

## STUDIES OF AMMONIA IN THE RAINBOW TROUT: PHYSICO-CHEMICAL PARAMETERS, ACID-BASE BEHAVIOUR AND RESPIRATORY CLEARANCE

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### SUMMARY

Ammonia ( $\text{NH}_3$ ) is only slightly more soluble (+3.4%) in the plasma of rainbow trout than in water, and its  $\text{pK}'$  is only 0.14 units higher than in fresh water at 15°C. Determination of these physico-chemical parameters together with measurements of blood and water pH and total ammonia concentrations allowed calculation of the mean resting partial pressure gradient across the gills ( $54 \times 10^{-6}$  Torr) and estimation of the gill permeability coefficient ( $D = 1.3 \times 10^{-5} \text{ cm}^{-1}$ ).

Under normal resting conditions of low external  $\text{NH}_3$  and pH, diffusive movement of  $\text{NH}_3$  appears to account adequately for ammonia excretion in the rainbow trout;  $90 \pm 10\%$  of the excreted ammonia appears to originate from the blood, rather than from *de novo* synthesis of ammonia in the gills.

During the high external ammonia treatments, the fish reached a steady state with a net inward ammonia gradient, which could be accounted for by a counter-balancing  $\text{Na}^+/\text{NH}_4^+$  exchange. Ammonium salt infusions or injections which stimulate  $\text{Na}^+$  influx are not sufficient demonstrations of  $\text{Na}^+/\text{NH}_4^+$  exchange, since acidosis is produced, and alternate interpretations of the  $\text{Na}^+$  flux stimulation are possible, such as enhanced  $\text{Na}^+/\text{H}^+$  exchange.

### INTRODUCTION

Although it has long been known that ammonia is produced in the metabolism of fishes, and that most of the ammonia is excreted across the gills (Smith, 1929), few studies have adequately considered the behaviour of ammonia in aqueous solutions. Like carbon dioxide (a volatile weak acid), ammonia is a volatile weak base, and under physiological conditions of temperature and pH, three equally important aspects of its behaviour must be taken into account: the respiratory transport of the dissolved gas form, free  $\text{NH}_3$ ; the acid-base and buffer effects of the  $\text{NH}_3/\text{NH}_4^+$  equilibrium; and the ionic behaviour of the conjugate acid,  $\text{NH}_4^+$ . A number of workers have

attempted to deduce which form accounts for the majority of ammonia movement across gills (Goldstein & Forster, 1961; Pequin, 1962; Pequin & Serfaty, 1963; Goldstein, Forster & Fanelli, 1964; Kormanik & Cameron, 1981; Goldstein, Claiborne & Evans, 1982; Claiborne, Evans & Goldstein, 1982), and others have cited experimental data in support of a proposed  $\text{Na}^+/\text{NH}_4^+$  exchange (Maetz & Garcia-Romeu, 1964; Kerstetter, Kirschner & Rafuse, 1970; Kirschner, Greenwald & Kerstetter, 1973; Payan & Matty, 1975; Evans, 1977; Payan, 1978), yet there have been no determinations of the solubility and  $\text{pK}'$  of the ammonia system in fish plasma, or at the temperatures and ionic strengths employed. Relatively small errors in the logarithmic  $\text{pK}'$  value could make rather large errors in the estimation of  $\text{P}_{\text{NH}_3}$  gradients.

The objective of the present study, then, was to determine the proper  $\text{pK}'$  and solubility values for use in studies of fish; to determine the normal resting values for the various forms of ammonia in the blood of the trout, the normal excretion rate, and the origin of excreted ammonia; and to conduct some experiments on the effects of changes in the ammonia gradient on subsequent ammonia movement.

#### MATERIALS AND METHODS

The experiments were carried out on rainbow trout, *Salmo gairdneri*, which were obtained from a Danish fish hatchery. They ranged from 1200–2000 g in weight, and were maintained at 15 °C on a regular diet of pelleted trout chow. In preparation for the experiments, the fish were anaesthetized quickly in a solution of 1:10 000 MS222, then transferred to an operating table where they were continuously ventilated with water containing 1:16 000 buffered MS222. Cannulae were placed in both the dorsal and ventral aortae, using a slight modification of the method of Soivio, Nyholm & Westman (1975). Polyethylene (PE50 or PE60) catheters with tapered tips were fitted over sharpened stainless steel wires, the wires aiding in insertion of the cannulae into the vessels. After insertion, the wires were withdrawn, and the cannulae advanced approximately 2 cm into the dorsal aorta or 5–8 mm into the ventral aorta. The short pieces inserted were connected to longer cannulae, filled with heparinized saline, and plugged. The ventral aorta was approached at about a 40° angle in the ventral midline, in such a way that the cannula penetrated the ventral aorta near its origin, or occasionally the bulbus arteriosus. The ventral cannula was secured by threading it under the skin for about 3 cm, and leading it out along the anterior margin of the pectoral fin, to which it could be securely tied. The dorsal aorta was approached in the usual manner through the roof of the buccal cavity.

#### *Experimental apparatus*

The chamber for the fish and its associated paraphernalia are shown in schematic form in Fig. 1. The fish were held in a darkened Lucite chamber, through which water was circulated by a pump. Water was passed through an oxygenation column, and also through temperature jackets on the oxygen electrode, as well as through the oxygen electrode's measuring chamber. By means of valves, the oxygenation column could be removed from the circuit, making the system closed to the air.

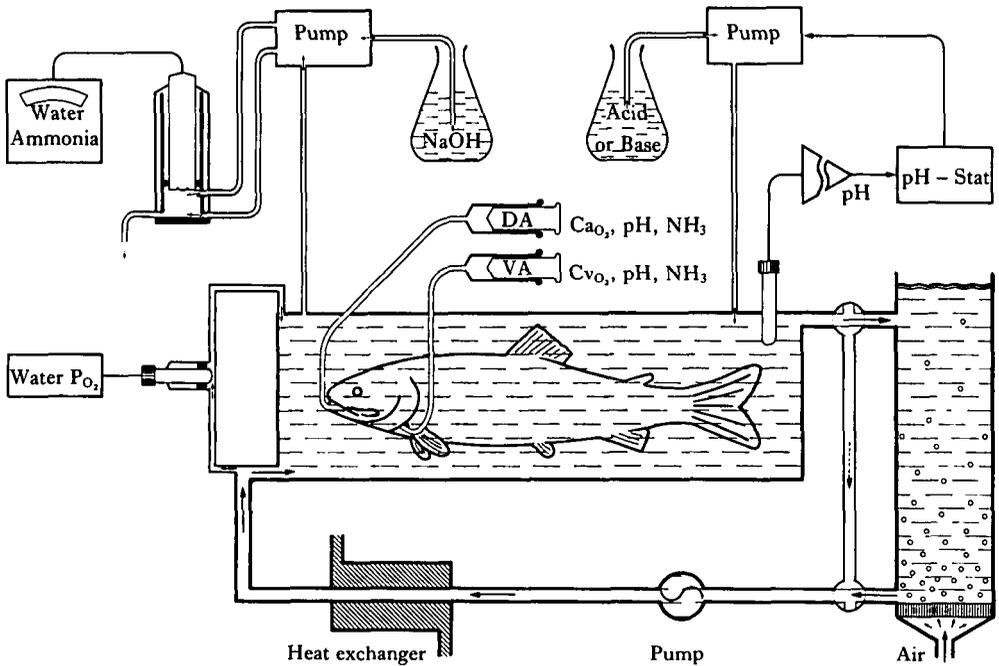


Fig. 1. Experimental apparatus used in the studies on rainbow trout. By use of the three-way valves at the right, the system could be used either as an open system with oxygenation, or as a closed system for measurement of oxygen consumption. The pH sensor, pH-stat and pump were used to control the pH of the water, the second pump (upper left) was used to supply a temperature-jacketed ammonia electrode chamber for measurement of ammonia excretion rates, and the lower pump recirculated the water, as well as supplying the temperature jacket and sensing chamber of the oxygen electrode system. Thermistor probes and a temperature regulator (not shown) controlled a flow of cold water to the heat exchanger in order to maintain the temperature of the system. DA, cannula to dorsal aorta; VA, cannula to ventral aorta.

The concentration of oxygen was maintained at 140 Torr or greater, except when oxygen consumption was being measured. During these intervals, the system was closed, and the rate of decline of oxygen measured either for 10 min, or until the partial pressure fell to 120 Torr. From the volume of the chamber (12–16 l), the temperature, and the oxygen solubility coefficient, the rate of oxygen consumption could be calculated.

Water was drawn from the system at a constant rate of  $1 \text{ ml min}^{-1}$ , mixed with  $0.2 \text{ ml min}^{-1}$  of  $1 \text{ N-NaOH}$ , and led through a temperature-controlled ( $30^\circ\text{C}$ ) chamber containing an ammonia electrode (HNU Systems, Inc.) for continuous measurement of the rate of ammonia excretion by the fish. As previous studies have shown that greater than 97% of the ammonia excretion is branchial, and only a small percentage renal (Smith, 1929; Cameron & Wood, 1978), the contribution of the urine was ignored.

The chamber also contained a pH probe, which was connected to a pH-stat titrator. In practice, the pH was maintained at either  $7.00$  or  $8.00 \pm 0.05$ . At the lower pH, the pH-stat controlled a pump connected to a reservoir of  $0.2 \text{ N-HCl}$ , which was added intermittently to maintain the pH. At the higher pH, the reservoir contained  $0.2 \text{ N-NaOH}$ . A temperature probe, also located in the fish chamber, was connected to a

regulator which controlled the flow of cold water to the heat exchanger (Fig. 1).

### *Protocol*

A minimum of 20 h recovery from surgery was allowed in the fish chamber, with a continuous flow of replacement water, and the temperature controlled at  $15 \pm 0.5^\circ\text{C}$ . The replacement water was then stopped, so that the excretion of ammonia could be measured continuously. Each series of measurements commenced with the switching of the chamber valves to recirculate the water as a closed system. During these short periods, samples were taken from both dorsal and ventral aortae within 1 min of each other for measurement of oxygen content, pH and total ammonia content. Activity of the fish could be observed through a small area in the rear of the chamber. After the short measurements of oxygen consumption and the blood sampling procedure, the chamber was again switched to the oxygenation circuit, and 20–60 min allowed to elapse before the next series of measurements.

When five or six complete series of control measurements had been taken at a water pH of 7.00 and external ammonia below 0.5 mM, the external pH was raised to 8.00 with NaOH, and the ammonia concentration raised to an average of 0.85 mM by addition of  $\text{NH}_4\text{Cl}$ . The sampling and measurement procedure described above was then continued, with samples varying slightly in time, but averaging 0.3, 1.2, 2.2 and 3 h after the change in conditions. At the end of this time, the pH of the water was titrated back to 7.00 with HCl, and the system was flushed with fresh water until the external ammonia concentration fell below 0.05 mM. After an hour at these control conditions, a final set of measurements was carried out. In a few experiments, either external pH was raised to 8.00 without addition of ammonia, or the ammonia concentration was raised without changing the external pH from 7.00.

### *Treatment of blood samples*

During the entire series, 0.5 ml was taken from each vessel at each sample period, and so the total of 10 measurement series required the withdrawal of 10 ml of blood, about 15% of the blood volume of the average weight fish. Immediately after sampling, a 40  $\mu\text{l}$  aliquot was used for the measurement of oxygen content ( $\text{CaO}_2$  or  $\text{CvO}_2$ ) in a galvanic cell oxygen analyser (Lexington Instruments), and an 80  $\mu\text{l}$  aliquot used for the measurement of pH in a thermostatted microelectrode cell (Radiometer-Copenhagen) calibrated with precision phosphate buffers. The remainder of each sample was immediately centrifuged for 1 min, the plasma separated and stored at  $4^\circ\text{C}$  until the ammonia analyses were performed. The total free ammonia in the plasma was assayed with a specific enzymatic assay (Sigma 170B), and all assays were performed within 8 h, usually less.

At the end of some experiments, before killing the fish, as much blood as possible was drawn from the catheters, centrifuged, and the decanted plasma saved for the determinations of  $\text{pK}'$  and solubility of ammonia. The plasma was stored at  $4^\circ\text{C}$ , but not frozen.

### *Physico-chemical measurements*

A special cell was constructed for the determinations of the  $\text{pK}'$  and relative solubility of ammonia in various solutions. The cell accommodated a pH electrode and a

ammonia electrode, a small magnetic stirring bar, inflow and outflow ports, and was constructed so that it could be immersed in a temperature bath. The ammonia electrode (HNU Systems) senses the partial pressure of ammonia gas in solution, aided by a highly permeable, hydrophobic membrane. The pH electrode used was either a low-temperature glass type (Ingold Type 401) or, for higher ionic strengths and pH values, a uranium glass electrode (Ingold Type U402-S7). The pH electrode was calibrated with the National Bureau of Standards phosphate buffer (pH 7.4) and the NBS borate buffer (pH 9.3), using the proper temperature correction values. The ammonia electrode was calibrated with alkalinized  $\text{NH}_4\text{Cl}$  solutions, brought to a pH at least 2 units above the  $\text{pK}'$ , so that >99% existed as free ammonia in the cell. After insertion of the electrodes, the cell volume was measured carefully by weighing, and averaged 17.5 ml.

#### *Determination of $\text{pK}'$*

The determination of  $\text{pK}'$  of ammonia in the following solutions was carried out: 10 mM- $\text{NH}_4\text{Cl}$ , 10 mM- $\text{NH}_4\text{Cl}$  plus 150 mM- $\text{NaCl}$ , 10 mM- $\text{NH}_4\text{Cl}$  plus 500 mM- $\text{NaCl}$ , and plasma. The procedure consisted of filling the calibrated electrode cell with the solution of interest, then titrating the solution with 1 N- $\text{NaOH}$  and/or 1 N- $\text{HCl}$  between pH values of approximately 7.5 to 11.5. The  $\text{pK}'$  was calculated from the  $\text{pH}/P_{\text{NH}_3}$  data pairs, and from the titrant volumes in several ways. The simplest method was to plot the titration curve ( $\text{pH}$  vs  $P_{\text{NH}_3}$ ) and estimate its inflection point by eye. This was generally not very precise, but yielded an estimate within about 0.02 pH units. The second method was to observe the maximum ammonia value at the highest pH, then find the pH value that corresponded to half the maximum ammonia value, since by definition, at the point where  $\text{pH} = \text{pK}'$ , half the ammonia is in the ionized form, and half is free ammonia. A third method was to calculate from each successive data pair the slope (or derivative,  $\Delta\text{NH}_3/\Delta\text{pH}$ ), plot these values as a function of pH, and estimate the peak of the resulting curve, since the first derivative of such titration curves is maximal at the inflection point, or  $\text{pK}'$  value. The fourth method used was that outlined by Bank & Schwartz (1960), of calculating the  $\text{pK}'$  at the points where sufficient titrant had been added to exactly titrate 1/10, 2/10 and so on, of the ammonia present. This approach could not be used for plasma, of course, since other buffers were present. For each of the other solutions, however, all four methods were used, and the results averaged. No series of determinations was used if the various estimates did not agree within 0.02 pH units. Each combination of temperature and ionic strength was tested at least three times.

For determination of the  $\text{pK}'$  in the plasma, the approach was slightly different. The total free ammonia content of the plasma sample was measured, then enough  $\text{NH}_4\text{Cl}$  to bring the total to between 1.0 mM and 1.5 mM was added. The sample was well mixed, then re-assayed so that the total ammonia content was precisely known. The plasma was then introduced into the calibrated electrode cell, and the titration carried out with 1 N- $\text{NaOH}$ . The titration was continued to a pH of 11.2, where about 97.5% of the ammonia is converted to free  $\text{NH}_3$ , which was taken into account in the  $\text{pK}'$  calculations. To make sure that the titration did not have disruptive effects on other plasma components that might have affected the  $\text{pK}'$  determination, the titration was then reversed with 1 N- $\text{HCl}$ . The same titration curve was obtained going up

the pH scale with base and going down with acid. The plasma titration process was carried out three times with different plasma samples, all at 15 °C.

Immediately after the end of each titration, the calibration of both electrodes was checked. For small drifts, corrections were made, but in a few cases where the drift was more than 0.04 pH units or more than 4 % of the ammonia calibration value, the data were discarded.

#### *Relative solubility measurements*

The measurement of solubility was made simple by the nature of the ammonia electrode, which is sensitive to the partial pressure of free ammonia in the solution presented to it. The total content of  $\text{NH}_3$  and partial pressure are related by:

$$C = \alpha \cdot P_{\text{NH}_3},$$

so for two solutions with identical content, the ratio of measured ammonia partial pressures is the inverse of the ratio of their solubility coefficients. The procedure, then, was to calibrate the ammonia electrode with the first solution, titrated to a pH value 2.00 units above its  $\text{pK}'$  (taking ionic strength and temperature into account). The chamber was then drained and filled with a second solution with identical content, but different ionic strength, and the resulting ammonia electrode reading noted. The process was generally repeated several times, alternating the solutions, and the average ratio of ammonia readings was taken as the reciprocal of their solubility ratio. In this way, the relative solubilities of the solutions given above (with 150 mM and 500 mM-NaCl added to 10 mM- $\text{NH}_4\text{Cl}$ ) were determined at 15 °C. The procedure was the same for plasma, using samples that had small amounts of  $\text{NH}_4\text{Cl}$  added to them to bring them to 1 mM- $\text{NH}_4\text{Cl}$ . This plasma was then compared to a solution of 1 mM- $\text{NH}_4\text{Cl}$  in distilled water. The absolute solubilities (Bunsen coefficients) could then be calculated for each solution using values for dilute ammonia solutions from the literature, and the relative solubilities determined as above.

## RESULTS

### *Physico-chemical parameters*

The  $\text{pK}'$  value for ammonia (10 mM) in distilled water was 9.505 at 15 °C, and had a temperature slope of  $-0.0296/^\circ\text{C}$ , which is similar to that reported earlier (Washburn, 1928; Bates & Pinching, 1949). With 150 mM-NaCl added, the value at 15 °C was 9.576, which is also close to the ionic strength effect reported by Bank & Schwartz (1960), and to that predicted by the Debye-Hückel theory. At an added NaCl concentration of 500 mM, the 15 °C value rose to 9.677, and the value for plasma at 15 °C was 9.648, somewhat higher than what would have been predicted on the basis of ionic strength alone. The relationships found between  $\text{pK}'$ , temperature and ionic strength were used to construct a nomogram, which is shown in Fig. 2.

Since a  $\text{pK}'$  value for sea water would have been of some biological interest, some measurements were attempted. The attempts were not successful, however, due to the precipitation of various components of the sea water at high pH values, and the consequently variable ionic strength of the titrated sample. The value almost certainly lies 0.01 or 0.02 of a pH unit above the value for 500 mM-NaCl.

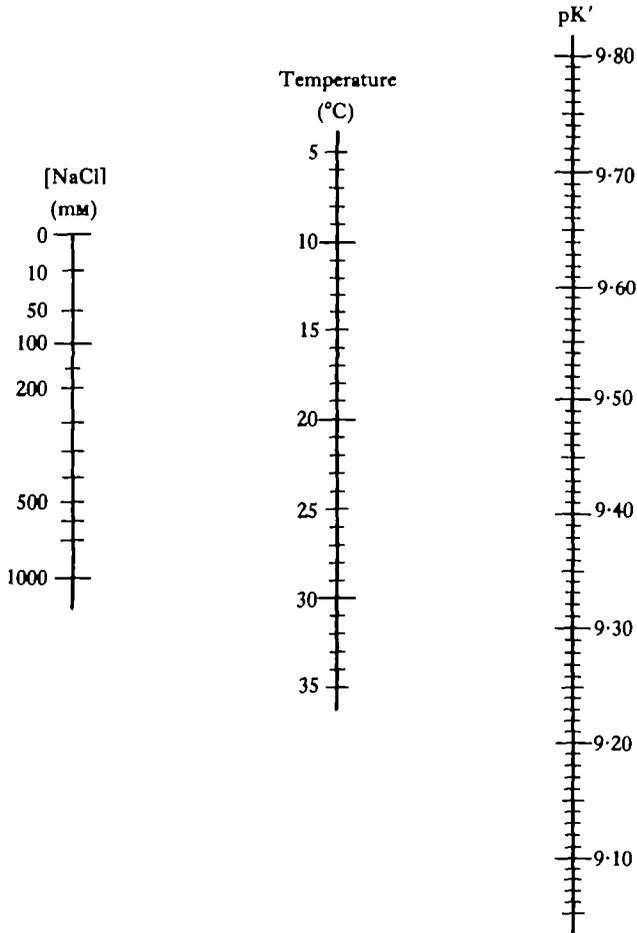


Fig. 2. Nomogram for determination of the  $pK'$  for ammonia in water, from the equation  $pH = pK + \log(NH_3/NH_4^+)$ . (Plasma at 15 °C behaves as if its ionic strength is equivalent to about 370 mM-NaCl.) Straight lines connecting values for sodium chloride concentration and temperature lead to the respective  $pK'$ .

Table 1. *Relative solubility of ammonia in various solutions at 15 °C, taking the value for 10 mM-NH<sub>4</sub>Cl in distilled water as the 100 % value*

|                 |         |
|-----------------|---------|
| Distilled water | 100.0 % |
| + 150 mM-NaCl   | 103.9 % |
| + 500 mM-NaCl   | 105.4 % |
| Plasma          | 103.4 % |
| Plasma water*   | 107.7 % |

\* Assuming a 4 % protein volume correction.

The relative solubility values for the solutions tested and for plasma are given in Table 1, taking the 10 mM-NH<sub>4</sub>Cl value as 100 %. Most respiratory gases are 'salted out', but as the data show, ammonia is salted in slightly, reaching a maximum of 5.4 %

Table 2. Table of solubility values of ammonia, using Bunsen coefficients given by Washburn (1928) and the relative solubilities from Table 1

| Temperature | Water<br>(l Torr <sup>-1</sup> ) | Water<br>(mmol Torr <sup>-1</sup> ) | Plasma<br>(mmol Torr <sup>-1</sup> ) |
|-------------|----------------------------------|-------------------------------------|--------------------------------------|
| 0           | 1.460                            | 66.093                              | 68.34                                |
| 10          | 1.174                            | 53.146                              | 54.95                                |
| 15          | 1.050                            | 47.533                              | 49.15                                |
| 20          | 0.933                            | 42.236                              | 43.67                                |
| 25          | 0.822                            | 37.211                              | 38.48                                |
| 30          | 0.719                            | 32.549                              | 33.66                                |

Table 3. Mean blood parameters from control measurements taken from nine fish ( $\bar{x} \pm$  s.e.)

|   |   |
|---|---|
| CaO <sub>2</sub>                            | 9.10 ± 0.79 vol%                                    |
| CvO <sub>2</sub>                            | 5.42 ± 0.61 vol%                                    |
| pHa   | 7.729 ± 0.029                                       |
| pHv   | 7.751 ± 0.027                                       |
| CaNH <sub>3</sub>                           | 0.177 ± 0.023 mmol l <sup>-1</sup>                  |
| CvNH <sub>3</sub>                           | 0.321 ± 0.049 mmol l <sup>-1</sup>                  |
| M <sub>NH<sub>3</sub></sub>                 | 0.333 ± 0.054 mmol kg <sup>-1</sup> h <sup>-1</sup> |
| M <sub>O<sub>2</sub></sub>                  | 61.3 ± 5.2 ml kg <sup>-1</sup> h <sup>-1</sup>      |
| Q̇ (from O <sub>2</sub> ) (cardiac output)  | 34.1 ± 3.8 ml kg <sup>-1</sup> min <sup>-1</sup>    |
| Q̇ (from NH <sub>3</sub> ) (cardiac output) | 43.4 ± 7.3 ml kg <sup>-1</sup> min <sup>-1</sup>    |

higher solubility in 500 mM-NaCl. Using these values for relative solubility and the Bunsen coefficients given by Washburn (1928), values for solubility in water and plasma in various units are given in Table 2 for temperatures between 0°C and 30°C.

#### Control measurements in vivo

The mean control values for arterial and venous pH, oxygen content and ammonia are given in Table 3. Also shown are the mean values for ammonia excretion and oxygen consumption, as well as the cardiac output ( $\dot{Q}$ ) values calculated from both ammonia and oxygen data. The values for  $\dot{Q}$  were obtained from the Fick equation:  $\dot{M} = \dot{Q} \cdot (C_a - C_v)$ , and were calculated from the mean data for each fish, rather than the grand means given in Table 3. If all the excreted ammonia originates from the free ammonia carried in the venous blood, then the two estimates of cardiac output should agree. Stated another way, the clearance (F) calculated as:

$$F = 100 \cdot [\dot{Q}_{O_2} \cdot (C_{vNH_3} - C_{aNH_3}) \cdot 60] / \dot{M}_{NH_3}$$

where  $\dot{Q}_{O_2}$  is in ml min<sup>-1</sup> and  $\dot{M}_{NH_3}$  in ml h<sup>-1</sup>, should equal 100%. Using the mean data from Table 3, a value of F = 88.5% is obtained. The same calculation was also performed for the mean data from each individual fish, and the mean ( $\pm$  s.e.) of these calculations was 86.9 ± 8.3%. This means that, within the limits of experimental error, approximately 88% of the ammonia excreted originates by clearance from the venous blood. Assuming that 2–3% is excreted *via* the kidneys (Cameron & Wood, 1978), this leaves some 9–10% which may originate in the gill tissue itself. The

Uncertainty of this calculation must not be ignored, however, since six independent measurements must be made simultaneously in order to make the calculation.

It has also to be considered that plasma ammonia concentrations have been used for these calculations instead of blood concentrations. However, since the twofold higher intraerythrocyte  $\text{NH}_4^+$  concentration (assuming a pH in the cells of  $\sim 0.3$  units lower than in plasma, equilibrium of  $\text{NH}_3$ , and little  $\text{NH}_4^+$  diffusion across the cell membrane) is at least partially compensated by the smaller water content of erythrocytes ( $\sim 2/3$ ), and since the red blood cell volume is small in fish, the above calculations are affected to only a minor extent, resulting in an overestimate of the gill tissue contribution by only about 2–4% of the total ammonia production.

#### *The effects of increased external pH and ammonia*

The results of five experiments on the effects of high pH (8.00) and external ammonia (0.85 mM) are shown in Fig. 3. After the usual recovery period, control data for 2–4 h are shown at the left side of each panel. At the time marked zero, the water conditions were changed as indicated at the top, maintained for 3 h, then returned to the control condition (pH = 7.00 and external ammonia less than 0.1 mM). The total ammonia concentrations of both venous and arterial blood rose rapidly during the first 30 min, then more slowly over the remainder of the period. There was a rapidly developing alkalosis in the blood, which was more pronounced on the arterial side than the venous and which appeared to be declining at the end of the third hour (Fig. 3, middle panel). Employing the  $\text{pK}'$  values from Fig. 2 and the solubility values for blood and water in Table 2, the partial pressures of free  $\text{NH}_3$  were calculated, and are shown in the lower panel of Fig. 3. Although the  $P_{\text{NH}_3}$  of the water was raised to about 550  $\mu\text{Torr}$ , the blood levelled off at just over 200  $\mu\text{Torr}$ , and there was thus a reversed  $P_{\text{NH}_3}$  gradient during the entire 3-h treatment period. Upon return to the control water conditions, the blood parameters rapidly returned to normal (Fig. 3, right-hand side).

#### *The effects of ammonium salt infusion*

Since some recent work has demonstrated the acid-base effects of ammonium salt injection or infusion (Cameron & Kormanik, 1982), and since such experiments have been cited in the past as evidence for  $\text{Na}^+/\text{NH}_4^+$  exchange, e.g. by Maetz & Garcia-Romeu (1964), we duplicated in two fish the approximate conditions of their 1964 goldfish experiment. The fish were fitted with dorsal aortic and intraperitoneal catheters, a series of control measurements was carried out in the usual manner, and then 2.5 mmol  $\text{kg}^{-1}$  of  $(\text{NH}_4)_2\text{SO}_4$  (= 5 mequiv  $\text{kg}^{-1}$   $\text{NH}_4^+$ ) was injected into the intraperitoneal catheter. The measurements of arterial pH and ammonia content were then continued.

The results of this experiment are shown in Fig. 4. In both fish there was a severe and almost immediate acidosis, accompanied by a very large rise in arterial ammonia concentration, to over 2 mM. The acidosis was fatal to one fish in 45 min (triangles), but produced a 0.45 unit pH depression in the other which had not been fully compensated after 22 h. Upon autopsy, the latter fish was found to have pulled the intraperitoneal catheter part way out, so that the infusion was largely into the muscular abdominal wall. Evidently the absorption of the load was more rapid when

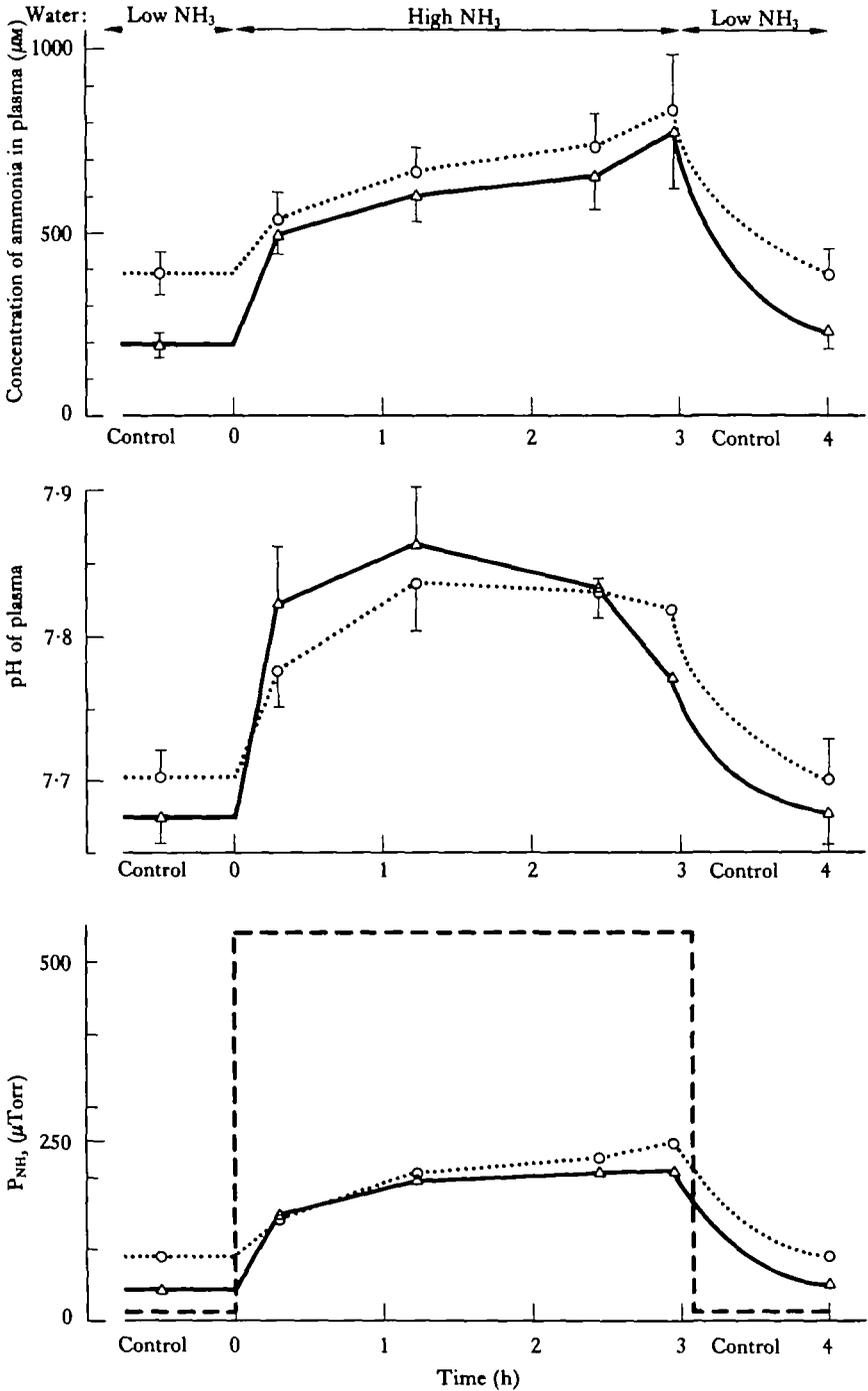


Fig. 3. The effects of increased external pH and ammonia on total ammonia concentration, and ammonia partial pressures in plasma and water, as well as on plasma pH. At the top, the water treatment periods are shown, where the 'Low NH<sub>3</sub>' period represents a water pH of 7, and an average ammonia concentration of less than 0.16 mM and the 'High NH<sub>3</sub>' represents a water pH of 8, and total ammonia averaging 0.85 mM. The solid line and open triangles represent arterial data, the dotted line and open circles venous data, and the dashed line (bottom panel) water. The error bars shown are  $\pm 1$  s.e.,  $N = 5$ .

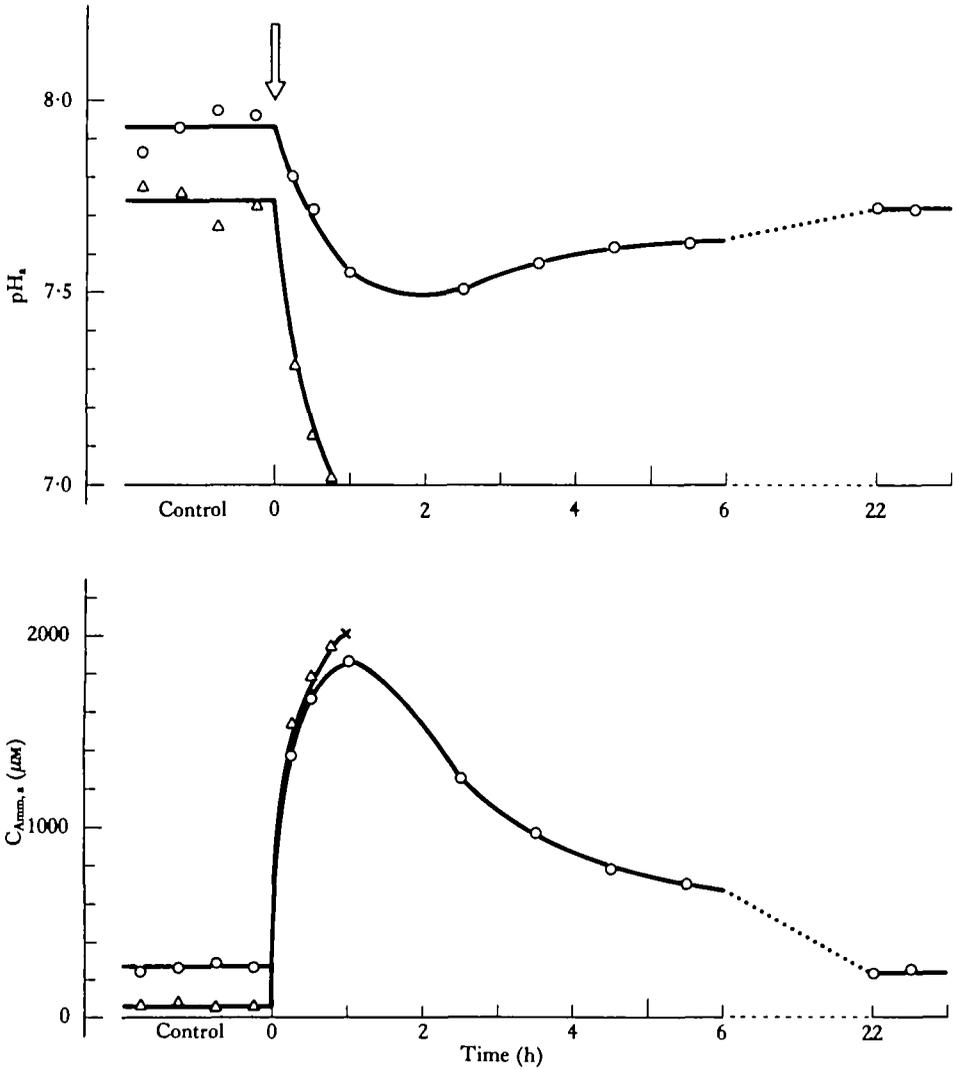


Fig. 4. Plasma total ammonia concentration ( $C_{Amm,a}$ ) and pH ( $pH_s$ ) of arterial blood before and after intraperitoneal infusion of  $2.5 \text{ mmol kg}^{-1}$  of  $(\text{NH}_4)_2\text{SO}_4$  in two rainbow trout. The fish represented by the triangles died 45 min after the infusion. The other probably received most of the infusion into the abdominal muscle wall.

fully injected into the peritoneum. These results were similar to those obtained from catfish with  $\text{NH}_4\text{Cl}$  infusions (Cameron & Kormanik, 1982), and the experiments were not continued.

DISCUSSION

*pK' and solubility*

The determination of the  $pK'$  values at various temperature and ionic strength

combinations was relatively straightforward and error-free, and produced a satisfactory agreement with other data from the literature. For example, our value reported here of 9.505 agrees well with Bates & Pinching's (1949) value of 9.564 for  $pK'$  at 15°C, taking into account the difference in ionic strength. In the calculation of  $P_{NH_3}$  gradients between blood and water in fresh water, failure to take into account the  $pK'$  difference of approximately 0.14 – due to the difference in ionic strength – would lead to a 34% over-estimate of the partial pressure of the blood.

The  $pK'$  value obtained for plasma (9.645 at 15°C) was higher than expected on the basis of ionic strength. Plasma, however, is far from an ideal solution, and there are many possible interactions between the ammonium ion and components of plasma which might alter its activity in solution, and hence the  $pK'$  observed. Weak complexes with other ions, and electrical interaction with protein charge centres are just two possibilities.

The increase in the solubility of ammonia ( $NH_3$ ) with increasing ionic strength was somewhat surprising, since most gases, and all the common respiratory gases, are 'salted out' at increasing ionic strength. There are, however, other examples of 'salting in' (Wilhelm, Battino & Wilcock, 1977), and the data were consistent. Nevertheless, our results show far less salting in than does the earlier study of Jacquez, Poppell & Jeltsch (1959), and do not agree with Maetz' (1973) use of a plasma-to-water solubility ratio of 1.75 to 1. We can find no compelling explanation for the large difference between our study and that of Jacquez *et al.* (1959), but we note that if there were non-reversible reactions with various plasma components during the long equilibration of blood at higher than normal ammonia concentrations, they would have obtained anomalously high apparent solubilities with their method of determination. Our method of measuring the partial pressure (effectively) with an electrode at normal physiological concentrations over short time periods may yield a more reliable estimate.

Only a few sample calculations are required to show that without accurate values for the  $pK'$ , the solubility, and both blood and water pH, it is futile to attempt to calculate the  $NH_3$  gradients across the gills of fish from the concentration difference. Errors of 0.1 for blood pH, 0.15 in the  $pK'$ , and 10% in solubility can produce a cumulative error of nearly 100%. Since this information has not been available until now, the importance of the  $NH_3$  gradient in ammonia excretion may be regarded as an open question.

#### *Normal resting values and the question of origin*

In Smith's (1929) studies of ammonia excretion in fishes, he assumed that ammonia originated in the various metabolizing tissues, and was cleared across the gills from blood entering on the venous side. Some years later, Goldstein and his colleagues (Goldstein & Förster, 1961; Goldstein *et al.* 1964) made the suggestion that some significant portion of the excreted ammonia might arise *de novo* in the gill tissue itself by deamination of amines, possibly glutamine. They attempted to measure the clearance of blood ammonia by estimating cardiac output, and measuring the a-v difference in ammonia, but the methods available at that time for estimation of cardiac output from non-catheterized fish (terminal cardiac puncture) could only produce inconclusive results. Pequin & Serfaty (1963) also addressed this question.

(and did not find much *de novo* production), but their methods for estimation of cardiac output were also quite approximate.

In the present study we show that most, if not all, of the ammonia excreted can be accounted for on the basis of a-v clearance, but there may be 5-8% that originates in the gill tissue. Since six independent measurements must be made simultaneously in order to calculate ammonia clearance (arterial and venous O<sub>2</sub> content, arterial and venous ammonia content, oxygen consumption and ammonia excretion), statistical analysis does not permit a more precise statement, nor is it likely that this approach will yield an improved estimate in the future. Our estimate of 5-8% of ammonia originating in the gills would yield a figure (from Table 3) of 16-27  $\mu\text{mol kg}^{-1} \text{h}^{-1}$ , which is not too different from Payan & Matty's (1975) estimate of 41  $\mu\text{mol kg}^{-1} \text{h}^{-1}$  'basal' ammonia production in perfused trout gills.

The normal a-v difference in ammonia concentration will have a slight effect on blood pH, which does not seem to have received notice before. Given a buffer value of about -12 mequiv per pH unit, the 0.144 mM difference in ammonia concentration should make the arterial pH approximately 0.012 units lower than the venous. This small difference is not likely to be detected under normal circumstances.

#### *Ammonia permeability*

In order to test the reasonableness of assuming ammonia movement by NH<sub>3</sub> diffusion, we may make that assumption, and see whether the calculated permeability is within reasonable limits. From the Fick equation:

$$\dot{M}_{\text{NH}_3} = \frac{-A \cdot D \cdot \alpha \cdot P_{\text{NH}_3}}{d},$$

where:  $\dot{M}_{\text{NH}_3} = -2.07 \times 10^{-3} \text{ cm}^3 \text{ kg}^{-1} \text{ s}^{-1}$  (present study),  $A = 2000 \text{ cm}^2 \text{ kg}^{-1}$  (Hughes, 1966),  $\alpha = 1.086 \text{ cm}^3 \text{ cm}^{-3} \text{ Torr}^{-1}$  (present study),  $d = 0.0006 \text{ cm}$  (Hughes, 1966), and  $P_{\text{NH}_3} = 54.4 \mu\text{Torr}$  (present study). Solving for D yields a value of  $1.05 \times 10^{-5}$ , compared to Washburn's values of  $1.7 \times 10^{-5}$  for CO<sub>2</sub> and  $1.9 \times 10^{-5}$  for O<sub>2</sub> at 18°C (cited in Randall, 1970). Our estimate is probably low, since about 20% of the secondary lamellar surface is underlain by pillar cells (Farrell, Sobin, Randall & Crosby, 1980), and therefore not part of the effective surface area (A), which would increase the value of D to  $1.31 \times 10^{-5}$ ; perfusion of only part of the secondary lamellae would increase the estimate further. The aqueous diffusion coefficients for NH<sub>3</sub> and CO<sub>2</sub> are nearly equal (Radford, 1964), and since diffusion through tissue is essentially a process of diffusion in aqueous solution, the finding that the permeability coefficients of CO<sub>2</sub> and NH<sub>3</sub> are similar may make it more reasonable to assume that ammonia can move across the gills in the free NH<sub>3</sub> form.

These results are in agreement with recent work on crabs and catfish (Kormanik & Cameron, 1981; and unpublished data), in which the flux of ammonia was found to be affected by changes in the NH<sub>3</sub> gradient, and in trout, where analysis of net ion movements between fish and environmental water during recovery from muscular activity indicated that a large proportion of the ammonia production was released by non-ionic diffusion (Holeton, Neumann & Heisler, 1983). The results are also consistent with work on the mammalian kidney (Pitts, 1973) and on single cells (Jacobs, 1940; Roos & Boron, 1981) which shows that tissues are generally quite permeable to



define a point on the upper right quadrant, which yields the partial pressure gradient from blood to water in units of  $\mu\text{Torr}$ . The open circles, for example, represent resting data from Fig. 3. The venous and arterial partial pressures have been averaged, and the average taken together with the partial pressure in the water defines a gradient value on the upper right quadrant,  $54 \mu\text{Torr}$ . Similarly, the closed circles are the 3-h data from Fig. 3, and show the reversed gradient of  $-300 \mu\text{Torr}$ .

*The effects of increased external ammonia*

Although the experiment depicted in Fig. 3 appears quite simple, and is similar to others reported on in the literature (e.g. Maetz, 1973), the interpretation of the results is actually quite complex. In the bottom panel, it is clear that the partial pressure of  $\text{NH}_3$  in the blood, though perhaps not quite at steady state after 3 h, is levelling off at less than half the partial pressure of  $\text{NH}_3$  in the water. In view of the rapid rise in the first 30 min, the explanation of this as a transient lag is not satisfactory. The prevailing gradients under these conditions are shown in Fig. 5 (filled circles), and assuming, again, that ammonia will diffuse along its partial pressure gradient (i.e. the permeability coefficient does not change), an inward diffusion of approximately  $1930 \mu\text{mol kg}^{-1} \text{h}^{-1}$  is predicted.

A hypothetical schematic model to attempt to account for the data of Fig. 3 is shown

Model of ammonia distribution in trout

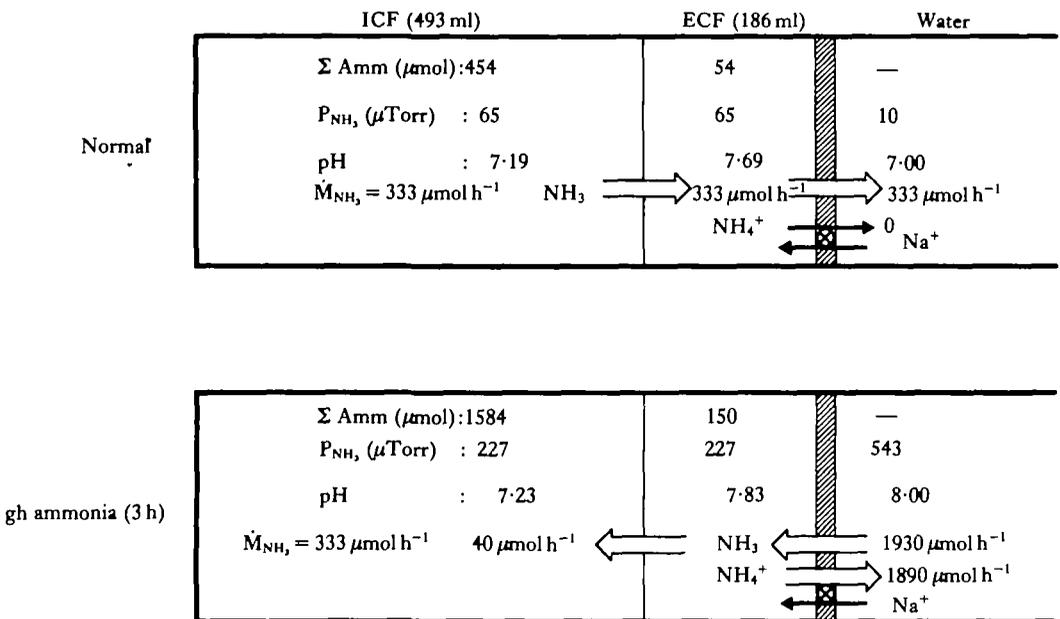


Fig. 6. A hypothetical model of ammonia distribution and movement based on data from Fig. 3. The ICF and ECF compartments are drawn roughly to scale of their relative volumes for a 1 kg fish (values for catfish, Cameron & Kormanik, 1982), with the gill tissue (hatched, right) separating the ECF from a water compartment of infinite volume. The principal assumptions are that ammonia production in tissues is constant, that ammonia movement across the gills is proportional to the  $\text{NH}_3$  gradient, and that only diffusion of  $\text{NH}_3$  takes place during the control period. For a complete description, see text.

in Fig. 6. In the upper portion, a three-compartment model of ammonia distribution has been constructed for the control conditions, with the relative volumes of ICF and ECF taken from a recent study of the freshwater catfish (Cameron, 1980). The total amount of ammonia in each of the internal compartments has been calculated from the concentration (control data, Fig. 3) and compartment volume, employing the assumption that the permeability of  $\text{NH}_3$  is much greater than that of  $\text{NH}_4^+$  (Jacobs, 1940; Pitts, 1973), and that it therefore distributes according to the difference in pH between the ICF and the ECF, much as the distribution of a weak acid such as DMO is calculated (Waddell & Butler, 1959; Roos & Boron, 1981). We further assumed that the  $P_{\text{NH}_3}$  was equal in ICF and ECF, and assumed a difference in pH of 0.5 units between ICF and ECF (Cameron, 1980; Heisler, 1980). Under the assumptions for the steady state control condition, the ammonia production of  $333 \mu\text{mol kg}^{-1} \text{h}^{-1}$  passes from ICF through the ECF to the water *via*  $\text{NH}_3$  diffusion, and the  $\text{Na}^+/\text{NH}_4^+$  exchange pathway is not needed.

In the lower panel, we have attempted to account for the data in Fig. 3 by assuming that the ammonia production rate is not changed (the oxygen consumption did not change), and that the ICF, being better buffered, would not experience as severe an alkalosis as the ECF (see Fig. 2, centre). Under these conditions, the inward diffusion of  $\text{NH}_3$ , given above, must be nearly balanced by extrusion of  $\text{NH}_4^+$  *via* the  $\text{Na}^+/\text{NH}_4^+$  pathway. The new quantity of ammonia in the ICF, calculated from the measured ECF concentration and the pH differential, is accounted for by the intrinsic production plus only a small net movement from water through ECF into the ICF ( $40 \mu\text{mol h}^{-1}$ ). The  $\text{NH}_4^+/\text{Na}^+$  exchange must then account for a net extrusion of  $1890 \mu\text{mol h}^{-1}$  in order to balance the compartments. Passive movement of  $\text{NH}_4^+$  would not help explain the results, since the gradient for  $\text{NH}_4^+$  is also directed inward (both electrically and chemically).

Two additional lines of evidence from the experiments support the model proposed in Fig. 6. One is that although the net ammonia excretion or uptake is very difficult to measure in the face of the large background concentration during high external ammonia treatment, there was consistently a small decrease in the external concentration of ammonia during the 3 h of treatment. As an aside, it is difficult to understand some reported measurements of ammonia excretion during high external ammonia treatment; one paper (Maetz, 1973) reports excretion rates of around  $30 \mu\text{mol h}^{-1}$  with a background concentration of nearly 7 mM, which implies an analytical accuracy for ammonia of better than 0.1%. The second line of evidence is that during the control treatment, a continual input of small amounts of acid by the pH-stat system was necessary to maintain pH. This is customarily the case, since the  $\text{H}^+$  ions are required to balance the bicarbonate which, in almost equimolar quantities, is either released together with  $\text{NH}_4^+$ , or is produced by ionization of  $\text{NH}_3$  after non-ionic diffusion into the water, when the fish is in acid-base steady state (Cameron, 1980; Heisler, 1980, 1982). During the high ammonia treatment, however, constant infusions of NaOH were required to maintain the pH, which is consistent with inward diffusion of  $\text{NH}_3$  and a stimulated uptake of  $\text{Na}^+$  in exchange for  $\text{NH}_4^+$ . In effect, then, there appears to be an  $\text{H}^+$  shuttle operating, with  $\text{NH}_3$  diffusing inwards, and  $\text{NH}_4^+$  being transported outwards, resulting in a net alkalosis in the fish (Fig. 3, middle panel). A quantification of the net  $\text{H}^+$  transfers and  $\text{Na}^+$  fluxes during control

and high external ammonia treatment would no doubt be a critical test for these hypotheses.

#### *Effects of high internal ammonia*

In numerous studies, the injection or infusion of ammonium salts has been used as one test for the presence of  $\text{Na}^+/\text{NH}_4^+$  exchange, the reasoning being that stimulation of  $\text{Na}^+$  uptake under these conditions provides support for the exchange (cf. Maetz & Garcia-Romeu, 1964; Evans, 1977). In recent work on catfish, however, it was clear that the infusion of  $\text{NH}_4\text{Cl}$  produced a severe acidosis (Cameron & Kormanik, 1982), and that the stimulation of a  $\text{Na}^+/\text{H}^+$  exchange by the acidosis was an equally viable interpretation. We repeated the experiment of Maetz & Garcia-Romeu (1964) simply to show that the infusion of the sulphate salt, as well as the chloride salt, does cause a substantial acidosis, and that there is no *a priori* reason to suppose that it is the  $\text{NH}_4^+$  rather than the  $\text{H}^+$  excess that in fact stimulates  $\text{Na}^+$  uptake. What these experiments do show unequivocally is that at least some substantial portion of the injected or infused ammonium salt must be excreted as  $\text{NH}_3$ ; otherwise no acidosis would result. This point seems to have been missed by some earlier workers (e.g. Payan & Maetz, 1973) although  $\text{Na}^+/\text{H}^+$  exchange was originally suggested by Krogh (1938) and supported by Kerstetter *et al.* (1970).

The study of ammonia in fish must take into account all three of its modes of behaviour in physiological solutions: its behaviour as a weak acid buffer, its behaviour as a respiratory gas, and its behaviour as an ion. In the trout under normal resting conditions of low external concentration and low partial pressure of ammonia, the diffusive permeability of the gills to ammonia may be adequate to account for the observed excretion rates. Most of the excreted ammonia appears to originate upstream from the gills and to be simply cleared during gill passage as a respiratory gas. The slight acid-base effects of the  $a-v$  ammonia difference are not normally detectable. Under abnormal experimental conditions of high external ammonia concentration and partial pressure of ammonia, the net inward movement of  $\text{NH}_3$  down its diffusive gradient must be balanced by an active extrusion mechanism, presumably a  $\text{Na}^+/\text{NH}_4^+$  ion exchange activated by the high internal ammonia. The activation of this ion exchange contributes significantly to the alkalosis already caused by increasing ammonia.

While our experiments do not prove that normal ammonia movement is by diffusion of  $\text{NH}_3$ , we have provided information crucial to the calculation of  $\text{NH}_3$  gradients, and have shown that a reasonable estimate of  $\text{NH}_3$  permeability is given by a model which assumes movement by  $\text{NH}_3$  diffusion. The acidosis that accompanies ammonium salt infusion and the alkalosis that accompanies high external ammonia are complicating factors in the interpretation of these experiments, which are usually interpreted as showing  $\text{Na}^+/\text{NH}_4^+$  exchange. In both cases, both diffusive movements of  $\text{NH}_3$  and exchange transport of  $\text{NH}_4^+$  are probably important.

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