

# A Mechanism for Na/Ca Transport

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**ABSTRACT** A model is developed which requires the binding of 4 Na<sup>+</sup> to a carrier before a Ca binding site is induced on the opposite side of the membrane. Upon binding Ca, this carrier translocates Na and Ca. The existence of partially Na-loaded but nonmobile forms for the carrier (NaX, Na<sub>2</sub>X, Na<sub>3</sub>X) suffices to explain both the activating and the inhibitory effects of Na on the Ca transport reaction. Analytical expressions for Ca efflux and influx in terms of [Na]<sub>o</sub>, [Na]<sub>i</sub>, [Ca]<sub>o</sub>, [Ca]<sub>i</sub>, and  $E_m$  are developed for the Na/Ca exchange system at equilibrium; these provide for a quantitative description of Ca fluxes. Under nonequilibrium conditions, appropriate modifications of the flux equations can be developed. These show a dependence of Ca efflux on [Ca]<sub>o</sub> and of Ca influx on [Ca]<sub>i</sub>. The large effect of internal ATP on Ca efflux and influx in squid axons, with no change in net Ca flux, can be understood on the single assumption that ATP changes the affinity of the carrier for Na at both faces of the membrane without providing an energy input to the transport reaction.

## INTRODUCTION

The experimental information presented in two recent papers (Requena et al., 1977; Brinley et al., 1977) makes it possible to propose some rather definite mechanisms by which Ca entry and exit take place in the squid giant axon. In developing an understanding of such mechanisms, one must note the impressive amount of experimental information showing that if the Na gradient across the cell membrane is large and inwardly directed, total axonal Ca is small and ionized Ca low, while if the gradient is reversed, or outwardly directed, total axonal Ca is high and Ca<sub>i</sub><sup>++</sup> high. By contrast, if the concentration of ATP is very small, Ca<sub>i</sub> is unaffected and an axon can recover from an imposed Ca load in the virtual absence of ATP, requiring only an inward Na<sup>+</sup> gradient. The idea that the source of energy for activated Ca<sup>++</sup> extrusion is the inward gradient of Na<sup>+</sup> was originated by Reuter and Seitz (1968).

The observations do not prove that a Na:Ca exchange mechanism is the sole basis for the regulation of axonal Ca, nor do they require that a single Na:Ca exchange mechanism be the only explanation of such experimental findings. An analysis of experimental data, however, shows that it is possible to fit all experimental observations into a single carrier mechanism that exchanges Na for Ca across the membrane.

In the total energy economy of an axon, Ca fluxes contribute relatively insignificantly since about 40 pmol/cm<sup>2</sup>s of Na enter vs. about 40 fmol/cm<sup>2</sup>s for Ca, or 1 one-thousandth as much. If there were no channels or other mecha-

nisms in the membrane leading to a Ca leak, then the Na/Ca exchange mechanism would use the Na gradient to extrude Ca and the Ca gradient to extrude Na in equal amounts. With a finite Ca leak, some Ca enters via this pathway and hence more Na must enter to extrude this Ca than that leaving the fiber in exchange for Ca. This extra entering Na will constitute a small extra load on the Na pump, which is an ATP-dependent mechanism; hence the extent of a Ca leak determines the indirect dependence of Ca homeostasis on ATP.

#### *The Nature of the Carrier*

Any model for translocating  $\text{Ca}^{++}$  must provide for its active transport. Therefore, the mechanism must include coupling to a source of energy. This model proposes to utilize the electrochemical gradient of  $\text{Na}^+$  for the source of free energy. The sole membrane constituent in the model is the  $\text{Na}^+/\text{Ca}^{++}$  carrier. Therefore, the carrier must provide a coupling of the electrochemical  $\text{Na}^+$  gradient to the movement of  $\text{Ca}^{++}$ . This requires the carrier to bind and move simultaneously 4  $\text{Na}^+$  inward and 1  $\text{Ca}^{++}$  outward, under the usual electrochemical gradients of these ions in axons.

While the original suggestion of Reuter and Seitz (1968) was that 2  $\text{Na}^+$  were moved inward per  $\text{Ca}^{++}$  extruded, a more detailed examination of conditions in squid axons (Mullins, 1976) showed that (a) the  $[\text{Ca}]$  gradient across the membrane is  $10^5$ , and (b) Ca fluxes are sensitive to membrane potential. More recently (Requena et al., 1977), it has been found that 180 mM  $\text{Na}_o$  is as effective as 450 mM  $\text{Na}_o$  in maintaining a physiological  $[\text{Ca}]_i$ . These findings suggest that Na/Ca exchange is not electroneutral and that the Na/Ca exchange mechanism cannot exploit a Na gradient when  $\text{Na}_o$  is in excess of  $\sim 180$  mM.

What is proposed to account for the foregoing is a system where the binding of 4 Na to a carrier induces a Ca binding site on the opposite side of the membrane. Such an induced Ca site could have an extremely high affinity for Ca, one that would disappear upon the dissociation of Na from the carrier. The arrangement proposed is shown in Fig. 1. This allows  $\text{Na}^+$  to be in thermodynamic equilibrium with the Na binding sites of the carrier while Ca binding is a function of whether or not the Na sites are fully occupied. Translocation requires that both the Na and Ca binding sites be fully occupied.

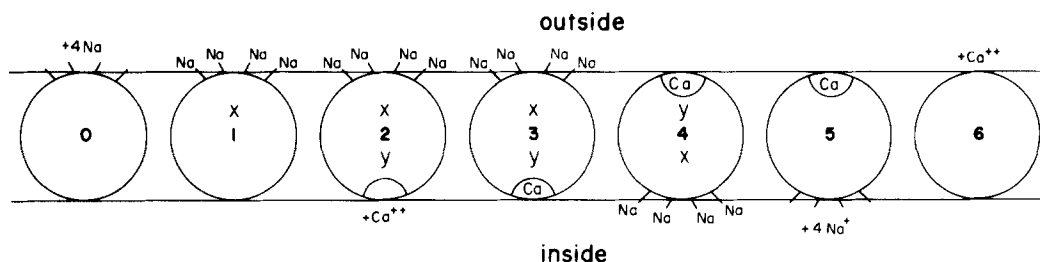
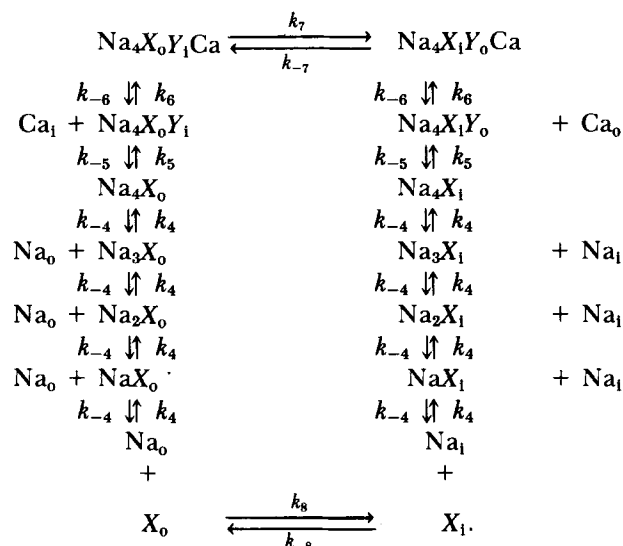


FIGURE 1. This shows the sequence of Na binding to the carrier, the induction of a Ca binding site (2), the translocation (4), and the dissociation of Ca (6). As drawn, the scheme represents Ca efflux; by interchanging the labels "outside" and "inside," it represents Ca influx.

*Reactions of the Carrier*

The equations involved are shown below:



If one reads this diagram from the lower left upward, it describes a sequence of reactions leading to the efflux of Ca; reading from the lower right upward describes reactions leading to the influx of Ca. In this scheme X is the Na binding site and Y is the induced Ca binding site, produced in step  $k_5$ . The translocation step is  $k_7$ , and  $k_8$  allows totally unloaded carriers to offer Na binding sites on either side of the membrane. The assumption of four Na binding steps is dictated both by energy considerations and by the sensitivity of Ca fluxes to membrane potential. A free movement of X across the membrane is necessary to account for net fluxes of Ca via the carrier system.

*Na:Ca Ion Exchange*

There are four Na binding reactions leading to the formation of the carrier complex  $\text{Na}_4\text{X}_0$ . For any assumed equilibrium constant for Na binding to the carrier, there will be a population of carriers with less than 4 Na bound to them and the fraction of the total carrier population in the  $\text{Na}_4\text{X}_0$  or  $\text{Na}_4\text{X}_1$  state will depend on the [Na] on each side of the membrane. With physiological values of  $\text{Na}_0$  and  $\text{Na}_i$  there will be a large difference between the number of carriers on each side of the membrane capable of binding Ca and the number that have less than 4 Na bound to them (making them stationary). The evidence from aequorin experiments is that an intact squid axon with normal ATP,  $\text{Ca}_i$  is normal when  $[\text{Na}]_0$  is 180 mM. Such an axon can be expected to have an  $[\text{Na}]_i$  of about 40 mM. Other experiments (Brinley et al., 1975; Blaustein and Russell, 1975) with dialyzed squid axons suggest that in the absence of ATP, a  $[\text{Na}]_i$  of 30 mM reduces Ca efflux to about half that found when  $[\text{Na}]_i = 0$ . This could mean that at an  $[\text{Na}]_i$  of 30 mM, the number of carriers in some nonmobile form of combination with Na ( $\text{NaX} \dots \text{Na}_3\text{X}$ ) is such that the carrier population

available for Ca efflux is half of that available when  $[\text{Na}]_i = 0$ . Note that a carrier with less than 4 Na is a poisoned carrier in the sense that it cannot carry Ca; it is also worth noting that  $\text{Na}_4\text{X}_0$  and  $\text{Na}_4\text{X}_1$  are also nonmobile forms of the carrier in the absence of  $\text{Ca}_i$  and  $\text{Ca}_o$ , respectively.

#### Carrier Loading

To understand this somewhat complex kinetic scheme, it is convenient to start by examining the behavior of the carrier in the absence of Ca in the system. A further simplification is to confine Na to only one membrane face; under these circumstances if  $K$  is the equilibrium constant for the formation of  $\text{NaX}$ , and  $(K/[\text{Na}]_o)$  is denoted by  $j$ , while  $X_T$  is the total carrier, then the various forms of the carrier are given by:<sup>1</sup>

$$[\text{Na}_4\text{X}] = 1/(1 + j + j^2 + j^3 + j^4), \quad (1)$$

$$[\text{NaX}] = 1/(1 + j + j^{-1} + j^{-2} + j^{-3}), \quad (2)$$

$$[\text{Na}_2\text{X}] = 1/(1 + j + j^2 + j^{-1} + j^{-2}), \quad (3)$$

$$[\text{Na}_3\text{X}] = 1/(1 + j + j^2 + j^3 + j^{-1}), \quad (4)$$

$$[\text{X}] = 1/(1 + j^{-1} + j^{-2} + j^{-3} + j^{-4}), \quad (4a)$$

$$[\text{X}]_T = [\text{X}] + [\text{NaX}] + [\text{Na}_2\text{X}] + [\text{Na}_3\text{X}] + [\text{Na}_4\text{X}]. \quad (5)$$

Hence the total concentration of carrier can be divided into a free carrier,  $[\text{X}]$ , nonmobile forms,  $[\text{NaX}] \dots [\text{Na}_3\text{X}]$ , and a Ca binding form,  $[\text{Na}_4\text{X}]$  that can translocate when it becomes  $\text{Na}_4\text{XY}$  and binds Ca. A plot of how these fractions of the carrier population vary with  $[\text{Na}]$  is shown in Fig. 2. A  $K$  of 140 mM has been selected somewhat arbitrarily for the reaction of the carrier with Na. This value has the property of making the *apparent*  $K_{1/2}$  for the rise of  $[\text{Na}_4\text{X}]$  lie at a value of  $\text{Na} = 200$  mM as compared with the value of  $[\text{Na}_4\text{X}]$  at  $\text{Na} = 450$  mM, and this is between the experimentally observed values of 160 mM (Blaustein et al., 1974) and 300 mM (Baker and McNaughton, 1976) for the activation of Ca efflux by  $[\text{Na}]_o$ . If Na at a concentration of 40 mM is introduced on the inside of the membrane, while the  $[\text{Na}]_o$  is fixed at 450 mM, a competition for carrier will be set up at both faces of the membrane. It is important to note from Fig. 2 that with an  $[\text{Na}]_i$  of 40 mM, virtually none of the complexed carrier at the inner surface is in a mobile, Ca-carrying form, but a substantial fraction is in a complexed but nonmobile form; these nonmobile forms at the inside of the membrane can only be obtained from the total carrier population by reducing  $[\text{Na}_4\text{X}_0]$ ; hence  $[\text{Na}]_i$  is inhibitory to Ca efflux.

#### Translocation

Assuming that carrier loading has been effected on both sides of the membrane, one has  $\text{Na}_4\text{X}_0\text{Y}_1\text{Ca}$  and  $\text{CaY}_0\text{X}_1\text{Na}_4$  as the translocating forms. If there were no membrane potential, these loaded carriers should move with equal velocities; with a membrane potential, the inward movement of two net charges (Na moving inward) is favored by the membrane field.

<sup>1</sup> For simplicity,  $[\text{Na}_4\text{X}]$  is assumed equal to  $[\text{Na}_4\text{XY}]$ , the actual Ca binding form.

The carrier, when fully loaded, has four positive charges on one side of the membrane and two positive charges on the other, or a total of six positive charges. This is not greatly different from the Na/K pump which is generally considered to bind 3 Na<sup>+</sup> on one side of the membrane and 2 K<sup>+</sup> on the other for a total of five positive charges per translocation.

*Kinetics*

The kinetic scheme for Na/Ca transport must also conform with the assumption that the sole source of energy for Ca transport is the Na electrochemical gradient; hence the behavior of the Na/Ca transport system must be such that at equilibrium

$$rzF(E_{Na} - E) - zF(E_{Ca} - E) = 0, \tag{6}$$

where  $r$  = coupling ratio Na/Ca, and  $E$  is membrane potential. If one assumes that  $r = 4$ , this expression can be readily transformed to

$$\frac{[Ca]_o}{[Ca]_i} = \frac{[Na]_o^4}{[Na]_i^4} \exp -2EF/RT. \tag{7}$$

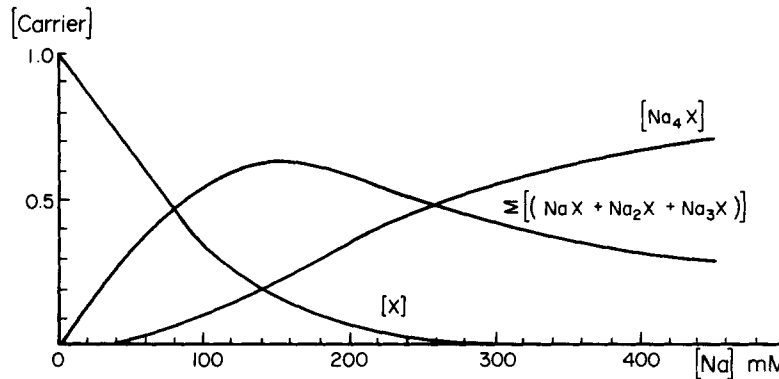


FIGURE 2. This shows the concentration of carrier in the free form [X], in the form [Na<sub>4</sub>X] which can carry Ca when it becomes Na<sub>4</sub>XY, and in nonmobile forms, as a function of [Na] on a single face of the membrane.

This equation implies that for flux balance

$$m_o^{Ca} = k_o[Ca]_i[Na]_o^4 = m_i^{Ca} = k_i[Ca]_o[Na]_i^4, \tag{8}$$

where  $m_o$  and  $m_i$  are efflux and influx, and  $k_o$  and  $k_i$  are constants that include the term in membrane potential.

The kinetic scheme shown earlier (*Reactions of the Carrier*) can be described by the following sets of equations, for the system at equilibrium.

For Na binding

$$\begin{aligned} k_4[Na]_i[X]_i &= k_{-4}[NaX]_i & k_4[Na]_o[X]_o &= k_{-4}[NaX]_o \\ k_4[Na]_i[NaX]_i &= k_{-4}[Na_2X]_i & k_4[Na]_o[NaX]_o &= k_{-4}[Na_2X]_o \\ k_4[Na]_i[Na_2X]_i &= k_{-4}[Na_3X]_i & k_4[Na]_o[Na_2X]_o &= k_{-4}[Na_3X]_o \\ k_4[Na]_i[Na_3X]_i &= k_{-4}[Na_4X]_i & k_4[Na]_o[Na_3X]_o &= k_{-4}[Na_4X]_o \end{aligned}$$

For induction and Ca binding

$$\begin{aligned} k_5[\text{Na}_4\text{X}]_i &= k_{-5}[\text{Na}_4\text{X}_1\text{Y}_0] & k_5[\text{Na}_4\text{X}]_o &= k_{-5}[\text{Na}_4\text{X}_0\text{Y}_1] \\ k_6[\text{Na}_4\text{X}_1\text{Y}_0][\text{Ca}]_o &= k_{-6}[\text{Na}_4\text{X}_1\text{Y}_0\text{Ca}] & k_6[\text{Na}_4\text{X}_0\text{Y}_1][\text{Ca}]_i &= k_{-6}[\text{Na}_4\text{X}_0\text{Y}_1\text{Ca}]. \end{aligned}$$

For translocation

$$k_7[\text{Na}_4\text{X}_0\text{Y}_1\text{Ca}] = k_{-7}[\text{Na}_4\text{X}_1\text{Y}_0\text{Ca}] \quad k_8[\text{X}]_o = k_{-8}[\text{X}]_i.$$

From the above, it follows that

$$[\text{Na}_4\text{X}_0\text{Y}_1\text{Ca}] = \frac{k_6}{k_{-6}} \frac{k_5}{k_{-5}} \left( \frac{k_4}{k_{-4}} \right)^4 [\text{Na}]_o^4 [\text{Ca}]_i [\text{X}]_o, \quad (9)$$

$$[\text{Na}_4\text{X}_1\text{Y}_0\text{Ca}] = \frac{k_6}{k_{-6}} \frac{k_5}{k_{-5}} \left( \frac{k_4}{k_{-4}} \right)^4 [\text{Na}]_i^4 [\text{Ca}]_o [\text{X}]_i. \quad (10)$$

It is also clear that

$$m_o^{\text{Ca}} = k_7[\text{Na}_4\text{X}_0\text{Y}_1\text{Ca}], \quad (11)$$

$$m_i^{\text{Ca}} = k_{-7}[\text{Na}_4\text{X}_1\text{Y}_0\text{Ca}], \quad (12)$$

hence from Eq. (7), (9), and (10)

$$\frac{k_7}{k_{-7}} = \exp -2EF/RT. \quad (13)$$

To obtain the unidirectional fluxes one can define

$$\begin{aligned} ([\text{X}]_o)_T &= [\text{X}]_o + [\text{NaX}]_o + [\text{Na}_2\text{X}]_o + [\text{Na}_3\text{X}]_o \\ &\quad + [\text{Na}_4\text{X}]_o + [\text{Na}_4\text{X}_0\text{Y}_1] + [\text{Na}_4\text{X}_0\text{Y}_1\text{Ca}], \end{aligned} \quad (14)$$

with

$$([\text{X}]_o)_T + ([\text{X}]_i)_T = ([\text{X}])_T, \quad (15)$$

and

$$[\text{X}]_o = [\text{X}]_i, \quad (16)$$

then with  $n_o = (k_4/k_{-4})[\text{Na}]_o$  and  $n_i = (k_4/k_{-4})[\text{Na}]_i$

$$[\text{X}]_o = \frac{([\text{X}])_T}{2 + n_o + n_i + n_o^2 + n_i^2 + n_o^3 + n_i^3 + n_o^4 + n_i^4 + \frac{k_5}{k_{-5}}(n_o^4 + n_i^4) + \frac{k_6 k_5}{k_{-6} k_{-5}} \left( \frac{k_4}{k_{-4}} \right)^4 ([\text{Na}]_o^4 [\text{Ca}]_i + [\text{Na}]_i^4 [\text{Ca}]_o)}. \quad (17)$$

Eq. (17) is equivalent to Eq. (4a) if  $0 = [\text{Ca}]_o = [\text{Ca}]_i = [\text{Na}]_i$  since  $n = 1/j$  and from Eq. (9), (10), and (17)

$$m_o^{\text{Ca}} = \frac{k_7 \frac{k_6 k_5}{k_{-6} k_{-5}} \left( \frac{k_4}{k_{-4}} \right)^4 [\text{Na}]_o^4 [\text{Ca}]_i ([\text{X}])_T}{2 + n_o + n_i + n_o^2 + n_i^2 + n_o^3 + n_i^3 + n_o^4 + n_i^4 + \frac{k_5}{k_{-5}}(n_o^4 + n_i^4) + \frac{k_6 k_5}{k_{-6} k_{-5}} \left( \frac{k_4}{k_{-4}} \right)^4 ([\text{Na}]_o^4 [\text{Ca}]_i + [\text{Na}]_i^4 [\text{Ca}]_o)}, \quad (18)$$

$$m_i^{\text{Ca}} = \frac{k_{-7} \frac{k_6 k_5}{k_{-6} k_{-5}} \left( \frac{k_4}{k_{-4}} \right)^4 [\text{Na}]_i^4 [\text{Ca}]_o ([\text{X}])_T}{2 + n_o + n_i + n_o^2 + n_i^2 + n_o^3 + n_i^3 + n_o^4 + n_i^4 + \frac{k_5}{k_{-5}}(n_o^4 + n_i^4) + \frac{k_6 k_5}{k_{-6} k_{-5}} \left( \frac{k_4}{k_{-4}} \right)^4 ([\text{Na}]_o^4 [\text{Ca}]_i + [\text{Na}]_i^4 [\text{Ca}]_o)}. \quad (19)$$

Eq. (7) and (18) describe all equilibrium states for the scheme shown earlier. In experiments on intact axons  $[Na]_o$  and  $[Ca]_o$  can be adjusted experimentally. Then  $m_o^{Ca}$ ,  $[Na]_i$ , and  $[Ca]_i$  can be measured to test the simultaneous validity of Eq. (7) and (18). However, most of the available data are from dialyzed axons in which  $[Na]_i$  and  $[Ca]_i$  can be adjusted experimentally. In such experiments there is a net flux of Ca, and steady-state equations are needed in place of Eq. (18). These equations are more general in that they contain the equilibrium states for which  $m_o^{Ca} = m_i^{Ca}$ .

*Properties of the Flux Equations*

It is useful to note that for Ca efflux the equation has a term in the numerator  $([Na]_o)^4[Ca]_i$ ; if this product is held constant, while also holding constant  $[Na]_i$  and  $[Ca]_o$ , it is possible to vary  $[Na]_o$  and  $[Ca]_i$  widely while still keeping the system at equilibrium (where the equation is valid). Fig. 3 shows a plot of the equilibrium  $[Ca]_i$  vs.  $[Na]_o$  for a constant product. For values of  $[Ca]_i$  less than 50 nM ( $[Na]_o$  200–450 mM) Ca efflux is linear with concentration because the absolute value of the terms in the denominator involving  $[Na]$  and  $[Ca]$  are small compared with the terms in  $n$ .

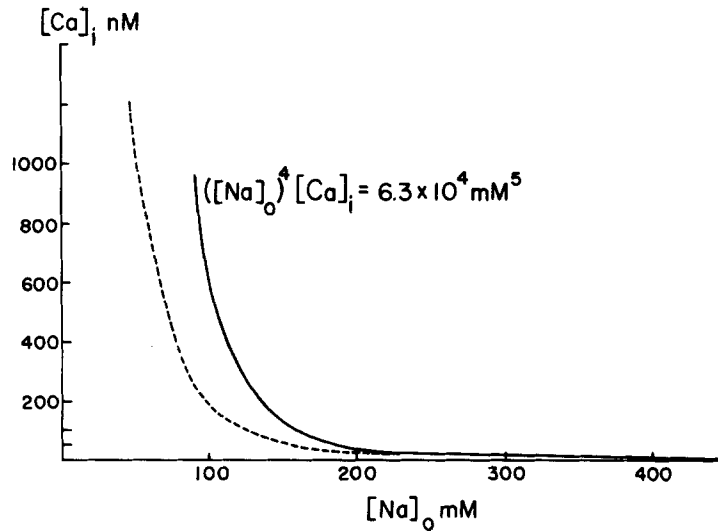


FIGURE 3. This is a plot of  $[Ca]_i$  vs.  $[Na]_o$  for the relation

$$[Ca]_i = \frac{[Ca]_o [Na]_i^4 \exp 2EF/RT}{[Na]_o^4},$$

(solid line), with the  $[Na]_o^4 [Ca]_i$  product as indicated on the curve, and for the case where the cation ( $C^+$ ) used as a diluent for  $Na_o$  is  $\frac{1}{10}$  as effective as Na. Dashed line is

$$[Ca]_i = \frac{[Ca]_o [Na]_i^4 \exp 2EF/RT}{([Na]_o + 0.1(450 - [Na]_o))^4}$$

$[Na]_i = 40$  mM,  $[Ca]_o = 3$  mM,  $E = -60$ mV.

In this range of  $[Na]_o$  Eq. (18) is approximated by

$$m_0^{Ca} = k_7 \frac{k_6}{k_{-6}} \frac{k_5}{k_{-5}} \left( \frac{k_4}{k_{-4}} \right)^4 [Ca]_i [X]_T, \quad (18a)$$

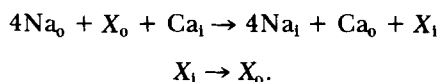
i.e.  $[Na_o X_o Y_i] \rightarrow [X]_T$  and the efflux rate is limited by the rate of binding  $[Ca]_i$ . Under these conditions, with  $[Na]_o$  and  $[Ca]_i$  in the physiological range, the efflux varies with  $[Ca]_i$  only. In particular, the efflux is insensitive to changes in  $[Ca]_o$ . This is in agreement with experiment when  $[Ca]_i$  is 30 to 50 nM (Brinley et al., 1975).

Making the membrane potential zero would decrease Ca efflux 11-fold, and increase Ca influx by a similar amount; since Ca efflux is close to linear with  $Ca_i$  in the physiological range,  $Ca_i$  would rise 122-fold to achieve a steady state. This is in agreement with the findings of Requena et al. (1977) that depolarization increases the steady-state  $Ca_i$ .

If  $Na_o$  is made equal to  $Na_i$  and the membrane potential abolished, which is equivalent to making the energy in the Na gradient zero, then the flux ratio (Ca influx/Ca efflux) =  $[Ca]_o/[Ca]_i$ , a result required by thermodynamic considerations.

#### Nonequilibrium Conditions

Many of the Ca flux data in the literature have been obtained under conditions where, for example,  $[Na]_i$  is zero. The equations presented earlier cannot be applied to such conditions but it is possible to develop steady-state (with respect to carrier), in contrast to equilibrium, equations for such conditions. On the assumption that  $[Na]_i$  is low, much of the transport reaction for Ca efflux is:



Under the conditions outlined above  $[X]_i$  cannot equal  $[X]_o$  since a gradient in X across the membrane must exist to sustain the Ca efflux reaction. The reaction above requires that the net flux of  $Na_4 X_o Y_i Ca$  be equal to the net flux of X and therefore

$$k_7 [Na_4 X_o Y_i Ca] - k_{-7} [Na_4 X_i Y_o Ca] = -k_8 [X]_o + k_{-8} [X]_i.$$

If we let  $\alpha_o = 1 + n_o + n_o^2 + n_o^3 + n_o^4 + (k_5/k_{-5})n_o^4$  and  $\alpha_i$  a similar summation in  $n_i$ , while  $\beta = k_6 k_5 k_4^4 / k_{-6} k_{-5} k_{-4}^4$ , and if it is assumed that the net flux in the steady state does not disturb the equilibrium binding reactions on each side of the membrane, then from Eq. (9) and (10) and the expression above

$$\frac{[X]_i}{[X]_o} = \frac{k_7 \beta [Na]_o^4 [Ca]_i + k_8}{k_{-7} \beta [Na]_i^4 [Ca]_o + k_{-8}}, \quad (20)$$

and

$$X_o = \frac{X_T}{\alpha_o + \beta [Na]_o^4 [Ca]_i + \frac{[X]_i}{[X]_o} (\alpha_i + \beta [Na]_i^4 [Ca]_o)}. \quad (21)$$



In Eq. (21) the term  $X_1/X_0$  can be evaluated from Eq. (20) and in the special case that the transition  $X_1 \rightarrow X_0$  is rate limiting in the transport reaction, the terms  $k_8, k_{-8}$  will be small compared with the other terms in Eq. (20), hence

$$\frac{[X]_1}{[X]_0} = \frac{k_7[Na]_0^{\alpha_1}[Ca]_1}{k_{-7}[Na]_1^{\beta_1}[Ca]_0} \quad (22)$$

This approximation cannot be used if  $[Na]_1$  or  $[Ca]_0 \rightarrow 0$ . From the substitutions that led to Eq. (18) one has

$$m_0^{Ca} = k_7\beta \frac{[Na]_0^{\alpha_1}[Ca]_1[X_T]}{\alpha_0 + \beta[Na]_0^{\alpha_1}[Ca]_1 + \left(\frac{k_7}{k_{-7}}\right) \frac{[Na]_1^{\beta_1}[Ca]_1}{[Na]_1^{\beta_1}[Ca]_0} (\alpha_1 + \beta[Na]_1^{\beta_1}[Ca]_0)} \quad (23)$$

The symmetry of this equation can better be shown by rearranging

$$m_0^{Ca} = k_7\beta \frac{[Na]_0^{\alpha_1}[Ca]_1[X_T][Na]_1^{\beta_1}[Ca]_0}{(\alpha_0 + \beta[Na]_0^{\alpha_1}[Ca]_1)[Na]_1^{\beta_1}[Ca]_0 + \left(\frac{k_7}{k_{-7}}\right) [Na]_0^{\alpha_1}[Ca]_1(\alpha_1 + \beta[Na]_1^{\beta_1}[Ca]_0)} \quad (23 a)$$

Eq. (23) is applicable to experimental conditions where  $[Ca]_1$  is high (micromolar) and  $[Na]_1$  is low. Under these circumstances, Ca efflux is large and is reduced if  $Ca_0$  is made low. The effect is usually called Ca:Ca exchange but it is clearly an expected mode of behavior of the Na/Ca carrier as Eq. (23) shows. In a recent paper, Blaustein (1977) has clearly defined the optimal conditions for this  $Ca_0$ -dependent Ca efflux as follows:  $[Li]_0$  450 mM,  $[Ca]_1 > 1 \mu M$ . In this medium Ca efflux is several-fold greater than it is in Na seawater and Ca efflux is largely abolished by the removal of  $Ca_0$ . The dashed line in Fig. 3 was drawn on the basis that  $Li^+$  had 0.1 effectiveness in combining with  $X$  as did  $Na^+$ . Thus the  $[Li]_0^{\alpha_1}[Ca]_1$  product for the conditions specified by Blaustein are virtually identical with an  $[Na]_0^{\alpha_1}[Ca]_1$  product where  $Na_0$  is 200 mM and  $Ca_1$  is 30 nM. The fluxes are larger in Li than in Na seawater because  $\alpha_0$  is much smaller in Li; Ca efflux is sensitive to  $Ca_0$  as specified in Eq. (23). A conclusion one draws from these sorts of experiments is that the (Na-Ca) products on either side of the membrane can never be zero since Li, and presumably other cations, have some ability to act like Na, and Blaustein has provided good evidence that  $K^+$  can act like Na inside the axon since replacing  $K^+$  with  $TMA^+$  reduces the Li-mediated Ca efflux.

An equation similar to Eq. (23) can be developed for Ca influx. This too has an explicit dependence of Ca influx on  $[Ca]_1$ .

It has been suggested above that ions used as Na substitutes have some ability to act like Na, and it also seems likely that  $Mg^{++}$ , present on both sides of the membrane in millimolar or greater concentrations, has some finite ability to substitute for Ca so that truly Ca-free conditions do not occur.

#### *Equilibrium Constants*

It should be recognized at the outset that experimental measurements of Ca fluxes have been carried out largely under conditions far from equilibrium. Hence any apparent affinity constants of the carrier for Na or Ca extracted from such measurements must be viewed with considerable caution. As an

example, if  $Ca_i$  is made 100  $\mu M$  (a saturating value) and  $Na_o$  is varied, Ca efflux declines with decreases in  $Na_o$  along a curve similar to that for  $Na_4X$  shown in Fig. 2. If on the other hand  $Ca_i$  is made 50 nM, there is no effect on Ca efflux of substituting  $Li^+$  for  $Na^+$  in the seawater (Brinley et al., 1975). The difference in these two experimental findings can be resolved by noting that at a  $Ca_i$  of 100  $\mu M$  the Na:Ca exchange system is very far from equilibrium and that the rate-limiting step in the reaction is most probably the delivery of  $Na_4X_oY_i$  for reaction with  $Ca_i$ . At a  $Ca_i$  of 50 nM, the exchange reaction is rather close to equilibrium and a reasonable inference is that it is not the rate of delivery of  $Na_4X_oY_i$ , but rather the rate of attachment of  $Ca_i$  to the carrier, that is rate limiting. If one assumes that  $Li_o^+$  allows a slow delivery of  $Li_4X_oY_i$  to bind Ca, then it is clear that at low  $Ca_i$  there will be no difference in Ca efflux whether  $Li^+$  or  $Na^+$  is present outside.

The equilibrium constants involving  $Na^+$  are  $(k_4/k_{-4})^4(k_5/k_{-5})$ . At high  $Ca_i$  it is easy to extract a value for  $(k_4/k_{-4})$  of  $1/140 \text{ mM}^{-1}$  as has been done for Fig. 2. By plotting activation curves for Ca efflux vs.  $Na_o$  at various values for  $k_5/k_{-5}$  it is possible to conclude that satisfactory fits can be obtained with values in the range 0.1–1.0. A value of 0.1 has been used arbitrarily for many trial computations.

The calcium equilibrium constants are  $(k_6/k_{-6})(k_7/k_{-7})$ . Again, these are phenomenological and no more easily separable than  $k_4, k_5$ . If  $Na_o$  is held at 450 mM and  $Na_i$  at 45 mM (values approximating a normal axon's [Na] gradient) and  $[Ca]_i$  is varied from perhaps 10 nM to 100,000 nM, one can assume that  $Na_o$  is a saturating [Na] and that the apparent  $K_{1/2}$  for the Ca efflux curve represents  $(k_6/k_{-6})k_7$ . Theoretical considerations presented earlier suggest that  $k_7 = 11.05x$  and  $k_{-7} = x/11.05$  so that a value for  $(k_6/k_{-6})$  can be extracted. This lies in the range 1–10  $\mu M k_{Ca}$  or  $k_6/k_{-6} = 1,000\text{--}100 \text{ mM}^{-1}$  (Brinley et al., 1975; Blaustein and Russell, 1975).

A final equilibrium constant is  $k_8/k_{-8}$ . Values for both  $k_8$  and  $k_{-8}$  are necessary to solve nonequilibrium equations under Ca-free conditions but, although we have assumed  $k_8/k_{-8} = 1$ , this hardly helps with quantitative solutions. One could argue that the translocation of X is the same as that of  $Na_4XYCa$  if allowance is made for X being uncharged. This assumption would allow  $k_7/k_{-7} = 122$  and  $k_8/k_{-8} = 1$  with  $k_7 = 11.05x$  and  $k_8 = 1x$ . Unfortunately, it is also possible that X has a formal charge so that  $X_o/X_i$  is not 1 under equilibrium conditions but is distributed unequally on the two sides of the membrane by virtue of its charge. Such an arrangement would not invalidate the thermodynamic arguments since the carrier would still translocate  $2^+$  charges inward per cycle; it would, however, substantially affect the kinetics.

In spite of the considerable uncertainties, I have used the values for constants cited above to show that with  $[Ca]_i$  high,  $[Na]_o$  activates Ca efflux along an S-shaped curve with a  $K_{1/2}$  of  $\sim 200 \text{ mM}$  and  $Ca_i$  vs. Ca efflux is a simple hyperbola with a  $K_{1/2}$  of 1–10  $\mu M Ca_i$ . The refinement of these constants is a problem of considerable complexity that will require both further data and appropriate computer techniques.

If  $[Ca]_i$ ,  $[Ca]_o$ , and  $[Na]_i$  are held constant then  $[Na]_o$  activates Ca efflux

along an S-shaped curve, according to Eq. (23a). The  $K_{1/2}$  depends upon the magnitudes of  $[Ca]_i$ ,  $[Ca]_o$ , and  $[Na]_i$ .

If  $[Ca]_o$ ,  $[Na]_o$ , and  $[Na]_i$  are held constant then  $m_o^{Ca}$  increases hyperbolically with increases in  $[Ca]_i$ , including an initial linear dependence in the nanomolar range. Again, the  $K_{1/2}$  depends upon the magnitude of the fixed concentrations of  $[Na]_i$ ,  $[Na]_o$ , and  $[Ca]_o$ .

These observations are in accord with experiments. However, if  $[Na]_o$ ,  $[Ca]_o$ , and  $[Ca]_i$  are held constant then Ca efflux increases along an S-shaped curve as  $[Na]_i$  increases, as expected from the symmetry of Eq. (23a). This conflicts with observations made at a  $[Ca]_o$  of 8 mM (Brinley et al., 1975). Re-examination of Eq. (20) in the denominator shows that with the value of  $\beta$  cited earlier ( $2.6 \times 10^{-8} \text{ mM}^{-5}$ ) and of  $K_{-7}$  ( $x/11.05$ ) then for  $Ca_o = 3 \text{ mM}$  and  $Na_i = 40 \text{ mM}$ , the value of the denominator is  $0.048x$  compared with an assumed value for  $K_{-8}$  of  $1x$ . Even at 80 mM Na, the value is 0.77 so that the approximation of Eq. (22) is invalid for low  $[Na]_i$ .

#### *The Effect of ATP on Ca Fluxes*

In principle, ATP could affect Ca fluxes by: (a) increasing the affinity of the carrier for Ca; (b) increasing the affinity of the carrier for Na; (c) increasing the rate of the translocation step; (d) activating a Ca pump separate from the Na:Ca exchange mechanism; (e) increasing  $[X]_i$ ; or by some combination of effects on each of properties (a)–(e).

While an increase in the affinity of the carrier for Ca easily explains experimental results showing that ATP increases Ca efflux, such a postulate ignores experimental findings showing that: (a) ATP has little effect on Ca efflux if  $[Na]_i$  is zero (DiPolo, 1976); and (b) the effect of ATP on Ca efflux at constant  $Na_i$  is much greater when  $Ca_i$  is low than when it is high but far from saturation (DiPolo, 1974, 1977).

Increasing the rate of the translocation step once the carrier is loaded with Ca and Na, as an explanation, suffers from the difficulty listed above, as does increasing  $[X]_i$ : it does not account for the lack of effect of ATP on Ca efflux in the absence of  $Na_i$ , and it fails to explain the virtual lack of effect of ATP on Ca efflux if  $Ca_i$  is high. A separate ATP-dependent Ca pump would seem to be unnecessary since the findings of Requena et al. (1977) are that  $[Ca]_i$  in an intact axon is not changed when apyrase has destroyed internal ATP and that such axons can recover from imposed Ca loads. The implications of this finding are that the Na gradient alone is capable of extruding Ca and maintaining a normal  $Ca_i$  and that an ATP-operated Ca pump is simply not necessary for Ca homeostasis.

A  $Ca_o$ -dependent Na efflux is not observed in axons dialyzed free of ATP (Brinley et al., 1975) so that one infers that Ca influx as well as Ca efflux is ATP dependent. The findings with respect to ATP can be related to the carrier mechanism developed above as follows. Because of the need for the carrier to bind 4 Na before it can develop a Ca binding site, at physiological  $[Na]_i$  some of the carrier can be expected to be in inactive forms such as  $NaX_1 \dots Na_3X_1$ ; an improvement in Na affinity will increase the fraction of the carrier than can

exist in a Ca-carrying form at any given  $[Na]$ , and thus facilitate Ca fluxes.

To test the idea that a change in affinity of the carrier for Na can explain the effects of ATP on Ca fluxes, Fig. 4 reproduces the curve shown in Fig. 2 for  $[Na_4X]$  as a function of  $[Na]$  with a  $K$  of 140 mM, together with a new curve with a  $K$  for Na of 30 mM. The curves are a reasonable representation of the data of Baker and McNaughton (1976) for the activating effects of  $Na_o$  on Ca efflux in squid axons with and without ATP. Additional points of interest are that at a  $[Na]$  of 40 mM (appropriate to a physiological  $Na_i$ ), the concentration of carrier with an induced  $Ca^{++}$  binding site is vanishingly small when  $K_m = 140$  mM (absence of ATP), while it has a value of 0.3 at a  $K_m$  of 30 mM (presence of ATP). These changes ought to relate to changes in Ca influx and hence to explain its ATP dependence. For the  $K = 30$  mM curve, over a substantial range of  $[Na]$ , the rise of  $[Na_4X]$  is proportional to  $[Na]^2$ , in agreement with the

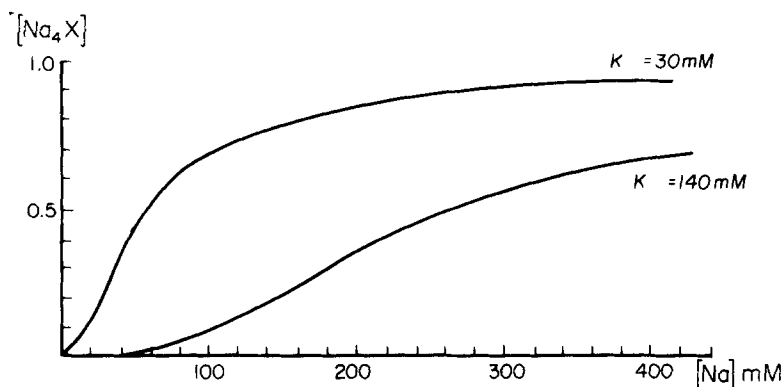


FIGURE 4. This shows the effect produced on  $[Na_4X]$  by a change in  $K$  from 140 mM to 30 mM, for the reaction of  $Na^+$  with  $X$ .

dependence of Ca influx on  $[Na]_i$  shown by Baker et al. (1969). Another feature of the  $K = 30$  mM curve is that  $[Na_4X]$  is virtually saturated at  $[Na] = 180$  mM, a finding in agreement with the results of Requena et al. (1977) that  $[Ca]_i$  is the same in an intact axon with ATP whether  $[Na]_o$  is 180 mM or 450 mM.

With respect to Ca efflux, one may note the following: with  $[Na]_o = 450$  mM there is very little difference in  $[Na_4X_o]$  whether  $K = 30$  mM or 140 mM. This agrees with experimental findings that in the absence of  $Na_i$ , Ca efflux is independent of ATP. The transport reaction is:  $4 Na + X_o \rightarrow Na_4X_oY_1 \rightarrow Na_4X_oY_1Ca \rightarrow Na_4X_1Y_oCa \rightarrow X_1 + 4 Na_i + Ca_o \rightarrow X_o$ , with  $X_1$  diffusing to  $X_o$ . If  $Na_i$  is greater than zero,  $X_1$  reacts with  $Na_i$  to form substantial amounts of nonmobile carrier and this depletes the available  $[X_o]$  and hence reduces Ca efflux. An alteration of the binding constant,  $K$ , from 140 mM to 30 mM greatly increases  $[Na_4X_1]$  at the expense of  $NaX \dots Na_3X$  and hence provides an alternative path for the return of carrier, viz.,  $Na_4X_1Y_oCa \rightarrow Na_4X_o + Ca_i \rightarrow 4 Na + X_o$ . This prevents the depletion of  $[X_o]$ .

A test of Eq. (18) using  $K = 30$  mM shows that Ca efflux is increased about fourfold by the change in equilibrium constant. This comes about because the

value of  $(k_4/k_{-4})^4$  increases more than the corresponding values for  $n$ . It is possible that  $(k_5/k_{-5})$  also increases, but given the lack of really precise information on Ca fluxes in the presence of ATP as a function of  $Ca_o$ ,  $Ca_i$ ,  $Na_o$ ,  $Na_i$ , it is possible to conclude that an adjustment solely in the binding constant for Na will explain the effects of ATP on both Ca influx and efflux.

Another effect of ATP is that it decreases the inhibitory effect that  $[Na]_i$  has on Ca efflux. This requires that  $K_{Na}$  increase, rather than decrease as has been argued above. A resolution of this apparent contradiction is possible if it is assumed that in the presence of ATP we have not a single value for  $k_4/k_{-4}$ , but rather four different binding constants for Na, some of which are smaller and some larger than the value assumed earlier.

#### *The Isolated Squid Axon*

The foregoing discussion has dealt largely with the highly artificial situation where Ca fluxes in an axon can take place solely via Na:Ca exchange. Actually, when an axon is stimulated, some Ca enters via the channels used to convey  $Na^+$  inward and it is likely that the membrane has some finite permeability to Ca apart from carrier-mediated processes. In addition, an isolated squid axon has a substantially lower membrane potential than that measured in the axon in the squid mantle so that it is reasonable to infer that the processes involved in isolating an axon induce an additional membrane leak of Ca as well as other ions.

The evidence from aequorin studies (DiPolo et al., 1976) is that an isolated squid axon can have a  $[Ca]_i$  of 30 nM in 3 mM Ca(Na) seawater; the level of Ca efflux in such an axon is 30–40 fmol/cm<sup>2</sup>s. Virtually all of this efflux must be Na:Ca transport since passive Ca efflux is vanishingly small. Influx must balance efflux since  $[Ca]_i$  is in a steady state, but some Ca influx is passive, noncarrier-mediated.

Since  $[Na]_o$ ,  $[Na]_i$ ,  $[Ca]_o$ , and  $E_m$  are all fixed by the homeostatic mechanisms of the squid, the equilibrium value for  $[Ca]_i$  is fixed, in the absence of a Ca leak, and is 1.5 nM. Since this is one-twentieth of the measured  $[Ca]_i$ , it suggests either that (a) too large a coupling ratio Na:Ca has been assumed; or (b) the Ca leak is the major factor in Ca influx when  $[Na]_o$  is normal. Two pieces of experimental information suggest that Ca influx is mainly leak: (a) Baker and McNaughton (1976) find that Ca influx from Na seawater is linear with  $[Ca]_o$  from micromolar to hundreds of millimolar, a result not characteristic of a carrier-mediated process; and (b) Requena et al. (1977) have shown that Ca influx from 37 mM Ca seawater is unaffected by removal of ATP from an axon while Ca influx produced by lowering  $[Na]_o$  is much inhibited.

If we take 1.5 nM as the *equilibrium* value of  $[Ca]_i$ , the corresponding value for Ca efflux is 1.5 fmol/cm<sup>2</sup>s. The measured Ca influx is 40 fmol/cm<sup>2</sup>s so that  $[Ca]_i$  will rise until flux balance occurs. One concludes therefore that in an isolated squid axon, the Na/Ca carrier flux ratio Ca efflux/Ca influx is of the order of 20.

Substantial  $[Ca]_i$  homeostasis is possible over a range of  $[Na]_o$  from 180–450 mM because (a)  $[Ca]_i$  is much higher than its equilibrium value, and (b) carrier-mediated Ca influx is small compared to leakage Ca influx.

The foregoing considerations argue strongly for the 4:1 coupling ratio that has been assumed since it is not the equilibrium value of  $[Ca]_i$  that is important but how much this value is perturbed to obtain a steady state.

In the presence of a passive inward "leak" of  $Ca^{++}$  the properties of the Na/Ca carrier mechanism are artificially modified. Under such circumstances the best tests of the carrier mechanism can be made in the absence of external  $Ca^{++}$ , eliminating the passive influx.

Then Eq. (20) becomes:

$$\frac{[X]_i}{[X]_o} = \frac{k_7\beta[Na]_o^4[Ca]_i + k_8}{k_{-8}}, \quad (20 a)$$

and Eq. (23 a) becomes:

$$m_o^{Ca} = k_7\beta \frac{k_{-8}[Na]_o^4[Ca]_i[X]_i}{k_{-8}[\alpha_o + \beta[Na]_o^4[Ca]_i] + \alpha_i[k_7\beta[Na]_o^4[Ca]_i + k_8]}. \quad (23 b)$$

It is satisfactory to note that this equation is consistent with the dependence of  $m_o^{Ca}$  on  $[Na]_o$ ,  $[Na]_i$ , and  $[Ca]_i$  reported by Blaustein et al. (1974) for tests made in Ca-free seawater.

#### CONCLUSIONS

While there is little doubt that a multivalent binding of Na to a carrier is a necessary condition for the induction of a Ca binding site, it is also possible that, once induced, the Ca binding site persists until all Na has dissociated from the carrier. This type of arrangement is not explicitly discussed because its quantitative description is difficult. It would make the Na:Ca carrier insensitive to the Ca gradient in the sense that this gradient could drive the carrier directly. Curiously, there is little experimental information that supports the notion that the Ca gradient can drive the carrier. Ca efflux is not decreased by increasing  $[Ca]_o$ .

A second point is that the multivalent nature of the Na binding sites suggests that these are provided by the dissociation of a proton in exchange for Na. In turn, this would make Ca transport both highly sensitive to pH and capable of transporting  $H^+$ .

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