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L-Alanine Uptake in Brush Border Membrane Vesicles from the Gill of a Marine Bivalve

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Summary. Brush border membrane vesicles were prepared from mussel gills using differential and sucrose density gradient centrifugation. These vesicles contained both the maximal Na^+-dependent alanine transport activity found in the gradient and the maximal activities of γ-glutamyl transpeptidase and alkaline phosphatase. Electron micrographs showed closed vesicles of approximately 0.1–0.5 μm diameter. Transport experiments using these vesicles demonstrated a transient 18-fold overshoot in intravesicular alanine concentration in the presence of an inwardly directed Na^+ gradient, but not under Na^+ equilibrium conditions. A reduced overshoot (10-fold) was seen with an inwardly directed K^+ gradient. Further studies revealed a broad cation selectivity, with preference for Na^+, which was characteristic of alanine transport but not glucose transport in these membranes. The apparent amino acid specificity of the uptake pathway(s) was similar to that of intact gills and supported the idea of at least four separate pathways for amino acid transport in mussel gill brush border membranes. The apparent Michaelis constant for alanine uptake was approximately 7 μM, consistent with values for K_t determined with intact tissue.

Key Words alanine transport · neutral amino acid transport · brush border membrane vesicles · marine bivalve gills · *Mytilus edulis* · epithelia · integument

Introduction

All soft-bodied marine invertebrates can accumulate amino acids from seawater (Jørgensen, 1976; Stephens, 1982). In marine bivalves, the epithelial cells of the gill are the primary site of uptake (Péquignat, 1973). Studies with both isolated gill tissue and intact animals have shown this transport to be a saturable process involving at least four separate pathways for the uptake of amino acids (Stewart, 1978; Wright, 1985). Furthermore, the rates of uptake seen in these animals suggest that this "integumental transport" can be significant in animal nutrition (Wright, 1982, 1987*a*).

Transport processes in marine bivalves are capable of net uptake of amino acids against very large concentration gradients. Intracellular amino acid concentrations in gill tissue are on the order of 100 mM (Wright & Secomb, 1986) and are thought to be involved in isosmotic volume regulation (Gilles, 1979). Recent studies have demonstrated net uptake of amino acids from concentrations as low as 10 nM (Manahan, Wright & Stephens, 1983; Wright & Secomb, 1986), indicative of transport against chemical gradients in excess of 1,000,000 to 1. In isolated gill tissue, amino acid transport has been shown to be Na^+-dependent (Stewart & Bamford, 1975; Wright, 1985), suggesting uptake according to the Na^+-gradient hypothesis (Crane, 1977). This would imply both a relatively large Na^+-coupling coefficient and efficient coupling between amino acid and Na^+ fluxes (Manahan et al., 1983). However, there is as yet no evidence of coupling between the flows of Na^+ and amino acids in bivalve gill epithelia.

The study of epithelial transport has benefited greatly from the introduction of preparations of isolated plasma membrane vesicles. These have permitted detailed tests of the Na^+-gradient hypothesis in several experimental systems (Murer & Kinne, 1980). To this end, the present study is a description of the procedures developed to isolate from the mussel gill a subcellular membrane fraction containing Na^+-dependent L-alanine transport. The transport activity in these membranes was associated with activity of apical membrane enzyme markers, suggesting that these membranes were of brush border origin. The kinetics and apparent structural specificity of alanine uptake paralleled closely those observed in previous studies of alanine uptake in intact gill tissue (Wright, 1985).

Materials and Methods

Animals

Specimens of the common blue mussel, *Mytilus edulis*, were purchased from the Bodega Marine Laboratory, Bodega Bay,

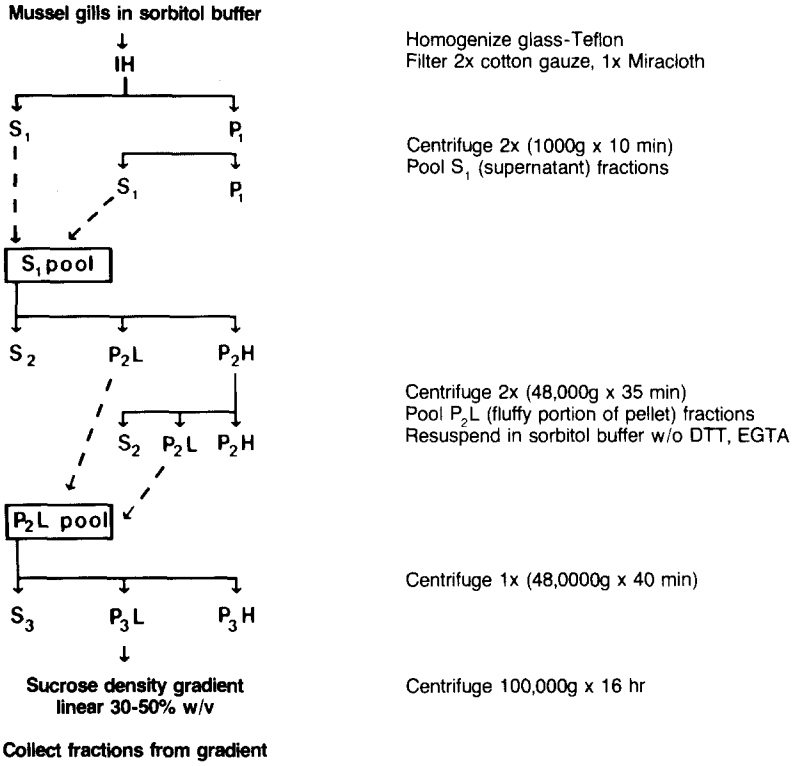

Fig. 1. Preparation of isolated brush border membrane vesicles from mussel gills

CA. They were maintained in refrigerated aquaria (12°C) containing recirculating, aerated artificial seawater (Instant Ocean). Animals were not fed and were used within four weeks of collection.

Membrane Preparation

Gill plasma membrane vesicles were prepared by a modification of the method developed by Ives, Yee and Warnock (1983) for isolation of brush border membranes from rabbit kidney. The modified method is illustrated in Fig. 1. Gill tissue from approximately 35 mussels was used for each preparation. Initial efforts at plasma membrane isolation were complicated by the large amount of mucus normally associated with the gills. This problem was relieved by presoaking the gill tissue (2 × 5 min) in an artificial seawater containing high concentrations of KCl (high K^+-ASW) and 1 mM dithiothreitol (DTT) at pH 8.0. The high K^+-ASW was prepared according to Cavanaugh (1956), except it contained a higher concentration of KCl (115 mM) and lower concentration of NaCl (319 mM) than the "normal" artificial seawater (i.e., 9 mM KCl, 425 mM NaCl). The high K^+ content of this solution served to stimulate mucus release from the goblet cells of the gill, while the DTT dispersed the mucus. The tissue was then blotted dry and weighed. A typical preparation used between 35 and 40 g (wet wt) of gills. The tissue was minced with scissors and homogenized gently using a glass-Teflon homogenizer (10 passes at 1200 rpm). The homogenization buffer contained 500 mM sorbitol, 1 mM DTT, 5 mM ethyleneglycol-bis(β-amino-ethylether)-N-N′-tetraacetic acid (EGTA), 5 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, buffered to pH 7.6 with tris(hydroxymethyl)-aminomethane (HEPES-Tris). Preliminary studies showed that the use of 300 mM sorbitol buffer or homogenization with an Omni-mixer (3 × 30 sec, with 1-min intervals) caused disruption of intracellular organelles as shown by high levels of succinate dehydrogenase (SDH) activity in all fractions. The homogenate was filtered twice through cotton gauze and once through Miracloth (Calbiochem). The resulting fraction was called the initial homogenate (IH).

Divalent cation precipitation has been used successfully to isolate brush border membranes from both mammalian (Booth & Kenny, 1974) and invertebrate (Hanozet, Giordana & Sacchi, 1980; Bajorat & Schlichter, 1983; Ahearn, Grover & Dunn, 1985) epithelia. In our preparation, however, neither $CaCl_2$ nor $MgCl_2$, alone or together, up to 50 mM in concentration, produced a selective precipitation of membranes. Consequently, several differential centrifugation steps (Beckman J2-21 centrifuge, JA-20 rotor) were used to prepare "crude plasma membranes" (*see* Fig. 1). The crude membrane preparation, P_3L (the fluffy portion of the final pellet), was then resuspended in 6 ml sorbitol buffer (500 mM sorbitol, 5 mM HEPES-Tris, pH 7.6) and layered onto two 35-ml linear 30–50% (wt/vol) sucrose density gradients buffered with 10 mM HEPES-Tris, pH 7.6. Approximately 15–20 mg protein were added to each gradient. The gradients were centrifuged overnight at 100,000 × g (Beckman L5-65 ultracentrifuge, SW-28 rotor). Fractions (3 ml) were collected from the gradients and were assayed directly for marker enzyme activity and protein content. For transport studies, the fractions were twice resuspended in 600 mM mannitol, 10 mM HEPES-Tris, pH 7.6, and centrifuged 1 hr at 48,400 × g. The final pellets were resuspended in a small volume of the same buffer. When it was necessary to equilibrate the membranes with Na^+- or K^+-containing solutions, the fractions were homogenized in NaCl or KCl buffer (300 mM, 10 mM HEPES-Tris, pH 7.6) and pelleted twice before the final resuspension. Membranes were then pre-equilibrated for 30 min in the Na^+- or K^+-containing buffer to which the cation-exchange ionophore, nigericin (25 μg/mg protein), had been added. Nigericin was also present in the transport buffer during the experi-

ments. Fractions containing the highest transport and brush border enzyme marker activity (fractions F_6–F_{10}) were pooled and either used fresh or were frozen in liquid nitrogen. There were no qualitative differences between fresh and frozen preparations. There was a gradual decline in the uptake rate of L-alanine over time (down to 60% of initial uptake rate by 7 days); therefore the membranes were used within three days of freezing. All kinetic studies were made using fresh membranes.

Electron Microscopy

Fractions F_6 through F_{10} from the sucrose density gradient were pooled and twice resuspended in 100 mM Na-cacodylate, 100 mM mannitol, pH 7.25, and centrifuged 1 hr at 48,400 × *g*. The final pellet was fixed overnight at 4°C in 2.5% glutaraldehyde-cacodylate (modified from Sabatini, Bensch & Barnett, 1963) and then rinsed twice in 100 mM Na-cacodylate, 100 mM mannitol, pH 7.25 at 4°C. The pellet was post-fixed at room temperature in 1% cacodylate-osmium tetroxide for approximately 2 hr and then rinsed twice with 100 mM Na-cacodylate buffer, pH 7.25. Subsequent steps included dehydration in a graded ethanol series and embedding in Embed/Araldite epoxy mixture (Mollenhauer, 1963). Thin sections, 60–80 nm, were cut and stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963).

Enzyme Assays

The distribution of marker enzyme activites was followed throughout the preparation. These enzymes were assayed according to established methods with modifications for different pH optima in mussel gills. The following methods were used (the pH used in our experimental incubations is shown in parentheses): alkaline phosphatase (pH 9.0), ouabain-inhibitable Na^+-K^+-ATPase (pH 8.0), succinate dehydrogenase (pH 8.0), acid phosphatase (pH 5.0) (Mircheff & Wright, 1976); γ-glutamyl transpeptidase (pH 7.4) (Naftalin et al., 1969); K^+-dependent paranitrophenyl phosphatase (pH 7.5) (modified from Ahmed & Judah, 1964); protein, BIORAD, using a γ-globulin standard. All assays were done at room temperature.

Transport Measurements

The transport of substrates into membrane vesicles was measured using a rapid filtration method described previously (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 Millipore filter (HAWP) and washed with 4 ml of cold mannitol buffer. The labeled substrate retained on the filter was extracted in scintillation vials containing 10 ml scintillation cocktail (Betaphase), and radioactivity was determined using a liquid scintillation counter (Beckman LSC-3801). Counts were corrected for variable quench and all uptakes were corrected for nonspecific binding to membranes and filters.

Isolated Gill Experiments

The uptake of ^{3}H-alanine into intact isolated gill tissue was determined by a method similar to the one outlined in Wright (1985) for the measurement of uptakes in gills from the mussel *Mytilus californianus*. However, the gills of *M. edulis* are more fragile than those of *M. californianus*, so several modifications were made. Demibranchs were isolated under an artificial seawater (ASW) (Cavanaugh, 1956) and cut into four pieces with a fresh razor blade. Five minutes before starting an experiment each piece of demibranch was gently transferred to an Erlenmeyer flask containing ASW. This solution also contained 10 μM 5-hydroxytryptamine (5-HT) to activate lateral cilia (Aiello, 1970; Wright, 1979). The flasks were placed on a gently rotating shaker (100 cycles per min). The ASW solution was poured out of the flasks, and 100 ml of ASW containing 10 μM 5-HT and 5 μM ^{3}H-alanine (with or without inhibitor) were added. The tissue was incubated with shaking at room temperature for 10 min. The reaction mixture was poured off, and ice-cold ASW was added as a stop solution. The flasks were left in ice 5 min to wash extracellular radioactivity from the gill pieces (Wright, 1985). The gill pieces were then blotted dry, weighed, and placed into scintillation vials with 1 ml 0.1 N HNO_3. Two hours later 10 ml of scintillation cocktail (Betaphase) were added to each vial and the radioactivity was counted. Uptake was expressed as μmol alanine accumulated per g wet gill wt.

Chemicals

^{3}H-alanine (50 Ci/mmol) was purchased from ICN. All other chemicals were obtained from Sigma Chemical Corp.

Results

Distribution of Enzyme and Transport Activity in the Sucrose Gradient

Figure 2 shows the distribution of enzyme markers and alanine transport in the sucrose density gradient. On the basis of enzyme marker distribution, there were two major membrane populations (Fig. 2*A*). One of these was found near the bottom of the gradient (approximately 43% sucrose) and contained the highest fraction activity of both γ-glutamyl transpeptidase (GGTP) and alkaline phosphatase. The second peak included both K^+-dependent para-nitrophenyl phosphatase (K-PNPPase) and Na^+,K^+-ATPase activities and was found near the top of the gradient (approximately 35% sucrose). The former enzymes are classical brush border markers in vertebrate (Mölbert, Duspiva & von Deimling, 1960; Rutenberg et al., 1969) and invertebrate (Momin & Rangneker, 1974; Schlichter, 1984) preparations, while the latter are often used as markers of the basal-lateral membrane of epithelia (Mayahara & Ogawa, 1980; Towle & Kays, 1986). The Na^+-sensitive alanine transport activity in the gradient (Fig. 2*B*) was associated with the brush border markers. The apical membrane of the gill epithelium, across which Na^+-dependent uptake of alanine occurs (Wright, 1985), has a rich microvil-

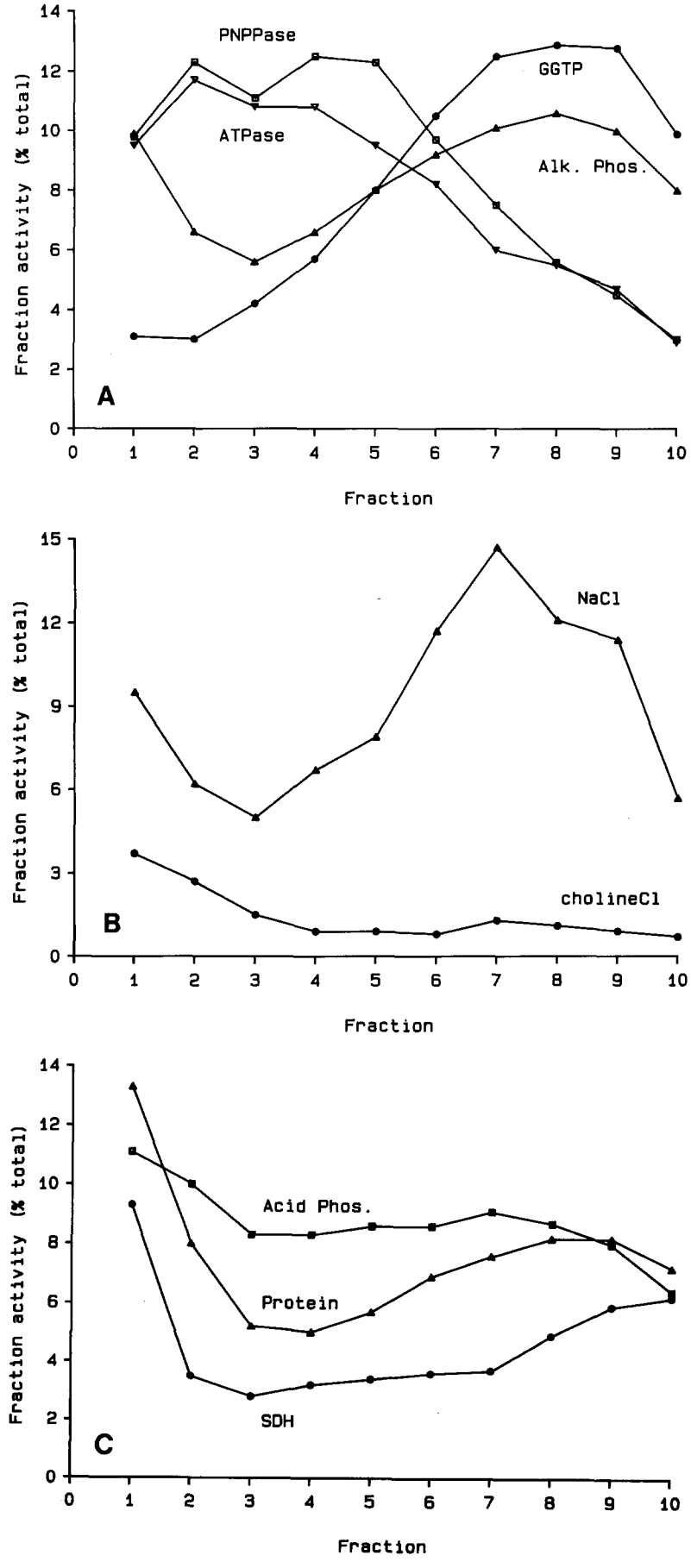

Fig. 2. (*A*) Distribution of marker enzymes in a linear 30–50% sucrose density gradient. Fraction 1 represents the top (30% sucrose) of the gradient. Activity is shown as the percentage of total recovered activity. Each curve represents the mean of 2 (*ATPase*), 3 (*PNPPase*, *GGTP*), or 4 (*Alk. Phos.*) membrane preparations. (*B*) Distribution of alanine transport activity in the sucrose density gradient. Vesicles contained 600 mM mannitol, 10 mM HEPES-Tris, pH 7.6. Transport buffers contained either 300 mM NaCl (upper curve, $n = 3$) or 300 mM cholineCl (lower curve, $n = 2$), with 10 mM HEPES-Tris, pH 7.6, and 5 μM ^{3}H-alanine. Activity in the presence of NaCl or cholineCl is expressed as a percentage of total recovered NaCl activity. Uptakes were run for 10 sec. (*C*) Distribution of protein ($n = 4$), succinate dehydrogenase (*SDH*; $n = 3$), and acid phosphatase (*Acid Phos.*; $n = 2$) in the sucrose density gradient

Table 1. Summary of specific activities, yields, and recoveries of marker enzymes and protein in membrane fractions during the preparation of brush border membranes from mussel gill

A Enzymes	*n*	Total recovery (% IH)[a]	IH (Sp act)[b]	P_3L		F_8		
				Sp act[b]	Yield (% IH)	Sp act[b]	Enrichment[c]	Yield (% IH)
GGTP	3	92.9 ± 7.2	1.4 ± 0.4	12.7 ± 1.4	28.2 ± 1.6	21.2 ± 2.6	18.5 ± 4.7	2.6 ± 0.7
Alkaline phosphatase	4	70.9 ± 4.2	83.2 ± 15.8	202.0 ± 30.2	6.8 ± 1.5	195.6 ± 31.4	2.8 ± 0.8	0.5 ± 0.1
K-PNPPase	3	99.3 ± 9.2	129.6 ± 16.7	230.5 ± 22.6	4.6 ± 0.1	229.6 ± 8.1	1.9 ± 0.4	0.27 ± 0.03
Na^+,K^+-ATPase	2	52.6 ± 3.7	67.0 ± 41.0	42.0 ± 29.0	0.8 ± 0.6	24.0 ± 17.0	0.4 ± 0.3	0.05 ± 0.04
SDH	3	89.4 ± 9.1	320.8 ± 8.4	56.9 ± 3.4	0.46 ± 0.02	12.0 ± 4.4	0.04 ± 0.02	0.003 ± 0.002
Acid phosphatase	2	112.9 ± 5.1	598.4 ± 35.2	651.3 ± 0.9	3.1 ± 0.2	2220 ± 51	4.4 ± 2.0	0.4 ± 0.01

B Protein	*n*	Total recovery (% IH)	IH (fraction protein (mg))	P_3L		F_8		
				Fraction protein (mg)	Yield (% IH)	Fraction protein (mg)	—	Yield (% IH)
Protein	4	102.6 ± 7.1	2667 ± 285	66.4 ± 5.9	2.5 ± 0.2	3.7 ± 0.2	—	0.14 ± 0.01

[a] Recovered activity expressed as % IH.
[b] Units of specific activity: GGTP μmol/mg protein-hr; others nmol/mg-hr.
[c] Specific activity of F_8 divided by specific activity of IH.

lous brush border (Wright, Secomb & Bradley, 1987). Therefore the Na^+-dependent alanine transport of the high-density membranes strongly suggests that this fraction includes the gill brush border. In intact mollusc intestinal epithelium, the effects of ouabain on transmural potential are limited to the serosal membrane (Gerencser, 1978), which suggests that the ouabain-inhibited Na^+,K^+-ATPase activity here included basal-lateral membranes. These are typically less dense than brush border membranes (e.g., Ives et al., 1983), consistent with their location in the gradient.

Most of the alanine uptake in the gradient was Na^+ sensitive. Alanine uptake in the presence of choline$^+$ (Fig. 2*B*) was distributed evenly through the gradient and represented a low proportion of the uptake seen in the presence of Na^+. Of interest was the absence of any peak of alanine transport, either Na^+ dependent or Na^+ independent, in the basal-lateral membrane fractions.

The activity of succinate dehydrogenase (SDH) was low in all fractions of the sucrose gradient (Fig. 2*C*) except for the bottom fraction (*not shown*), which contained approximately 45% of the total recoverable SDH activity. Acid phosphatase activity was distributed throughout the gradient (Fig. 2*C*). The protein distribution (Fig. 2*C*) resembled that of alkaline phosphatase except that the peak in fraction protein content at the higher sucrose density was smaller.

Enzyme Enrichments

The Table summarizes the marker enzyme activities in three fractions of the membrane preparation: the initial homogenate (IH), the crude plasma membrane fraction (P_3L) and the fraction representing the peak in brush border marker activity (F_8). The peak of the final membrane preparation (F_8) was enriched approximately 19-fold in GGTP activity and approximately threefold in alkaline phosphatase relative to the initial homogenate. Between 25 and 40% of the alkaline phosphatase activity in the initial homogenate was lost in the supernatant of the first high speed spin (S_2). Therefore a large proportion of the alkaline phosphatase in this tissue appears to be soluble rather than bound and accounts for the rather low enrichment in the F_8 fraction. This fraction was enriched approximately twofold in K-PNPPase, and approximately fourfold in the lysosomal marker, acid phosphatase. The contamination by mitochondria, as shown by 0.04-fold enrichment of SDH, was very low. The enrichment of Na^+,K^+-ATPase was also very low, 0.2-fold. However, the recovery of this enzyme was only 53%, which suggests a significant inactivation.

Since the yield of protein in F_8 was relatively low, fractions F_6 through F_{10} were pooled for transport studies. This increased the amount of protein from 3.7 ± 0.2 mg to 17.1 ± 1.0 mg (n = 4) and increased the yield of GGTP from 2.6 to 12.0% of

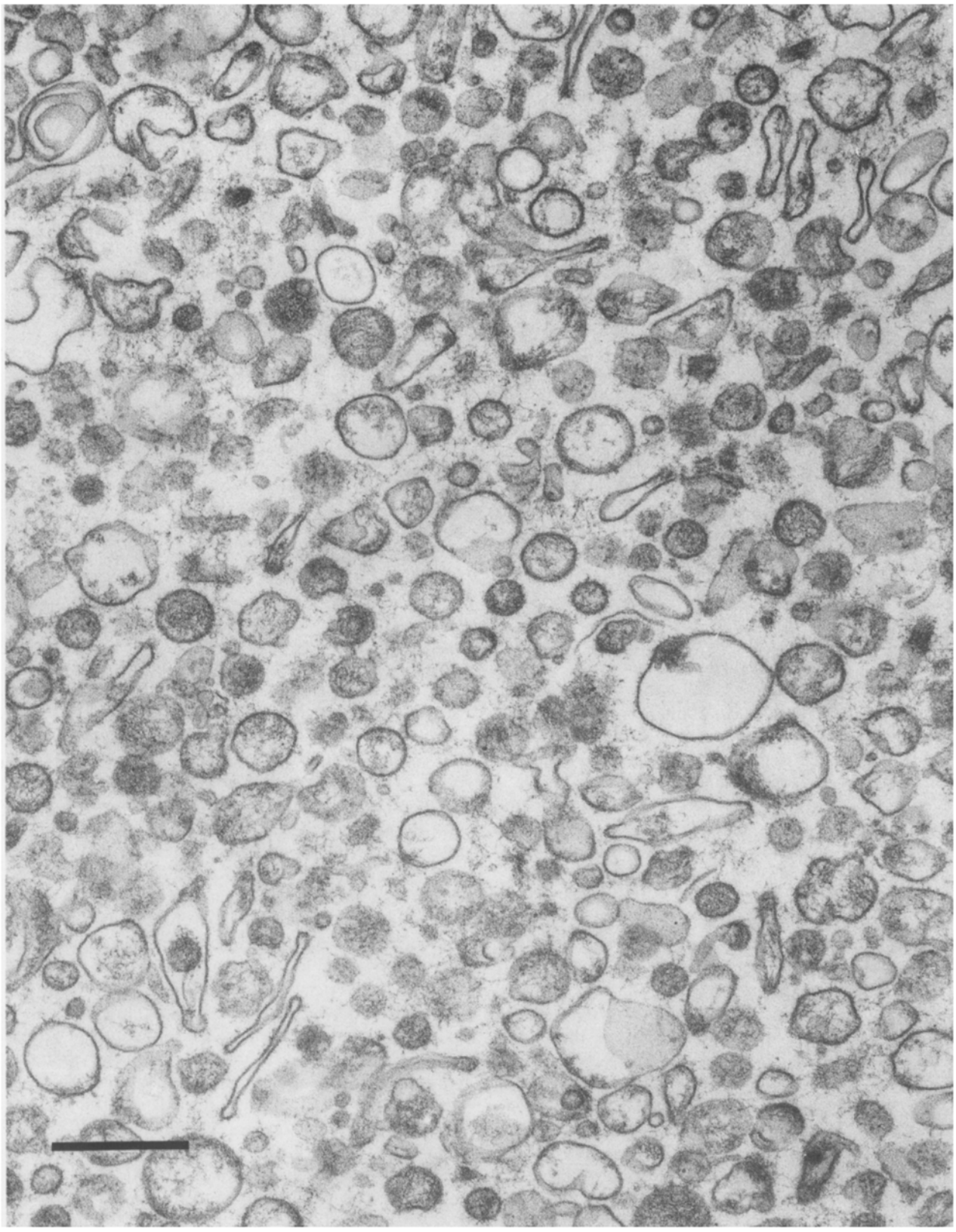

Fig. 3. Electron micrograph of the membranes used for transport studies, pooled fractions F_6–F_{10}. (×55,000). The bar represents 0.5 μm.

IH and the yield of alkaline phosphatase from 0.5 to 2.5% of IH. The enrichments of these enzymes in the pooled fractions changed slightly from those in fraction F_8: GGTP decreased from 18.5 to 17.8-fold and alkaline phosphatase increased from 2.8 to 3.8-fold enrichment. This pooled fraction is referred to as the brush border membrane vesicle (BBMV) fraction.

The total PNPPase activity was inhibited to the same extent (approximately 65%) by either a K^+-free reaction buffer (with Na^+ replacing K^+) or by 1 mM ouabain.

Electron Microscopy

Figure 3 shows an electron micrograph of the BBMV, i.e., the combined fractions F_6 through F_{10}. The vesicles appeared closed and ranged in size from approximately 0.1–0.5 μm. Associated with many of the vesicles was a glycocalyx-like layer, also seen in electron micrographs of the brush border of the intact mussel gill (Wright et al., 1987). Many of the vesicles also contained an electron-dense "core" material, possibly actin, which again suggests a brush border origin.

Time Course

Figure 4*A* shows the time course of alanine uptake into BBMV under four different conditions. With an inwardly directed NaCl gradient (300 mM NaCl out : 0 in), there was a transient "overshoot" in which the intravesicular alanine concentration exceeded that noted at 6 hr, the nominal "equilibrium" condition. The peak of the overshoot occurred at approximately 10 min, and in four experiments averaged 18.3 ± 3.6 times the uptake at 6 hr. Uphill alanine transport was still noted under Na^+-gradient, Cl^--equilibrium conditions (NaCl out : KCl in; no nigericin) with a 1.9-fold overshoot ($n = 1$; *results not shown*). These data indicate that a Na^+ gradient was sufficient to support concentrative alanine uptake into gill BBMV. Of interest was the observation that an inwardly directed K^+ gradient also supported uphill alanine uptake (Fig. 4*B*), although to a lesser extent (i.e., 9.5 ± 4.1-fold overshoot, $n = 4$) than that driven by a Na^+ gradient. An overshoot in alanine concentration, similar to that seen with K^+, was observed in the presence of an inwardly directed gradient of Li^+ (*results not shown*).

Initial attempts to measure alanine uptake under cation-equilibrium conditions employed homogenization and pre-equilibration in Na^+- and K^+-containing buffers in an effort to load the BBMV with these cations. Though this treatment did serve to reduce the rate of alanine uptake from Na^+- or K^+-containing transport buffers, it was not effective in eliminating transmembranous cation gradients as shown by the presence of overshoots in time courses of uptake into vesicles pre-equilibrated with NaCl (*data not shown*). Consequently, a cation-exchanging ionophore, nigericin, was used to facilitate preloading of the BBMV with Na^+ and K^+ (Beck & Sacktor, 1975). When vesicles were pre-equilibrated for 30 min in a cation-free buffer containing nigericin (25 μg/mg protein), the initial (6 sec) rate of alanine uptake from a buffer containing 300 mM NaCl plus nigericin was reduced by 40%, suggestive of a rapid collapse of the Na^+ gradient (Beck & Sacktor, 1975). When the pre-equilibration solution contained 300 mM NaCl plus nigericin, the initial rate of alanine uptake was reduced by 99.5%, and uphill accumulation of alanine (i.e., the overshoot) was eliminated, indicating that the nigericin was effective in completely collapsing the Na^+ gradient. Similar results were observed when nigericin was used to preload BBMV with K^+ (Fig. 4*B*).

Cation Effects

In light of the observed effects of K^+ on the time course of alanine uptake, a series of experiments was conducted to examine the cation selectivity of alanine transport. Figure 5 shows the 30-sec uptakes of alanine in the presence of inwardly directed gradients of different chloride salts. The greatest transport was seen in the presence of NaCl. LiCl and KCl could support 69 and 44%, respectively, of the transport seen with NaCl. Uptake rates were lower when Na^+ was replaced with mannitol or with the organic cations, choline$^+$ or N-methyl glucamine (NMG^+). However, approximately 95% of the uptake seen under these conditions was inhibited by 1 mM alanine. This suggests either a loose cation selectivity of the transporter or a cation-independent, carrier-mediated route for alanine uptake, or both. It would be surprising to find extensive facilitated diffusion transport of alanine on the brush border membrane of mussel gill cells in view of the very large intracellular amino acid concentrations which they maintain. Nevertheless, we have no evidence that this transporter can cotransport large cations, such as choline$^+$. Clearly, more work will need to be done to clarify this point. Initial rates were reduced by 99.5% under Na^+- and by 95% under K^+-equilibrium conditions, and these were lower than the uptake noted in the presence of mannitol.

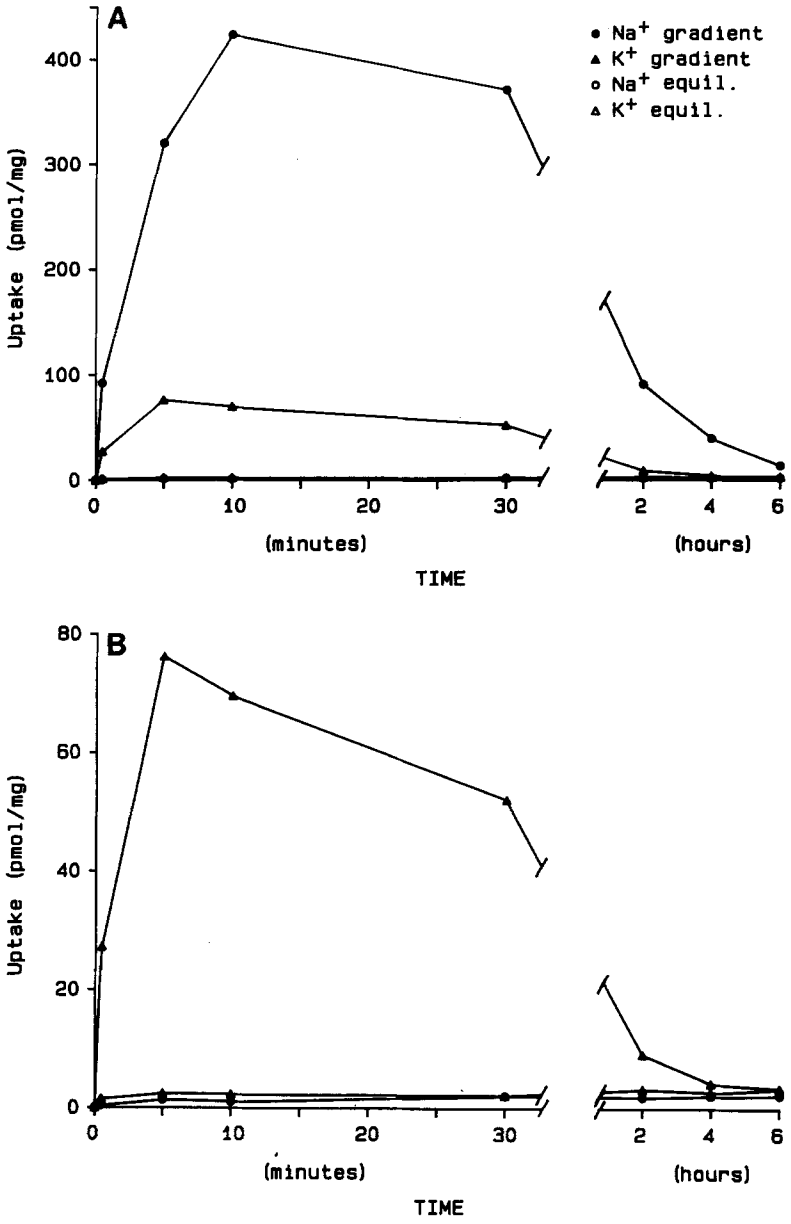

Fig. 4. (*A*) Time course of alanine uptake. Vesicles contained: 600 mM mannitol, 10 mM HEPES-Tris, pH 7.6 (gradient condition); 300 mM NaCl or KCl, 10 mM HEPES-Tris, pH 7.6 (Na^+ equilibrium or K^+ equilibrium condition). Transport buffers contained either 300 mM NaCl or KCl, with 10 mM HEPES-Tris, pH 7.6, and 5 μM ^{3}H-L-alanine. Overshoots were seen only in the presence of inwardly directed gradients of Na^+ (filled circles) or K^+ (filled triangles). The equilibrium conditions are shown with open circles (Na^+ equilibrium) and open triangles (K^+ equilibrium). Nigericin (25 μg/mg protein) was present in these groups. (*B*) shows in greater detail the time courses in the presence of a K^+ gradient and under equilibrium conditions. Symbols and transport buffers are the same as in *A*. Data points are the means of duplicate measures of uptake from a single membrane preparation

To examine whether the Na^+-stimulated and K^+-stimulated uptakes of alanine were occurring via independent carriers or by a common pathway or set of pathways, the following experiment was done (Fig. 6). The uptake of alanine in vesicles pre-equilibrated in 600 mM mannitol buffer (pH 7.6) was measured using transport buffers containing NaCl (300 or 150 mM), KCl (300 or 150 mM) or both NaCl and KCl at 150 mM each. The osmotic concentration was kept the same in each case with cholineCl. The initial rate (30 sec) of alanine uptake in the transport buffer containing both NaCl and KCl was greater than the uptake in each cation at 150 mM alone but not as great as the sum of the two. These observations support the contention that Na^+ and K^+ (and probably other cations) interact with a common set of transport pathways in the brush border of gill cells.

It was of interest to examine whether or not K^+ gradients could also support the uptake of substrates other than amino acids. Glucose uptake into intact bivalve gills is Na^+ dependent (Bamford & Gingles, 1974; Riley, 1981; S.H. Wright, *unpublished observations*). An inwardly directed Na^+ gradient was also found to stimulate the uptake of 10 μM D-glucose into BBMV from *Mytilus* gill. The initial 30-sec uptake rate was 168.7 ± 26.1 pmol/mg-min (n = 2). This uptake was inhibited 95% by 0.1

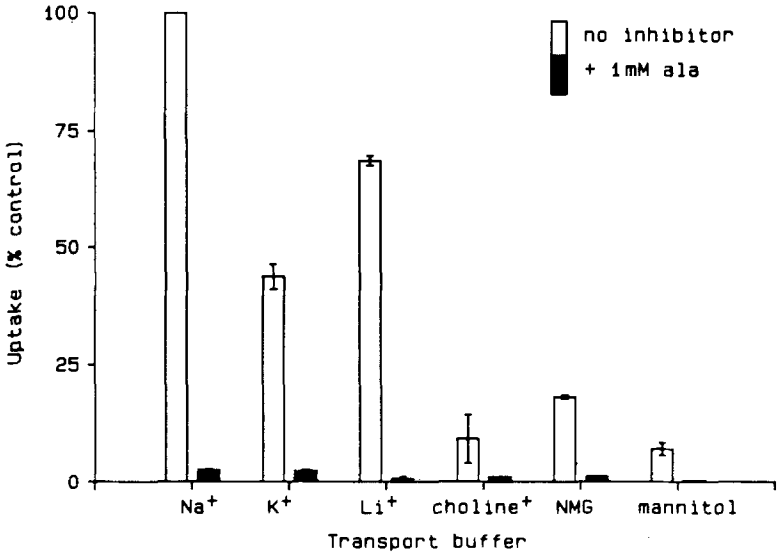

Fig. 5. Effects of cations on the uptake of alanine. Vesicles contained 600 mM mannitol, 10 mM HEPES-Tris, pH 7.6. Transport buffers contained 300 mM NaCl, KCl, LiCl, cholineCl, N-methyl-glucamine-HCl, or 600 mM mannitol with 10 mM HEPES-Tris, pH 7.6. The transport buffers also included 5 μM ^{3}H-alanine with (n = 1–2) or without (n = 2–7) 1 mM unlabeled alanine. Uptake is expressed as a percentage (±SE) of that seen in the presence of NaCl (146.8 ± 28.6 pmol/mg-min, n = 7). Uptakes were run for 30 sec

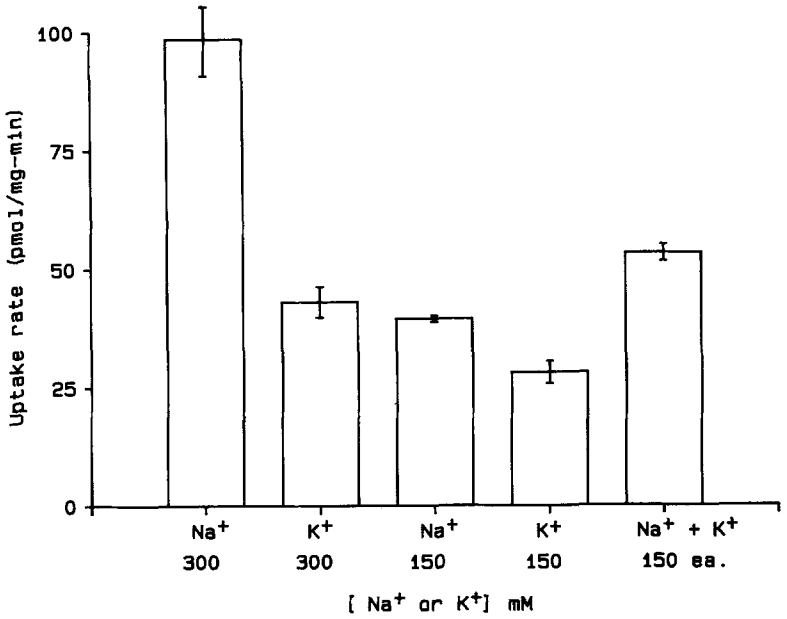

Fig. 6. Effects of Na^+ and K^+ on alanine uptake. Vesicles contained 600 mM mannitol, 10 mM HEPES-Tris, pH 7.6. Transport buffers contained: 300 mM NaCl or KCl *or* 150 mM NaCl or KCl with 150 mM cholineCl *or* both 150 mM NaCl and KCl. Each of these was buffered with 10 mM HEPES-Tris, pH 7.6, and contained 5 μM ^{3}H-L-alanine. Uptake times were 30 sec. The bars represent the uptake rates ± SE (triplicates from a single membrane preparation)

mM phlorizin and 95% by replacement of Na^+ with mannitol. Neither a choline$^+$ nor a K^+ gradient had a significant stimulatory effect on glucose uptake (inhibition by 98 and 99%, respectively), suggesting that the observed effects of K^+ on alanine uptake represent a specific interaction of this ion with the alanine transporter(s).

Amino Acid Specificity

Uptake of 5 μM ^{3}H-L-alanine into gill BBMV was inhibited by more than 88% by 100 μM unlabeled L-alanine, and by 98% by 1 mM L-alanine (Figs. 7, 8). A similar pattern of inhibition was seen with leucine (Figs. 7, 8). Glycine, a relatively polar neutral amino acid, was less effective as an inhibitor than either alanine or leucine, with 100 μM glycine reducing ^{3}H-L-alanine uptake by only 56% (Figs. 7, 8). However, 10 mM glycine was still capable of eliminating 95% of the alanine accumulation in the BBMV (*results not shown*). 100 μM D-alanine reduced uptake by only 50%, indicating that the transporter(s) is stereoselective for the L-isomer (Fig. 7). Neither taurine, a β-amino acid, nor aspartate, an anionic (acidic) amino acid, had any marked effect on alanine uptake (1 mM of taurine reduced alanine uptake only 10%, while 1 mM aspartate reduced it

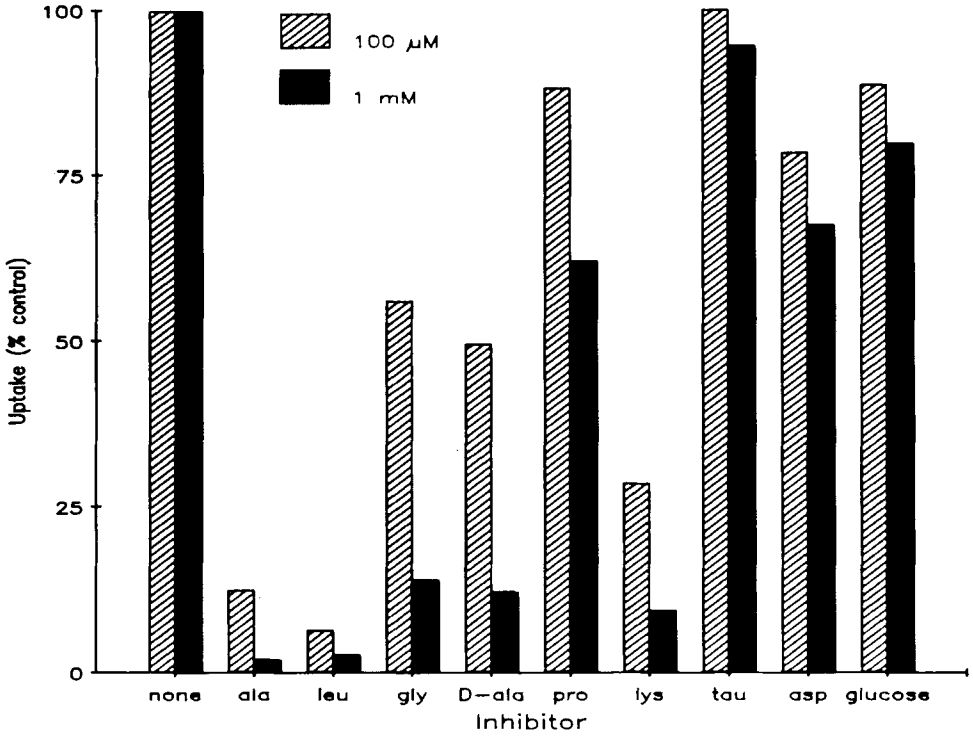

Fig. 7. Effect of 100 μM and 1 mM amino acids and glucose on alanine uptake. Vesicles contained 600 mM mannitol, 10 mM HEPES-Tris, pH 7.6. Transport buffers contained 300 mM NaCl, 10 mM HEPES-Tris, pH 7.6, 5 μM ^{3}H-L-alanine with or without amino acids or glucose. Uptakes were run for 30 sec. Uptake is expressed as a percentage of the uptake seen in the absence of added inhibitors (131.7 ± 21.5 pmol/mg-min, n = 6). Error bars were omitted for the purpose of clarity; standard errors ranged between 4 and 20% of the means, except in the experiments with alanine as an inhibitor where they were 40%. The results for D-alanine, aspartate, and glucose represent single membrane preparations; sample sizes for the other conditions ranged from 2 to 5

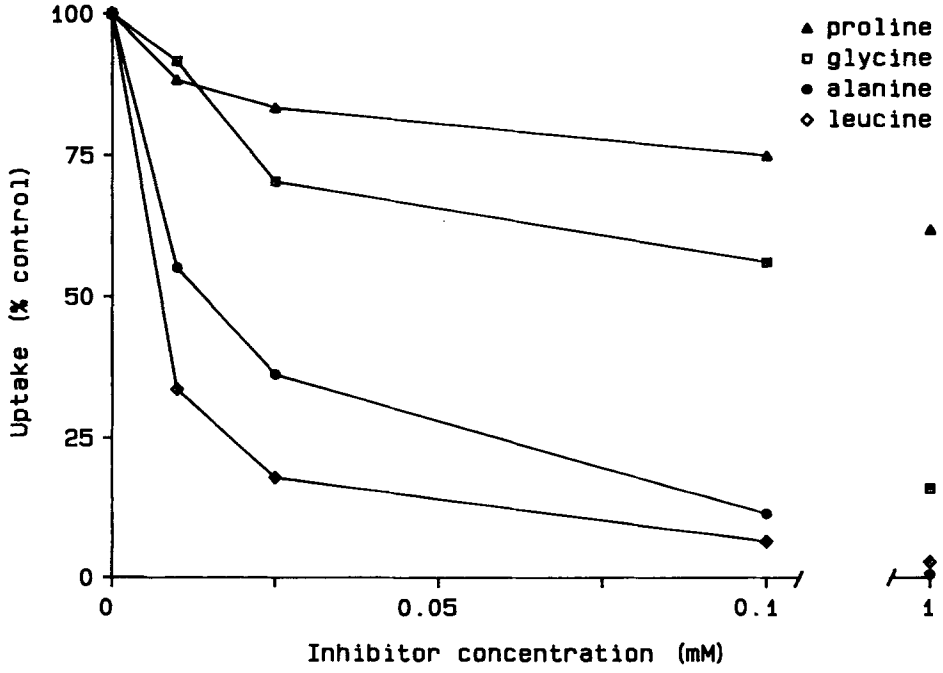

Fig. 8. Inhibition of 5 μM ^{3}H-alanine by unlabeled amino acids. Uptake times are 30 sec. Each curve shows the results of an experiment with a single membrane preparation. Conditions were the same as in Fig. 7

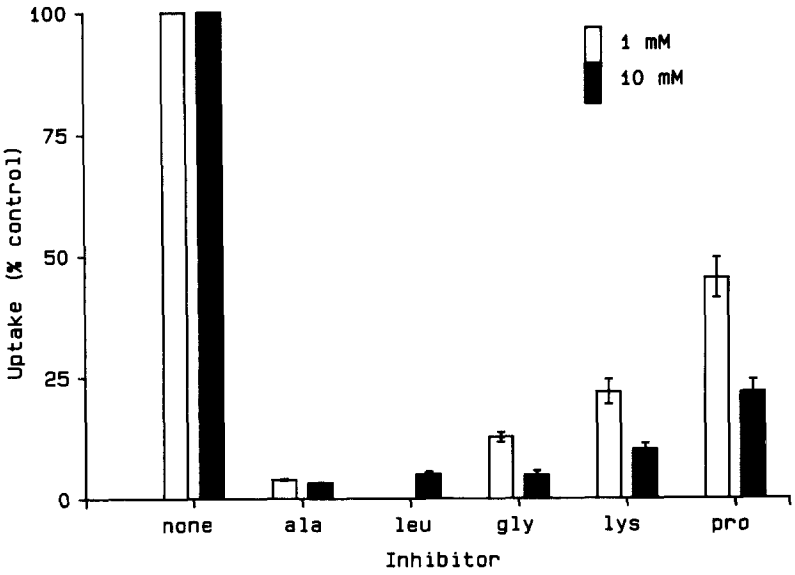

Fig. 9. Inhibition of 5 μM ^{3}H-alanine uptake by 1 and 10 mM unlabeled amino acids in intact gills. Results are expressed as percentage (±SE) of control uptake. Each point represents the mean of results from three animals. Gill pieces were exposed to label for 10 min. There were no results for 1 mM leucine

by 30%) (Fig. 7). Furthermore, in a separate experiment, 1 mM alanine inhibited the Na^+-dependent transport of 5 μM taurine less than 10% (10.6 *vs.* 9.6 pmol taurine/mg-30 sec). The cationic (basic) amino acid, lysine, was an effective inhibitor of alanine uptake, with 1 mM lysine reducing uptake 90% (Fig. 7). The imino acid, proline, also significantly reduced alanine uptake, with 1 mM proline inhibiting transport by 38% (Figs. 7, 8). However, increasing the proline concentration to 10 mM (*results not shown*) did not result in further inhibition of alanine uptake, suggesting the presence of proline-sensitive and proline-insensitive pathways for alanine uptake.

In intact gill tissue, a qualitatively similar pattern of apparent structural specificity for alanine was noted (Fig. 9). Both leucine and glycine were effective inhibitors of alanine uptake, though glycine was a more effective inhibitor in the intact gill than in the BBMV. Finally, both lysine and proline reduced alanine accumulation in the intact gill, though proline was a more potent inhibitor in the gill than in the BBMV.

Kinetics

As implied by the inhibitor study described above, alanine uptake into BBMV was saturable. Figure 10 shows the results of one experiment in which the effect of increasing alanine concentration on the initial rate of alanine accumulation was examined. The uptake was clearly saturable, and the kinetics of the process were adequately described by the Michaelis-Menten equation:

$$J = (J_{max} \cdot [A])/(K_t + [A])$$

where J is the initial rate of alanine uptake, J_{max} is the maximal rate of alanine uptake, [A] is the concentration of alanine, and K_t is the apparent Michaelis constant. It should be noted that in some vesicle preparations, alanine uptake appeared to include a nonsaturable component which behaved like passive diffusion, though may have represented a carrier-mediated pathway of low substrate affinity. The kinetics of uptake were described assuming the presence of two components $J = (J_{max} \cdot [A])/(K_t + [A]) + P[A]$, where P is an apparent diffusion coefficient. In six experiments, the J_{max} for alanine uptake was 363 ± 104 pmol/mg-min and the apparent K_t was 7 ± 2 μM. The value for P ranged between 1.5×10^{-8} and 7.9×10^{-8} liter/mg-sec.

Discussion

This report describes the preparation of plasma membranes from the epithelial cells of *M. edulis* gill. These membranes were clearly separated into two major populations including one enriched in Na^+-dependent L-alanine transport. This fraction was also enriched in the enzymatic markers, GGTP and alkaline phosphatase, which are commonly found in the brush border membrane of both vertebrate and invertebrate epithelia. Furthermore, studies with intact tissue have shown Na^+-dependent L-alanine uptake to be a feature of the brush border membrane of the gill (Wright, 1985). Therefore, we feel that the membrane fraction used in the transport experiments of the present study contains

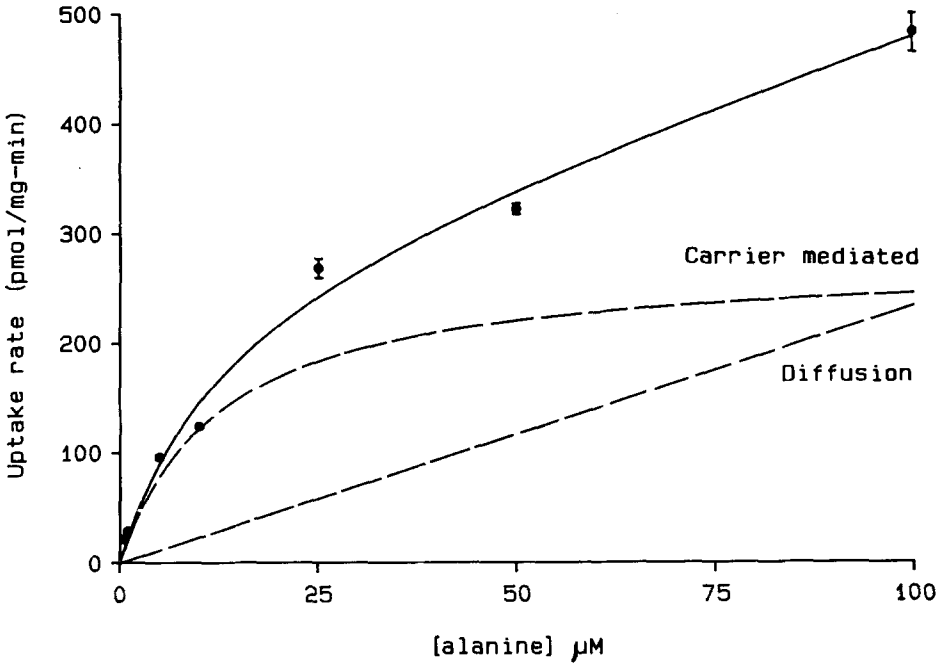

Fig. 10. Effect of increasing alanine concentration on the initial (6 sec) rate of alanine uptake. Vesicles contained 600 mM mannitol, 10 mM HEPES-Tris, pH 7.6. Transport buffers contained 300 mM NaCl, 10 mM HEPES-Tris, pH 7.6, with tracer ^{3}H-alanine and increasing concentrations of unlabeled alanine. The curve was generated using a nonlinear regression analysis for three parameter equations (T. Reedy, *personal communication*) with uptake presumed to occur by the sum of a carrier-mediated process and passive diffusion. The J_{max} was calculated to be 276 pmol/mg-min, the apparent K_t was 12 μM, and the apparent diffusion coefficient, P, was 4 × 10^{-8} liter/mg-sec. The points represent means ± SE of triplicate determinations from a single membrane preparation

brush border membranes. This is the first epithelial brush border preparation from a soft-bodied marine invertebrate to be used to characterize the Na^+-coupled transport of amino acids.

Studies with intact gill tissue have demonstrated net amino acid uptake against extreme chemical gradients (Manahan et al., 1983) by processes requiring Na^+ in the external medium (Wright, 1985). If the energy for such transport is derived solely from the Na^+ electrochemical gradient, then at least 3 Na^+ ions must be coupled to the transport of each amino acid (Manahan et al., 1983). However, until the present report, there has been no evidence for a coupling between the flows of Na^+ and amino acid. The appearance of a transient overshoot in intravesicular alanine concentration in the presence of an inwardly directed Na^+ gradient (Fig. 4) provides evidence for such a coupling.

The apparent coupling of K^+ and Li^+ with alanine transport in these studies was not expected. In intact gills, neither K^+ nor Li^+ is an effective substitute for Na^+, and complete replacement of Na^+ with other cations reduces uptake of amino acids, including alanine, by more than 95% (Wright, 1985, 1987*b*). However, interpretation of ion replacement studies with intact tissues must be done with care. Brief exposure (5 min) of isolated gills to Na^+-free seawater causes an inhibition of alanine uptake that takes up to an hour to recover upon reexposure to normal seawater (Wright, 1987*b*). Coincident with this inhibition of uptake is a 35% reduction in O_2 consumption that is also slow to recover. The exact nature of the ion and electrical gradients in intact tissues is not known, and it seems that the removal of Na^+ has effects on cells of the integument that go beyond a simple replacement of this cation at a cotransport site. Consequently, direct comparison of studies with intact tissues and isolated membranes may be premature.

It should be emphasized that there are precedents for cations other than Na^+ serving as cosubstrates in amino acid transport in both invertebrate (Hanozet et al., 1980; Giordana, Sacchi & Hanozet, 1982) and vertebrate epithelia (Berteloot, Khan & Ramaswamy, 1982). Of particular interest is the observation that electrochemical gradients of K^+, Li^+, and Na^+ are all effective in supporting uphill accumulation of phenylalanine and alanine in brush border vesicles from larval insect midgut, though K^+ was observed to produce both the highest rates and largest overshoots (Giordana et al., 1982; Hanozet et al., 1984). This comparatively poor cation selec-

tivity resembles the observations noted here for BBMV isolated from *M. edulis* gill.

The apparent structural specificity for alanine uptake in the BBMV was similar to that seen in intact gills. Previous studies with gills from *M. californianus* suggested the presence of at least two pathways for alanine transport (Wright, 1985), and the results of inhibition studies in both BBMV and isolated gills from *M. edulis* (Figs. 7, 8, 9) were consistent with this contention. These separate, parallel transport pathways appeared to be adequately described by the kinetics for a single carrier-mediated process (Fig. 10), though the situation is almost certainly more complex. Furthermore, the Na^+ concentration used in the studies reported here (300 mM) was considerably less than that of full-strength seawater (≃440 mM). Ambient Na^+ concentrations do influence the kinetics of amino acid uptake in intact gills (Wright, 1987*b*). Consequently, the kinetic parameters for alanine uptake in BBMV must be considered tentative until the effects of Na^+ are studied in greater depth. Nevertheless, they do support observations made with intact animals (Wright & Stephens, 1978) and isolated gills (Wright, 1985), which show that the transport processes for alanine and other amino acids have very high substrate affinities (K_t's between 1 and 10 μM). The transport capacity (i.e., J_{max}) of integumental transport processes is high when compared to the metabolic rate of intact mussels (Wright, 1982, 1987*a*). With these transporters exposed to environmental free amino acid concentrations on the order of 1 μM or less, high substrate affinities coupled with large transport capacities allow a significant accumulation of a sparse resource.

Amino acid transport in mussel gills has been shown to be extremely sensitive to the concentration in the external medium of divalent, as well as monovalent, cations. Reduction in the ambient Ca^{2+} and Mg^{2+} concentrations (from the normal levels in seawater of 10 and 50 mM, respectively) decreases rates of uptake and accelerates the efflux of amino acids from mussel gills (Swinehart et al., 1980). There is considerable evidence supporting a role for divalent cations in the regulation of cell membrane permeability to amino acids in isosmotic volume regulation (*see* Pierce, 1982). No effort was made in the present study to examine the influence of divalent cations on the uptake of alanine in gill BBMV, and we are not aware of any systematic study of the effect of divalent cations on amino acid permeability. However, this remains an important area for future study.

The mechanism of transport, be it dependent or independent of Na^+, in the basal-lateral membrane fractions is unknown at present. The distribution of transport activity in the sucrose density gradient suggests that the relative transport rate of basal-lateral membranes is low when compared with that in the brush border. Some form of carrier-mediated alanine transport is expected in the basal-lateral membranes. If epithelial uptake results in a trans-epithelial flux of substrate to support whole animal metabolic needs, then there must be avenues for efflux of amino acids from the cells to the hemolymph. In addition, the maintenance of a steep gradient of amino acid concentration between cells (≃ 100 mM) and the hemolymph (≃1 mM; Zurburg & De Zwaan, 1981) implies the presence of some form of concentrative capacity in the basal-lateral membrane. Our observations may have underestimated the presence of a basal-lateral carrier if the transport rate or substrate affinity in the basal-lateral membranes is very low. The latter might be expected with hemolymph free amino acid concentrations of 1 mM.

In summary, this study reports a method of preparation of brush border membrane vesicles from mussel gills and the initial characterization of alanine transport in these membranes. Uptake of alanine was shown to be coupled to a Na^+ chemical gradient, though other monovalent cations, notably K^+, were observed to support concentrative transport as well. The kinetics and amino acid specificity of this transport in BBMV were qualitatively similar to those seen with intact gill tissue. These results suggest that this membrane preparation is a useful one with which to study transport mechanisms involved in the maintenance of the large intracellular amino acid concentrations characteristic of mussel gills.

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