The Loss and Recovery of the Sodium Pump in Perfused Giant Axons

MITZY CANESSA-FISCHER, FERNANDO ZAMBRANO, and EDUARDO ROJAS

From the Laboratorios de Biofísica y Fisiología Celular, Facultad de Ciencias, Universidad de Chile, Clasificador 198, Santiago, Chile

INTRODUCTION

The experiments we are going to report are concerned with the following questions: (*a*) Does the cell membrane actively transport sodium when it is supplied with ATP as the only substrate? (*b*) Is it possible to correlate active transport with (Na + K)-activated ATPase-catalyzed reaction taking place in vitro in fragments of squid axon membranes?

We have approached these questions by studying sodium movements in the giant axons of the squid, in which by means of intracellular perfusion it is possible extensively to remove axoplasmic components and to replace them by solutions of known composition.

The following experiments demonstrate that intracellular perfusion causes the loss of the capacity of the axon to transport sodium against its electrochemical gradient with whatever anion is used to mimic axoplasm composition. The active efflux of sodium recovers on addition of ATP-Mg only when aspartate is present in the perfusing solution. This anion, which is one of the main free amino acids of invertebrate nerves, maintains the bioelectric properties of the fibers and an ATP-activated ouabain-sensitive Na outflux. However, there was no demonstrable correlation between the effect of ATP on sodium efflux and the hydrolytic breakdown of ATP in vivo or in vitro by axon membrane fragments.

The implications for a chemical mechanism of active transport operating in vivo and the stoichiometry of the conversion of phosphate bond energy into osmotic work are discussed.

MATERIALS AND METHODS

Squids (*Dosidicus gigas*) were caught at the Laboratorio de Fisiología Celular, Facultad de Ciencias, Renaca, Chile.

Microinjections were performed according to Brady, Spiropoulos and Tasaki (1958).

The Suction-Perfusion Technique This was carried out as described by Tasaki and Luxoro (1964), with 550-μ pipettes for suction and a 300 μ pipette for perfusion.

As sodium fluxes are proportional to the membrane area, axons were perfused over their entire length bathed in sea water. The perfusion procedure is different from that described by Tasaki and Luxoro, in which only part of the fiber immersed in sea water was internally perfused.

While the operation of microinjection and perfusion was being performed, action potentials were externally elicited and recorded. After the withdrawal of the outlet cannula, only internal recording of the action potential was performed, with a bare platinum wire. Standard electronic equipment was used for stimulation and recording: S-4 Grass stimulator, P-6 Grass differential amplifier (Grass Medical Instruments, Quincy, Mass.), and 502 Tektronix, Inc. (Beaverton, Ore.) oscilloscope.

Flux Measurements Sodium-22 was obtained either from Isoserve, Inc., Cambridge, Mass., or from the New England Nuclear Corp., Boston, Mass. Solutions for measurements of efflux and influx contained usually 10 and 25 μc/ml, respectively. Radioactivity of the samples

TABLE I

SODIUM FLUXES IN INTACT *DOSIDICUS* AXONS

	No. of experiments	Fluxes	Inhibition
		pmoles/cm²·sec	%
Effluxes			
10 K ASW	11	46.0±10	
Natural SW	7	67.0±12	
0 K ASW	6		58.2±9
10^{-5} M ouabain	8		82.2±9
6 mM amytal	4		57.2±12.0
3 mM cyanide	3		64.0
20 μg/ml rutamycin	4		56.6±22
Influxes	6	51.7±7.7	

Mean value ± SD. Temperature 20°C. Percent inhibition was calculated in every axon with respect to its own resting efflux, 60 min after the addition of the inhibitor to ASW. Artificial sea water composition: 420 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, 5 mM Tris Cl (pH 8.0).

was determined with a Nuclear-Chicago Corp. (Des Plaines, Ill.) automatic 2-inch crystal scintillation counter.

Intracellular ATP Hydrolysis The perfusate was collected from the suction cannula in small tared beakers, reweighed, and analyzed for inorganic phosphate as described elsewhere (Ernster, Zetterström, and Lindberg, 1950). The initial inorganic phosphate content of the perfusate was controlled, as well as the nonenzymic hydrolysis of ATP taking place in the perfusion solution prior to its passage through the axons. Samples of perfusate were collected usually for 10 min.

(Na + K)-Activated ATPase Assays These were performed according to Canessa-Fischer (1965) and Canessa-Fischer, Zambrano, and Riveros-Moreno (1967), with the 100,000 *g* fraction obtained from homogenates of optic nerves of *D. gigas*; it was found that this fraction had properties identical with those of the fraction obtained from giant axons. Phosphate was determined as described by Ernster et al. (1950).

Solutions and Reagents ATP was obtained from Mann Research Laboratories, Inc., New York, and solutions containing this nucleotide were prepared just before starting an experiment, its pH being adjusted to 7.4 with Tris Cl. Aspartic acid and glutamic acid (Calbiochem, Los Angeles, Calif., grade A) were used to prepare Na and K salts. These solutions were kept frozen at −10°C for no more than 1 wk and thawed just once for experiments.

TABLE II

THE EFFECT OF ANIONS ON RESTING SODIUM FLUXES IN PERFUSED GIANT AXONS

Internal ionic composition	No. of determinations	Efflux	No. of determinations	Influx
molar		*pmoles/cm²·sec*		*pmoles/cm²·sec*
0.5 KF, 0.1 NaF	21	3.7±1.3	26	50.5±9
0.5 KCl, 0.1 NaF	6	290	10	120
0.5 K glutamate, 0.1 Na glutamate	6	10.0±2	4	45.0
0.3 K aspartate, 0.1 Na isethionate 0.3 sucrose	7	23.3±8	—	—
0.45 K aspartate, 0.1 Na aspartate	20	13.8±7	2	60

Artificial sea water composition: 420 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, 5 mM Tris Cl (pH 8.0). Temperature 20°C.

Na isethionate was obtained from Eastman Kodak Co., Rochester, N. Y.; Tris 121 was obtained from Sigma Chemical Co., St. Louis, Mo.; sucrose from Merck, (Darmstadt, Germany).

RESULTS

The Active Transport of Sodium in Intact Axons of the Squid Dosidicus gigas

Table I summarizes the properties of sodium fluxes from giant axons of *Dosidicus gigas*. Sodium efflux to natural sea water from Montemar (Chile) is higher than the

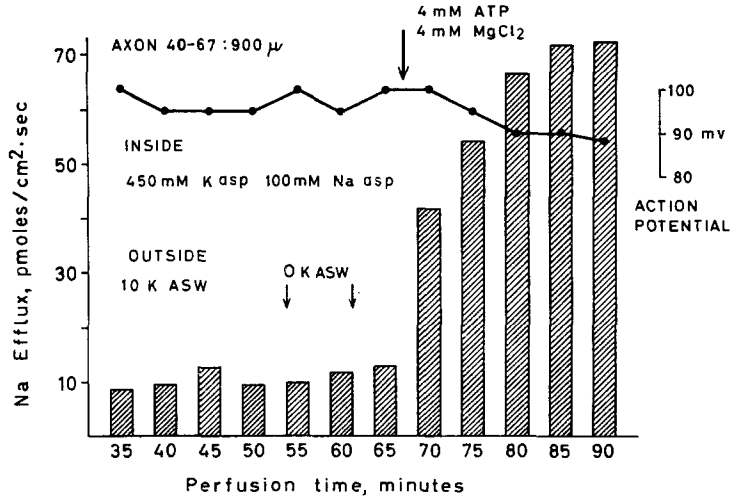

FIGURE 1. The effect of ATP-Mg on sodium efflux from a perfused giant axon. Artificial sea water composition: 420 mM NaCl, 50 mM $MgCl_2$, 10 mM KCl, 10 mM $CaCl_2$, 5 mM Tris Cl (pH 8.0). The artificial sea water was collected every 5 min as a single sample. Room temperature, 19°C; length of axon under perfusion, 2.2 cm. The solution with ATP was brought up to pH 7.3 with drops of Tris Cl buffer, pH 8.0.

sodium efflux to artificial sea water of the same osmotic pressure. The use of artificial sea water for effluxing giant fibers gives permeability (P) values of 4.6×10^{-7} cm/sec ($P = kd/4$, where k is the rate constant of sodium efflux, and d is the axon diameter); the flux was calculated assuming a mean value of 100 mM Na in the axoplasm for all axons.

The effect of various metabolic inhibitors upon sodium efflux (Table I) indicates that in *Dosidicus* axons as in *Loligo* an active transport of sodium coupled to potassium ions operates (Hodgkin and Keynes, 1955).

The Loss of the "Sodium Pump" by Intracellular Perfusion

The main problem in the study of sodium fluxes in perfused axons is the survival time of the excitability properties. The gradual loss of the ability to conduct action

TABLE III

THE EFFECT OF ATP ON SODIUM EFFLUX FROM PERFUSED AXONS

Internal ionic composition	No. of determinations	Na efflux	Action potential	Inhibition by ouabain
molar		*pmoles/cm²·sec*	*mv*	%
0.3 K aspartate, 0.1 Na isethionate, 0.3 sucrose	10	57.3±21.7	73.0±9.9	42.6±19.3
0.45 K aspartate, 0.1 Na aspartate	30	54.9±10	96.2±7	34.6±11
0.3 K glutamate, 0.1 Na isethionate, 0.3 sucrose	6	67.3±14.6	66.9±9.9	

Mean value ± SD. Artificial sea water, temperature 20°C. Maximal inhibition by ouabain was calculated 20–30 min after its addition to the sea water.

potentials is accompanied by a marked increase in passive permeability, making the active transport component of sodium fluxes unmeasurable. Thus our first step was to study sodium fluxes under optimal bioelectric conditions. For this reason we worked with fluoride, which according to Tasaki and Takenaka (1964) markedly increased the survival time of action potentials.

Table II shows that upon perfusion with fluoride as the only anion, sodium efflux decreases to 8% of the resting efflux measured in intact axons; the experimental flux ratios revealed that sodium fluxes can be accounted for by considering only electrochemical forces (Rojas and Canessa-Fischer, 1968). It has been observed that fluoride when placed externally is inhibitory of the transport mechanism of red blood cells and of the glycolytic production of ATP (Dunber and Passow 1950; Fruton and Simmonds, 1958). We found also that fluoride completely inhibited (Na + K)-activated ATPase of membrane fragments, as well as ATP hydrolysis in perfused fibers.

In order to find a suitable perfusion solution to allow ATP utilization by the cell membrane, we studied systematically the effect of various anions on sodium fluxes. Table II shows that chloride cannot be used for this purpose because it markedly increases passive fluxes. Other anions such as sulfate gave such a short survival time

as to make isotope flux measurements impossible. However, use of organic anions which are present in high concentration in squid axoplasm seemed more promising, especially in view of the suggestive and elegant results of Brinley and Mullins with dialyzed axons (1966, 1967).

It can be seen in Table II that glutamate and aspartate, used as the only anions of perfusing solutions, gave good survival times of action potential. The resting sodium efflux was 13.8 pmoles/cm^2·sec with aspartate, which means that the Na efflux is depressed to one-fifth of its normal value by removal of axoplasm and substrates. The resting sodium efflux of aspartate-perfused fibers was not dependent on external potassium ions or affected by the replacement of external sodium by lithium.

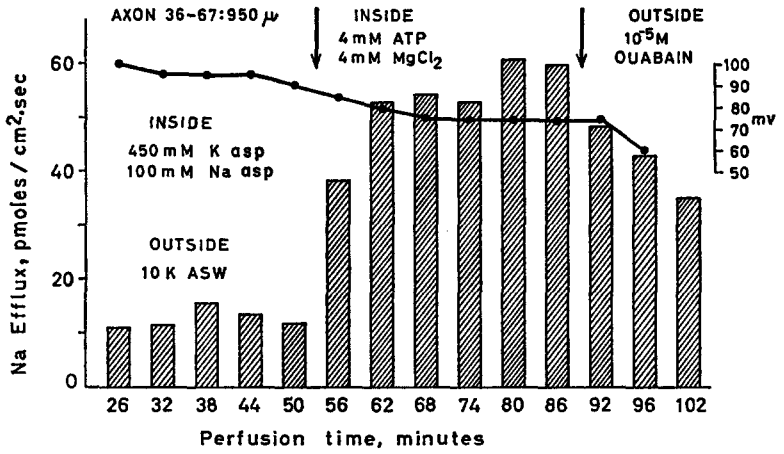

FIGURE 2. The effect of ATP on sodium efflux from a perfused giant axon. The perfusion was started with 0.6 M K aspartate and later changed to the mixture indicated in the figure.

The Recovery of the Sodium Pump by ATP in Perfused Fibers

In order to investigate the capacity of Mg-ATP for recovering the mechanism of sodium pumping, it was added to various types of perfusing solutions. Fig. 1 shows an experiment in which perfusion was started at zero time with 0.6 M K aspartate, pH 7.4, changed 15 min later to 0.45 M K aspartate, 0.1 M Na aspartate; the same solution containing ^{22}Na was introduced 5 min later and sea water was collected for radioactivity measurements 10 min later. It can be seen that resting sodium extrusion is very constant at a rate of 12 pmoles/cm^2·sec. Bioelectric properties are also well maintained, since at a very low threshold single pulses triggered action potential of normal size, with a rapid rate of rise. Later, the solution was changed to a new radioactive solution which contained in addition 4 mM ATP and 4 mM $MgCl_2$. The efflux very promptly increased to 42 pmoles/cm^2·sec and reached a steady-state level of 70 pmoles/cm^2·sec 20 min after ATP was added to the perfusing solution.

Table III summarizes results of experiments with ATP-activated efflux obtained with various anions. Aspartate gives the best results in maintaining good action

potentials and recovering the sodium pump. Solutions containing isethionate at low ionic strength, even though they enabled ATP to restore sodium efflux, did not maintain action potential of normal size (Fig. 2); it was found that action potentials of axons perfused with 0.6 M K aspartate, which usually were 100 mv, when the solution was changed to K aspartate, Na isethionate, and sucrose fell rapidly to about 80 mv.

Another observation which is worth mentioning is the tendency of action potentials to drop slightly upon perfusion with ATP-Mg; since resting potentials were not recorded in these experiments and no test was done for calcium traces present in ATP, there is not yet a satisfactory explanation for this finding. It can be noted

TABLE IV

THE EFFECT OF ANIONS ON THE RATE OF HYDROLYSIS OF ATP BY PERFUSED SQUID AXONS

Internal ionic composition	No. of experiments	Inorganic phosphate released
molar		*mμg/cm²·min*
0.5 KF, 0.1 NaF	2	None
0.5 KCl, 0.1 NaF	2	58.4
0.31 K_2SO_4, 0.035 NaCl, 0.36 sucrose	2	135.0
0.3 K glutamate, 0.1 Na isethionate, 0.3 sucrose	4	None
0.3 K aspartate, 0.1 Na isethionate, 0.3 sucrose	4	None
0.45 K aspartate, 0.1 Na aspartate	5	113.0

Artificial sea water, temperature 20°C. 0.004 M ATP, 0.004 M $MgCl_2$.

that perfusion with ATP restores sodium efflux to a value similar to the one determined in intact axons. However, it can be seen in Table III that the ATP-activated efflux was inhibited only 35% by 10^{-5} M ouabain in the sea water. This result is markedly different from that with intact fibers, whose sodium efflux is 82% inhibited by the glycoside (Table I); moreover, the addition of ouabain was usually accompanied by a decrease in action potential, which was never observed in intact fibers. Further investigations are required to clarify this point.

It should be mentioned that the ouabain-insensitive sodium efflux of perfused axons was not decreased by replacement of external sodium by lithium, a finding which may exclude an exchange diffusion component in the flux. A decrease in ouabain-insensitive Na efflux on replacement of Na by Li was seen only in giant axons which remained for long periods with very deteriorated action potential (20–40 mv) recorded intracellularly.

The Utilization of ATP by Perfused Giant Axons

Last year we attempted to restore outward sodium transport by perfusing with fluoride, chloride, and glutamate, with negative results.

The passage of solutions containing ATP-Mg through the intracellular compartment enabled us to determine whether or not this substrate was utilized by the membrane, by measuring the rate of release of inorganic phosphate in the perfusate

collected at the end of the sucking cannula. Baker and Shaw (1965) carried out experiments of this sort on eight axons; they used a sulfate-borate-sucrose solution to perfuse axons and they described a sodium-sensitive hydrolysis of perfused ATP in two axons and a ouabain-sensitive hydrolysis in three. However, no report is available on whether extrusion is activated by ATP under similar experimental conditions.

Table IV summarizes the effect of various anion mixtures on the rate of inorganic phosphate production by giant axons perfused with ATP. No measurable ATP

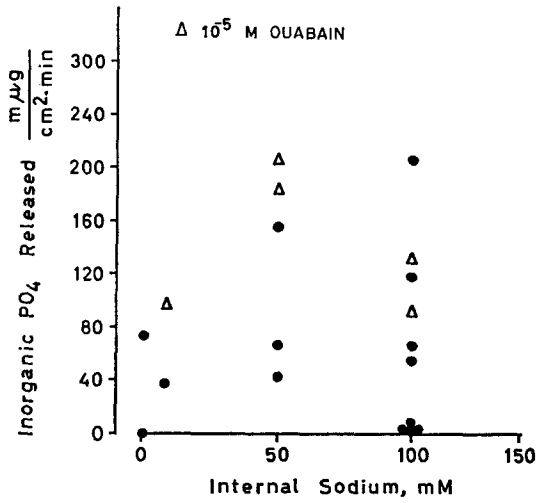

FIGURE 3. ATP breakdown in a perfused axon immersed in artificial sea water (circles); 10^{-5} M ouabain (Δ) was added to the ASW at various internal sodium concentrations. Perfusion fluids were (*a*) 0.6 M K aspartate (pH 7.3), 0.004 M Tris ATP, 0.004 M $MgCl_2$; (*b*) 0.55 M K aspartate, 0.05 M Na aspartate, 0.004 M ATP, 0.004 M $MgCl_2$; (*c*) 0.50 M K aspartate, 0.1 M Na aspartate, 0.004 M ATP, 0.004 M $MgCl_2$. Temperature 20°C.

hydrolysis occurred on perfusion with fluoride, glutamate-isethionate-sucrose, or aspartate-isethionate-sucrose solutions; the effect of fluoride is understandable, since a marked inhibition of ATP breakdown by membrane fragments isolated from the sheath of squid axons was found. Furthermore, the fluoride-perfused fiber did not recover sodium efflux when supplied with Mg-ATP. We do not yet have a clear explanation as to why no measurable inorganic phosphate release was detected upon perfusion with glutamate-isethionate-sucrose or aspartate-isethionate-sucrose solutions. It is noteworthy that the last-mentioned solution enabled a substantial recovery of sodium outflux, with no measurable ATP breakdown (Table III).

In those experiments where anions of the lyotropic series were used (chloride and sulfate), inorganic phosphate appeared in the perfusate coming out from the giant axons; however, when such solutions were used for measuring sodium fluxes, passive movements increased markedly, and excitability was lost in 15–20 min (Table II). This made the active transport component unmeasurable.

Upon perfusion with aspartate and Mg-ATP, considerable phosphate release occurred. This led us to study the dependence of nucleotide hydrolysis on the intracellular sodium concentration and the effect of ouabain.

Fig. 3 shows results obtained in 12 experiments in which internal sodium was varied from 0 to 100 mM. It can be seen that no clear relationship between the two variables could be demonstrated; this also occurred when sodium was increased in a single axon. The addition of 10^{-5} M ouabain to the artificial sea water, which inhibited 40% of the ATP-activated efflux, did not significantly inhibit the release of inorganic phosphate during perfusion with ATP. We can account for this hydrolysis by assuming that the residual axoplasm of the perfused fiber is 10–20%.

TABLE V

(Na + K)-ACTIVATED ATPase OF MEMBRANE FRAGMENTS OF SQUID AXONS

Ionic composition	Chloride	Aspartate
	μmoles/mg protein·hr	*μmoles/mg protein·hr*
Temperature 37°C		
0.004 M $MgCl_2$	53.6	21.4
0.004 M $MgCl_2$, 0.04 M K, 0.1 M Na	109.0	65.2
0.004 M $MgCl_2$, 0.04 M K, 0.1 M Na + ouabain	67.5	28.6
Temperature 20°C		
0.004 M $MgCl_2$	30.4	6.1
0.004 M $MgCl_2$, 0.04 M K, 0.1 M Na	48.8	14.6
0.004 M $MgCl_2$, 0.04 M K, 0.1 M Na + ouabain	44.0	11.0

Incubation media: 0.4 M Tris aspartate (pH 7.4), 0.004 M Tris ATP, 0.05 ml of enzyme suspended in 0.25 M sucrose, 0.03 M Tris Cl (pH 7.4), 0.001 M EDTA. Final volume 1 ml; preincubation time 10 min; incubation time 30 min. 10^{-5} M ouabain.

The Effect of Anions on the (Na + K)-ATPase Activity of Membrane Fragments of Squid Axons

We have previously reported the notable effect of anions on the ATP hydrolysis by membrane fragments isolated from the sheath of giant axons (Canessa-Fischer, 1965; Canessa-Fischer et al., 1967). It was found that anions could be ordered in a series in the direction of favoring ATP hydrolysis: Br, Cl, NO_3, SO_4, glutamate. This series was in the opposite direction from that favoring excitability properties of giant axons.

Table V shows the results obtained in assays of Na-K-ATPase activity using solutions which recovered the Na pump in perfused giant axons. It can be noticed that when aspartate is the main anion, the rate of ATP hydrolysis averages about one-third of that measured using chloride. These findings seem to indicate that the experimental conditions used to display ATPase activity of cell membrane in vitro do not allow the operation of an ATP-activated sodium efflux in vivo.

Table VI summarizes results of the whole series of experiments carried out in the past three years. The recovery of the active transport mechanism in giant axons by perfusion with ATP did not lead to measurable hydrolysis when aspartate-ise-

thionate-sucrose was used. The hydrolysis measured when only aspartate was introduced was not inhibited by ouabain, and it was not a function of the Na intracellular concentration; it could be accounted for residual axoplasm.

To pump sodium at a rate of 50 pmoles/cm^2·sec against the electrochemical gradient created by a concentration difference of 430 mM − 100 mM = 330 mM and by a potential difference of −55 mv, an ATP hydrolysis at a rate of 9 pmoles/sec would be required, assuming that the hydrolytic system has 100% efficiency and that the change in free energy for the reaction is $\Delta F = -7000$ cal per mole ATP. It should be pointed out that this rate of hydrolysis was not observed, although it was within the range of sensitivity of the analytical procedure used.

In summary, we have presented experimental data which answer our first initial question. Perfusion of giant axons with solutions supplying only ATP as substrate

TABLE VI

A SUMMARY OF FINDINGS LEADING TO THE RECOVERY OF SODIUM PUMP IN PERFUSED GIANT AXONS

	Aspartate	Aspartate-isethionate-sucrose	Chloride	Sulfate	Fluoride	Glutamate-isethionate-sucrose
ATP hydrolysis by perfused axons	++++	None	+++	++	None	None
ATP-activated Na efflux	++++	+++	None	—	None	++
(Na + K)-activated ATPase	++	—	++++	++	None	—
Action potential survival	+++	++	+	+	++++	++

restores the sodium pump only when a suitable anion is used to maintain intact the structure of the transport apparatus. However, the data do not support the view that the activity of the (Na + K)-activated ATPase correlates in vivo with the active transport of sodium.

Lehninger (1962) has pointed out that both myosin and mitochondrial membranes possess ATPase activity in a latent form. Hydrolysis of ATP is not a normal event in oxidative phosphorylation and perhaps not in the contractile event in the actomyosin that Davies, Kushmerick, and Larson (1967) recently examined. In both cases ATP hydrolysis is caused in vitro by an aberrant or side reaction in which a H_2O molecule replaces the normal reaction.

It should be pointed out that one of the main properties of the membrane enzyme system is to be cryptic. On the basis of its properties, (Na + K)-activated ATPase is part of the active transport mechanism present in the cell membrane; but in the light of these results obtained in giant axons, it appears that the system does not fully operate in the hydrolytic direction in vivo. This may be only a special property of excitable membranes of invertebrate nerves which have the unusually high concentrations of free aspartate which, as Lewis (1952) expected, seem to play an important physiological role enabling the operation of the excitation and active transport mechanism.

These findings raise acutely the problem of the mechanism by which ATP leads

to the recovery of the Na pump. Further investigations are required to establish more definitively whether or not degradation or transformation of entropy occurs to operate active sodium transport.

This research was supported by the University of Chile; by U. S. Air Force AFOSR grant 788-65, monitored by the Air Force Office of Scientific Research; and by U. S. Public Health Service grant NB-06503-02.

REFERENCES

BAKER, P. F., and I. T. SHAW. 1965. A comparison of the phosphorus metabolism of intact squid nerve with the isolated axoplasm and sheath. *J. Physiol.* (*London*). **180**:424.

BRADY, R. O., C. S. SPYROPOULOS, and I. TASAKI. 1958. Intraaxonal injections of biologically active materials. *Am. J. Physiol.* **194**:207.

BRINLEY, F. J., JR., and L. J. MULLINS. 1966. Sodium pumping by internally perfused squid axons. *Proc. Intern. Biophys. Congr., 2nd, Vienna.* 66.

BRINLEY, F. J., JR., and L. J. MULLINS. 1967. Sodium extrusion by internally dialyzed squid axons. *J. Gen. Physiol.* In press.

CANESSA-FISCHER, M. 1965. Properties of ATPase activities of membrane fractions from the sheath of squid giant axons. *J. Cellular Comp. Physiol.* **66**:165.

CANESSA-FISCHER, M., F. ZAMBRANO, and V. RIVEROS-MORENO. 1967. Properties of ATPase system from the sheath of squid giant axons. *Arch. Biochem. Biophys.* **22**:658.

DAVIES, R. E., M. KUSHMERICK, and R. E. LARSON. 1967. ATP activation and the heat of shortening of muscle. *Nature.* **214**:145.

DUNBER, E., and H. PASSOW. 1950. Verteilung von Anionen un Kationen bei Fluoridvergiftung menschlicher Erythrocyten. *Pflügers. Arch. Ges. Physiol.* **252**:542.

ERNSTER, L. ZETTERSTRÖM, and R. LINDBERG. 1950. A method for the determination of tracer phosphate in biological material. *Acta Chem. Scand.* **4**:942.

FRUTON, J., and S. SIMMONDS. 1958. General Biochemistry. John Wiley & Sons Inc., New York. 2nd edition. 472.

HODGKIN, A. L., and R. R. KEYNES. 1955. Active transport of cations in giant axons from *Sepia* and *Loligo*. *J. Physiol.* (*London*). **128**:28.

LEHNINGER, A. L. 1962. Horizons in Biochemistry. Academic Press Inc. New York. 431.

LEWIS, P. R. 1952. The free amino acids of invertebrate nerve. *Biochem. J.* **52**:330.

ROJAS, E., and M. CANESSA-FISCHER. 1968. Sodium movements in perfused squid giant axons: Passive fluxes. *J. Gen. Physiol.* In press.

TASAKI, I., and M. LUXORO. 1964. Intracellular perfusion of Chilean giant squid axons. *Science.* **145**:1313.

TASAKI, I., and T. TAKENAKA. 1964. Effects of various potassium salts and proteases upon excitability of intracellularly perfused squid giant axons. *Proc. Natl. Acad. Sci. U. S.* **52**:804.