the microinjection. This is seen in a series of 13 experiments in which $^{22}Na^{+}$ was simultaneously microinjected with $^{45}Ca^{++}$. Fig. 1 depicts one of these results. While the efflux of $^{22}Na^{+}$ is described by only one exponential, the kinetics of $^{45}Ca^{++}$ shows its typical biphasic behavior; as seen in sodium ions, at no moment during the experiment did the axon show any sign of leakage or contamination.

This conduct of microinjected $^{45}Ca^{++}$ was interpreted by Luxoro and Rissetti (4) in terms of a model which accounts for the known ability of calcium ions to be bound by axoplasmic components (5). Furthermore, in making their model, Luxoro and Rissetti assumed that this binding ability occurs in the bulk of the axoplasm and that $Ca^{++}$ must become unbound before leaving the nerve fiber. The model is shown in Fig. 2. A preliminary inspection of the experimental results showed that $k_{21}$ was much smaller than either $k_{12}$ or $k_{13}$, a fact which allows a simplification of the solution of the system of differential equations depicted in the figure. A somewhat better approxima-

tion than the one proposed previously (4) is

$$\text{Rate of } {}^{45}Ca^{++} \text{ efflux} = k_{13} X = k_{13} Xo\, e^{-(k_{13} + k_{12})t} + k_{21'} y \qquad (1)$$

Here, $k_{21'}$ is the same kinetic constant used by Hodgkin and Keynes (5) and by Luxoro and Rissetti (4) to describe the slow exponential. In terms of the model of Fig. 2:

$$k_{21'} = k_{21} \frac{k_{13}}{k_{12} + k_{13}} \qquad (2)$$

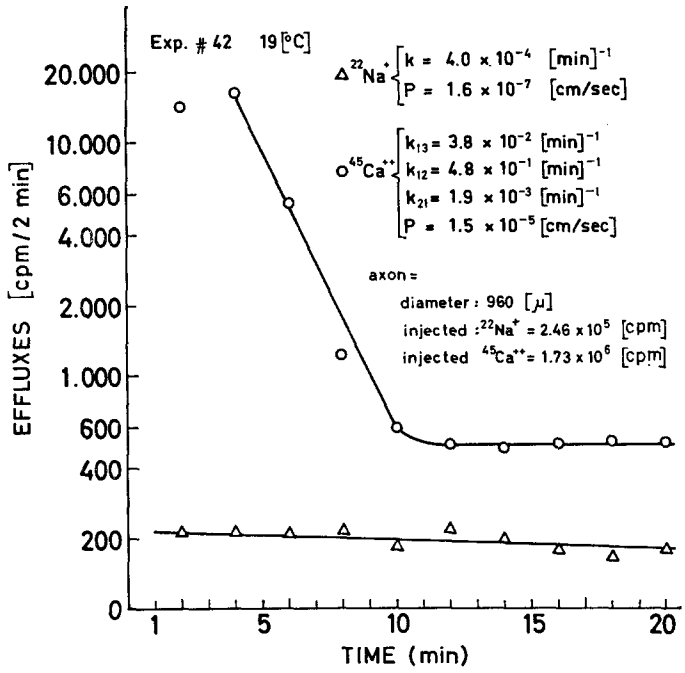
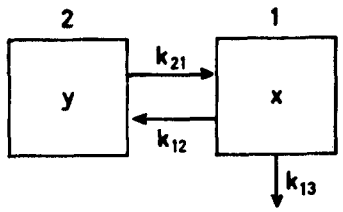



FIGURE 1. Simultaneous effluxes of $^{22}Na^{+}$ and $^{45}Ca^{++}$ microinjected in a giant axon Immediately after the microinjection of both isotopes, the external sea water was replaced every 2 min and counted. The second point in the curve for calcium efflux is the highest, owing to the slow diffusion of internal calcium. Semilogarithmic plot.

According to equation 1, the extrapolation of the semilogarithmic plot of Fig. 1 to zero time gives $k_{13}$ $Xo$, while the slope of the fast exponential gives $k_{13} + k_{12}$. From the knowledge of $Xo$, both kinetic constants can be calculated. It is involved, though, to obtain the extrapolated value (see reference 4). $k_{21'}$ is the ratio of the constant flux which one obtains after a long time has elapsed over the internal radioactivity which is detected at the end of the experiment. $k_{21}$ is calculated from $k_{21'}$ and equation 2.

Table I shows the results of nine experiments carried out at 19°C and nine experiments made at 2°C. Together with the kinetic constant defined in the model, the permeability coefficient "$P$," calculated from $k_{13}$ (6) is presented. The $Q_{10}$ for P turns out to be slightly inferior to 3.

With the knowledge of the three kinetic constants, the differential equations of Fig. 2, and the boundary conditions, the experimental results were reproduced in an analog computer (4). Although this is a required condition for the model to be right, it does not in itself constitute proof, since other systems behave according to the same equations. The required proof might be supplied if a direct identification of the kinetic constants were possible. Accordingly, direct evidence was gathered regarding the meaning of $k_{13}$ and $k_{21}$. This was done as follows.




$$\frac{dx}{dt} = k_{21}y - (k_{12} + k_{13})x$$

$$\frac{dy}{dt} = k_{12}x - k_{21}y$$

at $t = 0$, $x = x_o$ and $y = 0$

FIGURE 2. Compartmental analysis for the behaviour of microinjected $^{45}Ca^{++}$. Compartment 1 represents free, ionic calcium within the axon and compartment 2 represents the bound calcium. $X$ represents the amount of free ionic $^{45}Ca^{++}$ within compartment 1 and $Y$ represents the amount of bound $^{45}Ca^{++}$ within compartment 2. A microinjection was effected in compartment 1 at time $t = 0$, making $X = Xo$. Immediately after, $k_{13}X$, that is to say, the efflux of $^{45}Ca^{++}$ was measured in the external sea water. No allowance was made in the model diffusion within the axon.

TABLE I

KINETIC CONSTANTS FOR THE EFFLUX OF $^{45}Ca^{++}$ FROM AXONS, OBTAINED BY MICROINJECTION

External medium: artificial sea water.

| Temperature | $k_{13}$ | $k_{12}$ | $k_{21}$ | $k_{21}'$ | $P$ |
|---|---|---|---|---|---|
| °C | $min^{-1} \times 10^{2}$ | $min^{-1} \times 10^{1}$ | $min^{-1} \times 10^{2}$ | $min^{-1} \times 10^{4}$ | $cm/sec \times 10^{6}$ |
| 19 | 3.9 ± 1.5 (9) | 3.2 ± 2.0 (9) | 4.3 ± 3.8 (8) | 4.6 ± 2.7 (8) | 1.3 ± 0.3 (9) |
| 0 | 0.73 ± 0.22 (9) | 1.9 ± 0.5 (9) | 2.9 ± 0.6 (5) | 0.86 ± 0.45 (5) | 0.22 ± 0.09 (9) |

Values given in this table and throughout this work are mean ± SD. The numbers in parenthesis indicate the number of experiments. Four more experiments were done in sea water without potassium; these are not included here, even though they gave the same results.

If the intracellular level of $^{45}Ca^{++}$ is maintained constant by means of a rapid internal perfusion, the efflux of radioactivity should appear steady, that is to say, no fast exponential should be detected. Furthermore, from the efflux of $^{45}Ca^{++}$ and its concentration in the perfusion fluid (0.4 M K glutamate + 0.4 M glycerol + 0.005 M Tris, pH 7.3), a kinetic constant can be calculated, and this constant should be equal to $k_{13}$. It turns out that both predictions become true. Fig. 3 shows the results of an experiment in which $^{22}Na^{+}$ and $^{45}Ca^{++}$ were used simultaneously to perfuse internally a giant axon. No fast exponential for $^{45}Ca^{++}$ efflux is seen. In addition, this figure shows that while there is a significant increase in permeability to $^{22}Na^{+}$ associated with

impulse propagation, no similar changes are detected for $^{45}Ca^{++}$. Furthermore, the average value of the kinetic constants for the efflux of $^{45}Ca^{++}$ obtained from six experiments such as the one in Fig. 3 is $21 \pm 4 \times 10^{-2}$ $(min)^{-1}$ (equivalent to $P = 8.3 \pm 1.4 \times 10^{-5}$ [cm/sec]). This value compares well with $k_{13}$ depicted in Table I, although it is five times larger. This difference seems to reflect only how conservative we were in extrapolating to zero time. Fig. 3 also informs how much greater is the kinetic constant (or permeability) for the efflux of $Ca^{++}$ than for $Na^{+}$.

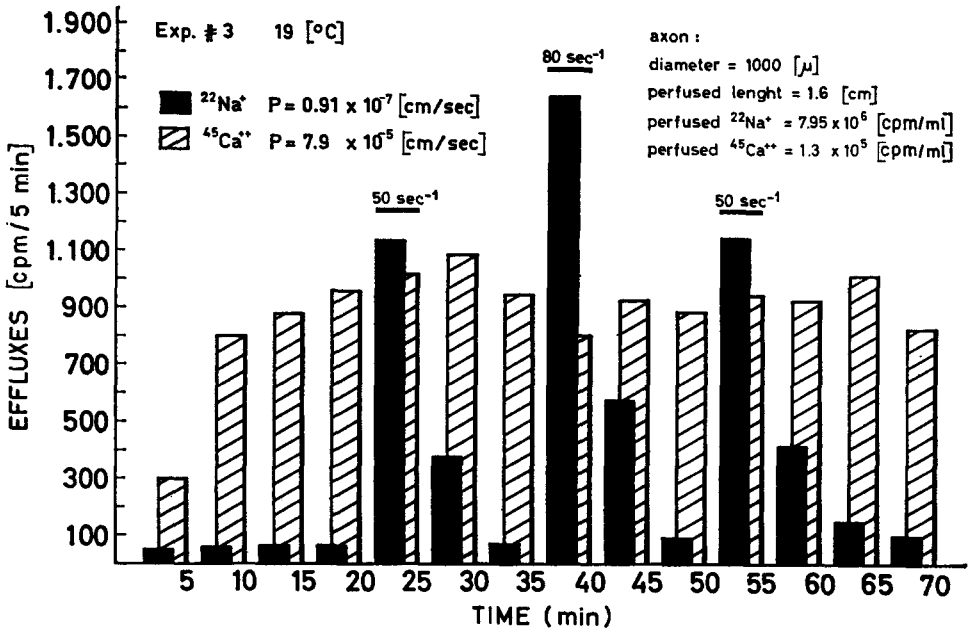



FIGURE 3. Simultaneous effluxes of $^{22}Na^{+}$ and $^{45}Ca^{++}$ internally perfused in a giant axon. Each bar represents the isotopic efflux (of $^{22}Na^{+}$ or $^{45}Ca^{++}$) into the external sea water which was collected every 5 min. The effect of stimulation (twice at 50/sec and once at 80/sec) on both isotopic effluxes is seen.

The identification of $k_{21}$, which in the model discussed here is related to the kinetics of dissociation of bound calcium, was done as follows. $^{45}Ca^{++}$ alone or mixed with $^{22}Na^{+}$ (dissolved in 0.6 M K glutamate, pH 7.3) was microinjected into a giant axon as usual; then the nerve fiber was repeatedly washed in sea water until the slow exponential was reached. Afterwards, the external $Ca^{++}$ which is deleterious to axoplasm was removed by rinsing twice in 0.6 M K glutamate, pH 7.3, and finally the internal content of the axon was extruded. This axoplasm was mounted inside of a collodium tube (1.5–2 mm in diameter) which previously had been perforated repeatedly (about 50 times/cm of length) with a pin which left holes of 120–150 $\mu$ in diameter. These collodium tubes did not constitute significant barriers to the movements of ions. After the protoplasm was in site, the efflux of $^{45}Ca^{++}$ (or $^{45}Ca^{++}$ and $^{22}Na^{+}$) from such artificial membraneless fibers into 0.6 M $K^{+}$ glutamate, pH 7.3, was studied. As a con-

trol, the outflow of $^{45}Ca^{++}$ from a nerve fiber into 0.6 M K glutamate was also investigated. No qualitative difference with the kinetics in sea water was detected. Fig. 4 describes the behavior of $^{45}Ca^{++}$ in the preparation of the collodium tube. Two experiments are shown in this figure. The efflux of $^{45}Ca^{++}$ is shown to be roughly constant. Table II, displays $k_{21}$ of eight of such experiments. In this table, six out of eight values are within the order of magnitude of the kinetic constant used earlier to describe membrane permeability (5) or the slow exponential (4) (see also Table I in this work). The small discrepancies can probably be ascribed to differences in the geometry of the systems and lack of $Ca^{++}$ in the external medium. The two values at variance (12 and 34) with the others of Table II were not considered in calculating the mean. A

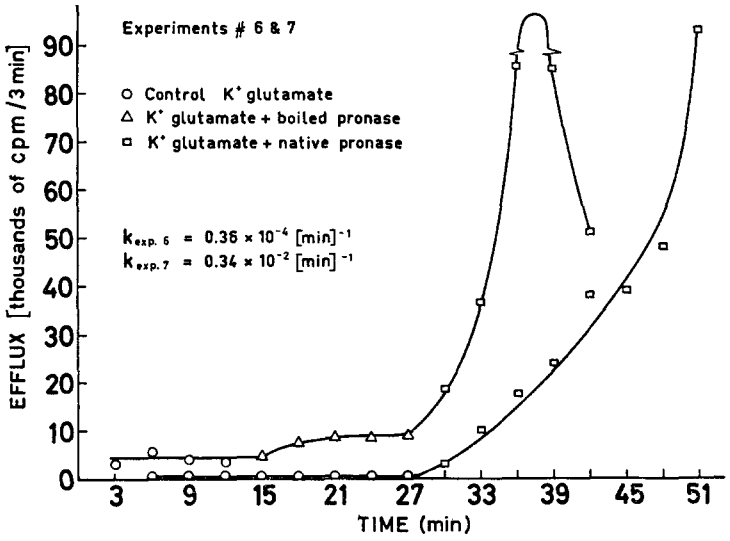



FIGURE 4. Efflux of $^{45}Ca^{++}$ from a perforated collodium tube filled with axoplasm: effect of pronase. External medium was 0.6 M K glutamate or glutamate plus pronase. When the protein is lysed by the enzyme, the efflux of $^{45}Ca^{++}$ is enormously increased showing that at this level the efflux of $^{45}Ca^{++}$ was limited only by the kinetics of dissociation of a calcium-protein complex.

small contamination of the axoplasm with the sea water calcium might explain their discrepancy. In any case, even these variant values are much smaller than $k_{13}$. The eight experiments of Table II were terminated by placing into the external medium (which could easily penetrate through the holes in the collodium) an agent which was thought to interfere with the binding of $^{45}Ca^{++}$. The agent was pronase, 2 mg/ml, or $CaCl_2$, 0.4 M. The effect of native and boiled (5 min) pronase is depicted by Fig. 4. The effect of pronase shows that the limiting step in the efflux of $^{45}Ca^{++}$ from the collodium tube is not a restriction imposed upon it by the perforated membrane but rather by the rate of dissociation of a complex formed between calcium ions and axoplasmic proteins.

The effect of $CaCl_2$ is seen in Fig. 5. The action of $CaCl_2$ shown in this experiment

is thought to be effected by competence with $^{45}Ca^{++}$ for the binding sites or/and by lysis of axoplasm produced by bulk calcium. The effect of $CaCl_2$ is specific for the $^{45}Ca^{++}$ kinetics, without altering the efflux of $^{22}Na^{+}$.

Once the validity of the model of Fig. 2 was established it became clear that the restrictions (or facilitation) imposed upon the movement of calcium ions are contained in $k_{13}$ and not in $k_{21}'$ as it is assumed in the current literature. From $k_{13}$, a permeability coefficient $P$, was calculated according to $P = k_{13}\frac{d}{4}$ where $d$ is the diameter of the axon (6). The $P$ calculated in this fashion do not take into account the opposing electrical gradient present across the axolemma. Active transport and ex-

TABLE II

KINETIC CONSTANT FOR THE EFFLUX OF $^{45}Ca^{++}$ FROM A PERFORATED COLLODIUM TUBE FILLED WITH AXOPLASM

External medium: 0.6 M, K-glutamate, pH 7.3

| Experiment No. | $k_{21}'$ | Terminating agent | Increased effiux of $^{45}Ca^{++}$* |
|---|---|---|---|
| | $min^{-1} \times 10^4$ | | *times* |
| 5 | 12 | Pronase‡ | 13 |
| 6 | 0.36 | Pronase | 780 |
| 7 | 34 | Pronase | 26 |
| 8 | 0.65 | Pronase | 190 |
| 9 | 0.70 | $CaCl_2$§ | 160 |
| 10 | 1.5 | $CaCl_2$ | 116 |
| 11 | 3.2 | $CaCl_2$ | 90 |
| 12 | 0.90 | $CaCl_2$ | 180 |

Mean 1.2, SD 1.1, as applied to values in column 2.
Experiments 5 and 7 were not considered in calculating the mean and SD.
Experiments 1–4 failed for faulty technique. No measurements were made.
* As a result of either pronase or $CaCl_2$.
‡ 2 mg/ml.
§ 0.4 M.

change diffusion of calcium ions, together with the electrical restrictions, are factors contained in the $P$ thus computed. A permeability coefficient defined in the same way was also obtained for sodium ions, this value being equal to $2.5 \pm 0.7 \times 10^{-7}$ (cm/sec). The ratio of $P_{Ca^{++}}/P_{Na^{+}}$ is about 50.

High permeability of the axon membrane to $Ca^{++}$ has been reported before (7). These data, though, are of little use since, as judged from the permeability to monovalent cations reported in this work, the axons seem to have been somewhat leaky. A biphasic efflux of $^{45}Ca^{++}$ has also been reported earlier (8, 9). Soloway, Welsh, and Solomon (8) found in whole crayfish nerves results similar to ours. They proposed two models which fit their data. One of these models is essentially the same one discussed herein. Tasaki, Teorell, and Spyropoulos (9), working in giant axons and using the technique of microinjection, reported data which are qualitatively the same as those published by Luxoro and Rissetti (4), although no precise interpretation was given.

The work of Hodgkin and Keynes, (5) which is the best known in this field, did not detect the fast exponential because their work was designed to measure slow movements of calcium and, accordingly, lacked the temporal resolution required.

The idea that calcium ions are extruded from the axon by means of some kind of active transport is not new (5, 10). The high $Q_{10}$ for $P_{Ca^{++}}$ reported in this work points in that direction. The effects of certain drugs are under study. Ouabain, at concentrations which reduce the sodium efflux by 80% or more, does not alter calcium movements. On the other hand, very preliminary experiments seem to show that both azide (3.0 mM) and dinitrophenol (0.2 mM) depress calcium efflux.

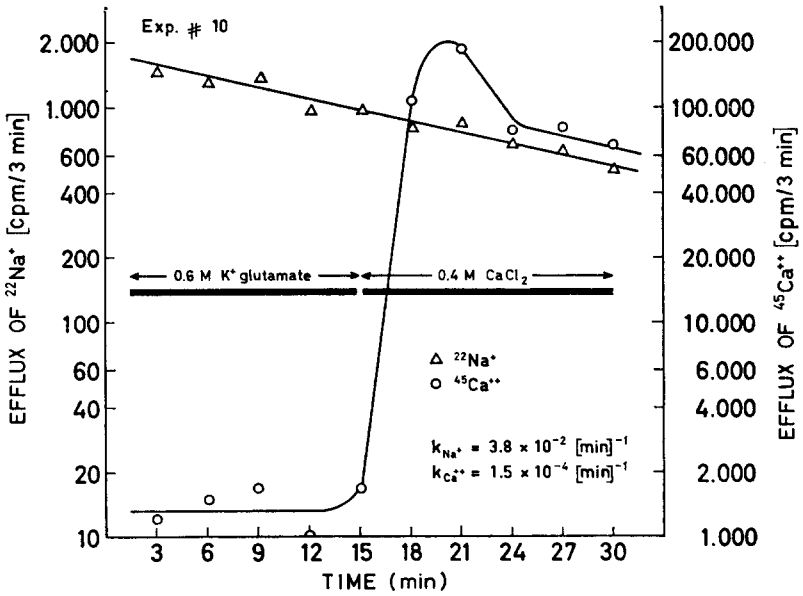



FIGURE 5. Simultaneous effluxes of $^{45}Ca^{++}$ and $^{22}Na^+$ from a perforated collodium tube filled with axoplasm: effect of $CaCl_2$. External medium was 0.6 M K-glutamate or 0.4 M $CaCl_2$. The efflux of $^{45}Ca^{++}$ is shown to increase when the kinetics of binding is interfered with $CaCl_2$. $^{22}Na^+$ efflux is not altered at all. Semilogarithmic plot.

With a clear understanding of the meaning of each kinetic constant, the possibility of studying the chemical reaction between calcium and nerve proteins and the factors which govern it has been opened. Luxoro, Rojas, and Wittig (10) showed that $Ca^{++}$ which enters a nerve fiber during an action potential reacts with axoplasmic proteins producing an uncoiling of them; ionizing groups thus are made available which normally are masked within the inner structure of the protein. In this work, it is shown that most of the internal calcium is bound to proteins. One wonders how important these reactions might be in controlling the triggering mechanism of the spike.

If the total intraaxonal concentration of calcium in *Dosidicus* is the same as reported in *Loligo* (0.42 mM) by Keynes and Lewis (11), an estimation of the internal concentration of free calcium and the total efflux can be made. In steady state, $k_{21}$ times

"bound calcium" = $k_{12}$ times "free calcium" since total calcium is closely equal to bound calcium: free calcium $= \frac{k_{21}}{k_{12}} 0.42 = 0.55 \times 10^{-2}$ mM.

It is worth noticing that Hodgkin and Keynes, on the basis of mobility determinations (5), placed an upper limit of $1 \times 10^{-2}$ mM for intracellular free calcium. The efflux of calcium can be computed from the intracellular concentration and the permeability coefficient given in this work: absolute efflux of calcium $= P_{Ca^{++}} \times [Ca^{++}]_i$. Using a range of permeabilities from 1.3 to $8.0 \times 10^{-5} \left[\frac{cm}{seg}\right]$, one obtains absolute calcium efflux $= 0.072\text{–}0.44 \left[\frac{p\ mole}{cm^2\ sec}\right]$.

The range of effluxes given in reference 5 (and calculated from the internal specific activity) is 0.075–0.50. This coincidence shows again the meaning of the coefficient $P$, as used in the present work, and the internal consistence of the model.

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## REFERENCES

1. ROJAS, E., and M. LUXORO. 1963. Microinjection of trypsin into axons of squid. *Nature.* **199**:78.
2. TASAKI, I., and M. LUXORO. 1964. Intracellular perfusion of chilean squid giant axons. *Science.* **145**:1313.
3. LUXORO, M., M. CANESSA, and F. VARGAS. 1965. Physiological properties of the giant axon from *Dosidicus gigas.* Proceedings of the 23rd International Congress of Physiological Science, Tokyo. Excerpta Medica Foundation, Amsterdam. 507.
4. LUXORO, M., and S. RISSETTI. 1967. Calcium efflux in giant axons. *Biochim. Biophys. Acta.* **135**:368.
5. HODGKIN, A. L. and R. D. KEYNES. 1957. Movements of labelled calcium in squid giant axons. *J. Physiol.* **138**:253.
6. KEYNES, R. D. 1951. The ionic movements during nervous activity. *J. Physiol.* **114**:119.
7. ROTHENBERG, M. A. 1950. Studies on permeability in relation to nerve function II. Ionic movements across axonal membranes. *Biochim. Biophys. Acta.* **4**:114.
8. SOLOWAY, S., J. H. WELSH, and A. K. SOLOMON. 1953. Studies on $^{45}Ca^{++}$ transport in crayfish nerve. *J. Cellular Comp. Physiol.* **42**:471.
9. TASAKI, I., T. TEORELL, and C. S. SPYROPOULOS. 1961. Movements of radioactive tracers across squid axon membrane. *Am. J. Physiol.* **200**:11.
10. LUXORO, M., E. ROJAS, and E. WITTIG. 1963. Effect of azide and Ca-ion on the reversible changes in protein configuration in stimulated nerves. *J. Gen. Physiol.* **46**:1109.
11. KEYNES, R. D., and P. R. LEWIS. 1956. The intracellular calcium contents of some invertebrate nerves. *J. Physiol.* **134**:399.