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Sodium Efflux at 0 °C in Single Barnacle Muscle Fibers

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Summary. A study has been made of the efflux of radiosodium in single barnacle muscle fibers cooled to 0 °C. Cooling from 24 to 0 °C results in a rapid fall in the Na efflux, the magnitude of which averages 81%. Rewarming leads to almost complete restoration of the Na efflux. The Arrhenius plot shows no "breaks" and gives an E_a value of 14.2 kcal/mol. External application of 10^{-4} M-ouabain following cooling to 0 °C causes a fall in the residual efflux (~8%). Rewarming results in partial restoration of the Na efflux. Lowering external pH (pH_e) results in a rise in Na efflux at 0 °C, which peaks and declines rather slowly. The magnitude of the stimulatory response to acidification is a function of pH_e over the pH_e 7.00–5.8 range, the theoretical threshold being pH_e 7.1. The magnitude of the response to acidification of cooled, ouabain-poisoned fibers suspended in Li-ASW is the same as that of fibers suspended in Na-ASW. Injection of pure protein kinase inhibitor into fibers maintained at 0 °C fails to reduce the size of the response to acidification. Benzolamide but not acetazolamide, ethoxzolamide or Cl 13,580 abolishes the response to acidification. It also reverses the response to acidification. SITS is also able to abolish the response to acidification. An additional new observation is that the Na efflux at 0 °C is stimulated following the injection of $CaCl_2$ in a concentration-dependent manner. A similar response is not seen with $MgCl_2$. Acidification (pH_e 5.8) following peak stimulation by injection of $CaCl_2$ is without effect. These results add up to a refutation of the concept that the Na efflux at 0 °C is wholly passive and that the response to acidification involves Na:Na or Na:Ca exchange. The results also weaken the argument that stimulation of the efflux by acidification is the result of activation of carbonic anhydrase.

Key words. Na efflux, 0 °C, external acidification, and barnacle muscle fibers.

There is a great deal to be learnt about Na movements at a low environmental temperature by employing a single cell preparation, e.g., muscle fibers from the barnacle, *Balanus nubilus*. One distinct advantage of this approach is that the kinetic results are discrete and less ambiguous than those obtained with a population of cells. Another is that it allows any existing differences between cells to be seen. Because of its size, the barnacle fiber can be readily dissected from its bundle and injected axially with a microinjector. Thus, radiosodium and other substances can be introduced rather rapidly and directly into the myoplasm. Hence, the fibers to be used are relatively fresh before chilling.

During recent years, studies involving barnacle muscle fibers in a warm environment, e.g., 24 °C, have shown that the Na efflux is always very sensitive to external acidification (Bittar & Tong, 1971) and that the stimulatory response fails to occur in the absence of external HCO_3^- (Bittar, Danielson, Lin & Richards, 1977). These studies have also led to evidence that the response is completely reversible and involves the ouabain-insensitive component of the Na efflux. More recently, a closer investigation has revealed that the response is abolished by benzolamide and injecting $KHCO_3$ (Schultz & Bittar, 1978*b*) but not by injecting pure protein kinase inhibitor (Bittar, Demaille, Fischer & Schultz, 1979). The latter finding is consistent with evidence that the cAMP and cGMP content of these fibers fails to rise following external acidification (Bittar et al., 1979).

The purpose of this paper is to describe experiments which were carried out at 0 °C in an attempt to characterize the loss of Na and to find out whether external acidification stimulates the residual Na efflux when metabolic processes are at a minimum. New information about the mechanisms underlying the response to acidification is also included. A preliminary report of a part of this work has already appeared (Chambers & Bittar, 1977).

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Materials and Methods

Barnacle specimens of the species *Balanus nubilus* were obtained from Mr. David King at Friday Harbor, Washington. They were shipped by air in insulated styrofoam containers with ice packs to maintain the animals closer to their natural environmental temperature. The animals were kept in an Instant Ocean aquarium containing seawater made up from dissolved salts and trace elements supplied by Instant Ocean, Inc., Eastlake, Ohio. The concentrations of the major cations were about (mM): Na, 465; K, 10; Ca, 10; and Mg, 60. These concentrations were determined by atomic absorption and flame emission analysis. The seawater was maintained at a temperature of ca. 12 °C throughout.

Isolation and Cannulation

The dissection of a barnacle was carried out by bluntly separating the specimen into two laterally symmetrical halves, each half containing three of the six depressor muscle bundles, as described by Hoyle and Smyth (1963). Each half was further dissected so as to split the shell retaining the insertions of each of the lateral and rostral bundles. The operculum was similarly divided with bone cutters to isolate the tendon inserts of each bundle.

Single fibers were isolated with microscissors by cutting away nerve and connective tissue of the bundle sheath and then cutting the tendon close to the operculum. The tendon was then held with forceps and lifted under tension so that the connective tissue adjoining other fibers could be cut away. The isolated fiber was then held almost vertically above the surface of the bathing medium and severed from the shell. Next, the cut-end of the fiber was drawn over the tip of a glass cannula with the help of a loop of fine thread. The cannula itself was anchored to a movable plexiglass platform attached to a Palmer screw stand. A small, approximately 50 mg metal weight, was tied to the fiber tendon to ensure that it hung vertically when suspended in the bathing medium. A mixture of petroleum jelly and mineral oil was applied at the cut end so as to prevent dessication and direct contact between myoplasm and external fluid.

Barnacle Ringer's Solution

Artificial seawater (ASW) used in this work was composed of (mM): NaCl, 465; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 10; and $NaHCO_3$, 10, at pH 7.8. Experiments involving the omission of either K or Ca from the ASW had osmotically equivalent amounts of NaCl added to the ASW. In those experiments where Li-ASW was used, substitution of 10 mM $KHCO_3$ for $NaHCO_3$ and 465 mM LiCl for NaCl was made. Unless otherwise indicated, standard ASW at room temperature was used for the dissection and during the initial period of experiments.

The Microinjector

The microinjector employed in this work was similar to that designed by Mr. Warren Lin in this laboratory. It consists of a glass capillary (OD 2 mm, ID 1 mm) pulled rapidly after heating to form a long thin profile (10–15 cm) having a tip diameter of 110–120 µm. The capillary was filled with water, and then a 1-cm column of silicone fluid, MS 200/1000 cs, was introduced under pressure. A nichrome wire closely fitting the inner capillary was threaded inwards, thus expelling water out of the capillary tip. The tip was then sealed off with some molten shellac and the nichrome plunger pushed farther until silicone fluid flowed backward along the wire. The capillary was then mounted in a glass tube well (OD 5 mm, ID 3 mm), tapered at one end to just allow the capillary to pass through. Molten shellac was applied at the tip of the well, so as to unite both pieces. The injector was then mounted on a micromanipulator and the plunger anchored down. Such a plunger usually delivers 0.3–0.4 µl of test fluid per a 3-mm excursion of the micromanipulator. This would mean approximately a 100-fold dilution by the myoplasm, since the intrafiber fluid volume of the fibers used in these experiments may be taken as about 40 µl (the dimensions of the fibers being ~4 cm in length and 1–1.5 mm in diameter). A rule of thumb is that the intrafiber fluid phase is roughly 10 µl per 1-cm length of the fiber.

Cooling the Cannulated Fiber

This was achieved by constructing a cold block of plexiglass with an inlet and outlet for the passage of chilled fluid. The block contained wells, 4 ml in capacity, that could be filled with ASW at 0 °C. Water containing 30% ethylene glycol was cooled in a water bath using a refrigeration unit with a submersible cold coil (Benchscale, Dayton, Ohio). Water in the bath was cooled to −2 °C (which was previously determined as the temperature of water necessary to maintain the cold block at 0±1 °C) and pumped through the cold block with the aid of a "Little Giant" submersible fluid pump having a fixed flow rate of 3 gallons per min. The coolant in turn was recycled to the water bath.

The effluent was collected with a Pasteur pipette at 5-min intervals and replaced with fresh ASW at 0 °C. The design of the cold block was such that it could be rapidly moved, so that fibers were transferred to wells containing fresh ASW, while fluid in the previously occupied wells was collected and replaced. Rewarming of the chilled fiber was accomplished by transferring it out of the well to ASW at 22–24 °C (room temperature).

Radiosodium Measurements and Calculations

^{22}Na was supplied by Amersham-Searle Corporation as the chloride salt at an activity of 0.2 mCi per 1 ml of aqueous solution. The solution was dried down and redissolved in 0.2 ml of distilled water. Injection of ~0.3 µl of the "hot solution" into a fiber gave an activity of ca. 6×10^5 cpm, which was sufficient for the type of experiments reported here. Serial collections of the ^{22}Na effluent were counted using a Beckman Biogamma counter. Each sample was counted for 2 min at a voltage setting of 220 V and the discriminators set at 50 and 1000 for the lower and upper windows, respectively. Residual fiber activity was determined by transferring the fiber to a counting vial at the end of the experiment.

The data output from the scintillation counter as recorded on tape, was processed by a Digital Corporation PDP-12 computer, programmed to provide net efflux rates (cts/min/min) and fractional rate constants for each of the 5 min effluents. These were calculated as follows:

$$^{22}\text{Na efflux rate} = \frac{\text{Net effluent activity/min}}{\text{collection time (min)}}$$

$$\text{Rate Constant} = \frac{\text{Efflux rate}}{\text{Fiber count} + {}^{1}/_{2}\ \text{efflux}}$$

^{22}Na efflux was plotted on semilog paper vs. the midpoint of each collection period. Rate constants were plotted on linear graph paper vs. collection time.

Estimation of stimulation or depression of Na efflux was arrived at on the basis of the rate constant plot. Size of the stimulation was calculated by taking the difference between the maximum rate constant observed during the stimulatory phase and the rate constant just prior to the onset of stimulation. The size of a second stimulatory response was estimated by subtracting the size of the

first from that of the two combined responses. The size of the inhibition was calculated by taking the difference between the rate constant immediately prior to inhibition and the rate constant at maximal depression or the value obtained following retropolation of the last points on the curve to the time of application of the test agent.

Measurement of the Ion Content of Fibers

Measurements were made of the Na, K, Ca, Mg and Cl content of cannulated fibers before and after cooling down to 0 °C as well as after acidification at 0 °C. Na, Ca and Mg were assayed by atomic absorption spectroscopy and K by flame emission, using a Jarell-Ash atomic absorption spectrophotometer (Model 82-500), as described by Bittar et al. (1972). Assay of Cl content of these fibers was done using a chloride titrator (chloridometer), as described by Bittar (1971). Correction was made for extrafiber water space by taking its value as 6%. Intra-fiber water content was estimated by oven drying at 120 °C for 15 hr.

Agents

Ouabain, HEPES, and ethylene glycol-bis-(β-amino ethyl ether) N,N′-tetraacetic acid (EGTA) were purchased from Sigma Chemical Company, St. Louis, Mo. SITS (4-acetoamino-4′-isothiocyano-2,2′ stilbene disulfonate) was purchased from K and K Laboratories, Inc., Plainview, N.Y. Pure protein kinase inhibitor was obtained as a gift from Drs. J. Demaille and E. Fischer of the Department of Biochemistry, University of Washington, Seattle, Wash. The source of the inhibitor was rabbit skeletal muscle, and the procedure used for its purification to homogeneity was that described by Demaille, Peters and Fischer (1977). Benzolamide was a gift from Dr. T.H. Maren of the Department of Pharmacology and Therapeutics, University of Florida at Gainesville. Diamox (acetazolamide) and CL 13,580 [2(O-chlorobenzenesulfon-amido)-1,3,4-thiadiazol-5-sulfonamide] were obtained from Lederle, American Cyanamid Company, Pearl River, N.Y. Ethoxzolamide was obtained from Upjohn Company, Kalamazoo, Mich. Amiloride HCl was obtained from Merck Sharp and Dohme, Westpoint, Pa.

Results

Effect of Reducing the Temperature from 22 to 0 °C on Na Efflux

Fibers cannulated and then injected with ^{22}Na were first allowed to reach a steady state of sodium efflux at room temperature. This was usually achieved in less than 30 min. Subsequently, the environmental temperature of the fibers was rapidly changed to 0 °C and the efflux allowed to reach a new steady state. In some experiments, fibers were bathed in cold ASW immediately following the injection of ^{22}Na and prior to efflux collection.

Figure 1 shows that upon cooling there is a rapid and marked fall in the efflux. The size of the effect averages 81 ± 1.7% ($n=20$). Also shown is that upon rewarming to 22 °C, the efflux is almost completely restored (86 ± 3.7%, $n=9$).

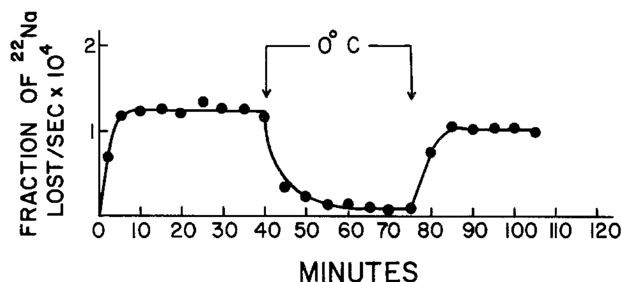


Fig. 1. Na efflux before and after cooling to 0 °C. Also shown is the response following rewarming to 22 °C (rate constant plot)

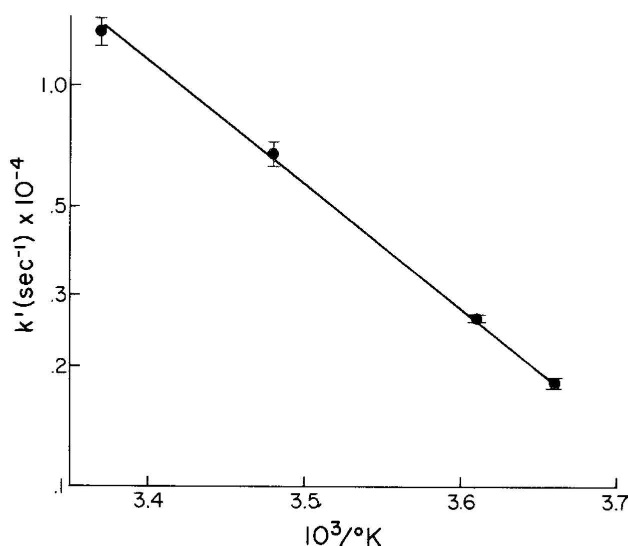


Fig. 2. Arrhenius plot of Na efflux over the temperature range 0 to 24 °C. Each plotted point is the mean value of 11 measurements

Arrhenius Plot for Na Efflux

The rate constant for Na efflux was determined in fibers that had been subjected to step-wise reduction in their environmental temperature. The Arrhenius plot obtained is shown in Fig. 2, where it can be seen that the plot gives a straight line with no break-points over the temperature range 0 to 24 °C. The slope of this line gives an E_a value of 14.2 kcal/mole. The complete absence of discontinuity in the Arrhenius plot is significant mainly because it strengthens the view that behavior of this kind is typical of the membranes of poikilotherms (Richardson & Tappel, 1962; McMurchie, Raison & Cairncross, 1973).

Ouabain Followed by Cooling and Vice Versa

As found by Bittar, Chen, Danielson and Tong (1973), the (Na^+-K^+)-ATPase inhibitor, ouabain, when applied externally causes a marked fall in the Na efflux in barnacle fibers maintained at room temperature. Figure 3*a* shows the effect of 10^{-4} M ouabain followed by cooling to 0 °C (74 ± 6.1% and 75 ±

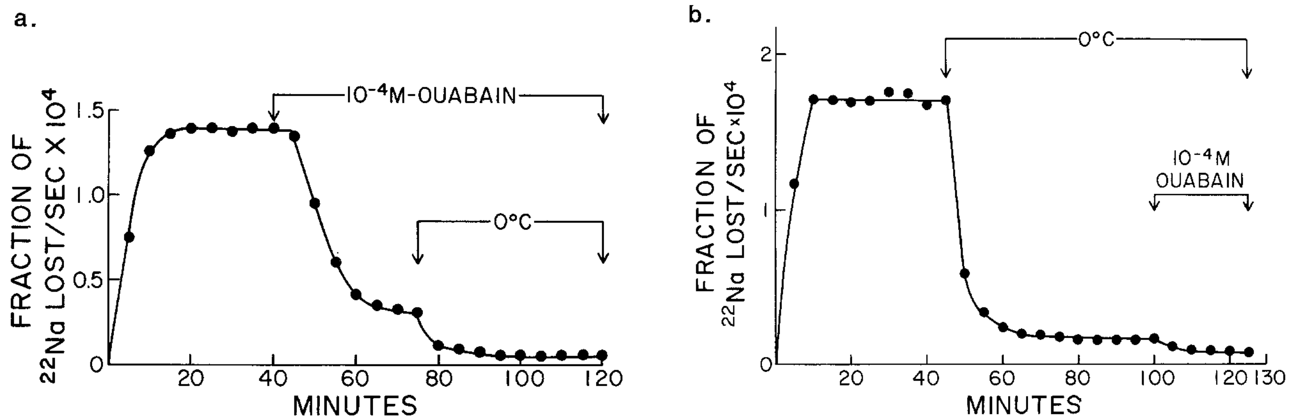


Fig. 3. (*a*): Response of the Na efflux to external application of 10^{-4} M ouabain, followed by cooling to 0 °C. (*b*): Response of the Na efflux to cooling to 0 °C followed by external application of 10^{-4} M ouabain

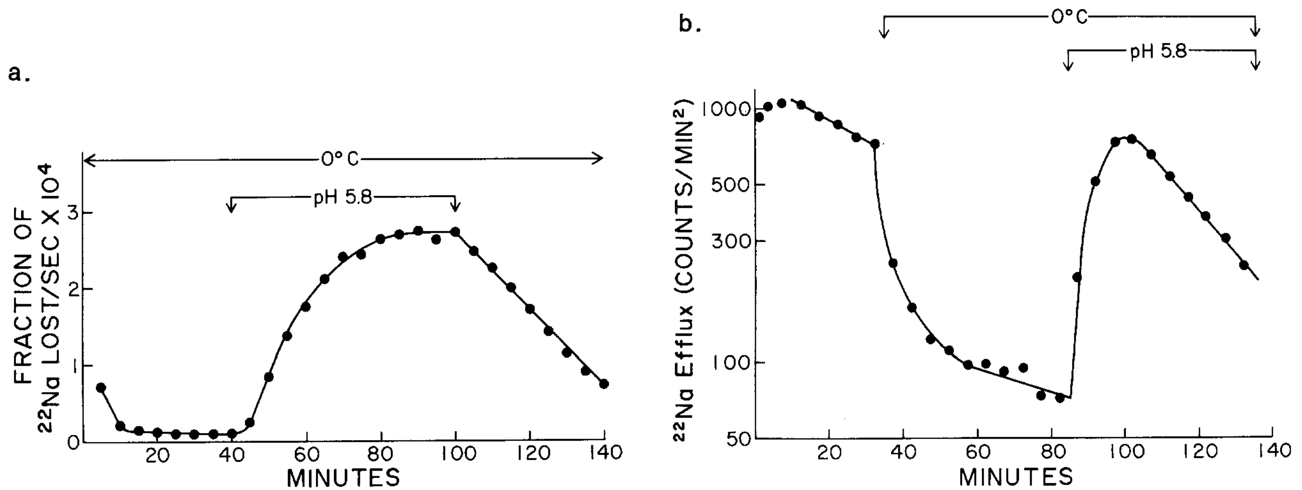


Fig. 4. (*a*): Response of the Na efflux at 0 °C to lowering of pH_e from 7.8 to 5.8, followed by restoring pH_e to 7.8. (*b*): Semilog plot of the Na efflux before and after acidification

4.2%, $n=6$, respectively). The combined fall is estimated as being 84 ± 5.7%. Figure 3*b* shows that external application of 10^{-4} M ouabain following cooling to 0 °C causes a decline in the residual efflux (7.9 ± 1.1%, $n=4$, the combined fall: 92 ± 1.7%). And upon rewarming to 22 °C, only partial restoration of the efflux occurs, the magnitude of the ouabain inhibition being 66.3 ± 11%, $n=8$. This value is comparable to that seen with 10^{-4} M-ouabain at room temperature, *viz.* 60.8 ± 5.6% ($n=6$).

Response of Na Efflux at 0 °C to External Acidification

As reported by Bittar et al. (1977), the component of the Na efflux that is sensitive to acidification of a HCO_3^--containing medium is ouabain-insensitive. Experiments were therefore done to explore the possibility that external acidification may also be stimulatory at 0 °C. As illustrated in Fig. 4*a*, a reduction in external pH (pH_e) from 7.8 to 5.8 results in a prompt rise in the Na efflux at 0 °C (averaging 1438 ± 256%, $n=11$) and return to pH_e 7.8 following peak stimulation results in almost complete reversal of the response. The kinetics of the rise and fall in the Na efflux at 0 °C following acidification are shown in Fig. 4*b*. The rise time in this particular group of fibers studied is 22 ± 1 min ($n=6$). However, a slower rise in fibers from other barnacle specimens is not unusual, e.g., 51 ± 4 min ($n=6$) and the $t\frac{1}{2}$ for the rate of decline (based on analysis of semilog plots of Na efflux in cts/min^2 *vs.* time in min) is 26 ± 2 min ($n=6$).

To determine whether the response to acidification depends on the presence of external HCO_3^-, experiments were done in which HCO_3^- was replaced by an inert buffer, e.g., HEPES (3 mM). The results of these experiments show a lack of effect following the lowering of pH_e from 7.8 to 5.8 ($n=8$). A similar

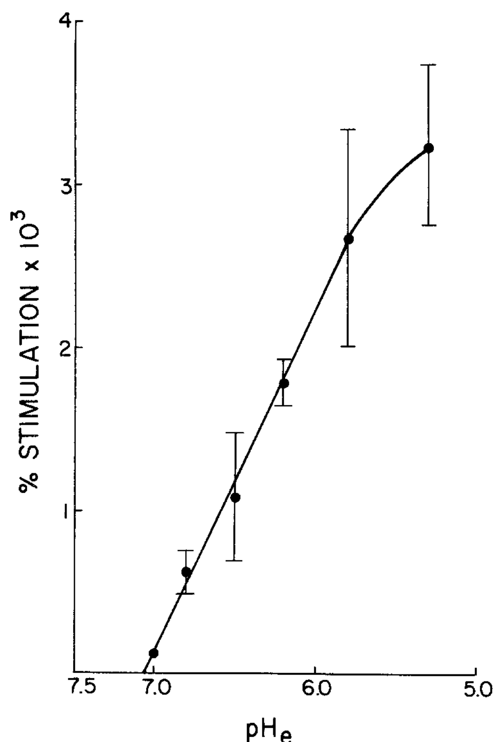


Fig. 5. Variation of Na efflux at 0 °C with pH_e. The fibers used were isolated from the same barnacle specimen. Each point represents the mean value of four measurements. Vertical bars indicate ±SE

result was reported for the Na efflux at room temperature (Bittar et al., 1977).

Variation of Na Efflux at 0 °C with pH_e

In view of the observed sensitivity of the Na efflux at 0 °C to external acidification, experiments were done by altering pH_e over the range 7.0 to 5.3. The results summarized in Fig. 5 indicate a linear relation between Na efflux and pH_e over the range 7.0 to 5.8. Extrapolation of the linear portion of the curve gives an intercept value of pH_e 7.1, which is the theoretical threshold of stimulation of the Na efflux at 0 °C. This value coincides with that found for ouabain-poisoned fibers at 22 °C (E.E. Bittar and E. Tong, *unpublished*).

Response to Acidification Following Ouabain and Cooling

The magnitude of the response of the Na efflux into 10^{-4} M-ouabain-ASW at 0 °C to lowering pH_e from 7.8 to 5.8 is found not to be significantly different from that observed in unpoisoned fibers at 0 °C (2413±256%, $n=11$, $P<0.2$). Also, the kinetics of the response resemble those of unpoisoned fibers.

Response at 0 °C to Acidification Followed by Rewarming

The response of fibers to sudden rewarming to 22 °C following peak stimulation by lowering pH_e from 7.8 to 5.8 is in the order of 93.7±5.2% ($n=7$). This was calculated by taking the difference between the maximum rate constant following rewarming and the rate constant immediately before acidification, and then subtracting the percentage increase due to acidification from the percentage (combined) rise. This rise upon rewarming differs from that usually seen upon rewarming in the absence of acidification in that it is initially transitory.

K Removal Followed by Cooling, Acidification, and Rewarming

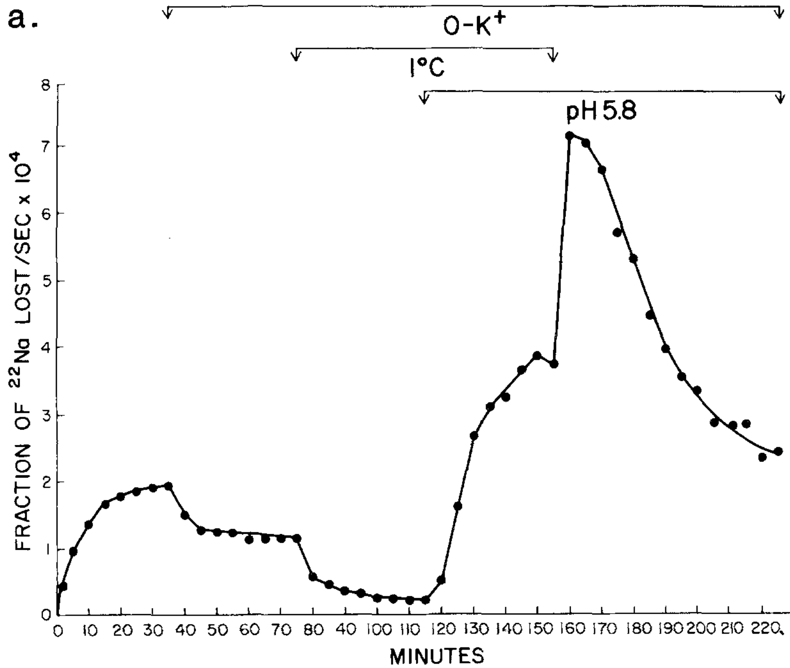
Shown in Fig. 6*a* is that cooling the fiber in a K-free medium leads to a further reduction in the Na efflux (80.3±1.8%, $n=8$) and that lowering pH_e from 7.8 to 5.8 causes a marked rise (2373±508%, $n=8$). This is further increased by rewarming to 22 °C after peak stimulation (2110±879%, $n=5$).

K Removal Followed by Ouabain, Cooling, and Acidification

Shown in Fig. 6*b* is the response to low pH_e of the Na efflux into K-free, 10^{-4} M ouabain-ASW at 0 °C. As can be seen, this also results in a marked rise of the efflux (5016±1004%, $n=6$). The value obtained is significantly greater than that obtained with unpoisoned fibers ($P<0.05$). It suggests that ouabain in the absence of external K enhances the response to acidification.

Response to Acidification of the Na Efflux into Li-ASW at 0 °C

It now became important to find out whether the response to acidification involves a mechanism of Na:Na exchange. Hence experiments were done with 465 mM Li-ASW. Shown in Fig. 7 is that a fiber suspended in 10^{-4} M ouabain-ASW at 0 °C is insensitive to the sudden omission of external Na, and to the addition of Li as the substitute. Lowering, however, pH_e from 7.8 to 5.8 causes a rapid and marked rise in the efflux (averaging 4363±1180%, $n=6$), which is not significantly different from that observed in controls suspended in regular ASW (2875±348%, $n=5$; $P>0.2$). Replacement of the external Li with Na is shown to cause a rather small stimulatory re-



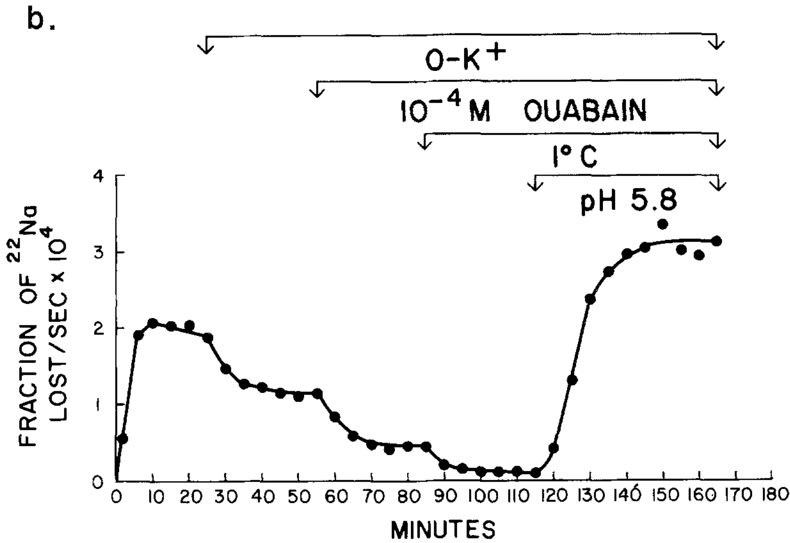


Fig. 6. (*a*): Response of the Na efflux into K-free ASW, followed by cooling to 0 °C and then lowering pH_e from 7.8 to 5.8 before rewarming. (*b*): The effect of 10^{-4} M ouabain on the response of the Na efflux into K-free ASW at 1 °C to external acidification

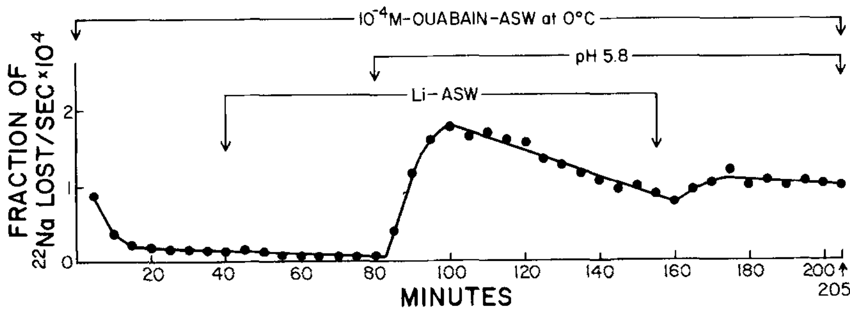


Fig. 7. Response of the Na efflux into 10^{-4} M ouabain-Li-ASW at 0 °C to external acidification, followed by restoration of external Na

sponse which is characterized by a rate of decline that is slower than that seen with Li-ASW. To exclude the possibility that Li *per se* might modify the response to acidification, experiments were carried out in which acidification of ASW was followed by replacement of Na with Li. This maneuver does not at all alter the course of the stimulated efflux ($n=3$). The obvious implication of these results is that the response to acidification is largely independent of external Na.

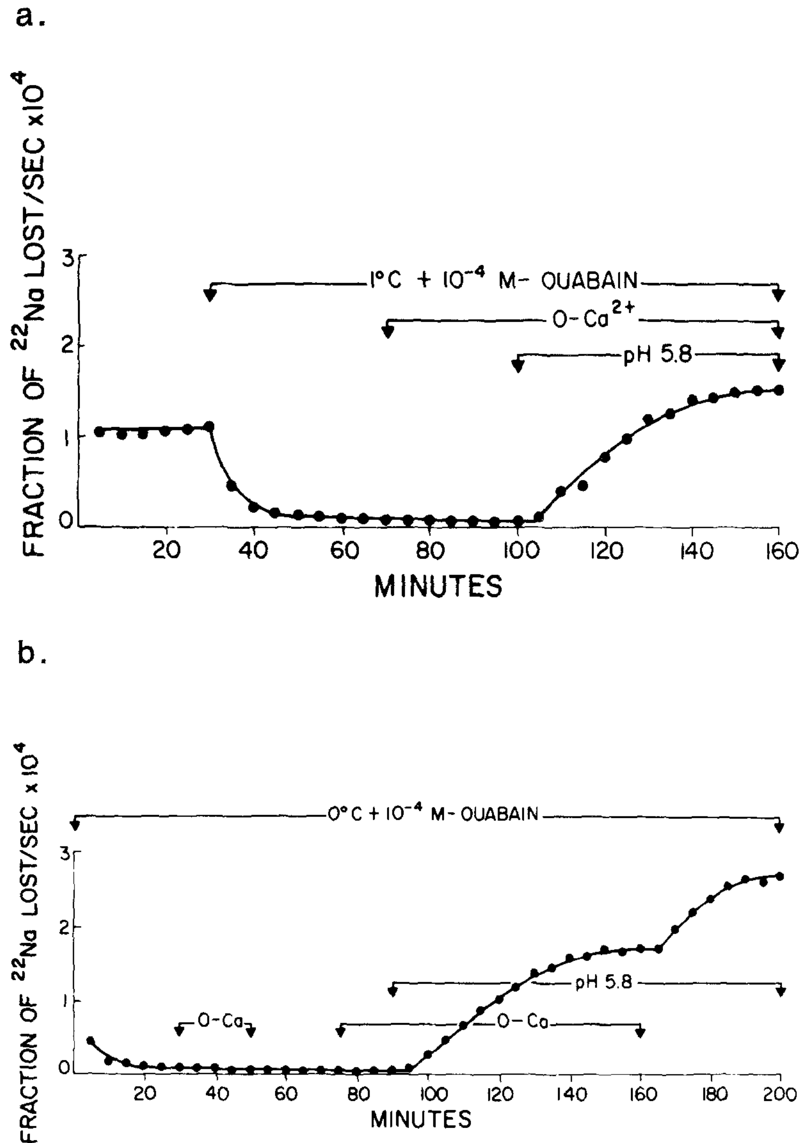


Fig. 8. (*a*): Response to external acidification of the Na efflux into Ca-free ASW at 1 °C, containing 10^{-4} M ouabain. (*b*): Lack of response of the Na efflux at 0 °C in the presence of 10^{-4} M ouabain to sudden omission and restoration of external Ca^{2+}, followed by external acidification in the nominal absence of external Ca^{2+}, followed by restoration of external Ca^{2+}

Amiloride Before Acidification

The pyrazine diuretic, amiloride, is thought to block Na transport, e.g., across frog skin (Eigler, Kelter & Renner, 1967) and toad bladder (Bentley, 1968) by preventing the entry of Na into the cell at the mucosal border. Keeping in mind the fact that the pK_a of amiloride is 8.7 (Cragoe, 1979) and that protonation of the guanidium NH_2 groups enhances the blocking action of the drug, fibers cooled to 0 °C were treated with 10^{-2} M-amiloride and found to show a response to acidification (pH_e 5.8) of 1333 ± 236% ($n=6$) *vs.* 2703 ± 591% in controls ($n=4$). However, the difference is not statistically significant, P being >0.05.

Response to Acidification in the Absence of $(Ca)_e$

The possibility that external calcium may be involved in the stimulatory response of the Na efflux at 0 °C to low pH_e was tested by cooling and applying 10^{-4} M ouabain, followed by Ca^{2+} omission and acidification. The results, as illustrated by Fig. 8*a*, show a stimulatory response of 5734 ± 1232% ($n=13$), as compared with a value of 2784 ± 393% found in controls ($n=8$). The difference is significant, P being <0.05. Parallel experiments were run in which external Ca was omitted for 20 min, restored for 25 min, and then omitted again before reducing pH_e from 7.8 to 5.8 and finally, restored after the onset of peak stimulation. $(Ca)_e$ restoration results in a rise averaging 2014 ± 286%, $n=8$ (Fig. 8*b*).

Lack of Effect of Injected EGTA on the Response to Acidification

The finding that the response to acidification partly depends on $(Ca)_e$ raised the question whether this involves a fall in myoplasmic pCa resulting from in-

creased Ca influx. Experiments were therefore carried out in which a 75 mM EGTA solution at pH 7.0 was injected into fibers already exposed to 10^{-4} M-ouabain at room temperature. The fibers were then cooled to 0 °C, and external Ca was omitted before acidifying the bathing medium. The results obtained show that the stimulatory response to a reduction in pH_e from 7.8 to 5.8 is in the order of $6667 \pm 1877\%$ ($n=4$), a value not significantly different from that obtained with controls, *viz.* $5349 \pm 740\%$ ($n=4$), P being > 0.5. In further experiments involving another barnacle specimen, ouabain-poisoned fibers were injected with 75 mM EGTA and cooled to 0 °C, and Ca was omitted before acidification, but the controls this time were left suspended in 10 mM Ca-ASW before reducing pH_e from 7.8 to 5.8. Th results show: $1696 \pm 405\%$ stimulation, $n=4$ *vs.* $828 \pm 170\%$, $n=4$, $P < 0.1$. Which means, then, that EGTA by stabilizing myoplasmic *p*Ca, tends to enhance the response to acidification when external Ca is present. A similar effect with EGTA is seen at 24 °C (Bittar et al., 1979). In additional experiments where fibers treated with 10^{-4} M ouabain were cooled to 0 °C, and injected with 100 mM EGTA following peak stimulation by acidification, no change in the course of the stimulated efflux is seen. Taken together, these results reinforce the conclusion that whereas myoplasmic *p*Ca plays no major role in the response to acidification, external Ca exerts a suppressing effect on the response.

Injection of Pure Protein Kinase Inhibitor Before Acidification

Bittar et al. (1979) found that injection of pure protein kinase inhibitor (PKI) fails to affect the response of the ouabain-insensitive Na efflux (at 22 °C) to external acidification. To see if this is also the case at 0 °C, 1.6×10^{-4} M-pure PKI was injected into fibers before cooling and external acidification. The magnitude of the response to reducing pH_e from 7.8 to 5.8 averages $2577 \pm 666\%$ ($n=4$), compared with $2660 \pm 614\%$ ($n=4$) in controls. It is hence concluded that the mechanism underlying the response to acidification does not involve newly formed cAMP (or cGMP).

Application of Acetazolamide and Benzolamide Followed by Acidification

There is evidence that barnacle muscle fibers possess carbonic anhydrase (T.H. Maren, *private communication*). There also is evidence that the powerful carbonic anhydrase inhibitor, benzolamide, is able to interrupt and reverse the response of the Na efflux to external acidification (Schultz & Bittar 1978*b*). To ascertain whether muscle fibers at 0 °C are similarly susceptible to blocking of the acidification response, initial experiments were done with acetazolamide (Diamox) by applying it externally and by injecting it before acidification. The results show that acetazolamide when applied externally in a concentration of 10^{-4} M to eight fibers and injected into six fibers in a concentration of 5×10^{-3} M is without effect on the size of the response to a reduction in pH_e from 7.8 to 5.8. This observation led to the next group of experiments which was carried out with benzolamide. The results obtained show that the response of the Na efflux at 0 °C in fibers pretreated externally with 5×10^{-4} M benzolamide averages $97 \pm 12\%$ ($n=4$), as compared with $1983 \pm 640\%$ found in controls ($n=4$) ($P < 0.05$). More experiments therefore were done but this time 5×10^{-4} M benzolamide was applied externally shortly after peak response to acidification. The results show that benzolamide causes prompt reversal and an almost complete return of the rate constant to the original baseline value ($n=12$). Application of benzolamide in a lower concentration, e.g., 10^{-6} M fails to appreciably reduce the response, no matter whether this is done before or after acidification ($n=6$). This is not the case with fibers in a warm environment, since $\sim 50\%$ inhibition was observed with 10^{-6} M benzolamide (Schultz & Bittar, 1978*b*). The reason for this difference in behavior towards benzolamide is obscure.

Additional experiments were carried out with two more derivatives of acetazolamide, ethoxzolamide, and CL 13,580. External application of 10^{-6} M-ethoxzolamide to two fibers cooled to 0 °C fails to reduce the magnitude of the response to acidification (pH_e 5.8). Trials with a 10^{-5} M-solution show a stimulatory response averaging $1356 \pm 178\%$ ($n=4$) *vs.* $1305 \pm 179\%$ in controls ($n=4$) (NS). Fibers treated externally with 10^{-5} M Cl 13,580 show a stimulatory response of $650 \pm 103\%$ ($n=4$) *vs.* $682 \pm 116\%$ in controls ($n=3$) (NS). It would thus appear that benzolamide is the only derivative among the four sulfonamides tested to have an effect and only when applied in high concentration.

Effect of SITS on the Response to Acidification

The disulphonic stilbenes, e.g., SITS, are powerful inhibitors of chloride transport in red blood cells (Cabantchik, Knauf & Rothstein, 1978), of Cl and H extrusion in the squid axon (Russell & Boron, 1976), Cl efflux in dialyzed barnacle muscle fibers (Russell & Brodwick, 1979) and undialyzed fibers (Bittar, Schultz & Tesar, 1980) and H efflux (Boron, 1977) in barnacle fibers. Fibers cooled to 0 °C and then

exposed to 2×10^{-4} M SITS show upon reducing pH_e to 5.8 a stimulatory response of 334 ± 92 ($n=6$), as compared with $1984 \pm 446\%$ in controls ($n=6$) ($P < 0.01$). SITS (10^{-4} M) was also applied following peak stimulation by acidification. The results show that SITS causes almost complete reversal of the response ($n=3$).

Response to Acidification Following $KHCO_3$ Injection

The stimulatory response to acidification in a warm environment is reduced in a concentration-dependent manner by injection of $KHCO_3$ and completely abolished by injecting a 2-M solution of $KHCO_3$ (Schultz & Bittar, 1978*b*). Fibers cooled to 0 °C and injected with 2M $KHCO_3$ show a reduced response to a lowering in pH_e from 7.8 to 5.8 ($448 \pm 209\%$ stimulation $n=4$ *vs.* $2845 \pm 801\%$ in controls, $n=4$, $P < 0.05$). Why total abolition does not occur is unclear.

The Ionic Profile of Fibers at 0 °C Before and After Acidification

Estimates were made of the Na, K, Ca, Mg and Cl content of cannulated fibers maintained at 0 °C for varying periods of time (3, 30, 60, 120 and 180 min). Determinations were also made at 10 and 50 min after external acidification (pH 5.8) on fibers cooled for 1 hr. The results obtained indicate no significant difference in water and electrolyte content between these fibers and uncannulated fibers selected for assay shortly before the test fibers were cooled.

Response to Injection of $CaCl_2$

Danielson, Bittar, Chen and Tong (1971) and Schultz and Bittar (1978*a*) reported that the Na efflux from unpoisoned and ouabain-poisoned fibers is stimulated in a dose-dependent manner by the injection of $CaCl_2$ and that the minimal concentration of $CaCl_2$ required for stimulating the ouabain-insensitive Na efflux is 10^{-6} M. The results of experiments at 0 °C show that the Na efflux rises rapidly following the injection of 1M $CaCl_2$ and that the response reaches a peak within 25 min and declines slowly afterwards. The magnitude of the stimulation averages $262 \pm 15\%$ ($n=6$). Fibers treated with 10^{-4} M ouabain and cooled to 0 °C fail to show a greater response to the injection of 1M $CaCl_2$, the stimulatory response being $206 \pm 52\%$ in size ($n=5$). In order to be certain that the active species is Ca^{2+} and not Cl^-, experiments were done in which 1M $MgCl_2$ was injected into cooled fibers ($n=6$). Similar experiments were also done by injecting 2M KCl ($n=4$). Both maneuvers fail to stimulate the Na efflux.

Additional experiments were done in which $CaCl_2$ in varying concentration was injected. The curve obtained shows that the minimal effective concentration injected lies in the region of 10^{-3} M, a value 3 orders of magnitude greater than that required in a warm environment. Thus the physiological significance of such data seems rather doubtful. Whether external Ca exerts a suppressing action on the response of $CaCl_2$ injection is not yet known.

$CaCl_2$ Injection Followed by Acidification

Fibers maintained at 22 °C are responsive to external acidification following injection of 1M $CaCl_2$ (Schultz & Bittar, 1978*a*). This, however, is not the case with cooled fibers, since lowering pH_e from 7.8 to 5.8 following peak stimulation by injecting 1M $CaCl_2$ results in no further rise in Na efflux ($n=6$). This also holds true of fibers pretreated with ouabain ($n=6$). On occasion, however, a slight stimulatory response to acidification after $CaCl_2$ injection in poisoned fibers at 0 °C is seen ($n=2$).

Discussion

The present results clearly show that sudden cooling to 0 °C leads to a rapid and marked fall in the Na efflux. The finding that this effect is almost completely reversed by rewarming is evidence that cooling of this degree does not adversely affect both the membrane and mechanism of Na extrusion. The absence of an overshoot in the Na efflux following rewarming to 22 °C is in line with the view that cooling fails to increase the internal Na concentration of the fiber, the wider view being that the outward and inward movement of electrolytes are affected by cooling to 0 °C to a similar extent. This is borne out by the relative constancy of the internal water content of the fibers studied at 0 °C.

Since ouabain reduces the Na efflux at 22 °C by 60%, and since cooling to 0 °C reduces the efflux by ~90%, it follows, then, that the Na efflux at room temperature can be divided, in an operational sense, into two major fractions, ouabain-sensitive and ouabain-insensitive. A similar division can be made of the efflux at 0 °C in view of the finding that the addition of ouabain after cooling causes a further fall in the efflux. Whether the residual efflux following the application of ouabain could be abolished by cooling below 0 °C is not yet known.

Convincing evidence has been brought forward that the Na efflux at 0 °C in the presence or absence of ouabain is markedly sensitive to a lowering of pH_e, provided the external buffer used is HCO_3, and that the threshold for activation of this response, pH_e

7.1, is the same as that found at 22 °C for the ouabain-insensitive Na efflux, as well as the efflux of Cl. Moreover, the observed response to acidification is rapidly and fully reversed by alkalinization, a situation resembling that seen at 24 °C (Bittar et al., 1977). As for the augmented response to acidification occurring when external Ca^{2+} is omitted, this can be accounted for not only in terms of the absence of the suppressing action of external Ca^{2+} but also membrane labilization. It should be mentioned, however, that an augmented response is not seen at room temperature (E.E. Bittar and R. Schultz, *unpublished*). The fact that fibers preinjected with EGTA and suspended in Ca^{2+}-free ASW show no change in sensitivity to acidification implies that the response is uninfluenced by myoplasmic pCa. The same is not true when external Ca^{2+} is present. It therefore seems very likely that the suppressing action of external Ca^{2+} involves an internal site. This reasoning is consistent with the evidence brought forward, showing that sensitivity to acidification is abolished by prior injection of calcium.

The key question now arising concerns the mechanism by which external acidification stimulates the efflux at 0 °C. Evidence though indirect, that the trigger event is more than just a fall in intracellular pH (pH_i) is provided by the observation that injection of a 2-M solution of $KHCO_3$ before acidification fails to obliterate the response. As has been argued previously, the requirement for the onset of a response is the addition of protons to a bathing medium which contains HCO_3^- and that the fixed threshold for activation of the Na efflux holds for pCO_2 but not for pH (Bittar et al., 1977; Schultz & Bittar, 1978*a*). That the response does not involve cyclic nucleotides is indicated by the fact that injection of pure protein kinase inhibitor fails to abolish or even reduce the response. This is in accord with the view held by Bittar et al. (1979) that the response to acidification observed at 24° C does not involve a rise in cAMP or cGMP content of the fiber. Failure of the sulphonamide derivatives tested, except for benzolamide in high concentration, to block the response, makes it highly unlikely that the carbonic anhydrase system plays any role here. This implies a change in thinking about the mode of action of benzolamide. Cousin, Motais and Sola (1975), working with erythrocytes, have concluded that the site of action of benzolamide is the cell membrane and not the carbonic anhydrase system. Support for a membrane site comes from the experiments carried out with SITS, which is known to act by binding to membrane protein.

There are grounds for thinking that the mechanism of exchange underlying the response to acidification does not in the main involve Na:Na or Na:Ca exchange. In the first place, the results obtained with LiASW argue against Na:Na exchange. By the same token, the experiments done with Ca-free ASW indicate that the response is independent of external Ca. This is in sharp contrast to the stimulatory response seen at 0 °C following the injection of cAMP since it wholly depends on external Ca (Bittar, Chambers & Schultz, 1976). A far more likely mechanism of exchange would seem to be Na:H exchange and/or outward movement of Na with Cl as the associated anion. Under such conditions a fall in pH_i due to the inward movement of H^+ is avoidable if Cl-HCO_3 exchange is possible. Not only has a mechanism of this type been proposed in work with barnacle fibers (Russell & Boron, 1976; Russell & Brodwick, 1979) but also evidence indicating that Cl efflux at 0 °C (and 24 °C) is markedly sensitive to acidification is available (Bittar et al., 1980). And secondly, the concept of Na:Na exchange can be tested by applying the flux ratio equation (Ussing, 1949) and seeing if the calculated voltage agrees with the measured value, assuming that the passive uphill outflux of Na equals the influx of Na. Taking $(Na)_e$ as 465 mM and $(Na)_i$ as 20 mM (at 0 °C), the Ussing equation leads to a voltage value of -75 mV. This is a departure from the recorded values of -35 to -40 mV for these fibers (E.E. Bittar and W. Lin, *unpublished; see also* DiPolo & Latorre, 1972; Fischbarg, 1972) and values of -20 to -25 mV following a lowering in pH_e from 7.8 to 5.8 for fibers whose E_m at 24 °C is about -60 mV (E.E. Bittar and W. Lin, *unpublished*).

Applying the Hodgkin-Katz equation (Hodgkin & Katz, 1949), the derived P_{Na} values at 24 and 0 °C are 370×10^{-9} cm/sec and 10×10^{-9} cm/sec for a fiber whose E_m is -60 mV at 24 °C and -30 mV at 0 °C, respectively. For the case where at 0 °C pH_e is reduced from 7.8 to 5.8, one would expect the rate constant for Na efflux to rise 14-fold. Taking, then, J_{Na} as ~ 14 pmol·cm^2·sec (the corrected absolute Na efflux at 0 °C being ~ 1 pmol·cm^2·sec), a P_{Na} value of ca. 82×10^{-9} cm/sec is obtained if the E_m of the fiber at 0 °C and pH_e 5.8 is -20 mV. Though the chemical basis of this marked increase in P_{Na} is far from clear, it suffices to say that on energetic grounds a pore rather than a carrier mechanism is the more likely. A pore requires an activation energy of 6 kcal/mole, whilst a carrier requires four times as much (Parsegian, 1969).

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