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MODES OF AMMONIA TRANSPORT ACROSS THE GILL EPITHELIUM OF THE DOGFISH PUP (*SQUALUS ACANTHIAS*)

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Summary

The modes of ammonia transport across the gill epithelium of the dogfish pup (*Squalus acanthias*) were investigated using an isolated, perfused head preparation. During perfusion for 2–3 h there was no obvious oedema of gill lamellae, the head was haemodynamically responsive to near-*in-vivo* concentrations of adrenaline, and the transepithelial potential was +1·9 mV (perfusate relative to irrigate) and was unaffected by any of the potential inhibitors used in these studies. The rate of ammonia efflux was somewhat above *in vivo* levels, was not due to structural leaks (which averaged less than 1 %), declined by 17 % during a second, 20-min efflux period, but was stable during a third efflux period. Addition of bumetanide to the perfusate inhibited ammonia efflux by 17 % compared with the control, but subsequent addition of ouabain had no effect. Amiloride added to the irrigate in the presence of ouabain in the perfusate had no effect on ammonia efflux. Ammonia efflux was stimulated by specific increases in perfusate P_{NH_3} much more (1100 times) than by specific increases in perfusate NH_4^+ concentrations. Given a pK of ammonia of 9·75, we calculate that 6 % of the total ammonia efflux is *via* ionic diffusion of NH_4^+, 77 % *via* non-ionic diffusion of NH_3, and 17 % *via* a bumetanide-sensitive $Na^+ + NH_4^+ + 2Cl^-$ cotransport system. Basolateral or apical Na^+/NH_4^+ exchange apparently is not involved in ammonia transport across this epithelium.

Introduction

Fish generally excrete ammonia as the major product of protein and nucleic acid catabolism (see reviews by Kormanik & Cameron, 1981; Cameron & Heisler, 1985; Evans, 1985; Evans & Cameron, 1986). Both in teleosts and in elasmobranchs, renal efflux of ammonia is vanishingly small compared with branchial loss (e.g. Evans, 1982).

The mechanisms of ammonia transport across the fish branchial epithelium are somewhat controversial (see Evans & Cameron, 1986, for a recent review),

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because one cannot experimentally separate the fluxes of NH_3 and NH_4^+, and manipulations of internal or external solutions are difficult *in vivo* and may alter ammonia fluxes secondary to perturbations of other transport steps. For example, alteration of external or internal ammonia concentrations can alter pH (assuming relatively high NH_3 permeance) and therefore putative Na^+/H^+ exchange, rather than Na^+/NH_4^+ exchange directly (e.g. Cameron & Heisler, 1983); addition of the inhibitor amiloride to external solutions can alter Na^+/H^+ exchange and therefore NH_3 gradients rather than Na^+/NH_4^+ exchange (e.g. Kirschner *et al.* 1973); and removal of external Na^+ can reduce paracellular cationic permeability rather than inhibit cationic exchange systems (e.g. Zadunaisky, 1984).

Despite these uncertainties, recent studies indicate that ammonia extrusion by the freshwater rainbow trout (*Salmo gairdneri*) is predominantly *via* non-ionic diffusion of NH_3, with a variable role played by Na^+/NH_4^+ exchange, depending on the experimental conditions (Cameron & Heisler, 1983; Wright & Wood, 1985). It also appears that branchial H^+/NH_4^+ exchange (external H^+ for internal NH_4^+) may play a role in catfish (*Ictalurus punctatus*) exposed to extremely high external ammonia concentrations (Cameron, 1986). Interestingly, ionic diffusion of NH_4^+ appears to play a major role in ammonia extrusion by the marine teleosts, *Myoxocephalus octodecimspinosus* (Longhorn sculpin) and *Opsanus beta* (Gulf toadfish) (Goldstein *et al.* 1982), but basolateral Na^+/NH_4^+ exchange also seems to be important in the toadfish (Claiborne *et al.* 1982).

Thus, these rather sketchy and often contradictory data indicate that ionic diffusion of NH_4^+ may be important in ammonia efflux from marine teleost species, but that non-ionic diffusion of NH_3 and/or Na^+/NH_4^+ exchange probably predominates in freshwater species. The structural bases for these apparent differences are unknown, but ion permeability is much higher in marine teleosts than in freshwater species (e.g. Evans, 1979). It may be that diffusion of NH_4^+ is a pathway for ammonia extrusion across the marine teleost gill epithelium simply because of a relatively high cationic permeability.

Modes of ammonia transport across the gill epithelium have rarely been studied in elasmobranchs, and may be especially interesting since these fish, despite their marine habitat, maintain a branchial cationic permeability of the same low order as that of the freshwater teleosts (Evans, 1979). The only published data supporting a role for Na^+/NH_4^+ exchange in the elasmobranchs (Payan & Maetz, 1973; Evans, 1982) suffer from the problems of secondary effects noted above, and our work on the little skate (*Raja erinacea*) could not demonstrate Na^+/NH_4^+ exchange (Evans *et al.* 1979). We have succeeded in perfusing the isolated head of the spiny dogfish pup (*Squalus acanthias*; Evans & Claiborne, 1983) and the present study was therefore an attempt to define more carefully the usefulness of this preparation and delineate and quantify various potential modes of ammonia transport across the gill epithelium of this species. The putative modes examined were: basolateral (ouabain-sensitive) and apical (amiloride-sensitive) Na^+/NH_4^+ exchange, basolateral (bumetanide-sensitive) $Na^+ + NH_4^+ + 2Cl^-$ cotransport, non-ionic diffusion of NH_3, and ionic diffusion of NH_4^+ (see Evans & Cameron,

1986, for a review of the data supporting the presence of these transport systems in the fish branchial epithelium).

Materials and methods

Near-term pups (40–70 g including the yolk sac) were removed from pregnant female spiny dogfish (*Squalus acanthias*) caught by gill nets in Frenchman Bay, Maine, and were either maintained in running sea water (12–15 °C) at the Mount Desert Island Biological Laboratory (MDIBL) or were air-freighted to Gainesville in insulated containers, and maintained at 12 ± 1 °C in a constant-temperature cold-room. Experiments were either performed at MDIBL at 12–15 °C using water-jacketed perfusion chambers [with irrigation solutions cooled *via* thermoelectric cold plates (Model TCP-2, Thermoelectrics Unlimited, Inc.)], or in the cold-room in Gainesville. The isolated, perfused pup head was prepared as before (Evans & Claiborne, 1983), except that: the dorsal aorta was not cannulated; afferent pressures were recorded on a Gilson Duograph; and the seawater irrigate (100 ml) was recirculated with a Masterflex pump. Irrigation and perfusion flow rates were maintained at 10 ml min^{-1} and approx. 650 μl min^{-1}, respectively. The perfusate (elasmobranch Ringer's solutions: ERS) was bubbled with 1 % CO_2 in air, and formulated as by Forster *et al.* (1972), with the addition of 80 mmol l^{-1} trimethylamine oxide and 10^{-7} mol l^{-1} adrenaline (L-hydrochloride). Preliminary studies (see Results) indicated that the pup head was haemodynamically sensitive to concentrations as low as 10^{-8} mol l^{-1} adrenaline, and spontaneous ventilation was usually prompted at 10^{-7} mol l^{-1} (unpublished results). Moreover, Butler *et al.* (1978, 1986) have found that resting levels of adrenaline are about 10^{-8} mol l^{-1} in *Scyliorhinus canicula* and reach nearly 10 times that level during repeated burst exercise or hypoxia. In addition, in the perfused head of *Salmo gairdneri* viability is increased and lamellar oedema is avoided when 10^{-8}–10^{-6} mol l^{-1} adrenaline is added to the perfusate (Part *et al.* 1982; Perry *et al.* 1984*b*). Finally, Ellis & Smith (1983) have suggested that lamellar oedema might also be associated with the lack of spontaneous ventilation of perfused gill preparations.

An earlier study (Evans & Claiborne, 1983) determined that the perfused shark pup head was sensitive to high concentrations (10^{-5} mol l^{-1}) of adrenaline, so, as part of an effort to examine more carefully the integrity and viability of this perfused-head system, we tested the haemodynamic sensitivity of the gill vasculature to levels of adrenaline more closely approximating those found *in vivo* (see above). In these experiments the adrenaline concentration of the perfusate was sequentially increased from 10^{-8} to 10^{-6} mol l^{-1}, after an initial control period with adrenaline-free perfusate.

To ensure that perfusion did not alter the gill morphology, as has been described for both the trout and eel (*Anguilla australis*) head preparations (Part *et al.* 1982; Ellis & Smith, 1983; Bornancin *et al.* 1985), gill morphology was examined by scanning electron microscopy. Arches were removed from non-perfused and

perfused heads and fixed for at least 24 h in 2% glutaraldehyde in 0·1 mol l^{-1} cacodylate-HCl buffer (pH 7·4) adjusted to the proper osmolarity (approx. 1000 mosmol l^{-1}) with sucrose. Fixed tissues were rinsed in distilled water, dehydrated through a graded ethanol series, critical-point dried in liquid CO_2, mounted on a specimen stub, coated with a gold–palladium alloy, and viewed on a Hitachi Model S-415A scanning electron microscope at an acceleration voltage of 15 kV.

Rates of ammonia transport across the perfused gill were measured by adding a known quantity of NH_4Cl to the perfusate and monitoring the rate of appearance of ammonia in the recycled, seawater irrigation solution. Perfusate and irrigate total ammonia concentrations were determined chemically by the phenol–hypochlorite method of Solorzano (1969). Actual perfusate ammonia concentrations were measured in each experiment because breakdown products from the urea (350 mmol l^{-1}) and colloidal osmolyte polyvinylpyrrolidone (PVP, 3%) generated perfusate ammonia concentrations in the range of 200–400 μmol l^{-1} in nominally ammonia-free perfusate. Control experiments consisted of three, 20-min efflux periods separated by approx. 10 min during which the irrigation solution was changed. At the end of this 'wash' period, a time zero sample (5 ml) of the irrigate was taken and the next 20-min efflux period was started. At the end of 20 min, a second 5-ml sample was taken, and the rate of ammonia efflux, in μmol 100 g^{-1} h^{-1}, was calculated from the difference in ammonia concentration of the T_0 *vs* T_{20} samples, corrected for the declining volume of the irrigation medium and the wet mass of the intact fish (measured before preparation of the perfused head). In other experiments (see below) the perfusate was also changed during the wash period between successive efflux periods so that the serosal side of the gill epithelium was in contact with the new perfusate for 10 min before the start of a new efflux period.

The role of basolateral (serosal) $Na^+ + NH_4^+ + 2Cl^-$ cotransport and basolateral Na^+/NH_4^+ exchange was investigated by perfusing the head with ERS containing 5×10^{-5} mol l^{-1} bumetanide during the second flux period (after an initial control efflux), and then ERS containing bumetanide plus 10^{-4} mol l^{-1} ouabain in the third period. In another series of experiments, the efflux of ammonia *via* apical (serosal) Na^+/NH_4^+ exchange was monitored, after an initial control flux period, by adding ouabain to the ERS in the second period, and 10^{-3} mol l^{-1} amiloride (in the presence of perfusate ouabain) to the irrigate in the third period. When amiloride was added, serosal ouabain was present to obviate potential effects of amiloride on basolateral Na^+,K^+-activated ATPase (see Discussion). Amiloride at such a high concentration is nearly insoluble in sea water so it was first dissolved in an equivalent of 1 mol l^{-1} HCl, added to 1 ml of hot (90–100°C) distilled water and then to 500 ml of hot sea water while stirring. The solution was then brought to the correct pH (approx. 8·1) by addition of 1 mol l^{-1} NaOH, and cooled slowly. Control analyses of ammonia in the presence of 10^{-3} mol l^{-1} amiloride indicated that the drug inhibited the phenol–hypochlorite reaction producing an apparent 30% decline in measured ammonia concentration. This value was subsequently

used to correct the measured ammonia concentrations in the irrigate solutions containing 10^{-3} mol l^{-1} amiloride.

The relative roles of non-ionic diffusion of NH_3 *vs* ionic diffusion of NH_4^+ were quantified using an experimental protocol similar to that of Goldstein *et al.* (1982) where the perfusate NH_3 and NH_4^+ concentrations were manipulated by specific alterations of the perfusate pH and ammonium chloride concentrations. It is obvious that diffusion of NH_3 will be down gradients of partial pressure, rather than of concentration. However, for comparative purposes (see Table 4) we have equated concentration with partial pressure, assuming constant solubility of the gas in the experimental solutions. Perfusate pH values were adjusted by altering HCO_3^- concentrations (2–20 mmol l^{-1} $NaHCO_3$). Actual perfusate and irrigate ammonia concentrations were determined for each experiment (see above) and the pH determined in the same samples to the nearest 0·01 on a Radiometer PHM 84 pH meter and Corning Model 150 pH meter, respectively. Both pH electrodes were maintained at 12 °C in a refrigerated incubator (Maine) or the cold room (Gainesville), and were calibrated with buffers in saline solutions with ionic concentrations similar to either Ringer's solution or sea water, respectively. Perfusate and irrigate NH_3 *vs* NH_4 concentrations were calculated by the Henderson–Hasselbalch equation, using a pK of 9·75, according to the calculations of Cameron & Heisler (1983). Perfusate contained 5×10^{-5} mol l^{-1} bumetanide plus 10^{-4} mol l^{-1} ouabain in these experiments to inhibit basolateral uptake steps (i.e. $Na^+ + NH_4^+ + 2Cl^-$ cotransport and Na^+/NH_4^+ exchange) which could potentially play a role in the NH_4^+-stimulated ammonia efflux. Amiloride was not added to the irrigate in these experiments because the perfusate NH_3 concentration never exceeded that used in the control (amiloride) experiments (see above), which determined that amiloride had no effect on ammonia transport (see Results). Since we found that the NH_3 permeability of the pup gill epithelium was relatively high (see Results), we corrected for changes in the NH_3 gradient across the gill produced in the NH_4^+-stimulation experiments, in order to separate NH_3 from NH_4^+ effects. Similar corrections were not necessary in the NH_3-stimulation experiments because of the relatively low NH_4^+ permeability of the pup gill, and the relatively small changes in the NH_4^+ gradients.

To assess the magnitude of ammonia loss *via* structural leaks, and as another test of the structural integrity of the perfused pup head preparation, most experiments included a final perfusion period with $2{\cdot}3\times10^{-4}$ mol l^{-1} erioglaucine (acid blue no 9, M_r 783) in the ERS. The efflux of dye over the 20-min flux period was determined by measuring its concentration in T_0 and T_{20} samples of the irrigation solution at 532 nm with a Beckman model 34 spectrophotometer. Percentage leak per 20 min was computed by comparing the change in absorbance of these samples of the experimental solutions with a standard curve prepared by adding known volumes of perfusate containing erioglaucine to 50 ml of irrigation solution.

Since the flux of NH_4^+ could be affected by the transepithelial potential (TEP) across the gill epithelium, and as a further test of the integrity of this preparation, we monitored the TEP in a separate series of experiments in which perfusate and

irrigate substitutions were made which mimicked those used in the ammonia efflux experiments. The TEP was measured *via* 2 % agar/3 mol l^{-1} KCl bridges placed in the irrigation solution surrounding the gills, and in the perfusate leaving the head. Stable potentials were only possible with the irrigation and perfusate flow momentarily stopped. The measuring bridges were connected to calomel electrodes in 3 mol l^{-1} KCl, and the potential between the electrodes was measured with a Keithley model 616 digital multimeter. Asymmetry and tip potentials, measured by placing the bridges into beakers containing irrigate and perfusate connected with a separate agar–KCl bridge, were subtracted from the TEPs measured across the gills. In another series of experiments, we measured the TEPs across the gills when the seawater irrigation solution was replaced with Na^+-free or Cl^--free artificial seawater solutions (see Claiborne & Evans, 1984, for solution formulation).

Adrenaline was manufactured by ESI Pharmaceuticals, amiloride and ouabain were obtained from Sigma Chemical Co., and bumetanide was kindly supplied through Dr Rolf Kinne by Leo Pharmaceutical Products.

Experimental results are given as means ± standard error (N) and statistical differences between experimental means were determined by Student's t-test (using paired data when appropriate). Calculations of slopes for NH_3-stimulated and NH_4^+-stimulated ammonia effluxes, and corrections for changes in the NH_3 gradient in the latter experiments were performed using Multiplan (Microsoft) on an Apple IIe computer.

Results

Structural and haemodynamic integrity of the perfused pup head

The surface morphology of representative gills taken from unperfused and perfused pups is shown in Fig. 1 and it is apparent that the gill lamellae of this near-term foetus are somewhat thicker than those found in the adults (De Vries & De Jager, 1984). Earlier studies (Evans *et al.* 1982; Kormanik & Evans, 1986) have demonstrated that the pups are capable of prolonged survival in sea water (either *intra utero* or outside the mother) and display normal blood ionic and acid–base parameters, so it is clear that these gills are functional in gas exchange and in ion and pH regulation. Comparison of the unperfused with the perfused gill indicates no obvious structural abnormalities (e.g. oedema) even after perfusion periods of approximately 2–3 h.

The concentration–response curve for the sensitivity of the perfused pup head branchial vasculature to adrenaline is shown in Fig. 2. Our experimental protocol does not permit accurate determination of the actual EC_{50} of this response, but it is clearly below 10^{-7} mol l^{-1}, especially since 10^{-5} mol l^{-1} adrenaline reduces the afferent pressure to some 60 % of the control (Evans & Claiborne, 1983).

The transepithelial electrical potential (TEP) measured between the perfusate and the irrigate of the perfused pup head was +1·9 ± 0·6 mV ($N = 6$; perfusate relative to irrigate) during irrigation with sea water. Subsequent irrigation with sea

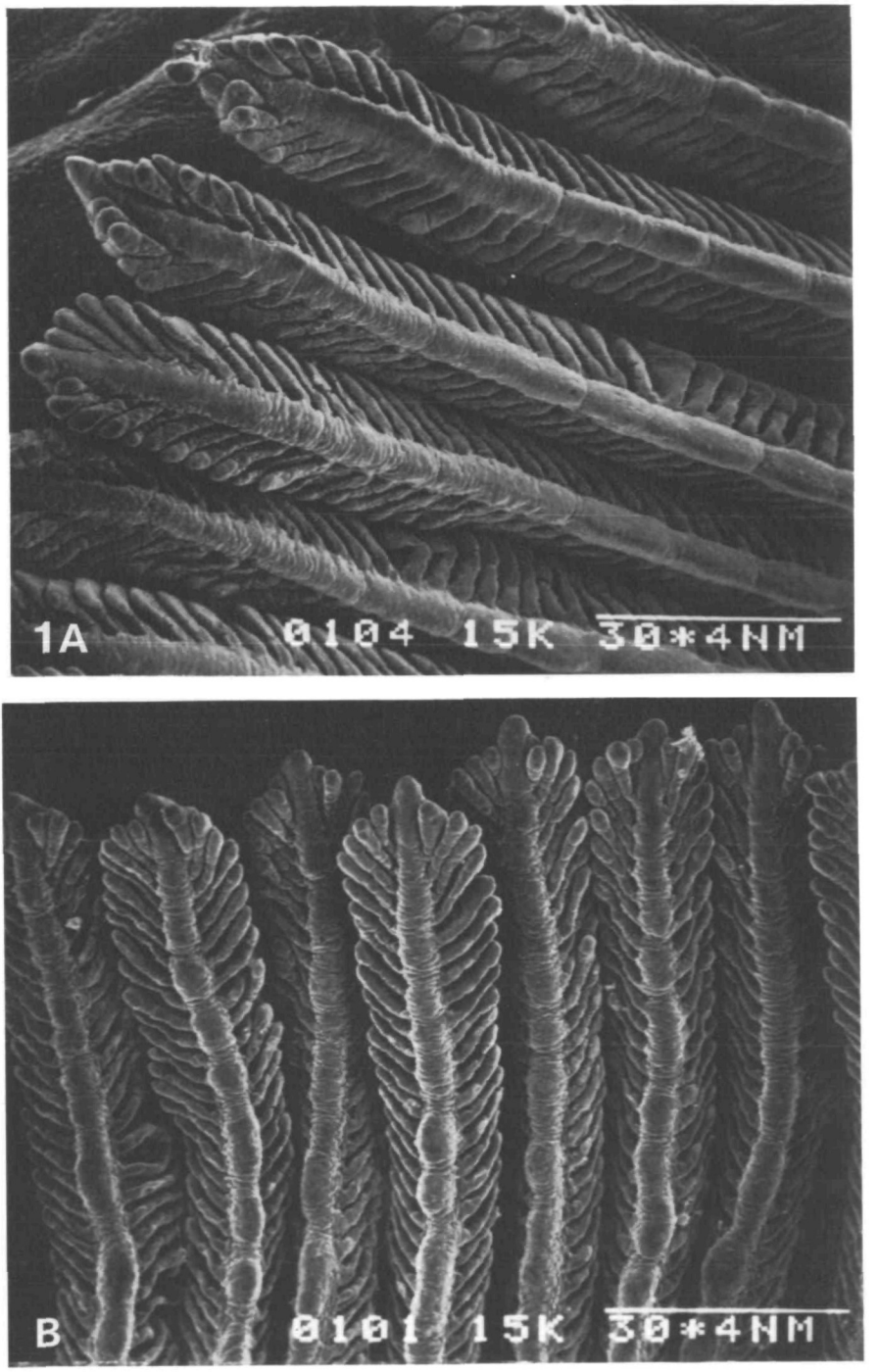

Fig. 1. Scanning electron photomicrograph of gills from unperfused (A) and perfused (B) heads of the pup of the dogfish shark *Squalus acanthias*. Gill filaments (6–7) contain lamellae intersecting each filament at right angles. Note that thickness of filaments and lamellae are not affected by 2–3 h of perfusion. Scale bar, 300 μm.

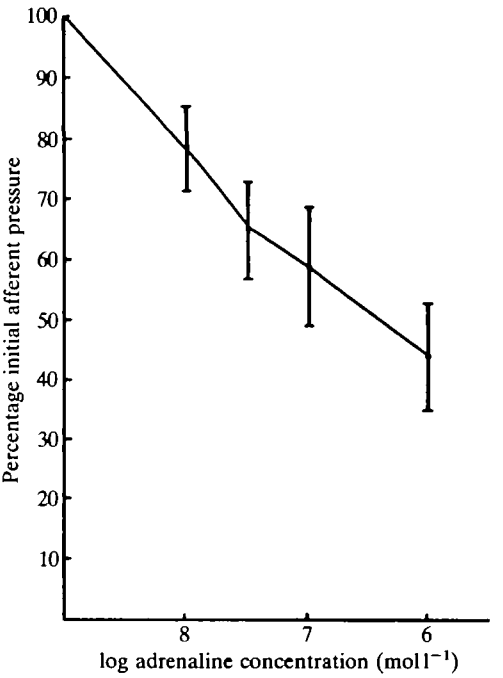

Fig. 2. Concentration–response curve for effect of adrenaline on afferent perfusion pressure of perfused shark pup head. All values are percentage of initial pressure in absence of adrenaline; mean ± S.E. ($N = 4$).

water in which the Na^+ had been replaced with choline depolarized the TEP to $-1{\cdot}0 \pm 0{\cdot}5$ mV ($N = 6$, $P < 0{\cdot}01$ compared with sea water, SW). When the head was irrigated with Cl^--free sea water (benzensulphonate) the TEP repolarized to $+5{\cdot}7 \pm 0{\cdot}5$ mV ($N = 6$; $P < 0{\cdot}01$ compared with SW and Na^+-free SW). Since Na^+ and Cl^- concentrations in the sea water were approximately equal (≈ 500 mmol l^{-1}) it appears that Cl^- conductance is greater than Na^+ conductance across the perfused shark pup head.

The erioglaucine 'leak' for the 53 perfused heads used in this study was $0{\cdot}66 \pm 0{\cdot}12$% of the perfusate for 20 min, indicating that structural leaks (and erioglaucine permeability) were vanishingly small across this preparation. In the current study, only two perfused heads displayed erioglaucine leaks greater than 3%, and data from these were not included in the results.

At the end of each experiment the gills were removed and examined under a dissecting microscope at 250× for completeness of perfusion. This examination was facilitated by the presence of the blue, erioglaucine dye. Perfusion was usually complete, but in a very few experiments the tips of a few lamellae remained red due to trapped erythrocytes. Thus perfusion was considered to be greater than 99%.

Table 1. *Total ammonia effluxes across the shark pup gill afferent perfusion pressures during successive 20-min periods*

	Period 1	Period 2	Period 3
Efflux (μmol 100 g^{-1} h^{-1})	19·4 ± 2·6	16·2 ± 2·1	16·1 ± 2·1
Pressure (mmHg)	21·2 ± 2·7	22·1 ± 2·4	22·7 ± 2·7

Mean ± S.E., $N = 9$.

Table 2. *The effect of bumetanide (5×10^{-5} mol l^{-1}) and bumetanide plus ouabain (10^{-4} mol l^{-1}) on total ammonia efflux across the shark gill and afferent perfusion pressures*

	Period 1 (Control)	Period 2 (Bumetanide)	Period 3 (Bumetanide+ ouabain)
Efflux (μmol 100 g^{-1} h^{-1})	19·1 ± 0·8	12·9 ± 1·3	10·7 ± 2·1
Pressure (mmHg)	22·3 ± 2·6	23·4 ± 3·1	27·7 ± 4·1
		$P > 0{\cdot}40$	
			$P < 0{\cdot}01$

$N = 6$.

Baseline, control efflux of ammonia

To establish the consistency of efflux over the time period utilized in subsequent experiments, we monitored ammonia efflux during three, sequential 20-min periods separated by the usual 10-min wash period (Table 1). Efflux fell slightly (17 %; $P < 0{\cdot}02$) in the second period, but did not decline further in the third period. These perfused heads also displayed a relatively stable afferent pressure over the course of the 90-min experiment (Table 1).

The roles of basolateral $Na^+ + NH_4^+ + 2Cl^-$ and Na^+/NH_4^+ transport

To examine whether basolateral $Na^+ + NH_4^+ + 2Cl^-$ and Na^+/NH_4^+ transports are involved in ammonia efflux across the pup gill we monitored the effect of addition of bumetanide and ouabain + bumetanide to the perfusate after an initial, control flux period (Table 2). Whereas bumetanide inhibited the efflux by 34 % ($P < 0{\cdot}05$), subsequent addition of ouabain in the presence of bumetanide did not inhibit the ammonia efflux further. Bumetanide did not affect the afferent pressure, but ouabain, in the presence of bumetanide, did increase the pressure slightly above the control period (+4·3 ± 1·6 mmHg; 1 mmHg = 133·3 kPa; Table 2). In an effort to quantify the potential effect of such an increase in afferent pressure, we examined the relationship between control ammonia fluxes and measured afferent pressures in heads which had not been treated with either

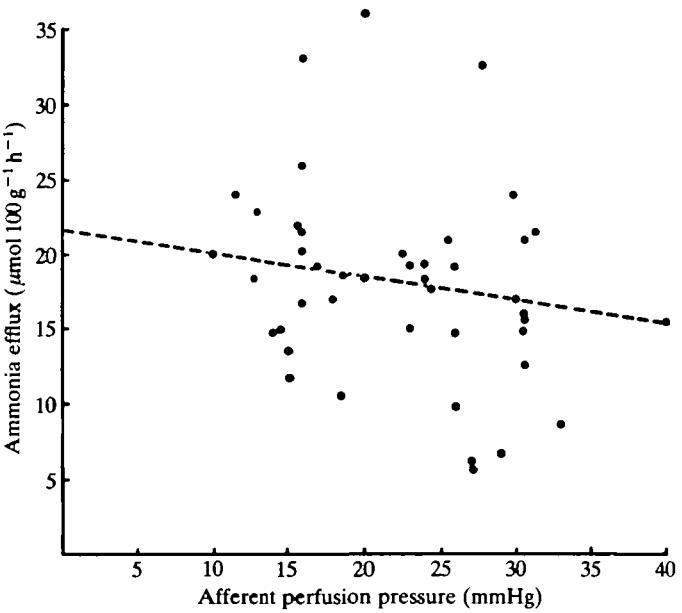

Fig. 3. The relationship between afferent perfusion pressure and ammonia efflux from shark pup heads perfused with normal elasmobranch Ringer's solution. Regression line: $y = 21{\cdot}5 - 0{\cdot}150x$; $r^2 = 0{\cdot}02$; $P > 0{\cdot}10$, indicating no significant correlation.

bumetanide or ouabain (Fig. 3). There appeared to be a slight negative relationship, but the correlation was not significant ($P > 0{\cdot}10$). Even if it were, a 4 mmHg change in pressure would only result in a fall in ammonia efflux of less than 1 μmol $100\,g^{-1}\,h^{-1}$, certainly too small to be significant in the present experiments. These data are consistent with the proposition that slight changes in afferent pressure in the perfused head are not associated with significant changes in ammonia efflux.

The role of apical Na^+/NH_4^+ exchange

To examine the importance of apical Na^+/NH_4^+ exchange, we added ouabain to the perfusate during the second flux period of another set of experiments, and amiloride to the irrigate (in the presence of perfusate ouabain) during the third period. Neither treatment affected the ammonia efflux from the perfused pup head (Table 3). Treatment with ouabain once again increased the gill resistance (Table 3), as did amiloride, but the final afferent pressures were still within the control range displayed in Fig. 3.

The roles of non-ionic diffusion of NH_3 vs *ionic diffusion of NH_4^+*

Increasing the perfusate NH_3 concentration, exclusive of changes in the perfusate NH_4^+ concentration, stimulated the total ammonia efflux significantly,

Table 3. *The effects of perfusate ouabain (10^{-4} mol l^{-1}) and ouabain plus external amiloride (10^{-3} mol l^{-1}) on the total ammonia efflux across the shark gill*

	Period 1 (Control)	Period 2 (Ouabain)	Period 3 (Ouabain+ amiloride)
Efflux (μmol 100 g^{-1} h^{-1})	20·9 ± 4·9	21·2 ± 3·6	21·5 ± 3·4
Pressure (mmHg)	21·2 ± 1·8	24·6 ± 3·4	29·7 ± 3·7
		$P < 0.02$	
			$P < 0.01$

$N = 6$.

with a slope of 0·56 ± 0·12 l 100 g^{-1} h^{-1} [μmol 100 g^{-1} h^{-1} (μmol l^{-1})$^{-1}$; $N = 22$] (Fig. 4). Specifically increasing the perfusate NH_4^+ concentration also stimulated the total ammonia efflux (Fig. 5), but millimolar concentrations were necessary (note the abscissa) so that the slope of the NH_4^+-stimulated ammonia efflux was 0·52 ± 0·15 × 10^{-3} l 100 g^{-1} h^{-1} ($N = 8$), less than 0·1 % of the slope of the NH_3-stimulated ammonia efflux. Clearly, the NH_3 permeability of the pup gill is significantly greater than the NH_4^+ permeability. Neither manipulation of perfusate ammonia concentration affected the afferent perfusion pressures (data not shown).

Effects of changes in the transepithelial electrical potential

None of the perfusate or irrigate manipulations in the experiments detailed above affected the TEP across the gills (data not shown).

Discussion

Structural integrity of the perfused head preparation

Perfusion of the pup head for as much as 3 h does not affect the gross lamellar structure (Fig. 1). Thus, this preparation does not suffer from the obvious structural abnormalities described for the eel, *A. australis*, holobranch (Ellis & Smith, 1983), or the rainbow trout, *S. gairdneri*, head perfused with adrenaline-free Ringer's solution (Part *et al.* 1982). More recently, it has been shown that the addition of at least 10^{-6} mol l^{-1} adrenaline and bovine serum albumin (0·2 % and 2 %, respectively) maintained normal lamellar anatomy for 30–45 min in the perfused trout head (Perry *et al.* 1984*b*; Bornancin *et al.* 1985), but these manipulations are not necessary with the perfused pup head.

The gill resistance of the perfused pup head is quite sensitive to the presence of adrenaline in the perfusate (Fig. 2), with an EC_{50} in the range of *in vivo* concentrations described for intact, exercising or hypoxic adult sharks (e.g. Butler *et al.* 1978, 1986). Since the perfused pup head retains functional cranial nerves,

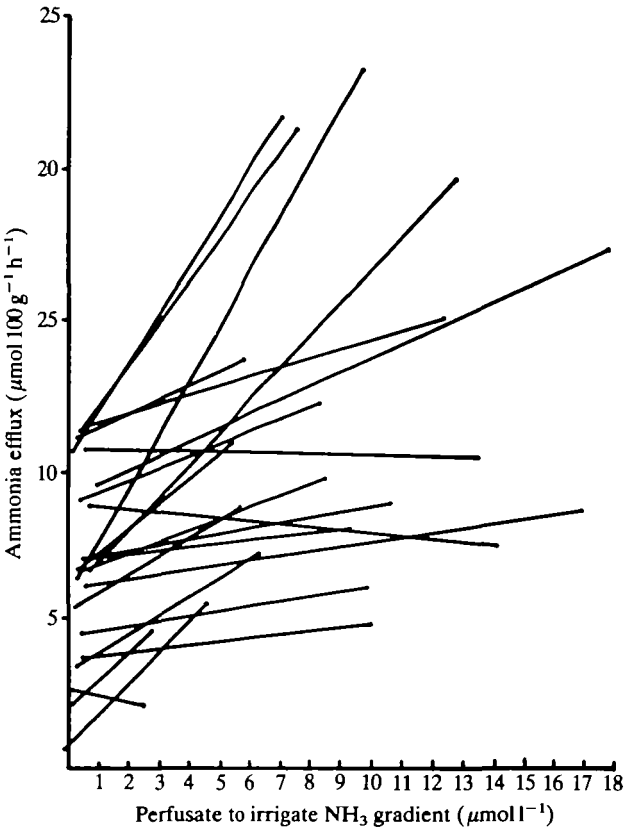

Fig. 4. The effect of specifically increasing perfusate NH_3 concentrations on total ammonia efflux from the perfused shark pup head. Each line connects data points from a single experiment. Note that the abscissa is in $\mu mol\,l^{-1}$ of NH_3.

specific concentration–response curves for adrenaline are probably impossible to obtain because of reflex vasoconstriction subsequent to hormone-mediated vasodilation. Indeed, in most cases, we observed partial reversal of the adrenaline-mediated fall in gill resistance within minutes after adding a given concentration of the catecholamine to the perfusate (unpublished observations). We added $10^{-7}\,mol\,l^{-1}$ adrenaline to the perfusate in subsequent experiments because it was the lowest concentration that usually elicited spontaneous ventilation by the head.

The transepithelial electrical potential (TEP) measured across the pup head was 6·3 mV more positive than the −4·4 mV measured in the intact pup (Evans *et al.* 1982). Only a very few measurements of TEP across perfused heads have been published. Claiborne & Evans (1984) found that the TEP across the head of the marine teleost *M. octodecimspinosus* was equivalent to that of the intact fish (+7·7 mV *vs* +7·2 mV), but Perry & Wood (1985) found that the TEP across the perfused trout head was −1·3 mV when perfused with irrigate containing 1·29 mequiv l^{-1} Ca^{2+} and Kerstetter *et al.* (1970) measured +6 mV across the

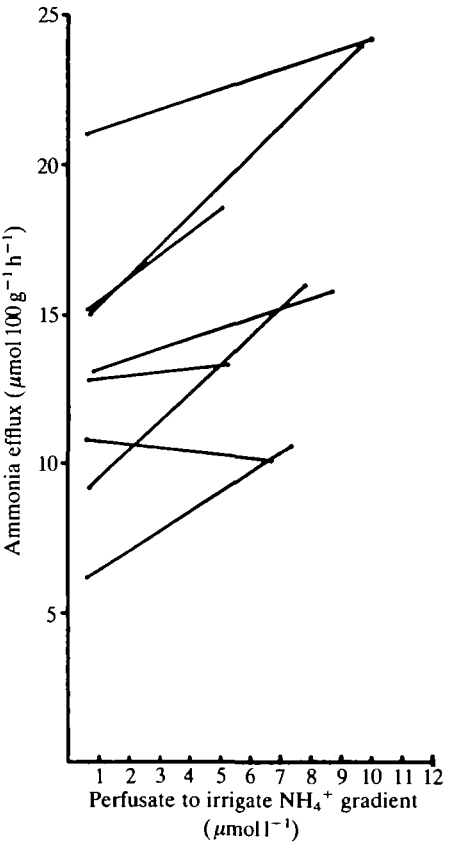

Fig. 5. The effect of specifically increasing perfusate NH_4^+ concentrations on total ammonia efflux from the perfused shark pup head. Each line connects data points from a single experiment. Note that the abscissa is in mmol l^{-1} of NH_4^+

intact fish under similar (1 mequiv l^{-1}) external Ca^{2+} conditions. One might argue that the TEPs measured across the perfused heads are better estimates of the true potentials than those measured *in vivo via* bridges in the peritoneal cavity and external medium; indeed, our unpublished observations indicate that changes in ionic composition of the irrigate produce nearly instantaneous changes in the TEP across the perfused head, but quite slow (minutes) TEP changes across the intact fish. However, if one assumes that the *in vivo* TEP values are the controls, then the data in Fig. 3 are consistent with the proposition that, like *Myoxocephalus octodecimspinosus* (Claiborne & Evans, 1984), the perfused pup head maintains a lower Cl^- conductance than normal. This proposition is supported by our finding that the depolarization produced by removal of Na^+ from the irrigate was 2·9 mV, compared with 2·6 mV *in vivo*; but the hyperpolarization produced by Cl^- removal was only 3·8 mV, compared with 13·5 mV *in vivo* (Evans *et al.* 1982). Despite this uncertainty about the 'normality' of the relative Cl^- and Na^+ conductances across the perfused pup head, it is clear that measurements of the

TEP are possible, and can be used to determine if ionic flux changes under other experimental conditions are secondary to changes in the electrochemical gradients for ionic species (see below).

Our preliminary experiments with the perfused pup heads determined that simple measurement of postbranchial perfusate outflow *vs* prebranchial inflow was sufficiently variable to preclude accurate estimation of the degree of structural leak of this preparation. Our data using erioglaucine to measure the sum of structural leak and erioglaucine permeability indicate that, under most circumstances (53 of 55 perfused heads), the leaks were minimal, less than 1 %. Assuming that the actual branchial permeability to erioglaucine is zero, and an average perfusate ammonia concentration of approximately 400 μmol l^{-1}, a perfusion flow of 650 μl min^{-1}, and an animal mass of 50 g, an erioglaucine leak of 0·7 % per 20 min would produce an apparent ammonia efflux of only 0·2 μmol 100 g^{-1} h^{-1}, vanishingly small compared with the fluxes described below. It is therefore clear that the perfused pup head is structurally 'tight', and any leak of perfusate cannot account for more than approximately 1 % of the measured ammonia efflux.

Control ammonia efflux

The data in Table 1 demonstrate that the ammonia efflux from the perfused pup head is relatively constant throughout the entire 90 min of perfusion. In addition, the gill resistance is stable because the afferent perfusion pressures do not change over this period. The control ammonia efflux from the perfused pup head is significantly higher than that described for the *in vivo* pup (2–10 μmol 100 g^{-1} h^{-1}; Evans *et al.* 1982). Since structural leaks cannot account for this relatively high ammonia flux (see above), it appears most likely that it is secondary to increased gill surface area and/or increased gill permeability to ammonia. There are no data on the *in vivo* extent of lamellar perfusion in the elasmobranchs, but data from trout indicate that *in vivo* perfusion in this species is only of the order of 60 %, but increases to 75 % when adrenaline is added (Booth, 1979). Adrenaline also stimulates lamellar recruitment in the catfish, *I. punctatus* (Holbert *et al.* 1979). Visual inspection of our perfused heads indicated virtually complete replacement of blood with perfusate so it is likely that at least some of the increased ammonia efflux from the perfused head is secondary to supranormal lamellar perfusion and, hence, increased gill surface area. The presence of significant ammonia in perfusate without added NH_4Cl (secondary to contamination from urea and PVP) precluded a determination of the role of branchial cell deamination *vs* perfusate clearance in total ammonia efflux.

Basolateral $Na^+ + NH_4^+ + 2Cl^-$ cotransport and Na^+/NH_4^+ transport

Although it has been generally accepted that ouabain-sensitive Na^+,K^+-activated ATPase can bind NH_4^+ at the K^+ site (e.g. Stekhoven & Bonting, 1981; Mallery, 1983), it is only recently that a sensitivity to NH_4^+ at the K^+ site of the bumetanide (furosemide)-sensitive $Na^+ + K^+ + 2Cl^-$ cotransporter has been described (Kinne *et al.* 1986*a*,*b*; O'Grady *et al.* 1987) in vesicles isolated from the

shark rectal gland and medullary thick ascending limb of the rabbit kidney. In fact, Good *et al.* (1984) demonstrated a furosemide-sensitive ammonia reabsorption in the thick ascending limb, but suggested that changes in the TEP secondary to this inhibition (e.g. Greger & Schlatter, 1984) could have altered diffusive movements of NH_4^+, rather than transport of the cation *via* the cotransporter. Ammonia is transported across the pup gill epithelium, at least in part, *via* a basolateral, bumetanide-sensitive system (Table 2), consistent with the proposition that a basolateral $Na^+ + NH_4^+ + 2Cl^-$ transporter is involved. However, the lack of a basolateral effect of ouabain (Tables 2 and 3) indicates that Na^+,K^+-activated ATPase is not directly involved. Neither inhibitor affects the gill resistance (Table 2) or TEP (data not shown) enough to obscure a direct effect on transport. Our finding that ammonia transport across the pup gill is insensitive to ouabain does not corroborate our previous data (Claiborne *et al.* 1982) on the perfused head of the marine teleost, *O. beta*. In addition, these earlier studies showed that increasing the perfusate K^+ concentration also inhibited ammonia efflux. Ouabain can inhibit the cotransport system indirectly by abolishing the Na^+ electrochemical gradient which drives the cotransport system (e.g. Frizzell *et al.* 1979; Greger & Schlatter, 1984), and perfusate K^+ could also compete with NH_4^+ for the cotransporter. However, our recent unpublished data indicate that ammonia transport across the *O. beta* gill is indeed sensitive to perfusate ouabain, but insensitive to perfusate bumetanide, so it appears that basolateral Na^+/NH_4^+ but not $Na^+ + NH_4^+ + 2Cl^-$ transport is involved in this marine, teleost species.

Our experiments do not, of course, allow estimates of the relative affinity of the $Na^+ + NH_4^+ + 2Cl^-$ cotransporter in the pup gill for NH_4^+ *vs* K^+. Kinne *et al.* (1986*b*) found relative affinities for the cotransporter in the thick ascending limb of the rabbit kidney for $NH_4^+ : K^+$ of 1:5. The relative perfusate concentrations in our experiments were approx. 1:10 ($400\,\mu mol\,l^{-1}$ NH_4^+ *vs* $6\,mmol\,l^{-1}$ K^+). If we make the simplifying assumption of similar affinities in the dogfish pup gill and the rabbit thick ascending loop, it appears that NH_4^+ has only a 1 in 50 chance of binding to the cotransporter in our experiments. One might suggest, therefore, the bumetanide-sensitive NH_4^+ transport is merely 'sloppiness' in the transport system, rather than a specific mode of extrusion. Data to be discussed below are consistent with this conclusion.

Our finding of a bumetanide-sensitive carrier on the basolateral aspect of the pup gill is of much more general interest than merely ammonia transport. Despite the rectal gland's obvious role in salt secretion (Evans, 1979), various experiments have shown that osmoregulation continues in elasmobranchs which have had the rectal gland ligated or removed (Chan *et al.* 1967; Haywood, 1975; Evans *et al.* 1982), supporting the proposition for another site for salt secretion. Cells have been described in the gill epithelium of elasmobranchs (Doyle & Gorecki, 1961; Laurent & Dunel, 1980; Laurent, 1984) which are similar to the chloride cells found in teleosts. Since chloride cells in teleosts are generally considered to be the site of active secretion of Cl^- *via* a basolateral $Na^+ + K^+ + 2Cl^-$ cotransporter (Zadunaisky, 1984), it is not surprising that we have found a bumetanide-sensitive

transporter in the gill epithelium of the dogfish pup. Nevertheless, it is the first physiological evidence for this putative carrier in an elasmobranch epithelium. A study of the effects of perfusate bumetanide on Na^+ and Cl^- efflux from the perfused pup head would be most interesting.

Apical Na^+/NH_4^+ transport

Previous studies on teleosts have demonstrated that ammonia efflux from intact fishes is at least partially dependent upon external Na^+ (Evans, 1977, 1982) and Payan (1978) found that ammonia efflux from the perfused trout head was also inhibited by removal of Na^+ from the irrigate, implicating apical Na^+/NH_4^+ exchange in ammonia extrusion. However, Evans & Cameron (1986) have recently pointed out that inhibition of apical Na^+/H^+ exchange, with a concomitant reduction in the NH_3 diffusion gradient, could account for these results. Moreover, removal of apical Na^+ has been shown to inhibit the paracellular, diffusive pathway for Na^+ across the chloride-cell-rich opercular epithelium of the teleost *Fundulus heteroclitus* (Zadunaisky, 1984). So, it is quite possible that removal of external Na^+ can affect diffusive, rather than carrier-mediated, movements of ammonia. Interestingly, removal of external Na^+ has only a slight, and non-reversible, effect on ammonia efflux from intact dogfish pups (Evans, 1982), and no effect on ammonia efflux from the intact skate, *Raja erinacea*, (Evans *et al.* 1979), despite complete, and reversible, cessation of acid efflux in Na^+-free artificial sea water in both species. These data are certainly consistent with the supposition that, not only is apical Na^+/NH_4^+ exchange not involved in elasmobranch ammonia extrusion, but that extrusion is also not affected by gradients produced by apical Na^+/H^+ exchange or by the control of paracellular, cation-selective diffusive pathways by external Na^+.

Amiloride (5×10^{-5}–10^{-4} mol l^{-1}) is effective in inhibiting ammonia efflux when added to the medium external to the trout (Kirschner *et al.* 1973; Payan, 1978; Wright & Wood, 1985), but is not effective (at 10^{-4} mol l^{-1}) in inhibiting ammonia efflux from the skate, *Raja erinacea*, in sea water (Evans *et al.* 1979). Since amiloride has been shown to be effective (at such high concentrations) in inhibiting Na^+/H^+ exchange, which can also include Na^+/NH_4^+ exchange (Kinsella & Aronson, 1981; Aronson, 1985), it appears from these data that the trout, but not the skate, is able to transport NH_4^+ *via* an apical Na^+/NH_4^+ exchanger. However, once again, one cannot rule out indirect effects of amiloride *via* changes in the diffusional gradient for NH_3 produced by inhibition of Na^+/H^+ exchange. Amiloride experiments have another complicating factor: amiloride may be a competitive inhibitor for Na^+ transport *via* channels or Na^+/cation exchange, so extremely high concentrations of amiloride (approx. 10^{-4}–10^{-3} mol l^{-1}) must be used in Ringer's solutions (e.g. Henderson *et al.* 1987; Moran, 1987) or sea water (e.g. Knakal *et al.* 1985) which contain approximately 150 and 500 mmol l^{-1} Na^+, respectively. At these concentrations amiloride has been shown to block paracellular cation channels (Balaban *et al.* 1979) and even basolateral Na^+,K^+-activated ATPase (Soltoff & Mandel, 1983), both of which

may play a direct or indirect role in ammonia efflux (present study; Evans & Cameron, 1986).

Given the potential inhibition of basolateral Na^+,K^+-activated ATPase by the high concentration of amiloride used in our experiments, we perfused the gills first with ERS containing ouabain, and then ouabain plus amiloride, in an attempt to cancel out basolateral events. Changes in the gill resistance in these experiments were slight, and ouabain did not, in itself, inhibit ammonia transport (Table 3). After correction for the inhibition of the phenol–hypochlorite reaction, it can be seen that 10^{-3} mol l^{-1} amiloride applied to the apical surface of the pup gill does not inhibit ammonia efflux (Table 3). This is certainly consistent with the conclusion that apical Na^+/NH_4^+ exchange does not play a measurable role in ammonia transport across the gill of the shark pup, although our data cannot exclude the possibility that 10^{-3} mol l^{-1} amiloride was ineffective because of competition with the 500 mmol l^{-1} Na^+ concentration in the irrigate.

Since we can find no evidence for transport of NH_4^+ *via* Na^+/NH_4^+ exchange across the apical membrane we are unsure of the mechanism whereby the NH_4^+ which enters the basolateral aspect of the branchial cell *via* $Na^+ + NH_4^+ + 2Cl^-$ cotransport (see above) leaves from the apical aspect of the cell into the sea water. NH_4^+ has a hydrated radius similar to that of K^+ (Kieland, 1937), and NH_4^+ has been shown to traverse K^+ channels in some tissues (see Zeiske & Van Driessche, 1983), so it is certainly possible that the NH_4^+ which enters the cell on the basolateral aspect leaves *via* putative potassium channels either on the apical surface directly into the sea water, or on the basolateral surface and subsequently *via* the paracellular pathway, which may be conductive to NH_4^+ (see below).

Non-ionic vs *ionic diffusion*

Until very recently it was generally assumed that, because of its lipid solubility, NH_3 was able to diffuse freely across biological membranes (e.g. Pitts, 1973; Valtin, 1983). However, recent determinations of the solubilities of NH_3 in lipid solvents such as chloroform and olive oil (J. N. Cameron, G. A. Kormanik & L. Goldstein, unpublished data), have confirmed a much older study which determined a chloroform:water partition coefficient of NH_3 of only 0·04 (Bell & Field, 1911), far below that of substances considered to be lipid-soluble. Nevertheless, Cameron & Heisler (1983) demonstrated that the net movement of ammonia followed the NH_3 partial-pressure gradients across the trout gill. Interestingly, in an earlier study of ammonia transport across the perfused heads of two marine teleosts (*O. beta* and *M. octodecimspinosus*), Goldstein *et al.* (1982) could find no evidence for NH_3 permeance, but specific NH_3 gradients were not measured, and our subsequent study of at least *O. beta* has demonstrated a significant NH_3 permeability (D. H. Evans & K. J. More, in preparation). Goldstein *et al.* (1982) described a significant NH_4^+ permeance across the gills of both teleost species, and this ionic permeability to NH_4^+ has been corroborated in more recent studies on the turtle bladder (Schwartz & Tripolone, 1983) and the rabbit renal proximal straight tubule (Garvin *et al.* 1987).

Table 4. *Apparent NH_3 and NH_4^+ permeabilities of various epithelial tissues*

Tissue	NH_3	NH_4^+	Authors
Turtle bladder	260	4·9	Schwartz & Tripolone (1983)
Turtle bladder	72	4·5	Arudda *et al.* (1984)
Rabbit proximal straight tubule	16000	45·0	Garvin *et al.* (1987)
Shark pup gill	420	0·4	Present study

All permeabilities are in 10^{-6} cm s^{-1}.

The present experiments indicate that the pup gill is permeable to both NH_3 and NH_4^+ (Figs 4, 5). The actual site(s) of these permeabilities is unknown, but may be paracellular because of the charge on NH_4^+ and possible low lipid-solubility of NH_3 (see above). Using the slopes of the respective experiments we can calculate that the NH_3 permeability is some 1100 times the NH_4^+ permeability. If we assume that the functional surface area of the perfused pup gill is of the same order as the structural surface area of the gill of the adult of the same species (3·7 $cm^2 g^{-1}$; Hughes & Morgan, 1973), we can calculate the respective 'apparent' permeabilities and compare them with the few similar calculations for other epithelia (Table 4). It is clear that the apparent permeability of the pup gill to NH_3 is significantly below that described for the rabbit proximal straight tubule, but of the same order as that found for the turtle bladder. The relative NH_4^+ permeability of the pup gill is only 10 % that of the turtle bladder and only 1 % that of the proximal tubule. One is tempted to correlate these relative permeabilities of the rabbit and turtle tissues with the evidence that the turtle bladder is a 'tight' epithelium, whereas the proximal tubule is considered to be 'leaky' (e.g. Fromter & Diamond, 1972; Erlij & Martinez-Palomo, 1978). Intact sharks certainly have a very low rate of Na^+ efflux, especially when compared with marine teleosts, and therefore their gill epithelium is generally considered to be rather impermeable to ions (Evans, 1979). Our data on apparent ammonia permeabilities support this supposition, but firm statements are only possible when true, functional surface areas are known for complex epithelia like those of the gill.

The data in Fig. 5 were generated with ouabain and bumetanide in the perfusate to obviate any stimulation of basolateral transport events by the increased perfusate NH_4^+ concentration. In four experiments, the perfusate did not contain either transport inhibitor and the slope of the NH_4^+-stimulated ammonia efflux was $0{\cdot}67 \pm 0{\cdot}21 \times 10^{-3}$ l 100 g^{-1} h^{-1}, not significantly different from that described for the slope of the NH_4^+-stimulated ammonia efflux when basolateral transport steps were inhibited. This indicates that increasing the perfusate NH_4^+ concentration by approximately 10-fold did not stimulate the bumetanide-sensitive cotransporter, supporting the notion that the bumetanide-sensitive component may be merely sloppiness in the transporter, rather than a directed, controlled transport of NH_4^+ (see above).

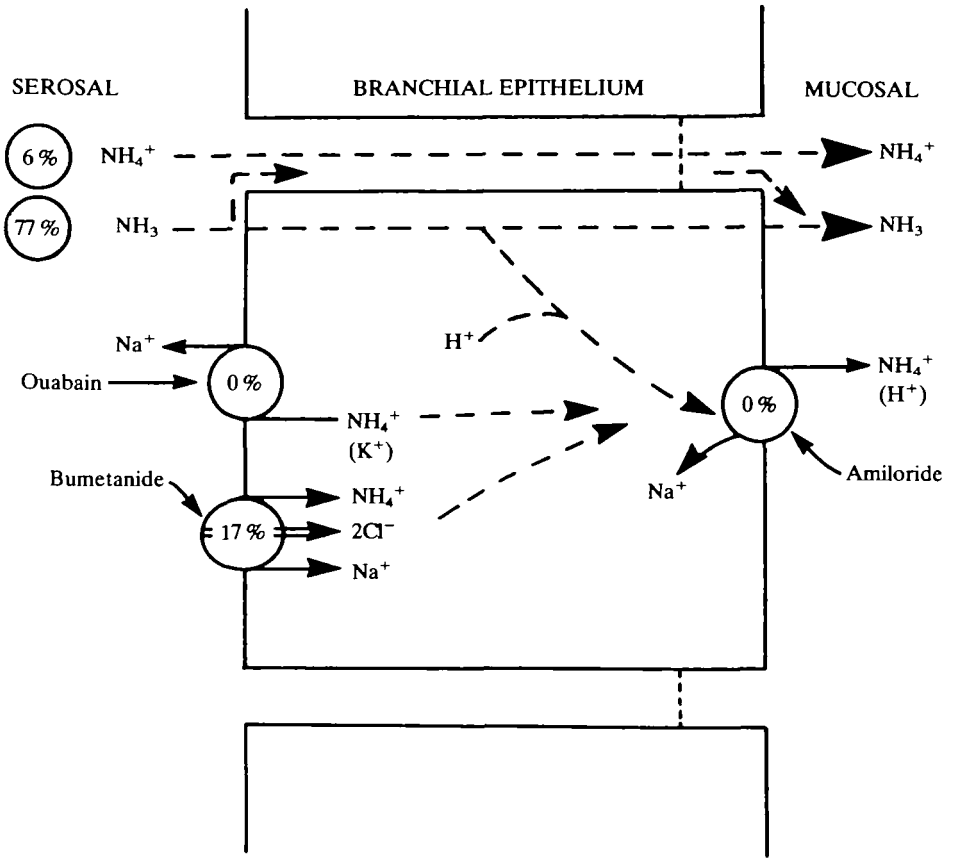

Fig. 6. Summary of relative roles played by various putative steps for transport of NH_3 or NH_4^+ across the shark pup gill epithelium. Redrawn and modified from Evans & Cameron (1986).

Relative roles of various transport pathways

Given the data presented here, and assuming an ammonia pK of 9·75 for elasmobranch Ringer's solution at 12°C (Cameron & Heisler, 1983), we can calculate the relative roles played by the various, putative transport pathways across the shark pup gill epithelium. At the pH of the perfusate (= shark blood ≈ 7·8) the concentration ratio of the two species is 0·011:1·0 (NH_3:NH_4^+). Since the actual relative efflux is the product of the relative permeability and the relative concentration (or partial pressure for a gas), the actual ratio of the effluxes for NH_3:NH_4^+ is 12:1, despite the 1100-fold difference in their respective permeances across the gill epithelium. Since bumetanide inhibited the ammonia efflux by 17 % (below the second control period), we can calculate that the other 83 % of the total efflux is diffusion of NH_3 and NH_4^+. Fig. 6 presents a summary of these calculations and indicates that, despite the dominance of non-ionic diffusion of NH_3, basolateral Na^++NH_4^++2Cl cotransport and ionic diffusion of NH_4^+

play measurable roles in the transport of ammonia across the gill epithelium of the pup of *Squalus acanthias*.

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