Coupling Ratio of the Na-K Pump in the Lobster Cardiac Ganglion

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ABSTRACT The electrogenic Na-K pump coupling ratio in the large neurons of the lobster cardiac ganglion was determined by two different electrophysiological techniques. A graphical analysis plotting exp(E_mF/RT) vs. [K], after the pump was blocked by ouabain was used to determine values for [K], P_Na/P_K, and the pump coupling ratio. These measurements were made 4–8 h after the cells were penetrated with microelectrodes, and thus represent non-Na-loaded steady state values. The value obtained for the pump coupling ratio under these conditions was 1.44 ± 0.06 (n = 9) or close to 3 Na for 2 K. The second technique used to measure the coupling ratio was to iontophoretically inject Na ions into the neuron. Neurons were penetrated with three microelectrodes, two of which were filled with 2 M Na-citrate; the third electrode contained either 2 M K-citrate or 3 M KCl. By passing current between the Na salt-containing electrodes, Na was injected into the cell soma. The injection system was calibrated by injecting ^24Na-citrate into counting vials from representative microelectrodes (calculated ^24Na transport = 0.92). By knowing the Na load injected into the cells, and by measuring the time-current area produced by the Na activation of the Na-K pump, the coupling ratio was calculated to be 1.54 ± 0.05 (n = 19), which is not significantly different from the value obtained by the first method. This value represents a Na-loaded experimental situation. When Na was removed from the external bathing solution, the coupling ratio shifted to 2 Na to 1 K (2.0 ± 0.07, n = 4). These results suggest that the pump normally operates with a 3:2 ratio both in steady state and under Na load but that in the absence of external Na, it can operate with less than a full complement (2) of K on the external surface of the pump.

INTRODUCTION

The lobster cardiac ganglion is a unit of nine neurons that control the rhythmic contraction of the lobster heart. Five of the nine neurons are large enough to be penetrated with up to three microelectrodes. These cells have been previously shown to have an electrogenic Na-K pump (Livengood and Kusano, 1972), which under some conditions can influence the discharge rate of the cardiac ganglion and consequently influence the heart rate of the lobster (Livengood and Kusano, 1973). The electrogenicity of the Na-K pump in nerve cells has been well estab-
lished (see Thomas, 1972). This electrogenicity is a function of the relative ratio of the Na ions pumped out to the K ions pumped into the cell. The Na-K coupling ratio has been the subject of some controversy. In red blood cells the ratio seems to be fixed at 3 Na to 2 K ions (Post and Jolly, 1957). The ratio in nerve cells is less clear. Mullins and Brinley (1969) have suggested that the coupling ratio may be variable in squid axons, but Abercrombie and De Weer (1978) have presented evidence indicating that the electrogenic coupling ratio in squid axon seemed to be fixed at 3:2. Gorman and Marmor (1974a) have found that the electrogenic coupling ratio in Anisodoris neurons was 2:1 in a steady state non-Na-loaded condition. An electrogenic pump coupling ratio of 2:1 was also found in Aplysia neurons (Cooke et al., 1974). These results differ from the findings of Thomas (1969) and Lambert et al. (1974), who found that the coupling ratio in Helix neurons was 3:2. Gorman and Marmor (1974a) suggested that one explanation for these differences might be the level of Na loading of the different nerve cells.

The present experiments in lobster cardiac ganglion cells attempted to resolve this issue using two distinctly different electrophysiological methods to estimate the electrogenic pump coupling ratio in both a Na-loaded and a non-Na-loaded condition. Evidence from the two methods is internally consistent; it indicates that in the nerve cells of the lobster cardiac ganglion, the electrogenic pump coupling ratio in both a Na-loaded and a steady state non-Na-loaded condition is 3:2 when the ganglion is bathed in artificial seawater containing a normal level of Na and K. However, if the external Na is replaced with a nonpermeant cation, the coupling ratio is raised to a value close to 2:1. A preliminary report has appeared previously (Livengood, 1981).

METHODS

The cardiac ganglion of the North American lobster, Homarus americanus, was used for these experiments. Dissection of the cardiac ganglion has been described previously (Hagiwara and Bullock, 1957; Connor, 1969). The excised Y-shaped ganglion was pinned with stainless-steel pins on a layer of Sylgard (Dow Chemical Co., Indianapolis, IN) in a chamber whose volume was 2 or 3 ml, depending on the height of the suction standpipe at the opposite end of the chamber from the solution entry port. The maximum solution perfusion rate through the recording chamber was 36 ml/min. A minimum of 100 ml was perfused through the chamber for each change of salt concentration. When the time of response of the ganglion to solution changes was not essential, solutions were sometimes exchanged at less than a maximum rate to minimize loss of solution and possible disturbances of microelectrode penetration by solution flow vibration.

The maximum rate of solution change in the recording chamber was determined by the rate of clearance of methylene blue from the bath. Samples were collected over 1 min at 3-s intervals. Samples were collected in disposable tubes from the outlet suction tube from the recording chamber. Methylene blue absorbance was measured on a Spectronic 21 (Bausch & Lomb Inc., Rochester, NY).

Measurements from two washout curves were determined. The log of the percentage of dye in each sample tube, as compared with the dye solution added to the bath, was plotted against time in seconds. The goodness of the fit of the data was
determined by linear regression analysis. The correlation coefficient for this line was 0.99. The time for 90% change of the bath was calculated to be 13 s.

Membrane potentials were measured differentially between an intracellular microelectrode and an extracellular 3 M KCl-agar-filled bridge with a cone-shaped tip and small-diameter opening (Strickholm, 1968). Intracellular recording microelectrodes were filled with either 3 M KCl or 2 M K-citrate. K-citrate electrodes were necessary in experiments requiring Cl-free solutions. The intracellular electrodes and the extracellular bridge were connected to the system electronics by means of a 3-M KCl/AgCl/Ag junction. The bath was connected to a virtual ground current meter by a second cone-shaped 3 M KCl-agar-filled bridge through a KCl/AgCl/Ag junction. The electronic drift of the system was <0.5 mV in 24 h. Drift of the system with 3 M KCl microelectrodes was also <0.5 mV in 24 h. The system drift with K-citrate electrodes was generally the same. However, it was observed that on occasion drift could be much higher than this (as much as 1 mV/h); therefore, care was taken to check for electrode drift before the start of the experiment and at the end of each experiment. In Na injection experiments, Na was iontophoretically injected by passing current between two of three microelectrodes inserted into the cell (Thomas, 1969; Livengood and Kusano, 1972). The amount of Na injected into the cell was determined by calculating the area under the current-time curve (charge) resulting from passing current between the two iontophoretic microelectrodes and then dividing this value by the Faraday constant.

The amount of Na moving out of the neuron that was responsible for producing the Na pump-activated membrane hyperpolarization was calculated by measuring the area under the time-voltage curve and dividing this value by the calculated membrane resistance, as well as by the Faraday constant. The areas were determined by tracing the curve displacements on tracing paper, cutting out the paper, and weighing them on an analytical balance. Three to four measurements were made for each value and the average was used for further calculations. Typically the measurements were within 5% of each other. Table I shows the compositions of solutions used in these experiments. Various concentrations of K were obtained by mixing appropriate amounts of solutions B and C or, in the Cl-free experiments, by mixing solutions E and F. All solutions were adjusted to pH 7.4. The solution temperature was held at 17-18°C for most of the experiments described here by means of a refrigerated circulating water bath (RTE-4; Neslab Instruments, Inc., Portsmouth, NH), which circulated cold water around the recording chamber and the solution input lines. Some of the injection experiments were performed at room temperature at the Marine Biological Laboratory, Woods Hole, MA.

**Determination of the Na Transport Number**

The amount of Na injected by iontophoretic current passed between two microelectrodes was determined using 24Na-citrate-filled microelectrodes (24Na-citrate was produced in the Armed Forces Radiobiology Research Institute's TRIGA reactor). 1 g of crystalline Na-citrate was weighed out in a plastic tube and placed in the reactor. The Na was activated in the reactor at 50,000 W for 1 h. 24Na-labeled Na-citrate was removed to a hot lab where 1.7 ml of distilled water was added to the crystalline Na-citrate to produce a 2-M Na-citrate solution. The activity of the solution was \( \sim 8 \times 10^{10} \) cpm per equivalent of Na. A series of fiber-filled microelectrodes was filled with the 24Na-citrate solution. A matched pair of these microelectrodes was inserted into a counting vial containing 250 mM K isethionate to approximate the cytoplasm of a lobster cardiac ganglion neuron. A current of 25 nA was passed
RESULTS

Steady State Experiments in Normal Saline

The rhythmic pacemaker activity of the lobster cardiac ganglion is a function of the coordinated firing of the nine neurons forming the ganglion. The regular, spontaneous, non-overshooting burst potentials produced by these cells are between 15 and 50 mV in amplitude (Livengood and Kusano, 1972). The soma itself is electrically inexcitable, and the burst potential reflects the passive spread of action potentials from the active area of the axon (Hagiwara, 1961). In addition to the rhythmic discharge of the ganglion, the soma membrane also displays an active electrogenic Na-K pump (Livengood and Kusano, 1972). Fig. 1 shows a typical recording from one of the five large neurons. In this cell the burst potentials (upward deflections) were 30–35 mV in amplitude, and the resting potential was approximately −55 mV. The ganglion was exposed to a K-free saline (first arrow) for a period of 25 min. Exposure to a K-free saline inhibits the Na-K pump and leads to Na loading (Thomas, 1972; Gorman and Marmor, 1970; Livengood and Kusano, 1972). The return to normal K saline (second arrow) results in a membrane hyperpolarization (22 mV), which reflects the activation of an electrogenic Na pump (Livengood and Kusano, 1972). Similar findings have been obtained in molluscan neurons (Gorman and Marmor, 1970; Thomas, 1969, 1972). Previous experiments in lobster demonstrated that the electrogenic pump

![Figure 1](image_url)

**Table I**

| Mm | NaCl | MgCl₂ | MgSO₄ | CaCl₂ | KCl | HEPES | Cl | Na methyl-sulfate | Ca(OH)₂ | K methyl-sulfate | Methyl-sulphonic acid |
|----|------|-------|-------|-------|-----|-------|----|------------------|--------|------------------|--------------------|
| A  | 450  | 4     | 4     | 25    | 15  | 10    | 0  | 0                | 0      | 0                | 0                  |
| B  | 465  | 4     | 4     | 25    | 0   | 10    | 0  | 0                | 0      | 0                | 0                  |
| C  | 355  | 4     | 4     | 25    | 100 | 10    | 0  | 0                | 0      | 0                | 0                  |
| D  | 0    | 4     | 4     | 25    | 15  | 10    | 0  | 450              | 0      | 0                | 0                  |
| E  | 0    | 0     | 8     | 0     | 0   | 10    | 0  | 465              | 25     | 0                | 66                 |
| F  | 0    | 0     | 8     | 0     | 0   | 10    | 0  | 360              | 25     | 100              | 66                 |

between the two microelectrodes for a number of different test periods. The amount of ^24^Na in the counting vials was determined by counting in a gamma counter.
activity is dependent on internal Na and external K or K-like cations (Rb, Cs, Li), and is sensitive to temperature and cardiac glycosides (Livengood and Kusano, 1972).

In maintaining a steady state internal Na concentration, the Na pump will extrude Na ions in an amount equal to that acquired as a result of the membrane Na permeability. Therefore, under steady state conditions, the contribution of the electrogenic Na pump to the membrane potential is a function of the coupling ratio of the pump and the rate of pumping, which in turn is a function of the Na permeability (the influx of Na). For determining the Na-K pump coupling ratio and the contribution of the electrogenic Na pump to the steady state resting potential, it is convenient to use the analysis of Mullins and Noda (1963) (see reviews by Thomas, 1972; De Weer, 1975). This same approach has been used previously by Gorman and Marmor (1974) and by Lambert et al. (1974). The Mullins-Noda relationship (Mullins and Noda 1963, Eq. 12) was expressed as follows:

\[ E_m = \frac{RT}{F} \ln \frac{r[K^+]_o + (P_{Na}/P_K)[Na^+]_o}{r[K^+]_i + (P_{Na}/P_K)[Na^+]_i}, \quad (1) \]

where \( r \) gives the value for the active coupling ratio between Na and K, and the other values have their usual meanings. The basic limitation to this equation, as pointed out by Mullins and Noda, is the assumption that [Na] and [K] are in a steady state and therefore are not changing. If the Na-K pump is inactivated, for example, with a cardiac glycoside, then the following constant field relationship applies:

\[ E_\ast = \frac{RT}{F} \ln \frac{[K^+]_o + (P_{Na}/P_K)[Na^+]_o}{[K^+]_i + (P_{Na}/P_K)[Na^+]_i}, \quad (2) \]

where \( E_\ast \) is the membrane potential after the pump is blocked. This relationship is valid only if other ion constituents, such as Cl, either have a low permeability or are rapidly and passively distributed. The assumption that Cl plays only a minor role was tested, and will be described later. Since \( (P_{Na}/P_K)[Na^+] \ll [K^+] \), Eq. 2 can be rewritten as follows:

\[ \exp \left( \frac{E_\ast F}{RT} \right) = \frac{[K^+]_o + (P_{Na}/P_K)[Na^+]_o}{[K^+]_i + (P_{Na}/P_K)[Na^+]_i}. \quad (3) \]

Considered as a function of [K]o, this is a linear equation whose slope is the reciprocal of the internal K concentration, and whose intercept is a function of the \( P_{Na}/P_K \) ratio (Geduldig, 1968; Moreton, 1968). Taking the difference between the Eqs. 1 and 2 yields the following equation:

\[ E_m - E_\ast = E_p = \frac{RT}{F} \ln \frac{1 \frac{r[K^+]_o + (P_{Na}/P_K)[Na^+]_o}{r[K^+]_i + (P_{Na}/P_K)[Na^+]_i}}, \quad (4) \]

where \( E_p \) is equal to the contribution of the electrogenic pump to the resting potential. Since the values for the external Na concentration and external K concentration are known, by applying the calculated value for \( P_{Na}/P_K \), the coupling ratio \( r \) can be determined:
\[
\frac{r}{(P_{Na}/P_{K}[Na]_o)}\left[\left(K_o + \frac{P_{Na}/P_{K}[Na]_o}{K_o}\right)\exp\left(FE_p/RT\right) - K_o\right]^{-1}.
\]  

Eqs. 3 and 4 are valid only if the constant field approximations hold and the permeability ratio does not change appreciably with the change in membrane potential produced by the ouabain depolarization. The current-voltage relationships of individual neurons of the lobster cardiac ganglion are well behaved, which shows a linear current-voltage relationship for hyperpolarizing pulses and for depolarizing pulses of <10–15 mV (D. R. Livengood, unpublished observations) and is similar to current-voltage relationships from cardiac ganglion neurons from other decapod crustaceans (Haigiwara et al., 1959). The validity of Eq. 3 also requires that \(P_{Na}/P_{K}[Na]_o \ll [K]_o\) and that [K]_o not change appreciably during the time course of the experiment. After the Na pump is blocked by ouabain, the cell is no longer in steady state (Mullins and Noda, 1963, Eq. 2, Table VI) and is constantly losing K and gaining Na. Therefore, Eqs. 2–5 are no longer steady state equations. Cells that continuously gain an appreciable amount of Na and lose K after ouabain would depolarize. Data from cells that show an appreciable depolarization during the course of the experiment following ouabain treatment would therefore be unusable.

Since it was necessary for the cells under study to be in a steady state condition, they were penetrated with a single microelectrode at least 4 h before any experimental manipulation. Fig. 2 is an example of the experimental protocol. On penetration, the membrane potential was a little over \(-61\) mV, and remained between \(-61\) and \(-62\) mV for the next 6 h. Membrane potential measurements were sampled at half-hour intervals. After 6 h, the solution was changed to one containing \(10^{-4}\) M ouabain and 15 mM K. After 15–20 min, the membrane potential stabilized at a potential \(~2\) mV positive to the resting potential obtained before addition of ouabain. The bathing solution was then changed to other concentrations of external K (2.5, 10, and 5 mM). An interval of 15–20 min was allowed between each solution change in this experiment and the electrode was withdrawn at the end of the change to 5 mM K. In most experiments, however, the solution was cycled several times through a series of K concentrations, including 2.5, 5, 7.5, 10, and 15 mM. The average time for membrane potential stabilization for all solution changes in the nine experiments reported in this section was \(4.2 \pm 0.5\) min (SE) with a range of 1–19 min. The average time for the stabilization of the membrane potential after the solution changes from 15 mM K to 2.5 mM K was \(6 \pm 1.5\) min (SE) with a range of 1–19 min. The longer time involved in this change may be due to the washout time of K from the ganglion sheath. Because of the loss of validity of Eq. 3 if the internal K concentration changed appreciably, data were discarded for cells showing any obvious polarization drift with repeated solution changes to the same K concentration. Subsequent experiments not shown in this paper have indicated that measurements taken 1–2 h after electrode penetration yield comparable results. Fig. 3 is a plot of \(\exp(E_{\text{rev}}F/RT)\) vs. the external K concentration for two cardiac ganglion cells. From Eq. 3, the reciprocal of
The slope of the least-squares fit for the data for one cell (Fig. 3, closed circles) gave a calculated internal K concentration of 247 mM. The K value calculated for the second cell from the reciprocal of the slope (Fig. 3, open circles) was 208 mM. The $P_{Na}/P_K$ values for the two cells were calculated to be 0.029.
and 0.009, respectively. A summary of the values obtained from nine neurons is shown in Table II. This table lists the membrane potential obtained immediately before the addition of ouabain, the ouabain-induced membrane depolarization, and the calculated values for the internal K concentration, the $P_{Na}/P_K$ ratio, and the electrogenic pump coupling ratio. The pump coupling ratio of 1.44 is not significantly different statistically from the value expected for a pump coupling ratio of 3 Na to 2 K (1.50).

**Steady State Experiments in Cl-free Saline**

Validity of the assumption that Cl does not contribute appreciably to the membrane potential was tested by a series of experiments in which Cl was replaced by the impermeant anion methylsulfate (see solutions E and F in Table I). Fig. 4 shows a recording from a single cardiac ganglion cell in Cl-containing saline and in Cl-free saline during the change to K-free saline and then the return to K-containing saline. In Fig. 4A, the solution was changed from 15 mM K-containing saline to K-free saline at the first arrow. At the second arrow, 15 mM K saline was reintroduced, resulting in the activation of a pump-mediated membrane hyperpolarization of $\sim 36$ mV. After the membrane potential stabilized, the solution was changed to a Cl-free saline (Fig. 4B, at arrow), which resulted in a depolarization of the membrane of several millivolts. In 11 solution changes in 7 neurons, the average depolarization in response to the change to Cl-free saline was 8 mV. However, the quantification of this depolarization is complicated by the change in the pacemaker activity of the ganglia, as can be seen at 2 min after the change to Cl-free saline. Part of the depolarization was apparently caused by the increase in firing activity. Burst potentials were frequently seen to depolarize and then remain so for some period of time. However, the membrane potential recovered to near its steady state level in 15–20 min. The trace in Fig. 4C shows a change to a K-free medium at the first arrow in a Cl-free
condition. After 20 min exposure to Cl-free K-free saline, the solution was again changed back to 15 mM K, Cl-free saline, which in this case resulted in a 40-mV hyperpolarization. The previously described method for calculating [K]ᵢ, P₉/K, and the pump coupling ratio was used to analyze the data from the Cl-free experiments. To assure a steady state condition before the addition of ouabain, the cells were in normal saline for 2 h followed by 2 h in Cl-free saline before any experimental manipulation. The K concentrations used in these experiments were 2.5, 5, 10, 15, and 20 mM. Experimental values obtained for seven neurons measured in this fashion are shown in Table III. The mean values of resting potential, internal K concentration, the P₉/K ratio, and the electrogenic pump coupling ratio were not significantly different at the 5% level from the values obtained in Cl-containing saline. The difference in the means was tested using an analysis of variance.

**TABLE III**

Values Calculated for [K]ᵢ, the P₉/K Ratio, and the Pump Coupling Ratio in Cl-free Saline

| Experiment | Resting potential (mV) | Ouabain depolarization (mV) | Correlation coefficient | [K]ᵢ (mM) | P₉/K | Coupling ratio |
|------------|------------------------|-----------------------------|------------------------|------------|------|----------------|
| MN121091   | -51                    | 2.0                         | 0.96                   | 179        | 0.017| 1.3            |
| MN121095   | -51                    | 3.0                         | 0.94                   | 242        | 0.040| 1.3            |
| MN121193   | -62                    | 3.9                         | 0.98                   | 232        | 0.018| 1.7            |
| MN121191   | -64                    | 1.5                         | 0.99                   | 264        | 0.012| 1.5            |
| MN120693   | -52                    | 4.5                         | 0.89                   | 181        | 0.028| 1.6            |
| MN120403   | -64                    | 2.3                         | 0.96                   | 166        | 0.013| 1.45           |
| MN110785   | -60                    | 2.0                         | 0.98                   | 222        | 0.021| 1.24           |

-57.7±2.3 2.7±0.4 212±14 0.021±0.004 1.41±0.07
after a log transformation of data presented as ratios. However, the ouabain depolarizations in the Cl saline and Cl-free saline were significantly different ($P < 0.05$). While the values for the average resting potential, internal K concentration, and the $P_{\text{Na}}/P_{\text{K}}$ ratio in Cl-free saline are not significantly different from the values obtained in Cl-containing saline, their averages are all slightly lower than the values obtained in Cl-containing saline. Although these differences are slight, a possible explanation may be that the Cl replacement ion decreased $P_{\text{Na}}$ (reflected in the decrease in the $P_{\text{Na}}/P_{\text{K}}$ ratio), thereby reducing the internal [Na] and consequently the pump contribution to the membrane potential. This would also account for the slightly depressed membrane potential as well as the slightly decreased internal K concentrations. However, regardless of this speculation and the total pump contribution to the membrane potential, the electrogenic pump coupling ratios measured in the two different salines were essentially the same.

**Na Injection Experiments in Normal Saline**

The second approach used to determine the electrogenic pump coupling ratio was the interbarrel injection technique described by Thomas (1969). This technique was used by Thomas to demonstrate a nearly 3:2 coupling ratio of Na to K in *Helix* neurons under a Na-loaded condition. Under similar Na-loaded conditions, a 3:2 ratio was reported previously in lobster neurons using a similar injection technique (Livengood and Kusano, 1972). Experiments described here augment that study using the same injection technique.

A major reservation with the interbarrel injection technique has been the inability to determine the exact amount of Na injected from the microelectrode into the neuron (De Weer, 1975). Thomas (1976, 1982) showed that $\sim 0.93$ of the current that passed through a Na acetate electrode was carried by Na. Na-citrate is a somewhat more complicated salt in that it has 3 Na per citrate anion. To determine the amount of Na injected from a Na-citrate electrode, an isotopic method was used (see Methods). Current was passed between two matched 24Na-citrate electrodes whose tips were immersed in a vial containing $0.4 \text{ ml of } 250 \text{ mM K isethionate}$. The amount of Na injected into test vials by the microelectrodes was plotted against the Na concentration calculated from the charge transferred by the electrodes (Fig. 5). The solid line in Fig. 5 represents the value that would be obtained if the Na transfer number were 1. Linear regression analysis on the data from four representative microelectrodes yielded a slope of 0.92 with a correlation coefficient of 0.99. Therefore, $\sim 92\%$ of the current passed through a Na-citrate microelectrode is carried by Na. The factor 0.92 was used in subsequent calculations of the amount (load) of Na injected into lobster neurons.

Fig. 6 shows a representative experiment involving iontophoretic injection of sodium into a lobster neuron. Downward deflections are voltage responses to injected current pulses that were used to determine the membrane input resistance of the neuron. An injection of 40 nA of current for 70 s produced a Na load of $\sim 29 \text{ peq}$. This Na load produced a 6-mV hyperpolarization resulting from activation of the electrogenic Na pump. In the injection experiments, it is assumed that the cell will pump out all the injected Na until
it returns to a steady state Na level equivalent to the level that existed before the Na injection. The electrogenic pump coupling ratio can be calculated from this type of experiment by the following relationship:

$$CR = \frac{TLI}{(TLI - LUC)} = \frac{Na}{K},$$

**Figure 5.** Plot of the equivalents of $^{24}\text{Na}$ ejected from four different $^{24}\text{Na}$-citrate microelectrodes vs. the theoretical number of Na equivalents as calculated by measuring the charge passed by the electrode. Current was passed between two microelectrodes inserted in a vial containing 0.4 ml of 25 mM K isethionate. The line represents the theoretical value for a one-to-one correspondence.

**Figure 6.** Strip-chart recording of the membrane potential change recorded in a lobster cardiac ganglion cell in response to injection of a measured quantity of Na into the cell. The upper trace indicates the current passed between two of three microelectrodes inserted into the cell. The downward deflection of $\sim 40 \text{nA}$ and 70 s represents a load of 29 peq. The middle trace is the membrane potential. The bottom trace indicates the transmembrane current injected to measure the membrane input resistance. The current calibration mark is the same for both current traces.
where $CR =$ electrogenic pump coupling ratio, $TLI =$ the total load of Na injected into the cell, and $LUC =$ the component of the extruded Na not coupled to the uptake of K, which is therefore the net charge transfer across the cell membrane, which is responsible for the electrogenic pump hyperpolarization. $LUC$ is calculated by determining the time-voltage area determined by the electrogenic pump hyperpolarization and then dividing this value by the calculated membrane resistance. Since $TLI$ is the total amount of Na injected and since $LUC$ is the uncoupled part of the extruded Na responsible for the membrane potential change, $TLI - LUC$ must equal the amount of coupled change transported across the membrane and would therefore be the equivalent of the K (presumably) taken up by the pump.

Another problem associated with the injection technique (De Weer, 1975) is that in the process of injecting Na, anions may also be injected into the cell. With Na-citrate microelectrodes, 92% of the current delivered by the anode was calculated to be carried by Na. The remaining part of the current is carried by an unknown mix of anions and cations from both the microelectrodes as well as cellular ions. As the major counterion in the microelectrodes some citrate ions may also be injected into the cell. In the absence of experimental evidence, the amount of citrate injected into the cell is unknown. Any injected anion, however, may cause passive diffusion potentials. Several approaches were used to test this possibility. The first approach is illustrated in Fig. 7. A current was passed between a Na-citrate electrode and a K-citrate electrode in one direction to inject Na-citrate into the cell (Fig. 7A), which resulted in a pump-activated hyperpolarization. The current flow

![Figure 7](image-url)
was then reversed in direction between the two electrodes to inject K-citrate into the cell (Fig. 7B). Any long-lasting potential changes seen after the K-citrate injection may have been a result of an anion diffusion potential. In this experiment there was no long-lasting change in potential due to the K-citrate injection. The second test for anion effects is shown in Fig. 8. Na-

![Figure 8](image)

**Figure 8.** Strip-chart recording of the response of a cardiac ganglion cell to four different injections of Na under different external conditions. The upper trace represents the injection current, and the lower trace represents the voltage response of the ganglion cell. (A) Injection of 38 peq while the chamber containing the ganglion was perfused with 15 mM K saline. (B) Injection of 38 peq after the bathing saline had been changed to K-free saline for 8 min. (C) Injection of 34 peq after the ganglion had been perfused with K-free saline for 1 h. At the arrow, the bathing saline was changed to 15 mM K saline. (D) Injection of 38 peq after the cell had been returned to a 15 mM K saline for ~1 h.

citrate was injected in the presence and in the absence of external K-containing saline. In Fig. 8A, in normal lobster saline containing 15 mM K, an injection of 38 peq of Na into the cell yielded an 8-mV hyperpolarization of the membrane potential. 10 min after switching to a K-free lobster saline, which perfused the chamber at a low rate, a 38-peq injection of Na-citrate produced a much reduced change in membrane potential (3 mV). 1 h after changing to K-free saline, an injection of 34 peq of Na-citrate into the cell
produced no change in membrane potential. The arrow in Fig. 8 shows when the solution was rapidly changed back to one containing 15 mM K. This resulted in the onset of a long-duration membrane hyperpolarization because of the K activation of the electrogenic Na-K pump. In Fig. 8D, after the stabilization of the membrane potential following the return to 15 mM K, a subsequent injection of 36 peq of Na-citrate into the neuron once again resulted in an 8-mV membrane hyperpolarization. Experiments presented in Figs. 7 and 8 indicate that citrate as a counterion produced no net diffusional potential. On the other hand, injection experiments in the lobster ganglia

\[
\text{Table IV}
\]

| Experiment| Resting potential| Input resistance (MΩ)| Load injected (peq)| Uncoupled load pumped out (peq)| Coupling ratio |
|-----------|------------------|----------------------|-------------------|-----------------------------|--------------|
| 1 167     | −55              | 1.2                  | 44.2              | 14.9                        | 1.51         |
|           |                  | 1.2                  | 24.3              | 11.2                        | 1.85         |
| 1 185     | −53              | 2.8                  | 35.0              | 10.5                        | 1.43         |
| 1 186     | −50              | 0.6                  | 26.5              | 10.9                        | 1.70         |
|           |                  | 0.6                  | 37.5              | 19.1                        | 2.04         |
|           |                  | 0.6                  | 39.7              | 11.0                        | 1.38         |
| 1 W23     | −60              | 1.3                  | 25.9              | 7.3                         | 1.44         |
|           |                  | 1.4                  | 32.2              | 9.2                         | 1.40         |
|           |                  | 1.4                  | 36.8              | 9.0                         | 1.32         |
| 1 W16     | −55              | 2.0                  | 38.6              | 13.5                        | 1.54         |
|           |                  | 2.0                  | 35.0              | 11.2                        | 1.47         |
|           |                  | 2.0                  | 35.0              | 16.0                        | 1.84         |
|           |                  | 2.0                  | 58.9              | 22.5                        | 1.62         |
| 1 W24     | −60              | 1.3                  | 27.2              | 8.9                         | 1.49         |
|           |                  | 0.8                  | 47.3              | 15.7                        | 1.50         |
|           |                  | 2.4                  | 31.5              | 6.1                         | 1.24         |
| 1 W76-1   | −60              | 2.9                  | 36.7              | 8.7                         | 1.28         |

(Average) −56.1 ± 1.5 (Average) 1.54 ± 0.05

using other anions did on occasion yield results that could be interpreted as being due to an anionic diffusion potential. It was previously shown that an injection of potassium and lithium acetate salts into the lobster cardiac ganglia neurons can result in a long-lasting, several-millivolt depolarization of the membrane potential (Livengood and Kusano, 1972) (see Fig. 10). The results of 19 injections in 7 different preparations are shown in Table IV. The average coupling ratio calculated for the 19 injections presented here was 1.54, or ~3 Na pumped out for 2 cations pumped in, presumably K. The range of injections was 24–59 peq. The average volume for 20 visually measured cells was 1.4 × 10⁻⁶ cm². A range of injections of 24–59 peq would be expected to increase the internal Na concentration of an average cell by...
17–42 meq/liter. However, no significant effect of Na load could be observed on the pump coupling ratio.

**Na Injection Experiments in Na-free Saline**

In a small number of experiments, a comparison was made of electrogenic pumping in the presence and absence of external Na in the bathing saline. An example of such an experiment is presented in Fig. 9. In Fig. 9A, an injection of 36.7 peq of Na into a lobster neuron resulted in an 8-mV hyperpolarization. After changing the bathing saline to one in which Na was replaced with choline, an injection of 28.2 peq of Na resulted in a 15-mV hyperpolarization. The small upward deflection in the bottom trace of Fig. 9, A and B, reflects a small transmembrane leakage of current (1 nA) caused by a slight mismatch of the two constant current generators used to pass the iontophoretic current. This mismatch amounted to ~2% of the 50 Na of
current passed between the two iontophoretic electrodes. This caused a deflection in the voltage trace of ~3 and 2.3 mV, respectively. The current pulse and the membrane response used to calculate the input resistance of the cell were taken a few minutes before the section shown in this figure. Calculation of the pump coupling ratio obtained from four such injections in two preparations is presented in Table V. The average coupling ratio was 2, which corresponds to a 2:1 exchange of Na for K. This ratio is significantly different ($P < 0.05$) from the coupling ratios determined in the previous experiments; an analysis of variance following a log transformation of the ratios was used. In addition, the rate of relaxation of the pump potential (Fig. 9) was faster in the Na-free condition than in the Na-containing condition. This is shown graphically in Fig. 10 as a log linear plot of the decay of the electrogenic pump potential, averaged for four injections in Na-contain-

### Table V

| Experiment | Resting potential (mV) | Input resistance (M$\Omega$) | Load injected (peq) | Pumped out (peq) | Coupling ratio |
|------------|------------------------|-----------------------------|---------------------|-----------------|---------------|
| 1W16       | -52                    | 1.4                         | 66.6                | 35.1            | 2.12          |
|            |                        | 1.7                         | 60.8                | 32.0            | 2.11          |
| 1W76-1     | -58                    | 2.37                        | 28.15               | 12.7            | 1.82          |
|            |                        | 2.2                         | 18.6                | 9.0             | 1.94          |

(Average) 2.00±0.07

The rate of decay of the pump potential in the Na-free saline is approximately twice as fast as the decay rate in Na-containing saline, which indicates a faster clearing of the Na load.

### Discussion

The results from two different and independent methods indicate that under physiological or near-physiological conditions, the coupling ratio of the electrogenic Na-K pump is not significantly different from a 3:2 ratio. The values for the coupling ratio obtained in the ouabain experiments in the presence and absence of external chloride were not significantly different from those obtained after Na injection in the presence of external Na. Both the ouabain experiments and the three-electrode interbarrel injection experiments demonstrate that the electrogenic Na-K pump ratio seems to be independent of the internal Na load under a narrow range of increasing Na concentration. In addition, the small amount of citrate that is injected into the cell does not seem to cause any perceptible transient diffusional potential. The assumption that either Cl is passively distributed or that Cl permeability is low in comparison to K permeability seems justified by the results from the
Cl-free experiments. When the solutions were changed to Cl-free, methylsulfate-substituted saline, the average resting potential depolarized slightly. This change in the average membrane potential was approximately the same as the decrease in the average ouabain depolarization in the Cl-free saline. One possible explanation for this observation is that methylsulfate has a slightly inhibitory effect on the Na permeability and therefore on the total pump activity. This could also account for the slight difference in the calculated internal K concentrations, which, although it is not significant, indicates the possibility of a reduction in the internal K concentration caused by the decrease in pump activity in the Cl-free methylsulfate-substituted saline.

![Figure 10](image-url)
The values obtained for the internal K concentration from the ouabain experiments fit well within a range obtained from other marine animals. Table VI shows some representative values for $P_{Na}/P_{K}$ ratios, internal K concentrations, and the electrogenic pump coupling ratios. In addition, the undershoot following the burst potential in the lobster cardiac ganglion is due primarily to an increase in K permeability (Livengood and Kusano, 1972). Using the reversal potential for the undershoot measured in 15 mM K saline of 69.5 mV, a value for the internal K concentration of 249 mM was calculated. This is close to the value for $[K]$ determined in the ouabain experiments and therefore gives a check on the validity of these measure-

**Table VI**

| Preparation                 | $[K]$ | $P_{Na}/P_{K}$  | Pump ratio | Reference                       |
|-----------------------------|-------|----------------|------------|---------------------------------|
| Anisodoris                 | 207.2 | 0.0478 (12°C)  | 1.9        | Gorman and Marmor (1974b)       |
| Aplysia                    | 232   |                |            | Sato et al. (1967)              |
| Aplysia                    | 197*  | 0.13           | 1.5        | Eaton et al. (1975)             |
| Aplysia                    |       | 0.10           |            |                                 |
| Lobster axon               | 235†  | 0.01           | 2          | Cooke et al. (1974)             |
| Lobster abdominal ganglion | 85.4  |                |            | Brinley (1965)                  |
| Lobster cardiac ganglion   | 254   | 0.03           | 1.5        | Zollman and Gainer (1971)       |
| Squid                      | 267-369‡ | 0.02-0.01   | 1.5        | Livengood (present paper)       |
| Squid                      |       |                |            | Abercrombie and De Weer (1978)  |

Concentration values are in millimolar except where noted.
* Activity.
† Millimoles per kilogram of axoplasm.

ments. The $[K]$ value calculated for the lobster cardiac ganglion neurons presented here (254 mM) is higher than the value obtained by Zollman and Gainer (1971) for lobster abdominal ganglia neurons (85.4 mM). However, Zollman and Gainer observed little or no activity of an electrogenic pump in these cells. The value obtained for the $P_{Na}/P_{K}$ ratio in the cardiac ganglion (0.03) also fits within a range observed for other marine animals (0.01–0.13; see Table VI).

The requirement for $[K]$ remaining relatively unchanged during the course of the experiment after the addition of ouabain is extremely important for the validity of the ouabain depolarization approach. For example, if the membrane permeability of a cell penetrated with a single microelectrode were the same as that of cells penetrated with three microelectrodes, then the Na load of the cells penetrated with a single electrode could be calculated. A plot of the amplitude of the pump potential vs. the load injected for the 19 injections shown in Table IV would suggest an average 9-mV potential for a 50-peq load. For an average cell volume of $1.4 \times 10^{-6}$ cm$^3$, this would suggest a potential change of 9 mV for an increase in [Na] of 36 meq. In
single-electrode experiments (Livengood and Kusano, 1972), the average pump potential for an exposure to 20 min in K-free saline was also 9 mV. A comparison of these two studies would suggest a cell loading of Na and a corresponding displacement of internal K amounting to 36 meq by 20 min after the Na pump was blocked. If the pump were blocked for a total of 80 min, the Na load would increase by 144 meq/liter with a corresponding shift of internal K by 144 meq. This would lead to a membrane depolarization of the cell by ~20 mV in control saline from the beginning of the experiment until the end. This is clearly not the case in these experiments (Fig. 3). Typically the potential was within 5 mV for each return to control saline. The explanation for this is twofold. First, the cells are selected for those that do not depolarize continuously (deviation from linearity). In this particular series of experiments, a total of 23 cells were stable enough during the control period to perform the ouabain-blocked experiment. Of these, seven were rejected for lack of linearity in response to the changes in [K]<sub,o</sub> after ouabain application. The second reason is that the cells that were penetrated by three microelectrodes are much "leakier" than cells penetrated by single microelectrodes, as is indicated by the difference in their average resting membrane potential, and therefore the load calculation for the single-electrode studies is an overestimate of the actual Na load. It should be obvious, however, that the ouabain block approach will not work for cell types that lose an appreciable amount of their internal K over the time course of the experiment.

The best-studied Na-K pump stoichiometry has been in red cells, first reported by Post and Jolly in 1957 to have a ratio of 3:2. Garrah and Glynn (1967) obtained values for the pump coupling ratio of 1.2 and 1.35, but when corrected for K-K exchange, the apparent Na-K coupling ratio was revised from 1.35 to 1.84. In a very careful study in Helix neurons using essentially the ouabain depolarization approach, Lambert et al. (1974) obtained a coupling ratio of 3:2. Thomas (1969), using the interbarrel injection technique in Helix, also obtained a value of close to 3:2 under Na-loaded conditions. Simchowitz et al. (1982) obtained a value for the pump coupling ratio in neutrophils that was much closer to a 3:2 ratio. Lieberman (1979), working with crayfish axons, reported a variable pump ratio dependent on external K concentration. Mullins and Brinley (1969) found a pump coupling ratio in squid that varied with changes in internal Na concentration from 1:1 at low concentrations of Na to 3:1 at high concentrations. Their value was close to 3:2 at physiological concentrations of internal Na. On the other hand, Abercrombie and De Weer (1978) found a pump coupling ratio in squid axon that was apparently fixed at 3:2 over a range of external K concentrations from 0 to 20 mM. Gorman and Marmor (1974a) found a pump coupling ratio of 1.9 in Anisodoris, which is a ratio of nearly 2 Na for 1 K. They argued that the differences in the coupling ratio in Anisodoris compared with the coupling ratio found in Helix by Thomas and by Lambert et al. might be due to the level of internal Na loading obtained by the different approaches used by the various authors. Gorman and Marmor were
very careful to ensure that the cells had reached a steady state condition before they made their measurements. In contrast to the ratio obtained by Gorman and Marmor, the present study reveals a 3:2 coupling ratio, with internal Na ranging from steady state to Na loaded. It is difficult to explain this difference, but it may indicate an inherent difference in the pump of these different cells. Cooke et al. (1974) also demonstrated an apparent electrogenic pump coupling ratio of 2:1 in Aplysia giant neurons. However, the difference between the result obtained in the present study and that reported by Cooke et al. may be explained by the fact that the latter experiments were performed in Na-free saline. When the lobster ganglia cells were exposed to Na-free saline, there also seemed to be a shift in the coupling ratio to 2:1.

In addition to the apparent increase in the electrogenic coupling ratio in Na-free saline, an increase also occurred in the rate of clearing of the Na load from the cell. The rate of clearing of Na in Na-free saline appears to be twice as fast as that in Na-containing saline. This may indicate a reduced activation of the Na-K pump in the presence of Na (Baker et al., 1969). Both the increase in the pump coupling ratio and the increase in the rate of Na clearance could be accounted for if the pump could cycle with an external configuration wherein the K sites are not saturated in the Na-free saline. If this hypothesis is correct, then in Na-containing saline, the external face of the pump molecule must be loaded with 2 K before it can cycle, because of inhibition by external Na; in contrast, in the Na-free condition, the pump can cycle either loaded or partially loaded, because of the removal of Na inhibition. Hobbs (1982) recently suggested from ouabain inhibition studies that in Na-free media the pump could cycle in squid axon with less than its full complement of K ions. The pump coupling ratio in the absence of external Na would be somewhat K dependent and would vary from 3:1 to 3:2 with changes in external K concentration. In this regard, Smith and Robinson (1981) found that in Ehrlich ascites tumor cells, the pump coupling ratio was variable depending on the external cations present; the ratio increased with an increase in external K and decreased with an increase in external Na.

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