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APICAL MEMBRANE PERMEABILITY OF *MYTILUS* GILL: INFLUENCE OF ULTRASTRUCTURE, SALINITY AND COMPETITIVE INHIBITORS ON AMINO ACID FLUXES

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SUMMARY

The apical membrane of gill integumental cells from the mussels *Mytilus edulis* and *M. californianus* serves as a permeability barrier separating sea water from a cytoplasm rich in amino acids and other small organic molecules. Morphometric analysis of transmission electronmicrographs indicates that the membrane area of these cells is increased between 10- and 18-fold by the presence of a microvillous brush border. The microvilli do not appear to influence the kinetics of solute transport across the cell apex, as determined using a mathematical model of the relationship between membrane structure and the kinetics of transport. Rates of amino acid loss from the integument were low, and estimates of the upper limit of the passive permeability of the apical membrane to amino acids ranged from 0·5 to 10×10^{-10} cm s^{-1}. Abrupt exposure of intact mussels or isolated gill tissue to 60 % sea water (19‰ salinity) resulted in a transient, 40- to 80-fold increase in the rate of loss of all amino acids from integumental tissues. Upon exposure to full-strength sea water, efflux rates returned to near control values. Exposure to 60 % sea water also inhibited the carrier-mediated accumulation of amino acid: uptake of 0·5 μmol l^{-1} [^{14}C]alanine and [^{14}C]taurine was reduced by 80 % compared to control uptake in 100 % sea water. This inhibition was not adequate to account for the increase in net efflux of taurine from gill tissue into 60 % artificial sea water (ASW), though the inhibition of alanine uptake may have contributed significantly to the increased loss of this amino acid. Efflux of discrete structural classes of amino acid occurred when integumental tissues were exposed to 50 μmol l^{-1} concentrations of structurally related analogues. It is concluded that the apical membrane of gill cells has a very low passive permeability to amino acids, and that the overall permeability of the gill can be increased in a reversible fashion by exposure to reduced salinity or to high external concentrations of amino acid.

Key words: transport, amino acid, integument, *Mytilus*, gill, epithelia.

INTRODUCTION

Normal ciliary activity of the lamellibranch gill results in a flow of water through the mantle cavity that is associated with gas exchange, acquisition of particulate foodstuffs and the removal of wastes from the animal. During this activity, the gill is continuously exposed to sea water which has much lower concentrations of small organic solutes than the cytoplasm. Among these compounds are the amino acids which are used by bivalve tissues in isosmotic volume regulation (Gilles, 1979). Intracellular concentrations of amino acids in the gill are greater than 100 mmol l^{-1} cell water (Zurburg & De Zwaan, 1981; Wright & Secomb, 1986), whereas the concentration of free amino acids in the near-shore environment ranges from 20 nmol l^{-1} to 2 μmol l^{-1} (Manahan & Arnold, 1983; Braven, Evens & Butler, 1984; Siebers & Winkler, 1984). These extremes of concentration are separated by the apical membrane of the gill epithelium, and movements of solute across this membrane may play a role in cell volume regulation (Crowe, 1981) and in the acquisition of nutrients (Wright, 1982).

The permeability of the apical membrane is influenced, at least in part, by the presence of several separate, carrier-mediated pathways for amino acid transport (Wright, 1985). However, though the gill can effect a net accumulation of amino acid from dilute solution (Manahan, Wright, Stephens & Rice, 1982; Manahan, Wright & Stephens, 1983; Siebers & Winkler, 1984; Wright & Secomb, 1986), the very large intracellular concentration of these substrates has led to the suggestion that the passive loss of amino acids from integumental tissues may be large (Hammen, 1968). It has been proposed that apical transport processes cause a reduction in the loss of endogenous substrates from the integument (Gomme, 1981), and recent evidence has supported this suggestion in marine bivalves (Wright & Secomb, 1986). It has also been suggested that such processes could be influenced by the presence of the microvillous brush border of the apical membrane (Gomme, 1981), though this has not been examined critically.

Flux of amino acids across the membranes of bivalve integumental tissues has been shown to be influenced by several factors. Reduction of ambient salinity has been shown to decrease rates of amino acid uptake into isolated gill tissue (Anderson, 1975; Anderson & Bedford, 1973; Bamford & Campbell, 1975) and to increase rates of efflux from intact animals (Livingston, Widdows & Fieth, 1979) and isolated tissues (Henry & Mangum, 1980). However, it is not clear whether such factors influence general membrane permeability, or preferentially influence the flux of separate classes of these compounds. A specific loss of taurine from integumental tissues of *Mytilus edulis* can be produced by acute exposure to submillimolar concentrations of structural analogues of taurine (Wright & Secomb, 1986).

In the present study we have examined the structure of the apical membrane of gill cells, and have considered the relationship between membrane structure and the kinetics of integumental transport using a mathematical model. The results indicate that the microvillous brush border membrane of gill cells does not influence the

kinetics of transport in these cells, though it is clear that an appropriate combination of morphological and physiological parameters could result in a significant role for the brush border in influencing solute exchange. We have also examined the effect on amino acid efflux from the gill of (i) reduced salinity and (ii) exposure to high concentrations of specific structural classes of amino acid. The former procedure resulted in a transient increase in the general apical membrane permeability to amino acid, while the latter treatment increased the permeability to specific structural classes of these compounds.

MATERIALS AND METHODS

Animals

Mussels (*M. edulis* and *M. californianus*) were purchased from the Bodega Bay Marine Biological Laboratory, and were kept in a refrigerated (13°C) aquarium containing filtered artificial sea water (Instant Ocean). Encrusting organisms were scraped from the valves with a wire brush. The animals were not fed and were usually used within 4 weeks of collection.

Efflux studies

Experiments in which intact animals were exposed to reduced salinity were performed as follows. Valves were propped open by insertion of a 0·7 mm glass tube (i.d. 0·2 mm) into the mantle cavity ventral to the exhalant aperture. Large rubber bands secured the valves such that the tubes remained in place. By means of a small plastic cannula (P.E. 90), fed through the glass tube, and a peristaltic pump, the mantle cavity could be continuously irrigated with the test medium in which the animal was placed. Pump-driven flow was approximately 25 ml min^{-1} for a 10- to 30-g animal (wet mass soft-body parts). The incubation volume was usually 500 ml, and all test solutions were based on the artificial sea water (ASW) described by Cavanaugh (1956). Efflux of amino acid from animal tissue was measured by sampling the medium and measuring the rate of appearance of total amino acid as determined using the chromatographic procedure described below. Control experiments were performed to verify that efflux of amino acid was dependent upon exposure of the medium to the mantle cavity; incubations of the test chamber or of animals completely sealed with rubber bands had no effect on amino acid levels in the test solution.

Studies of the effect of exogenous application of amino acid on amino acid efflux used a protocol similar to that described above, except it was not necessary to prop the animals open; the animals were permitted to gape and pump normally.

For studies on isolated gill tissue, gills were removed from specimens of *M. californianus* and a piece of 000 silk suture tied to one end of each demibranch. The tissue was held in ASW for 30 min prior to any experimental incubation. Experiments were started by suspending a demibranch in a test solution (usually 50 ml) that was gently mixed with a magnetic stirrer. Test solutions, and the medium used for the last 5 min of the preincubation, contained 10 μmol l^{-1}

5-hydroxytryptamine (5-HT) to activate lateral cilia (see Wright, 1979). Fluxes of amino acid were determined as described for experiments using intact animals.

Influx studies

Accumulation of ^{14}C-labelled amino acids was measured using procedures described previously (Wright, 1985). Isolated demibranchs from *M. californianus* were suspended in 200 ml of ASW of appropriate composition, containing 10 μmol l^{-1} 5-HT and 0·5 μmol l^{-1} labelled amino acid. After 5 min, the tissue was removed and rinsed in ice-cold ASW for 5 min. Disks (7 mm) of tissue were cut from the rinsed tissue, and accumulated radioactivity extracted in 0·7 ml of 0·1 mol l^{-1} HNO_3. After 2 h, 10 ml of scintillation cocktail was added and radioactivity measured in a scintillation counter (Beckman 3801). All counts were corrected for quench using H-number analysis.

Amino acid analysis

High performance liquid chromatography (HPLC) was used to measure amino acids in samples of ASW. The procedure was a modification of that described by Lindroth & Mopper (1979) and Manahan *et al.* (1983). The reagent *o*-phthaldialdehyde (OPA) was used to make fluorescent derivatives of the amino acids in experimental samples. The derivatives were separated on a 3 μm C18 reverse-phase column (Beckman ODS Ultrasphere) and monitored with a fluorescence detector. Individual peaks were identified and quantified by comparison of retention times and peak areas to those of appropriate standards. The reproducibility of standard retention times and peak areas was better than 5 %. The limit of detection and quantification for taurine was 0·7 pmol, which corresponded to a concentration in the test solutions of 4 nmol l^{-1}. The limit for alanine, aspartate, glutamate, glycine and serine was approximately 1 nmol l^{-1}.

Electron microscopy and morphometry

Specimens of *M. edulis* were dissected in sea water. Gill tissue was removed intact and prepared for fixation for transmission electron microscopy. Tissues were initially 'quick fixed' for 20 s (Bradley & Satir, 1981) in a 1:1 mixture of 2 % osmium tetroxide and 1·1 mol l^{-1} sodium cacodylate + 0·6 mol l^{-1} sucrose. Tissues were then rinsed in buffer and placed for 1 h in a buffered glutaraldehyde solution containing 4 % glutaraldehyde, 0·05 mol l^{-1} cacodylate buffer (pH 7·3) and 0·3 mol l^{-1} sucrose. Tissue was postfixed in 1 % osmium for 1 h in the above cacodylate buffer and dehydrated in an ethanol gradient and propylene oxide. While in propylene oxide, smaller pieces of gill were removed from the fixed intact tissue and then embedded in Epon 812.

Thin sections were taken perpendicular to the long axis of the gill filaments. Randomly selected thin sections containing circumferential cross-sections of gill filaments were sequentially photographed at ×6600, recording the entire circumference of the gill filaments. Using a Zeiss digital analyser MOP 3 at a final magnification of ×16 500, linear measurements were made of the gill surface

membrane, excluding the surface area represented by the microvilli. Measurements were then made of the microvillar surface area, i.e. the highly folded apical membrane itself. These data were separated into three categories, each representing a distinct region of the cross-section, i.e. frontal, sublateral and abfrontal. Data for each region, collected from three filaments, were combined and analysed statistically.

Analysis of the percentage of gill surface area occupied by intramicrovillar and extramicrovillar space was conducted on the micrographs using a linear analysis on the Zeiss MOP 3 at the level of the glycocalyx. Estimations of intermicrovillar distances were made by measuring the distance between adjacent microvillar profiles at the level of the glycocalyx.

Chemicals

OPA was purchased from Cal-Biochem. [^{14}C]alanine (150 mCi mmol^{-1}) was purchased from ICN-Radiochemicals. [^{14}C]taurine (97·5 mCi mmol^{-1}) was purchased from New England Nuclear. All other chemicals were purchased from standard sources and were the highest grade available.

RESULTS

Ultrastructure and morphometric analysis of the apical membrane of gill cells

The gills of *Mytilus* produce a unidirectional flow of water through the mantle cavity. The general architecture of the gill and the pattern of water movement through this tissue are described in several excellent discussions (e.g. White, 1937; Bayne, Thompson & Widdows, 1976; Jørgensen, 1976). For the present purpose it is sufficient to emphasize that all the water that passes through the animal must pass through the ostial network of the gill, during which time this water briefly (<0·2 s) comes within approximately 20 μm of the external surface of gill cells. The lamellar surfaces of the demibranchs through which the inhalant water passes consist of numerous parallel gill filaments which are attached to their neighbours *via* junctions, either ciliary (in the case of *M. edulis*) or fleshy (in the case of *M. californianus*).

Each gill filament is a tube whose outer surface is exposed to sea water while the inside is bathed by haemolymph. In the subsequent discussion, each filament is subdivided into three distinct regions: Frontal, Sublateral and Abfrontal (Fig. 1). Transmission electronmicrographs of filaments from *M. edulis* revealed that each region consists of three distinct layers (Figs 2, 3): (i) an outer layer formed by a sheet of epithelial cells which sits on (ii) a fibrous lamina of collagen-like material (the conchiolin of White, 1937) and (iii) an inner layer of thin endodermal cells that appear to provide an incomplete lining of the branchial blood vessel.

The epithelial cell layer, composed of the cells facing sea water, represented the predominant cellular mass of the gill. The Frontal region was composed of: three ciliated cell types, named on the basis of the type of cilia borne by each, i.e. frontal, laterofrontal and lateral; goblet cells; and columnar epithelial cells. The Abfrontal region included a ciliated cell type, as well as goblet and columnar cells. The cells of

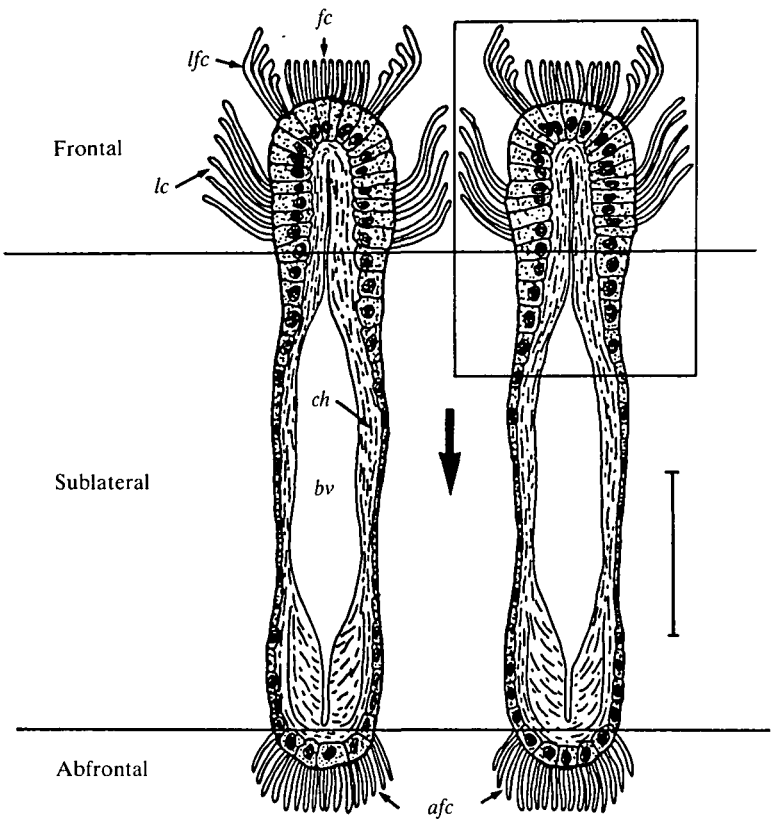
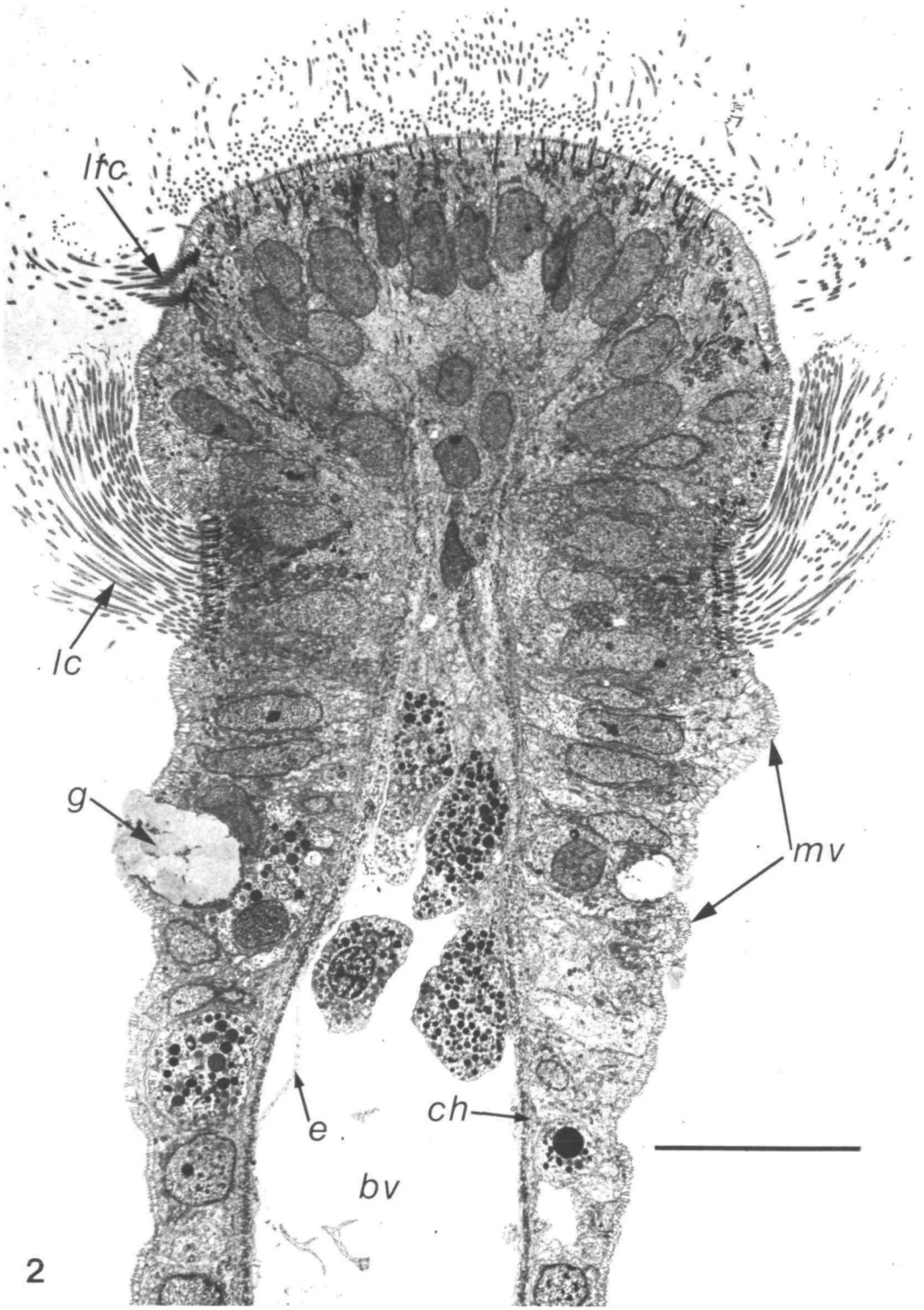


Fig. 1. Schematic representation of a cross-section of two adjacent gill filaments from *Mytilus edulis*. Three regions of the gill are indicated: Frontal, Sublateral and Abfrontal. The arrow indicates the direction of water movement between filaments produced by activity of the lateral cilia (*lc*). Other abbreviations: *fc*, frontal cilia; *lfc*, laterofrontal cilia; *afc*, abfrontal cilia; *bv*, branchial blood vessel; *ch*, 'conchiolin' (acellular structural material). The rectangle over the upper portion of the right filament shows the region pictured in the electronmicrograph of Fig. 2. Scale bar, 50 μm.

the sublateral region (the largest percentage of total gill surface) became progressively squamous in form (Figs 2, 3). There were no ciliated cells in this region, but goblet cells were noted.

Microvilli were a structural element of the apical membranes of all the filament surfaces (Figs 2, 3). Morphometric analyses were performed on the 'brush border' of the three regions of the gill (Fig. 1): (i) frontal, i.e. the region including the lateral ciliated cells and the tissue above them; (ii) abfrontal, i.e. the region of the ciliated cells of the abfrontal aspect of the filaments; and (iii) sublateral, i.e. the area of the sides of the filament below the lateral ciliated cells and above the first ciliated cells of the abfrontal region. No significant differences were found to exist in the diameter

Fig. 2. Transmission electronmicrograph of a cross-section of the gill filament from *Mytilus edulis*. A microvillous brush border (*mv*) is evident on the apical face of the gill epithelium. Abbreviations as noted in Fig. 1 with the addition of: *g*, goblet cell; *e*, endothelial cell layer. Scale bar, 20 μm.



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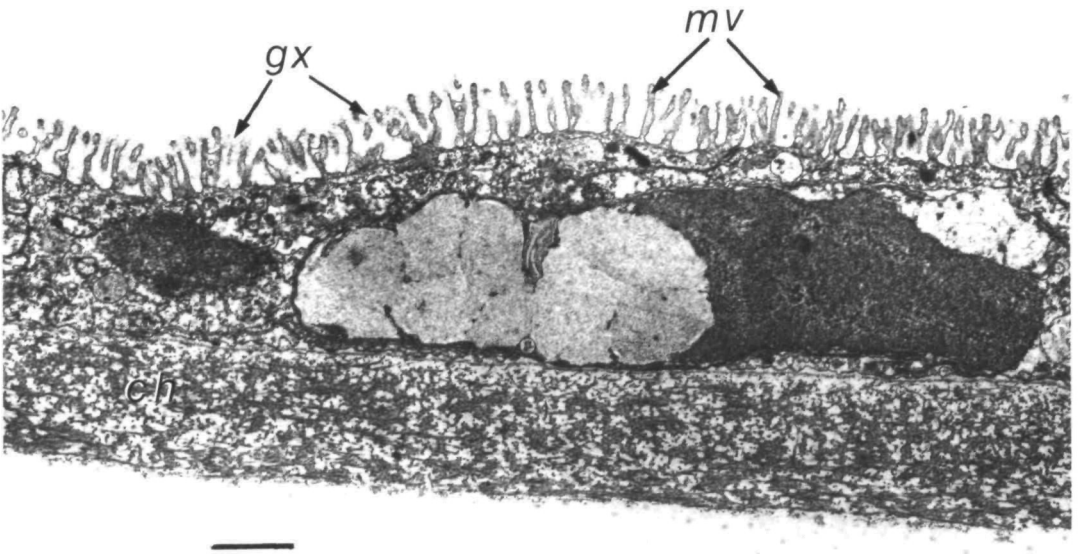

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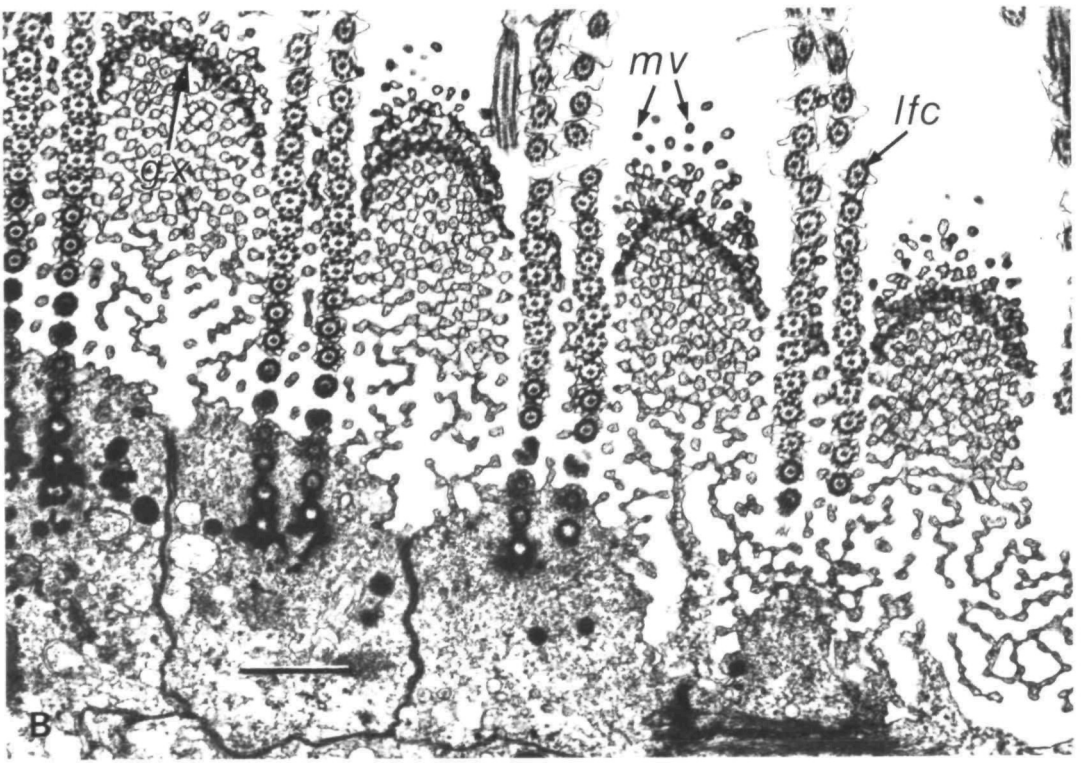

Table 1. *Morphometric parameters of the apical membrane of* Mytilus edulis *gill*

Parameter	Frontal	Sublateral	Abfrontal
Apical perimeter (μm)	147 ± 15·8	374 ± 33·9	107 ± 44·4
Intermicrovillar distance (μm)	0·09 ± 0·010	0·11 ± 0·013	0·10 ± 0·015
Microvillar length (μm)	1·16 ± 0·080	0·56 ± 0·050	1·07 ± 0·100
Microvillar diameter (μm)	0·10 ± 0·008	0·093 ± 0·007	0·11 ± 0·005
Fractional area*	0·39 ± 0·030	0·43 ± 0·042	0·41 ± 0·005
Microvilli: apex area†	18·5×	10·3×	16·5×

* Fraction of a plane through the microvilli parallel to the apical surface of the cells that is occupied by microvilli.

† Increase in apical membrane area due to the presence of microvilli; calculated from the assumption that microvilli are cylinders of the diameter and height indicated in the Table, and that they occupy the indicated fractional area of the apical membrane.

and spacing of microvilli in the three regions. However, the Sublateral microvilli were significantly shorter ($P < 0·05$) than those of the Frontal and Abfrontal aspects of the filaments (0·6 *vs* 1·2 and 1·1 μm, respectively). This explains, at least in part, why the microvilli of the Sublateral region caused a comparatively smaller increase in apical cell area than the Frontal and Abfrontal microvilli, i.e. 10·3-fold *vs* 18·5- and 16·5-fold, respectively. Nevertheless, given the length of this perimeter, the Sublateral aspect of the filaments still provided 46 % of the gill surface area (Table 1). We previously estimated that there are approximately 550 cm^2 of cell apex area per gram wet mass of mussel gill tissue (approximately equal for both *M. edulis* and *M. californianus*; Wright & Secomb, 1984). The present morphometric measurements indicate that total apical area due to microvilli is actually of the order of 7300 $cm^2 g^{-1}$ gill tissue.

There was a glycocalyx associated with the microvilli of all three regions, and it was particularly evident towards the tips of the microvilli (Fig. 3A). Fig. 3B shows a tangential section through the apex of several cells bearing laterofrontal cilia. The condensation of glycocalyx is evident as the section moves towards the tips of the microvilli in this region. At this level of the brush border, microvillar area represented approximately 40 % of the total of extracellular area plus microvillar area (Table 1).

Amino acid content of the integument

Taurine constituted between 57 and 86 % of the free amino acid pool of the gill and mantle from *M. edulis* and *M. californianus* (Table 2). Taurine, in conjunction with aspartate, glutamate, serine, glycine and alanine, made up in excess of 95 % of the non-ammonia, OPA-sensitive material that was resolved chromatographically. The

Fig. 3. Transmission electronmicrographs of gill filaments from *Mytilus edulis*. (A) Cross-section of squamous epithelial cells from the Sublateral region of the filament. Microvilli (*mv*) and an associated glycocalyx (*gx*) are evident on the apical surface of the cells. There is no significant infolding of the basal-lateral aspect of the cells. (B) Tangential section passing through the apical membrane and microvilli (*lfc*) of laterofrontal ciliary cells. The apparent condensation of the glycocalyx is evident as a dark band near the tips of the microvilli. Scale bars, 1 μm.

Table 2. *Amino acid content of gill and mantle tissue from* Mytilus edulis *and* M. californianus

Species	Asp	Glu	Ser	Gly	Tau	Ala
Gill						
M. edulis	4·8 ± 0·6	2·2 ± 0·1	0·4 ± 0·1	6·4 ± 0·9	61·0 ± 2·1	3·9 ± 0·2
M. californianus	8·3 ± 1·4	2·8 ± 0·4	0·8 ± 0·3	4·5 ± 0·7	114 ± 11·4	2·3 ± 0·3
Mantle						
M. edulis	4·4 ± 2·5	4·0 ± 2·3	1·4 ± 0·4	28·5 ± 6·8	56·6 ± 14·9	3·8 ± 0·7
M. californianus	5·6 ± 0·9	5·5 ± 0·1	3·4 ± 1·1	13·3 ± 2·3	99·2 ± 2·8	17·5 ± 3·6

Content in μmol g^{-1} wet mass, ±1 S.E.M. (for *M. edulis*, $N = 3$; for *M. californianus*, $N = 4$).

total, extractable pool of amino acid from the gill was 79 μmol g^{-1} wet mass for *M. edulis* and 133 μmol g^{-1} for *M. californianus*.

Efflux of amino acid from intact mussels

When intact specimens of *M. edulis* and *M. californianus* were placed into amino-acid-free ASW there was a gradual increase in taurine concentration of the medium which reached a steady state generally within 30–60 min. The steady-state concentrations for taurine averaged 17 ± 8·6 nmol (S.E., $N = 4$) and 26 ± 5·7 nmol ($N = 6$) for *M. edulis* and *M. californianus*, respectively, in close agreement with previous measurements (Wright & Secomb, 1986).

The measured rate of loss in these experiments of the 'non-taurine' constituents of the integumental amino acid pool was more equivocal. The limit of resolution of our chromatographic procedures permitted quantification of amino acids other than taurine at concentrations as low as 1 nmol l^{-1}. However, this level is within the range of the background contamination of our system for amino acids such as serine, glycine, alanine, aspartate and glutamate. It also approximated the steady-state concentration for these substrates. For example, in seven experiments with *M. californianus*, there was no significant difference between the concentrations of the non-taurine amino acids in the zero-time ASW blank and the concentration of these substrates after 60 min. Taurine concentration, on the other hand, was significantly greater after 60 min ($P = 0·02$; Wilcoxon matched-pairs signed-ranks test). Nevertheless, our observations do permit assigning an upper level to the steady-state concentration for the non-taurine amino acids (i.e. $[\text{amino acid}]_{ss}$ in *M. californianus* ($N = 7$; in nmol l^{-1}): $[\text{Asp}]_{ss} < 6·9$; $[\text{Glu}]_{ss} < 4·2$; $[\text{Ser}]_{ss} < 12·1$; $[\text{Gly}]_{ss} < 13·4$; $[\text{Ala}]_{ss} < 5·6$. Upper limits for steady-state concentrations for *M. edulis* were ($N = 4$): $[\text{Asp}]_{ss} < 13·8$; $[\text{Glu}]_{ss} < 3·6$; $[\text{Ser}]_{ss} < 17·6$; $[\text{Gly}]_{ss} < 16·5$; $[\text{Ala}]_{ss} < 9·5$.

Effect on salinity on amino acid efflux from intact mussels

When intact *M. edulis* were exposed to a step decrease in salinity, from 32‰ to 19·2‰ (i.e. from 100 % to 60 % ASW), there was an immediate increase in the rate of

loss of amino acid from the animals (Fig. 4). For example, during a 15-min exposure to 60 % ASW taurine loss increased more than 44-fold; the average loss of taurine went from the control value of $0{\cdot}52 \pm 0{\cdot}179\,\text{nmol}\,g^{-1}$ wet mass soft-body parts to $23{\cdot}4 \pm 7{\cdot}69\,\text{nmol}\,g^{-1}$. The result of this increase in loss of taurine from mussels was the development of ambient taurine concentrations that ranged from 0·2 to $3{\cdot}0\,\mu\text{mol}\,l^{-1}$ ($N = 4$). Expressed per gram of gill tissue, the change in taurine efflux was from $6{\cdot}3 \pm 1{\cdot}13$ (S.E., $N = 4$) to $333 \pm 131{\cdot}6\,\text{nmol}\,g^{-1}\,15\,\text{min}^{-1}$. Though this latter set of figures is likely to overestimate the actual loss of taurine from the gill, the gill does represent approximately 85 % of the total surface area of the mantle cavity (Jørgensen, 1983), and normalizing efflux data to gill mass can be expected to

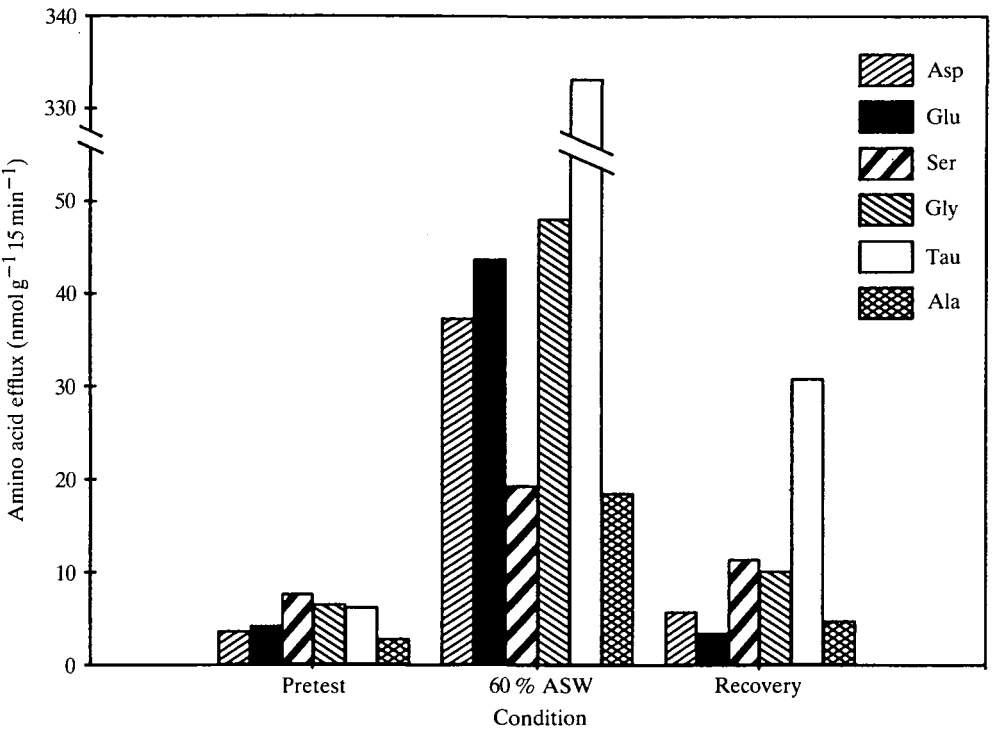

Fig. 4. Effect of acute exposure to 60 % artificial sea water (ASW) on amino acid efflux from intact *Mytilus edulis*. Glass tubes were used to prop open mussels, and the mantle cavity was irrigated through the tube using a perfusion pump, resulting in a continuous recirculation of the test solution (500 ml total volume) through the cavity. The experimental protocol was as follows. Exposure to 100 % ASW for 15 min (Pretest); replacement of the medium with 60 % ASW followed by a 15-min exposure to this test solution (60 % ASW); replacement of the medium with 100 % ASW, followed by a 1-h recovery period; replacement with fresh 100 % ASW, followed by a 15-min incubation (Recovery). Efflux is expressed per gram wet mass of gill tissue. The height of individual bars reflects the mean loss of amino acid to the medium during the test exposures, determined from four separate experiments with different mussels. Error bars are omitted for purposes of clarity; the standard errors ranged from 30 to 50 % of the means. In every experiment, exposure to 60 % ASW resulted in an increase in the efflux of each of the amino acids monitored, and re-exposure to 100 % ASW resulted in a decrease in efflux. Note the break in the units of the ordinate for taurine.

provide a reasonable approximation of the effect of an acute exposure to a hypotonic medium on taurine loss from the gill.

Exposure to 60% ASW also resulted in a loss of the non-taurine constituents of the integumental free amino acid pool (Fig. 4). In four experiments with *M. edulis*, the exposure to reduced salinity increased efflux (compared to control values) by: Asp, 10·1-fold; Ala, 6·5-fold; Gly, 7·3-fold; Glu, 10·3-fold; and Ser, 2·5-fold. Identifiable peaks of Tau, Asp, Glu, Ser, Gly, Ala and NH_3 accounted for $79{\cdot}8 \pm 2{\cdot}95\%$ ($N = 4$) of the OPA-positive material on the chromatograms of the samples collected after the 15-min exposure to 60% ASW.

The increased efflux of amino acid resulting from exposure to reduced salinity was at least partially reversible. Following the exposure to 60% ASW, the mussels were exposed to 100% ASW for 1 h, after which the medium was changed and loss to the medium determined after 15 min (Fig. 4). Though in most cases the 'recovery' rates of efflux were higher than the control rates (approximately one- to three-fold), they represented a 41–92% decrease in the maximal efflux noted during exposure to reduced salinity. Therefore, the loss of amino acid induced by exposure to hypotonic medium was not due to irreversible damage to the apical surface of integumental cells.

Exposure to a hypo-osmotic shock resulted in a similar, reversible increase in amino acid efflux from intact *M. californianus*. In two experiments, loss of taurine was increased 88-fold over the control rate [$15{\cdot}1 \pm 8{\cdot}90$ (the range of two experiments) to $1330 \pm 179\,\text{nmol}\,g^{-1}\,15\,\text{min}^{-1}$], and recovery was 95% complete (i.e. recovery period efflux of $76{\cdot}4 \pm 18{\cdot}20\,\text{nmol}\,g^{-1}\,15\,\text{min}^{-1}$). Effluxes of the other major constituents were as follows (in $\text{nmol}\,g^{-1}\,15\,\text{min}^{-1}$, pre-test to 60% ASW to recovery): Asp, 2·7 to 108·4 to 7·6; Glu, 2·9 to 57·0 to 2·7; Ser, 4·1 to 46·8 to 6·0; Gly, 4·3 to 146·0 to 12·4; Ala, 1·4 to 176·8 to 9·4.

Effect of salinity on fluxes of amino acid in isolated gill tissue

Gill tissue isolated from *M. californianus* lost amino acids to 100% ASW at rates higher than those noted in studies with intact mussels. For example, the apparent efflux of taurine during the first 5 min ($5{\cdot}8 \pm 0{\cdot}65 \times 10^{-7}\,\text{mol}\,g^{-1}\,h^{-1}$; $N = 6$) was approximately 10 times higher than the steady-state loss of taurine from gills in intact mussels (Wright & Secomb, 1986). This difference was probably due to the presence of damaged tissue at the cut edges of the *in vitro* preparation. The non-taurine amino acids were also lost from gill tissue, although at 2–5% of the rate observed for taurine, which was consistent with their contribution to the total free amino acid pool in gill tissue.

Isolated gills could be given a controlled exposure to the experimental solutions. Isolated gills treated with 5-HT retained lateral ciliary activity so that all gill surfaces were exposed to the test solution.

When gills from *M. californianus* were exposed for 5 min to a step decrease in salinity (100% to 60% ASW) there was a rapid, reversible increase in the rate of loss of all the major pool amino acids (Fig. 5). The increase in efflux caused by exposure of the gill to 60% ASW was greatest for taurine: a 36-fold increase (from a control

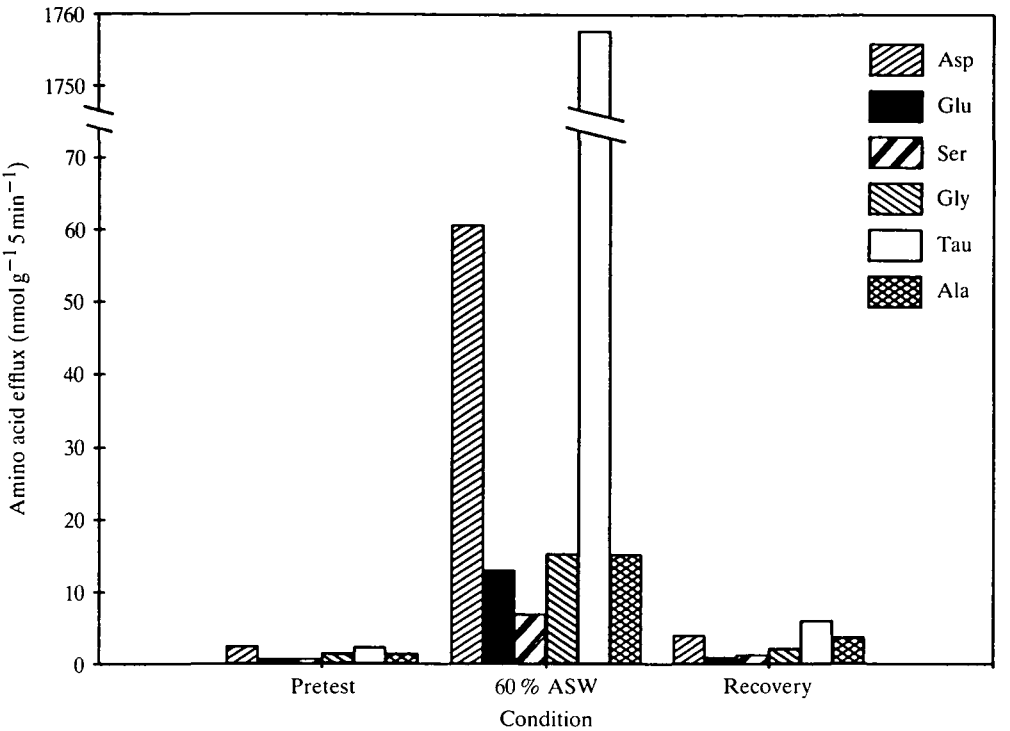

Fig. 5. Effect of acute exposure to 60 % artificial sea water (ASW) on amino acid efflux from gill tissue isolated from *Mytilus californianus*. Individual demibranchs were exposed to three successive 5-min, 50 ml rinses in 100 % ASW, with the efflux noted in the last rinse as 'Pretest' efflux. The tissue was then placed for 1 h into a 50 ml 100 % ASW rinse, after which it was placed into a fresh 100 % ASW rinse for the determination of the 5-min 'Recovery' efflux. The height of each bar reflects the mean efflux of amino acid determined in six experiments with tissue from different mussels. Error bars were omitted for purposes of clarity; the standard errors ranged from 15 to 50 % of the means. Note the break in the range of units on the ordinate for taurine.

rate of $48{\cdot}2 \pm 5{\cdot}39$ to 1760 ± 238 nmol g^{-1} 5 min^{-1}). For the other major amino acids, in decreasing order, the increases in efflux were : Asp, $27{\cdot}7 \pm 6{\cdot}9$; Gly, $13{\cdot}9 \pm 4{\cdot}0$; Glu, $9{\cdot}8 \pm 1{\cdot}9$; Ala, $9{\cdot}4 \pm 1{\cdot}7$; Ser, $3{\cdot}4 \pm 0{\cdot}5$. Two experiments in which gill tissue was exposed to 60 % ASW for 15 min indicated that the rate of net loss of these amino acids was not a linear function of time for more than 5 min, but appeared to approach a steady state after approximately 10–15 min (data not shown). Thus our estimates of the rate of amino acid loss induced by exposure to 60 % ASW (Fig. 5) may underestimate the initial efflux across the apical membrane of gill cells. The 5-min loss of these amino acids represented a maximum of 2·3 % (for taurine) and a minimum of 0·9 % (for glutamate) of the total pool of each in the gill.

There was a progressive decline in the amount of amino acid lost from isolated gill tissue when it was exposed to 60 % ASW for five successive 5-min washes. In the experiment shown in Fig. 6, the initial exposure to 60 % ASW resulted in a 36-fold increase (compared to the control period) in the rate of taurine efflux from the tissue. This was reduced to a 12-fold increase during the fifth wash. The non-taurine amino

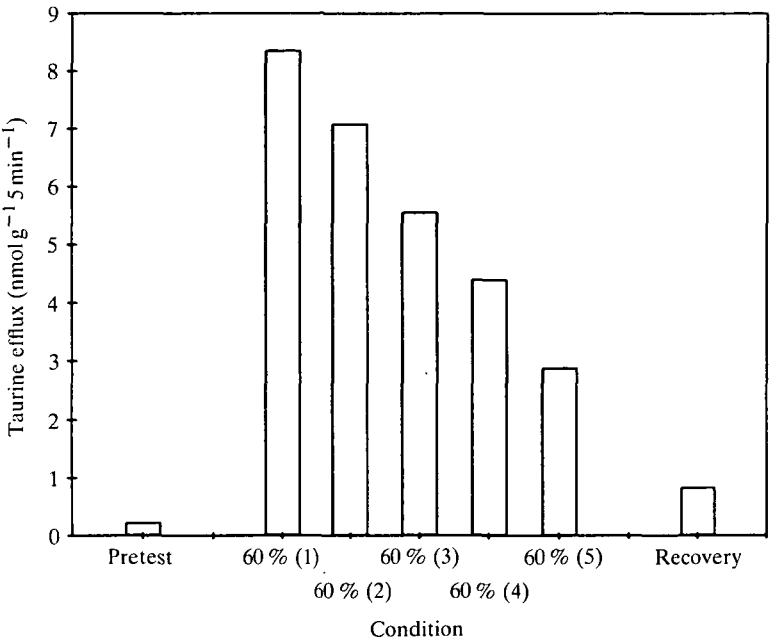

Fig. 6. Effect of successive exposures to 60 % artificial sea water (ASW) on amino acid efflux from gill tissue isolated from *Mytilus californianus*. The protocol for exposure to 60 % ASW was as indicated in the legend to Fig. 5, with the addition that the first 5-min exposure to 60 % ASW was followed by four successive 5-min rinses in 60 % ASW. 'Recovery' efflux was then measured as described in Fig. 5. This experiment was performed using gill tissue from three different mussels with similar results.

acids showed a similar progressive reduction in the rate of efflux induced by exposure to the hypo-osmotic medium. Less than 4 % of the total tissue taurine was lost during the five rinses ($3{\cdot}5\,\mu mol\,g^{-1}$), suggesting that the decline in taurine efflux noted in Fig. 6 was not due to a simple depletion of this compound from the tissue.

Exposure to a hypo-osmotic medium also inhibited the influx of amino acid into isolated gill tissue. Accumulation of $0{\cdot}5\,\mu mol\,l^{-1}$ [^{14}C]alanine during a 5-min exposure to 60 % ASW was reduced by 79 %, compared to the control condition in 100 % ASW ($0{\cdot}35 \pm 0{\cdot}06\,\mu mol\,g^{-1}\,h^{-1}$ *vs* $1{\cdot}73 \pm 0{\cdot}36\,\mu mol\,g^{-1}\,h^{-1}$; $N = 4$). Uptake of $0{\cdot}5\,\mu mol\,l^{-1}$ [^{14}C]taurine was reduced 82 % by the same treatment ($0{\cdot}19 \pm 0{\cdot}08$ *vs* $1{\cdot}01 \pm 0{\cdot}27\,\mu mol\,g^{-1}\,h^{-1}$; $N = 5$). Consequently, the increased net loss of amino acid from gill tissue to 60 % ASW must have involved, at least in part, the reduced capacity of several, separate influx pathways to reaccumulate this material (see Wright & Secomb, 1986). The degree to which reduced 'recycling' of substrates influenced the observed loss of amino acids was presumably a function of the rate of loss of each compound and the kinetics of its uptake. In the case of taurine, the 36-fold increase in net loss caused by exposure to 60 % ASW led to an efflux that exceeded by two-fold the maximal rate of influx noted in a previous study (Wright, 1985). Such a rate of loss cannot easily be ascribed to an inhibition of taurine uptake. However, the 10-fold increase in alanine loss corresponds to a net efflux ($0{\cdot}18\,\mu mol\,g^{-1}\,h^{-1}$) that was less than 1 % of the maximal rate of influx of this amino

acid ($\approx 40\,\mu mol\,g^{-1}\,h^{-1}$; Wright, 1985). Therefore, inhibition of alanine uptake could have contributed significantly to its loss in 60% ASW.

Effect of competitive inhibitors on efflux

We previously found that addition of a specific competitive inhibitor of taurine uptake (γ-aminobutyric acid; GABA) resulted in an increase in the rate of efflux of taurine from integumental tissue, while having no significant effect on the loss of other amino acids (Wright & Secomb, 1986). It was concluded that the increase in taurine efflux was the combined result of (i) the competitive inhibition of the reaccumulation of taurine lost from integumental tissues by normal, steady-state processes, and (ii) the accelerative heteroexchange of endogeneous taurine for GABA. Thus it was of interest to examine the effect of adding to test solutions substrates which have been shown to inhibit the transport of other structural classes of amino acid. When intact mussels were exposed to $40\,\mu mol\,l^{-1}$ L-leucine there was an immediate increase in the rate of loss of other neutral (i.e. zwitterionic) α-amino acids, in particular alanine, glycine and serine (Fig. 7B). This increase was transient; after rising to between 40 and $190\,nmol\,l^{-1}$, the concentration of these neutral α-amino acids decreased to less than $10\,nmol\,l^{-1}$ after 2 h. Over the same period, integumental uptake of the ambient leucine caused its concentration to decrease from $38\,\mu mol\,l^{-1}$ to $21\,nmol\,l^{-1}$ (Fig. 7A). Of particular importance was the observation that only neutral α-amino acids were lost when leucine was added; there was no appreciable change in loss of taurine (a neutral β-amino acid; Fig. 7C), or of aspartate or glutamate (anionic α-amino acids; Fig. 7D), compared to the steady-state concentrations of each of these substrates. These latter structural classes of amino acid are transported by separate pathways (Wright, 1985), and therefore the failure of leucine to affect the efflux of these amino acids was expected.

The cationic α-amino acid lysine is a partial inhibitor of neutral α-amino acid uptake (Wright, 1985). In two experiments with *M. californianus*, addition of $50\,\mu mol\,l^{-1}$ lysine resulted in a small, transient increase in the concentration of alanine and glycine (alanine, maximum ambient concentration of $55\,nmol\,l^{-1}$ *vs* $[Ala]_{ss}$ of $4\,nmol\,l^{-1}$; glycine, maximum of $49\,nmol\,l^{-1}$ *vs* $[Gly]_{ss}$ of $8{\cdot}3\,nmol\,l^{-1}$), with comparatively little effect on the concentrations of serine, taurine, aspartate and glutamate (data not shown). Finally, the addition of $50\,\mu mol\,l^{-1}$ aspartate had no effect on the efflux of either neutral α- or β-amino acids (the presence of the large aspartate peak in chromatograms from these experiments prevented observations of any effects of aspartate on the flux of glutamate; data not shown).

DISCUSSION

The integumental epithelium of the bivalve gill is routinely exposed to surrounding sea water. As a result, the apical membrane of gill cells serves as a permeability barrier, separating sea water from cytoplasm and, in conjunction with the basal-lateral membrane of these cells, the branchial haemolymph. The present study has examined several aspects of apical membrane permeability to amino acids.

The microvillous brush border of the apical membrane of gill cells increases the area exposed to sea water, consistent with the idea that these cells may have an absorptive function. The permeability of this barrier, as shown by the rate of loss of amino acid to the surrounding medium, is very low and influenced by at least two experimental manipulations: (i) reduction in ambient salinity results in a general increase in the permeability to amino acids; and (ii) exposure to large, external concentrations of neutral amino acid causes a specific increase in the loss of endogenous amino acids.

Structural and functional correlates of the apical membrane

It is a general axiom that the microvillous brush border of epithelial cells serves to increase the absorptive area of the cells' apical surface (Fawcett, 1962). Morphological measurements of the microvilli of *M. edulis* gill verified that the brush border amplifies the total apical area exposed to sea water by 10- to 18-fold (Table 1). This is considerably less, however, than the effect on surface area noted for microvilli from several other systems. For example, the brush border of cells in the mammalian

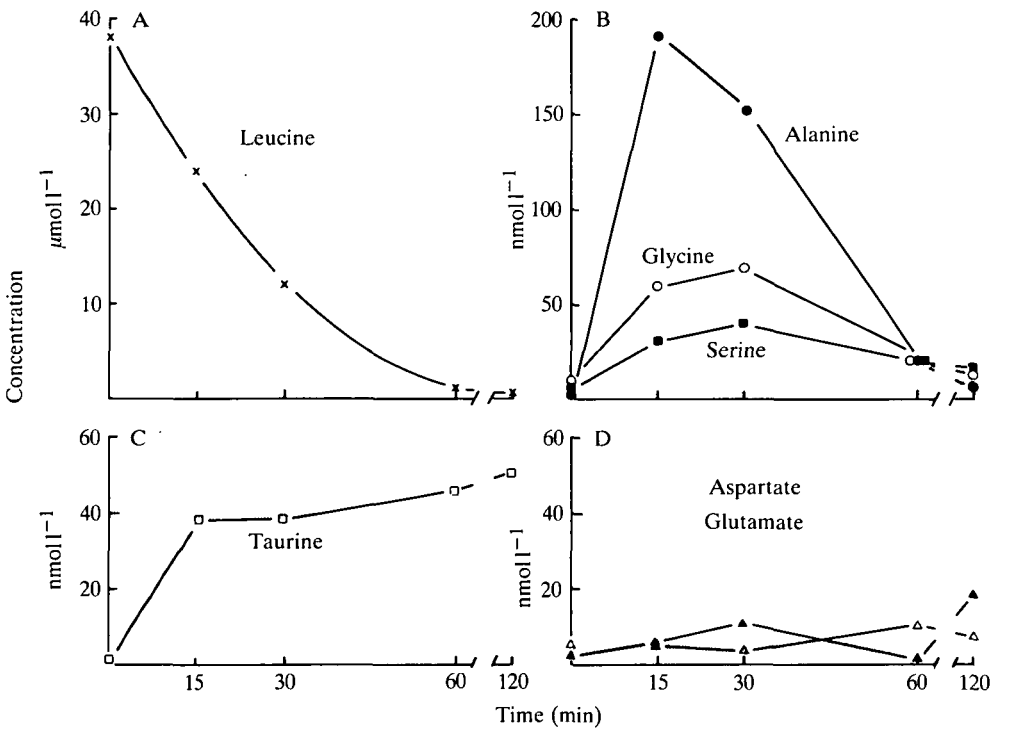

Fig. 7. Effect of L-leucine on efflux of amino acid from intact *Mytilus californianus*. The mussel was placed into 500 ml of amino-acid-free 100% artificial sea water (ASW). The steady-state level of each structural class of amino acid was noted, the range of which is shown as the stippled horizontal bar in B–D. The medium was then replaced with 100% ASW containing 40 μmol L-leucine. Samples of the medium were collected at time zero, and periodically thereafter for 2 h. Note that the scales differ and that in A the concentration is in μmol l^{-1}, while in B–D it is in nmol l^{-1}. This experiment was performed using three different animals with similar results.

proximal convoluted tubule increases the luminal area by a factor of up to 36 (Welling & Welling, 1975), and the brush border of the rat intestine increases apical area by a factor of 24 (Palay & Karlin, 1959). As discussed below, the comparatively small area of microvillous membrane may assist in reducing the overall permeability of the apical surface, thereby reducing the diffusional loss of endogenous substrates to surrounding sea water.

Though the presence of microvilli clearly results in an amplification of cell membrane area, it is less clear what effect the geometry of microvilli has on the kinetics of solute exchange across the membrane. It is conceivable, for example, that the combination of microvillous structure and the kinetic characteristics of transporters in the apical membrane could serve to reduce significantly the amount of endogenous material lost from the cell to the overlying medium (see also Gomme, 1981). To examine this possibility, we have developed a theoretical model for the effect of microvillous structure on reaccumulation of amino acids by gill cells. It incorporates the geometric characteristics of the apical membrane and the kinetics of membrane transport (see Appendix for the development of the model). For the purposes of this discussion, we have considered the following hypothetical situation: (i) uniform distribution of the active transport process along the sides of the microvilli and (ii) localization of the 'leak' to the basal area of the apical membrane, between the microvilli (see Fig. 8A). Solute lost across the apical membrane must 'run the gauntlet' between the microvilli, during which time it can serve as substrate for the transporters and thereby be recovered. This particular configuration maximizes the opportunity for the microvillous brush border to reduce the amount of endogenous material lost from gill cells to surrounding sea water. Results from the theoretical model will therefore provide an upper limit on this effect for a given set of transport and morphological parameters. The parameters associated with the calculations are (i) the ratio of J_{max} to K_t, i.e. the maximal rate of carrier-mediated transport, expressed per unit area of microvillous membrane, to the Michaelis constant for the transporter*; (ii) the rate of loss of amino acid, expressed per unit area of the base of the microvilli; (iii) the height of the individual microvilli; and (iv) the perimeter and area density of the microvilli overlying the apical base of the gill cell.

Details of the theoretical model are given in the Appendix. Fig. 8B depicts the computed variation of the rate at which solute diffuses along the spaces between microvilli from the base ($z = 0$) to the tips of the microvilli ($z = z_m$). The flux at $z = z_m$ represents the apparent leakage rate from the brush border. Comparison of the three curves shows that this leakage depends strongly on the relationship between the linear (i.e. first-order) uptake rate and the diffusive rate, as represented by the parameter λz_m defined in the Appendix, which depends on the geometric and

* This ratio, which has units of cm s^{-1}, is a measure of the carrier-mediated permeability in the first-order range of substrate(s) uptake, i.e. when $[S] \ll K_t$. The presumption that the transporter is exposed to such concentrations is supported by the low rates of steady-state leakage of amino acids from integumental tissues, and the submicromolar concentrations of amino acids often found in near-shore waters.

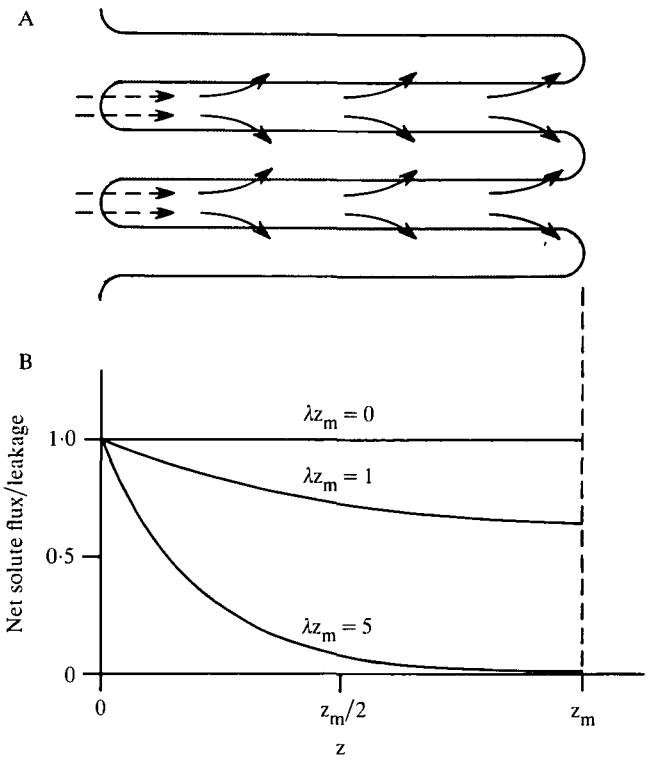

Fig. 8. (A) Idealized structure of a section of brush border. Dashed arrows indicate the passive, diffusional leak of solute at the base of the microvilli; solid arrows indicate the carrier-mediated uptake of solute across the microvillous membrane. (B) Variation of net outward solute flux across planes parallel to the cell surface (normalized with respect to leakage), when outer solute concentration is zero. The dimensionless parameter λz_m depends on the ratio of rate parameters for active uptake (i.e. J_{max}/K_t), and on diffusional and geometric parameters (see Appendix). Specific cases: (i) $\lambda z_m = 0$, diffusion is rapid compared to the capacity of membrane transport to reaccumulate solute, so there is no reabsorption; (ii) $\lambda z_m = 1$, there is moderate interplay between the transport capacity, configuration of the membrane and the rate of solute diffusion; (iii) $\lambda z_m = 5$, the combination of membrane configuration and transport capacity is such that reabsorption is essentially complete.

transport parameters listed above. The value of λz_m calculated for the apical brush border of *M. edulis* gill is 0·26 (see Appendix). According to the model, approximately 97 % of the solute leaked at $z = 0$ emerges from the brush border without being reaccumulated under these conditions. The assumption that leakage occurs only at the base maximizes the opportunity for solute reabsorption. Therefore we conclude that the combination of the kinetics of uptake, configuration of microvilli, and rate of solute diffusion are such that the geometrical configuration of the microvilli has little effect on the apparent leakage of solute from the cells. Once in bulk solution, however, taurine of endogenous origin can be reaccumulated. The efficiency of this reaccumulation is such that approximately 30 % of the material lost from the integumental surface can be recovered (Wright & Secomb, 1986).

It is of interest to note that, just as the structure of the apical membrane appears to have no direct effect on the kinetics of solute efflux, it can likewise be shown that it should not influence the influx of solute. This observation is of interest because previously we estimated the kinetics of amino acid uptake into the pumping gill using a model that took into account the influence on uptake of gill structure and water flow: the 'convection–diffusion' model (Wright & Secomb, 1984; Wright, 1985). However, the model did not take into account the effect of microvilli on the kinetics of transport. The present results suggest that the simple convection-diffusion model is adequate to describe transport in the gill, and indicate that our estimates for the Michaelis constants for amino acid uptake into this organ (i.e. 1–5 μmol l^{-1}) provide reasonable estimates of the 'true' value for the K_t of each of these membrane processes.

It is worthwhile to emphasize that, though the dimensions of the microvilli in the bivalve gill are such that they would appear to have little impact on the kinetics of solute exchange, this need not be the case for microvilli in other systems. Microvilli can exceed 4·5 μm in length, and the brush border can have a fractional density of 80 % (Smith *et al.* 1969; see also Berridge & Oschman, 1972). For example, if the gill brush border had such dimensions, the presence of microvilli would result in recovery of approximately 32 % of the taurine lost from gill. Thus, the structure of microvilli can play a role in defining the effective kinetics of solute exchange between an epithelial cell and the overlying medium, given an appropriate set of morphological and kinetic parameters.

Permeability of the apical membrane of gill cells

The low steady-state concentrations for the non-taurine amino acids suggest that the permeability of the apical membrane to these compounds is very low. At steady state, the rate of efflux from the animal is balanced by the rate of influx *via* integumental pathways. The kinetics of uptake for glycine into intact, actively pumping *M. californianus* have been determined: the J_{max} is $19{\cdot}2\times10^{-12}$ mol $cm^{-2}\,s^{-1}$, with a K_t of 4·9 μmol l^{-1} (Wright, 1985)*. The ambient concentration at which glycine efflux is equivalent to influx in *M. californianus* is $\leqslant$6·8 nmol l^{-1}. Efflux should then be $\leqslant[(19{\cdot}2\times10^{-12})\ (6{\cdot}8\times10^{-9})]/[(4{\cdot}9\times10^{-6})+(6{\cdot}8\times10^{-9})]$ or $\leqslant2{\cdot}8\times10^{-14}$ mol $cm^{-2}\,s^{-1}$. Given the tissue content of glycine (Table 2), the intracellular concentration of this compound in gill cells is probably at least 2 mmol l^{-1}. Assuming that this approximates the intracellular activity of this compound, the steady-state efflux of glycine reflects an upper limit of the apical membrane permeability to this molecule of $1{\cdot}4\times10^{-8}$ cm s^{-1}. A similar calculation for the permeability of *M. californianus* gill to aspartate results in an estimated permeability coefficient of $0{\cdot}6\times10^{-9}$ cm s^{-1}. These values are similar to our previously reported estimate of 1×10^{-9} cm s^{-1} for the permeability of *M. edulis* gill to taurine (Wright & Secomb, 1986). The actual permeability of the apical brush border will be

* The mass-specific J_{max} for glycine uptake in isolated gill tissue is 38·3 μmol $g^{-1}\,h^{-1}$ (Wright, 1985, and unpublished observations). The conversion to an area-specific uptake was based on an estimate of 550 $cm^2\,g^{-1}$ gill tissue.

approximately 13-fold lower than these figures (i.e. $10{\cdot}8–0{\cdot}5\times10^{-10}$ cm s^{-1}), as they do not take into account the effect of microvilli on increasing apical surface area. Finally, to the extent that the efflux of these amino acids is the result of backflux through mediated pathways or paracellular pathways, flux across the epithelial surfaces of the mantle, or excretion, the actual passive permeability of the apical surface of the gills will be lower than our calculations suggest.

It is worthwhile to consider how these estimates for gill membrane permeability compare to estimates from other epithelial systems. Unfortunately, though a number of studies have addressed the question of 'transepithelial' membrane permeability to amino acids (e.g. Dantzler & Silbernagl, 1976; Barfuss & Schafer, 1979), there have been few studies examining the passive permeability of the apical membrane of epithelial cells to these substrates. However, studies with isolated brush borders can be used to estimate passive permeability coefficients. Uptakes into isolated membranes are usually expressed per milligram of membrane protein. Permeability coefficients (P) from such studies, therefore, have units of $l\,mg^{-1}\,s^{-1}$ (e.g. Stevens, Ross & Wright, 1982). For purposes of comparison, these permeabilities must be converted into units of cm s^{-1}. This calculation can be made assuming that the average diameter of a brush border vesicle is $0{\cdot}2\,\mu$m (e.g. Haase, Schafer, Murer & Kinne, 1978; Hopfer, Crowe & Tandler, 1983). Combined with the measured value of the 'equilibrium' volume of the brush border vesicle suspension (where $1\,\mu l$ of 'apparent space' mg^{-1} membrane protein $= 1\times10^{-9}$ $cm^3\,mg^{-1}$), the conversion of P values to units of cm s^{-1} becomes apparent. For example, Stevens *et al.* (1982) reported P values of 1·6 and $4{\cdot}5\times10^{-8}\,l\,mg^{-1}\,s^{-1}$ for proline and phenylalanine, respectively, in brush border membranes from rabbit jejunum. The equilibrium volume of their vesicle preparation was $1\,\mu l\,mg^{-1}$, resulting in estimated P values of 5 and 15×10^{-8} cm s^{-1} for proline and phenylalanine, respectively. These values are 50–3000 times greater than the conservative values for P in the bivalve gill estimated in the present study. The apparent passive permeability of the gill approaches the values reported for amino acids in liposomal membranes (i.e. $\approx1\times10^{-11}$ cm s^{-1}; Young & Ellory, 1977). This very low permeability of gill membranes, in conjunction with a comparatively sparse apical brush border, may in part account for the ability of gill cells to sustain such large intracellular amino acid concentrations in the face of the constant exposure of the apical membrane to sea water.

Exposure to an abrupt decrease in ambient salinity resulted in a dramatic increase in the rate of loss of amino acid from gill tissue, and served to illustrate that gill cells contain amino acids which are osmotically active rather than bound or compartmentalized. The present set of observations cannot distinguish between specific, carrier-mediated efflux of amino acids and a general increase in non-specific membrane permeability to these compounds. However, the reversibility of the process (Figs 4, 5) suggests that the exposure to a hypo-osmotic medium did not result in irreversible damage to the apical membrane of the gill epithelium. Furthermore, the transient nature of the loss of amino acid from *M. californianus* gill tissue (Fig. 6) suggests that membrane permeability may be regulated. It should also be stressed that the similarity of the responses to reduced salinity of *M. edulis* and

M. californianus was expected. While the euryhalinity of *M. edulis* is widely documented (e.g. Lange, 1963), the capacity of *M. californianus* to adapt to reduced salinity is not always appreciated. Though largely confined to the comparatively stenohaline environment of the open, rocky coast, this restriction appears to be the result of the stenohalinity of the larvae of *M. californianus*; adult *M. californianus* survive for many months at salinities of 20‰ (Young, 1941).

There are several other reports of an increase in loss of amino acids from bivalves upon exposure to dilute sea water. For example, Livingston *et al.* (1979) reported that abrupt exposure of *M. edulis* acclimated to 30‰ to 15‰ sea water resulted in an increase in the rate of 'excretion' of amino acid (i.e. primary amines), from approximately 0 to a maximum of 3 μg N g^{-1} dry mass h^{-1}. For purposes of comparison, this rate can be expressed per gram wet mass of gill tissue, or approximately 0·3 μmol amino acid g^{-1} h^{-1}. This is probably an underestimate of the 'initial' rate of amino acid loss from these animals, as they were allowed to pump in the 200 ml test solution for 2 h before samples were collected for analysis; our data suggest that amino acid loss in a restricted volume is not linear for 2 h. Henry & Mangum (1980) reported that a hypo-osmotic shock caused a rapid, though transient, loss of amino acid from gills of the estuarine clam, *Rangia cuneata*. It is not clear from either of these studies whether the loss of amino acid from integumental tissues plays a quantitatively significant role in the reduction of cellular amino acid levels associated with isosmotic volume regulation in these animals.

The observation that exposure to leucine and lysine caused a specific loss of neutral amino acids (e.g. Fig. 7) is consistent with earlier observations that exposure to GABA results in the specific loss of taurine from integumental tissues (Wright & Secomb, 1986). The integumental transport of taurine and neutral amino acids involves separate transport pathways (Wright, 1985), suggesting that the efflux caused by exogenous amino acids involves interaction of the external amino acid with its specific carrier. We suggested that the increase in taurine loss to sea water upon addition of GABA was due to two separate phenomena: (i) a competitive inhibition that prevents the reaccumulation of taurine lost from the animal; and (ii) an accelerative heteroexchange of external GABA for endogenous taurine. In the present series of experiments, the rapid appearance of neutral amino acids observed upon the addition of leucine (and to a lesser extent, lysine) was probably due primarily to heteroexchange. The extremely low steady-state concentrations for the neutral amino acids are consistent with routine efflux rates that account for less than 20% of the observed alanine lost in the presence of leucine. It was of interest that alanine, though present in the gill at approximately half the concentration of glycine, was lost four times faster (e.g. Fig. 7). This could be explained if the affinity of the alanine/glycine transporter(s) at the intracellular, or *trans*, face of the membrane was greater for alanine than for glycine. This possibility is supported by the observation that alanine (and other lipophilic neutral amino acids) is a better inhibitor of neutral transport pathways than glycine (and other hydrophilic amino acids) (Wright, 1985).

In conclusion, the apical membrane of cells from the integumental gill epithelium has a microvillous brush border that serves to increase the apical area by approximately 13-fold, though the dimensions of these microvilli are such that they should not influence the kinetics of solute exchange across the apical membrane. The apical poles of gill cells serve as a barrier for the passive loss of endogenous amino acids to surrounding sea water. The apparent permeability of these cells to amino acids is much lower than that noted in mammalian systems. This low permeability, when combined with the presence of several separate pathways for the accumulation of amino acid from sea water, results in a low rate of loss of amino acid from the tissue, and thereby helps to maintain the very large intracellular concentrations of amino acid used by integumental cells in isosmotic volume regulation. Exposure to dilute sea water causes a general increase in the permeability of the gill to amino acids. This increase is transient and reversible, and may play a part in volume regulation.

APPENDIX

Analysis of concentration gradients within the brush border

In order to analyse the potential for reabsorption of leaked substrate within the brush border, we consider the configuration shown in Fig. 8A. Uniform cylindrical microvilli of fixed length project from a base plane. Leakage is assumed to occur in this plane only, i.e. across the apical membrane at the base of the microvilli, and reabsorption is considered to be uniformly distributed along the sides of the microvilli, and to be described by Michaelis–Menten kinetics. As already indicated, this hypothetical configuration was chosen to provide an upper boundary for reabsorption, compared to cases in which leakage is distributed more uniformly over the microvillar surface.

It is assumed that the fluid contained between the microvilli is stationary, so that solute transport occurs by diffusion and not convection. A further important simplification is made possible by the fact that the spaces between microvilli are relatively long and narrow. Concentration variations across these spaces are therefore generally negligible compared with the longitudinal variations. In this approximation, the concentration is given by $C(z)$ $(0 \leqslant z \leqslant z_m)$ where z is distance from the base plane, and z_m is the length of the microvilli. Similarly, the outward solute flux per unit area of the base plane is represented by $Q(z)$.

Since the microvilli are assumed to be uniform in cross-section, the fraction of each plane parallel to the base plane which lies outside the microvilli is a constant (a_g), as is the perimeter of the microvilli cut by such planes, per unit base plane area (p_m). The outward flux of solute is then given by:

$$Q = -a_g D \frac{dC}{dz}, \tag{1}$$

where D is the diffusion coefficient. The variation of flux along the microvilli

depends on the rate of uptake at the wall:

$$\frac{dQ}{dz} = -p_m \frac{J_{max} C}{K_t + C}, \tag{2}$$

where J_{max} is the maximum uptake rate per unit microvillar area and K_t is the Michaelis constant. Values for J_{max} used in these calculations were determined in studies of amino acid uptake into isolated gill tissue under conditions in which solute availability was not limiting and the transport processes was clearly saturated (i.e. substrate levels of >50 times the apparent K_t; Wright, 1985). We have also assumed that uptake is distributed uniformly over the microvillar surface. Estimates of K_t values were made using the convection–diffusion model described elsewhere (Wright & Secomb, 1984; Wright, 1985). The boundary conditions on this pair of coupled ordinary differential equations are:

$$Q = a_g l \text{ when } z = 0 \text{ and } C = C_{ext} \text{ when } z = z_m, \tag{3}$$

where l is the leakage rate in the base plane area between microvilli and C_{ext} is the solute concentration in the external stream over the brush border.

For concentrations much lower than the Michaelis constant, the nonlinear equation (equation 2) may be replaced by a linear approximation:

$$\frac{dQ}{dz} = -p_m T_m C$$

where

$$T_m = J_{max}/K_t \tag{4}$$

and T_m is the effective first-order kinetic parameter for uptake. Equations 1, 3 and 4 represent a standard problem of one-dimensional diffusion (e.g. Bird, Stewart & Lightfoot, 1960, p. 288) which may be solved analytically:

$$C = C_{ext} \frac{\cosh \lambda z}{\cosh \lambda z_m} + \frac{l}{\lambda D} \frac{\sinh \lambda (z_m - z)}{\cosh \lambda z_m}, \tag{5}$$

where

$$\lambda = \left[\frac{p_m T_m}{D a_g} \right]^{1/2}. \tag{6}$$

The net rate of efflux from the brush border is given by:

$$Q(z_m) = l' - T'_m C_{ext}, \tag{7}$$

where the apparent leak l' and the apparent parameter T'_m are given by:

$$l' = a_g l \operatorname{sech} \lambda z_m$$

and

$$T'_m = p_m z_m T_m \frac{\tanh \lambda z_m}{\lambda z_m}. \tag{8}$$

The variation of these parameters with λz_m is shown in Fig. 9. When λz_m is very small, diffusion along the microvilli is so rapid that the apparent leak and uptake are

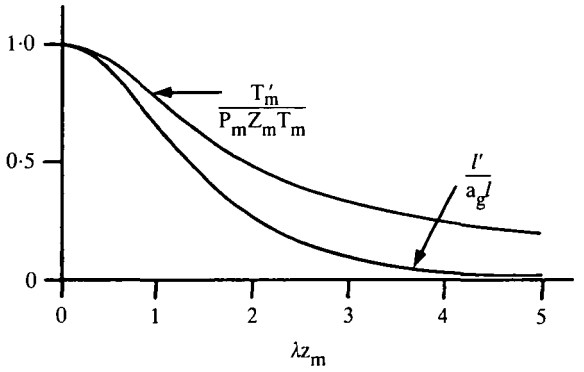

Fig. 9. Variation of the apparent linear uptake parameter T'_m and apparent leakage, l', with λz_m. Both are normalized with respect to their values in the case of rapid diffusion ($\lambda z_m = 0$).

equal to their actual values multiplied by factors representing areas available for leakage and uptake. However, as λz_m increases, both apparent leakage and apparent uptake rate are reduced due to diffusional resistance in the spaces surrounding the microvilli. Apparent leakage is seen to fall off substantially more rapidly than the apparent uptake parameter.

The dimensionless parameter λz_m may also be expressed as:

$$\lambda z_m = \left[\frac{J_{fmax}/K_t}{a_g D/z_m} \right]^{1/2}, \tag{9}$$

where $J_{fmax} = p_m z_m J_{max}$ is the effective maximum uptake rate per unit frontal area of the brush border. The quantity in brackets represents a ratio of linear uptake to rate of diffusion along the microvilli. Thus, if uptake is rapid and diffusion slow, λz_m is large and concentration gradients within the brush border substantially alter apparent transport behaviour. Conversely, if diffusion is sufficiently rapid, then λz_m is small and the fluid surrounding the microvilli may be considered well-mixed. This analysis is based on the assumption that the solute concentration is everywhere small compared to the Michaelis constant. If this is not the case, then deviations from the well-mixed situation will be smaller than the above analysis predicts, as the ratio J_{fmax}/K_t then overestimates the transport capacity.

For the microvilli lining the gill epithelia studied here, typical parameter values are: $a_g = 0{\cdot}60$, $J_{fmax} = 19{\cdot}3 \times 10^{-12}$ mol cm^{-2} s^{-1}, $K_t = 4{\cdot}9$ μmol l^{-1}, $D = 10^{-5}$ cm^2 s^{-1}, $z_m = 1$ μm. The resulting estimate is $\lambda z_m = 0{\cdot}26$. This value is sufficiently small that the fluid surrounding the microvilli may be considered well-mixed. However, it is clear that geometrical and transport parameters of the same order of magnitude could readily lead to values of z_m around unity. For instance, for the brush border in insect intestinal epithelium, $a_g \approx 0{\cdot}2$ and $z_m \approx 4{\cdot}5$ μm (Smith *et al.* 1969). If the same transport parameters are assumed as for the gill epithelia, then $\lambda z_m \approx 0{\cdot}94$.

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REFERENCES

ANDERSON, J. W. (1975). The uptake and incorporation of glycine by the gills of *Rangia cuneata* (Mollusca: Bivalvia) in response to variations in salinity and sodium. In *Physiological Ecology of Estuarine Organisms* (ed. F. J. Vernberg), pp. 239–258. Columbia, S. Carolina: University of S. Carolina Press.

ANDERSON, J. W. & BEDFORD, W. B. (1973). The physiological response of the estuarine clam, *Rangia cuneata* (Gray), to salinity. II. Uptake of glycine. *Biol. Bull. mar. biol. Lab., Woods Hole* **144**, 229–247.

BAMFORD, D. R. & CAMPBELL, E. (1976). The effect of environmental factors on the absorption of L-phenylalanine by the gill of *Mytilus edulis*. *Comp. Biochem. Physiol.* **53**A, 295–299.

BARFUSS, D. W. & SCHAFER, J. A. (1979). Active amino acid absorption by proximal convoluted and proximal straight tubules. *Am. J. Physiol.* **236**, F149–F162.

BAYNE, B. L., THOMPSON, R. J. & WIDDOWS, J. (1976). Physiology: I. In *Marine Mussels: Their Ecology and Physiology* (ed. B. L. Bayne) pp. 121–206. Cambridge: Cambridge University Press.

BERRIDGE, M. J. & OSCHMAN, J. L. (1972). *Transporting Epithelia*. New York: Academic Press. 91pp.

BIRD, R. B., STEWART, W. E. & LIGHTFOOT, E. N. (1960). *Transport Phenomena*. New York: John Wiley & Sons. 780pp.

BRADLEY, T. J. & SATIR, P. (1981). 5-Hydroxytryptamine-stimulated mitochondrial movement and microvillar growth in the Malpighian tubules of the insect *Rhodnius prolixus*. *J. Cell Sci.* **49**, 139–161.

BRAVEN, J., EVENS, R. & BUTLER, E. I. (1984). Amino acids in sea water. *Chem. Ecol.* **2**, 11–21.

CAVANAUGH, G. M. (1956). *Formulae and Methods,* IV, *of the Marine Biological Laboratory Chemical Room*. Woods Hole, MA: Marine Biological Laboratory. 61pp.

CROWE, J. H. (1981). Transport of exogenous substrate and cell volume regulation in bivalve molluscs. *J. exp. Zool.* **215**, 363–370.

DANTZLER, W. H. & SILBERNAGL, S. (1976). Renal tubular readsorption of taurine, γ-aminobutyric acid (GABA), and β-alanine studies by continuous microperfusion. *Pflügers Arch. ges. Physiol.* **367**, 123–128.

FAWCETT, D. W. (1965). Physiologically significant specializations of the cell surface. *Circulation* **26**, 1105–1125.

GILLES, R. (1979). Intracellular organic osmotic effectors. In *Mechanisms of Osmoregulation in Animals* (ed. R. Gilles), pp. 111–154. New York: John Wiley & Sons.

GOMME, J. (1981). Recycling of D-glucose in collagenous cuticle: a means of nutrient conservation? *J. Membr. Biol.* **62**, 47–52.

HAASE, W., SCHAFER, A., MURER, H. & KINNE, R. (1978). Studies on the orientation of brush-border membrane vesicles. *Biochem. J.* **172**, 57–62.

HAMMEN, C. S. (1968). Aminotransferase activities and amino acid excretion of bivalve mollusks and brachiopods. *Comp. Biochem. Physiol.* **26**, 697–705.

HENRY, R. P. & MANGUM, C. P. (1980). Salt and water balance in the oligohaline clam, *Rangia cuneata*. III. Reduction of the free amino acid pool during low salinity adaptation. *J. exp. Zool.* **211**, 25–32.

HOPFER, U., CROWE, T. D. & TANDLER, B. (1983). Purification of brush border membrane by thiocyanate treatment. *Analyt. Biochem.* **131**, 447–452.

JØRGENSEN, C. B. (1976). On gill function in the mussel *Mytilus edulis* L. *Ophelia* **13**, 187–232.

JØRGENSEN, C. B. (1983). Patterns of uptake of dissolved amino acids in mussels (*Mytilus edulis*). *Mar. Biol.* **73**, 177–182.

LANGE, R. (1963). The osmotic function of amino acids and taurine in the mussel, *Mytilus edulis*. *Comp. Biochem. Physiol.* **10**, 173–179.

LINDROTH, P. & MOPPER, K. (1979). High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn derivatization with *o*-phthaldialdehyde. *Analyt. Chem.* **51**, 1667–1674.

LIVINGSTON, D. R., WIDDOWS, J. & FIETH, P. (1979). Aspect of nitrogen metabolism of the common mussel *Mytilus edulis*: adaptation to abrupt and fluctuating changes in salinity. *Mar. Biol.* **53**, 41–55.

MANAHAN, D. T. & ARNOLD, K. E. (1983). Microspatial variability of free amino acids and ammonia in sea water. *Trans. Am. geophys. Union (EOS)* **64**, 1020.

MANAHAN, D. T., WRIGHT, S. H. & STEPHENS, G. C. (1983). Simultaneous determination of transport of 16 amino acids into a marine bivalve. *Am. J. Physiol.* **244**, R832–R838.

MANAHAN, D. T., WRIGHT, S. H., STEPHENS, G. C. & RICE, M. A. (1982). Transport of dissolved amino acids by the mussel, *Mytilus edulis*: demonstration of net uptake from natural seawater. *Science* **215**, 1253–1255.

PALAY, S. L. & KARLIN, L. J. (1959). An electron microscopic study of the intestinal villus. I. The fasting animal. *J. biophys. biochem. Cytol.* **5**, 363–371.

SIEBERS, D. & WINKLER, A. (1984). Amino-acid uptake by mussels, *Mytilus edulis*, from natural sea water in a flow-through system. *Helogolander wiss. Meeresunters.* **38**, 189–199.

SMITH, D. S., COMPHER, K., JANNERS, M., LIPTON, C. & WITTLE, L. W. (1969). Cellular organization and ferritin uptake in the midgut epithelium of a moth, *Ephestia kuhniella*. *J. Morph.* **127**, 41–72.

STEVENS, B. R., ROSS, H. J. & WRIGHT, E. M. (1982). Multiple transport pathways for neutral amino acids in rabbit jejunal brush border vesicles. *J. Membr. Biol.* **66**, 213–225.

WELLING, L. W. & WELLING, D. J. (1975). Surface area of brush border and lateral cell walls in the rabbit proximal nephron. *Kidney Intl* **8**, 343–348.

WHITE, K. M. (1937). Mytilus. In *L.M.B.C. Memoirs on Typical British Marine Plants and Animals*, vol. 31 (ed. R. F. Daniel). Liverpool: University Press of Liverpool. 117pp.

WRIGHT, S. H. (1979). Effect of activity of lateral cilia on transport of amino acids in gills of *Mytilus californianus*. *J. exp. Zool.* **209**, 209–220.

WRIGHT, S. H. (1982). A nutritional role for amino acid transport in filter-feeding marine invertebrates. *Am. Zool.* **22**, 621–634.

WRIGHT, S. H. (1985). Multiple pathways for amino acid transport in *Mytilus* gill. *J. comp. Physiol.* B **156**, 259–267.

WRIGHT, S. H. & SECOMB, T. W. (1984). Epidermal taurine transport in marine mussels. *Am. J. Physiol.* **247**, R346–R355.

WRIGHT, S. H. & SECOMB, T. W. (1986). Epithelial amino acid transport in marine mussels: role in net exchange of taurine between gills and sea water. *J. exp. Biol.* **121**, 251–270.

YOUNG, J. D. & ELLORY, J. C. (1977). Red cell amino acid transport. In *Membrane Transport in Red Cells* (ed. J. C. Ellory & V. L. Lew), pp. 301–325. New York: Academic Press.

YOUNG, R. T. (1941). The distribution of the mussel (*Mytilus californianus*) in relation to the salinity of its environment. *Ecology* **22**, 379–386.

ZURBURG, W. & DE ZWAAN, A. (1981). The role of amino acids in anaerobiosis and osmoregulation in bivalves. *J. exp. Zool.* **215**, 315–325.