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A NEW *IN VITRO* ASSAY FOR CARBON DIOXIDE EXCRETION BY TROUT RED BLOOD CELLS: EFFECTS OF CATECHOLAMINES

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Summary

A new *in vitro* assay was developed and critically characterized to measure the rate of CO_2 excretion by trout red blood cells (RBCs) from HCO_3^- in their natural plasma under normal *in vivo* conditions of acid–base status. The assay is based on the addition of [^{14}C]bicarbonate to the whole blood and collection of the resultant $^{14}CO_2$ in the overlying gas phase. The assay simulates the exposure of blood passing through the gills, and measured CO_2 excretion rates are representative of those occurring *in vivo*. Rates are linear over the 3 min time course of the assay, related to haematocrit in a non-linear fashion, elevated by the addition of carbonic anhydrase, reduced by blockade with acetazolamide, and sensitive to variations of equilibration P_{CO_2}. Large variations in plasma [HCO_3^-] have only a small effect on CO_2 excretion rates when the blood is chronically equilibrated at these levels. Acute elevations in [HCO_3^-], however, create a non-equilibrium situation, resulting in large increases in CO_2 excretion. When the blood is acidified, to duplicate typical post-exercise metabolic acidosis, adrenaline causes a marked inhibition of RBC CO_2 excretion. The response is transient, reaching a peak 5–8 min after addition of adrenaline and disappearing by 30–60 min. The magnitude of the adrenergic inhibition is correlated with the magnitude of the RBC pHi regulatory response, expressed as the RBC transmembrane pH difference (pHe−pHi). These results support the 'CO_2 retention theory' explaining observed increases in blood P_{CO_2} *in vivo* after exhaustive exercise and catecholamine infusions in fish.

Introduction

Carbon dioxide excretion at the gills of teleost fish follows the standard vertebrate scheme whereby (i) plasma HCO_3^- enters the red blood cell (RBC) in electroneutral exchange for Cl^-; (ii) the HCO_3^- is dehydrated to CO_2 by

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erythrocytic carbonic anhydrase; and (iii) the resultant CO_2 diffuses from the RBC through the plasma and respiratory epithelium to the external environment (reviewed by Perry, 1986; Perry and Laurent, 1990). The flux is driven by the P_{CO_2} gradient from RBC to water. However, after strenuous exercise, fish routinely exhibit large increases in Pa_{CO_2} without a corresponding decrease in Pa_{O_2} (e.g. Turner *et al.* 1983; Milligan and Wood, 1986; McDonald *et al.* 1989). The response is curious because a decrease in Pa_{O_2} would be expected if the phenomenon were due to a simple diffusive or convective limitation on gas exchange. To explain this observation, several years ago we proposed that the mobilization of catecholamines into the acidotic bloodstream after exhaustive exercise produces an inhibition of the HCO_3^- entry step, thereby causing CO_2 to back-up in the system until the rise in P_{CO_2} is sufficient to restore the flux (Wood and Perry, 1985).

Since that time, this 'CO_2 retention theory' has proved controversial. On the one hand, chronic intra-arterial infusions of adrenaline have been shown to cause simultaneous elevations of Pa_{CO_2} and Pa_{O_2} (Perry and Vermette, 1987; Vermette and Perry, 1988) and intra-arterial injections of carbonic anhydrase have been shown to reduce the post-exercise increase in Pa_{CO_2} without altering Pa_{O_2} (Perry and Wood, 1989). On the other hand, neither Steffensen *et al.* (1987) nor Playle *et al.* (1990) were able to demonstrate a transient fall in relative rate of carbon dioxide production ($\dot{M}_{CO_2}$) (i.e. a decrease in the respiratory exchange ratio, RE) after exercise or catecholamine injections, in contrast to the predictions of the theory. Furthermore, Tufts *et al.* (1988) were unable to replicate a key observation on which the theory was based, namely that catecholamines cause an inhibition of CO_2 excretion by intact trout RBCs *in vitro*.

Both the original observation (unpublished data of S. F. Perry and T. A. Heming presented by Wood and Perry, 1985) and the contrasting results of Tufts *et al.* (1988) were based on modifications of the manometric 'boat' assay for carbonic anhydrase, first used for RBCs by Booth (1938). In the assay, the RBCs are exposed to unphysiological buffer systems and pH values, and massive HCO_3^- pulses. Booth concluded that it was impossible to use this assay to measure CO_2 excretion rates in intact erythrocytes, and over the years its use for that purpose with fish RBCs has been plagued with controversy (e.g. Haswell and Randall, 1976; Obaid *et al.* 1979; Cameron, 1978; Heming and Randall, 1982). Unfortunately, other available methods involve similar disturbances, such as the use of artificial buffers, large pulses of acid or base, filtration separation of the RBCs, cell lysis or the addition of carbonic anhydrase to the extracellular fluid (e.g. Dirken and Mook, 1931; Piiper, 1969; Lambert and Lowe, 1978; Crandall *et al.* 1978; Maren and Couto, 1979). We therefore decided that an entirely different approach was required, one in which net CO_2 excretion from the unmodified plasma through the intact RBCs could be quantified under conditions closely approximating those *in vivo*.

In the present paper we describe and characterize a sensitive new method, based on the use of [^{14}C]bicarbonate, for measuring net CO_2 excretion by trout RBCs in their normal plasma *in vitro*. The P_{CO_2} gradients and acid–base conditions are

similar to those encountered by blood passing through the gills *in vivo*. Using this technique, we have confirmed the adrenergic inhibition of RBC CO_2 excretion, and identified the conditions under which it occurs. The companion paper (Perry *et al.* 1991) presents a detailed analysis of the mechanism of this inhibition.

Materials and methods

Experimental animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum); 150–400 g] of either sex were obtained from Thistle Springs Trout Farm (Ashton, Ontario). Fish were maintained indoors in large rectangular fibreglass tanks supplied with flowing, vigorously aerated, dechlorinated City of Ottawa tapwater ($[Na^+]$=0.12 mmol l^{-1}; $[Cl^-]$=0.15 mmol l^{-1}; $[K^+]$=0.03 mmol l^{-1}; pH 7.5–8.0; temperature 9–12°C; photoperiod 12 h light:12 h dark). Fish were fed daily *ad libitum* on floating commercial trout pellets (Purina), but were not fed for 48 h prior to experimental procedures.

To avoid endogenously elevated catecholamine and lactate levels, all blood was drawn from chronically cannulated fish. Trout were anaesthetized in a 1:10000 (w/v) solution of ethyl-*m*-aminobenzoate (MS 222; Sigma) adjusted to pH 7.5 with $NaHCO_3$ and then placed onto an operating table which permitted continuous retrograde perfusion of the gills. An indwelling cannula (Clay–Adams PE 50) filled with modified Cortland saline ($[HCO_3^-]$=5 mmol l^{-1}; Wolf, 1963) was implanted into the dorsal aorta (Soivio *et al.* 1975). After surgery, fish were placed in darkened Perspex boxes (volume 3 l) served with continuously flowing acclimation water at the experimental temperature (10±1°C). Fish were allowed to recover for at least 48 h after surgery before blood sampling. Cannulae were flushed daily with 0.2–0.3 ml of heparinized saline (10 i.u. ml^{-1} ammonium heparin; Sigma).

Sampling and handling of blood

Trout were initially infused with 0.5 ml of heparinized (50 i.u. ml^{-1}) saline; blood was then withdrawn slowly until the fish showed the first signs of struggling or disorientation, at which point sampling ceased. This procedure generally yielded 2–3 ml per fish, though occasionally large trout yielded up to 5 ml. Depending on the size of the experiment, blood from at least three, and up to 12, fish was pooled, additionally heparinized (150 i.u. ml^{-1}), and stored on ice for 1–2 h prior to use. At this point, the pooled blood was routinely assayed for whole-blood pH (pHe=7.8–8.0), true plasma carbon dioxide content (C_{CO_2}= 4–6 mmol l^{-1}), plasma catecholamines (adrenaline plus noradrenaline always below 7 nmol l^{-1}) and haematocrit (15–25 %).

In preparation for the CO_2 excretion assay, the pooled heparinized blood was split into 1.0 ml samples in individual 20 ml glass scintillation vials. In experiments where haematocrit or plasma HCO_3^- levels (long-term changes) were manipulated, the adjustments were performed immediately prior to the splitting. For the

former, the pooled blood was very lightly centrifuged (500 *g* for 1 min), and an appropriate volume of homologous plasma added or removed. Plasma was also obtained in this fashion for 'plasma only' assays. Adjustments of plasma [HCO_3^-] were performed by very gradual additions of 140 mmol l^{-1} HCl or 140 mmol l^{-1} $NaHCO_3$ to whole blood, care being taken to avoid haemolysis. Addition of 140 mmol l^{-1} NaCl served as a control. The vials were then stoppered with rubber septa, gas-equilibrated in an open, flow-through system, as described by Walsh *et al.* (1990), and shaken for 2 h in a constant-temperature bath at 10±1°C. The normal gassing medium was a humidified mixture with P_{CO_2}=0.25 kPa (1.91 mmHg), P_{O_2}=20.7 kPa (155 mmHg), balance N_2 provided by a Wösthoff model M 301a/f gas-mixing pump. This P_{CO_2} was virtually identical to resting Pa_{CO_2} measured in the same batch of trout (Wood *et al.* 1990). Gas equilibration was continued until the start of the CO_2 excretion assay.

The CO_2 excretion assay

The assay is illustrated diagrammatically in Fig. 1, and is based on the addition of [^{14}C]bicarbonate to the whole blood (i.e. to the extracellular compartment) and collection of the resultant $^{14}CO_2$ in the overlying gas phase. Theoretically, this $^{14}CO_2$ may be generated by two routes: (i) dehydration of [^{14}C]bicarbonate in the plasma (presumably at the uncatalysed rate); and (ii) dehydration of [^{14}C]bicarbonate in the RBCs (presumably catalysed by erythrocytic carbonic anhydrase) subsequent to entry *via* Cl^-/HCO_3^- exchange. The net CO_2 flux is driven by the P_{CO_2} gradients between the respective blood compartments and the gas phase. The blood is initially equilibrated to normal *in vivo* P_{CO_2}, but in the assay vial it is exposed to a gas phase in which P_{CO_2} is close to zero, owing to the presence of a CO_2 trap; the situation is similar to that of blood passing through the gills, where the external water P_{CO_2} is close to zero.

In practice, 2 μCi (10 μl of 200 μCi ml^{-1}) of sodium [^{14}C]bicarbonate (in 5 mmol l^{-1} HCO_3^- Cortland saline) was added to each 1 ml of pre-equilibrated blood (or plasma). The vial was then immediately resealed with a new rubber septum from which was suspended a plastic well containing a trap for CO_2 (Fig. 1; Walsh *et al.* 1988), returned to the shaker bath, and timing commenced. Assays were routinely run for exactly 3 min. The trap consisted of a fluted filter paper (Whatman GF/A 2.4 cm glass microfibre filter) impregnated with 150 μl of 1 mol l^{-1} hyamine hydroxide in methanol. At the termination of the assay, the filter was immediately removed and assayed for ^{14}C activity, and the blood drawn into a Hamilton gas-tight syringe. Whole blood (or plasma) pHe was measured, and the remaining blood immediately centrifuged (12 000 *g* for 2 min). The plasma was decanted anaerobically for C_{CO_2} determination (100 μl) and ^{14}C counting (50 μl); the packed red cell pellet was frozen in liquid N_2 for later determination of RBC pHi by the freeze–thaw lysate method (Zeidler and Kim, 1977).

The CO_2 excretion rate for each assay vial was calculated by dividing filter paper ^{14}C activity by plasma specific activity and time. Note that specific activity was measured at the end, rather than at the start, of the assay. The calculated rate was

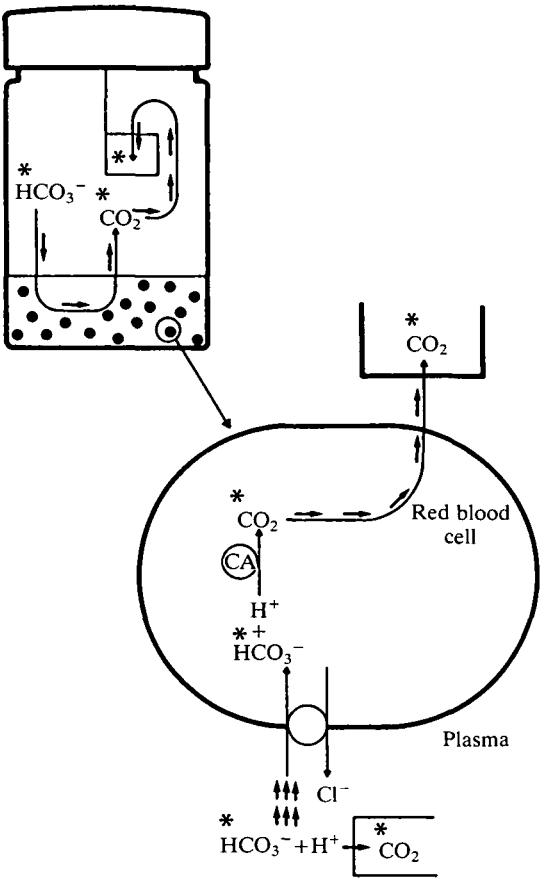

Fig. 1. Diagrammatic representation of the CO_2 excretion assay. Radioactive [^{14}C]bicarbonate (marked with *) is added to the whole blood (i.e. extracellular compartment) and $^{14}CO_2$ is collected in the gas-phase trap. The inset shows the pathway of $^{14}CO_2$ flux through a single red blood cell (RBC) and surrounding plasma. Excreted $^{14}CO_2$ collected in the trap may originate either from uncatalysed dehydration in the plasma (minor pathway) or from dehydration in the RBC catalysed by erythrocytic carbonic anhydrase (CA) subsequent to entry *via* Cl^-/HCO_3^- exchange (major pathway). See text for additional details.

then corrected for the efficiency of CO_2 trapping by the hyamine hydroxide filter. Trapping efficiency was measured over a wide range of CO_2 evolution rates by acidifying various 1 ml HCO_3^- standards (^{14}C-labelled, in plasma) with 100 μl of 35 % $HClO_3$, and then running the assay for 3 min. Trapping efficiency was always 74 %, regardless of the CO_2 evolution rate.

The following drugs were used in various tests: acetazolamide sodium U.S.P (Diamox; Lederle); bovine erythrocytic carbonic anhydrase (2500 Wilbur–Anderson units per mg; Sigma) (1 Wilbur–Anderson unit causes the pH of 0.012 mol l^{-1} veronal to drop from 8.3 to 6.3 in 1 min at 0°C); L-adrenaline

bitartrate (Sigma); and L-noradrenaline bitartrate (Sigma). Drug additions to the assay vials were made in 50 μl samples of 140 mmol l^{-1} NaCl, the vehicle alone being added to control vials.

Analytical procedures

Haematocrit was determined by centrifuging 80 μl of blood in a heparinized capillary tube for 10 min at 5000 *g*. RBC pHi and pHe (whole blood or plasma) were determined with a micro-capillary pH electrode (G299A) thermostatted to the experimental temperature in a BMS3 Mk2 blood micro-system, and displayed on a PHM-71 acid–base analyzer (all Radiometer). Plasma C_{CO_2} was determined in a few early experiments by the method of Cameron (1971), and in later experiments using a Corning 965 CO_2 analyzer. Plasma P_{CO_2} and [HCO_3^-] were calculated using the Henderson–Hasselbalch equation and appropriate constants listed in Boutilier *et al.* (1984). Adrenaline and noradrenaline levels were determined on alumina-extracted plasma samples using high performance (pressure) liquid chromatography with electrochemical detection according to the method of Woodward (1982).

Plasma and filter paper ^{14}C activities were determined by liquid scintillation counting (LKB Rackbeta) and automatic quench correction. Plasma (50 μl) was counted in 10 ml of commercial cocktail (ACS II; Amersham), and filter papers in 10 ml of a customized cocktail containing 2.0 g of PPO plus 0.1 g of POPOP dissolved in 0.8 l of toluene plus 0.2 l of 95 % ethanol.

Statistical analyses

Results are reported as means±1 S.E.M. (*N*), or representative experiments, as appropriate. Significant differences were detected using factorial analysis of variance followed by Fisher's LSD multiple-comparison test; 5 % was taken as the fiducial limit of significance.

Results

Time course of the assay

Initial trials were run at a range of assay durations. The cumulative CO_2 excretion of whole blood increased in an almost linear fashion with time up to about 10 min, and thereafter deviated only gradually from linearity (Fig. 2A). This basic linearity up to 10 min was seen both in tests run with the endogenous HCO_3^- levels in the equilibrated blood (about 5 mmol l^{-1}) and in tests where the plasma [HCO_3^-] was acutely doubled at the start of the assay so as to elevate the CO_2 excretion rates (see below).

Fig. 2B illustrates the change in acid–base status of the blood plasma over time in the assay, as measured at the immediate termination of each run. The P_{CO_2} fell progressively and plasma [HCO_3^-] declined as CO_2 was excreted. The trap kept the gas-phase P_{CO_2} close to zero, so the fall in blood P_{CO_2} reflected equilibration with the gas-phase. When plotted on a pH–HCO_3^- diagram, the change in

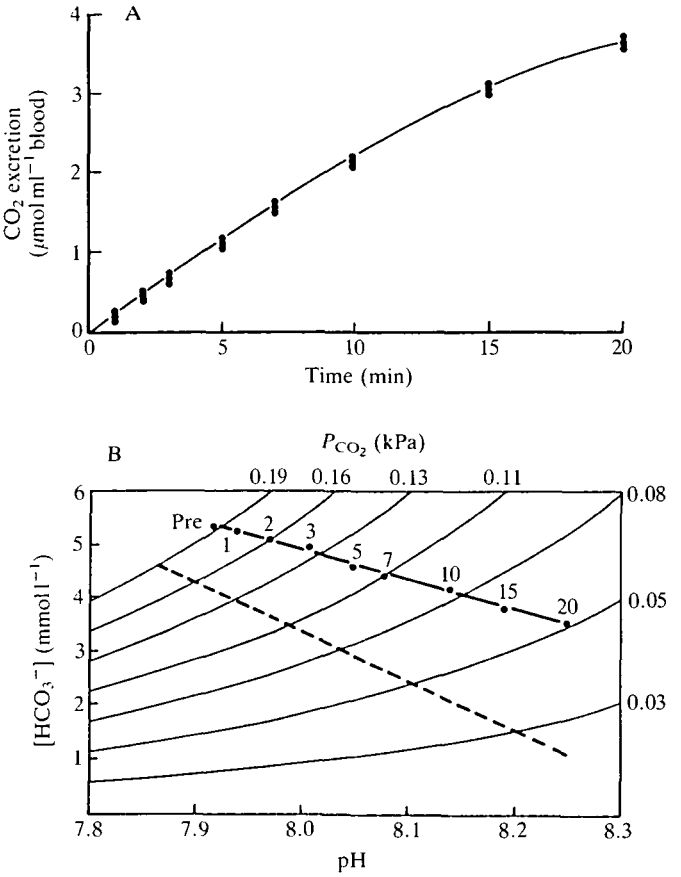

Fig. 2. (A) Representative experiment showing the time course of CO_2 excretion in the assay system. Triplicate assays were run on a common pool of trout blood (haematocrit=20%) over various durations ranging from 1 to 20 min. (B) A pH–HCO_3^- diagram displaying the mean acid–base status of true plasma measured immediately at the end of the assay for each time point in the same experiment. The non-HCO_3^- buffer line (dashed, β=9.2 mequiv l^{-1} pH unit^{-1}) for this haematocrit, based on the relationship of Wood *et al.* (1982), is included for reference.

acid–base status virtually paralleled the non-HCO_3^- buffer line, confirming that it was due to P_{CO_2} decline alone. The decrease in P_{CO_2} gradient from blood to air (the net driving force for the CO_2 excretion process) undoubtedly contributed to the slight deviation from linearity at longer durations. The efficiency tests demonstrated that it was not due to saturation of the trap. Based on these results, a standard assay duration of 3 min, in the strictly linear region, was selected for all further trials.

Validation tests

The rate of CO_2 excretion increased with haematocrit in tests run at both endogenous and acutely elevated levels of plasma $[HCO_3^-]$ (Fig. 3). The increase

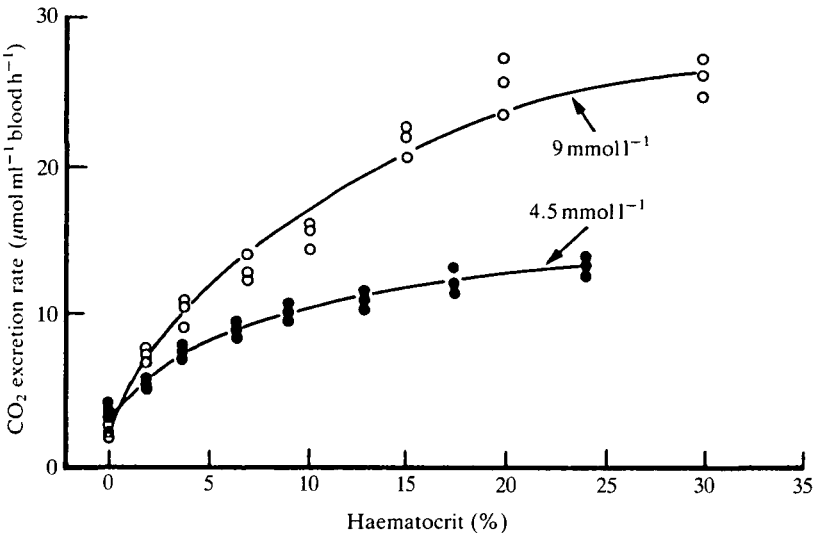

Fig. 3. The effects of haematocrit and of acute elevations in plasma [HCO_3^-] on the CO_2 excretion rate of trout whole blood. Triplicate assays were run on pools of blood made up to different haematocrits by removal or addition of homologous plasma. In the lower curve, the blood was equilibrated at control P_{CO_2} with endogenous plasma HCO_3^- concentration (4.5 mmol l^{-1}) for 2 h prior to assay. In the upper curve, the blood was equilibrated under the same conditions for 2 h. However, at the immediate start of the assay, the [HCO_3^-] was acutely doubled by addition of 10 μl of 472 mmol l^{-1} $NaHCO_3$.

was not directly proportional to haematocrit, but tended to attenuate at higher RBC levels. At a normal haematocrit of 25 % and normal blood acid–base status (plasma [HCO_3^-]=5 mmol l^{-1}, pHe=7.9), the CO_2 excretion rate of the whole blood was about 14 μmol ml^{-1} h^{-1} or about 5 times the rate of true plasma.

The addition of 0.2 mg of bovine carbonic anhydrase (500 Wilbur-Anderson units) to 1 ml of true plasma raised its CO_2 excretion rate to a value slightly greater than that of whole blood (Fig. 4). The addition of 10^{-4} mol l^{-1} acetazolamide (Diamox) either to whole blood or to plasma plus carbonic anhydrase lowered their CO_2 excretion rates back to those of plasma alone. Finally, as a check that catecholamines did not affect carbonic anhydrase activity directly, 10^{-6} mol l^{-1} adrenaline was added to plasma plus carbonic anhydrase. The CO_2 excretion rate remained equal to that of plasma plus carbonic anhydrase alone. From these tests we conclude that the assay is responsive to changes in CO_2 excretion mediated either by the erythrocytes or by artificial intervention, and that the plasma rate can be subtracted from the whole blood rate to yield the rate of CO_2 excretion mediated by the RBCs alone.

The influence of plasma [HCO_3^-] and P_{CO_2}

The effects of alterations in plasma [HCO_3^-] at constant P_{CO_2} differed greatly, depending on whether the blood had been pre-equilibrated at the various HCO_3^-

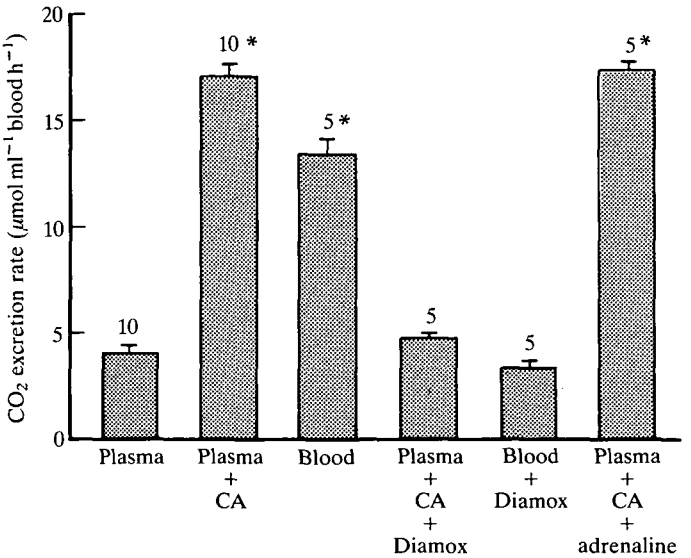

Fig. 4. CO_2 excretion rates in rainbow trout plasma, plasma plus 0.2 mg of bovine carbonic anhydrase (CA), whole blood (mean haematocrit=24%), plasma plus carbonic anhydrase plus 10^{-4} mol l^{-1} acetazolamide (Diamox), whole blood plus Diamox, and (vi) plasma plus carbonic anhydrase plus 10^{-6} mol l^{-1} adrenaline, all at control acid–base status. Drugs were added at least 1 h prior to assay. Means+1 S.E.M., *N*. Asterisks indicate means significantly different from plasma value ($P<0.05$).

levels or exposed to acute changes. Fig. 5 illustrates an experiment where the plasma HCO_3^- levels were adjusted by addition of HCl or $NaHCO_3$, and then the blood was equilibrated at a normal P_{CO_2} for 2 h prior assay. Under these circumstances, the CO_2 excretion rate was markedly unresponsive to the plasma HCO_3^- concentration. Indeed, over a 45-fold range in $[HCO_3^-]$ (0.6–27.3 mmol l^{-1}), and accompanying 1.9 unit increase in pHe, the whole-blood CO_2 excretion rate increased by only 35%. Within a more physiological range of plasma HCO_3^- concentrations (2–12 mmol l^{-1}) there was no detectable effect in several experiments.

In contrast, the RBC CO_2 excretion rate was very responsive to acute changes in plasma $[HCO_3^-]$, as illustrated by Fig. 3. Here the plasma $[HCO_3^-]$ was abruptly elevated at the start of the assay from the equilibration level of 4.5 mmol l^{-1} to 9.0 mmol l^{-1} (at normal P_{CO_2}), thereby creating a non-equilibrium situation. RBC CO_2 excretion rate increased in almost direct proportion to the rise in plasma $[HCO_3^-]$, and the response was related to the haematocrit.

The CO_2 excretion rate was also very responsive to the P_{CO_2} to which the blood had been equilibrated prior to assay. The data of Table 1 are from four separate experiments with pools of blood of slightly different haematocrits. Nevertheless, it is clear that increases in the equilibration P_{CO_2} greatly elevated the CO_2 excretion rate. Quantitative interpretation was complicated by the fact that the greater the

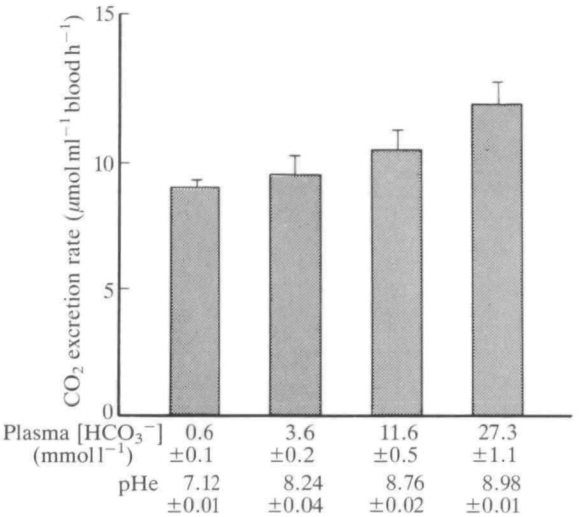

Fig. 5. The effects of chronic adjustments of plasma HCO_3^- concentration on the CO_2 excretion rate of trout whole blood (mean haematocrit=25 %). Plasma [HCO_3^-] was adjusted by addition of 140 mmol l^{-1} HCl or 140 mmol l^{-1} $NaHCO_3$ (with appropriate balancing volumes of 140 mmol l^{-1} NaCl), followed by at least 2 h of equilibration at control P_{CO_2} prior to assay. Acid–base status was measured at the immediate end of each assay. Means ±1S.E.M. (N=4–5). Only rates at the lowest and highest [HCO_3^-] are significantly different from one another (P<0.05).

Table 1. *The influence of the equilibration of* P_{CO_2} *on the* CO_2 *excretion rates of whole blood*

P_{CO_2}* (kPa)	Haematocrit (%)	CO_2 excretion rate† (μmol ml^{-1} blood h^{-1})
0.05	17.5	6.31±0.33 (4)
0.25	17.5	14.64±1.19 (6)
1.00	23.3	24.97±2.52 (5)
1.50	21.1	49.00±2.30 (5)

Values are mean±1 S.E.M. (N).

* Experiments were performed on different pools of blood at each P_{CO_2}; the blood was equilibrated to the relevant P_{CO_2} for at least 2 h prior to assay.

† The rates at each P_{CO_2} were all significantly different from one another (P<0.05).

starting P_{CO_2}, the greater was the fall in P_{CO_2} during the 3 min assay, so this subject was not pursued further.

The influence of catecholamines

In a series of preliminary experiments run at normal acid–base status, the addition of adrenaline or noradrenaline (10^{-8}–10^{-6} mol l^{-1}) to the blood

30–120 min prior to assay had small and inconsistent effects on CO_2 excretion rates. In general, the effects were inhibitory, but they were neither consistent nor statistically significant in most trials. However, *in vivo* the inhibitory effect after strenuous exercise is hypothesized to occur at the time of catecholamine mobilization, which is also a time of severe metabolic acidosis (see Introduction). Furthermore, recent studies on another trout RBC response, the regulation of RBC pHi *via* adrenergic activation of Na^+/H^+ exchange, have shown that this phenomenon becomes markedly sensitized at acidotic pHe values (see Discussion). Therefore, an experiment was performed to evaluate the possible time-dependency of both the RBC pHi and CO_2 excretion responses, using blood acidified to about pH 7.4 with HCl to simulate the metabolic acidosis component ($\Delta H_m^+ = 7\,mmol\,l^{-1}$) recorded in trout immediately after exhaustive exercise (Turner *et al.* 1983; Milligan and Wood, 1986).

Under these conditions, adrenaline ($10^{-6}\,mol\,l^{-1}$) caused a rapid rise (0.1 unit) in RBC pHi and fall (0.4 unit) in pHe, measured 3 min after addition (Fig. 6C,D). The peak pHi response occurred at 3 min, and the peak pHe response at 8 min, after which both declined. By 33 and 63 min, the pHi effect had disappeared, though the fall in pHe was still highly significant.

Adrenaline ($10^{-6}\,mol\,l^{-1}$) had no immediate effect on RBC CO_2 excretion rate (measured 0–3 min after addition; Fig. 6A). However, by 5–8 min, CO_2 excretion rate had declined significantly by 35 %, coincident with the maximum reduction in pHe−pHi. At 15–18 min, the inhibition, while still significant, had attenuated to 12 %. At 30–33 min and 60–63 min there was no longer any significant effect, despite the persistence of pHe depression. The cumulative influence of inhibited CO_2 excretion was clearly reflected in the plasma C_{CO_2} levels measured at the end of each 3 min assay (Fig. 6B).

These results indicated that adrenaline caused a clear, though transient, inhibition of RBC CO_2 excretion under acidotic conditions. The results further suggested that the effect might in some way be associated with the pHe−pHi response. All subsequent experiments were performed with similarly acidified blood (pHe=7.40±0.02, $[HCO_3^-]=1.90\pm0.07\,mmol\,l^{-1}$ at $P_{CO_2}=0.25\,kPa$), with assays conducted at 5–8 min after catecholamine addition. Both adrenaline and noradrenaline caused a concentration-dependent reduction in pHe−pHi over the range 10^{-8}–$10^{-6}\,mol\,l^{-1}$. In individual samples (treated with either catecholamine at 10^{-8}–$10^{-6}\,mol\,l^{-1}$ or a control saline addition) there was a highly significant correlation ($r=0.76$, $P<0.001$, $N=62$) between pHe–pHi and the RBC CO_2 excretion rate (Fig. 7). The greater the effect of catecholamines on pHe–pHi, the greater was the inhibition of CO_2 excretion.

Discussion

The CO_2 excretion assay

[^{14}C]bicarbonate-based techniques are widely used in algal physiology for

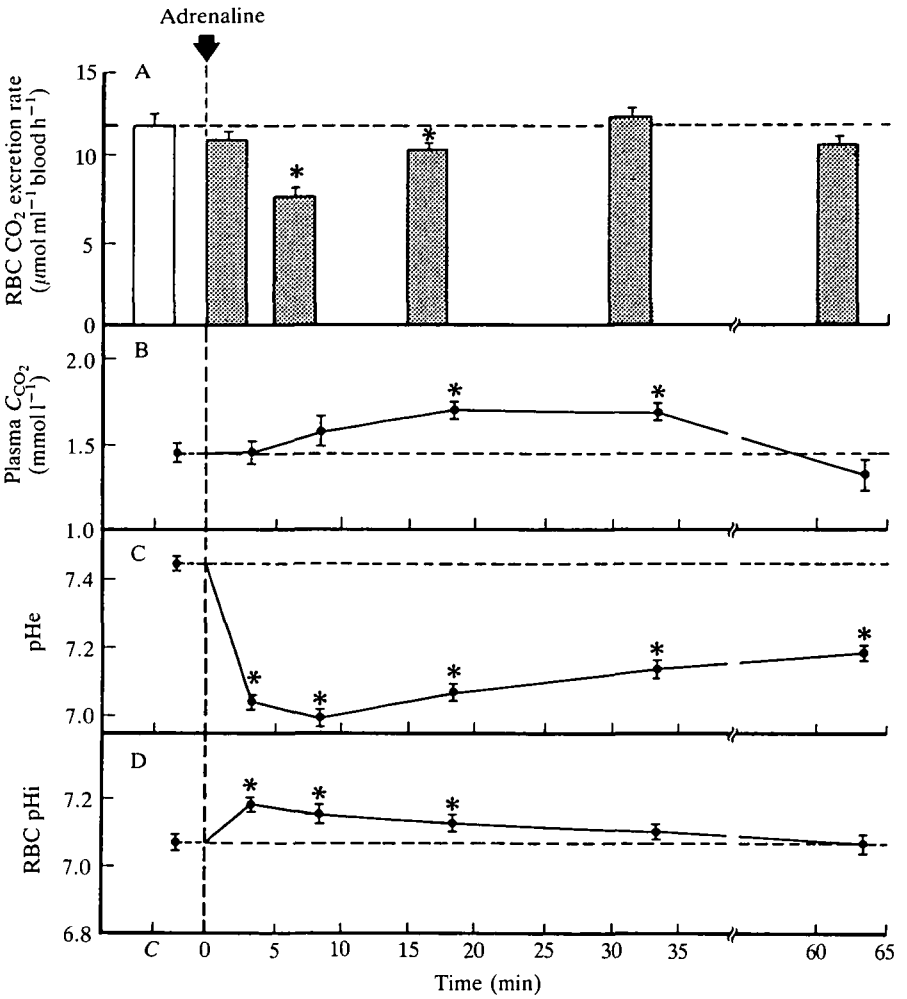

Fig. 6. The time-dependent effects of addition of adrenaline (10^{-6} mol^{-1}) to acidified blood (mean haematocrit=21 %) on (A) red blood cell (RBC) CO_2 excretion rate; (B) true plasma total CO_2 content; (C) extracellular pH; and (D) red blood cell intracellular pH. Adrenaline was added at time 0, and assays were run before addition (*C*), and at 0–3 min, 5–8 min, 15–18 min, 30–33 min and 60–63 min after addition. Acid–base status was measured at the immediate end of each assay. Means±1 S.E.M. (N=5). Asterisks indicate means significantly different (P<0.05) from the pre-adrenaline control.

assessing the role of carbonic-anhydrase-mediated HCO_3^- dehydration in photosynthesis (e.g. Tsuzuki *et al.* 1980; Shiraiwa and Miyachi, 1985). However, to our knowledge, they have not previously been used to monitor analogous processes in vertebrate RBCs. In developing a new *in vitro* assay for RBC CO_2 excretion, our prime concerns were that the assay be sensitive and reproducible, that the assay conditions be as representative of the *in vivo* situation as possible, and that the

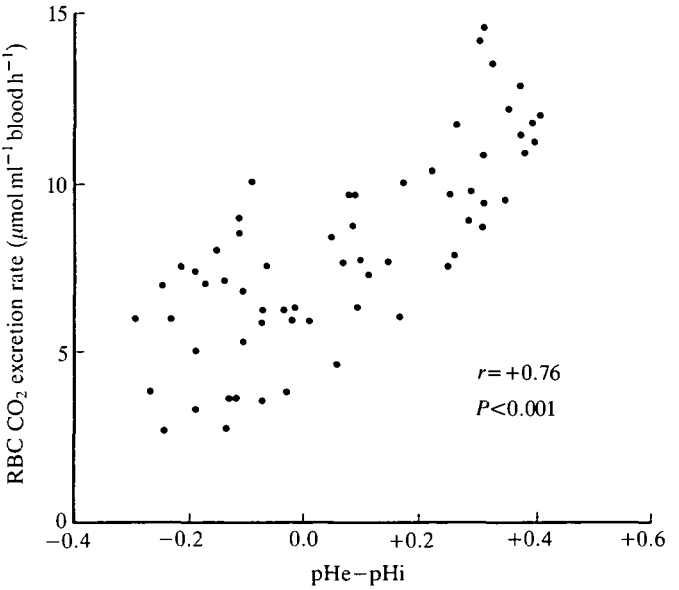

Fig. 7. Correlation between red blood cell (RBC) CO_2 excretion rate and the RBC transmembrane pH gradient (pHe – pHi) in 62 acidified blood samples treated with various doses (10^{-8}–10^{-6} mol^{-1}) of adrenaline or noradrenaline or 140 mmol l^{-1} NaCl. Rates were measured 5–8 min after addition, and the pHe−pHi gradients were measured at the immediate end of each assay.

assay should yield rates that are physiologically realistic. The present assay fulfills all three criteria.

By virtue of the use of radiotracer, the assay can measure rates in 3 min or less with a coefficient of variation (CV=S.D./mean) generally less than 10% for multiple assays run on a common pool. In our experience, the largest source of error is the accuracy of timing with respect to insertion and removal of the CO_2 trap. When particular care is taken with this step, the CV can be reduced below 5%. In contrast to other techniques (see Introduction), the assay is run with intact RBCs in their normal plasma (i.e. whole blood) under *in vivo* conditions of pHe, P_{CO_2} and plasma [HCO_3^-]. The exposure to a gas phase of zero P_{CO_2} simulates the conditions encountered by blood as it passes through the gills. At a normal haematocrit of 25%, the CO_2 excretion rate of whole blood in the assay at 10°C was about 14 μmol ml^{-1} h^{-1}. Assuming a normal resting cardiac output of 18 ml kg^{-1} min^{-1} for the rainbow trout at this temperature (Cameron and Davis, 1970; Kiceniuk and Jones, 1977), the estimated whole-animal $\dot{M}_{CO_2}$ would be about 15 mmol kg^{-1} h^{-1}. Measured *in vivo* $\dot{M}_{CO_2}$ was about 20% of this figure (Wright *et al.* 1986; Lin and Randall, 1990). Given the nature of the extrapolation, the probable greater diffusion barriers *in vivo* (e.g. unstirred layers) and the fact that the equilibrating water P_{CO_2} cannot be truly zero *in vivo*, owing to regional ventilation–perfusion mismatch, the agreement is not unreasonable.

We cannot entirely eliminate the possibility that there is some component of

diffusion limitation in the present assay. However, given the fact that the CO_2 excretion rate of whole blood in the assay appeared to be somewhat higher, rather than lower, than *in vivo* rates, this is unlikely to be a serious problem. The assay can measure experimentally induced elevations in rate well above normal control levels (e.g. Table 1, Fig. 3), as well as inhibitions below control levels (Figs 6, 7; Perry *et al.* 1991). We have also used the assay to measure large changes in CO_2 excretion rate of blood resulting from changes in the physiological condition of the fish from which the blood was sampled (C. M. Wood, unpublished results).

It is of interest to compare the performance of the present assay with that of the 'boat' assay, which has been commonly employed until now for trout blood (Haswell and Randall, 1976; Heming and Randall, 1982; Tufts *et al.* 1988). The boat assay itself can be run in as little as 1 min, but only after a 3–10 min settling period for initial pressure equilibration. As CO_2 is evolved into the gas phase, the back-pressure on the system progressively rises, so the assay quickly becomes non-linear. The influence of this back-pressure at the gas/fluid interface probably contributes to the foaming artefact reported to block CO_2 excretion by some workers (Haswell and Randall, 1976; Heming and Randall, 1982), but not by others (Tufts *et al.* 1988) when the assay is run with plasma or whole blood. The coefficient of variation has not been reported, but would appear to be about 10 % based on recalculation of published data. The most serious drawback of the boat assay is that the acid–base status of the sample is grossly perturbed. The blood is initially incubated in a pH 6.8 phosphate buffer (HCO_3^--free and Cl^--free) followed by acute exposure to 100 mmol l^{-1} $NaHCO_3$ plus 10 mmol l^{-1} NaOH. Normal plasma is no longer present, pH and P_{CO_2} must be greatly altered, and substrate concentration ($[HCO_3^-]$) is suddenly dramatically elevated. In the light of the present findings on the effects of acute HCO_3^- elevation on RBC CO_2 excretion rates (Fig. 3, discussed below), it is not surprising that the CO_2 excretion rate reported for trout blood of 25 % haematocrit by Tufts *et al.* (1988) was about 1000 μmol ml^{-1} h^{-1}, 70 times greater than the present values and 350 times greater than *in vivo* rates.

Factors affecting RBC CO_2 excretion

In the light of the large amounts of carbonic anhydrase normally present in erythrocytes (Maren, 1967; Perry and Laurent, 1990), it was initially surprising that whole-blood CO_2 excretion rates were only about fivefold higher than plasma rates. Nevertheless, this is in accord with findings of the boat assay for trout blood when wetting agents were used to overcome the foaming artefact (Heming and Randall, 1982), and also with the haematocrit *versus* $\dot{M}_{CO_2}$ relationship established by Perry *et al.* (1982) *in vivo*. The presence of large amounts of carbonic anhydrase in the RBCs is not expressed as vastly elevated whole-blood CO_2 excretion rates relative to that of plasma. This is because the rate of net HCO_3^- entry into the RBCs (*via* Cl^-/HCO_3^- exchange), rather than carbonic anhydrase activity itself, is thought to be the rate-limiting step in CO_2 excretion (Perry, 1986).

Nevertheless, if this were the only factor involved, we would expect a linear

relationship between haematocrit and CO_2 excretion rate, whereas this clearly did not occur (Fig. 3). Instead, rates tended to saturate at higher haematocrits. The only other examination of this relationship in fish blood, by Tufts *et al.* (1988) using the boat assay, found exactly the opposite, a virtually exponential increase with haematocrit (the rate increased more than eightfold as haematocrit rose from 10 to 30 %). Although it is difficult to account for such an exponential relationship, there are several possible explanations for the saturating relationship seen in the present study.

One obvious possibility is substrate limitation – i.e. depletion of plasma $[HCO_3^-]$ over the course of the assay by the greater number of RBCs. However, this appears to be unlikely, because measured plasma HCO_3^- levels at the end of the 3 min assay were essentially the same at all haematocrits within a pool. In any event, under equilibrium conditions the blood CO_2 excretion rate was only marginally dependent on plasma $[HCO_3^-]$ (Fig. 5). A more likely explanation is some sort of physical blocking phenomemon associated with more concentrated RBCs – e.g. interference with substrate access to Cl^-/HCO_3^- exchange sites, a greater volume occupied by unstirred layers on the RBC surfaces, viscosity effects on convective mixing or lower diffusion rates through the RBCs or at the gas phase/blood interface (Gros and Moll, 1971; Klocke, 1988). It would be interesting to know whether such effects occur *in vivo* or are an artefact of the assay conditions.

The dependence of the CO_2 excretion rate on the pre-equilibration P_{CO_2} of the blood (Table 1) was the expected result, since the P_{CO_2} gradient from RBC to gas phase provides the net driving force for the CO_2 excretion process in the assay, as *in vivo*. The relative independence of the CO_2 excretion rate from the pre-equilibration plasma $[HCO_3^-]$ (Fig. 5), but its sensitivity to acutely altered plasma $[HCO_3^-]$ (Fig. 3), is more interesting. In the blood of resting trout (i.e. no adrenergic stimulation) under equilibrium conditions, HCO_3^- and Cl^- (and H^+) are probably passively distributed across the RBC membrane according to a Gibbs–Donnan equilibrium (Wood *et al.* 1982; Heming *et al.* 1986; Nikinmaa *et al.* 1987). Thermodynamically, there will be no driving force for net HCO_3^- entry, regardless of the plasma $[HCO_3^-]$, until the assay is actually started by exposure to a gas phase P_{CO_2} of zero, thereby lowering intracellular $[HCO_3^-]$ by catalysed conversion to CO_2. At this point, the HCO_3^- distribution ratio will no longer be dictated by the membrane potential, and net HCO_3^- entry/Cl^- exit will occur because of the electrochemical disequilibrium. This disequilibrium will tend to be slightly greater with higher extracellular $[HCO_3^-]$, explaining the shallow dependence of CO_2 excretion rate on plasma $[HCO_3^-]$ (Fig. 5).

In contrast, when the plasma $[HCO_3^-]$ is acutely raised at the start of the assay, the assay starts under non-equilibrium conditions. Immediately, there is a large driving force for net HCO_3^- entry, and the CO_2 excretion rate is very sensitive to this non-equilibrium plasma HCO_3^- level (Fig. 3). This phenomenon undoubtedly accounts for the very high CO_2 excretion rates in the boat assay (see above), where the blood is acutely exposed to 100 mmol l^{-1} $NaHCO_3$.

The influence of catecholamines on RBC CO_2 excretion

When trout blood was acidified to simulate the metabolic acidosis normally seen after exhaustive exercise (ΔH_m^+=7 mmol l^{-1}; Turner *et al.* 1983; Milligan and Wood, 1986), the addition of adrenaline or noradrenaline (10^{-8}–10^{-6} mol^{-1}) caused a clear inhibition of RBC CO_2 excretion (Figs 6, 7). This is in accord with the original observation (unpublished data of S. F. Perry and T. A. Heming presented by Wood and Perry, 1985), and supports the 'CO_2 retention theory'. The time course of the inhibitory effect (maximal at 5–8 min after catecholamine addition, persistent at 15–18 min and over by 30–60 min; Fig. 6) is not dissimilar to the time course of Pa_{CO_2} elevation *in vivo* after exhaustive exercise in the rainbow trout (Wood and Perry, 1985).

The negative results of Tufts *et al.* (1988) are probably attributable to a combination of factors: the limitations of the boat assay discussed earlier, the transitory nature of the response (Fig. 6), and the general lack of response at normal blood pH (see also Perry *et al.* 1991, for additional data on this point). Our original reasons for tests at reduced pHe were three-fold: (i) this is the relevant situation *in vivo* when 'CO_2 retention' occurs; (ii) the original experiments of S. F. Perry and T. A. Heming (unpublished results) reported by Wood and Perry (1985) were conducted with trout RBCs in Cortland saline, which probably had a lower pHe; and (iii) it is now known that adrenergic activation of RBC Na^+/H^+ exchange (the RBC pHi regulatory response) is markedly sensitized at acidotic pHe values (Nikinmaa *et al.* 1987; Borgese *et al.* 1987; Cossins and Kilbey, 1989; Milligan *et al.* 1989).

The magnitude of the inhibition of RBC CO_2 excretion by catecholamines was clearly correlated with the magnitude of the pHe–pHi response (Fig. 7). It is possible that the two responses share a common pathway, and there are theoretical reasons why the two could be mechanistically related. However, the time courses were certainly not identical (i.e. the pHe–pHi effect lasted long after the inhibition of RBC CO_2 excretion had disappeared; Fig. 6), and correlation (Fig. 7) does not prove causation. The mechanism of the inhibitory response is addressed in the subsequent paper (Perry *et al.* 1991).

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