J. Membrane Biol. 107, 77–88 (1989)

The Journal of
Membrane Biology

Sodium D-Glucose Cotransport in the Gill of Marine Mussels: Studies with Intact Tissue and Brush-Border Membrane Vesicles

Ana M. Pajor,* Debra A. Moon, and Stephen H. Wright
Department of Physiology, College of Medicine, University of Arizona, Tucson, Arizona 85724

Summary. Glucose transport was studied in marine mussels of the genus *Mytilus*. Initial observations, with intact animals and isolated gills, indicated that net uptake of glucose occurred in mussels by a carrier-mediated, Na^+-sensitive process. Subsequent studies included use of brush-border membrane vesicles (BBMV) in order to characterize this transport in greater detail. The highest activity of Na^+-dependent glucose transport was found in the brush-border membrane fractions used in this study, while basal-lateral membrane fractions contained the highest specific binding of ouabain. Glucose uptake into BBMV showed specificity for Na^+, and concentrative glucose transport was observed in the presence of an inwardly directed Na^+ gradient. There was a single saturable pathway for glucose uptake, with an apparent K_t of 3 μM in BBMV and 9 μM in intact gills. The kinetics of Na^+ activation of glucose uptake were sigmoidal, with apparent Hill coefficients of 1.5 in BBMV and 1.2 in isolated gills, indicating that more than one Na^+ may be involved in the transport of each glucose. Harmaline inhibited glucose transport in mussel BBMV with a K_i of 44 μM. The uptake of glucose was electrogenic and stimulated by an inside-negative membrane potential. The substrate specificity in intact gills and BBMV resembled that of Na^+-glucose cotransporters in other systems; D-glucose and α-methyl glucopyranoside were the most effective inhibitors of Na^+-glucose transport, D-galactose was intermediate in its inhibition, and there was little or no effect of L-glucose, D-fructose, 2-deoxy-glucose, or 3-O-methyl glucose. Phlorizin was an effective inhibitor of Na^+-glucose uptake, with an apparent K_i of 154 nM in BBMV and 21 nM in intact gills. While the qualitative characteristics of glucose transport in the mussel gill were similar to those in other epithelia, the quantitative characteristics of this process reflect adaptation to the seawater environment of this animal.

Key Words Glucose transport · Na-glucose cotransport · brush-border membrane vesicles · marine bivalve gills · *Mytilus edulis* · integument

Introduction

Soft-bodied marine invertebrates can accumulate dissolved organic matter, such as amino acids and sugars, from seawater directly into the cells of their integument (Jørgensen, 1976; Stephens, 1988). In marine mussels, the primary site for this uptake is the epithelium of the gill (Pequignat, 1973). There has been considerable speculation about the potential role of this transport, particularly in animal nutrition (Wright, 1988) and as a mechanism to reduce diffusional loss of sugars and amino acids to the environment (Gomme, 1981; Wright & Secomb, 1984, 1986).

Increasing attention is being addressed to the mechanism and energetics of this process. For example, net uptake of amino acids occurs from ambient concentrations in seawater of less than 1 μM (Manahan, Wright & Stephens, 1983), yet the cellular amino acid pool of marine invertebrate tissues exceeds 0.1 M (Zurburg & DeZwaan, 1981). Consequently, integumental transport processes typically move substrate against chemical gradients in excess of 10^5 to 1, and marine bivalves have been shown to sustain gradients of as much as 10^7 to 1 (Manahan et al., 1983; Wright & Secomb, 1986). The energy to support this transport has been proposed to come from the inwardly directed electrochemical gradient for Na^+ (*see* review by Wright, 1988), and evidence for a direct coupling of alanine and Na^+ fluxes in the bivalve gill has recently been reported (Pajor & Wright, 1987).

In contrast to the numerous reports of amino acid transport, there have been few studies on the transport of sugars in marine bivalves, despite indications that seawater concentrations of carbohydrates may exceed those of amino acids by as much as tenfold (Williams, 1975). The uptake of glucose into oyster, *Crassostrea gigas,* gill tissue is sensitive to the concentration of Na^+ in the external solution, suggesting that the mechanism of integumental glucose transport also involves coupling with Na^+ (Bamford & Gingles, 1974). This paper describes the characterization of glucose transport in

* *Present address:* Department of Physiology, UCLA School of Medicine, Los Angeles, California 90024.

mussels of the genus, *Mytilus*, through studies that made use of whole animals, intact gill tissue, and brush-border membrane vesicles isolated from the gill. Glucose transport was found to involve an electrogenic, Na^+-specific cotransport process, qualitatively similar to Na^+-dependent glucose transport in many other epithelial systems (*see*, e.g., Hopfer, 1987). However, the quantitative characteristics of this process in marine mussels were unique, and appear to represent adaptations to the conditions found in the near-shore environment of these animals.

Materials and Methods

Animals

Specimens of the common blue mussel (*Mytilus edulis*) were purchased from Sea Life Supply, Sand City, CA. California coastal mussels (*Mytilus californianus*) were purchased from Bodega Marine Biological Laboratory, Bodega Bay, CA. They were maintained in refrigerated aquaria (12°C) containing recirculating, aerated artificial seawater (Instant Ocean). Animals were not fed and were used within two weeks (*M. edulis*) to six weeks (*M. californianus*) of collection.

Intact Mussel Experiments

The procedure used in studies involving intact *M. californianus* was described by Wright and Secomb (1986). Each animal was incubated in an artificial seawater (ASW; Cavanaugh, 1956) containing between 3.4 and 4.0 μM ^{14}C-D-glucose. Samples of the test medium were collected at intervals and assayed for radioactivity by liquid scintillation counting (Beckman LSC-3801), and for total D-glucose concentration by the fluorimetric assay of Hicks and Carey (1968).

Isolated Gill Experiments

The uptake of glucose into isolated *M. californianus* gill tissue was measured as described by Wright (1985). Excised gills were suspended in ASW. The gills were preincubated 30 min, and then transferred to the test solution, which contained, unless noted in figure legends, 0.5 μM ^{14}C-D-glucose and 10 μM serotonin (to activate lateral cilia; Wright, 1979). Uptake was stopped by rinsing with ice-cold ASW. A modification of this method was used for studies involving *M. edulis* (Pajor & Wright, 1987), since the gills of this mussel are more fragile than those of *M. californianus*. In these studies, rather than being suspended in stirred solutions, pieces of demibranchs were incubated in test solutions on a gently rotating shaker. In both procedures, the gill pieces were blotted dry, weighed, and placed into scintillation vials with 1 ml extraction medium (95% ethanol or 0.1 N HNO_3). Several hours later 7 ml of scintillation cocktail (Betaphase) were added to each vial, and the radioactivity was determined using a liquid scintillation counter. All counts were corrected for variable quench. Uptake was expressed as μmol glucose accumulated per gram wet gill weight.

Membrane Preparation

Brush-border membrane vesicles (BBMV) were prepared from *M. edulis* gills by differential and sucrose density gradient centrifugation as described in a previous study (Pajor & Wright, 1987). Briefly, the gills were soaked in an artificial seawater containing a high concentration of K^+ (115 mM) and 1 mM dithiothreitol (DTT) in order to remove mucus. The gills were then homogenized in a buffer composed of 500 mM sorbitol, 5 mM ethyleneglycol-bis(β-amino-ethylether)-N-N'-tetraacetic acid (EGTA), 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffered to pH 7.6 with tris(hydroxymethyl)-aminomethane (HEPES-Tris). A crude plasma membrane fraction was isolated by several differential centrifugation steps. Brush-border membranes were separated from other membranes using a linear 30 to 50% (wt/vol) sucrose density gradient. The purity of the final brush-border membrane preparation was assessed by monitoring the activity of enzymatic membrane markers, as well as by measuring rates of cation-dependent amino acid transport. In a previous study (Pajor & Wright, 1987), it was shown that the final brush-border vesicle fraction had the highest enrichment (relative to the initial homogenate) of the apical membrane marker, γ-glutamyl transpeptidase (GGTP; 18-fold), and the lowest enrichment of the basal-lateral membrane markers, Na,K-ATPase (0.4-fold) and K-dependent para-nitrophenyl phosphatase (PNPPase; 1.8-fold), in addition to the highest activity of cation-dependent alanine transport.

For most of the studies outlined here, the final brush-border membrane fraction was resuspended as described previously (Pajor & Wright, 1987) in a buffer containing 600 mM mannitol and 10 mM HEPES-Tris at pH 7.6. In the experiments with vesicles containing KCl, the final membrane fraction was resuspended in 300 mM KCl, 10 mM HEPES-Tris at pH 7.6 and left on ice approximately 6 hr, followed by 30 min at room temperature.

Transport Measurements with BBMV

The transport of substrates into membrane vesicles was measured using a rapid filtration method described by Wright et al. (1983). The transport reaction was run at room temperature, stopped with 1 ml ice-cold isosmotic mannitol buffer (600 mM mannitol, 10 mM HEPES-Tris, pH 7.6), and the reaction mixture rapidly filtered with suction through a 0.45 μm filter (Millipore HAWP) and washed with 4 ml of cold mannitol buffer. The labeled substrate retained on the filter was extracted in scintillation vials containing 6 ml of scintillation cocktail (Betaphase) and radioactivity was determined. All uptakes were corrected for nonspecific binding to membranes and filters.

Binding Measurements

The equilibrium binding of tracer ^{3}H-ouabain (1.5×10^6 dpm) was measured in the same buffer used for measurement of Na,K-ATPase activity (Mircheff & Wright, 1976): 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 3 mM EDTA, 3 mM ATP, 83.5 mM Tris, pH 8.0. Ninety microliters of this buffer were combined with 10 μl BBMV, and binding was allowed to proceed for 10 min. The incubations were stopped and filtered as in transport experiments. Specific binding to ouabain binding sites on the membrane was determined from the difference between binding in the presence and absence of 1 mM unlabeled ouabain.

Chemicals

^{3}H-D-glucose (83 Ci/mmol), ^{14}C-D-glucose (359 mCi/mmol), ^{14}C-α-methyl-D-glucopyranoside (150 mCi/mmol) and ^{3}H-ouabain (40 Ci/mmol) were purchased from NEN. All other chemicals were obtained from Sigma Chemical Corporation. Valinomycin, FCCP, phloretin, and cytochalasin B were added to transport buffers as stock solutions in ethanol and the final concentration of ethanol in these buffers was 1%.

Results

Net Uptake of Glucose in Intact Mussels

Figure 1 shows the results of an experiment in which the rate of disappearance of ^{14}C-D-glucose from the medium, a relative measure of influx, and the amount of chemically-determined D-glucose in the medium, an indication of net flux, were monitored over time. Over the course of the 30-min test period, the influx and net flux of glucose were similar. This implies that there was little efflux of glucose from the mussel, and that the uptake of radiolabeled glucose was a good measure of net uptake.

In experiments with three animals, the mean initial rate of influx was 2.83 ± .007 μmol/g (wet wt gill tissue)-hr (n = 2), while the net rate of glucose clearance was 3.49 ± 0.18 μmol/g (wet wt gill tissue)-hr (n = 3).

Time Course of Glucose Uptake in Gills

The rate of glucose uptake into intact, isolated gills of *M. californianus* was linear for at least 10 min (Fig. 2, inset). There was extensive metabolism of the accumulated glucose during this period. Chromatograms of 80% ethanol extracts of gills pulse-labeled for 10 min had no demonstrable peak of ^{14}C-glucose. As much as 16% of the accumulated radioactivity was released to the medium as $^{14}CO_2$, as shown by trapping of acid-volatile material in hyamine hydroxide (*data not shown*). In contrast, chromatograms of extracts of gills pulse-labeled for 10 min with the glucose analog, ^{14}C-α-methyl-D-glucopyranoside (α-MG), resulted in almost complete recovery (99.6%) of accumulated radiolabel from a single peak that coincided with that of the stock ^{14}C-α-MG. In other epithelial systems, α-MG has been shown to be transported by the Na^+-glucose cotransporter (Kimmich & Randles, 1981). We elected to use a 5-min incubation in our subsequent studies of glucose uptake into intact gill tissue, though the above results indicate that this may have represented an underestimate of the initial rate of substrate influx.

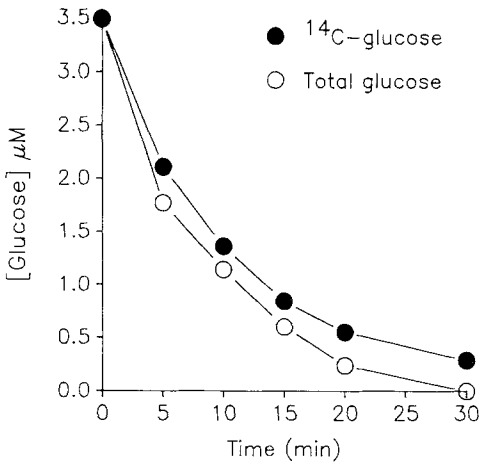

Fig. 1. Influx and net flux of glucose in an intact *M. californianus*. The animal was placed into 500 ml of artificial seawater containing 3.5 μmol ^{14}C-D-glucose. Samples of this medium were collected and analyzed for radioactivity (filled circles) or total glucose concentration (open circles)

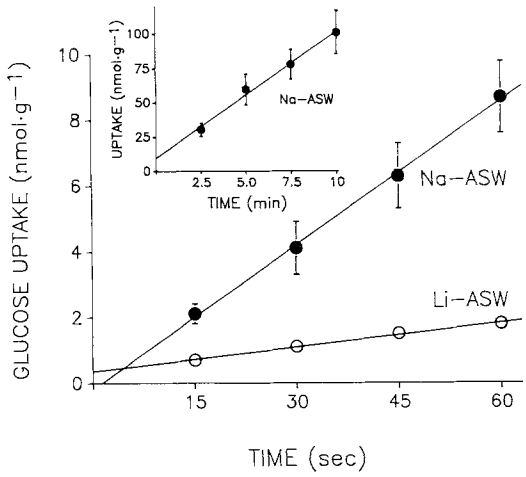

Fig. 2. Time course of 0.5 μM ^{14}C-D-glucose uptake into isolated gills of *M. californianus*, over 60 sec and (inset) 10 min. Test solutions consisted of either Na^+-containing artificial seawater (Na-ASW) or ASW in which the Na^+ was replaced with Li^+ (Li-ASW). Each point represents the mean ± SE of experiments with gills from four separate animals

Figure 2 also shows the uptake of glucose into intact gills in Na^+-free seawater (the Na^+ was replaced by Li^+). Under these conditions, the rate of glucose uptake was reduced by 96%. Figure 3 summarizes the effects of Na^+ replacement by monovalent cations in both *M. californianus* and *M. edulis*. The initial rate of glucose uptake in Li^+-, K^+-, or choline$^+$-ASW was reduced by more than 85%, rel-

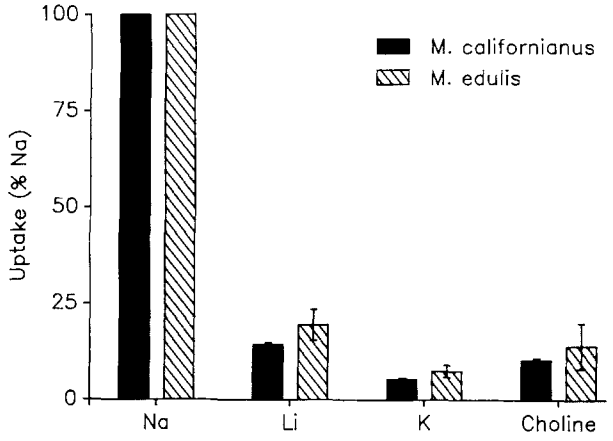

Fig. 3. Effect of replacing Na^+ in seawater with Li^+, K^+, or choline$^+$ on the 5-min uptake rate of 0.5 μM ^{14}C-D-glucose into isolated gill tissue of *M. californianus* and *M. edulis*. The bars represent means ± SE of experiments conducted with three animals

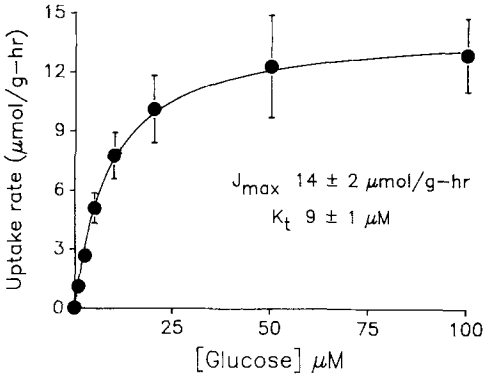

Fig. 4. Kinetics of uptake of ^{14}C-D-glucose in intact gills of *M. californianus*. Test solutions contained ASW, 10 μM serotonin, and up to 100 μM ^{14}C-D-glucose. The points represent means ± SE of experiments conducted with gills from four separate animals. The kinetic constants shown were calculated from nonlinear regressions for the best fit to the Michaelis-Menten equation (Duggleby, 1981)

ative to the rate of glucose uptake in Na^+-containing ASW.

Kinetics in Intact Gills

Glucose uptake into intact gills of *M. californianus* was saturable and adequately described by the Michaelis-Menten equation

$$J = \frac{J_{max} \cdot [S]}{K_t + [S]} \quad (1)$$

where J is the initial rate of uptake from an external glucose concentration of [S], J_{max} is the maximal rate of uptake, and K_t is the concentration of substrate at which the rate of glucose uptake is $\frac{1}{2} J_{max}$.

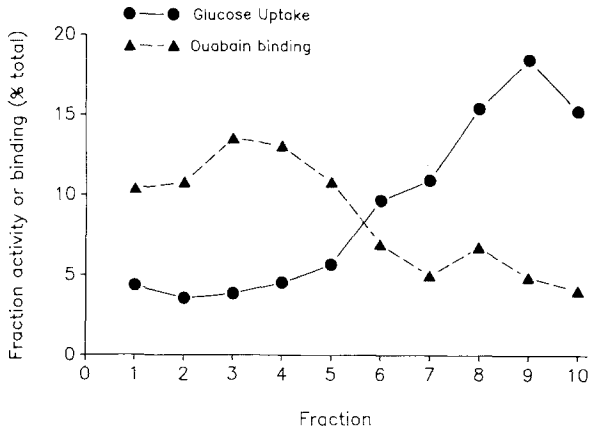

Fig. 5. Distribution of Na^+-dependent glucose transport and specific ouabain binding in a linear 30–50% sucrose density gradient. Fraction 1 represents the top (30%) of the gradient. The transport activity or amount of binding is shown as a percent of the total activity or binding in the gradient. Vesicles contained 600 mM mannitol, 10 mM HEPES-Tris, pH 7.6. Each point represents the mean of two determinations. For measurement of glucose transport, transport buffers contained 60 mM mannitol, 260 mM NaCl or KCl, plus 10 mM HEPES-Tris, pH 7.6, 0.5 μM ^{3}H-D-glucose, 25 μM FCCP. Ten-sec uptakes were measured. The Na^+-dependent glucose transport shown represents the difference between transport in NaCl and KCl. Other conditions are described in Materials and Methods

In experiments with four animals, the J_{max} was 14 ± 2 μmol/g-hr and the apparent K_t was 9 ± 1 μM (Fig. 4). There was no indication of any parallel, nonsaturable pathways for glucose uptake in the intact gill.

Distribution of Transport Activity and Binding in the Sucrose Gradient

The preceding experiments showed that there was net uptake of glucose in mussel gills by a carrier-mediated, Na^+-sensitive process. It was decided to characterize further the uptake of glucose in mussel gills using a preparation of isolated brush-border membranes. In a previous study (Pajor & Wright, 1987), two major membrane populations were separated by the sucrose density gradient. The higher density population near the bottom of the gradient contained the highest activity of GGTP and highest rate of cation-dependent alanine transport. This fraction was concluded to be enriched in brush-border membranes. The lower density population near the top of the gradient was enriched in Na,K-ATPase and K-PNPPase activity and was thought to represent basal-lateral membranes. As shown in Fig. 5, the distribution of Na^+-dependent glucose uptake showed a peak near the bottom of the gradient in fractions previously concluded to represent the brush-border membranes. The distribution of Na^+-dependent taurine and lysine transport also

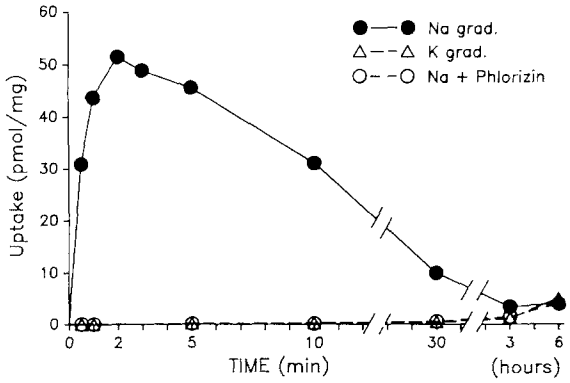

Fig. 6. Time course of D-glucose uptake into brush-border membrane vesicles from mussel gills. The vesicles were resuspended in 600 mM mannitol, 10 mM HEPES-Tris, pH 7.6. The transport buffers contained 0.5 μM ^{3}H-D-glucose, 60 mM mannitol, 10 mM HEPES-Tris, pH 7.6 and either 260 mM NaCl, 260 mM KCl, or 260 mM NaCl plus 10 μM phlorizin. Data points are the means of triplicate measures of uptake from a single membrane preparation

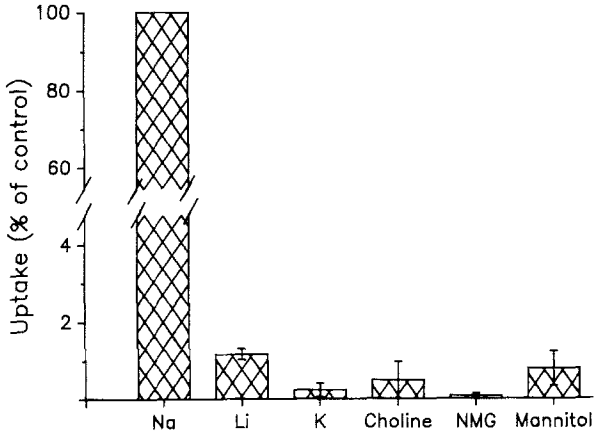

Fig. 7. Cation specificity of glucose uptake in BBMV. Initial rates (10 sec) of uptake were measured. Vesicles were resuspended in 600 mM mannitol, 10 mM HEPES-Tris, pH 7.6. Transport buffers contained 0.5 μM ^{3}H-D-glucose, 60 mM mannitol, 10 mM HEPES-Tris, pH 7.6, 25 μM FCCP and either 260 mM NaCl, LiCl, KCl, CholineCl, N-methyl D-glucamineCl (NMG) or 600 mM mannitol. The uptake is expressed as a percentage of that seen in NaCl. The data represent the means ± SE of experiments with three membrane preparations

showed peaks near the bottom of the gradient (*results not shown*). The specific binding of ouabain (Fig. 5) was distributed with a peak near the top of the gradient, and coincided with the distribution of Na,K-ATPase (Pajor & Wright, 1987).

Time Course of Glucose Uptake in BBMV

The time course of glucose uptake in BBMV is shown in Fig. 6. In the presence of an inwardly directed sodium gradient there was a transient overshoot in intravesicular glucose concentration, which reached a maximum at approximately 2 min. The glucose concentration in the vesicles then decreased slowly and reached an apparent equilibrium by 6 hr. The peak of the overshoot exceeded the equilibrium value by 9.4 ± 1.8-fold (n = 3). When Na^+ was replaced with K^+, or when phlorizin, an inhibitor of Na^+-dependent glucose transport in the gill (*see* Fig. 15), was added, the overshoot in glucose concentration was abolished and intravesicular glucose concentrations rose slowly, presumably by diffusion, to reach apparent equilibrium by 6 hr. These results support the hypothesis that the transport of glucose and Na^+ is coupled, since concentrative uptake of glucose (the "overshoot") was seen only in the presence of an inwardly directed gradient of Na^+.

The uptake of glucose occurred into an osmotically active space. The apparent equilibrium space (i.e., uptake at 6 hr) was progressively reduced when time course experiments were carried out in solutions of increasing osmotic concentration (prepared using 260 mM NaCl, 60 mM mannitol, 10 mM HEPES-Tris and concentrations of polyethylene glycol (PEG) 400 ranging from 0 to 600 mM) (*data not shown*). The equilibrium space in the presence of 600 mM PEG was 19% of the control.

Cation Specificity of Glucose Uptake in BBMV

The uptake of glucose in BBMV was dependent upon the presence of Na^+ (Fig. 7). The replacement of Na^+ by Li^+, K^+, choline$^+$, N-methyl D-glucamine (NMG) or by mannitol reduced the initial rate of glucose uptake by more than 98%.

Kinetics of Glucose Uptake in BBMV

Preliminary experiments showed that the rate of glucose uptake in BBMV at 0.5 μM was linear through 15 sec and at 25 μM, linear up to 10 sec. Therefore, 10-sec uptakes were used to calculate initial rates of glucose uptake in the following kinetic experiments.

The kinetics of glucose transport under voltage-clamped conditions (the effects of membrane potential on glucose transport are discussed in detail in a later section) are presented in Fig. 8 for a representative membrane preparation. The uptake of glucose was saturable (inset, Fig. 8) and the kinetics were adequately described by the Michaelis-Menten equation [Eq. (1)]. The Woolf-Augustinsson-Hofstee plot (Fig. 8) of the data was linear suggesting the presence of a single saturable process. In experiments with membranes from four separate preparations, the J_{max} for glucose transport was 480 ± 84 pmol/mg-min with an apparent K_t of 3 ± 1 μM.

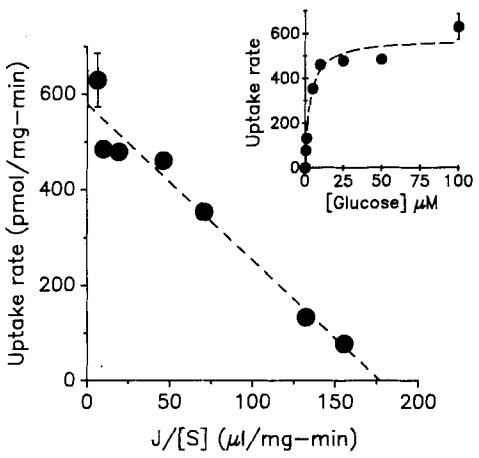

Fig. 8. Kinetics of glucose uptake in BBMV. Woolf-Augustinsson-Hofstee plot of the initial rate (10 sec) of glucose uptake (J) at different glucose concentrations [S]. The insert shows the same data, presented as a Michaelis-Menten plot. Vesicles were resuspended in 600 mM mannitol, 10 mM HEPES-Tris, pH 7.6. Transport buffers contained ^{3}H-D-glucose (from 0.5 to 100 μM), 260 mM NaCl, 60 mM mannitol, 10 mM HEPES-Tris, pH 7.6 and 25 μM FCCP. Each point represents the mean ± SE (some of the points are larger than the error bars) of triplicate determinations from a single membrane preparation. The line in the main figure was determined using a linear regression, but the kinetic parameters (J_{max} 580 pmol/mg-min, K_t 3 μM) were determined from a nonlinear regression of the data fit to the Michaelis-Menten equation (Duggleby, 1981)

Na^+-Activation of Glucose Uptake

The activation of glucose uptake in BBMV appeared to be a sigmoid, rather than hyperbolic, function of the extravesicular Na^+ concentration (Fig. 9*A*). Similar results were obtained in studies of the effect of Na^+ on glucose uptake in intact gills (Fig. 9*B*). These observations suggest that more than one Na^+ ion is involved in the transport of each glucose molecule across the brush-border membrane of the gill. For the purposes of comparison with other studies, Hill analyses of the data were carried out. In experiments with four separate preparations of BBMV, the apparent Hill coefficient, n, was 1.5 ± 0.1, the K_{Na^+} was 97 ± 10 mM, and the J_{max} for uptake of 0.5 μM glucose was 66 ± 9 pmol/mg-min. In the isolated gill, n was 1.2 ± 0.3, the K_{Na} was 142 ± 65 mM, and the J_{max} was 0.9 ± 0.2 μmol/g-hr.

Inhibition of Glucose Transport by Harmaline

Harmaline has been shown to inhibit Na^+-glucose cotransport in rabbit kidney BBMV by competing with Na^+ for binding at the transporter (Aronson & Bounds, 1980). Figure 10*A* shows a Dixon plot of harmaline inhibition of Na^+-glucose transport in mussel gill BBMV at two different concentrations of Na^+. The intersection of the two lines above the x-axis indicated that harmaline interacted with the glucose transporter as a competitive inhibitor of Na^+ (*see* Segel, 1975) with a K_i of 44 μM (Fig. 10). The interaction between harmaline and the transporter was hyperbolic, as shown by the linear Dixon plot in Fig. 10, implying that glucose uptake was inhibited when a single Na^+ binding site on the transporter was blocked. Harmaline inhibition of glucose uptake by intact gills in full-strength seawater (425 mM Na^+) was also hyperbolic (Fig. 10*B*). Under this condition, the concentration of harmaline which inhibited glucose uptake by 50% (the IC_{50}) was 560 ± 100 μM (n = 5).

Effect of Membrane Potential on Glucose Uptake in BBMV

The cotransport of Na^+ with glucose should be electrogenic, bringing a net positive charge into the vesicles. To test this prediction, the effects of membrane potential difference (PD) on glucose transport were examined. The PD of the vesicles was manipulated in three ways. First, diffusion potentials of anions having different permeabilities in other epithelia were used (Gunther, Schell & Wright, 1984; Kimmich et al., 1985). The initial rate of Na^+-dependent glucose transport was stimulated in the presence of the permeant anion, thiocyanate (SCN^-), and inhibited in the presence of the impermeant anion, isethionate, relative to the uptake in Cl^- (Fig. 11). These voltage-dependent differences in uptake rate were abolished by addition of the protonophore, carbonyl cyanide p-trifluoromethoxy phenylhydrazone (FCCP), which should have clamped the PD at 0 mV, given equal pH inside and outside the vesicles (pH 7.6). These observations support the contention that Na^+-dependent glucose uptake in these membranes is electrogenic and stimulated by an inside-negative membrane potential. In later studies, 25 μM FCCP, and equal pH inside and outside the vesicles, was routinely used to voltage-clamp the vesicles at PD = 0.

The second approach involved manipulation of PD through the use of K^+ diffusion potentials, established with K^+ gradients and the ionophore, valinomycin. Figure 12*A* shows the time course of glucose uptake into KCl-loaded vesicles, under control conditions or with the K^+-selective ionophore, valinomycin, present in the transport buffer. There was a marked stimulation of both the initial rate and the height of the overshoot in the

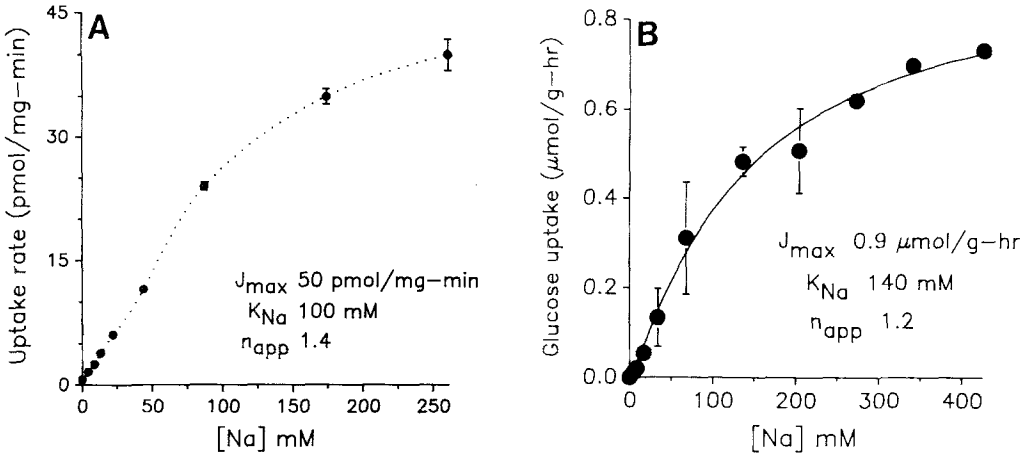

Fig. 9. The activation of the initial rate of glucose uptake by increasing concentrations of Na^+. (*A*) BBMV: Vesicles were resuspended in 600 mM mannitol, 10 mM HEPES-Tris, at pH 7.6. Transport buffers contained 0.5 μM ^{3}H-D-glucose, 60 mM mannitol, 10 mM HEPES-Tris, at pH 7.6, 25 μM FCCP, and NaCl from 0 to 260 mM (the solutions were kept isoosmotic with LiCl). Ten-second uptakes were measured. Each point represents the mean ± SE of triplicate determinations from a single membrane preparation. (*B*) Intact gills from *M. californianus:* Demibranchs were incubated 5 min. in ASW containing 0.5 μM ^{14}C-D-glucose and increasing concentrations of Na^+, with Li^+ used as the replacement ion for Na^+. The points represent means ± range of determinations using two animals. The kinetic parameters were determined by a computer program for the best fit to the Hill equation

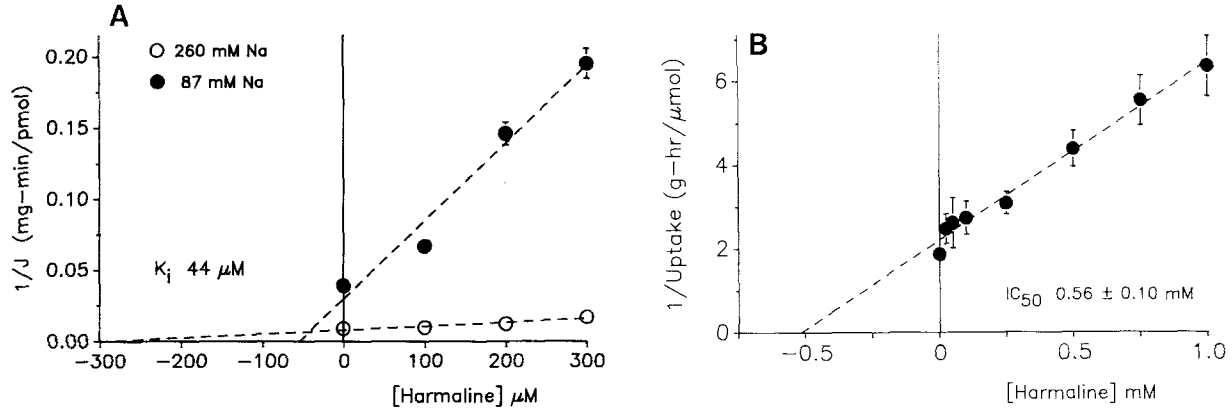

Fig. 10. (*A*) Dixon plot of the initial rate (10 sec) of glucose uptake (*J*) at increasing concentrations of harmaline in BBMV. Vesicles contained 600 mM mannitol, 10 mM HEPES-Tris, pH 7.6. Transport buffers contained 0.5 μM ^{3}H-D-glucose, 60 mM mannitol, 260 or 87 mM NaCl, 10 mM HEPES-Tris, pH 7.6, 25 μM FCCP and up to 300 μM harmaline. Individual data points (triplicate determinations from a single membrane preparation) are shown. The equations of the lines were determined from least-squares linear regressions of the data, and the K_i shown is the negative *x*-intercept of the intersection of those lines. (*B*) Inhibition of glucose uptake by harmaline in intact *M. californianus* gills. Gills were incubated 5 min in full-strength ASW (425 mM Na^+) with 0.5 μM ^{14}C-D-glucose, and up to 1 mM harmaline. The data points represent means ± SE of determinations using gills from five separate animals. The IC_{50} shown is the mean ± SE of the *x*-intercepts from five individual plots

presence of valinomycin (i.e., an inside-negative membrane potential). Figure 12*B* shows the effect of adding valinomycin to KCl-containing vesicles at the peak of the overshoot. Since the leak pathways for glucose in these vesicles are very small (*see* Figs. 7 and 8), the overshoot represents the point at which the energy in the inwardly directed Na^+ electrochemical gradient is balanced by the outwardly directed glucose gradient (*see* Aronson, 1981). Addition of valinomycin at this point produced an increase in the height of the overshoot, suggesting that the additional energy from the inside-negative membrane potential could drive uphill transport of glucose. The reverse experiment, adding valinomycin in a transport buffer containing 200 mM NaCl and 100 mM KCl to mannitol-loaded vesicles, resulted in a decrease in the initial rate of 68 ± 1% (n = 3), a 77% decrease in the height of the overshoot

when valinomycin was added at time = 0, and a more rapid decrease in intravesicular glucose concentration when valinomycin was added at the overshoot (1 min after addition of valinomycin the uptake was reduced by 60%). Preliminary experiments with valinomycin in NaCl showed that there was no direct effect of the ionophore on the initial rate of glucose transport (*results not shown*).

In the third approach, the electrogenicity of glucose transport in gill BBMV was also demonstrated in experiments which utilized the diffusion of protons via the proton ionophore, FCCP, to manipulate membrane potential (Hopfer, Lehninger & Thompson, 1968). The initial rate of glucose uptake in NaCl was stimulated 1.5-fold in the presence of an outwardly directed pH gradient (7.6 inside, 8.5 outside) when FCCP was added. In the presence of an inwardly directed pH gradient (7.6 inside, 6.0 outside) plus FCCP, the initial rate of glucose uptake was only 70% of the control. In the absence of a pH gradient (pH 7.6) the addition of FCCP had no effect on glucose transport.

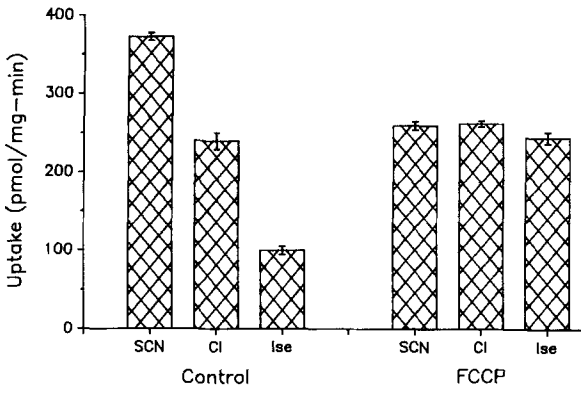

Fig. 11. The effects of anions and FCCP on the initial rate (10 sec) of glucose uptake in BBMV. Vesicles were resuspended in 600 mM mannitol, 10 mM HEPES-Tris, pH 7.6. Transport buffers contained 10 μM ^{3}H-D-glucose, 60 mM mannitol, 10 mM HEPES-Tris, pH 7.6 and 260 mM of either NaCl, NaSCN, or NaIsethionate. The control groups (shown on the left of the figure) received ethanol, while the FCCP groups (shown on the right) received 25 μM FCCP in ethanol. The final ethanol content of the transport buffers was 1%. Each bar represents the mean ± SE of triplicate determinations from a single membrane preparation

Substrate Specificity

The inhibitory effects of a series of structural analogs of glucose on Na^+-dependent glucose transport in mussel gill BBMV are shown in Fig. 13. The transporter showed stereospecificity for the D-isomer of glucose: 100 μM D-glucose inhibited uptake of 0.5 μM ^{3}H-D-glucose by 97% while 100 μM L-glucose had no effect. A modification of the structure of glucose at the C1 position had no effect on transport; α-MG was as effective an inhibitor as D-glucose. Modification of the glucose structure at position C2 or C3 abolished its ability to interact with the Na^+-glucose transporter; there were no significant inhibitory effects of either 2-deoxy-glucose or 3-O-methyl-glucose. D-galactose (100 μM) reduced uptake of D-glucose by 84%, indicating that a modification of the structure of glucose at C4 reduced the affinity of substrates for the glucose transporter. D-fructose did not inhibit glucose transport.

The effects of 100 μM concentrations of these sugars on the transport of 0.5 μM ^{14}C-glucose in intact gills of *M. edulis* and *M. californianus* were very similar to their effects in the BBMV (Fig. 14).

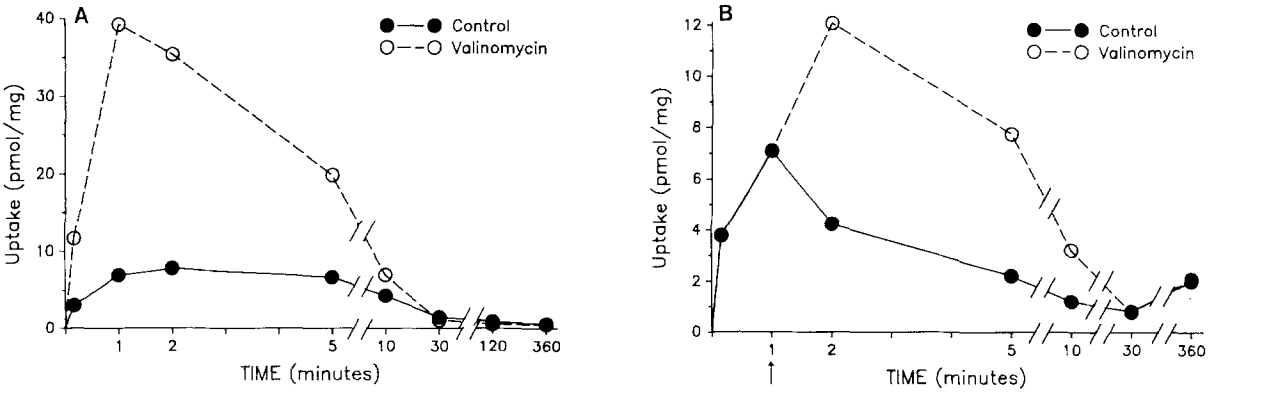

Fig. 12. Time courses of glucose uptake in brush-border membrane vesicles resuspended in 300 mM KCl, 10 mM HEPES-Tris, pH 7.6. Transport buffers contained 0.5 μM ^{3}H-D-glucose, 60 mM mannitol, 260 mM NaCl, 10 mM HEPES-Tris, pH 7.6 and either ethanol (control) or 25 μg/ml valinomycin in ethanol. (*A*): Time courses in which the vesicles were preincubated with ethanol or valinomycin and the transport buffers also contained ethanol or valinomycin from the start. (*B*): Time courses that were started without ethanol or valinomycin. The ethanol or valinomycin was added at 1 min (indicated by the arrow). Each point represents the mean of triplicate determinations from a single membrane preparation

As in the BBMV, D-glucose and αMG were the most effective inhibitors (greater than 80% inhibition) of D-glucose transport. D-galactose, which reduced glucose uptake by 84% in the BBMV, inhibited glucose uptake by less than 50% in the gill. There were minor effects (less than 20% inhibition) of 2-deoxy-glucose and 3-O-methyl-glucose on glucose transport in the gill. Finally, L-glucose and D-fructose did not inhibit the uptake of glucose in the intact gill.

Phlorizin was an effective inhibitor of glucose transport in both BBMV and intact gills. The kinetics of the interaction of phlorizin with the glucose transporter are shown as modified Dixon plots in Fig. 15*A* and *B*. The mean apparent K_i for phlorizin in BBMV was 154 ± 17 nM (n = 3), and in intact gills, was 21 ± 9 nM (n = 6). The differences in apparent K_i between preparations may be related to the different concentrations of Na^+ used (260 mM

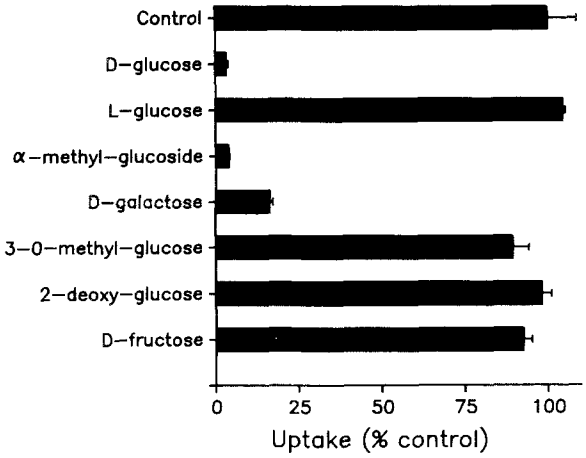

Fig. 13. Effect of glucose analogs on Na^+-glucose transport in mussel gill brush-border membrane vesicles. Vesicles were resuspended in 600 mM mannitol, 10 mM HEPES-Tris, pH 7.6. Initial rates (10 sec) were measured. Transport buffers contained 0.5 μM ^{3}H-D-glucose, 260 mM NaCl, 60 mM mannitol, 10 mM HEPES-Tris, pH 7.6, 25 μM FCCP and either distilled water (control) or 100 μM inhibitor. The uptake seen in the presence of inhibitor is expressed as a percentage of control and each bar represents the mean ± SE of experiments from three different membrane preparations

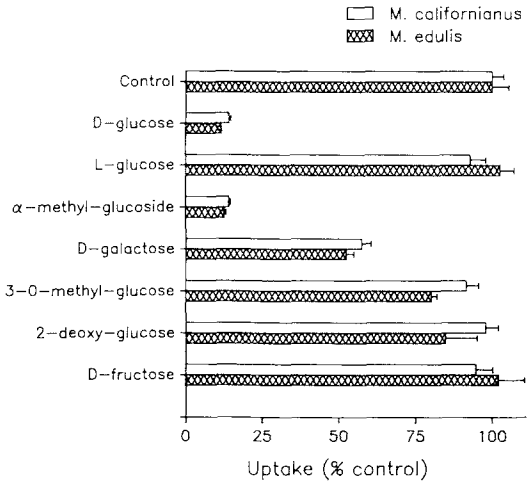

Fig. 14. Effect of glucose analogs on Na^+-glucose transport in isolated gills from *M. californianus* and *M. edulis*. The test solutions contained: 0.5 μM ^{14}C-D-glucose, 100 μM inhibitor (control solutions contained water), and 10 μM serotonin in ASW. Uptakes were measured for 5 min. The bars represent means ± SE of experiments conducted with three animals

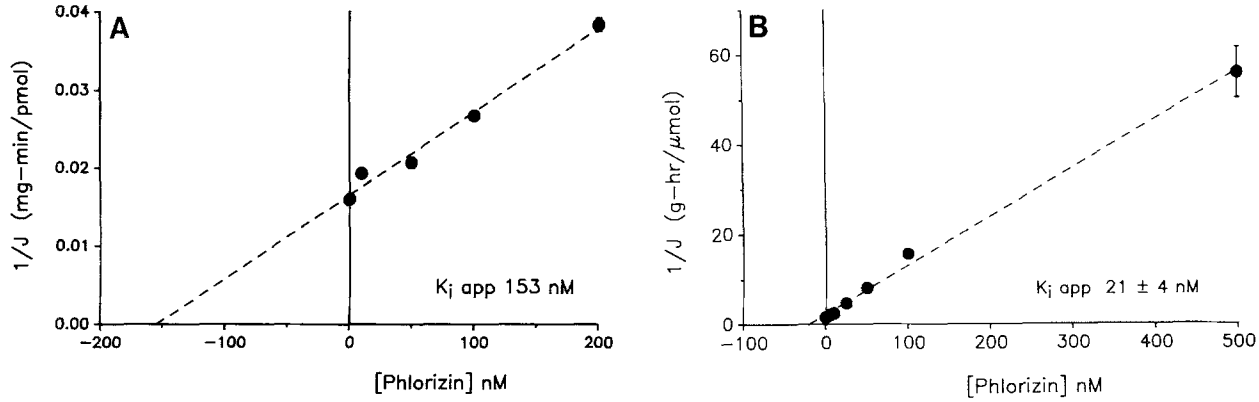

Fig. 15. Modified Dixon plots of the initial rates of glucose uptake (J) at increasing concentrations of phlorizin. The dashed lines were determined from least-squares linear regressions of the data and the apparent K_i's shown are the negative x-intercepts of those lines. (*A*) BBMV: Vesicles contained 600 mM mannitol, 10 mM HEPES-Tris, pH 7.6. Transport buffers contained 0.5 μM ^{3}H-D-glucose, 260 mM NaCl, 60 mM mannitol, 10 mM HEPES-Tris, pH 7.6, 25 μM FCCP and up to 200 nM phlorizin. Uptakes were measured for 10 sec. Means ± SE of triplicate determinations from a single membrane preparation are shown. (*B*) Isolated gills from *M. californianus:* The test solutions contained 0.5 μM ^{14}C-D-glucose, up to 100 μM phlorizin, and 10 μM serotonin in ASW. Five-minute uptakes were measured. The points represent means ± SE of determinations using six animals.

for BBMV *vs.* 425 mM for gills). Phloretin and cytochalasin B, inhibitors of Na^+-independent glucose transport (Basketter & Widdas, 1978), had comparatively minor inhibitory effects on Na^+-dependent glucose transport in gill BBMV. In a single experiment, 1 μM phlorizin inhibited the initial rate of 0.5 μM glucose transport by 91%, while 1 μM phloretin or 1 μM cytochalasin B only inhibited glucose transport by 15 and 12%, respectively.

Discussion

The transport of D-glucose in mussel gills resembles the concentrative uptake of glucose in other transporting epithelia, such as the mammalian small intestine and kidney (*see* reviews by Ullrich, 1986; Hopfer, 1987). This uptake occurs by a secondary active process involving cotransport of sodium and glucose. As shown here, this process is capable of a net accumulation of glucose into the cells of the integument from ambient concentrations in seawater of less than 100 nM.

Two related species of mussels were used for these studies: *Mytilus edulis* and *Mytilus californianus*. The results presented here showed that there were no qualitative differences in glucose transport between these two species. However, each of these species had unique characteristics that dictated their use for the different aspects of this study. The gills of *M. californianus* are ideally suited for intact gill experiments because the stable junctions between adjacent filaments prevent damage to the gill during the handling associated with transport measurements. In contrast, *M. edulis* gills have very fragile interfilamentous junctions, and so were easily disrupted during the gentle homogenization procedures used in the preparation of brush-border membrane vesicles.

The epithelial cells of mussel gills, and of other marine invertebrate integuments (Ahearn & Gomme, 1975), rapidly metabolize transported glucose. However, since the concentrations of glucose in seawater are extremely low, perhaps on the order of 50 nM (Vaccaro et al., 1968), the transmembrane glucose gradient is still likely to be large and outwardly directed (*see also* Gomme, 1981). Initial observations of glucose transport in intact mussel gills showed that net uptake of glucose occurred by a Na^+-sensitive, carrier-mediated process. This suggested that the mechanism of uphill glucose transport in the gill involves a coupling of the movements of Na^+ and glucose, as in vertebrate epithelia. In order to address this question, studies were performed using brush-border membrane vesicles prepared from mussel gills.

The transient concentrative uptake of glucose (the "overshoot") in BBMV seen in the time course experiment (Fig. 6) provides evidence that the energy for active transport of glucose in the gill comes from an inwardly directed gradient of sodium, and requires coupling between the movements of glucose and sodium. Furthermore, this transport shows specificity for Na^+, since replacement of Na^+ by other cations decreased the initial rate of uptake and abolished the overshoot. The Na^+ requirement for glucose uptake observed here in mussel gills, as well as in other bivalve gill preparations (Bamford & Gingles, 1974), supports the suggestion that cotransport with Na^+ is a general feature of concentrative glucose transport in animal cells. In contrast, alanine transport in mussel gill BBMV involves a component that exhibits a low cation selectivity, since concentrative transport was supported by gradients of Li^+ and K^+ as well as Na^+ (Pajor & Wright, 1987).

The transport of glucose in gill BBMV was electrogenic, and was affected by changes in membrane potential (Figs. 11 and 12). It should be emphasized that changes in initial rates of transport produced by manipulations of membrane potential do not in themselves provide evidence that transport is electrogenic; changes in initial rates may simply reflect a catalytic effect of membrane potential on the transporter. Nevertheless, the time course experiments show that an inside-negative membrane potential can provide energy for uphill transport (Fig. 12). The peak of the overshoot in a time course experiment, in the absence of leak pathways, signifies the point at which the energy in the inwardly directed sodium gradient is equal to the outwardly directed glucose gradient (*see* Aronson, 1981). Beyond this point there is no net concentrative uptake of glucose and intravesicular glucose concentrations fall toward equilibrium. In the experiment shown in Fig. 12*B*, the generation of an inside-negative diffusion potential in these vesicles at the time of the overshoot resulted in an increase in the height of the glucose overshoot. This indicated that the energy in such an electrical gradient was additive to that in the Na^+ chemical gradient, evidence that the membrane potential can provide energy for uphill transport of glucose. This is consistent with a transport mechanism that involves cotransport of sodium and glucose, with a net transfer of positive charge.

The brush border of the mussel gill probably contains only a single carrier-mediated pathway for the uptake of glucose (Figs. 4 and 8), based on the linear relationship of the Woolf-Augustinsson-Hofstee plot. This transporter appears to have a very high affinity substrate binding site, as shown by an

apparent K_t for glucose ranging from 3 μM in BBMV to 9 μM in intact gills, and apparent K_i for phlorizin of 150 nM in BBMV and 21 nM in intact gills. In contrast, the major Na^+-glucose transport system in mammals has K_t values between 0.2 and 4 mM (*see* review by Hopfer, 1987). The high substrate affinity in mussels appears to reflect the low glucose concentrations in the near-shore waters, less than 1 μM (Vaccaro et al., 1968), to which the brush-border membrane of the integument is exposed.

The large outwardly directed glucose gradient across the mussel gill brush-border membrane could conceivably be overcome by coupling more than one Na^+ to the transport of each glucose (*see* Aronson, 1981). There are several reports of Na^+-glucose cotransporters in vertebrate epithelia with coupling coefficients of two or more (e.g., Kimmich & Randles, 1980; Turner & Moran, 1982; Kaunitz & Wright, 1984). The Na^+-glucose cotransporter in the late portion of the renal proximal tubule (Turner & Moran, 1982) has a coupling stoichiometry of 2 : 1. This has been suggested to represent an adaptation permitting the accumulation of the last traces of glucose in the nephron against large concentration gradients.

The kinetics of the interaction of Na^+ with the glucose transporter in mussel gills suggested a coupling coefficient of at least 2 : 1. The energy from the chemical gradient of Na^+, together with the −60 mV potential difference (Murakami & Takhashi, 1975), across the gill brush-border membrane, could potentially support uptake against up to a 90,000-fold gradient of glucose if two Na^+ ions were coupled to the movement of each glucose (*see* discussion in Manahan et al., 1983). Furthermore, the kinetics of glucose uptake suggest that the passive permeability of the brush-border membrane to glucose is low. This would serve to reduce passive leaks from the gill in the face of a large outwardly directed glucose gradient.

The substrate specificity of glucose transport in mussel gill BBMV was very similar to that of the intact gill (Figs. 13 and 14). The effects of glucose analogs on sugar transport by the gills of other marine bivalves, *Ostrea edulis* (Riley, 1981) and *Crassostrea gigas* (Bamford & Gingles, 1974), resembled their effects on transport in the mussel gill. A similar pattern of inhibition of glucose transport by sugars was observed for the integument of the polychaete worm, *Nereis diversicolor,* although L-glucose, which did not affect transport in bivalve gills, did inhibit D-glucose transport in *Nereis* (Albrechtsen & Gomme, 1984). It appears, therefore, that integumental glucose transporters in general have substrate specificities, which are very similar to those in mammalian epithelia (Turner & Silverman, 1978). In all preparations studied to date, α-methyl glucoside is an effective inhibitor of Na^+-dependent D-glucose transport, D-galactose is intermediate in its effects, and removal of the OH group at C2 of glucose removes any inhibitory effects.

The Na^+-dependent glucose transport activity of mussel gills was predominantly found in brush-border membrane fractions. There was no evidence of Na^+-independent transport of glucose in the BBMV, so we conclude that the brush border probably contains a single Na^+-dependent glucose transporter. There was no peak in the distribution of Na^+-independent glucose uptake in the sucrose density gradient, which may suggest that this pathway is absent from basal-lateral membranes. However, if the Na^+-independent transport has a high K_t for glucose, for example in the millimolar range, the existence of this pathway in the basal-lateral membrane fractions may have been overlooked with the micromolar substrate concentrations used in the present set of experiments.

In conclusion, the transport of D-glucose in intact gills and in BBMV isolated from the gills of the mussel, *Mytilus,* resembled quite closely Na^+-glucose cotransport in vertebrate epithelia. Of interest, though, are the quantitative characteristics of the process in the bivalve integument. The high substrate affinity and coupling of multiple Na^+ ions to the flux of each glucose molecule suggests that this transporter is well-adapted for operation at the extremely low environmental glucose levels to which it is exposed.

This study was funded in part by a NIH predoctoral training grant to AMP (HL-07249) and by NSF Grant DCB85-17769.

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Received 27 June 1988, revised 18 August 1988