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IN VIVO AND *IN VITRO* EFFECTS OF ADRENERGIC STIMULATION ON CHLORIDE/BICARBONATE EXCHANGE IN RAINBOW TROUT ERYTHROCYTES

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Summary

In vitro and *in vivo* experiments were carried out to determine the effect of catecholamines on erythrocytic chloride/bicarbonate exchange in the rainbow trout. A further modified boat assay is described and was used to measure bicarbonate flux through intact erythrocytes. Catecholamines had no significant effect on the bicarbonate flux *in vitro*. The erythrocytes were sensitive to adrenergic stimulation, however, since the agonists used caused a decrease in the pH gradient across the erythrocyte membrane. Exhaustive exercise was associated with an increase in bicarbonate flux through the intact erythrocytes. The mechanism for this increase is not clear, but it is evidently not adrenergic in origin.

Introduction

The regulation of oxygen transport in fish *via* adrenergically mediated changes at the level of the erythrocyte has been well characterized. There are a very limited number of studies, however, that examine the effect of catecholamines on carbon dioxide transport by fish erythrocytes. Wood & Perry (1985) have reported that chloride/bicarbonate exchange in rainbow trout erythrocytes is inhibited *in vitro* by catecholamines. *In vivo*, this inhibition would be expected to result in CO_2 retention since plasma bicarbonate to be excreted as CO_2 enters fish erythrocytes *via* the chloride/bicarbonate exchanger (Cameron, 1978; Obaid *et al.* 1979). Steffensen *et al.* (1987), however, were unable to find any adrenergic inhibition of CO_2 excretion in the rainbow trout *in vivo*. Thus, the role of catecholamines in modulating CO_2 excretion in trout is unclear since the *in vivo* and *in vitro* results appear to conflict. In the present study, we examine the role of catecholamines in modulating CO_2 transport in fish in an attempt to resolve this conflict.

Materials and methods

In vitro *experiments*

The blood used in these experiments was obtained from anaesthetized rainbow

Key words: exercise, catecholamines, erythrocytes, trout, chloride/bicarbonate exchange.

trout (150–250 g) *via* caudal vessel puncture, except in the experiments that examined the effects of adrenergic stimulation, when blood was obtained from resting fish *via* a dorsal aortic cannula that had been surgically implanted 48 h prior to the experiment. In all experiments, the collected blood was pooled and equilibrated in an intermittently rotating glass tonometer at 10°C with humidified gas mixtures containing 0·2 % CO_2 in air or 1 % CO_2 in air (Wösthoff gas-mixing pumps, Bochum, FRG).

The first series of experiments examined the effect of haematocrit on carbon dioxide evolution using the modified boat assay described below. In these experiments, the blood pool was equilibrated with 0·2 % CO_2 in air for at least 1 h, at which time a blood sample to be assayed was removed. The haematocrit of the sample was adjusted (to 10, 15, 20, 25 or 30 %) with an appropriate volume of plasma from the same blood pool and the final volume of the whole blood sample to be assayed was always 0·4 ml. For each whole blood assay, there was an additional assay which measured the rate of carbon dioxide evolution from a true plasma sample from the same blood pool. The volume of plasma assayed was equivalent to the plasma volume of the preceding whole blood sample. A second series of experiments examined the effect of the anion exchange inhibitors DIDS (4,4-diisothiocyanostilbene-2,2-disulphonic acid) and SITS (4-acetamido-4-isothiocyanatostilbene-2,2-disulphonic acid) and the carbonic anhydrase inhibitor acetazolamide on the rate of carbon dioxide evolution from intact erythrocytes. In these experiments, 2 ml samples of whole blood (adjusted to 25 % haematocrit) were removed from the blood pool and equilibrated in separate tonometers in the presence of 0·1 mmol l^{-1} DIDS, SITS or acetazolamide for 30 min prior to the boat assay. A control set of experiments was also performed in which only the saline vehicle for the above solutions (100 μl of Cortland's saline) was added to the tonometer.

In the final series of experiments, the effects of the adrenergic agonists isoproterenol, adrenaline and noradrenaline on carbon dioxide evolution from intact erythrocytes were investigated. This series of experiments was similar to the second series with the following exceptions. (1) The equilibration gas mixture was 1 % CO_2 in air since the effects of adrenergic agents on trout erythrocytes are enhanced at lower pH (Nikinmaa *et al.* 1987; Heming *et al.* 1987). (2) Isoproterenol, adrenaline or noradrenaline was added to the tonometers to reach a final blood concentration of 10^{-4} mol l^{-1}. (3) The vehicle for these agonists, acting as a control, was 100 mmol l^{-1} perchloric acid, diluted to 4 vol % with Cortland saline; final volume 100 μl. (4) The agonist or control was also added to the buffer solution in the boat for both the whole blood and true plasma assays to prevent the dilution of agonists during the equilibration period in the boat. (5) The extracellular and erythrocyte pH were also determined following the 30 min equilibration period.

In vivo *experiments*

In these experiments, the fish were chronically cannulated in the dorsal aorta and allowed at least 24 h to recover from the surgery in light-proof chambers at

10 °C. Following the recovery period, a resting blood sample (2 ml) was removed *via* the cannula into a Hamilton gas-tight syringe. An equivalent volume of Cortland saline was injected back into the fish. The haematocrit and extracellular pH of the blood sample were then measured immediately. In addition, a 400 μl sample of whole blood and the appropriate volume of plasma were immediately assayed in the boat. Two 25 μl samples of this whole blood sample were also saved for later analysis of haemoglobin concentration (Sigma assay no. 525). Rates of carbon dioxide evolution (boat assay) are given as $\mu l\,g\,Hb^{-1}\,min^{-1}$ in these experiments, to eliminate the influence of haematocrit. Finally, a portion of the sample was centrifuged in an Eppendorf tube and the red cell pellet was frozen in liquid nitrogen after the plasma had been removed. The pellet was later used to measure the erythrocyte pH with the method of Zeidler & Kim (1977).

Following the resting sample, fish were subjected to one of four protocols. The first protocol served as a control, in which the fish remained undisturbed except for a sham injection and the removal of a second blood sample 10 min after the injection. In the second protocol, the fish was vigorously exercised to exhaustion. The exercise was immediately followed by the sham injection and the second sample was taken 10 min later. This procedure was repeated in the third and fourth protocols, but in these experiments either isoproterenol or propranolol (final concentrations of $10^{-5}\,mol\,l^{-1}$) was injected into the fish immediately following the exercise period. The isoproterenol or propranolol was dissolved in Cortland saline, which was also used as the sham. Injected volumes were approximately 250 $\mu l\,100\,g^{-1}$.

Modified boat assay

The evolution of CO_2 from erythrocytes took place in a 50 ml Ehrlenmeyer flask which had been partitioned along the bottom by a raised glass ridge. 2 ml of bicarbonate solution (200 mmol l^{-1} $NaHCO_3$ in 20 mmol l^{-1} NaOH) was placed on one side of the ridge and 2 ml of phosphate buffer solution (61 % 200 mmol l^{-1} Na_2HPO_4 and 39 % 200 mmol l^{-1} KH_2PO_4) was placed on the other side. It is noteworthy that in a recent study, Nikinmaa *et al.* (1986) included 20 mmol l^{-1} NaCl in the bicarbonate solution. The effect of sodium chloride addition was, therefore, examined in the present study (Fig. 1), but was found to have no significant effect on the assay between 0 and 200 mmol l^{-1} NaCl. Thus, no NaCl was added in the present experiments. The material to be assayed was added to the side containing the buffer solution.

The top of the boat was fitted with a ground-glass joint connected to a differential pressure transducing system (Validyne DP 103 with CD 16 carrier demodulator) by latex tubing. The output from the pressure transducing system was displayed on a chart recorder (Gould TA 600). The boat was also connected to a shaker. Partial immersion of the boat in a constant-temperature bath as in previous studies (Haswell & Randall, 1976; Heming & Randall, 1982) was found to cause a temperature-related artefact in the system. Under these conditions, the pressure in the system fell initially prior to increasing as the reaction proceeded.

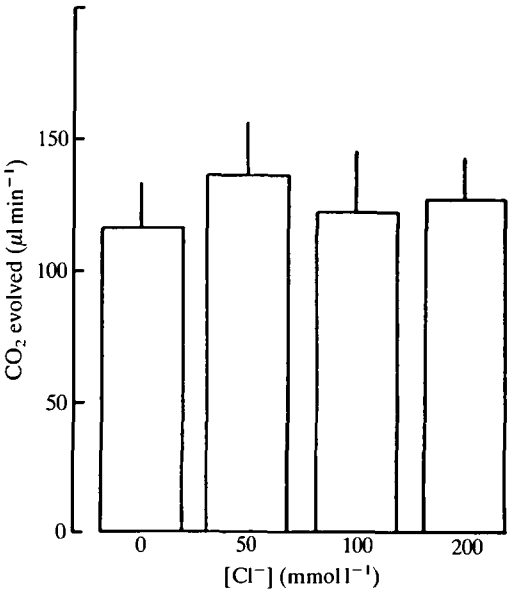

Fig. 1. The effect of extracellular chloride concentration on the carbon dioxide evolution by rainbow trout erythrocytes. Values are means + standard error ($N = 6$).

The entire system was, therefore, kept at 10°C in a constant-temperature room. This eliminated the initial fall in pressure and increased the reproducibility of the assay.

Upon addition of a sample to the buffer solution, the boat was sealed and left for 5–10 min until the output from the recorder stabilized (manual sealing of the vessel caused a temporary temperature-related pressure change). The shaker was then turned on and the pressure in the system was followed as the dehydration reaction proceeded. The system was calibrated with known volumes of air from 50 to 400 μl at the experimental temperature. The increase in pressure was linear over this range and there was no detectable leakage in the system.

Reaction rate

Reaction rates (μl CO_2 evolved min^{-1}) were calculated from the time required for the sample mixture to evolve 200 μl of gas. The rate due to erythrocytes was obtained by subtraction of each sample's true plasma rate from the whole blood rate. This eliminated any error that could be attributed to variations in endogenous plasma factors and/or haemolysis.

Results

The rate of CO_2 evolution was proportional to the number of erythrocytes in the boat (Fig. 2). At a haematocrit of 10%, the rate of carbon dioxide evolution was $30{\cdot}9 \pm 13{\cdot}8$ μl min^{-1}. As the haematocrit increased, the rate of CO_2 evolution also

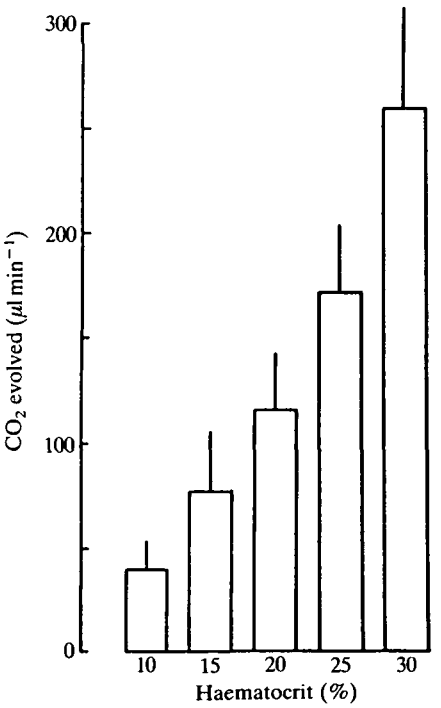

Fig. 2. Effect of changing haematocrit on carbon dioxide evolution by rainbow trout erythrocytes. Values are means + standard error ($N = 6$ in all cases except haematocrit = 10 % where $N = 5$).

increased, and at a haematocrit of 30 % the CO_2 evolution had reached $259 \pm 48{\cdot}2\ \mu l\ min^{-1}$.

The anion exchange inhibitors DIDS and SITS caused the CO_2 evolution to decrease from the control value of $214{\cdot}7 \pm 13{\cdot}4\ \mu l\ min^{-1}$ to $93{\cdot}7 \pm 17{\cdot}0$ and $126 \pm 18{\cdot}9\ \mu l\ min^{-1}$, respectively (Fig. 3). DIDS, therefore, caused a 54 % reduction in CO_2 evolution and SITS inhibited the system by 41 %. This inhibition was due to a change in the characteristics of the erythrocytes, since the rate of CO_2 evolution from plasma was not significantly different after addition of these blockers. It is noteworthy that in four separate experiments (unpublished results) DIDS was also added to the buffer solution in the boat prior to the assay of both whole blood and true plasma. In these experiments, the degree of inhibition was enhanced (78 %). This indicated that some dilution of the blockers may have occurred in the boat when the blocker was added only to the blood and not to the buffer solution in the boat. The carbonic anhydrase inhibitor, acetazolamide, decreased the rate of CO_2 evolution to 14·7 % of the control rate (95 % inhibition) (Fig. 3).

Adrenaline, isoproterenol and noradrenaline had no significant effect on the rate of CO_2 evolution from the erythrocytes (Fig. 4). The control rate was $150{\cdot}9 \pm 13{\cdot}2\ \mu l\ min^{-1}$ and the rates with the agonists were $151{\cdot}5 \pm 13{\cdot}8$,

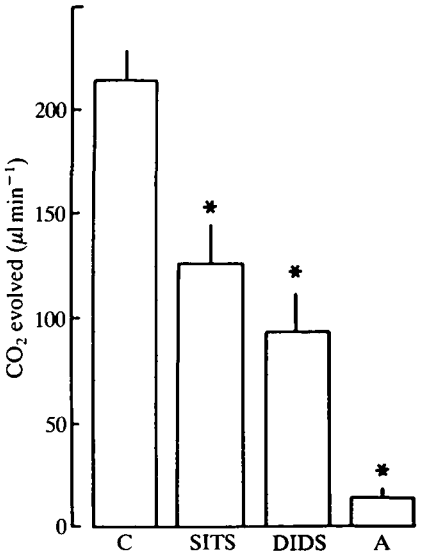

Fig. 3. The effect of SITS (0·1 mmol l^{-1}), DIDS (0·1 mmol l^{-1}) or acetazolamide (A, 0·1 mmol l^{-1}) on the carbon dioxide evolution of rainbow trout erythrocytes (haematocrit 25 %). Values are means + standard error ($N = 6$). Unpaired t-tests were used to compare blocker values with control values. Differences were accepted as significant at the $P < 0{\cdot}05$ level and are indicated by an asterisk. C, control.

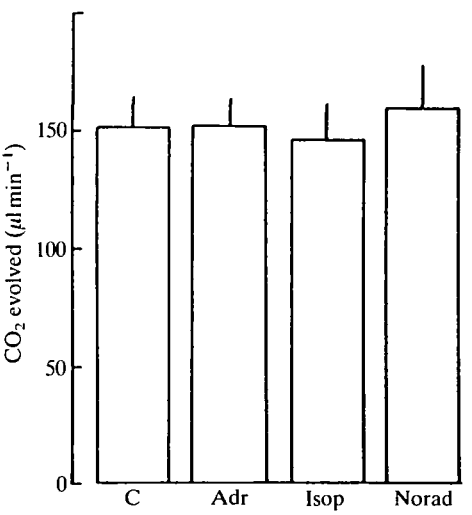

Fig. 4. The effect of adrenaline (Adr, 0·1 mmol l^{-1}), isoproterenol (Isop, 0·1 mmol l^{-1}) and noradrenaline (Norad, 0·1 mmol l^{-1}) on the carbon dioxide evolution of rainbow trout erythrocytes (haematocrit 25 %). Values are means + standard error ($N = 8$). Unpaired t-tests were used to compare agonist values with control values. No values differed from each other at the $P < 0{\cdot}05$ level. C, control.

Table 1. *Effect of adrenaline, isoproterenol and noradrenaline on the plasma pH (pHe), erythrocyte pH (pHi) and on the pH gradient (ΔpH) of rainbow trout blood*

Treatment	pHe	pHi	ΔpH
Control	7·601 ± 0·049	7·273 ± 0·029	0·328
Adrenaline	7·405 ± 0·028*	7·300 ± 0·016	0·105
Isoproterenol	7·371 ± 0·029*	7·323 ± 0·011	0·048
Noradrenaline	7·446 ± 0·029*	7·323 ± 0·020	0·123

Values are means ± one S.E.M.
Asterisk denotes significant (unpaired *t*-test; $P < 0{\cdot}05$) difference from control.

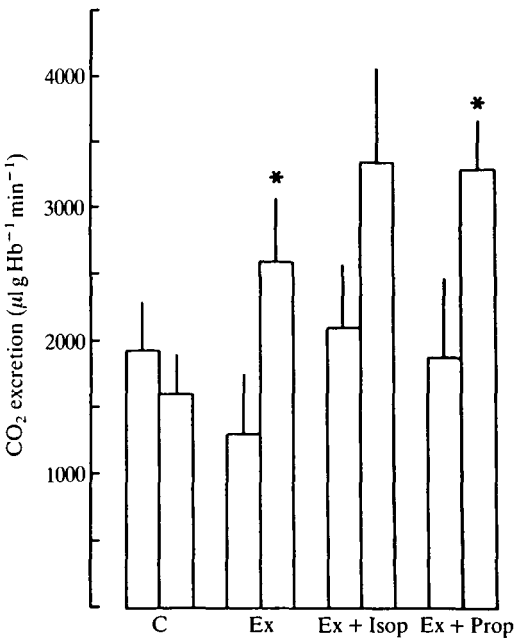

Fig. 5. Carbon dioxide evolution of rainbow trout erythrocytes before and after the following *in vivo* conditions: Control (C), exercise (Ex), exercise plus isoproterenol infusion (Ex + Isop) and exercise plus propranolol infusion (Ex + Prop). Values are means + standard error ($N = 6$). Paired *t*-test was used to compare before and after samples. Significant ($P < 0{\cdot}05$) differences are indicated by an asterisk.

145·5 ± 15·8 and 160·5 ± 18·1 μl min^{-1}, respectively. These agonists did, however, have a marked effect on the pH gradients across the erythrocyte membrane (Table 1). In each case, the erythrocyte pH increased whereas the extracellular pH decreased.

The effect of the *in vivo* protocols on the evolution of carbon dioxide from trout erythrocytes is illustrated in Fig. 5. In the control group, carbon dioxide evolution decreased slightly (17%), but not significantly. Exercise, however, increased the

Table 2. *Extracellular pH (pHe), erythrocyte pH (pHi) and the pH gradient (ΔpH) in the rainbow trout blood during the specified* in vivo *conditions*

Treatment	pHe	pHi	ΔpH
Control			
Before	8·011 ± 0·030	7·494 ± 0·018	0·517
After	8·040 ± 0·035	7·522 ± 0·020	0·518
Exercise			
Before	8·048 ± 0·039	7·451 ± 0·017	0·597
After	7·737 ± 0·042*	7·285 ± 0·024*	0·452
Exercise+isoproterenol			
Before	7·934 ± 0·042	7·442 ± 0·038	0·492
After	7·705 ± 0·060*	7·464 ± 0·040	0·241
Exercise+propranolol			
Before	8·068 ± 0·034	7·381 ± 0·033	0·687
After	7·725 ± 0·061*	7·275 ± 0·040*	0·450

Values are means ± one S.E.M.

Asterisk denotes significant (paired *t*-test; $P < 0·05$) difference between before and after samples.

carbon dioxide evolution from the erythrocytes in all cases. These increases were significant in both the sham- and propranolol-injected fish. In the sham-injected fish the percentage increase was 102%, and in the propranolol-injected fish the percentage increase was 75%. The extracellular pH did not change significantly in the control group, but decreased significantly in all three exercise groups (Table 2). In both the sham- and propranol-injected fish, this was associated with significant decreases in the erythrocyte pH. In the isoproterenol-injected fish, however, the erythrocyte pH remained constant (Table 2).

Discussion

Critique of methods

The present study employed the carbonic anhydrase boat technique, first described in detail for mammalian erythrocytes by Booth (1938) and later modified for studies of CO_2 excretion by fish erythrocytes (Haswell & Randall, 1976; Heming & Randall, 1982). Difficulties encountered in the previous studies on fish made it imperative to examine the system thoroughly before investigating the effects of catecholamines on CO_2 excretion in rainbow trout erythrocytes. The first of these control experiments was to determine if the evolution of CO_2 was dependent on the number of cells present, as it clearly was (Fig. 2). Not only was this further confirmation that fish erythrocytes are bicarbonate-permeable (Cameron, 1978; Obaid *et al.* 1979; Heming & Randall, 1982), but it also demonstrated the linearity of the system in the chosen range of volume and

haematocrit used for the subsequent experiments. In addition, inhibition of CO_2 evolution from whole blood by acetazolamide indicated that the present assay was indeed measuring the carbonic anhydrase activity of erythrocytes (Fig. 3). Moreover, the marked degree of inhibition obtained when the anion exchange inhibitors DIDS and SITS were added to the blood (Fig. 3) provided evidence that the rate of carbon dioxide evolution in this assay was dependent on erythrocytic chloride/bicarbonate exchange. Taken together, these results indicated that the present system could be employed to examine the effects of catecholamines on chloride/bicarbonate exchange in trout erythrocytes.

There are important sources of error in this assay which need to be addressed. First, any haemolysis that went undetected in the boat assay would lead to a considerable overestimate of the CO_2 excretion of the erythrocytes. This problem was avoided in the present experiments by subtracting the rate of CO_2 evolution of the true plasma from that of the whole blood to obtain the erythrocyte rate. We found that it was imperative to use the blood's true plasma, in the same volume as in the blood sample, for these purposes. This also ensured that the effects of pharmacological agents seen in the assay were the result of changes in the characteristics of the intact erythrocytes and not merely an effect of these agents on the free enzyme (or other endogenous substances) in the plasma. In previous studies, only the whole blood rate was determined and this was compared to a saline control rather than to the blood's true plasma. Thus, if any haemolysis occurred prior to or during the assay, it would go undetected. In preliminary experiments, we attempted to determine whether the mechanical agitation and/or chemistry of the assay itself caused any haemolysis. Initially, we centrifuged the boat solution after every blood and true plasma sample, and analysed the supernatant for haemoglobin concentration and carbonic anhydrase activity as indicators of haemolysis. There was never any indication that haemolysis occurred as a result of the assay itself.

In previous studies in which CO_2 excretion of fish erythrocytes was measured using the boat technique, the erythrocytes were often suspended in saline after having been washed several times. In our hands, it was never possible to 'wash' erythrocytes in saline without causing some detectable haemolysis, and therefore extracellular carbonic anhydrase activity. This problem could only be eliminated if the 'true saline' rates of the erythrocyte suspension were subtracted from the red cell rates. We also found no evidence of the foaming problem noted by Heming & Randall (1982) and indeed found that defoaming agents exacerbated accurate measurements by causing variable degrees of haemolysis.

Reproducibility of the method is also vastly improved by equlibrating the samples (e.g. blood) at known CO_2 tensions prior to assay, thereby ensuring comparable pH values between series of measurements. Because the assay is so pH-sensitive (Booth, 1938), it is also important that each of the plasma controls be 'true' rather than 'separated' plasma. In the present experiments, we also monitored the erythrocyte and plasma pH of each of the samples to verify the acid–base conditions of each assay.

Effects of catecholamines on CO_2 excretion of erythrocytes

In the present experiments, catecholamines had no effect on bicarbonate flux through rainbow trout erythrocytes (Fig. 4). This is in sharp contrast to the results cited by Wood & Perry (1985). Indeed, according to the data in the study reported by Wood & Perry (1985), the dosages of adrenergic agonists used in the present study should have caused a large reduction in the evolution of CO_2 from the erythrocytes. The discrepancy between studies could be explained if, for example, the adrenergic receptors on the erythrocytes in the present study were for some reason 'down regulated'. However, the agonists did have a marked effect on the pH gradient across the erythrocyte membrane (Table 1) as described before (Nikinmaa, 1982; Nikinmaa & Huestis, 1984; Primmett *et al.* 1986; Heming *et al.* 1987), demonstrating that the erythrocytes were adrenergically stimulated.

Using the methodological approach described here, we are therefore unable to support the contention that catecholamines will inhibit CO_2 excretion at the level of the red blood cell, and that this might limit overall CO_2 excretion following exercise in fish (Wood & Perry, 1985). Moreover, recent studies on intact fish have shown that neither exercise nor adrenaline infusion had any demonstrable effect on the CO_2 excretion or respiratory exchange ratio of freely swimming trout (Steffensen *et al.* 1987). Indeed, in the present studies, exhaustive exercise was associated with a rise in the evolution of CO_2 from rainbow trout erythrocytes (Fig. 5). Exhaustive exercise usually leads to large increases in the circulating levels of catecholamines in rainbow trout (Primmett *et al.* 1986; Butler *et al.* 1986; Milligan & Wood, 1987; Tang & Boutilier, 1988). In the sham-injected trout, however, the exercise caused a marked drop in erythrocyte pH similar to that which occurred in propranolol-injected fish. This suggests that the catecholamine secretion or the sensitivity of the erythrocytes to catecholamines may have been reduced in these animals since some regulation of erythrocyte pH would be anticipated (Primmett *et al.* 1986; Milligan & Wood, 1987; Y. Tang & R. G. Boutilier, in preparation). This does not explain, however, the absence of adrenergic inhibition of carbon dioxide evolution from these erythrocytes. In the isoproterenol-injected fish, erythrocyte pH was regulated, but carbon dioxide evolution from these cells was also increased rather than inhibited.

According to Booth (1938), a decrease in erythrocyte pH will cause an acceleration of bicarbonate flux through intact erythrocytes. This may have contributed to the increases in the sham- and propranolol-treated fish (Table 2; Fig. 5). However, other factors must also have contributed to the increased bicarbonate throughput since, in the isoproterenol-treated fish, the rates of erythrocytic CO_2 evolution were also elevated (Fig. 5) despite the fact that red cell pH was maintained at a constant level (Table 2). An elevated rate of bicarbonate flux through the erythrocytes of exercising fish would seem to be adaptive in increasing the overall transepithelial conductance of CO_2 from fish to environmental water. Indeed, it could be construed as part of a suite of cardiorespiratory adaptations aimed at facilitating gas exchange during periods of high O_2 demand (e.g. Randall & Daxboeck, 1984; Wood & Perry, 1985). It is also possible that the

increased evolution of CO_2 would facilitate greater uptake of oxygen at lamellar gas exchange sites and, ultimately, to the delivery of oxygen to the respiring tissues.

In summary, the present study demonstrates that catecholamines do not inhibit bicarbonate flux through intact rainbow trout erythrocytes incubated *in vitro* or through erythrocytes obtained from exhaustively exercised animals. Indeed, the bicarbonate throughput in these erythrocytes is accelerated rather than inhibited, and though the mechanism for this acceleration is not clear, it is evidently not adrenergic in origin.

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