Axoplasmic Free Magnesium Levels and Magnesium Extrusion from Squid Giant Axons

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ABSTRACT The free magnesium concentration in the axoplasm of the giant axon of the squid, *Loligo pealei*, was estimated by exploiting the known sensitivity of the sodium pump to intracellular Mg^{2+} levels. The Mg-citrate buffer which, when injected into the axon, resulted in no change in sodium efflux was in equilibrium with a Mg^{2+} level of about 3–4 mM. Optimal $[Mg^{2+}]$ for the sodium pump is somewhat higher. Total magnesium content of axoplasm was 6.7 mmol/kg, and that of hemolymph was 44 mM. The rate coefficient for ^{28}Mg efflux was about 2×10^{-3} min^{-1} for a 500-μm axon at 22–25°C, with a very high temperature coefficient (Q_{10} = 4–5). This efflux is inhibited 95% by injection of apyrase and 75% by removal of external sodium, and seems unaffected by membrane potential or potassium ions. Increased intracellular ADP levels do not affect Mg efflux nor its requirement for Na_o^+, but extracellular magnesium ions do. Activation of ^{28}Mg efflux by Na_o^+ follows hyperbolic kinetics, with Mg_o^{2+} reducing the affinity of the system for Na_o^+. Lanthanum and D600 reversibly inhibit Mg efflux. In the absence of both Na^+ and Mg^{2+}, but not in their presence, removal of Ca^{2+} from the seawater vastly increased ^{28}Mg efflux; this efflux was also strongly inhibited by lanthanum. A small (10^{-14} mol cm^{-2}) extra Mg efflux accompanies the conduction of an action potential.

INTRODUCTION

Magnesium ions are required in many biochemical reactions, in particular those involving phosphate compounds. For example, the membrane-bound (Na + K)ATPase, generally identified with the active sodium-potassium pump, has an absolute requirement for magnesium ions, and its activity goes through a maximum as the magnesium concentration is varied (Skou, 1957). The complete kinetic description of an *in situ* sodium-potassium pump, therefore, will require knowledge of prevailing intracellular free magnesium levels, and the pursuit of that information was one of the incentives behind the present investigation. It is known that active sodium extrusion by the squid giant axon is inhibited by excess intracellular magnesium (De Weer, 1970). Mullins and Brinley (unpublished) have also found that complexation of intracellular Mg with diaminocyclohexane tetraacetate inhibits sodium efflux. Clearly, there must exist an optimum free magnesium concentration which may or may not be identical with the naturally prevailing axoplasmic free magnesium concentration. In the present investigation this dependence of sodium transport on intracellular magnesium levels is exploited as an indicator for the determination of intracellular free magnesium

levels. It is also known that the active extrusion of magnesium ions is stimulated by extracellular sodium ions (Ashley and Ellory, 1972; Baker and Crawford, 1972). Hence, the possibility exists that a sodium-for-magnesium exchange takes place across the cell membrane. Such Na influx, if not taken into account, could complicate studies on the kinetics of the sodium pump proper. An efficient and selective inhibitor of Na-stimulated Mg efflux would also be desirable.

Part of this work has been briefly presented elsewhere (De Weer, 1974 *a*).

METHODS

Preparation

These experiments were performed at the Marine Biological Laboratory, Woods Hole, Massachusetts, during the summers of 1972 through 1975. Live specimens of the squid, *Loligo pealei*, were decapitated and their hindmost stellar nerves were removed and either used immediately or stored in ice-cold seawater for periods up to 12 h. The giant axons were carefully cleaned and mounted in a vertical microinjection chamber.

Isotopes

^{22}Na injection and efflux measurements were performed as described before (De Weer, 1970). ^{28}Mg (half-life 21.3 h) was obtained as the chloride salt in dilute HCl from Brookhaven National Laboratory, Upton, N. Y. It was concentrated under a stream of warm, dry N_2, neutralized with Tris-HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid) buffer containing phenol red, and microinjected in the usual manner. The amount injected was such that even on the third day of use, axoplasmic Mg level was never raised by more than 2.5 mM, and usually by only 1–2 mM. Efflux of ^{28}Mg was monitored by collecting the artificial seawater flowing past the axon, and measuring its radioactivity by liquid scintillation counting (Bray, 1960).

Artificial Seawater

Two kinds of artificial seawater (ASW) solutions were used. For ^{22}Na efflux measurements, the ASW had the following composition (in mM): NaCl: 425; KCl: 10; $MgSO_4$: 25; $MgCl_2$: 25; $CaCl_2$: 10; EDTA: 0.2; Tris-HEPES: 5 (pH 7.8). Because the ^{28}Mg efflux experiments called for ASW's with high and low Mg, Ca, and Na levels, as well as the use of lanthanum, a series of sulfate-free ASW's was prepared that allowed for isosmotic substitution of Mg, Ca, or Na by choline. Table I lists the composition of these solutions. Their ionic strength ranged from 0.56 to 0.74. (Na)ASW solutions that were Ca free and Mg free were supplemented with 10^{-7} M tetrodotoxin to prevent spontaneous firing of the axons. When appropriate, $LaCl_3$ was added to the ASW immediately before its use.

Mg Determinations

Hemolymph was collected from live squid by cardiac puncture. Axoplasm was rolled out from partially cleaned axons and quickly weighed. Suitable aliquots were taken up in a 1% $LaCl_3$ solution and assayed for Mg by atomic absorption spectrometry. Standards for hemolymph assays included suitable amounts of NaCl, KCl, and $CaCl_2$; those for the axoplasm assays included 400 mM potassium isethionate.

Microinjections

Apart from ^{22}Na or ^{28}Mg, some axons received a second injection of, for example, apyrase, NaCl, isethionate, K-aspartate, arginine hydrochloride, K_3citrate, or $MgSO_4$.

Solutions of these substances were prepared in 5 mM Tris-HEPES buffer, pH 7, containing 1 mM phenol red. The method employed for the determination of free magnesium levels involves injection of various Mg/citrate mixtures that are theoretically at equilibrium with a given free $[Mg^{2+}]$ level, and observation of the effect, if any, these injections have on ^{22}Na efflux. Obviously, any artifact (mechanical, osmotic) due to the injection itself (which adds about 10% to the axoplasmic volume) would seriously compromise the usefulness of this technique. Therefore, various solutions of different osmotic pressure were injected into axons whose ^{22}Na efflux was being monitored. As shown in Fig. 1, there is little effect from injections of solutions that are up to about isotonic with seawater (~0.93 osM). The depression of ^{22}Na efflux after injections of hypertonic NaCl or Na isethionate solutions may be due to osmotic effects, saturation of the sodium pump, or both. The point of interest here is that solutions with osmolarity up to 0.5 can be injected without producing any osmotic artifacts. For this reason $MgSO_4$ and K_3citrate injections

TABLE I

COMPOSITION OF THE ARTIFICIAL SEAWATER SOLUTIONS USED IN STUDIES OF ^{28}Mg EFFLUX

Seawater symbol	NaCl	Choline Cl	$MgCl_2$	$CaCl_2$	KCl
	mM	*mM*	*mM*	*mM*	*mM*
(Na)ASW	400	75	50	10	10
(Chol)ASW	–	475	50	10	10
100 Mg(Na)ASW	400	–	100	10	10
100 Mg(Chol)ASW	–	400	100	10	10
0 Mg(Na)ASW	400	150	–	10	10
0 Mg(Chol)ASW	–	550	–	10	10
0 Mg, 0 Ca(Na)ASW	400	150	–	–	10
0 Mg, 0 Ca(Chol)ASW	–	550	–	–	10
0 Mg, 20 Ca(Na)ASW	400	120	–	20	10
0 Mg, 20 Ca(Chol)ASW	–	520	–	20	10
150 K, 0 Mg(Na)ASW	400	–	–	10	150
150 K, 0 Mg(Chol)ASW	–	400	–	10	150

In addition to the major components listed, all solutions contained EDTA, 0.2 mM, and Tris-HEPES buffer, 5 mM, adjusted to pH 7.8.

were made with 0.25 and 0.125 M solutions, respectively, and mixtures were simply made by combining these two solutions.

Free Mg^{2+} in Equilibrium with Injected Mg-Citrate Mixtures

As mentioned above, the principle of the method employed here for determining intracellular free magnesium levels is that Mg/citrate injections into squid axons should have no effect on the sodium pump if the free magnesium level in the injected solution exactly matches that prevailing in the axoplasm. The following remarks are pertinent to this procedure, and are intended to illustrate the calculations upon which the theoretical curve of Fig. 4 is based. Assume an axoplasm volume a, with free magnesium concentration $[Mg^{2+}]_a$, buffered by magnesium-binding ligand(s) L. The ratio of bound to free ligand(s), $[MgL]/[L]$, determines $[Mg^{2+}]_a$ via the appropriate equilibrium constant(s). Now an additional isotonic volume b is injected, containing free Mg^{2+} at exactly the same level, or $[Mg^{2+}]_b = [Mg^{2+}]_a$. The bound and free axoplasmic ligand species will be diluted by a factor $a/(a + b)$, *but their ratio will be unchanged.* Hence, no consumption or release of free Mg^{2+} by the ligand buffer mixture takes place. Had the injected solution contained,

in addition, a Mg-binding ligand L' (citrate in the present case), this ligand would also have been diluted by a factor $b/(a + b)$, again *without changing the* $[MgL']/[L']$ *ratio*. Consequently, at the true null point one needs an exact match between axoplasm $[Mg^{2+}]_a$ on the one hand, and free $[Mg^{2+}]_b$ of the extra solution, *before injection*, on the other hand. For this reason, the free $[Mg^{2+}]$ values along the abscissa of Fig. 4 are those calculated for the Mg-citrate mixtures *before* injection.

Since, in the experiments to be described, the injected solutions were only a little more than half isotonic, the additional effect of osmotic equilibration, shortly after the injection of a hypotonic solution, should be considered. Injection of a half-isotonic Mg solution with no citrate is equivalent to injecting half the volume with twice the Mg concentration. The points at the extreme right of Fig. 4 may thus be off by a factor of almost 2 on the abscissa. In the region of the lower null point (2–4 mM free Mg^{2+}), however, a twofold

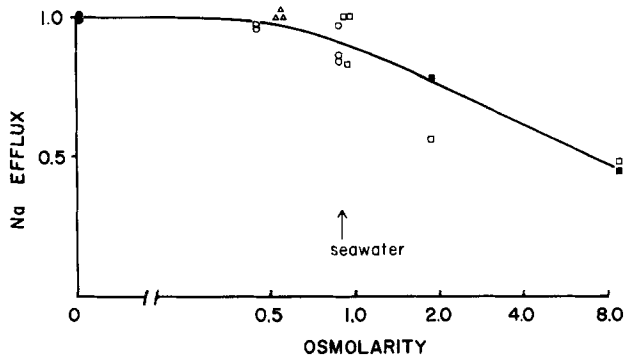

FIGURE 1. Effect of injecting solutions of various osmolarities into squid axons upon ^{22}Na efflux from these axons. Phenol red 1 mM, Tris-HEPES 5 mM (●); K aspartate (○); mannitol (△); NaCl (□); and Na isethionate (■). Ordinate gives ^{22}Na efflux after the injection relative to that before the injection. Osmolarity of artificial seawater solutions is about 0.93. Osmolarities were measured with a dewpoint osmometer, except for the highest four points, which were calculated from Robinson and Stokes (1959) on the assumption that Na isethionate and NaCl solutions have similar osmotic coefficients. The injection apparatus delivers 0.18 μl of fluid per centimeter of axon. Average axon diameter was 510 μm.

dilution or concentration of the injected Mg/citrate mixture affects the free Mg^{2+} concentration by less than 5%, well within the error of the method. For this reason further corrections in the calculation of the abscissa values of the points in Fig. 4 were dispensed with.

Calculations

Isotope effluxes are expressed as rate coefficients (in min^{-1}) and, where appropriate, converted to $pmol \cdot cm^{-2} \cdot s^{-1}$. Averages are given as mean ± standard error of the mean (SEM). The free $[Mg^{2+}]$ levels in equilibrium with given mixtures of K_3citrate and $MgSO_4$ were calculated by Newton-Raphson iteration of the appropriate simultaneous equations. An arbitrary third-degree polynomial was least-squares fitted by computer to the data of Fig. 4. In addition, four nonlinear least-squares data-fitting programs were written to fit: *(a)* simple rectangular hyperbolae to the data of Fig. 11, *(b)* a monoprotic pH titration curve to the absorbance measurements of Fig. 2A, *(c)* Eq. 1 (page 163) to the data of Fig.

2 B (top three curves), and *(d)* an implicit equation expressing changes in metal indicator absorbance as a function of *total* [Mg] in the presence of a fixed concentration of citrate (lower curve in Fig. 2 B). The last program was patterned after a data-fitting program written for implicit equations encountered previously in a study of Mg binding to adenine nucleotides (De Weer and Lowe, 1973).

RESULTS

Dissociation Constants of Mg-Eriochrome Blue SE and Mg-Citrate Complexes

Since no published data are available on the dissociation constant of Mg-citrate at the high ionic strength prevailing in squid axoplasm, the constant was determined in this laboratory, using the dye 3[(5-chloro-2-hydroxyphenyl)azo]-4,5-dihydroxy-2,7 naphthalene disulfonic acid (Mordant Blue 13; Plasmocorinth B; Eriochrome Blue SE), which was shown by Scarpa (1974) to have spectral and other properties that make it a Mg^{2+} indicator suitable for use in media of biological interest. Although a good indicator for Mg^{2+}, Eriochrome Blue SE is very sensitive to pH in the region of biological interest. A careful preliminary study of the Mg-indicator properties of the dye, as affected by pH in the neighborhood of 7, was therefore undertaken. Eriochrome Blue SE undergoes acid dissociation around pH 7.3 (see Fig. 2 A) so that the apparent dissociation constant for the Mg-indicator complex, $K_{d(\text{app})}^{MgE}$, will be a sensitive function of pH in the range 6–8. In addition, the differential molar absorptivity $\Delta\epsilon_{550}^{H}$ (protonated vs. deprotonated), is about double that of the Mg complex, $\Delta\epsilon_{550}^{Mg}$, at 550 nm (the differential absorbance maximum for the Mg complex).

Figure 2 A illustrates the effect of pH on A_{550} of a solution of Eriochrome Blue SE at high ionic strength. Least-squares fitting of the data to a monoprotic dissociation curve yielded pK = 7.30 ± 0.03 and $\Delta\epsilon_{550}^{H}$ = 7.4 (± 0.3) × 10^3 $M^{-1}\cdot cm^{-1}$. The top three curves in Fig. 2 B were obtained as follows. At the three pH values, 6.9, 7.0, and 7.1, the effect of Mg^{2+} on the absorption of Eriochrome Blue SE at 550 nm was determined. The complete set of data (184 points) was then fitted by a least-squares computer program to a single expression of the form:

$$\Delta A = \frac{\Delta A_{max}[Mg^{2+}]}{K_d^{MgE}(1 + 10^{(pK-pH)})}, \quad (1)$$

where K_d^{MgE} represents the dissociation constant of the Mg complex with the deprotonated form of Eriochrome Blue SE, and the expression $K_d^{MgE}(1 + 10^{(pK-pH)})$ represents the apparent dissociation constant of the Mg-Eriochrome Blue SE complex at any arbitrary pH in the vicinity of pK. The reasonable fit supports the assumption that Mg^{2+} binds practically exclusively to the deprotonated form of the dye. Least-squares value for K_d^{MgE} was 2.41 ± 0.10 mM. Given a pK of 7.30, this yields apparent dissociation constants of 8.50 mM at pH 6.9, 7.25 mM at pH 7.0, and 6.25 mM at pH 7.1. Prevailing pH affects not only the apparent dissociation constant (in predictable fashion), but $\Delta\epsilon_{550}^{Mg}$ as well (this has also been noted by Scarpa, 1974). Since the initial (Mg-free) absorbance of the indicator varies with pH according to Fig. 2 A, it was assumed for simplicity that

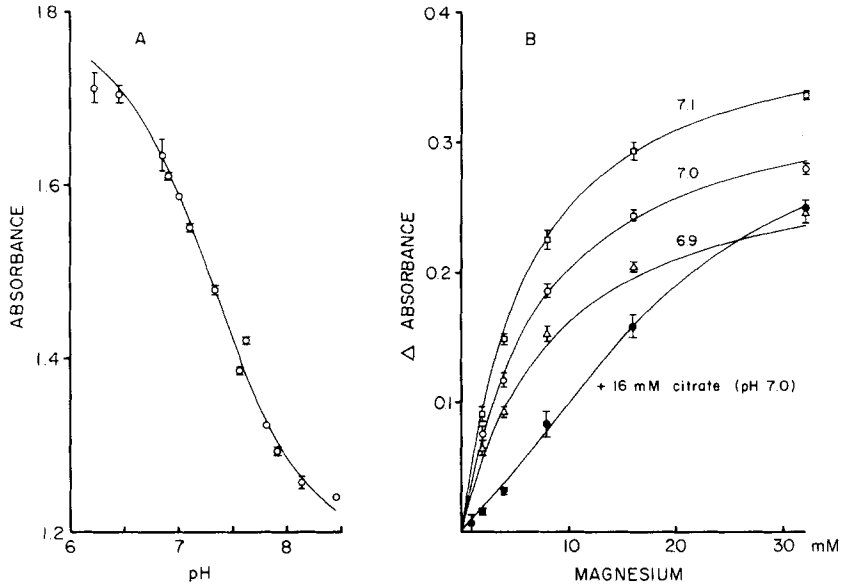

FIGURE 2. (A) Absorbance at 550 nm of an 82 μM solution of Eriochrome Blue SE in 425 mM KCl, 25 mM K-HEPES buffer adjusted to various pH values. The experimental points are averages, ± SEM, of from 4 to 11 determinations. In most cases SEM was smaller than the radius of the symbol. The smooth line is a monoprotic titration curve of the form $A = A_0 + \Delta A/(1 + 10^{pH-pK})$, fitted by a least-squares computer program to the 100 individual experimental points, with pK = 7.30 and $\Delta\epsilon^{H}_{550} = 7.4 \times 10^3\ M^{-1}\cdot cm^{-1}$. (B) Differential absorbance at 550 nm of an 82 μM solution of Eriochrome Blue SE in KCl-HEPES solutions similar to those used in Fig. 2 A. The symbols represent averages ± SEM of from 7 to 16 determinations. Open symbols (top three curves): effect of varying amounts of $MgCl_2$ at three selected pH values. The three curves were generated by least-squares fitting of Eq. 1 to 184 individual points, using the parameters (pK and $\Delta\epsilon^{H}_{550}$) obtained in Fig. 2A; the rectangular hyperbolae have apparent dissociation constant values of 8.50, 7.25, and 6.25 mM at pH 6.9, 7.0, and 7.1, respectively. ΔA_{max} of Eq. 1 was assumed to be a function of pH similar, except for the sign, to that depicted in Fig. 2A (but the assumption of linear dependence on pH gave an equally satisfactory fit over the limited pH range explored). Least-square values for $\Delta\epsilon^{Mg}_{550}$ are 3.6×10^3, 4.3×10^3, and $4.9 \times 10^3 M^{-1}\cdot cm^{-1}$ at pH 6.9, 7.0, and 7.1, respectively. Closed symbols (bottom curve): effect of varying amounts of $MgCl_2$, at pH = 7.0, in the presence of 16 mM potassium citrate. The curve was generated by a computer program designed to find the Mg-citrate dissociation constant for "best fit" to the 56 experimental points. The least-squares value found was $K_d = 3.7 \pm 0.6$ mM.

a similar equation governed the dependence of $\Delta\epsilon^{Mg}_{550}$ on pH, although, over the limited pH range used here, a linear dependence fitted the data equally well. Least-squares values for $\Delta\epsilon^{Mg}_{550}$ at pH 6.9, 7.0, and 7.1 were $(3.6 \pm 0.1) \times 10^3$, $(4.3 \pm 0.1) \times 10^3$, and $(4.9 \pm 0.1) \times 10^3\ M^{-1}\cdot cm^{-1}$, respectively.

The lower curve in Fig. 2 B shows the effect of varying (total) Mg concentration on the differential absorption of the indicator dye at pH 7.0 in the presence

of 16 mM K_3citrate. Using the indicator dye constants determined above, it was then possible to least-squares fit these data to the implicit functions relating total Mg concentration to free Mg^{2+} concentration, and hence to the absorbance measurements. The "best fit" curve shown in Fig. 2 B required a Mg-citrate dissociation constant of 3.7 ± 0.6 mM. This value was subsequently used in connection with the determination of axoplasmic free Mg^{2+} levels (see below). It should be noted that, since the third pK of citric acid (~5.6) is rather removed from neutral pH, variations of medium pH in the range 7.0–7.5 will have very little effect on the apparent dissociation constant of the Mg-citrate complex.

Magnesium Content of Hemolymph and Axoplasm

The total magnesium concentration in *Loligo pealei* hemolymph was 43.5 ± 1.9 mM (n = 5), which compares with the figure of 50 mentioned by Blaustein (1974). The magnesium content of extruded axoplasm averaged 6.7 ± 0.5 mmol/kg (n = 17). A similar figure of 6.4 ± 0.8 was found by Baker and Crawford (1972) in *Loligo forbesi* axons. Clearly, these data indicate that either a very substantial fraction of hemolymph magnesium must be bound, or active extrusion must take place, or both.

Intracellular Free Magnesium Levels

It is known that some 98% of the sodium efflux from squid giant axon represents active transport (Mullins, 1972). The sodium pump is known to require magnesium ions. On the other hand, excess Mg^{2+} inhibits ^{22}Na efflux (De Weer, 1970). This property was exploited in order to determine free magnesium. The principle of the method is as follows: injection of excess Mg^{2+} into an axon inhibits the sodium pump (see Fig. 3 A), and so does the injection of citrate ions, since the latter will reduce the free Mg^{2+} level through complexation (see Fig. 3 B). If now various mixtures of Mg and citrate are injected, some may be at equilibrium with a free Mg^{2+} concentration exactly equal to that prevailing inside the axon, and their effect on the sodium pump will be nil (see Fig. 3 C). The validity of the underlying assumptions is supported by the example of Fig. 3 C: had either citrate or $MgSO_4$ been injected, sodium efflux would have been depressed, yet in combination these two inhibitors have no effect; the plausible interpretation is that the two inhibitors neutralized one another. Operationally, then, this technique employs a null method. It should be realized, however, that the *optimum* Mg^{2+} level for the operation of the sodium pump may not coincide with the *actual* Mg^{2+} level inside the axon.

Thirty-one injections, similar to those depicted in Fig. 3, were made into axons whose ^{22}Na efflux was being monitored, and their effect upon ^{22}Na efflux was noted. Fig. 4 is a plot of the relative magnitude of ^{22}Na efflux after the injection as compared to the control efflux, against [Mg^{2+}] calculated to be present *in the injected mixture*. This free magnesium concentration was computed using the Mg-citrate complex dissociation constant (3.7 mM) derived from the data shown in Fig. 2 B. The arbitrary polynomial which fits the data merely represents [Mg^{2+}] in the injection mixture *before injection*, and not the intracellular free magnesium content after the injected fluid has mixed with the axoplasm. Only if [Mg^{2+}] of

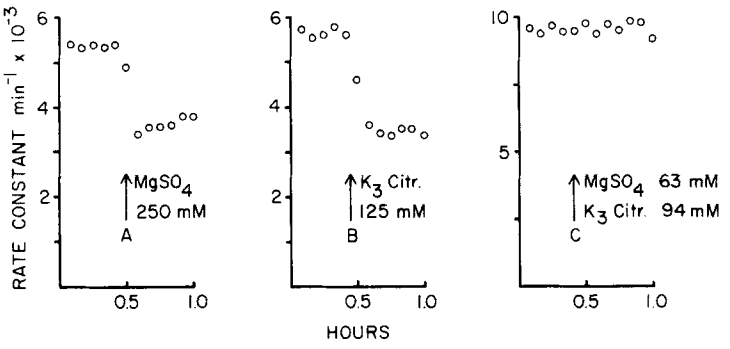

FIGURE 3. Effect of injecting $MgSO_4$, K_3citrate, or a mixture of both, on ^{22}Na efflux from squid giant axon. The injection apparatus delivers 0.18 μl of fluid per centimeter of axon. (A) Injection of $MgSO_4$ to final axoplasmic concentration of 23.9 mM causes 36% inhibition (axon diameter, 490 μm). (B) Injection of K_3citrate to final concentration of 11.5 mM causes 39% inhibition (axon diameter, 500 μm). (C) Injection of $MgSO_4$ to a final concentration of 7.5 mM *plus* K_3citrate to a final concentration of 11.1 mM has no noticeable effect (axon diameter, 440 μm).

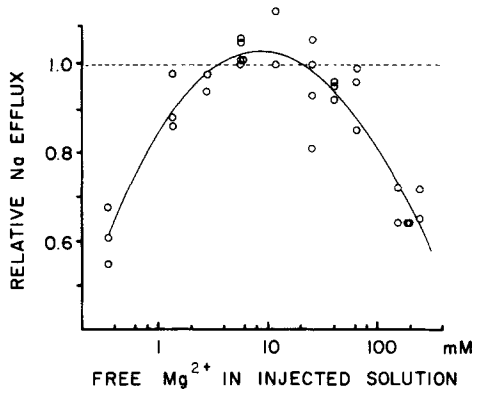

FIGURE 4. Effect of injections of various Mg/citrate mixtures upon ^{22}Na efflux from squid giant axon. Ordinate is ^{22}Na efflux after injection, relative to that before injection. In each instance, the abscissa gives the calculated free magnesium concentration in the injected mixture, using a Mg-citrate dissociation constant of 3.7 mM. In order to include the three points representing pure 125 mM potassium citrate injections on the same logarithmic scale with the other 28 points, it was arbitrarily assumed that a small quantity (10 mM) of $MgSO_4$ was also present. The smooth curve, described by the arbitrary polynomial: Relative Na efflux = 0.27 + 0.69 pMg − 0.13 $(pMg)^2$ − 0.011 $(pMg)^3$, where pMg = $-\log_{10}[Mg^{2+}]$, was found by least-squares fitting. This curve is not to be construed as a description of dependence of pump activity on *prevailing* free magnesium concentrations; see text for further discussion.

both axoplasm and injected fluid are identical will axoplasmic $[Mg^{2+}]$ remain unchanged; otherwise it will acquire a value intermediate between the resting one and that of the injected mixture, due to exchange of magnesium between intrinsic axoplasmic buffering system(s) and the extrinsic (citrate) buffer. The

curve of Fig. 4 suggests that optimal sodium pump activity, as affected by $[Mg^{2+}]$, is somewhat higher than in resting axons and that, consequently, two null points can be identified. In other words, two different Mg/citrate mixtures, when injected into axons, result in no change in sodium pump activity: one where $[Mg^{2+}] \simeq 3.6$ mM, and another where $[Mg^{2+}] \simeq 20$ mM. Clearly, the null point at the higher $[Mg^{2+}]$ levels can be discarded as having no physiological meaning since it exceeds *total* axoplasmic Mg content (7 mM). The lower null point must therefore represent physiological $[Mg^{2+}]$ present in resting axons. That we are dealing with a genuine null point can be verified by considering in its immediate vicinity the experimental data. The five points at 1.4 and 2.8 mM all show inhibition; of the five injections at 5.7 mM, none inhibited ^{22}Na efflux. The difference between these two sets of experiments is statistically significant ($P < 0.01$), so that the conclusion seems warranted that, for some $[Mg^{2+}]$ value between 2.8 and 5.7 mM, no effect on ^{22}Na efflux will be observed. Some measure of the reliability of this null point can be obtained from the following considerations. The least-squares curve drawn in Fig. 4 was generated using a Mg-citrate dissociation constant of 3.7 mM, and its null intercept was 3.6 mM. Standard error on the dissociation constant itself was 0.6 mM, so that two additional least-squares polynomials can be generated in Fig. 4, using 3.7 ± 0.6 mM for K_d of Mg-citrate. The (lower) null intercepts of these additional polynomials were 3.2 and 4.0 mM, respectively. The most likely value for resting axoplasmic $[Mg^{2+}]$, as measured by the method developed here, thus seems to be in the range of 3–4 mM.

^{28}Mg Efflux

The rate coefficient for ^{28}Mg efflux from 24 axons (average diameter 434 ± 12 μm) into 50 Mg(Na)ASW was $2.31 \pm 0.28 \times 10^{-3}$ min^{-1} at room temperature (22–25°C). Normalizing to a uniform axon diameter of 500 μm, one finds $1.96 \pm 0.22 \times 10^{-3}$ min^{-1}. Assuming complete isotopic equilibration inside the axon, this rate coefficient is equivalent to a Mg efflux of about 3 pmol·cm^{-2}·s^{-1}. However, half of these axons were tested in 1974, and the other half in 1975. When considered separately, the average (normalized) rate coefficients differed by a factor of about two, being $2.73 \pm 0.20 \times 10^{-3}$ min^{-1} for the 1974 axons, as compared to $1.19 \pm 0.22 \times 10^{-3}$ min^{-1} for the 1975 axons. Although the temperature coefficient for ^{28}Mg efflux is remarkably high, the average room temperatures were not sufficiently different to account for this discrepancy. Other characteristics of ^{28}Mg efflux (dependence on external Na^+, inhibition by lanthanum, etc.) were not different in the two groups. I have no suggestion for the possible origin of the difference in rate coefficient other than that the 1974 axons were tested in June, and the 1975 axons in July, and that the former group may thus have contained a larger proportion of older animals (Summers, 1971).

The temperature dependence of ^{28}Mg efflux was studied in two 1975 axons by subjecting them to a series of temperature steps (four different temperatures each) in the range 7–27°C. Both axons were tested at the common temperature of 13°C, and the results are normalized accordingly in Fig. 5. The temperature dependence is quite steep indeed: between 8 and 18°C, the value of Q_{10} amounts to about 4–5, comparable to the value of 3–4 found by Ashley and Ellory (1972) in

barnacle muscle fibers. In a *Loligo forbesi* axon that had just recovered from cyanide poisoning, Baker and Crawford (1972) found a fourfold decrease in ^{28}Mg efflux upon cooling from 20 to 0°C.

Effect of Apyrase and Electric Stimulation

Injection of the nonspecific ATP hydrolyzing enzyme apyrase is a rapid and convenient method for reducing intracellular [ATP] to low levels (De Weer, 1970). Fig. 6 shows the dramatic effect that an apyrase injection has on ^{28}Mg efflux. In three such injections (final axoplasmic concentration ~5 U/ml), average inhibition was 95 ± 2%. Using cyanide and 2,4-dinitrophenol as inhibitors of oxidative phosphorylation, Baker and Crawford (1972) found 85% inhibition of ^{28}Mg efflux from *Loligo forbesi* axons. These findings are compatible with the idea that active magnesium extrusion, like sodium-potassium pumping, is energized by the hydrolysis of ATP. Definitive proof, however, can only come from experiments utilizing internal perfusion or dialysis. Also shown in Fig. 6 is the extra efflux of ^{28}Mg which can be elicited by electrical stimulation. This quantity is very small indeed, amounting, in three axons injected with apyrase, to a little less than 10^{-14} mol·cm^{-2}·(impulse)$^{-1}$; it is likely to be even smaller in untreated axons, where more magnesium is probably bound to nucleotides.

Effect of Removing External Sodium Ions, and of Changing the Intracellular ATP/ADP Ratio

Substitution of choline for sodium ions in the seawater bathing the cell causes a sizable and reversible reduction of ^{28}Mg efflux from barnacle muscle fiber (Ashley and Ellory, 1972) and squid axon (Baker and Crawford, 1972). In 17 *Loligo pealei* axons tested in the present investigation, magnesium efflux was reduced to 27 ± 2% of control when external sodium was removed. This phenomenon is reminiscent of the reduction of Na efflux observed when K^+ ions are removed from the medium. The dependence of Na efflux on external K^+ is more variable, however, and can be modified by altering the intracellular ATP/ADP ratio. One of several ways in which the ATP/ADP ratio can be altered (De Weer, 1970), is by microinjection of L-arginine, which will consume ATP and produce ADP via the arginine phosphokinase reaction: ATP + L-Arg ADP + ArgP. This technique was used here in order to ascertain whether the dependence of ^{28}Mg efflux on external Na, like that of ^{22}Na efflux on external K, was sensitive to alterations of the intracellular ATP/ADP ratio. In two such experiments, one of which is shown in Fig. 7, L-arginine injections had little or no effect on ^{28}Mg efflux or on its requirement for external Na^+. Since some axoplasmic ATP must be consumed in the phosphorylation of arginine, the absence of any effect on ^{28}Mg efflux in Fig. 7 must also mean that normal axoplasmic [ATP] is far in excess of the concentration required to saturate the Mg extrusion mechanism. Another possibility is that ADP can substitute for ATP in stimulating Mg extrusion.

Reversible Inhibition by La and D600

^{45}Ca efflux from squid giant axon is inhibited when lanthanum ions are present in the seawater (van Breemen and De Weer, 1970), and so is ^{28}Mg efflux from

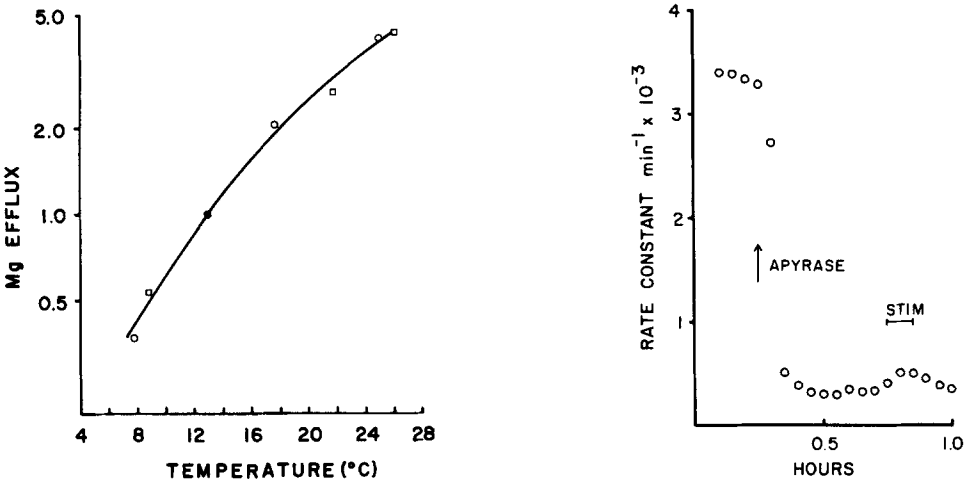

FIGURE 5. Temperature dependence of ^{28}Mg efflux from squid giant axon. Efflux is plotted relative to that at 13°C (reference point). Open squares and circles represent two different axons.

FIGURE 6. Effect of apyrase injection and electrical stimulation upon ^{28}Mg efflux from squid giant axon. At the time indicated by the arrow, apyrase was injected to produce a final axoplasmic concentration of 4–5 U/ml. During the time indicated by the horizontal bar, the axon conducted 24,000 action potentials.

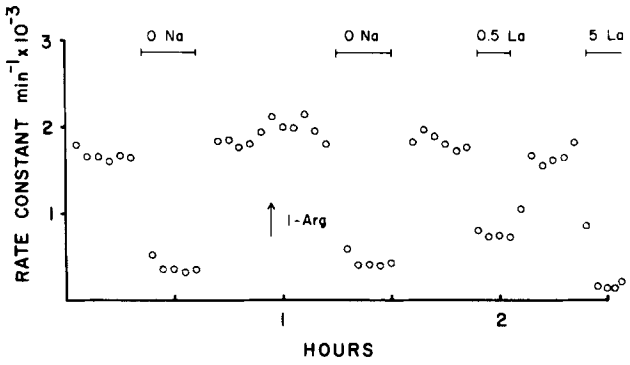

FIGURE 7. ^{28}Mg efflux dependence on external Na^+, and inhibition by lanthanum. The axon was exposed to (Na)ASW, except during the periods indicated, when it was exposed to (Chol)ASW (choline artificial seawater) or to (Na)ASW containing lanthanum chloride. At the time indicated by the arrow, enough L-arginine hydrochloride was injected to produce a final axoplasmic concentration of 12 mM.

barnacle muscle fibers (Ashley and Ellory, 1972). Fig. 7 shows the readily reversible inhibitory effect of lanthanum ions on ^{28}Mg efflux from giant axons. A nominal concentration of 5 mM lanthanum chloride in the seawater inhibited ^{28}Mg efflux by 94 ± 1% (n = 4), and half-maximal inhibition occurred at about 0.35 mM (Fig. 8). Another substance known to interfere with calcium fluxes is

the compound D600, a methoxy derivative of iproveratril (Kohlhardt et al., 1972). Fig. 9 illustrates that this compound, at 1 mM, also reversibly inhibits ^{28}Mg efflux from squid axon. From the *rate* of inhibition at 0.2 mM D600 (two experiments, not shown), a forward rate coefficient of approximately 150 $M^{-1}min^{-1}$ was calculated for the binding of D600. If one assumes that the recovery rate coefficient (0.04 min^{-1}, see Fig. 9) is determined solely by the dissociation of the drug from its receptor, the K_m for the binding of D600 is computed to be around 0.25 mM.

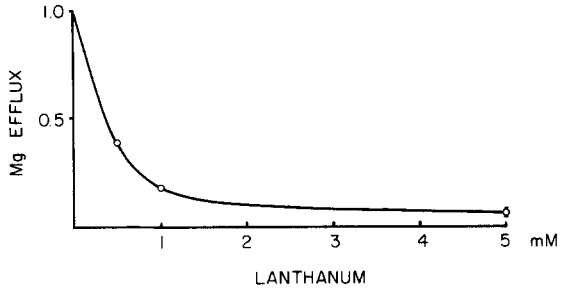

FIGURE 8. Inhibition of ^{28}Mg efflux by lanthanum. Plotted is the efflux, relative to control, against the nominal $LaCl_3$ concentration present in the (Na)ASW bathing the axon.

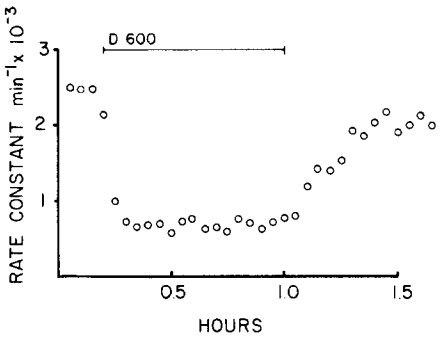

FIGURE 9. Reversible inhibition of ^{28}Mg efflux by D600. During the time period indicated the (Na)ASW contained 1 mM D600. The axon became inexcitable shortly after exposure to the drug, and remained so until the end of the experiment.

Effect of Membrane Potential on ^{28}Mg Efflux

When axons were depolarized to about −30 mV by exposing them to seawater containing 150 mM potassium ions (Fig. 10), only a very small, if any, effect on the rate of ^{28}Mg efflux was observed, both in the presence and absence of sodium ions. This finding is in contrast to the observation (Brinley and Mullins, 1974; Blaustein et al., 1974; Mullins and Brinley, 1975) that ^{45}Ca efflux from squid axon is markedly decreased by membrane depolarization. Here, if anything, a small increase is observed, at least in the absence of extracellular sodium. Another conclusion to be drawn from the experiment shown in Fig. 10

is that, unless external K^+ and membrane depolarization have opposing effects on ^{28}Mg efflux, K^+ is a poor substitute for Na^+ in its ability to promote ^{28}Mg efflux.

Effect of Na^+, Mg^{2+}, and Ca^{2+} on ^{28}Mg Efflux

It has already been mentioned that ^{28}Mg efflux was greatly decreased when Na^+ ions were removed from the seawater (see Figs. 7 and 10). Baker and Crawford (1972) have shown that, in *Loligo forbesi* axons, the kinetics of the fraction of ^{28}Mg efflux stimulated by external Na^+ conform to a rectangular hyperbola. Similar Michaelis-type kinetics were found in the present work on *Loligo pealei*. In addition, it was found that the apparent K_m for ^{28}Mg efflux activation by external Na^+ is affected by the concentration of Mg^{2+} in the seawater.

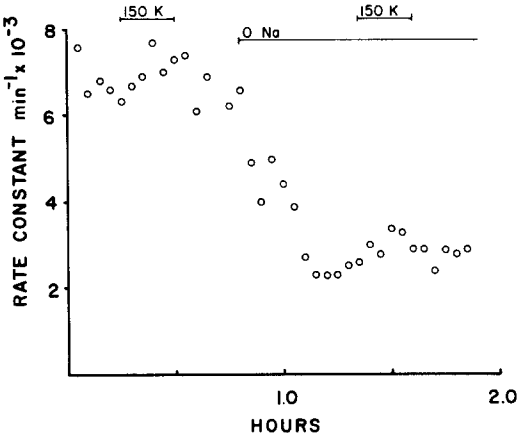

FIGURE 10. Effect of high-potassium seawater on ^{28}Mg efflux from squid axon. During the periods indicated by the horizontal bars, the seawater bathing the axon contained 150 mM K^+. This is known to depolarize axons by about 30 mV.

Table II lists the relative magnitude of ^{28}Mg efflux from squid axons bathed in seawater solutions containing various concentrations of Na^+ and Mg^{2+} ions. The same data are summarized in graphic form in Fig. 11, where ^{28}Mg efflux (relative to that in 50 Mg(Na)ASW) is plotted against $[Na^+]$ in the seawater, for three different Mg^{2+} concentrations (0, 50, and 100 mM). The Na-stimulated fractions of magnesium efflux are fitted well by rectangular hyperbolae. Two points of interest should be noted: first, all three curves tend toward maxima that are not significantly different; and second, as $[Mg^{2+}]$ in the seawater is reduced, the apparent K_m for activation by Na^+ is lowered.

Raising the Ca^{2+} concentration in the seawater from 10 to 20 mM had little, if any, effect on ^{28}Mg efflux, either in the presence or absence of Na^+. For this reason, data from experiments with 20 mM Ca seawater were included in Table II as well as in Fig. 11, and the curve-fitting for Fig. 11 was done to all points including those obtained in 20 mM Ca ASW. On the other hand, the effect of *removing* Ca^{2+} from the seawater depended on the presence of Mg^{2+} and/or Na^+

TABLE II

EFFLUX OF ^{28}Mg FROM SQUID GIANT AXON INTO SEAWATER SOLUTIONS WITH VARIOUS Na^+ AND Mg^{2+} CONCENTRATIONS*

			Efflux relative to that into 50 Mg(Na)ASW													
Axon	Diameter	Rate coefficient‡	[Mg] = 0 [Na] = 0	0 50	0 100	0 200	0 400	0 0 [Ca] = 20	50 0	50 50	50 100	50 200	100 0	100 50	100 200	100 400
	μm	$min^{-1} \times 10^{-3}$														
71674B	390	2.25	–	–	–	–	1.29	–	0.44	–	–	0.84	–	–	–	0.76
71774A	420	3.90	–	0.89	–	–	–	–	0.30	0.51	–	–	–	–	–	–
71774B	390	4.90	–	–	–	–	–	–	0.18	0.44	0.63	–	–	–	–	–
71774D	420	3.88	–	–	–	–	–	–	0.23	0.48	–	–	0.15	0.40	–	0.92
71874A	430	1.72	–	–	–	–	–	–	0.16	–	–	–	–	–	–	–
71874B	450	2.25	–	(0.47)§	(0.96)‖	–	–	0.15	0.27	(0.39)¶	0.69	–	–	–	–	–
71874C	420	3.75	–	–	–	–	1.17	–	0.24	–	–	–	0.18	–	–	0.91
71874D	370	4.20	–	–	–	–	–	–	0.25	–	–	–	–	–	–	–
71874E	520	2.37	–	–	–	–	1.16	–	–	–	–	–	–	–	–	–
62575A	530	0.88	0.09	–	–	–	1.22	–	0.27	–	–	–	0.20	–	–	0.87
62575B	500	1.13	0.16	–	–	1.0	1.13	–	0.34	–	–	0.79	0.28	–	0.72	0.91
62575C	410	1.67	0.08	–	–	1.11	1.16	–	0.22	–	–	0.73	0.18	–	0.65	0.85
62575D	390	1.15	0.08	–	–	–	1.14	–	0.30	–	–	–	–	–	–	–
62675A	400	0.60	0.22	–	–	–	–	0.22	0.42	–	–	–	0.36	–	–	–
62675B	390	0.33	0.06	–	–	–	1.07	0.06	0.27	–	–	–	–	–	–	–
62675C	400	0.68	0.14	–	–	–	1.17	0.29	0.25	–	–	–	–	–	–	–
62675D	420	1.29	–	–	–	–	1.11	–	0.23	–	–	–	–	–	–	–
62675E	430	1.42	–	–	–	–	–	0.05	0.28	–	–	–	–	–	–	–
Mean	427	2.13	0.12			1.06	1.16	0.15	0.27	0.48	0.66	0.79	0.23		0.69	0.87
SEM	11	0.33	0.02			0.06	0.02	0.05	0.02	0.02	0.03	0.03	0.03		0.04	0.02
n	18	18	7			2	10	5	17	3	2	3	6		2	6

* [Ca] 10 mM except where indicated.

‡ Into 50 Mg, 400 NaASW.

§ 0 Mg, 20 Ca, 25 NaASW.

‖ 0 Mg, 20 Ca, 100 NaASW.

¶ 50 Mg, 25 NaASW.

ions. In 50 Mg ASW (with or without 400 mM Na), or in Mg-free 400 Na ASW, removal of Ca^{2+} had little effect on ^{28}Mg efflux (not shown). However, in seawater that was both Mg *and* Na free, removal of Ca^{2+} resulted in a 10–20-fold stimulation of ^{28}Mg efflux (see Fig. 12). Furthermore, this efflux was extremely sensitive to lanthanum ions, being 99% inhibited by as little as 1 mM La^{3+}.

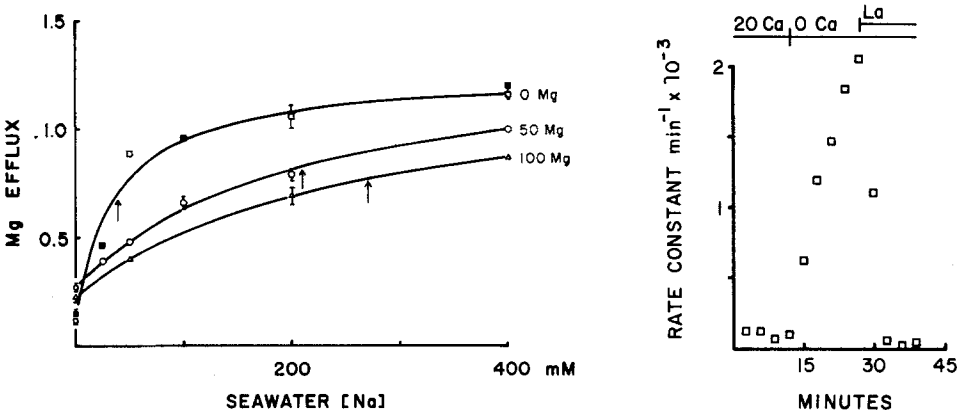

FIGURE 11. Stimulation of ^{28}Mg efflux from squid giant axons by Na^+ present in the seawater, at three different levels of Mg^{2+}: 0 mM (squares), 50 mM (circles), and 100 mM (triangles). Efflux is expressed relative to that observed into seawater containing 400 mM Na^+, 10 mM Ca^{2+}, and 50 mM Mg^{2+}. NaCl and $MgCl_2$ were replaced with choline chloride on an isosmolar basis. Most of the data were obtained in seawater containing 10 mM Ca^{2+} (open symbols). A few experiments (filled squares) were done in seawater containing 20 mM Ca^{2+}. The curves were least-squares fitted to the three families of points by computer, and are of the form $\phi_{tot} = \phi_o + (\phi[Na^+]/(K_m + [Na^+]))$, where total efflux ($\phi_{tot}$) is the sum of a sodium-independent fraction (ϕ_o) and a saturable, sodium-stimulated fraction of maximal magnitude ϕ. The concentration at which Na^+ activates half maximally is given by K_m. For the three Mg^{2+} concentrations tested: 0, 50, and 100 mM, K_m for activation by Na^+ was 39, 210, and 270 mM, respectively. These values are indicated by arrows next to the curves. All three curves tend toward similar maxima: 1.27 ± 0.02, 1.39 ± 0.04, and 1.30 ± 0.21 for 0, 50, and 100 mM Mg^{2+}, respectively.

FIGURE 12. Effect of Ca^{2+} and La^{3+} on ^{28}Mg efflux from squid axon into Na-free, Mg-free ASW. Choline chloride replaced NaCl, $MgCl_2$, and $CaCl_2$.

DISCUSSION

Although divalent ion activities can be measured by means of liquid ion exchange electrodes (Rechnitz, 1975), this technique has not yet been adapted to intracellular measurements. One problem is the need to fabricate microelectrodes containing the ion exchanger, and another is the sizable interference from monovalent cations, which would be especially serious in the case of marine organisms. Apart from ion-exchange methods, there are several possible ways in which intracellular Mg ion activities can be measured or calculated. All have limitations, and this dearth of unambiguous methodology explains the repeated

attempts at measuring intracellular free Mg^{2+} levels. I will enumerate six different methods that have been described in the literature, including the one used here. Four of these have been, or are being, applied to the problem of free Mg^{2+} in squid giant axon.

Probably the first attempt at calculating intracellular free Mg^{2+} levels was made by Nanninga (1961). The principle of his method was to list all possible Mg-complexing agents present in the cell, with best estimates for respective concentrations as well as Mg-complex dissociation constants, and to solve the set of simultaneous equations describing the multiple equilibria. This method, refined to include insoluble and soluble high-molecular weight ligands, has been applied most recently by Veloso et al. (1973) to several tissues of rat. It has not been used in the case of squid axon. A second method, also not yet employed in squid axon, has been used by C. C. Ashley in barnacle giant muscle fibers (quoted in Ashley and Ellory, 1972). The method exploits the fact that Mg ions have a quenching effect on the light emitted by the Ca-sensitive photoprotein, aequorin (Hastings et al., 1969). A third method (Baker and Ellory, 1972), the first to be applied to squid (*Loligo forbesi*) axon, makes inferences about the free magnesium levels from a knowledge of total magnesium concentration, and measurement of the diffusion coefficient and electrophoretic mobility of radioactive tracer. Since the magnitude of these two parameters was about half of that expected, the authors concluded that about half of the total magnesium content was free, i.e., about 2–3 mM. Unfortunately, there is no simple relation between mobility and degree of complexation, especially if the complexes themselves are relatively mobile, or if they carry different or even opposite charges. A fourth method, proposed by Scarpa (1974), makes use of the Mg-sensitive indicator dye Eriochrome Blue SE and dual-wavelength spectroscopy. In their application of this method to squid giant axon, Brinley and Scarpa (1975) dialyzed solutions containing different Mg^{2+} levels against axoplasm preloaded with the dye, and determined the Mg^{2+} concentration which produced no change in differential absorption at selected wavelengths. The value they found was 3–4 mM. A possible limitation of the Eriochrome Blue method is that both apparent dissociation constant and $\Delta\epsilon_{550}^{Mg}$ are extremely pH sensitive (see Fig. 2) so that any mismatch between dialysis fluid pH and axoplasm pH may induce errors. On the other hand, this null method does not require an accurate knowledge of the Mg-indicator dissociation constant. For example, K_d for the Mg-Eriochrome Blue SE complex quoted by Brinley and Scarpa (1974) is 25 mM, several times higher than the value found here. A fifth method is based on the well-established principle that the apparent equilibrium constant of many biochemical reactions is influenced by metal ions, due to complex formation between the metal ion and the equilibrating species. For example, the citrate-isocitrate equilibrium catalyzed by aconitase is determined by the free Mg^{2+} level in the medium, due to preferential binding of the metal to citrate. This property was first used by England et al. (1967) in an attempt to determine free Mg^{2+} levels in rat heart. More recent work, on the same preparation, was done by Veloso et al. (1973). The Mg dependence of the citrate-isocitrate equilibrium in media of high ionic strength has been determined in this laboratory (De Weer, unpublished),

and experiments are under way to apply this method to the squid giant axon. Possible drawbacks are: the problem of whether the system under study is indeed at equilibrium in the cell (the remedy is to inject a large excess of aconitase enzyme), and the difficulty of assaying rather low levels of isocitrate and citrate. Finally a sixth method is the one proposed in the present paper, where the *activity* of the sodium pump is taken as an indicator of prevailing free Mg^{2+} levels. So far, this method has only been applied to squid axon. Its most obvious drawback is the danger of osmotic or mechanical artifacts due to the injection maneuver.

The value found here for free Mg^{2+} in *Loligo pealei* axoplasm, 3–4 mM, is somewhat lower than the figure of 4 mM given in an earlier communication (De Weer, 1974 *a*). The present figure rests upon a more accurate redetermination of the Mg-citrate dissociation constant, using a metal indicator dye. It is comparable to the value of 3.0–3.5 mM obtained by Brinley and Scarpa (1975) for the same species of squid. However, the method developed by these authors involves the use of internal dialysis fluids containing a sizable amount of aspartate, which is likely to bind a fraction of the magnesium ions present. The value of 2–3 mM suggested by Baker and Crawford (1972) for *Loligo forbesi* axons is also comparable to the one found here. In view of the vast difference in principles underlying these three methods, their agreement is remarkable.

With regard to the extrusion of ^{28}Mg from squid giant axon, some of its properties merit comparison with those of Na^+ fluxes. One of the more striking features of ^{28}Mg efflux is its very large temperature coefficient; in the temperature range below 12°C, the value of Q_{10} exceeds 5, indicating an activation energy of nearly 25 kcal/mol. This is far in excess of the values reported for Na^+ efflux from *Sepia* (Hodgkin and Keynes, 1955) or squid giant axons (De Weer, 1970). The effect of apyrase offers a second basis for comparison. Injection of similar amounts of apyrase as employed here reduces active Na^+ extrusion to about 15% of control (De Weer, 1970), whereas residual ^{28}Mg efflux is only about 5% of control. If, as seems likely, inhibition in both cases results from lack of ATP, one may expect that K_m (ATP) in the case of Mg^{2+} extrusion will be about three times higher than K_m (ATP) for Na^+ extrusion. It will be recalled that the well-known dependence of Na^+ efflux on external K^+ ions is reversibly abolished whenever intracellular ADP levels are elevated; it has been suggested that this observation can be interpreted on the basis of a kinetic model in which ADP is released from the transport enzyme before inward K^+ transport occurs (De Weer, 1970, 1974 *b*). The fact that the dependency of ^{28}Mg efflux on external sodium ions seems unaffected by intracellular ADP levels (Fig. 7) suggests that a similar biochemical reaction scheme will probably not be applicable to Mg^{2+} transport. Finally, since a major fraction of Mg^{2+} extrusion requires the presence of extracellular Na^+, it is plausible that a Na/Mg exchange reaction may take place across the cell membrane. If this is so, then one should find a saturable component of Na influx, dependent on internal ATP and internal Mg^{2+}, and inhibited by external La^{3+} or D600. In addition, K_m for external Na would be a function of the external Mg concentration (see Fig. 11). An ATP-dependent, strophanthidin-insensitive Na influx has been described in dialyzed

squid axons (Mullins and Brinley, 1969; Mullins, 1972), but its magnitude (25 pmol·cm^{-2}·s^{-1}) is larger than the flux predicted here (about 3 pmol·cm^{-2}·s^{-1} on a 1:1 exchange basis).

The energetics of active Mg^{2+} extrusion deserve some comment. With axoplasmic $[Mg^{2+}] \simeq 4$ mM and hemolymph $[Mg^{2+}] \simeq 40$ mM, $[Na]_i/[Na]_o = 0.1$, and $V_m \simeq -60$ mV, the question arises whether active Mg^{2+} extrusion may be driven by the downhill movement of Na^+ ions down their electrochemical gradient, with ATP playing only a "catalytic" role. Such mechanisms have been proposed, for example, in the case of Ca^{2+} transport (for a review see Blaustein, 1974). If Na:Mg counter-transport occurs with 1:1 coupling, then the equilibrium concentration ratio for Mg will be

$$\frac{[Mg]_i}{[Mg]_o} \geq \frac{[Na]_i}{[Na]_o} \cdot \exp(VF/RT) \simeq 0.3.$$

Hence, one-to-one coupling would not seem sufficient to achieve the Mg activity ratio observed. If, however, two sodium ions enter for every Mg^{2+} exported, the relationship becomes

$$\frac{[Mg]_i}{[Mg]_o} \geq \frac{[Na]_i^2}{[Na]_o^2} \simeq 0.01.$$

A two-to-one countertransport mechanism could therefore account for the Mg concentration ratio observed, and, being electroneutral, would be insensitive to changes in membrane potential. On the other hand, the kinetics of ^{28}Mg efflux activation by $[Na]_o$, described by a simple rectangular hyperbola (Fig. 11), do not plead for 2:1 coupling. Although admittedly no ATP consumption, linked to Mg extrusion, has been demonstrated, the pronounced inhibitory effect of apyrase on ^{28}Mg efflux suggests at this time that ATP breakdown provides the energy source for active Mg extrusion.

The interaction between mono- and divalent cations and their effect on ^{28}Mg efflux are quite complex. As a first approximation, the data of Table II and Fig. 11 can be summarized by stating that a large fraction of ^{28}Mg efflux is activated in Michaelis-Menten fashion by Na^+, and that this activation is competitively inhibited by Mg^{2+}: at very high $[Na^+]$ levels, ^{28}Mg efflux tends toward the same value regardless of the magnesium concentration in the seawater. If the action of Na^+ ions seems kinetically fairly simple, that of extracellular Mg^{2+} is not. In the absence of sodium ions, increasing concentrations of Mg^{2+} first stimulate (50 mM), then inhibit (100 mM) ^{28}Mg efflux (see Table II and the ordinate of Fig. 11). The most economical model at this point seems to be one with two external sites to which magnesium ions can bind: a stimulatory site with higher affinity, and an inhibitory one with lower affinity. In this scheme, sodium ions would be Mg^{2+} analogs on the first site, and competitors on the second, while La^{3+} would act at the inhibitory site. The role of Ca^{2+} in Mg^{2+} transport is not clear at this time. Further experimentation is required to characterize these complex interactions.

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