8-1987

Sodium/Proton Exhange in Membrane Vesicles From Crab (Callinected Sapidus and Carcinus Maenas) Gill

Robert Eric Shetlar

Abstract

Amiloride-sensitive Na^+/H^+ exchange in plasma membrane vesicles prepared by sucrose density gradient centrifugation of mitochondria-rich posterior gill cells in two species of crabs (*Callinectes sapidus* and *Carcinus maenas*) was examined by acridine orange fluorescence quenching. Sodium-loaded vesicles injected into sodium-free incubation medium (pH_{out} = pH_{in}) caused a quench in fluorescence indicating inward flux of H^+. Addition of Na^+, Li^+ or K^+ but not tetramethylammonium$^+$ to the external medium relieved the quench, indicating outward flux of H^+. Amiloride (0.5 mM) partially inhibited relief by Na^+ or K^+. Vesicles prepared from blue crabs (but not green crabs) acclimated to 5 ppt seawater showed greater total exchange activity per mg protein than vesicles from animals acclimated to 35 ppt seawater, suggesting that activation of Na^+/H^+ exchange may be part of the mechanism by which blue crabs are able to adapt to reduced salinities.

SODIUM/PROTON EXCHANGE IN MEMBRANE VESICLES FROM CRAB (*CALLINECTES SAPIDUS* AND *CARCINUS MAENAS*) GILL

By

Robert E. Shetlar

Approved:

David W. Towle
Chairman, Thesis Committee

John W. Ramsey
Member, Thesis Committee

Member, Thesis Committee

Richard W. Tipton
Member, Thesis Committee

Examining Committee

William S. Woodcott
Barbara A. Whitman
W. Robert Hayden
F. B. Loftfield
R. Dean Becker

Wm. R. West, Jr.
Milton R. Denney

SODIUM/PROTON EXCHANGE IN MEMBRANE VESICLES FROM CRAB (*CALLINECTES SAPIDUS* AND *CARCINUS MAENAS*) GILL

By

Robert Eric Shetlar

B.S., Emporia State University, 1985

A Thesis

Submitted to the Graduate Faculty

of the University of Richmond

in Candidacy

for the degree of

Master of Science

in

Biology

August, 1987

Richmond, Virginia

Acknowledgements

I would like to thank Dr. David W. Towle for guiding me through this project. His insight, assistance and friendship were of great value during the last two years. Thanks also are due to the members of my thesis committee, Professor Janet Nolin, Dr. John Bishop and Dr. Richard Topham, for suggestions concerning improvement of this work. I also would like to thank my colleague, good friend and roommate of the past two years, Torstein Hølleland, who made a special trip to the shores of Maine to collect crabs for this project. In addition I wish to extend my thanks to the University of Richmond graduate school for granting me the full assistantship which allowed me to come to Richmond and persue this degree.

Finally a very special note of gratitude to my family in Kansas for their spiritual, emotional and financial support throughout the last 28 years.

Table of Contents

Introduction

The existence of a sodium-ion-for-hydrogen-ion exchange mechanism in the membranes of certain epithelial cells has been demonstrated by many investigators (Murer et al., 1976; Tsai et al., 1984; Seiler et al., 1985). This mechanism has been shown to exchange one Na^+ for one H^+ (Aronson & Igarashi, 1986) and is thought to be more prominent in the brush-border membranes (Murer et al., 1976; Reenstra et al., 1981; Ives et al., 1983) of mammalian kidney tubules and intestinal tissues than in basolateral membranes of these tissues. The roles the exchanger plays in the cell may include intracellular pH regulation (Boron, 1986a,b; Montrose & Murer, 1986; Doppler et al., 1986) and cell volume regulation (Grinstein et al., 1986). Isolated membrane vesicles provide a simple experimental tool with which to study the transport properties of plasma membranes. This method allows problems associated with whole cell or animal studies, such as interfering metabolic reactions and tissue complexities, to be overcome rather easily. Additionally the composition of the intravesicular and the extravesicular medium can be changed to accommodate the experimental design (Hopfer, 1978; Sachs et al., 1980).

A model of Na^+ transport in the crab gill may include at least two components, the Na^+/H^+ exchanger in the apical membrane and the Na^++K^+-ATPase in the baso-lateral membrane. These two systems may work in concert to bring sodium first into the cell from the surrounding environment and next into

the blood from the cell (Figure 1). Recent work has demonstrated the existence of the Na^+/H^+ exchanger in membrane vesicle preparations of the gill tissue of the blue crab Callinectes sapidus (Rathbun), and of the green shore crab, Carcinus maenas, (Linnaeus) (Shetlar & Towle, 1986; Shetlar & Towle, 1987).

Many different methods exist that allow measurement of the activity of the Na^+/H^+ antiporter (the term antiporter implies that transport takes place in opposite directions, i.e. Na^+ inside is exchanged for H^+ outside or vice versa). One of the newest and fastest methods involves the use of pH-sensitive fluorescent dyes (Lee & Forte, 1978; Burnham et al., 1982; Sabolic & Burckhardt, 1983). These dyes distribute themselves across the vesicular membrane according to a pH gradient (Binder & Murer, 1986) and can therefore be used to monitor changes in intravesicular pH. When sodium-loaded vesicles are injected into a sodium-free incubation medium containing the dye acridine orange (pH inside the vesicle is the same as pH outside the vesicle, $pH_i=pH_o$) an immediate decrease in relative fluorescence intensity is observed (Figure 2). This decrease (termed fluorescence quench) is directly attributable to the action of the Na^+/H^+ exchanger (Sabolic & Burckhardt, 1983; Tsai et al., 1984; Barros et al., 1986). As sodium-loaded vesicles come in contact with the sodium-free medium the antiporter works to exchange sodium ions inside the vesicle for protons in the external

medium. This action leads to the formation of a pH gradient ($pH_o > pH_i$), and the dye molecules follow the pH gradient and move into the vesicle (Burnham et al., 1982; Warnock et al., 1982; Sabolic & Burckhardt, 1983; Ives, 1985; Sacktor & Kinsella, 1986). This effect can at least be partially reversed by adding excess sodium ion to the external medium forcing the exchanger to work in a reverse manner. The incomplete relief of fluorescence quench (Figure 2) may be explained by dye binding to the vesicle interior once it has traversed the membrane and is protonated, or by dye binding to the vesicle exterior (Binder & Murer, 1986). This method can be used to gather both qualitative and quantitative information about the Na^+/H^+ exchanger and could provide a valuable tool for further examining the mechanisms of this exchanger in the crab gill. The goals of this study were 3-fold: 1) to compare Na^+/H^+ exchange activity in two species of crab adapted to dilute and full-strength sea water; 2) to learn more about the cation specificity of the exchanger in these animals; and 3) to determine some kinetic parameters for the system in the blue crab.

Materials and Methods

Green shore crabs were collected from intertidal and subtidal areas adjacent to the Mount Desert Island Biological Laboratory, Mount Desert Island, Maine. Blue crabs were obtained from the Chesapeake Bay and its tributaries through

Virginia seafood suppliers. The crabs were maintained at 20 C (blue crabs) or 12 C (green crabs) on a 12 hour light/dark cycle in recirculated artificial seawater (Instant Ocean) at a salt concentration of 10 ppt (green crabs), 5 ppt (blue crabs) or 35 ppt (both crabs) for at least 1 week in each concentration prior to use. The water was filtered through a biological filter which was incorporated into the recirculation system. The animals were fed commercial squid once a week.

To prepare membrane vesicles, crabs were placed on ice for 30 minutes before sacrifice by bisection. Tissue regions containing mitochondria-rich cells from gills 7-9 (counting anterior to posterior) in the green crab and gills 6-7 in the blue crab were quickly dissected and immediately placed in ice cold homogenizing medium [0.25 M sucrose, 6 mM disodium ethylenediaminetetraacetic acid (EDTA), 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), adjusted to pH 6.8 with tris(hydroxymethyl) aminoethane (Tris)]. The tissue was then blotted, weighed, and placed in fresh homogenizing medium (5 ml/g tissue) containing 0.1% (w/v) sodium deoxycholate. The tissue was homogenized in a Teflon glass homogenizer. Six to eight ml of homogenate were layered on continuous sucrose density gradients (10-40%, 32 ml each) and centrifuged for 30 min at 100,000 x g in a swinging bucket rotor. The resulting gradients were inspected visually and the two membrane bands were carefully removed, corresponding to 12-15 ml from the center of each gradient.

The membrane fraction was diluted 10-fold with dilution-loading-resuspension medium (56 mM sucrose, 20 mM HEPES, 100 mM sodium gluconate, pH 6.8 with Tris), and was centrifuged at 100,000 x g for 1 h to isolate the membranes. The supernatant was decanted from the centrifuge tubes and further removed by carefully wiping the inside of each tube with a Kimwipe wrapped around the end of a wooden cotton tipped applicator. The pellets were resuspended in the dilution-loading-resuspension medium (at 5-10 mg/ml protein) using a Teflon microhomogenizer. This results in sodium-loaded vesicles with an internal pH assumed to be 6.8. Total protein in the preparation was determined by the method of Bradford (1976).

Assays of Na^+/H^+ exchange were conducted in a Na^+-free incubation medium containing 10 uM acridine orange, 20 mM HEPES, 36 mM sucrose and 100 mM tetramethylammonium (TMA) gluconate, pH 6.8 with Tris. Sodium-loaded vesicles (10 ul), prepared as described above, were injected into a cuvette containing 2 ml of the Na^+-free incubation medium ($pH_i=pH_o$) and changes in fluorescence were continuously monitored using either an Aminco-Bowman spectrofluorometer coupled to a strip chart recorder or a Gilford Instruments Fluoro IV spectrofluorometer and plotter (excitation at 493 nm and emission at 525 nm). The assay mixture was stirred throughout the experiment using a stirring motor attached to the top of the cuvette. The pH gradient which developed across the vesicle membrane

was collapsed by injection of 75 mM (final concentration) sodium gluconate into the cuvette.

Amiloride, a diuretic drug known to specifically inhibit the Na^+/H^+ exchanger, was used to distinguish between true Na^+/H^+ activity and other ion pathways which may be present in the membranes and contribute to the overall translocation of Na^+ or H^+ (Kruwlick, 1983). In whole animal studies on blue crabs, amiloride (included in the dilute seawater to which the crabs were acclimated) has been shown to partially inhibit Na^+ uptake (Cameron, 1979; Pressley et al., 1981). The amiloride sensitivity of the Na^+/H^+ exchange system in the present study was determined by adding amiloride (0.5 mM final concentration from water stock) to the cuvette 0.5-1.5 min after the addition of vesicles, followed by injection of Na^+ gluconate. The amiloride-sensitive portion of exchange was determined by subtracting the rate of quench relief observed in the presence of amiloride from that obtained in the absence of amiloride. Appropriate amounts of distilled water were added to controls.

To calibrate the dye response to a pH gradient, vesicles were loaded with pH 6.8 buffer as described earlier except the buffer contained 100 mM TMA gluconate instead of sodium gluconate. The vesicles were injected into 2 ml of incubation medium of pH 8.4, 7.5, or 7.1. The pH variation was brought about by altering the final concentrations of Tris and HEPES while all other components remained as previously noted.

This treatment results in vesicles with an internal proton concentration greater than that of the incubation medium into which they are injected. Thus dye would be expected to move into the vesicle giving a quench of fluorescence signal. In this way it was possible to construct a calibration curve relating pH to fluorescence quench.

In order to relate fluorescence quench to amount of vesicle protein, sodium-loaded vesicles from green and blue crabs were injected into 2 ml sodium-free incubation medium in 4 ul increments (up to 16 ul). Readings of the magnitude of quench were then taken after 2 min and plotted as relative fluorescence versus amount of protein added to the cuvette. This treatment made it possible to determine where the response to protein was linear, and this information was subsequently used as a starting point for further experiments.

Experiments were undertaken to determine the possible salinity dependence of the exchange activity as well as to compare the activity between the two species of crab. For these studies crabs were adapted to varying salinities as previously described. After an acclimation period of at least one week assays were conducted to determine exchange activity and comparisons were made between the groups.

Cation specificity studies were conducted to examine the exchanger's ability to substitute one cation for another. Sodium, lithium, potassium (all chloride salt) and TMA (as the gluconate salt) ions were also tested for their ability

to induce a collapse of the pH gradient and subsequent relief of the initial fluorescence quench. Tetramethylammonium ion was chosen because it is thought to be an inert cation which would not be expected to participate in any carrier mediated transport process, and as such would provide a control for the effect of adding large amounts of ion to the incubation medium. Cation specificity experiments were conducted using Callinectes sapidus adapted to dilute sea water.

In an attempt to better understand the kinetic properties of the blue crab gill Na^+/H^+ exchanger, vesicles from crabs acclimated to reduced salinity were used to examine the effects of increasing concentrations of sodium gluconate on the release of fluorescence from within the vesicle. Sodium-loaded vesicles were injected into 2 ml sodium-free incubation medium and the pH gradient was allowed to develop. After a period of 1 min, sodium gluconate was injected and the relief of quench was monitored as described previously. This was done repeatedly using increasing amounts of sodium gluconate (from 7.5 mM to 75 mM final concentration). In order to determine the rate, a tangent to the curve was drawn at 2 seconds after addition of sodium gluconate. These data were plotted as rate versus substrate concentration.

All chemicals used in these experiments were obtained from Sigma Chemical Co., St. Louis, MO.

Results

To calibrate dye response, pH differences were applied across the membrane. When sodium-free vesicles with an internal pH of 6.8 were added to 2 ml of incubation medium, which was alkaline with respect to vesicle interior, an immediate drop in fluorescence was noted indicating dye uptake into the vesicle. This drop was recorded six seconds after addition of vesicles and the values plotted as relative fluorescence quench against difference in pH (out-in). In this way it was determined that the magnitude of quench could be used to estimate the difference in pH across the membrane (Figure 3). This finding is in agreement with the work of other investigators (Burnham et al., 1982; Sabolic & Burckhardt, 1983), and indicates that acridine orange may be used as a probe of pH gradients in these vesicles.

The amount of protein in the vesicle preparations was found to have a direct effect on the magnitude of initial fluorescence quench (Figure 4). Sodium-loaded vesicles injected into 2 ml of sodium-free incubation medium showed a protein-dependent quench of fluorescence which was linear over a broad range (up to 100 ug protein). Similar results have been reported for vesicles from mammalian renal and intestinal systems (Hackovic et al., 1986). The curvature of the line above 100 ug protein may be explained by vesicular aggregation at these elevated protein concentrations. Such aggregation would tend to decrease the amount of vesicular

membrane in contact with the incubation medium and may thus cause a drop in the magnitude of quench below what might be expected. For further experiments the amount of vesicles added to the cuvette was kept within this linear range.

With the results of these preliminary experiments it was possible to begin looking at some specific aspects of Na^+ transport by the Na^+/H^+ exchange mechanism. In order to compare the exchange in two species of crabs, vesicles were derived from each as described in the methods section. Figure 5 depicts a typical experiment using sodium-loaded vesicles derived from Callinectes sapidus. The upper curve represents vesicles from crabs acclimated to 35 ppt salinity and the lower from crabs acclimated to 5 ppt salinity. Figure 6 is representative of experiments with sodium-loaded vesicles from Carcinus maenas acclimated to 35 ppt and 10 ppt salinity. While it appears at first glance that there is a difference, the reverse of what was seen in C. sapidus, statistical analysis reveals that there is no significant difference in transport rates between C. maenas acclimated to 35 ppt and C. maenas acclimated to 10 ppt (Table 1).

The amiloride sensitivity of the exchanger was determined by injecting vesicles into incubation medium and allowing the pH gradient to develop, followed by addition of 0.5 mM amiloride (final concentration). This was followed by injection of 75 mM sodium gluconate to induce fluorescence

quench relief. Amiloride itself has some optical properties which cause a further fluorescence quench upon addition to the incubation medium (Figure 7). More importantly amiloride does seem to inhibit relief of the quench thus allowing separation of Na^+/H^+ exchange from other transport phenomena which may contribute to overall Na^+/H^+ translocation. This inhibition was determined by measuring the rate of quench relief following injection of sodium gluconate in the presence of amiloride (Figure 7).

Figure 8 and table 1 present the results of the salinity study and the comparison between the two species of crabs. As already noted there was no significant difference in quench relief in C. maenas acclimated to either high or low salinity [P = 0.29 (student's t-test with significance set at P = 0.05 level of probability)]. On the other hand there was a significant difference in transport rates between blue crabs acclimated to either high or low salinity (P = 0.013). In comparing species it was found that blue crab vesicles from low salinity showed no significant difference from green crab vesicles from low salinity (P = 0.52) while those from high salinity did show a significant difference (P = 0.037).

Cation specificity studies revealed that the exchanger can use cations other than Na^+ in the process of proton translocation. In order to account for possible osmotic effects of adding large concentrations of ions to the incubation medium, sodium-loaded vesicles derived from C. sapidus were

injected into 2 ml incubation medium and a pH gradient was allowed to form. Then, either 45 mM sodium gluconate or 45 mM TMA gluconate was injected into the cuvette. Sodium caused a typical relief of quench while TMA pushed fluorescence lower (Figure 9). Further experiments along these lines revealed that the exchanger may use Li^+ or K^+ equally as well as Na^+. Figure 10 shows that there is no difference in mean quench relief regardless of the ion employed. Sodium gluconate did however exhibit a larger relief when used in this manner compared to the chloride salts of the other ions. This effect may be due to some other transport mechanisms involving chloride (e.g., Cl^-/HCO_3^- exchange) which may in some way reduce the magnitude of the pH gradient developed by the Na^+/H^+ exchanger and by so doing affect the amount of dye that moves into the vesicle. Further cation specificity experiments centered on the ability of potassium ion to produce effects similar to those observed with sodium ion. When potassium-loaded vesicles, made as previously described except that potassium gluconate replaced sodium gluconate, were injected into 2 ml of incubation medium a quench in fluorescence was observed. The relief of this quench was found to be amiloride-sensitive regardless of the ion employed to initiate the relief (potassium or sodium). Figure 11 illustrates these findings with vesicles from Carcinus acclimated to low salinity. Similarly, in experiments with sodium-loaded vesicles it was found that potassium ion (from potassium gluconate) produced a relief

of quench not unlike that observed with sodium ion. The potassium effect was also found to be amiloride-sensitive.

In an attempt to better understand the kinetics of the blue crab gill Na^+/H^+ exchanger an experiment was done which allowed the measurement of the effect of increasing amounts of sodium ion on the relief of fluorescence quench. It was determined that the system does not follow simple Michaelis-Menten kinetics, rather sigmoid kinetics were found (Figure 12). These data gave a $K_{0.5}$ (rather than a K_m) value of approximately 26 mM and a V_{max} of approximately 11.4 fluorescence units/sec/mg protein.

Discussion

The mechanisms of ionic regulation in osmoregulating aquatic animals has been an area of active research for many years, and it is generally accepted that the Na^++K^+-ATPase plays a central role in this scenario by extruding Na^+ from the cell into the blood (Epstein et al., 1967; Towle, 1984). This system is most likely part of a larger system which would include other transport phenomena working together to facilitate the movement of ions from the environment into the blood or from the blood to the environment. Membrane vesicles have been widely used to study these processes in a variety of organisms (Kinne-Saffran et al., 1982; Warnock et al., 1982; Lee & Pritchard, 1985; Barros et al., 1986). In the blue crab, vesicles have proven to be a valuable tool for exploring the

mechanisms of sodium ion transport (Towle & Hølleland, 1987). These and other studies have focused on the role of the Na^++K^+-ATPase in ionic regulation in animals subjected to varying salinities in hopes of better understanding the role this enzyme may play in adaptation to a euryhaline environment (Towle et al., 1976; Neufeld et al., 1980).

In the posterior gill of the blue crab and the green crab, Na^++K^+-ATPase has been shown to predominate in the basolateral membranes of a patch of thick epithelial cells (Towle & Kays, 1986). This finding suggests that other transport processes are also taking place here, and some evidence has been collected in support of this hypothesis (Towle, 1985). Of principle interest has been the Na^+/H^+ exchanger. This transporter has been demonstrated in all epithelial tissues which have been examined for its presence (Aronson, 1985). In mammalian renal and intestinal systems the Na^+/H^+ exchanger has been shown to be more predominant in the apical membranes implicating it in the entry of sodium ion into the cell and the movement of protons out of the cell. The separation of apical from basolateral membranes in the crab has as yet not been achieved. However, amiloride sensitive Na^+/H^+ exchange has been demonstrated by the present study in membrane vesicles from both the green crab and the blue crab.

The blue crab is a hyperregulating animal and thus maintains its blood concentration of sodium ion at a value greater than that of the surrounding medium when challenged with reduced

salinity (Ballard & Abbott, 1969). There is evidence that at least part of the mechanism of this regulation involves an increase in Na^++K^+-ATPase activity above that observed in animals acclimated to full strength seawater (Towle et al., 1976; Neufeld et al., 1980). The participation of other transport mechanisms in the overall ionic regulation in these animals then becomes an interesting problem with principal attention given to the Na^+/H^+ exchanger as a mechanism for sodium ion entry into the cell. It has been shown in the present study that this transporter may indeed play a role in the overall process. Blue crabs acclimated to reduced salinity exhibit a significant increase in amiloride-sensitive Na^+/H^+ exchange activity when compared to animals from high salinity. The baseline, or amiloride-insensitive portion of activity, remains unchanged (Figure 8). This observation lends support to the idea of an increase in Na^+/H^+ exchange as a mechanism by which the animal may compensate for reduced ion concentrations in the environment. Thus, the crab adapted to 5 ppt salinity may express an increase in transport sites within the membrane or a "turning on" of existing transport sites which are masked until the animal is in need of them.

Carcinus maenas, on the other hand, exhibited no significant transport rate difference in relation to acclimation salinity (Figure 8). This point is rather puzzling since this crab is also an osmoregulator though not as strong an osmoregulator

as is Callinectes. Experiments relating acclimation salinity to Na^++K^+-ATPase activity in this animal have given results not unlike those from the blue crab, i.e. reduction in environmental salinity leads to an increase in this enzyme's activity (Siebers et al., 1982). It might therefore be expected to show Na^+/H^+ exchange results similar to those observed in Callinectes though possibly not of as great a magnitude.

In comparing the species it was found that no significant difference existed between low salinity adapted animals ($P = 0.52$). There was however a significant difference between those animals from a high salinity environment ($P = 0.037$, Figure 8 and Table 1). The reason for the apparent difference in transport rates between Carcinus and Callinectes may be due to Carcinus membranes possessing less non-functional protein than the membranes from Callinectes. Carcinus membrane preparations were found to be consistently lower in protein when compared to identically prepared membranes from Callinectes. If Carcinus membranes possess more functional transport sites per mg protein (i.e. less non-functional protein) than membranes from Callinectes it may tend to make Carcinus transport rates appear artificially high (for comparison, rates are divided by the mg protein present in the sample).

The cation specificity of the Na^+/H^+ exchanger has been investigated in rabbit renal brush border membranes (Kinsella & Aronson, 1981), and rat renal brush border membranes

(Sabolic & Burckhardt, 1983). From these studies it is generally concluded that the exchanger is able to substitute Li^+ and NH_4^+ (but not K^+) for Na^+ or H^+ (Aronson & Igarashi, 1986). In the present study it was found that the Na^+/H^+ exchanger of Callinectes gill could also use Li^+ to induce relief of fluorescence quench (Figure 10). In addition, the exchanger in these membranes can apparently substitute K^+ for Na^+. This finding is rather curious in light of previously reported results for other Na^+/H^+ exchange systems. To distinguish Na^+/H^+ exchange that is able to utilize K^+ as well as Na^+ from a separate amiloride-insensitive K^+/H^+ exchange mechanism, which has been identified in rat ileum apical membranes (Binder & Murer, 1986), amiloride sensitivity studies were performed on potassium-loaded and sodium-loaded vesicles (data not shown). The amiloride sensitivity of the fluorescence quench relief, regardless of whether Na^+ or K^+ was used to induce relief, suggests a common exchange mechanism for both of these ions. Tetramethylammonium ion was unable to produce a relief of quench (Figure 9). The enhancement of quench by TMA may be due to osmotic effects on the vesicles. This finding is in agreement with results of Binder and Murer (1986) where rat ileal membranes were used. Experiments with NH_4^+ have as yet been inconclusive and additional experiments are necessary to fully understand the substitution of this ion.

Kinetic studies of Na^+/H^+ exchange with the fluorescence method in rat, rabbit and mouse has revealed $K_{0.5}$ values that are all seemingly close to one another [rat colon = 20 mM (Dudeja et al., 1986); rabbit kidney = 19 mM (Tsai et al., 1984); mouse kidney = 16 mM (Mackovic et al., 1986); blue crab, this study = 26 mM]. All of the above mentioned mammalian systems exhibit typical Michaelis-Menten kinetics indicating simple saturation of the transporter by increased levels of sodium ion. Results from experiments with the blue crab indicate another type of kinetics operating in this system, namely sigmoid kinetics (Figure 12). This observation seems to indicate that positive cooperativity is a factor in the blue crab Na^+/H^+ exchanger, and it would thus appear that there is perhaps a Na^+ binding site on the molecule other than the transporting site that can modify the exchanger in a way which enhances transport ability. Aronson et al. (1982) reported finding H^+ playing a modifier role in the allosteric activation of Na^+/H^+ exchange in rabbit renal cortex membranes. Further, Aronson raises the idea that the allosteric modification of the exchanger by proton may be helpful in extruding acid loads, and as such contribute to the regulation of pH. Perhaps the allosteric modification seen with sodium in the present work serves a similar function by enabling the animal to adjust more efficiently to ionic changes in the environment.

Acridine orange, a pH sensitive fluorescent dye, seems to be a valuable tool for investigating trans-membrane pH

gradients generated by the Na^+/H^+ exchanger in membrane vesicles from crab gill epithelium. This method has revealed that blue crab acclimation to reduced salinity is accompanied by an increase in amiloride-sensitive Na^+/H^+ exchange activity, and that the exchanger in blue and green crab membranes is capable of utilizing Li^+ or K^+ as well as Na^+. In addition, the sigmoid kinetics observed for the Na^+/H^+ exchanger in the blue crab indicate allosteric modification may play a role in activation of the transporter in this organism.

Literature Cited

Aronson, P.S. 1985. Kinetic properties of the plasma membrane Na^+-H^+ exchanger. Ann. Rev. Physiol. **47**:545-560.

Aronson, P.S., Nee, J. and Suhm, M.A. 1982. Modifier role of internal H^+ in activating the Na^+-H^+ exchanger in renal microvillus membrane vesicles. Nature. **299**:161-163.

Aronson, P.S. and Igarashi, P. 1986. Molecular properties and physiological roles of the renal Na^+-H^+ exchanger. Curr. Top. Memb. Trans. **26**:57-75.

Ballard, B.S. and Abbott, W. 1969. Osmotic accommodations in Callinectes sapidus Rathbun. Comp. Biochem. Physiol. **29**:671-687.

Barros, F., Dominguez, P., Velasco, G. and Lazo, P.S. 1986. Na^+/H^+ exchange is present in basolateral membranes from rabbit small intestine. Biochem. Biophys. Res. Comm. **134**:827-834.

Binder, H.J. and Murer, H. 1986. Potassium/proton exchange in brush-border membrane of rat ileum. J. Memb. Biol. **91**:77-84.

Boron, W.F. 1986a. Intracellular pH regulation in epithelial cells. Ann. Rev. Physiol. **48**:377-388.

Boron, W.F. 1986b. Approaches for studying intracellular pH regulation in mammalian renal cells. Curr. Top. Memb. Trans. **26**:15-33.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248-254.

Burnham, C., Munzesheimer C., Rabon, E. and Sachs, G. 1982. Ion pathways in renal brush border membranes. Biochim. Biophys. Acta **685**:260-272.

Cameron, J.N. 1979. Effects of inhibitors on ion fluxes, trans-gill potential and pH regulation in freshwater blue crabs Callinectes sapidus (Rathbun). J. Comp. Physiol. **133**:219-225.

Doppler, W., Maly, K. and Grunicke, H. 1986. Role of the Na^+/H^+ antiport in the regulation of the internal pH of Ehrlich ascites tumor cells in culture. J. Memb. Biol. **91**:147-155.

Dudeja, P.K., Foster, E.S., and Brasitus, T.A. 1986. Regulation of Na^+-H^+ exchange by transmethylation reactions in rat colonic brush-border membranes. Biochim. Biophys. Acta **859**:61-68.

Epstein, F.H., Katz, A.I. and Pickford, G.E. 1967. Sodium-and potassium-activated adenosine triphosphatase of gills: role in adaptation of teleosts to salt water. Science Wash. D. C. **156**:1245-1247.

Grinstein, S., Cohen, S., Goetz, J.D., Rothstein, A., Mellors, A. and Gelfland, E.W. 1986. Activation of the Na^+-H^+ antiport by changes in cell volume and by phorbol esters; possible role of protein kinase. Curr. Top. Memb. Trans. **26**:115-134.

Hopfer, U. 1978. Transport in isolated plasma membranes. Amer. J. Physiol. **234**:F89-F96.

Ives, H.E., Yee, V.J. and Warnock, D.G. 1983. Asymmetric distribution of the Na^+/H^+ antiporter in renal proximal tubule epithelial cell. J. Biol. Chem. **258**:13513-13516.

Ives, H. 1985. Proton/hydroxyl permeability of proximal

tubule brush border vesicles. Amer. J. Physiol. **248**:F78-F86.

Kinne-Saffran, E., Beauwens, R. and Kinne, R. 1982. An ATP-driven proton pump in brush-border membranes from rat renal cortex. J. Memb. Biol. **64**:67-76.

Kinsella, J.L. and Aronson, P.S. 1981. Interaction of NH_4^+ and Li^+ with renal microvillus membrane Na^+-H^+ exchanger. Amer. J. Physiol. **241**:C220-C226.

Kruwlick, T.A. 1983. Na^+/H^+ antiporters. Biochim. Biophys. Acta **729**:245-264.

Lee, H.C. and Forte, J.G. 1978. A study of H^+ transport in gastric microsomal vesicles using fluorescent probes. Biochim. Biophys. Acta **508**:339-356.

Lee, S-H. and Pritchard, J.B. 1985. Bicarbonate-chloride exchange in plasma membranes of blue crab. Amer. J. Physiol. **249**:R544-R550.

Mackovic, M., Zimolo, Z., Burckhardt, G. and Sabolic, I. 1986. Isolation of renal brush-border membrane vesicles by low speed centrifugation; effect of sex hormones on Na^+/H^+ exchange in rat and mouse kidney. Biochim. Biophys. Acta **862**:141-152.

Montrose, M.H. and Murer, H. 1986. Regulation of intracellular pH in LLC-PK1 cells by Na^+/H^+ exchange. J. Memb. Biol. **93**:33-42.

Murer, H., Hopfer U. and Kinne, R. 1976. Sodium/proton antiport in brush-border-membrane vesicles isolated from rat small intestine and kidney. Biochem. J. **154**:597-604.

Neufeld, G.J., Holliday, C.W. and Pritchard, J.B. 1980. Salinity adaptation of gill Na,K-ATPase in the blue crab, Callinectes sapidus. J. Exp. Zool. **211**:215-224.

Pressley, T.A., Graves, J.S. and Krall, A.R. 1981. Amiloride sensitive ammonium and sodium ion transport in the blue crab. Amer. J. Physiol. **241**:R370-R378.

Reenstra, W.W., Warnock, D.G., Yee, V.J., and Forte, J.G. 1981. Proton gradients in renal cortex brush-border membrane vesicles. Demonstration of rheogenic proton flux with acridine orange. J. Biol. Chem. **256**:11663-11666.

Sabolic, I. and Burckhardt, G. 1983. Proton pathways in rat renal brush-border and baso-lateral membranes. Biochim. Biophys. Acta **734**:210-220.

Sachs, G., Jackson, R.J. and Rabon, E.C. 1980. Use of plasma membrane vesicles. Amer. J. Physiol. 238:G151-G164.

Sacktor, B. and Kinsella, J.L. 1986. Hormonal regulation of renal Na^+-H^+ exchange activity. Curr. Top. Memb. Trans. 26:223-244.

Seiler, S.M., Cragoe E.J. and Jones, Jr., L.R.. 1985. Demonstration of a Na^+/H^+ exchange activity in purified canine cardiac sarcolemmal vesicles. J. Biol. Chem. 260:4869-4876.

Shetlar, R.E. and Towle, D.W. 1986. Sodium/proton exchange in membrane vesicles from crab (Carcinus maenas and Callinectes sapidus) gill. Bull. Mt. Des. Isl. Biol. Lab. 26:00-00.

Shetlar, R.E. and Towle, D.W. 1987. Sodium/proton exchange in membrane vesicles from crab gill: Response to salinity change. (Abstract) Fed. Proc. 46:1274.

Siebers, D., Leweck, K., Markus, H. and Winkler, A. 1982. Sodium regulation in the shore crab Carcinus maenas as related to ambient salinity. Marine Biol. 69:37-43.

Towle, D.W. 1984. Regulatory functions of Na^++K^+-ATPase in marine and esturine animals. In: Osmoregulation in Esturine and Marine Animals. Edited by A. Pequeux, R. Gilles, L.Bolis. Berlin: Springer-Verlag, 1984, p. 157-170.

Towle, D.W. 1985. Amiloride sensitive Na^+/H^+ exchange in membrane vesicles from crab (Callinectes sapidus) gill (Abstract). Am. Zool. 25:31.

Towle, D.W. and Hølleland, T. 1987. Ammonium ion substitutes for K^+ in ATP-dependent Na^+ transport by basolateral membrane vesicles. Amer. J. Physiol. 252:R479-R489.

Towle, D.W. and Kays, W.T. 1986. Basolateral localization of Na^++K^+-ATPase in gill epithelium of two osmoregulating crabs, Callinectes sapidus and Carcinus maenas. J. Exp. Zool. 239:311-318.

Towle, D.W., Palmer, G.E. and Harris III, J.L. 1976. Role of gill Na^++K^+ dependent ATPase in acclimation of blue crab (Callinectes sapidus) to low salinity. J. Exp. Zool. 196:315-322.

Tsai, C-J., Ives, H.E., Alpern R.J., Yee V.J., Warnock D.G. and Rector,Jr, F.C. 1984. Increased Vmax for Na^+/H^+ antiporter activity in proximal tubule brush border vesicles

from rabbits with metabolic acidosis. Amer. J. Physiol. **247**:F339-F343.

Warnock, D.G., Reenstra W.W. and Yee V.J. 1982. Na^+/H^+ antiporter of brush border vesicles: studies with acridine orange Amer. J. Physiol. **242**:F733-F739.

TABLE 1
COMPARISON OF CRAB SPECIES AND SALINITY DEPENDENCE RESULTS
ALL VALUES (MEAN N=3) REPORTED AS QUENCH RELIEF/MG PROTEIN
10 SECONDS AFTER INJECTION OF 75 mM SODIUM GLUCONATE

	C. maenas 10 ppt	*C. maenas* 35 ppt	*C. sapidus* 5 ppt	*C. sapidus* 35 ppt
Total No amiloride	70.1	93.2	80.8	52.2
Amiloride present	18.9	18.3	25.1	24.2
Amiloride sensitive Total-amiloride	51.2	74.9	55.7	28

Statistical analysis: t-test with significance set at P = 0.05 level of probability

Salinity dependence results:
C. sapidus = significant difference between crabs acclimated to 35 ppt and 5 ppt (P = 0.01)
C. maenas = no significant difference between crabs acclimated to 35 ppt and 10 ppt (P = 0.3)

Figure 1. A model for sodium transport in crab gill epithelium.

MODEL OF NA+ UPTAKE

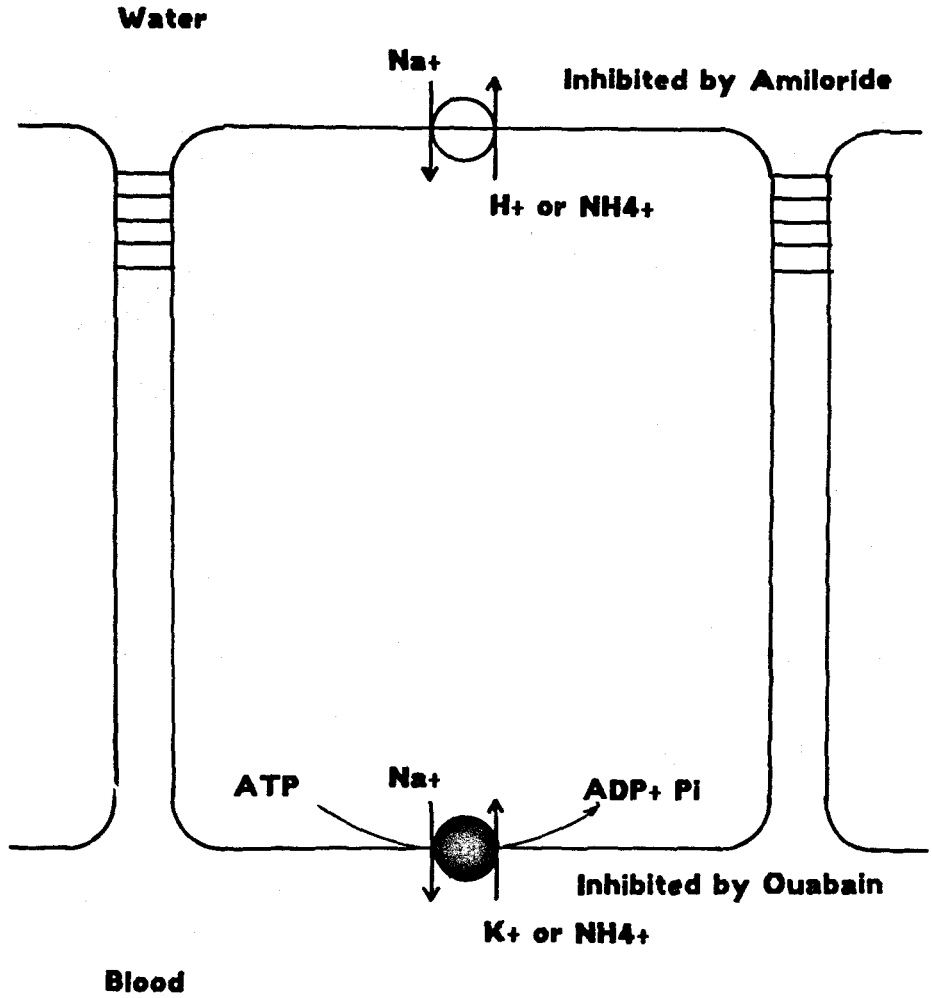


Figure 2. Typical quench curve obtained by injecting 10 ul C. sapidus membrane vesicles (A) from crabs acclimated to high salinity into 2 ml acridine orange-containing incubation medium. Injection of 75 mM sodium gluconate (B) causes partial recovery of fluorescence signal.

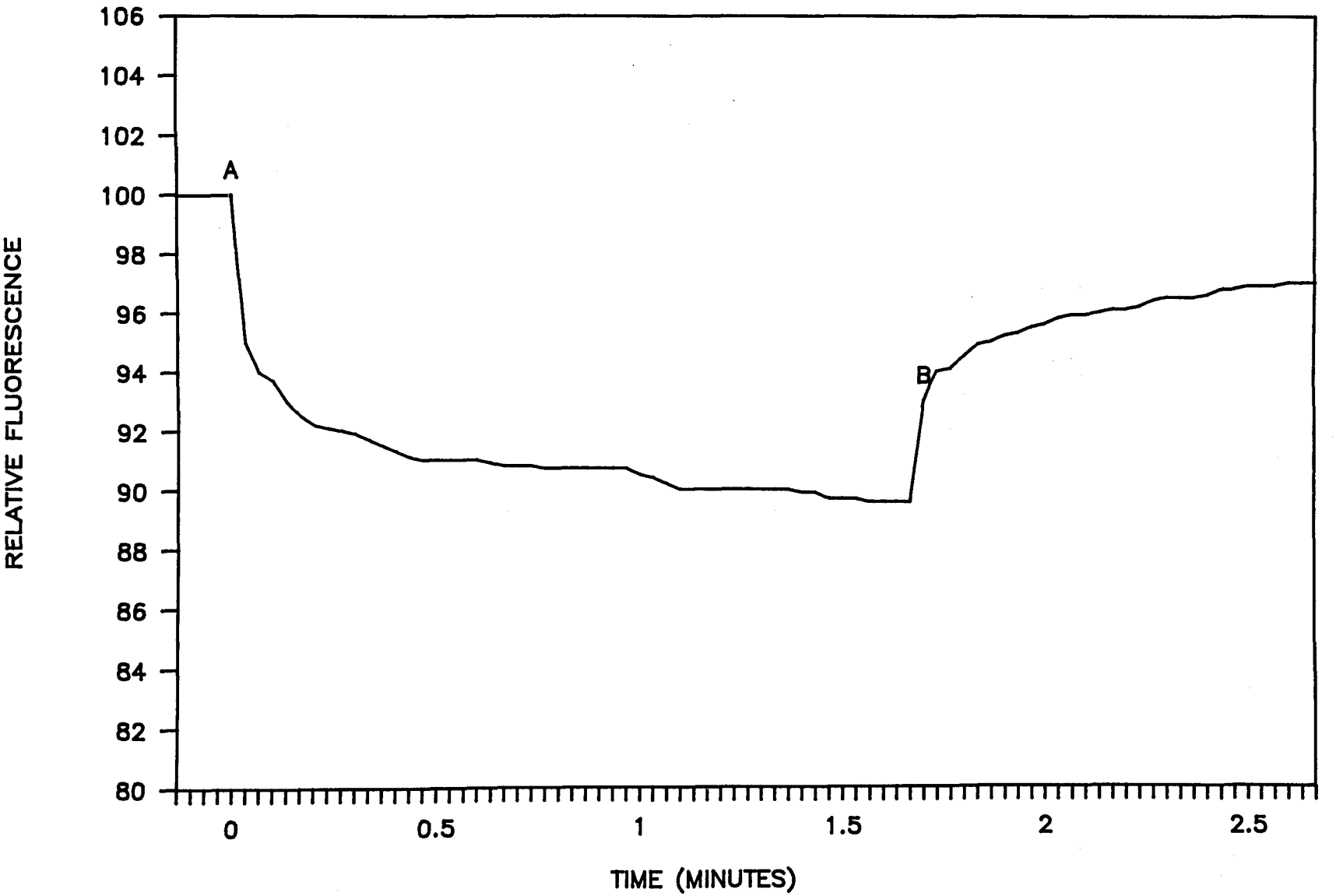

106
104
102
100
98
96
94
92
90
88
86
84
82
80
A
B
RELATIVE FLUORESCENCE
0
0.5
1
1.5
2
2.5
TIME (MINUTES)

Figure 3. Calibration curve for magnitude of quench in relation to difference in pH (out - in). Points are means [n = 3 ± standard error of mean (SEM)] of replications with the same preparation of vesicles.

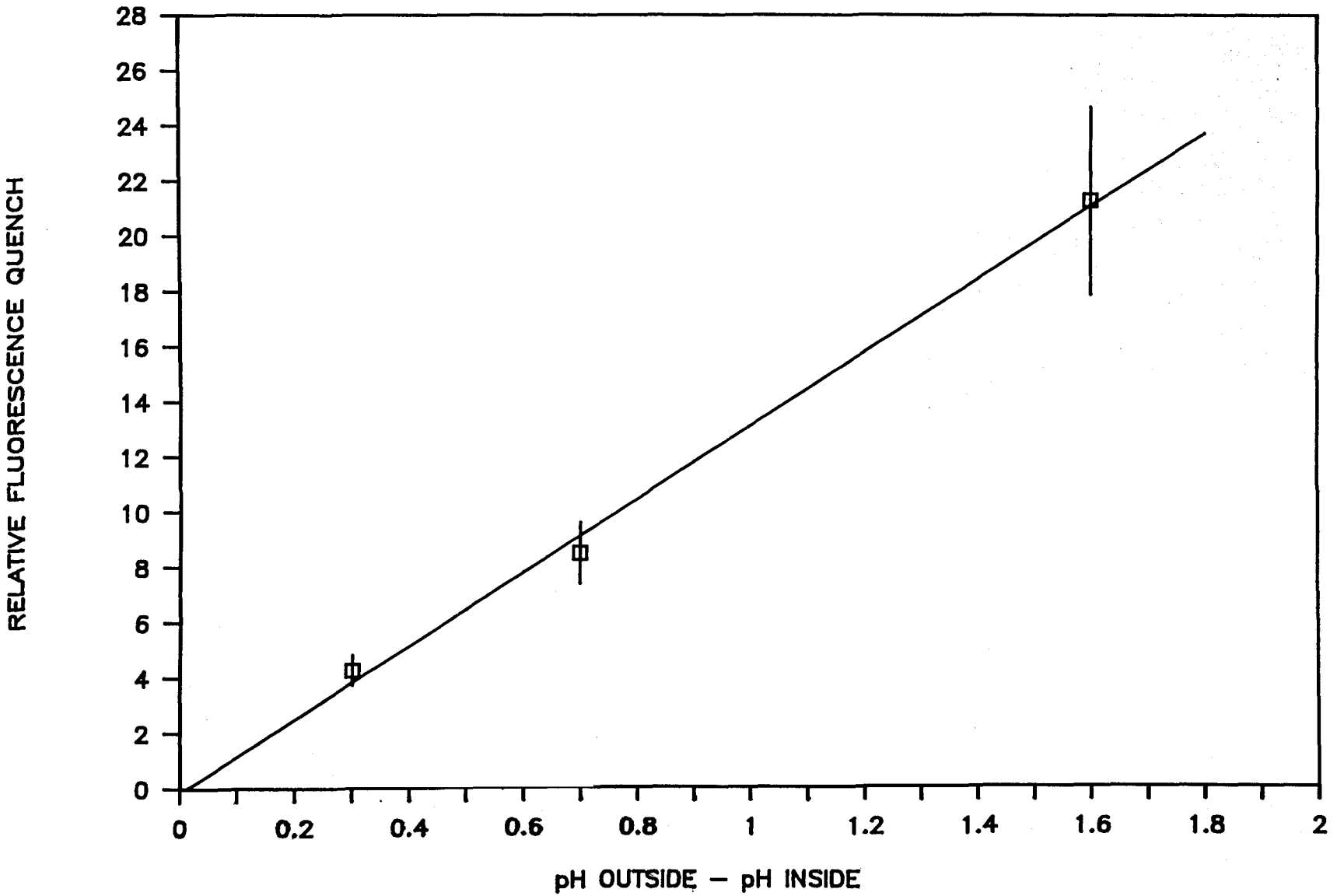

RELATIVE FLUORESCENCE QUENCH
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
pH OUTSIDE – pH INSIDE

Figure 4. Quench of fluorescence in relation to the amount of vesicle protein added to the cuvette. Crosses are C. maenas, squares are C. sapidus.

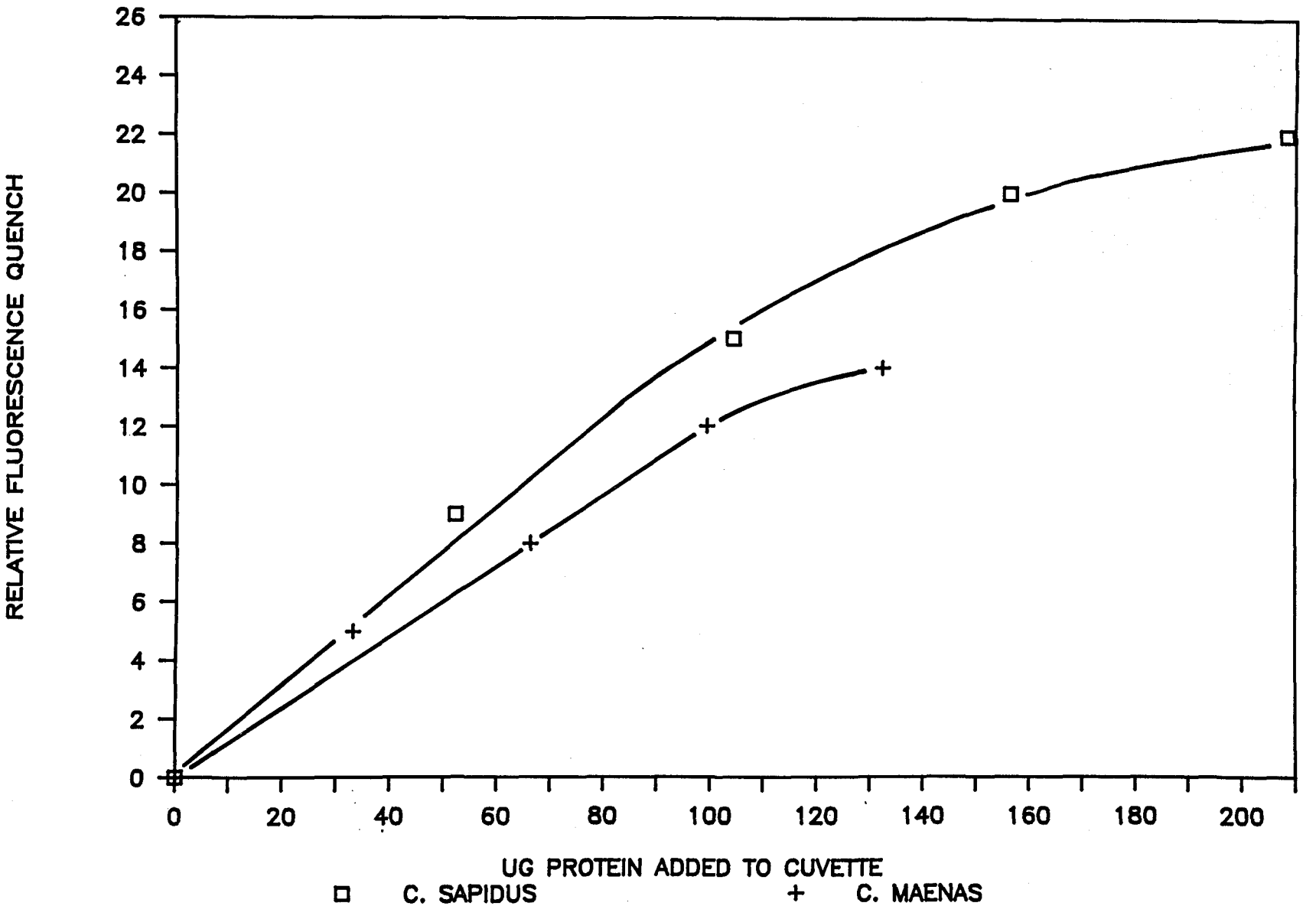

RELATIVE FLUORESCENCE QUENCH
26
24
22
20
18
16
14
12
10
8
6
4
2
0
0
20
40
60
80
100
120
140
160
180
200
UG PROTEIN ADDED TO CUVETTE
C. SAPIDUS
C. MAENAS

Figure 5. Acclimation salinity comparison of C. sapidus. Vesicles injected at A (lower curve = animals from 5 ppt, upper curve = animals from 35 ppt). Sodium gluconate (75 mM) injected at B to induce collapse of pH gradient.

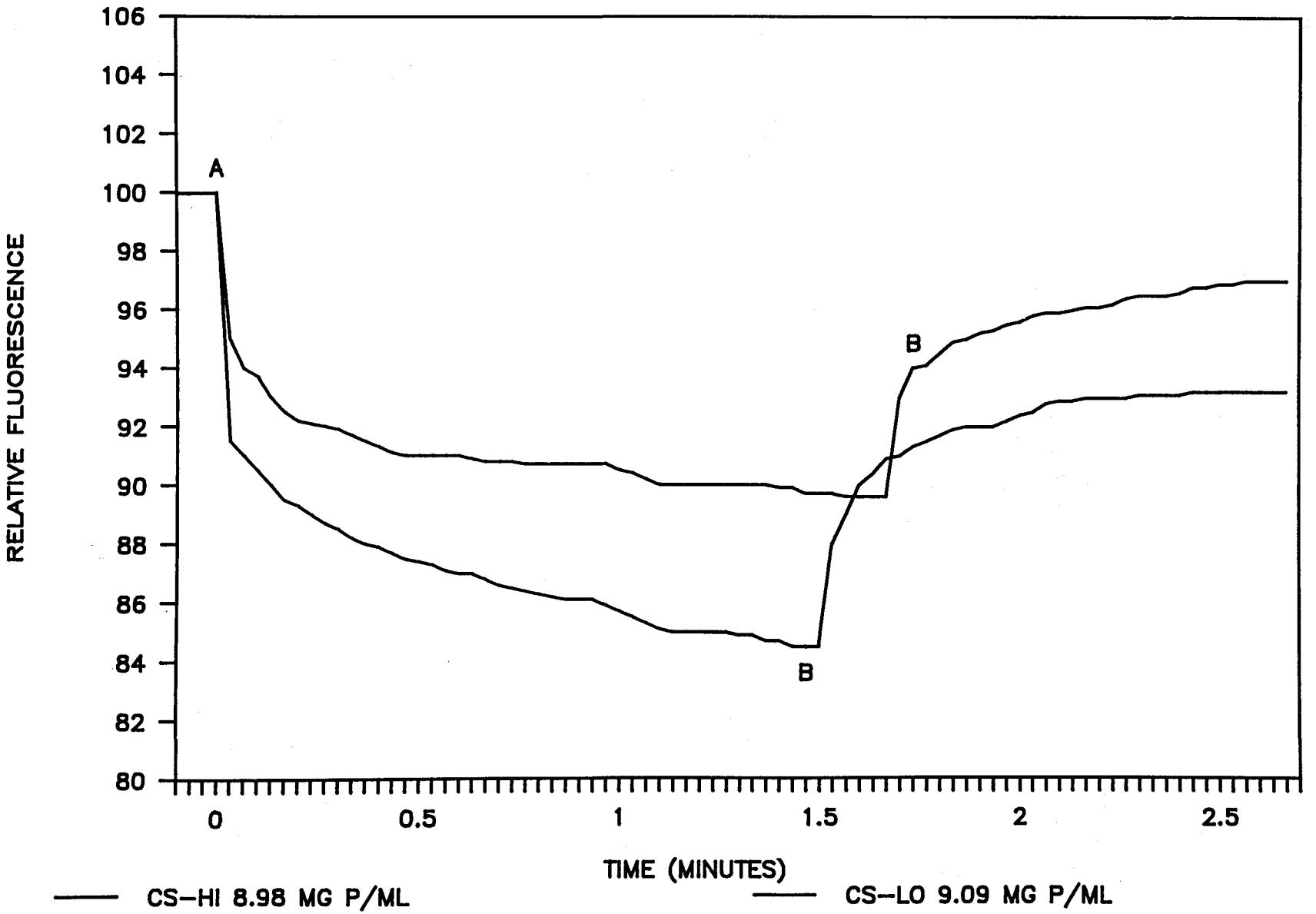

106
104
102
100
98
96
94
92
90
88
86
84
82
80
RELATIVE FLUORESCENCE
A
B
B
0
0.5
1
1.5
2
2.5
TIME (MINUTES)
CS-HI 8.98 MG P/ML
CS-LO 9.09 MG P/ML

Figure 6. Acclimation salinity comparison of C. maenas. Vesicles injected at A (lower curve = animals from 35 ppt, upper curve = animals from 10 ppt). Sodium gluconate (75 mM) injected at B to induce collapse of pH gradient.

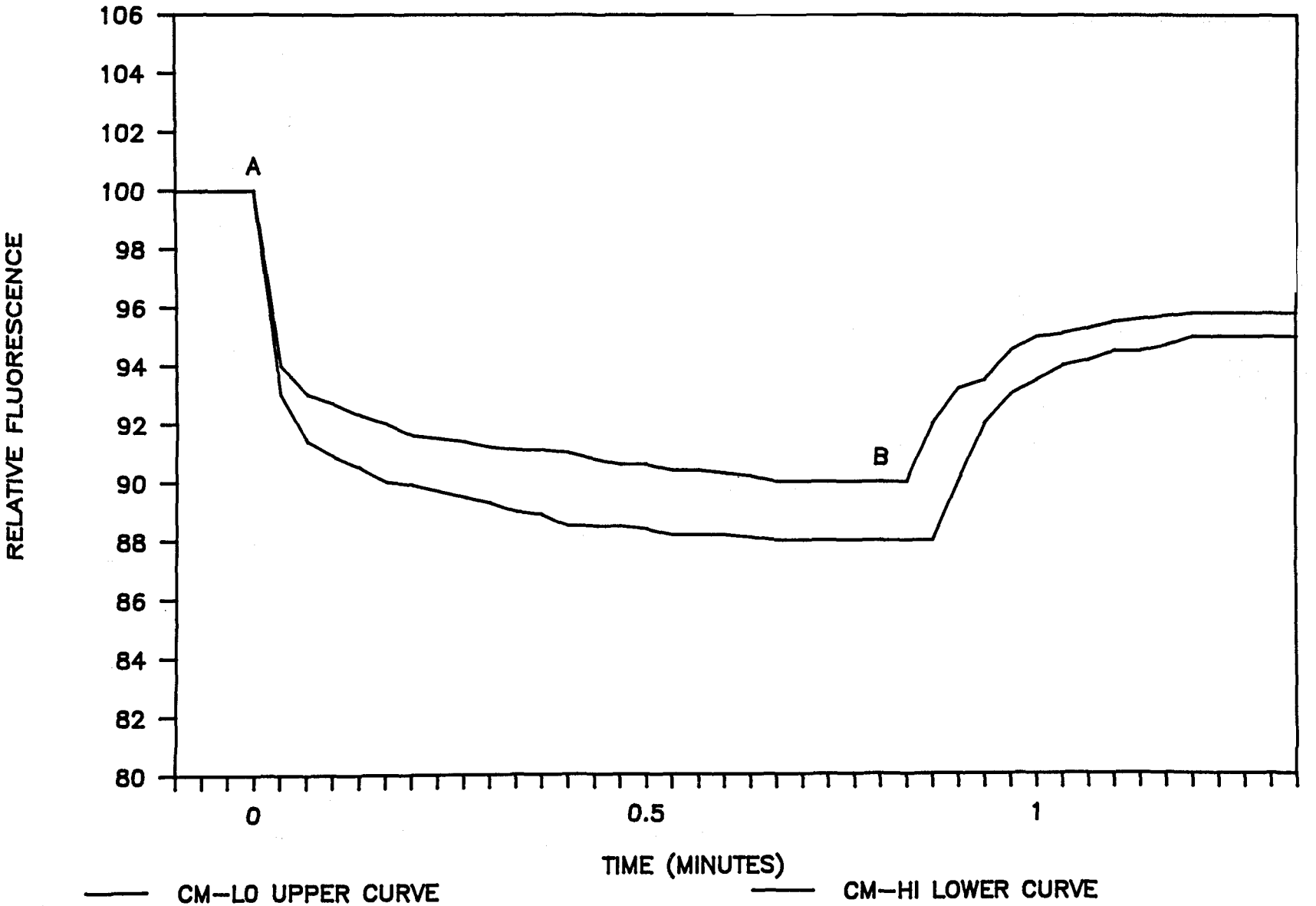

106
104
102
100
98
96
94
92
90
88
86
84
82
80
A
B
RELATIVE FLUORESCENCE
0
0.5
1
TIME (MINUTES)
CM–LO UPPER CURVE
CM–HI LOWER CURVE

Figure 7. Effect of amiloride on relief of quench. Vesicles from C. sapidus acclimated to 35 ppt injected into cuvette at A. Addition of amiloride (0.5 mM upper curve causes fluorescence to drop further) or an identical volume of distilled water (lower curve causes no change in fluorescence) at C, followed by injection of (75 mM) sodium gluconate at B. The difference in rate of quench relief is taken as the amiloride-sensitive portion of Na^+/H^+ exchange.

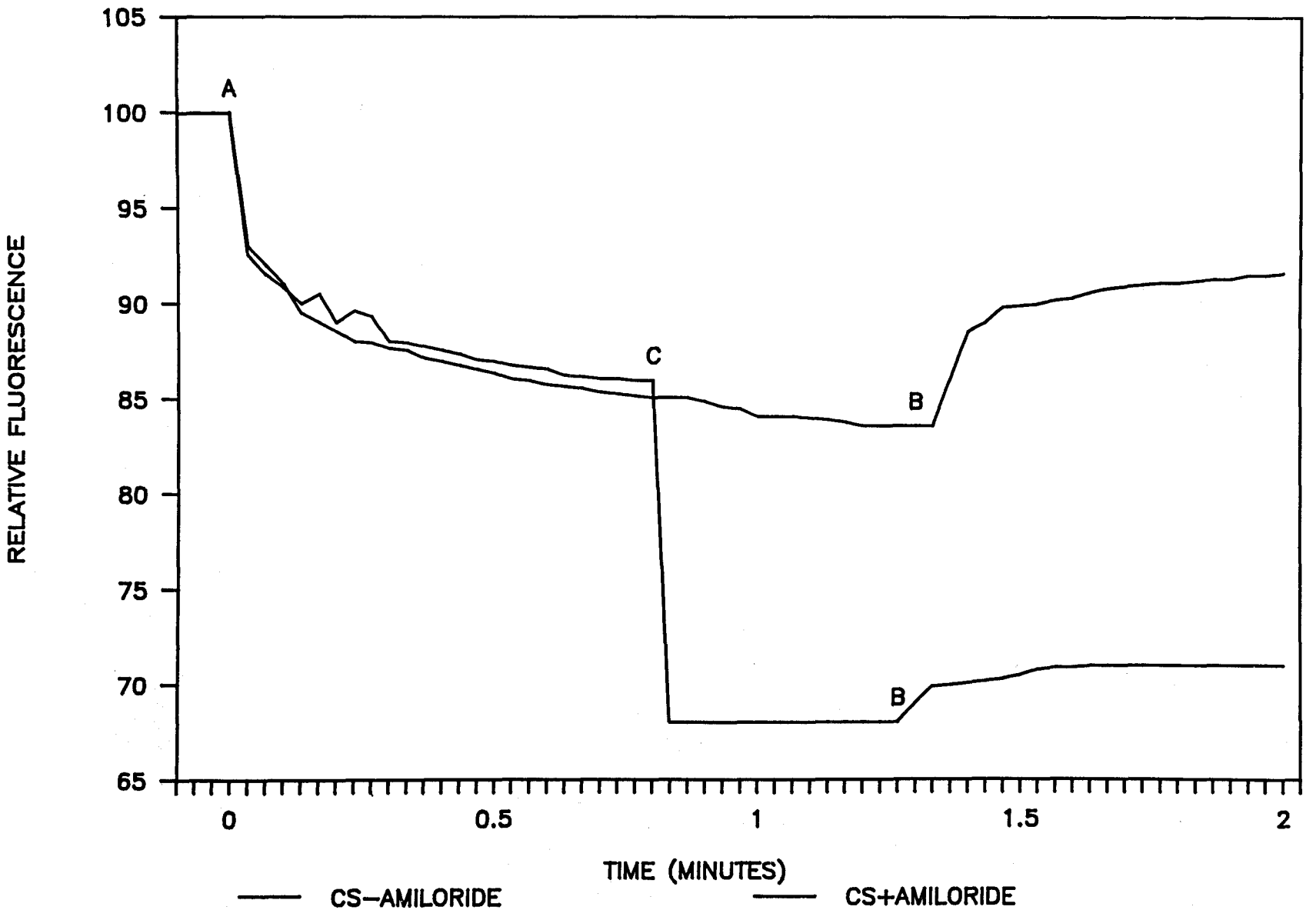

105
100
95
90
85
80
75
70
65
RELATIVE FLUORESCENCE
A
C
B
B
0
0.5
1
1.5
2
TIME (MINUTES)
CS−AMILORIDE
CS+AMILORIDE

Figure 8. Summary of results of salinity and species comparison. CS = C. sapidus, CM = C. maenas, H = animals from high salinity, L = animals from low salinity. Totals are means [± standard error of mean (SEM)] from two separate experiments (n = 6). Amil-insens = amiloride insensitive portion while amiloride-sens = amiloride sensitive portion.

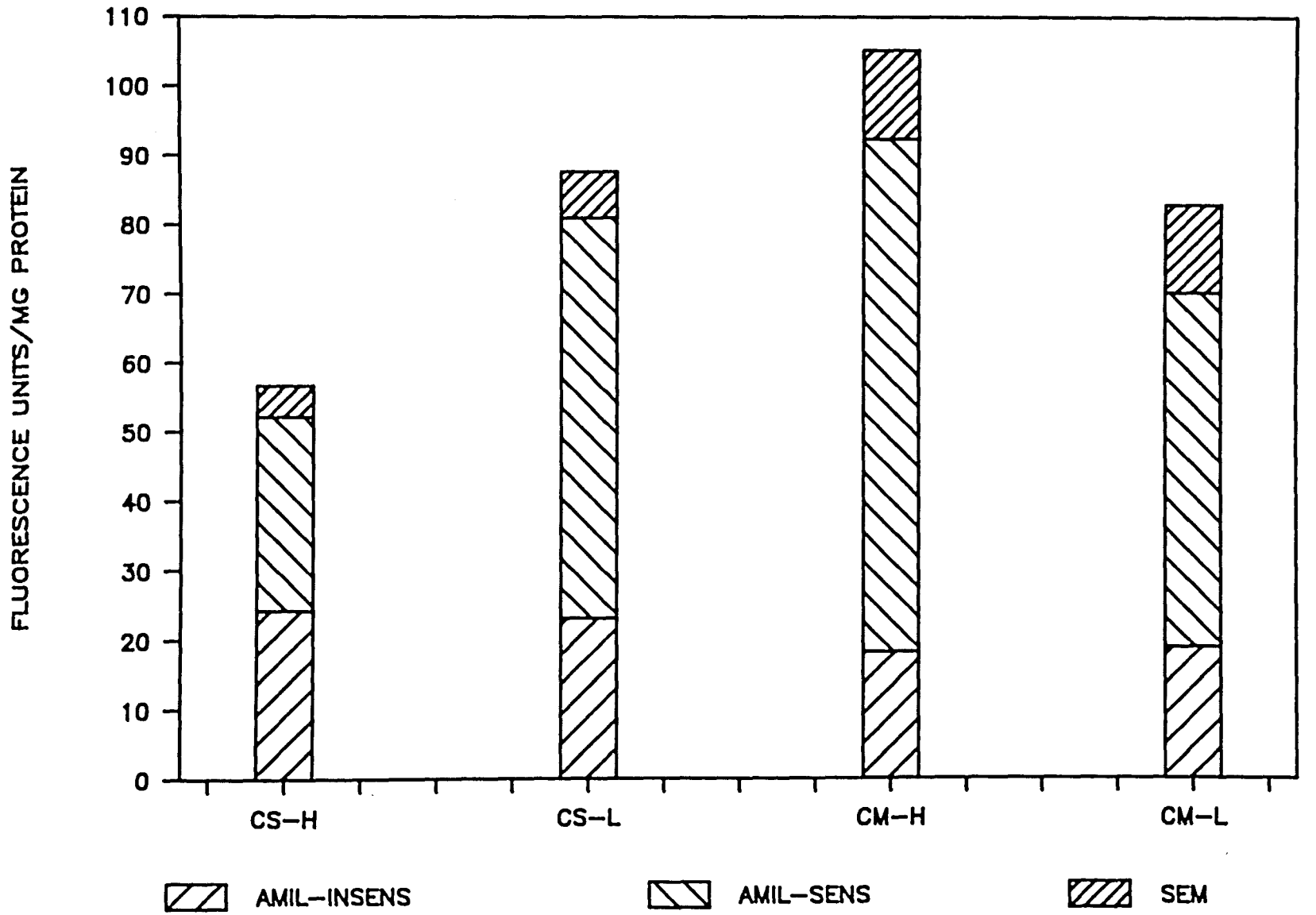

FLUORESCENCE UNITS/MG PROTEIN
110
100
90
80
70
60
50
40
30
20
10
0
CS–H
CS–L
CM–H
CM–L
AMIL–INSENS
AMIL–SENS
SEM

Figure 9. Effect of TMA (tetramethylammonium) ion on fluorescence and quench relief. C. sapidus vesicles from animals acclimated to 5 ppt seawater injected at A. Either 45 mM sodium gluconate (injected at upper B) or 45 mM TMA gluconate (injected at lower B) was added to the cuvette.

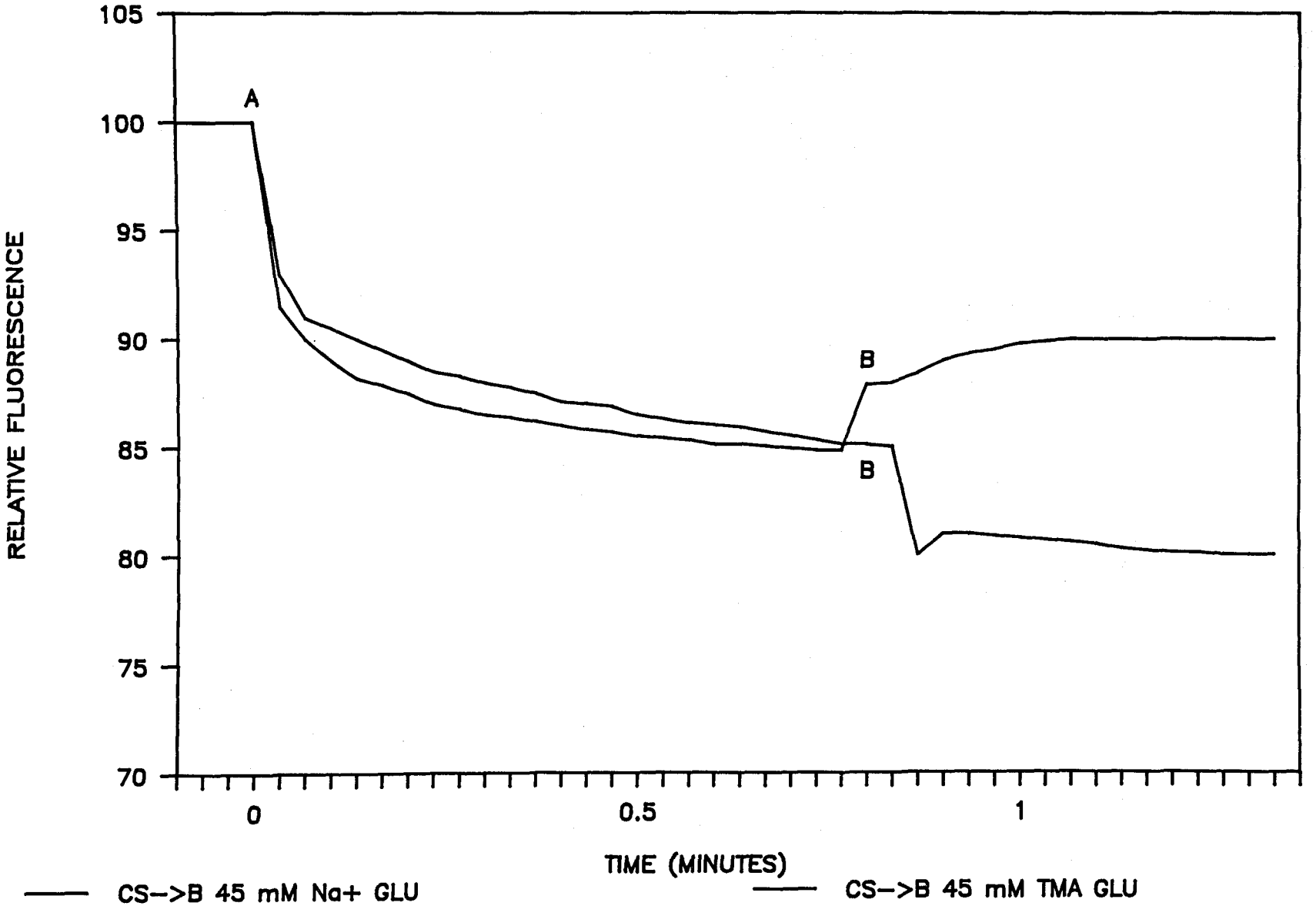

105
100
95
90
85
80
75
70
A
B
B
0
0.5
1
RELATIVE FLUORESCENCE
TIME (MINUTES)
CS–>B 45 mM Na+ GLU
CS–>B 45 mM TMA GLU

Figure 10. Effect of different cations on quench relief. Totals are means [n = 3 ± standard error of mean (SEM)] from a single preparation of C. sapidus vesicles from animals acclimated to 5 ppt seawater.

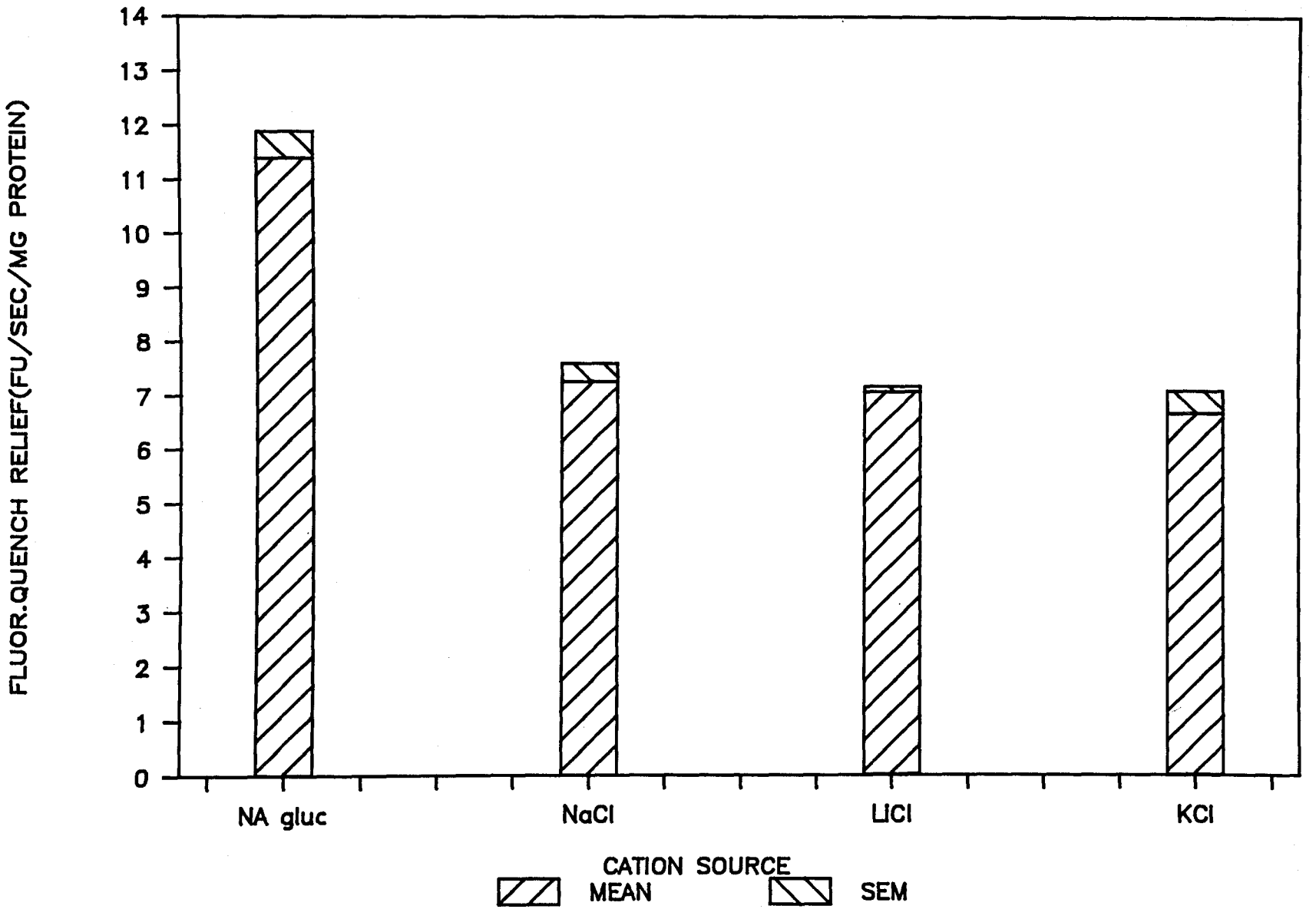

FLUOR.QUENCH RELIEF(FU/SEC/MG PROTEIN)
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
NA gluc
NaCl
LICl
KCl
CATION SOURCE
MEAN
SEM

Figure 11. Effect of potassium-loading. Potassium gluconate-loaded vesicles from C. maenas acclimated to 10 ppt seawater injected at A. Either 75 mM potassium gluconate (lower curve) or 75 mM sodium gluconate (upper curve) injected at B to induce collapse of the pH gradient.

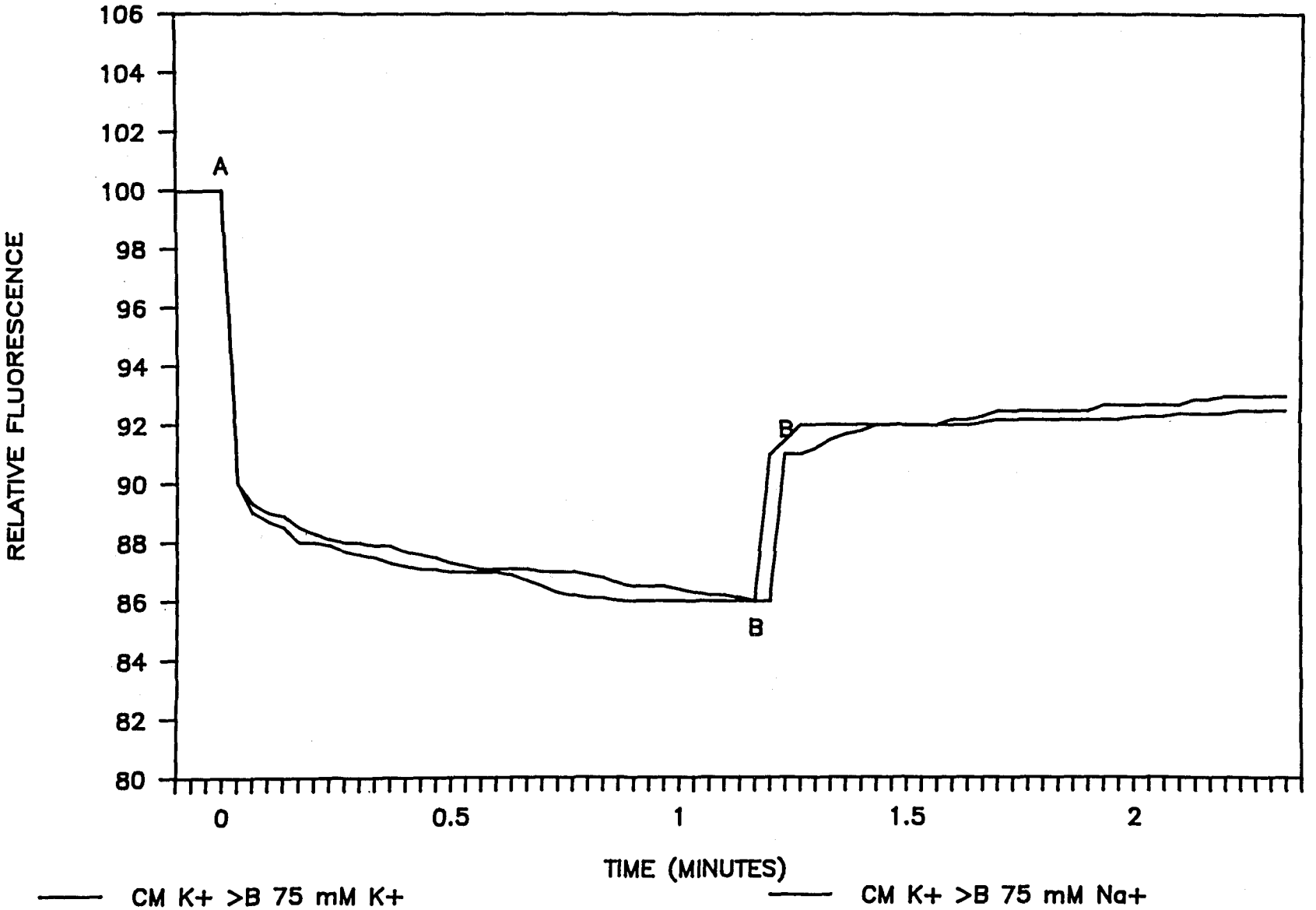
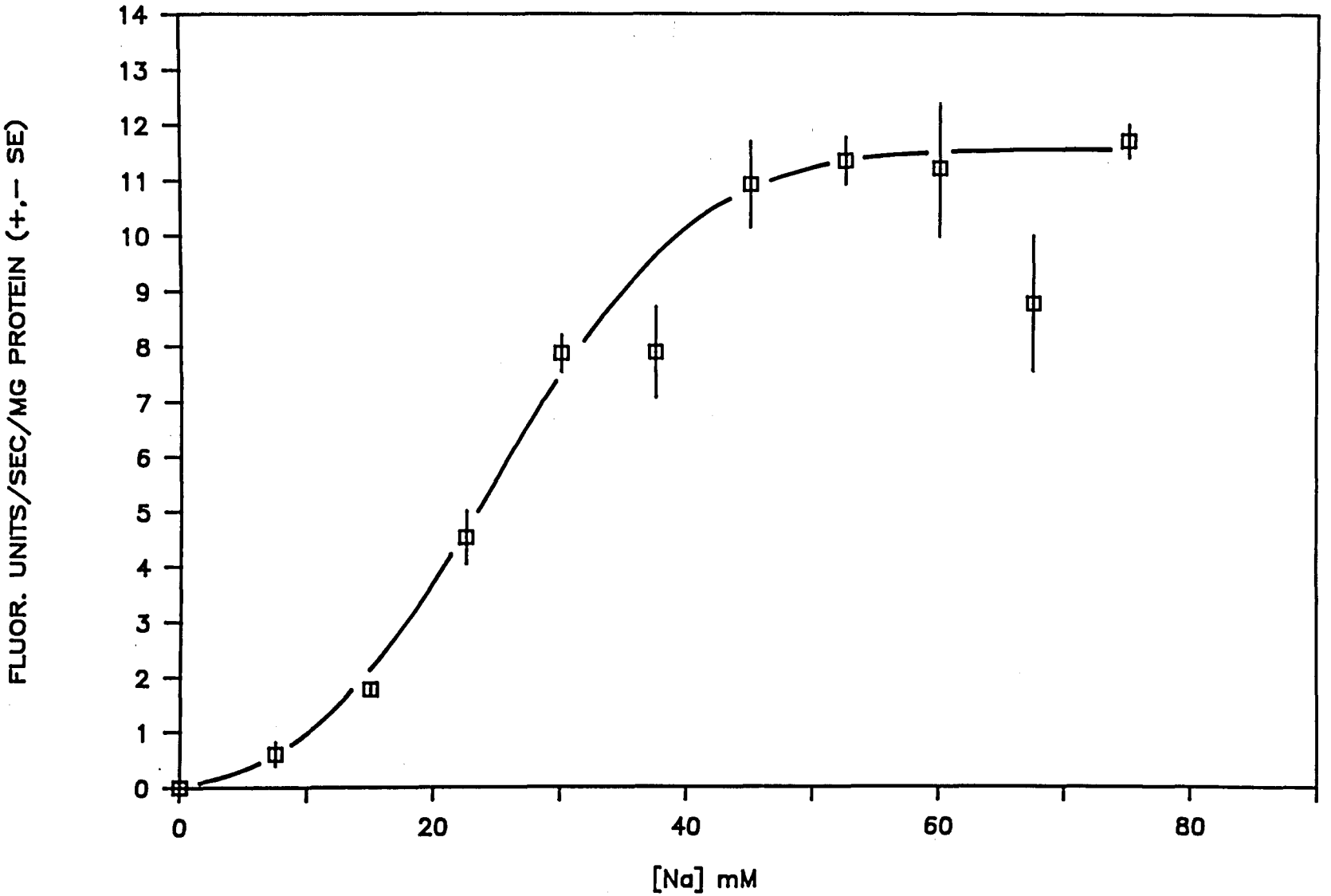

106
104
102
100
98
96
94
92
90
88
86
84
82
80
A
B
B
RELATIVE FLUORESCENCE
0
0.5
1
1.5
2
TIME (MINUTES)
CM K+ >B 75 mM K+
CM K+ >B 75 mM Na+

Figure 12. Results of kinetic experiment. Increasing concentrations of sodium gluconate (7.5 to 75 mM) were used to induce collapse of the pH gradient. Rates were determined as described in the materials and methods section. Each point represents the mean (n = 3 $\pm$ standard error of mean (SEM)] of replications done with a single preparation of C. sapidus vesicles from animals acclimated to 5 ppt seawater. $K_{0.5}$ = 26 mM and V_{max} = 11.4 fluorescence units/sec/mg protein.


FLUOR. UNITS/SEC/MG PROTEIN (+,- SE)
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
0
20
40
60
80
[Na] mM

Vita

Robert Shetlar was born in San Angelo, Texas in 1959. He went to school in Kansas, graduating from Winfield High School in Winfield, Kansas in 1977. After a four hiatus he attended Emporia State University in Emporia, Kansas and graduated in May 1985 with a Bachelor of Science in biology. Robert moved to Richmond in August 1985 for the express purpose of attending graduate school and working with Dr. David W. Towle. He finished his Master's requirements in May 1987 and will start work on a Ph.D. sometime within the next year.