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Ammonia movement and distribution after exercise across white muscle cell membranes in rainbow trout

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Ammonia movement and distribution after exercise across white muscle cell membranes in rainbow trout. Am. J. Physiol. 271 (Regulatory Integrative Comp. Physiol. 40): R738-R750, 1996.—Manipulations of pH and electrical gradients in a perfused preparation were used to analyze the factors controlling ammonia distribution and flux in trout white muscle after exercise. Trout were exercised to exhaustion, and then an isolated-perfused white muscle preparation with discrete arterial inflow and venous outflow was made from the posterior portion of the tail. The tail-trunks were perfused with low (7.4)-, medium (7.9)-, and high (8.4)-pH saline, achieved by varying HCO$_3$ concentration ([HCO$_3$]) at constant P$_{CO_2}$. Intracellular and extracellular pH, ammonia, CO$_2$, K$^+$, Na$^+$, and Cl$^-$ were measured. Muscle intracellular pH was not affected by changes in extracellular pH. Increasing extracellular pH caused a decrease in the transmembrane NH$_3$ partial pressure (P$_{NH_3}$) gradient and a decrease in ammonia efflux. When extracellular K$^+$ concentration was increased from 3.5 to 15 mM in the medium-pH group, a depolarization of the muscle cell membrane potential from -92 to -60 mV and a 0.1-unit depression in intracellular pH occurred. Ammonia efflux increased despite a marked reduction in the P$_{NH_3}$ gradient. Amiloride (10$^{-4}$ M) had no effect, indicating that Na$^+/H^+$ NH$_+_1$ exchange does not participate in ammonia transport in this system. A comparison of observed intracellular-to-extracellular ammonia distribution ratios with those modeled according to either pH or Nernst potential distributions supports a model in which ammonia moves as NH$_3$ according to NH$_3$ partial pressure (P$_{NH_3}$) gradients and a decrease in ammonia efflux.

In mammals, ammonia movement and distribution across muscle cell membranes traditionally follow the theory of nonionic diffusion (20); i.e., ammonia moves as NH$_3$ according to NH$_3$ partial pressure (P$_{NH_3}$) gradients and therefore distributes according to transmembrane pH gradients, because NH$_3$ permeability (P$_{NH_3}$) is so much higher than NH$_+_1$ permeability (P$_{NH_+_1}$; 13, 17). As illustrated in Fig. 1A, this will lead to distribution ratios (intracellular to extracellular) of ~4.0. However, several recent studies have questioned this hypothesis because of the finding of significant disagreement between the measured muscle to plasma distribution ratio for ammonia and that predicted by the pH gradient (9, 10). In fish muscle, the situation is also unclear (24, 34). Most experimental studies (27, 29, 38, 39), but not all (19), have indicated that intracellular ammonia levels after exercise are far higher than explicable by a pH-driven distribution, but very close to those predicted by a Nernst distribution between intracellular (ICF) and extracellular fluid (ECF), assuming a muscle cell membrane potential ($E_m$) in the range of -60 to -100 mV. As illustrated in Fig. 1B, this will result in a distribution ratio of ~30.0. These studies suggest that the influence of $E_m$ on NH$_+_1$ is the dominant factor governing transmembrane distribution, so that PNH$_+_1$ must be appreciable. However, this concept has been strongly criticized on theoretical grounds (11), most notably because an “NH$_+_1$ shuttle” could result in an inwardly directed flux of H$^+$ into the cells (38), creating an intolerable load for the intracellular pH (pH$_i$) regul-
Higher pH extracellular fluid, respectively. ApHi+, change in intracellular to extracellular pH; [ThJi, nia, respectively; E,, membrane potential; ICF, ECF, intra- and extracellular fluid. A: scenario when membrane is only permeable to NH₃. Note very different distribution ratios that arise. B: scenario when membrane also has a significant permeability to NH₄. The situation is further complicated by the finding of Tang et al. (27) on postexercise trout in vivo that, despite an ammonia distribution between ICF and ECF apparently in accordance with in vivo studies. In particular, the preparation is characterized and employed to block the possible involvement of Na⁺/H⁺-NH₄ carriers.

**MATERIALS AND METHODS**

**Experimental Animals**

Rainbow trout (500–600 g) were obtained from Spring Valley Trout Farm (Petersburg, Ontario) and then raised for 2–6 mo in a 800-liter fiberglass cylindrical tank with flowing dechlorinated Hamilton tap water (in meq/l: 0.6 Na⁺, 0.8 Cl⁻, 1.8 Ca²⁺, 0.5 Mg²⁺, 0.04 K⁺; pH 8.0, temperature 5–12°C) until the desired size (800–1,000 g) was reached. During this period the fish were fed with high-protein trout grower floating pellets (Aquaculture Zeigler Brothers) three times a week. Before the experiment, fish were acclimated to 15 ± 1°C for 5–7 days without feeding to standardize metabolic status. Exercised fish were used in this perfusion study to elevate muscle ammonia levels that naturally occur via adenylate breakdown. Before perfusion, the fish were manually chased for 6 min to exhaustion in a 150-liter cylindrical tank (29). Immediately on cessation of exercise, the trout was transferred to a dark acrylic box containing 8 liters aerated water and anesthetized with MS-222 (0.5 g/l neutralized with NaOH). This resulted in loss of equilibrium and cessation of ventilation within 1 min without struggling. This method has been proven to cause minimum metabolic and acid-base disturbances (26, 30). The tail was cut off at the level of the anus and weighed before perfusion. The unconscious fish was killed immediately after the tail was removed.

**Experimental Protocols**

**Experimental design and perfusate preparation.** This study was designed to investigate how changes in pH and electrical gradients between ICF and ECF of muscle affect transmembrane ammonia flux and distribution. Cortland salmonid saline with 3% bovine serum albumin (BSA; fraction V, Sigma) was used as the basic perfusate. Ammonia already present in the distilled water and component salts was sufficient to provide normal resting arterial levels of total ammonia (Tₐₐ₉₉, ~50 μmol/l) in the perfusate (27, 29, 30, 39). Pco₂ was kept constant at ~2 Torr to represent the typical resting value of trout arterial blood in vivo (29). NaHCO₃ levels were adjusted accordingly to manipulate perfusate pH, in different treatment groups (Table 1). Changes in the pH gradient were achieved by altering perfusate pH, while the electrical gradient was depressed by elevating perfusate potassium concentration ([K⁺]) from the normal level of 3 mEq/l to 15 mEq/l.

**Table 1. Expected inflow saline pH, Pₐ₉₉, HCO₃⁻, and K⁺ in the four experimental treatment groups**

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>pH</th>
<th>Pₐ₉₉, Torr</th>
<th>HCO₃⁻, mM</th>
<th>K⁺, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low pH</td>
<td>7.4</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Medium pH (control)</td>
<td>7.9</td>
<td>2</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>High pH</td>
<td>8.4</td>
<td>2</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td>High K⁺ (depolarized)</td>
<td>7.9</td>
<td>2</td>
<td>7</td>
<td>15</td>
</tr>
</tbody>
</table>
The study comprises four treatment groups (6-11 fish per group): low, medium, and high pH, and high potassium (at medium pH), as shown in Table 1. The medium-pH group was used as the control group, with normal pH (~7.9), which mimics resting trout arterial plasma pH (29). The low-pH group (pH ~7.4) simulates the typical trout arterial plasma pH after exhaustive exercise (29). The high-pH group (pH ~8.4) was chosen to create an elevated transmembrane pH gradient, which could occur under some circumstances such as high environmental pH (31). The pH and HCO₃ concentration ([HCO₃⁻]) of the high-K⁺ group ([K⁺] ~15 mM) were kept at the control level. Changes in saline [HCO₃⁻] were achieved by reciprocal changes in NaHCO₃ and NaCl so as to maintain Na⁺ concentration ([Na⁺]) constant in various treatment groups. However, in the high-K⁺ group, because 12 mmol more KCl was added to elevate the perfusate K⁺ level, the NaCl level was reduced correspondingly to avoid introducing excessive Cl⁻ into the saline.

On the basis of the results of these studies, it was apparent that one of the drug treatments used in our parallel study on lactate and metabolic acid transport in the perfused postexercise trunk preparation (Wang et al., unpublished results) could provide useful information on ammonia transport. Methods were identical to that of the low-pH treatment, except that 10⁻⁴ M amiloride HCl (Sigma) was present in the perfusate throughout the second 30 min of perfusion. Perfusate and tissue samples from these trunk (n = 11) were analyzed for all ammonia-relevant parameters; these data are presented in the current study.

**Perfusion and sampling.** Immediately after the tail trunk was severed from the body and weighed (10-15 g), catheters (Clay-Adams PE-90 tubing) were implanted to a depth of 1.5-2.0 cm into the caudal artery and caudal vein and secured by a ligature in a deep incision around the vertebral column. This deep insertion and ligation ensured that there was no leakage from the caudal vessels themselves and, furthermore, prevented perfusion of segmental arteries close to the plane of section, thereby minimizing leakage through the cut surface. With this technique, all of the venous outflowing perfusate that was collected from the caudal vein passed through the arterial-to-venous circuit of the preparation. Therefore, venous perfusate represented the true outcome of perfusion. The terminal muscle samples (see below) were also obtained from the area away from the cut surface where muscle tissue was fully perfused.

Perfusion was started immediately at 2 ml·min⁻¹·100 g tail wt⁻¹ with Cortland saline plus BSA of the appropriate pH and [K⁺]. The saline was heparinized with 50 IU/ml sodium heparin (Sigma) to prevent blood clotting. The tail trunk was submerged in a temperature-controlled saline bath (15 ± 0.5°C) during the entire perfusion period, while the perfusate was pumped through a heat-exchange coil in a 15°C water bath. Sampling ports were placed in arterial and venous catheters to allow subsequent collection of inflowing and outflowing perfusate samples, respectively, and the outflow of the venous catheter was set to the level of the saline bath, i.e., zero pressure.

The perfusion preparation setup is shown schematically in Fig. 2. The perfusate was gassed with 0.25% CO₂, balance O₂, bath. Sampling ports were placed in arterial and venous catheters to allow subsequent collection of inflowing and outflowing perfusate samples, respectively, and the outflow of the venous catheter was set to the level of the saline bath, i.e., zero pressure.
Gas Mixing Pump

Oxygenator

Peristaltic Pump

15 °C Water bath

Windkessel

Chart Recorder

Pressure transducer

Arterial sample port

Venous sample port

SDR Production

analysis. For perfusate Cl− measurements, undiluted perfusate was used. Both Na+ and K+ were determined with flame atomic absorption spectrometry (Varian AA-1275), whereas Cl− was analyzed by coulometric titration (Radiometer CMT10).

Perfusate pH was determined with the same acid-base apparatus used for muscle pH. Perfusate PO2 was measured at 15°C with a Radiometer PO2 electrode (E5046) connected to a Cameron Instrument OM-200 oxygen meter. Perfusate TCO2 was determined on a Cameron Instrument Capn-Con total CO2 analyzer (model II). PCO2 and [HCO3−] were calculated by manipulation of the Henderson-Hasselbalch equation using appropriate constants (aCO2 and pK′) for rainbow trout true plasma at 15°C (3). Total protein and water content of saline was measured with an American Optical refractometer.

The perfusate T↓Amn content was measured by the glutamate dehydrogenase assay using a Sigma kit. Along with tissue T↓Amn analysis, these enzymatic measurements were conducted on an LKB UltraspecPlus 4053 spectrophotometer.

**Perfusate buffer capacity.** The nonbicarbonate buffer capacities (β) of each of the four perfusates were determined. In brief, perfusate samples (4 ml) were placed in tonometer vessels (Instrumentation Laboratories 287) and equilibrated with 1, 2, 4, and 8 Torr PCO2, balance O2, for 20 min at 15°C, then analyzed for pH and TCO2 with aforementioned methods. [HCO3−] of the samples were calculated, and β in various treatment groups was determined from the slope of the regression of HCO3− against pH

\[
\beta = \frac{\Delta[HCO_3^-]}{\Delta pH}
\]  

Calculations and Statistics

The perfusion pressure has been expressed as net inflow pressure exerted on the tail trunks only, calculated as the difference between the perfusion pressure with and without the tail trunks in the system (i.e., correcting for cannula resistance). Inasmuch as the perfusion rate (2 ml·100 g tail wt−1·min−1) was constant in each preparation, the perfusion pressure reflected the resistance of the tail trunk.

The ECF volume (ECFV, ml/g) and ICF volume (ICFV, ml/g) of white muscle in this perfusion preparation were estimated by [H2O], and Na+, K+, and Cl− concentrations of the muscle and ECF (venous perfusate water in this case) using the “Cl−-K+ space” approach of Conway (5)

\[
ICFV = [H_2O]_i - ECFV
\]
ECFV = \frac{[K^+]_t[Cl^-]_t - ([H_2O]_t)[Cl^-]_t[K^+]_t}{[K^+]_e[Cl^-]_e + [Cl^-]_e[K^+]_e - 2[H_2O]_e[Cl^-]_e[K^+]_e} \tag{3}

where the subscripted \( t \) and \( e \) are the concentrations in whole muscle tissue (mmol/kg for ions, ml/g for \( H_2O \)) and in ECF (mmol/l perfusate \( H_2O \)), respectively. The estimate of ECFV was made on the basis of the assumption that ions in ECF have been fully equilibrated with venous perfusate after 60 min of perfusion.

The ICF ions and \( T_{\text{Amn}} \) concentrations (\( [S]_i \)) were calculated as

\[ [S]_i = \frac{[S]_e - [S]_e \times \text{ECFV}}{\text{ICF}} \tag{4} \]

where the subscripted \( i \) is the concentration in ICF, and \( S \) represents ions or \( T_{\text{Amn}} \).

Oxygen consumption (\( M_{O_2} \)) and \( CO_2 \) efflux were calculated with the use of the Fick principle from the perfusion rate and the differences of gas content between inflow and outflow perfusate, e.g.

\[ \text{Mo}_2 (\text{mmol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}) = \text{perfusion rate} \times \alpha_{O_2} \times \Delta P_{O_2} \tag{5} \]

where \( \Delta P_{O_2} \) is \( O_2 \) partial pressure difference between arterial and venous perfusate, and \( \alpha_{O_2} \) (1.77 mmol·1·Torr⁻¹) is the solubility coefficient of \( O_2 \) at 15°C in water of appropriate ionic strength to match the perfusate (3). An analogous equation, with the corresponding \( \Delta CO_2 \) values substituted for \( \text{O}_2 \times \text{O}_2 \), was applied in the CO₂ efflux calculation.

Ammonia, as a metabolic substrate and an end product, exists as an anion, a respiratory gas, and a weak base (pK = 9.7 at 15°C). \( T_{\text{Amn}} \) is the sum of the ionic and nonionic forms of this substance

\[ T_{\text{Amn}} = [NH_4^+] + [NH_3] \tag{6} \]

\[ P_{NH_3} = \alpha_{NH_3} \tag{7} \]

where \( \alpha_{NH_3} \) is taken from Cameron and Heisler (4).

At physiological pH (6.0–8.0), according to the following equation

\[ \text{pH} = \text{pK} + \log \frac{[NH_3]}{[NH_4^+]} \tag{8} \]

over 98% of ammonia exists as \( NH_4^+ \), with pK again from Cameron and Heisler (4). If the muscle cell membrane is permeable only to \( NH_3 \) and \( P_{NH_3} \) is in equilibrium across the membrane, then the total ammonia distribution will be a function of the transmembrane pH gradient only

\[ \frac{T_{\text{Amn}}}_{\text{ICF}} = \frac{1 + 10^{pK - pH_s}}{1 + 10^{pK - pH}} \tag{9} \]

Therefore, the relatively lower pH, will trap more \( NH_4^+ \) in the ICF (Fig. 1A).

However, if the cell membrane is permeable only to \( NH_3 \), ammonia will be distributed according to membrane Nernst potential (\( E_{\text{nm}} \)) only

\[ E_{\text{nm}} = \frac{RT}{Z^F} \ln \frac{[NH_4^+]_e}{[NH_4^+]_c} = \frac{RT}{Z^F} \ln \frac{T_{\text{Amn}}}_e - [NH_3]_c \tag{10} \]

where \( R, T, Z, \) and \( F \) are the gas constant, the absolute temperature, the valence, and Faraday’s constant, respectively. Under equilibrium conditions, the negatively charged ICF will trap a great deal more \( NH_4^+ \) (Fig. 1B) than if pH were governing the distribution. The \( E_{\text{nm}} \) of the white muscle was estimated from the measured intra- and extracellular concentrations of \( K^+, Na^+, \) and \( Cl^- \) according to the Goldman-Hodgkin-Katz equation

\[ E_{\text{nm}} = \frac{RT}{Z^F} \ln \frac{PK^+[K^+]_{\text{ICF}} + PNa^+[Na^+]_{\text{ICF}} + PCl^-[[Cl^-]_{\text{ICF}}}{PK^+[K^+]_{\text{ECF}} + PNa^+[Na^+]_{\text{ECF}} + PCl^-[[Cl^-]_{\text{ECF}}} \tag{11} \]

where \( PK^+ \), \( PNa^+ \), and \( PCl^- \) are relative permeability coefficients, from Hodgkin and Horowicz (12).

Under the situation where the cell membrane has significant permeability to both ionic and nonionic forms of ammonia, \( T_{\text{Amn}} \) distribution will be a function of both transmembrane pH and Eₙ gradients. According to Boron and Roos (2)

\[ \frac{T_{\text{Amn}}}_{\text{ICF}} = \frac{[NH_4^+]_e + K}{[NH_4^+]_c + K} \tag{12} \]

If the pHₙ gradient and the \( E_{\text{nm}} \) are known, then the \( [T_{\text{Amn}}]_{\text{ICF}}/\text{[T}_{\text{Amn}}]_{\text{ECF}} \) ratio will be a function of the permeability ratio \( P_{NH_3}/P_{NH_4^+} \) as outlined by Wright et al. (38) and Wood et al. (37).

All data are reported as means ± SE. Significant differences between means in the four different perfusate groups were evaluated by one-way analysis of variance (ANOVA). If the ANOVA indicated significance \( (P < 0.05) \), then post hoc comparison by means of Duncan’s multiple-range and critical-range test \( (P < 0.05) \) was performed, with reference to the medium-pH group as the control. Student’s paired t-test was used to evaluate differences within treatment groups between 30- and 60-min values. Simple unpaired t-tests were used to evaluate differences between the low-pHₙ treatment and the low-pHₙ plus amiloride treatment.

### RESULTS

#### Condition of the Perfused Tail-Trunk Preparation

The perfusion pressure started at ~15 cm\( H_2O \) and, thereafter, slowly declined by ~5 cm\( H_2O \) as the perfusion proceeded for the first 30 min. However, this trend was reversed during the second 30 min, such that perfusion pressures at 60 min were 16–19 cm\( H_2O \).
Table 3. \( \text{[H}_2\text{O}]_{\text{t}} \), ICFV, and ECFV after 60 min of perfusion in the four experimental treatment groups

<table>
<thead>
<tr>
<th></th>
<th>Low pH (Control)</th>
<th>Medium pH (Control)</th>
<th>High pH</th>
<th>High K(^+) (Depolarized)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[\text{H}<em>2\text{O}]</em>{\text{t}}]</td>
<td>0.795 ± 0.005</td>
<td>0.784 ± 0.001</td>
<td>0.779 ± 0.002</td>
<td>0.769 ± 0.009</td>
</tr>
<tr>
<td>ICFV</td>
<td>0.719 ± 0.005</td>
<td>0.703 ± 0.008</td>
<td>0.698 ± 0.008</td>
<td>0.735 ± 0.012*</td>
</tr>
<tr>
<td>ECFV</td>
<td>0.076 ± 0.005</td>
<td>0.081 ± 0.008</td>
<td>0.080 ± 0.008</td>
<td>0.034 ± 0.016*</td>
</tr>
<tr>
<td>(n)</td>
<td>7</td>
<td>11</td>
<td>8</td>
<td>6</td>
</tr>
</tbody>
</table>

Values are means ± SE for each treatment group (ml/g wet tissue); \[\text{H}_2\text{O}]_{\text{t}}\), white muscle tissue water content; ICFV, intracellular fluid volume; ECFV, extracellular fluid volume. *Significantly different from corresponding control values.

Table 4. Intracellular \( \text{Na}^+ \), \( \text{K}^+ \), and \( \text{Cl}^- \) concentrations in trout white muscle after 60 min of perfusion in the four experimental treatment groups

<table>
<thead>
<tr>
<th></th>
<th>Low pH (Control)</th>
<th>Medium pH (Control)</th>
<th>High pH</th>
<th>High K(^+) (Depolarized)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[\text{Na}^+]</td>
<td>154.7 ± 8.8</td>
<td>165.8 ± 5.2</td>
<td>163.8 ± 5.9</td>
<td>142.5 ± 1.7</td>
</tr>
<tr>
<td>[\text{K}^+]</td>
<td>3.08 ± 0.12</td>
<td>2.96 ± 0.16</td>
<td>2.94 ± 0.12</td>
<td>14.05 ± 0.71</td>
</tr>
<tr>
<td>[\text{Cl}^-]</td>
<td>3.60 ± 0.22*</td>
<td>3.52 ± 0.19*</td>
<td>3.29 ± 0.10</td>
<td>12.40 ± 0.76*</td>
</tr>
</tbody>
</table>

Values are means ± SE for each treatment group (mmol/l perfusate water). Subscript \(a\) and \(v\), arterial and venous, respectively. *Significantly different from corresponding arterial values.

There were no significant differences in pressure between the four groups at either 30 or 60 min (Table 2). There was no visible red color in the outflow perfusate after 20 min of perfusion, indicating thorough washout of red blood cells. There were also no detectable differences in protein concentration (~3%) between inflowing and outflowing perfusate at any time. The \[\text{H}_2\text{O}]_{\text{t}}\) of each treatment group after 60 min perfusion (Table 3) was not significantly different from that of initial muscle samples (0.786 ± 0.016 ml/g wet tissue, \(n = 32\), pooled total of 4 groups) and was, therefore, not affected by the experimental treatments. However, the high-K\(^+\) treatment caused a marked redistribution of internal fluid volumes, resulting in a 58% decrease in ECFV and a 5% increase in ICFV (Table 3). Similarly, white muscle ICF \(\text{Na}^+\), \(\text{K}^+\), and \(\text{Cl}^-\) showed no significant changes as pH\(_a\) varied, but Na\(^+\) and Cl\(^-\) were altered markedly in response to high-K\(^+\) perfusion (Table 4). ICF \(\text{Na}^+\) and \(\text{Cl}^-\) increased by 35 and 280%, respectively, relative to the control group, whereas K\(^+\) remained unchanged despite the large increase of ECF \(\text{K}^+\) (≈15 mM; Tables 4 and 5). It was not possible to compare these values with ICFV and ICF ion concentrations before perfusion because ECF measurements required for the intracellular calculations were not obtained when the initial muscle samples were collected. However, the ICFV and ECFV values of the three pH treatment groups, as well as the intracellular ion concentrations after 60 min perfusion, were comparable to those of our previous in vivo study on exercised trout (29).

There were no significant differences of [Na\(^+\)] or [Cl\(^-\)] between inflow and outflow perfusate (arterial-venous (a-v) difference) in any of the three pH treatment groups after 60 min of perfusion (Table 5). Despite the large increases in ICF [Na\(^+\)] and [Cl\(^-\)] in the high-K\(^+\) treatment, there were again no significant a-v differences in these two ions. However, [K\(^+\)], increased by almost 20% relative to [K\(^+\)]\(_a\) in both the low-pH\(_a\) and the medium-pH\(_a\) (control) groups, indicating a net efflux of K\(^+\) at 60 min (Table 5). This did not occur in the high-pH\(_a\) treatment. In contrast, the high-K\(^+\) group exhibited a 10% decline in [K\(^+\)]\(_a\), relative to [K\(^+\)]\(_a\), indicating a net uptake of K\(^+\) at 60 min (Table 5). The trend for lower absolute ECF [Cl\(^-\)] with increasing pH in the three pH treatments and the lower absolute ECF [Na\(^+\)] in the high-K\(^+\) treatment was due to the original makeup of the salines (see MATERIALS AND METHODS).

\(E_m\) was not affected by perfusate acid-base status and averaged about ~90 mV in the three pH treatments (Table 6). The increase in perfusate [K\(^+\)] from 3 to 15 mM in the high-K\(^+\) treatment caused the intended partial depolarization (35%), reducing white muscle \(E_m\) to about ~59 mV (Table 6). In this group, slight muscle twitching was observed at the start of perfusion with high K\(^+\) saline. In all treatments, \(E_m\) remained unchanged when calculated with respect to either the arterial or venous ECF perfusate compositions (Table 6).

Typically, there was a 250- to 350-Torr decrease in \(P_{O_2}\) from the arterial to the venous perfusate, and \(O_2\) uptake remained relatively stable over the course of perfusion in all four treatment groups (Table 7). The \(O_2\) supply did not seem to be exhausted, because venous \(P_{O_2}\) (>75 Torr) was far from being depleted. \(O_2\) uptake
Table 7. In- and outflowing perfusate $P_{O_2}$, $O_2$ consumption, and $CO_2$ efflux rate of the tail-trunk preparation after 60 min perfusion

<table>
<thead>
<tr>
<th></th>
<th>Low pH</th>
<th>Med pH (Control)</th>
<th>High pH (Depolarized)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_{O_2}$, Torr</td>
<td>321.4 ± 24.0*</td>
<td>399.0 ± 16.3</td>
<td>413.8 ± 14.0</td>
</tr>
<tr>
<td>$P_{O_2}$, Torr</td>
<td>85.0 ± 10.4</td>
<td>115.6 ± 22.8</td>
<td>105.5 ± 12.1</td>
</tr>
<tr>
<td>$MO_2$, mmol·kg⁻¹·h⁻¹</td>
<td>0.48 ± 0.03*</td>
<td>0.60 ± 0.03</td>
<td>0.66 ± 0.03</td>
</tr>
<tr>
<td>$CO_2$ efflux, mmol·kg⁻¹·h⁻¹</td>
<td>1.45 ± 0.02*</td>
<td>1.20 ± 0.15</td>
<td>0.69 ± 0.23*</td>
</tr>
</tbody>
</table>

Values are means ± SE for each treatment group. *Significantly different from corresponding control values.

Table 7 shows that $P_{O_2}$ and $CO_2$ efflux were significantly affected by pH treatment. Low pH treatment resulted in a 20% decrease in $P_{O_2}$ compared to the control, while high pH treatment caused a 70% increase in $CO_2$ efflux. These changes were consistent with the predicted effects of pH on muscle metabolism and were in agreement with the pH data obtained from the trunk and intracellular fluid.

Acid-Base Status

The intended differences in pH among treatments were achieved (Fig. 3, see Table 1), and nonbicarbonate buffer capacities (β) of perfusate used in the four treatment groups were not significantly different. The overall mean value was 5.54 ± 0.15 mM [HCO₃⁻] pH/unit. As arterial pH (pHₐ) increased from 7.4 to 8.4, the a-v difference ($ΔpH_{av}$) also increased from 0.13 to 0.34 units (Fig. 3). However, the substantial differences in pHₐ among treatments (−1.0 unit between high- and low-pH perfusates) had no effect on muscle pHₐ, which remained at about 6.6 in this postexercise preparation. Therefore, the overall transmembrane pH gradient ($ΔpH_{m}$) varied linearly with pHₐ, from 0.70 at low pHₐ to 1.50 at high pHₐ (Fig. 3). It should be noted that pHₐ measured in initial muscle samples taken immediately before the start of perfusion was 6.596 ± 0.026 (n = 30) and therefore identical to the 6.6 measured after 60 min in the three pH treatments.

In contrast to the pH treatments, the 35% reduction of the muscle cell Eₘ induced by higher extracellular [K⁺] resulted in a significant depression in pHₐ to approximately 6.4 (Fig. 3). However, the overall $ΔpH_{m}$ (1.20) did not change in comparison to the control group ($ΔpH_{m}$ = 1.16). Venous pH also fell significantly, so $ΔpH_{mv}$, almost doubled relative to the control group.

pH Effects on Ammonia Distribution and Efflux

Intracellular ammonia concentration ($T_{Amnl}$) was ~9,000 μM in this postexercise preparation, and there were no significant differences among the three pH treatment groups after 60 min of perfusion (Fig. 4). This may be compared with an initial value of 7,712 ± 355 μM (n = 30) measured in initial muscle samples taken before the start of perfusion. Thus there was a significant depression in pHₐ to ~6.4 with perfusion.
small but significant increase in $T_{Amn,i}$ over the course of the 60 min perfusion.

Compared with the control group, there was a 47% decrease in venous ammonia ($T_{Amn,v}$) in the high-$pH_e$ group but no significant change in $T_{Amn,v}$ in the low-$pH_e$ group (Fig. 4). In accord with the unchanged $T_{Amn,i}$ and $pH_e$, there were no significant differences in intracellular $P_{NH_3}$ among the three $pH$ treatment groups; intracellular $P_{NH_3}$ averaged ~150 μTorr (Fig. 5). Nevertheless, as extracellular $pH$ increased, transmembrane $P_{NH_3}$ gradients (intracellular to venous) dropped from 132.7 to 88.4 μTorr (Fig. 5). This decreased transmembrane $P_{NH_3}$ gradient was mainly due to the elevation of venous $P_{NH_3}$ induced by higher $pH_e$.

In parallel to the depressed transmembrane $Γ_{NH_3}$ gradient, as $pH_e$ increased, $T_{Amn}$ efflux tended to decrease (Fig. 6). The high-$pH_e$ treatment caused a significant 47% decrease in $T_{Amn}$ efflux relative to the control group, whereas the 12% increase at low-$pH_e$ was not significant (Fig. 6).

The measured transmembrane $T_{Amn}$ ratio ($T_{Amn,i}/T_{Amn,e}$) was calculated relative to the venous end, a conservative approach maximizing the potential for equilibration. The ratio increased markedly as $pH_e$ increased (Fig. 7). The lower venous $T_{Amn}$ (Fig. 4) contributed primarily to the higher ratio. For comparison, the transmembrane $pH$ gradients at the venous end were used to estimate the $T_{Amn}$ distribution ratios (Eq. 9) assuming that the white muscle membrane is effectively permeable only to $NH_3$. It is clear that $T_{Amn}$ ratios calculated in this manner from the $pH$ gradients were far from matching the measured ratios and indeed greatly underestimated them in all three $pH$ treatment groups (Fig. 7). Nonetheless, as transmembrane $pH$ gradient increased, the $pH$ estimated ratio increased in parallel with the elevation in the measured $T_{Amn}$ ratio (Fig. 7). The absolute differences between the measured and the $pH$ estimated ratios remained almost unchanged as $pH_e$ increased.

**E, Effects on Ammonia Distribution and Eflux**

The membrane depolarization caused by the increase in extracellular $K^+$ did not alter $T_{Amn,i}$ significantly (Fig. 4) but caused substantial elevations in both $T_{Amn,v}$ (Fig. 4) and $T_{Amn}$ efflux (Fig. 6) relative to the control.
To look at the matter from another perspective, Nernst potentials were calculated from the measured intra- and extracellular [NH₄⁺] concentrations on the assumption that the muscle cell membranes are permeable only to NH₄⁺ (Eq. 10). The NH₄⁺ estimated Nernst potentials agreed closely with recorded \( E_m \) values in the low-pH₆ and medium-pH₆ treatments, but were significantly more negative than \( E_m \) in the high-pH₆ and high K⁺ treatments (Fig. 8). In general, these findings agree with the analysis based on distribution ratios (Fig. 7).

### Amiloride Effects on Ammonia Distribution and Efflux

In general, the \( T_{\text{Amm}} \) distribution ratios predicted from \( E_m \) by Eq. 10, assuming that the white muscle cell membrane was effectively permeable only to \( \text{NH}_4^+ \), agreed with the observed distribution ratios much better than did those predicted from pH gradients by Eq. 9, assuming effective permeability to \( \text{NH}_3 \) (Fig. 7). This agreement was strongest in the low-pH₆ treatment. We therefore evaluated whether amiloride (10⁻⁴ M), an inhibitor of both \( \text{Na}^+/\text{H}^+/-\text{NH}_4^+ \) exchange and \( \text{Na}^+ \) channels at this concentration (1), influenced the distribution and efflux of ammonia in the low pH₆ treatment.

Amiloride had no significant effect on pH gradients or \( E_m \) in the preparation (data not shown). Amiloride also had no significant effect on either the measured \( T_{\text{Amm}} \) distribution ratio or the measured \( T_{\text{Amm}} \) efflux rate (Table 8).

### DISCUSSION

**Evaluation of the Tail-Trunk Perfusion Preparation: Comparison With in Vivo Studies**

The present perfused trout trunk preparation is similar to that of Moen and Klungsoyr (18). In contrast to whole trunk preparations (16, 28, 33), only the postanus region of the tail was perfused, thereby avoiding involvement of the kidney and allowing discrete collection of venous outflow from the caudal vein. In this region, white muscle makes up ~90% of the total soft tissue volume (8), the balance being mainly red muscle in a discrete band under the lateral line. The preparation therefore facilitates the measurement of metabolite fluxes between white muscle and ECF, but cannot preclude small contributions from red muscle. The perfusion flow rate of 2 ml·min⁻¹·100 g tail wt⁻¹ was chosen as a compromise between \( O_2 \) delivery requirements and measurement accuracy for \( \Delta T_{\text{Amm}} \) and other metabolites between inflow and outflow.
flow. This flow rate is about twice the blood flow rate to white muscle recorded during aerobic exercise in trout, or about three- to fourfold resting flow rates (23, 32). The only estimates of white muscle blood flow in trout after exhaustive exercise of the type used here are those of Neumann et al. (21), who reported a 1.5-fold increase from resting levels. Perfusion pressure was somewhat lower than in vivo levels, reflecting the absence of sympathetic tone in the preparation.

In preliminary tests, we found that the use of 3% HSA to provide colloid osmotic pressure was a major improvement relative to previous preparations employing polyvinylpyrrolidone or dextran as an oncotic agent (16, 18, 28, 33); vascular reactivity was sustained, edema did not occur, and the preparation exhibited fluid volumes and intracellular ion, pH, and T_Amm levels all comparable with those of our in vivo study on exhaustively exercised trout (29). The rate of MO2 (Table 7) was ~25% of that measured in vivo on whole trout at rest or ~10% of that seen after exhaustive exercise (36). In view of the low metabolic rate of white muscle relative to aerobic tissues such as liver and gills, these figures seem quite reasonable. Certainly, the relatively high venous PO2 (70-120 Torr; Table 7) indicated that O2 supply was not compromised. In our preliminary tests, when the perfusion rate was reduced to one-third of the above rate or the arterial PO2 was decreased to ~200 Torr, venous PO2 could be depressed to as low as 10-20 Torr. This also suggests that the O2 delivery to the preparation was far from insufficient.

Acid-Base Status

pH in the perfused trunk preparation (~6.6; Fig. 3) was very similar to that measured in the white muscle of exhaustively exercised trout in vivo and may be compared with resting values of ~7.2 both in vivo (25, 27, 29, 36) and in vitro (16). As extracellular "respiratory" acid-base status was maintained constant at a resting level of arterial PCO2 in the postexercise tail-trunks, the acidotic pH was due to intracellular "metabolic acidosis" (low HCO3−, confirmed Wang et al., unpublished results). This was undoubtedly due to II+ generation from lactate production and ATP breakdown during exercise as documented in many studies, such as those cited above. The significantly lower pH in the high K+ group was likely caused by additional muscle twitching induced by membrane depolarization; these trunks had higher intracellular lactate levels.

In the present experimental design, changes in extracellular acid-base status were achieved by purely "metabolic" adjustments (i.e., changes in HCO3−) at constant PCO2 (Table 1), whereas in vivo after exhaustive exercise, both factors change: increased PCO2 and decreased HCO3−. White muscle pH remained constant at the postexercise level independent of extracellular HCO3− and pH, in both this in vitro study (Fig. 3) and in trout in vivo infused with a large dose of HCO3− after exhaustive exercise (27). These results suggest that metabolic acid-base disturbance in the ECF has minimal influence on intracellular acid-base status in white muscle, in contrast to the well-documented influence of ECF respiratory acid-base disturbance on muscle pH, both in vitro (16) and in vivo (35).

Ammonia Distribution and Efflux

Assuming that passive movements of NH3 and NH4+ are the only routes by which ammonia can be released from muscle cells, then changes in P_NH3 gradients should dictate movements of the former, and changes in the net electrochemical gradients for NH4+ should dictate the latter. Our experimental design manipulated the former by changing pH (Fig. 3) and the latter by changing E_m (Table 6). The results show clearly that both factors had a significant influence on T_Amm efflux from the preparation. Thus, in the absence of changes in E_m (Table 6), T_Amm efflux decreased as pH increased (Fig. 6), and therefore the P_NH3 gradient decreased (Fig. 5). Conversely, T_Amm efflux increased with a partial depolarization by high K+ (Fig. 6; Table 6), despite a marked reduction in the P_NH3 gradient. Specifically, a 47% decrease in efflux at high pH relative to medium pH was associated with an apparent 27% decrease in the P_NH3 gradient from ICF to venous perfusate. Conversely, a 77% increase in efflux in the high-K+ treatment was associated with a 203% increase in the electrochemical gradient for NH4+ and a 67% decrease in the P_NH4+ gradient. Although both NH3 and NH4+ movements are clearly important, it is impossible to calculate the exact contribution of each component without knowledge of exactly how the gradients are distributed along the arterial to venous pathway in muscle capillaries and whether the absolute permeabilities change.

An alternative approach to the same general question is to examine the intracellular to extracellular distribution ratio of T_Amm (34, 37, 38), with the use of basic principles elaborated by Boron and Roos (2). With the use of Eq. 10, if P_NH3 predominates, then a relatively high [T_Amm]i/[T_Amm]e dictated by E_m will result (see Fig. 1B); whereas if P_NH4+ predominates, by Eq. 9, a relatively low [T_Amm]i/[T_Amm]e dictated by the pH-e-pHc gradient will result (e.g., Fig. 1A). These ratios have fixed values for any fixed values of pH-e-phc and E_m, but will change when pH-e-phc and E_m vary. In the situation where both permeabilities are significant, the distribution ratio will be intermediate between the asymptotes set by pH-e-phc (low value of the ratio) and by E_m (high value of ratio; see Fig. 1 of Ref. 34 for a graphical representation of the relationship). The exact value will be dictated by the exact value of P_NH3/P_NH4+ as outlined in Eq. 12. In absolute terms P_NH4+ does not have to quantitatively exceed P_NH3 for the distribution ratio to be dictated by E_m. Because there is so much more NH3 than NH4+ in solution at physiological pH, even if P_NH3 is only 10% of P_NH4+ (i.e., P_NH4+ ~ 10), then the ratio will approach the asymptote dictated by E_m. It is important to note that this analysis assumes steady-state conditions and that all ammonia is freely diffusible.

Application of this approach to the present data set revealed several interesting features (Fig. 7). First, it
reinforces the conclusion that both the pH gradient, acting on NH$_3$ distribution, and E$_m$, acting on NH$_4^+$ distribution, are important in setting the distribution of T$_{Amn}$. Thus as pH$_r$ increased at constant E$_m$, the measured distribution ratio increased in parallel to the pH-predicted distribution ratio. Conversely, as E$_m$ was lowered by partial depolarization by high K$^+$, the measured distribution ratio decreased in parallel to the E$_m$ predicted ratio. These changes occurred although the ratios predicted by the reciprocal controlling factors did not change significantly. Second, at least at low pH$_r$ and medium pH$_m$, the observed ratios were not significantly different from the E$_m$-predicted values, but remained far above the pH-predicted values. Third, under all conditions, the measured distribution ratios were greater than the maximum ratios predicted by E$_m$, although these differences were only significant in the high-pH$_r$ and high-K$^+$ treatments (Fig. 7). This situation precluded calculation of P$_{NH_3}$/PNH$_4^+$ from Eq. 12.

This latter situation can only occur if steady-state conditions were not achieved and/or if some of the intracellular ammonia was not freely diffusible. Both explanations are possible. Although the tail-trunk was perfused for 1 h before measurements were made and calculation was based on the distribution ratios with respect to the venous outflow, it remains possible that disequilibrium persisted, especially if intracellular ammonia production was continuing. This is suggested by the fact that T$_{Amn}$ actually increased over the 60 min of perfusion. Recent data (summarized in Fig. 9) from our study (R. Henry, Y. Wang, and C. M. Wood, unpublished results) on perfused tail-trunks from resting trout cast some light on the situation. In that investigation, ammonia released from resting trunks was negligible, suggesting that production did not occur, and the measured distribution ratio was far below the maximum value predicted by E$_m$. Alternately or additionally, there is considerable evidence from mammalian studies that a portion of intracellular ammonia may be protein bound or otherwise compartmentalized (see Ref. 38 for a detailed discussion) and therefore not available for free diffusive exchange. An additional explanation might be that ammonia is actively transported into the ICF; however amiloride had no effect. Local pH gradients, which can be different from bulk gradients, may also complicate the analysis.

Amiloride was tested in the low-pH$_r$ treatment where the observed distribution ratio most closely matched that expected from the effect of E$_m$ on NH$_4^+$. The complete lack of effect of this drug (Table 8) at a concentration (10$^{-4}$ M) that should block both Na$^+$/H$^+$-NH$_4^+$ exchangers and Na$^+$ channels (1) suggests that carrier mediation of this type is not involved in the movement of NH$_4^+$ across the white muscle cell membranes.

**Comparison With in Vivo Studies**

Overall, the present results indicating an ammonia distribution after exhaustive exercise close to that predicted by E$_m$, at least under the physiologically realistic condition of low pH$_r$, agree well with a number of investigations on exhaustively exercised teleost fish in vivo (27, 29, 38, 39). All of these studies have indicated that ammonia is distributed between white muscle ICF and ECF approximately according to E$_m$ and not according to pH$_r$-pH$_m$ after exercise; i.e., P$_{NH_3}$ effectively predominates. Nevertheless, just as in the present study, artificially raising pH$_r$ by HCO$_3^-$ infusion after exhaustive exercise in vivo markedly increased the distribution ratio, inasmuch as more T$_{Amn}$ accumulated in the ICF and less appeared in the blood plasma (27). Therefore, both in vivo and in vitro, P$_{NH_3}$ is sufficiently large relative to P$_{NH_4^+}$ after exhaustive exercise that ammonia distribution is largely governed by E$_m$ acting on NH$_4^+$. Nonetheless, P$_{NH_3}$ is substantial, so ammonia efflux responds sensitively to the pH$_r$-pH$_m$ gradient. These statements are not contradictory, for flux and distribution ratio are not the same quantity. Thus, in the dynamic postexercise situation at low pH$_r$, the Nernst potential for NH$_3^+$ is almost identical to E$_m$ (Fig. 8), so there is no net driving force for NH$_4^+$ to leave the cells, but there is a large P$_{NH_3}$ gradient for NH$_3$ to leave the cells (Fig. 5).

Under resting conditions, intracellular production of ammonia is presumably very small due to an absence of adenylate breakdown. Nevertheless, most in vivo studies on resting teleost fish have indicated that ammonia is again distributed according to E$_m$ (27, 31, 38, 39). Under these conditions, the electrochemical gradient for inward movement of NH$_3$ would be balanced by the P$_{NH_3}$ gradient for outward movement of NH$_3$ (Fig. 1B). Heisler (11) has objected to this conclusion on theoretical grounds, specifically that the resulting inward “H-
shuttle" would place an intolerable load on the pH regulatory mechanisms of the cells.

Figure 9 illustrates that, in the perfused trout trunk under resting conditions (Henry et al., unpublished results) and in our recent in vivo study on trout (29), the $[\text{TAmn}]_{i}/[\text{TAmn}]_{e}$ distribution ratio was much lower than in all previous studies, far lower than that predicted by $E_{m}$ and, in fact, approximated that predicted by $pH_{i}-pH_{e}$. The reason for the discrepancies between our resting data and those of many previous studies is unclear, but it should be noted that resting ratios are subject to the greatest error because of the low plasma $T_{\text{Amn}}$ concentrations, the greatest bias because of arterial versus venous sampling, and the difficulty of obtaining muscle samples from resting fish without ammonia production from adenylate breakdown (30). These problems do not apply in the resting perfused trunk preparation.

The in vitro and in vivo patterns of Fig. 9 show remarkable agreement and suggest that the pattern may change from a pH-dictated distribution at rest to an $E_{m}$-dictated distribution during recovery from exhaustive exercise. As pointed out earlier (29), the advantages of this switchover could be considerable. At rest, the costly H+ shuttle would be avoided, whereas, after exercise, the $E_{m}$-dictated distribution would help retain much higher levels of ammonia in the muscle for greater intracellular buffering and ATP resynthesis. If this is the case, then the $\text{PNH}_{2}/\text{PNH}_{4}$ ratio of the white muscle cell membranes must decrease between rest and postexercise; acidosis itself might be a controlling factor.

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