THE ROLE OF FISH ERYTHROCYTES IN TRANSPORT AND EXCRETION OF CARBON DIOXIDE

by

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ABSTRACT

Involvement of the red blood cells of fish in CO_2 exchange was investigated by examination of the CO_2 transport properties of fish blood, of ion movements (HCO_3^-, Cl^-, H^+) across the red cell membrane, and of erythrocyte carbonic anhydrase activity. Adrenergic modulation of erythrocyte function *in vitro* and the effects of catecholamines on branchial CO_2 exchange *in vivo* also were studied.

Approximately 92% of the CO_2 content of venous blood of rainbow trout (*Salmo gairdneri*) was HCO_3^-, which at an haematocrit of 25% was distributed between the plasma water space and the red cell water space in a ratio of about 9:1. Plasma HCO_3^- accounted for some 82% of the CO_2 excreted during branchial blood transit, while erythrocyte HCO_3^- accounted for only 9%. The remainder of CO_2 excreted at the gills was derived from, in descending order of importance, carbamino compounds ($R-NHCO_2^-$), molecular CO_2, and CO_3^{2-}.

The erythrocyte of rainbow trout was freely permeable to HCO_3^-, Cl^- and H^+, all of which were distributed passively across the red cell membrane. HCO_3^- traversed the erythrocyte membrane in an one-for-one exchange with Cl^- via a SITS-sensitive mechanism analogous to the Band 3 anion exchange pathway of mammalian red cells. The transmembrane equilibrium distributions of HCO_3^-, Cl^- and H^+, however, were complicated by the presence of a cell nucleus. The nuclear compartment of trout erythrocytes

appeared to be more acidic and to contain less HCO_3^- and Cl^- than the cytosol.

The kinetics of the uncatalysed HCO_3^-:CO_2 conversion were found to be at least one order of magnitude too slow to account for the observed branchial CO_2 movements. Fish erythrocytes however, contained sufficient carbonic anhydrase activity to catalyse the interconversion of HCO_3^- and CO_2, increasing the rate of reaction by several orders of magnitude.

Fish plasma contained inhibitors of carbonic anhydrase which were active against the enzyme activity of both erythrocyte and gill homogenates. These inhibitors lacked access to intracellular carbonic anhydrase and had no direct effect on membrane transport of anions. It is suggested that these inhibitors probably function to immobilize carbonic anhydrase released into the plasma during the normal destruction of erythrocytes or during injury, but have no effect on intact red cells.

These data, together with evidence that the basolateral membrane of the gill is largely impermeable to HCO_3^- (Perry *et al.* 1982), clearly indicate that the principal pathway for CO_2 excretion in fish is via the movement of plasma HCO_3^- into the red cell by way of a 'chloride shift'. This bicarbonate then is rapidly dehydrated to form CO_2 in the presence of erythrocyte carbonic anhydrase. The resultant CO_2 diffuses down its concentration gradient out of the red cell and across the gill epithelium. CO_2 loading of fish blood during tissue capillary transit involves a simple reversal of these transport and chemical mechanisms. The present information conclusively

refutes the gill model of CO_2 excretion (Haswell *et al.* 1980) which asserts that fish erythrocytes have no functional role in branchial CO_2 exchange. Evidence was found which indicated that the original studies of Haswell and coworkers suffered from technique-related artifacts.

Catecholamines had profound effects on both erythrocyte function and branchial CO_2 exchange. Beta-adrenergic agonists appeared to stimulate coupled Na^+/H^+ and Cl^-/HCO_3^- exchangers on the cell membrane of trout erythrocytes, similar to the ion exchangers involved in volume-regulatory ion movements in amphibian red cells (Cala 1980). The adrenergic responses of rainbow trout erythrocytes included a net cellular gain of Na^+, Cl^- and H_2O, a net cellular loss of H^+ and HCO_3^-, a pronounced cell swelling, and a functional reduction in net HCO_3^- flux through the red cell. *In vivo*, these adrenergic responses were accompanied by a transient reduction in CO_2 excretion, an increase in body CO_2 stores, and a disruption of the HCO_3^-:CO_2 chemical equilibrium in arterial blood immediately downstream of the gill. Oxygen uptake was unaffected by adrenaline. The adrenergic responses of fish red cells probably are important in regulating erythrocyte pH during periods of stress, and hence serve to maintain O_2 transport to the tissues under such conditions. At the same time, these responses slow net HCO_3^- flux through the red cells during branchial blood transit, and thus serve to maintain an extracellular pool of HCO_3^- in stress which then may be used to enhance the intracellular buffering capacities of other tissues.

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GENERAL INTRODUCTION

Carbon dioxide is produced by tissues as an end-product of oxidative metabolism. Transport of CO_2 in blood and its exchange at the gas exchange organ is more complex chemically than that of other respiratory gases (O_2, NH_3) and is less well understood, especially in the lower vertebrates. The aim of this thesis was to examine the mechanisms underlying the transport and excretion of CO_2 in fish, with emphasis on the role of the red blood cell in those processes.

CO_2 is both relatively soluble and readily hydrated in aqueous solutions. Carbonic acid, formed by CO_2 hydration, dissociates to yield hydrogen, bicarbonate and carbonate ions (for review see Edsall 1969) as follows

(1) $CO_2 + H_2O \leftrightarrows H_2CO_3 \leftrightarrows H^+ + HCO_3^- \leftrightarrows 2H^+ + CO_3^{2-}$.

The reaction

(2) $CO_2 + OH^- \leftrightarrows HCO_3^- \leftrightarrows H^+ + CO_3^{2-}$

also is important at pH values of 8 and above, as are found in fish plasma. Moreover, CO_2 binds directly to the terminal amino groups of proteins, including haemoglobin, forming carbamino compounds ($R-NHCO_2^-$). Bicarbonate ion is the predominant species present within the physiological pH range of vertebrate blood.

As a result of the aqueous CO_2-bicarbonate-carbonate equilibrium, carbon dioxide behaves as a weak acid in biological systems; increases in CO_2 tension (P_{CO_2}) decrease pH whilst decreases in P_{CO_2} increase pH. A balance between CO_2 production and excretion is necessary, therefore, to maintain a stable internal pH. Regulation of internal acid-base status is critical in preserving the 'correct' net charge state of proteins (Reeves 1977), which in turn is crucial to the biochemical functioning of proteins.

In general, vertebrates regulate their acid-base status by adjustments to blood CO_2 levels. Air-breathing vertebrates typically regulate blood pH in the short term by modulating blood P_{CO_2} via changes in ventilation (for review see Comroe 1974 and Davenport 1974). Hyperventilation increases the diffusion gradient for dissolved CO_2 between aveolar gas and pulmonary blood, and thus decreases blood P_{CO_2} and raises blood pH. Hypoventilation has the opposite effect. Long-term pH regulation in air-breathers is achieved by adjustments to blood HCO_3^- levels mainly via renal function (Davenport 1974).

A similar tight coupling between ventilation, CO_2 excretion and acid-base regulation is absent in water-breathing vertebrates. The oxygen capacitance coefficient and hence the oxygen concentration of water is only about 0.03-0.05 times that of air, while the CO_2 capacitance coefficient of water is some 20-30 times greater than that of O_2 (Dejours 1975). Consequently, the ventilatory strategies of water-breathers are directed toward maintenance of adequate O_2 uptake rather than modulation of CO_2 excretion (for theoretical discussion see Rahn 1966). Indeed, blood O_2 levels set the main ventilatory drive in

water-breathers (Dejours 1973, Randall and Jones 1973), rather than blood CO_2 levels as occurs in air-breathers (Comroe 1974). As a direct consequence of the difference between the oxygen and carbon dioxide capacitance coefficients of water, ventilatory water flows that are adequate to satisfy O_2 demands hyperventilate the gills with respect to CO_2 excretion. In other words, dissolved CO_2 is washed out of fish blood during branchial blood transit. As a result, blood P_{CO_2} levels in fish are extremely low (2-5 torr above ambient water levels, Rahn 1966, Randall 1970a), are dependent primarily upon diffusion and reaction kinetics at the gill, and are independent of all but immense changes in ventilatory flow (Cameron and Polhemus 1974, Wood and Jackson 1980). A corollary of this loose coupling between ventilation and CO_2 excretion is that fish regulate blood pH by modulating blood HCO_3^- levels while allowing blood P_{CO_2} to parallel environmental levels. Retention of plasma HCO_3^- increases extracellular pH while decreases in plasma HCO_3^- have the opposite effect. The associated respiratory protons (see equation 1) are free to cross the gill epithelium (McWilliams and Potts 1978) and they do so independent of CO_2 movements (van den Thillart *et al.* 1983). Regulation of plasma HCO_3^- levels as a means of pH regulation has been observed in response to temperature changes (Randall and Cameron 1973), environmental hypercapnia (Cameron and Randall 1972, Eddy *et al.* 1977), and acid stress (McDonald *et al.* 1980). The mechanisms involved in retention and/or resorption of HCO_3^- are not yet fully understood. Moreover, it is uncertain whether the regulatory

mechanism acts upon HCO_3^-, OH^-, H^+, NH_4^+ or a combination of these ions. Excretion of H^+ or retention of OH^- are functionally the same as retention of HCO_3^- (see equations 1 and 2). Excretion of NH_4^+ effectively removes a H^+ since ammonia is produced metabolically as NH_3.

The gill is the principal site of CO_2 excretion in trout. Thus, the gill plays a paramount role in acid-base and ionic regulation (Cameron 1978a, Randall *et al.* 1982, Heisler 1984). Other organs, including the kidney, gut and skin, are generally of little importance in pH regulation. Two conflicting models have been developed to describe the pathway for CO_2 excretion in fish, and hence the relationship between branchial CO_2 movements and ionic/pH regulation.

The original theory, the red cell model of CO_2 excretion, combines the accepted mammalian information regarding CO_2 excretion (cf. Roughton 1964) with the known $Na^+/H^+(NH_4^+)$ and $Cl^-/HCO_3^-(OH^-)$ exchangers present on the apical membrane of the gill epithelium (Maetz 1971, Evans 1975). In this model, plasma HCO_3^- enters the red cell during branchial blood transit where it is catalytically converted to CO_2 in the presence of erythrocyte carbonic anhydrase. The resultant CO_2 diffuses down its concentration gradient out of the red cell and across the gill epithelium. A small proportion of this CO_2 (3-4%, Cameron 1976) is back-converted to HCO_3^- and H^+ in the presence of gill carbonic anhydrase. These ions then serve as counter ions for the apical ion exchangers. Modulation of these exchangers is believed to be the primary pH-regulating mechanism in fish (Cameron 1978a, Heisler 1984). The rate-limiting step in CO_2

excretion in this model is the entry of plasma HCO_3^- into the erythrocyte. The possibility that modulation of this step, bicarbonate entry into the red cell, also is involved in acid-base regulation largely has been ignored.

A more recent theory, the gill model of CO_2 excretion (Haswell and Randall 1978, Haswell *et al.* 1980), has been developed around the assertion that fish erythrocytes are functionally impermeable to HCO_3^- *in vivo* (Haswell and Randall 1976, Haswell *et al.* 1978). In this model, plasma HCO_3^- and H^+ enter the gill epithelium directly and then either serve as counter ions for the apical ion exchangers or are catalytically converted to CO_2 by gill carbonic anhydrase and excreted as such. The rate-limiting step in CO_2 excretion is proposed to be the entry of plasma HCO_3^- into the gill epithelium; CO_2 excretion in this model is independent of erythrocyte function. Acid-base regulation involves a combination of modulation of the apical ion exchangers and the entry of plasma HCO_3^- across the basal membrane of the gill.

The objectives of the present studies were to examine CO_2 transport and excretion in fish in light of the red cell and gill theories of CO_2 exchange. In so doing, the role of the erythrocyte in catalysing the interconversion of HCO_3^- and CO_2 was studied. The functioning of the red blood cell in this regard entails the net movement of HCO_3^- across the red cell membrane, as well as, the activity of intracellular carbonic anhydrase. The role of catecholamines in modulating the net flux of HCO_3^- through the red cell, and hence in regulation of acid-

base status, also was investigated *in vitro* and *in vivo*. Overall, these studies shed light on the relationship between CO_2 movements and acid-base regulation in lower vertebrates.

GENERAL MATERIALS AND METHODS

Rainbow trout (*Salmo gairdneri*), weighing between 200 and 500 g, were obtained from Sun Valley Hatchery (Mission, B.C.). The fish were held outdoors at the University of British Columbia in natural light in flowing, dechlorinated Vancouver tap water. Temperature of this water varied seasonally between 6 and 13 C. The fish were fed to satiation daily with a commercial fish food. Food was withheld for at least 24 h prior to, as well as, during all studies.

Experimental animals were anaesthetized in an aerated solution of tricaine methane sulphonate (66.7 $mg \cdot L^{-1}$ MS-222, pH buffered to about 7.5 with 133.3 $mg \cdot L^{-1}$ $NaHCO_3$) maintained at the ambient water temperature. The animals then were transferred to a surgical table where an aerated anaesthetic solution (50 $mg \cdot L^{-1}$ tricaine methane sulphonate, 100 $mg \cdot L^{-1}$ $NaHCO_3$) at 5-10 C was recirculated over the gills. The dorsal aorta and, in some cases, the ventral aorta were cannulated through the mouth . A catheter (Sovereign indwelling canine catheter, 2 inch, 18 gage) was used to make a blind puncture of the dorsal aorta in the caudad direction at the midline of the branchial basket between the first and second gill arches. A cannula of polyethylene surgical tubing (PE-50) was fed down the catheter and into the aorta. The catheter then was removed leaving the cannula chronically implanted in the aorta. The cannula exited the fish through the roof of the mouth in front of the nares by way of a

flanged section of surgical tubing (PE-200). The ventral aorta was cannulated in a similar manner. Cannulation of the ventral aorta involved a blind puncture of the vessel made laterally beneath the tongue between the first and second gill arches. Ventral aortic cannulae exited the fish through the floor of the mouth by way of a flanged section of surgical tubing (PE-200). Both dorsal and ventral aortic cannulae were secured with sutures to the walls of the oral cavity. Cannulae were flushed daily with Cortland saline (Wolf 1963, 10,000 U.S.P. units·L^{-1} sodium heparin) to maintain their patency. Following surgery, the fish were transferred to light-proof acrylic boxes supplied with a 3 $L \cdot min^{-1}$ flow of aerated water. Fish were allowed at least 48 h of post-operative recovery before studies were undertaken.

Data Treatment and Presentation

Statistical treatment of data included regression analysis by the method of least squares, analysis of covariance to test homogeneity of regression coefficients, and standard and paired Student's t-tests (Steel and Torrie 1960, Snedecor and Cochran 1967). In all cases, differences present at the 5% level of probability ($P \leq 0.05$) were judged to be significant. Experimental data are presented as arithmetic means $\pm$ 1 standard error (S.E.), whereas statistically-derived data (ie. regression coefficients) are presented with their 95% confidence intervals. Mean pH values were calculated directly, as recommended by Boutilier and Shelton (1980), without transformation back to H^+ concentrations.

Chapter 1.

CO_2 TRANSPORT PROPERTIES OF BLOOD OF *SALMO GAIRDNERI*

A primary physiological function of vertebrate blood is transport of the respiratory gases, carbon dioxide and oxygen, between the gas exchange organ(s) and the tissues. The effectiveness of this transport is determined not only by the flow of exchange media (air/water and blood) through the gas exchange organ(s), but also by the transport properties of the blood under consideration. The objective of this chapter is to elucidate the CO_2 transport properties of *Salmo gairdneri* blood in order to provide a framework upon which subsequent examinations of piscine gas exchange function can be made.

Carbon dioxide is transported in blood primarily in chemically-bound states, as dissociated salts of carbonic acid. Bicarbonate is the predominant species present within the physiological pH range of vertebrate blood. By convention, the relationship between dissolved and bound CO_2 is expressed in the form of a Henderson-Hasselbach equation

$$(3) \quad pH = pK_{app} + \log \left(C_{CO_2} / (\alpha CO_2 \cdot P_{CO_2}) - 1 \right)$$

where pK_{app} is the apparent dissociation constant of carbonic acid, C_{CO_2} is total CO_2 content, αCO_2 is the solubility coefficient of CO_2, and P_{CO_2} is CO_2 partial pressure. The derivation and limitations of this equation are dealt with at

length by Albers (1970). Bound CO_2 is represented by the difference between C_{CO_2} and $[\alpha CO_2 \cdot P_{CO_2}]$, and generally is termed simply HCO_3^-. For the purpose of this thesis, however, bound CO_2 is referred to as apparent bicarbonate or $bHCO_3^-$ since it includes all species of chemically-bound CO_2. Given appropriate values for pK_{app} and αCO_2, equation 3 can be used to calculate C_{CO_2}, $bHCO_3^-$, P_{CO_2} or pH when any two of C_{CO_2}, P_{CO_2} and pH are measured simultaneously. Values of pK_{app} and αCO_2 have been measured for mammalian plasma with great accuracy (Severinghaus *et al.* 1956, Bartels and Wrbitzky 1960, Siggaard-Andersen 1962, Austin *et al.* 1963, Rispens *et al.* 1968, among others). Similar values are absent for most fish species. Simple extrapolation of mammalian values is not satisfactory (Nicol *et al.* 1983, Boutilier *et al.* 1984a), considering the length of temperature extrapolation required and the differences in ionic strength between fish plasma (0.15 - 0.70, see Albers 1970 for references) and human plasma (0.167).

CO_2 behaves as a weak acid in aqueous solution in as much as increases in CO_2 tension necessarily reduce pH via equation 3, whilst reductions in CO_2 tension necessarily increase pH. A corollary of this CO_2:pH relationship is that blood is acidified during tissue capillary transit and is alkalinized during branchial/pulmonary capillary transit. Variations in blood pH resulting from CO_2 transport are buffered primarily by blood proteins, the most important of which is haemoglobin (Albers 1970, Comroe 1974). This buffering action facilitates formation of the dissociated moiety of carbonic acid via the 'Law of Mass Action', and thus is an important determinant of blood CO_2

transport properties.

The buffering capacity of haemoglobin enhances CO_2:HCO_3^- conversion inside the red blood cell. In mammalian erythrocytes, intracellular CO_2 reactions are followed by exchange of HCO_3^- between the red cell and extracellular plasma (Roughton 1964). Mammalian red cells are freely permeable to most anions and H^+, all of which are distributed passively across the cell membrane according to a Gibbs-Donnan equilibrium (Jacobs and Stewart 1947, Gunn *et al.* 1973, Freedman and Hoffman 1979). Little is known of the Donnan equilibrium in fish erythrocytes. Donnan ratios for chloride and/or $bHCO_3^-$ have been determined in a small number of fishes (Ferguson *et al.* 1938, Ferguson and Black 1941, Albers *et al.* 1969, Haswell *et al.* 1978, Wood *et al.* 1981). As in mammalian erythrocytes, the Donnan ratios of fish erythrocytes vary inversely with plasma pH. For the most part, however, Donnan ratios of fish red cells do not agree well with values predicted on the basis of a passive distribution (Jacobs and Stewart 1947, Freedman and Hoffman 1979). A passive distribution of anions and H^+ in fish erythrocytes thus is open to question, especially in light of the polemical work of Haswell and Randall (1976, 1978) indicating that fish red cells are functionally impermeable to HCO_3^-.

Studies in this chapter examined the CO_2 transport properties of rainbow trout blood in terms of *in vitro* dissociation curves, that is, the relationship between CO_2 content and partial pressure. The physico-chemical properties of blood with respect to CO_2 transport, pK_{app} and αCO_2, also were

determined. Partitioning of C_{CO_2} between plasma and erythrocytes, and the effects of CO_2 on transmembrane ion equilibria were investigated in detail. Finally, *in vivo* determinations of arterial and venous CO_2 and acid-base states were coupled with results of the *in vitro* studies to produce a model of arterial and venous CO_2 transport in rainbow trout.

MATERIALS AND METHODS

A. Solubility of CO_2 and Apparent Dissociation Constant of H_2CO_3

CO_2 solubility coefficients were determined using separated plasma obtained by centrifugation of blood withdrawn from the dorsal aortas of chronically cannulated rainbow trout (see General Materials and Methods). One-mL aliquots of this plasma were acidified slowly to about pH 2.5 by addition of concentrated lactic acid and then were transferred to 50-mL tonometer flasks. All samples were kept on ice until used. Acidified samples were equilibrated at 5, 10 and 15 C with a humidified gas mixture containing 40% CO_2 in air delivered by a Wostoff gas mixing pump. Samples were gassed for at least 60 min. Total CO_2 content (C_{CO_2}) of the plasma then was measured in duplicate with a CO_2 electrode (Radiometer, type E5036) using the methods of Cameron (1971). Partial pressure of CO_2 (P_{CO_2}) in the plasma was calculated from that in the gas mixture taking into account the prevailing barometric pressure, water vapour pressure, equilibration temperature and CO_2 content of air (0.033%, Glueckauf 1951). CO_2 solubility coefficients (αCO_2) were calculated from Henry's Law, where

(4) $$\alpha CO_2 = C_{CO_2} / P_{CO_2} .$$

As a check of the technique, CO_2 solubility coefficients of normal distilled water and of distilled water acidified as above

with lactic acid also were measured.

The apparent dissociation constant of carbonic acid (pK_{app}) was determined using a mixed pool of whole blood obtained from the dorsal aortic cannulae of several rainbow trout. Two-mL aliquots of pooled blood were distributed to 50-mL tonometer flasks on ice and then were equilibrated at 10 C with humidified gas mixtures containing 0.2, 0.5 and 1.0% CO_2 in air (Wostoff pumps). Samples were gassed for at least 60 min and then were centrifuged anaerobically at room temperature for 2-4 min. Plasma pH was measured anaerobically with a glass capillary electrode (Radiometer, type G297/G2) thermostatted at the equilibration temperature (10 C) and calibrated before and after each pH determination with precision buffers (Radiometer, S1500, S1510). Plasma C_{CO_2} and P_{CO_2} were determined as before. Values of pK_{app} were calculated from measured pH, C_{CO_2}, P_{CO_2} and aCO_2 values using a rearrangement of the Henderson-Hasselbach equation for carbonic acid

$$(5) \qquad pK_{app} = pH - \log([C_{CO_2}/(aCO_2 \cdot P_{CO_2})] - 1) \ .$$

B. *In Vitro* Respiratory and Acid-base Characteristics of Blood

Blood samples were withdrawn from the dorsal aortic cannulae of several rainbow trout and were pooled on ice. Two-mL aliquots of pooled blood were equilibrated at 5, 10 and 15 C with humidified gas mixtures containing 0.25, 0.50, 1.00 and 2.00% CO_2 in air (Wostoff pumps). Samples were gassed for at least 45 min before their respiratory and acid-base states were assessed.

Blood pH was measured with Radiometer glass capillary

electrodes thermostatted at the prevailing equilibration temperature and calibrated as described previously. Intraerythrocytic pH was measured on an anaerobically-obtained pellet of red cells using the freeze-thaw techniques of Zeidler and Kim (1977) and employing the same pH electrode as above. Blood P_{CO_2} was measured using a CO_2 electrode (Radiometer, type E5036) thermostatted at the prevailing equilibration temperature and preconditioned to the expected P_{CO_2} value as recommended by Boutilier *et al.* (1978). Each P_{CO_2} determination was bracketed with calibration gases delivered via Wostoff pumps. C_{CO_2} of blood and of plasma obtained from the blood by anaerobic centrifugation at room temperature was measured as described in the CO_2 solubility study. The apparent bicarbonate concentration ($bHCO_3^-$) of samples was calculated from measured C_{CO_2}, P_{CO_2} and αCO_2 values as

$$(6) \qquad bHCO_3^- = C_{CO_2} - (\alpha CO_2 \cdot P_{CO_2}) \ .$$

It should be noted that $bHCO_3^-$ calculated in this way does not distinguish between HCO_3^-, CO_3^{2-} or carbamino ($R\text{-}NHCO_2^-$). Haematocrit measurements were made in triplicate. Water contents of blood and plasma were determined by weighing samples to the nearest 0.1 mg, first wet and then again after the samples had been dried to constant weight at 60 C. Additional aliquots of equilibrated blood and plasma were stored frozen for later analyses of ions. Cl^- concentrations of blood and plasma were measured with a Butler-Cotlove amperometric titrator using the

methods of Cotlove (1963). Plasma Na^+, K^+, Mg^{2+} and Ca^{2+} concentrations were measured by flame photometry as described by Annio (1964) using a Perkin-Elmer atomic absorption spectrophotometer (model 2380). Lactate was assayed enzymatically (Sigma procedure no. 826-UV); blood samples used in lactate assays were deproteinated with chilled perchloric acid immediately upon sampling. Intraerythrocytic levels (IL) of Cl^-, $bHCO_3^-$ and water were calculated from the relation,

$$(7) \quad IL = [\text{blood value} - (\text{plasma value})(1 - \text{haematocrit} \cdot 10^{-2})] / (\text{haematocrit} \cdot 10^{-2}) .$$

Ion concentrations were expressed per kilogram of red cell or plasma water in calculation of Donnan ion ratios.

The CO_2-combining properties of separated plasma were examined in an independent series of experiments. Separated plasma was obtained by centrifugation of blood withdrawn from dorsal aortic cannulae. One-mL aliquots of separated plasma were equilibrated at 10 C with humidified gas mixtures containing 0.2, 1.0 and 2.0% CO_2 in air (Wostoff pumps). Samples were gassed for at least 60 min. Plasma pH and C_{CO_2} then were measured as described above. Plasma P_{CO_2} was calculated from that in the gas mixtures as described in the CO_2 solubility study.

C. Arterial and Venous Transport of CO_2 *In Vivo*

Blood samples were withdrawn simultaneously from both the dorsal and ventral aortas of cannulated rainbow trout. Ambient water temperature was 10 C. A portion of each blood sample was

used to measure haematocrit in duplicate. The remainder was centrifuged anaerobically at room temperature for 1-2 min. All subsequent measurements were made on plasma. Arterial and venous values of C_{CO_2} and pH were measured as before. P_{CO_2} values were calculated from measured C_{CO_2}, pH, pK_{app} and αCO_2 values using a rearrangement of the Henderson-Hasselbach equation for carbonic acid, where

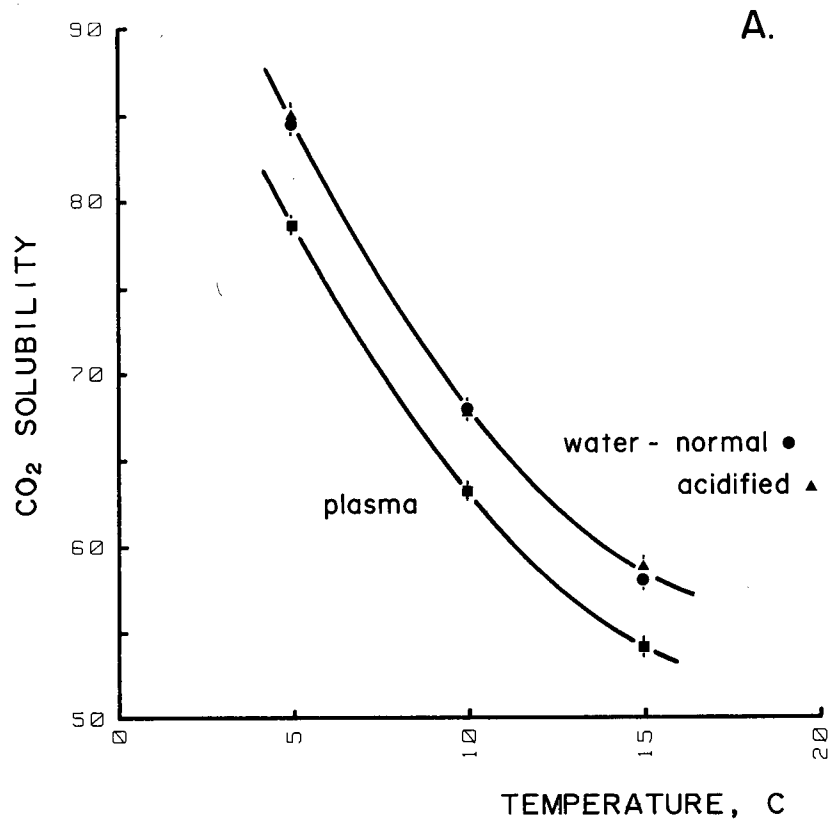
(8) $$P_{CO_2} = C_{CO_2} / ([10^{pH-pK_{app}} + 1] \cdot \alpha CO_2) .$$

Values of $bHCO_3^-$ were calculated using equation 6.

RESULTS

CO_2 solubility coefficients (αCO_2) of rainbow trout plasma and distilled water are presented in Figure 1 and Table 1. The mean measured values for water were within 2.7 $\mu mol \cdot L^{-1} \cdot torr^{-1}$ of those reported by Murray and Riley (1971). Comparable data for the αCO_2 of trout plasma were not found in the literature. CO_2 solubility was a function of temperature and ionic strength; αCO_2 decreased when either temperature or ionic strength increased (distilled water, I=0; plasma, I=0.15). Comparison of αCO_2 values of normal and acidified distilled waters (Fig. 1, Table 1) suggests that the acidification process had little direct effect on measured αCO_2 values. However, Pleschka and Wittenbrock (1971) noted that acidification of the plasma of dogfish (*Scyliorhinus canicula, S. stellaris*) resulted in precipitation of plasma proteins, altering plasma osmolarity and hence its CO_2 solubility. In the present study, initial attempts to acidify trout plasma also resulted in precipitation of proteins which occurred sporadically, independent of the rate of acidification or plasma temperature. Protein precipitation was found to be related to the use of heparin as an anticoagulant. In an independent experiment, it was found that acidification of plasma containing more than 10,000 U.S.P. units$\cdot L^{-1}$ sodium heparin resulted in precipitation of proteins, while no precipitation was present in plasma containing less than that concentration of heparin. This effect probably was attributable

Figure 1. Physico-chemical parameters pertaining to CO_2 transport in *Salmo gairdneri* blood. Panel A. CO_2 solubility ($\mu mol \cdot L^{-1} \cdot torr^{-1}$) of water and trout plasma at 5, 10 and 15 C. Values are means $\pm$ 1 S.E. (n=6). Least squares regression lines are presented in Table 1. Panel B. Effect of pH on the appparent dissociation constant of carbonic acid (pK_{app}) in trout plasma at 10 C. Least squares regression line is pK_{app} = 6.974 - 0.098 pH (r=-1.000, n=3). Values are means $\pm$ 1 S.E. (n=8).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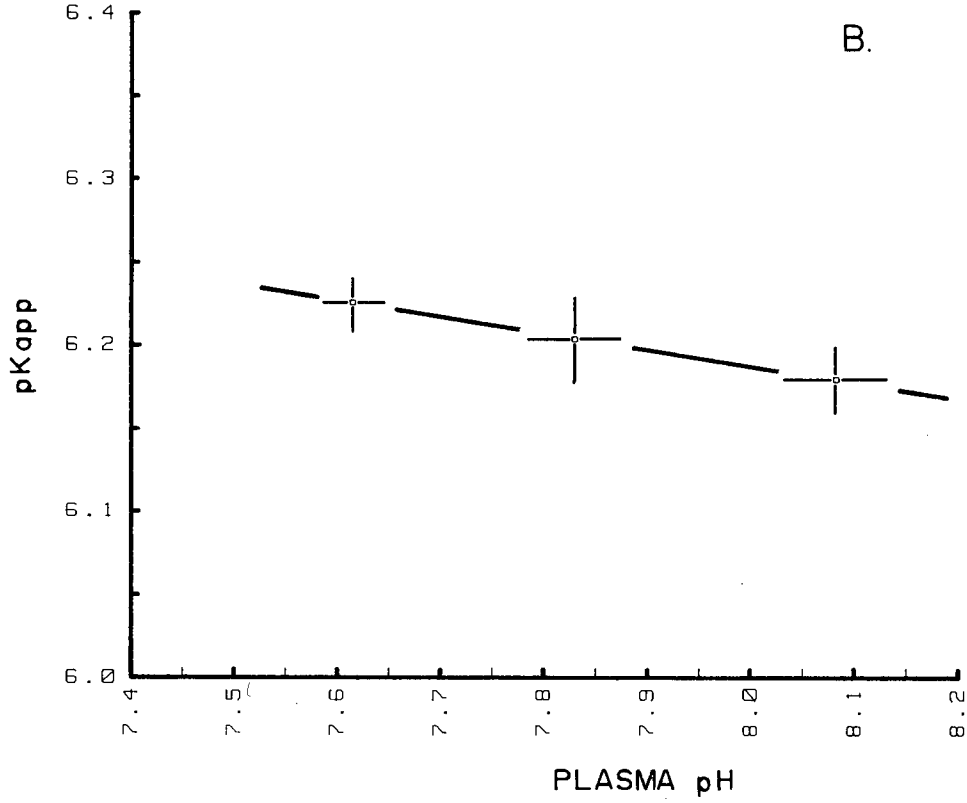
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8.2
PLASMA pH

Table 1. Least squares regression lines describing the effect of temperature (T, C) on CO_2 solubility (αCO_2, $\mu mol \cdot L^{-1} \cdot torr^{-1}$) of water and *Salmo gairdneri* plasma, where $\alpha CO_2 = a + bT + cT^2$. Regression coefficients are presented ± 95% confidence intervals (n=18).

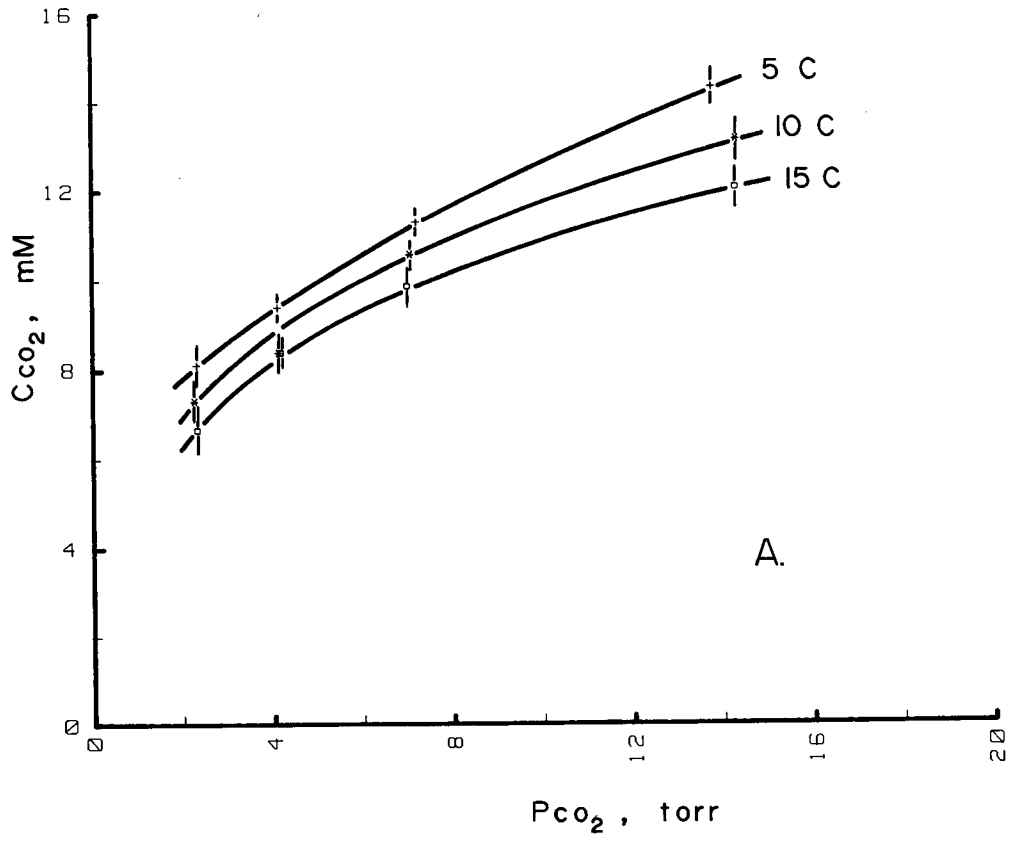
	a	b	c	r
distilled water	110.2 ± 2.6	-5.87 ± 0.59	0.163 ± 0.029	-0.998
acidified water	107.5 ± 4.7	-5.26 ± 1.07	0.131 ± 0.053	-0.995
plasma	100.5 ± 3.0	-5.08 ± 0.68	0.132 ± 0.034	-0.997

to the action of heparin on the structure of plasma lipoprotein aggregates (Loewe *et al.* 1953). Use of heparin was minimized in studies reported here and no protein precipitation was evident.

The apparent dissociation constant of carbonic acid (pK_{app} = -log $[bHCO_3^-][H^+]$ / [free CO_2]) in trout plasma varied with plasma pH (Fig. 1); pK_{app} decreased when plasma pH increased. This pH-effect was relatively small (-0.098 pK_{app} units per pH unit), but it was significant statistically (paired t-test: difference between pK_{app} at pH 8.1 and pH 7.9, t=2.955 with 7 d.f., $P \leq 0.02$ sign considered; difference between pK_{app} at pH 8.1 and pH 7.6, t=3.371 with 7 d.f., $P \leq 0.01$ sign considered). True dissociation constants are not influenced by the concentrations of their reactants or products. Hence, the finding that pK_{app} was pH-dependent emphasizes that pK_{app} has no thermodynamic meaning; it is nothing more than an empirically derived factor which relates plasma pH, free CO_2 and $bHCO_3^-$.

The CO_2-combining properties of rainbow trout blood were a function of blood temperature and haematocrit (Fig. 2). At a constant P_{CO_2}, cold blood had a higher C_{CO_2} than warmer blood. Red cells enhanced the CO_2-combining affinity of blood; the mean CO_2 capacitance, βCO_2, calculated by linear regression of C_{CO_2} against P_{CO_2} over the physiological range of P_{CO_2} in trout blood (2-7 torr, Table 2), varied directly with haematocrit (Fig. 2). CO_2 capacitance reflects CO_2-combining affinity in the same way that the slope of an oxygen saturation curve reflects O_2-binding affinity. Effects of temperature on βCO_2 were not evident (Fig. 2).

Figure 2. CO_2 dissociation curves and CO_2 capacitances of oxygenated whole blood of *Salmo gairdneri* determined *in vitro*. Panel A. Effect of temperature on CO_2 dissociation curves. Values are means $\pm$ 1 S.E. (n=5–7). Panel B. Effect of haematocrit on mean CO_2 capacitance ($bCO_2 = dC_{CO_2}/dP_{CO_2}$) between 2 and 7 torr P_{CO_2} at temperatures of 5 (+), 10 (*) and 15 (o) C. Least squares regression line, ignoring temperature, is bCO_2 = 0.232 + 0.022 haematocrit (r=0.757, n=18).


16
12
8
4
0
C_{CO_2}, mM
5 C
10 C
15 C
A.
0
4
8
12
16
20
P_{CO_2}, torr

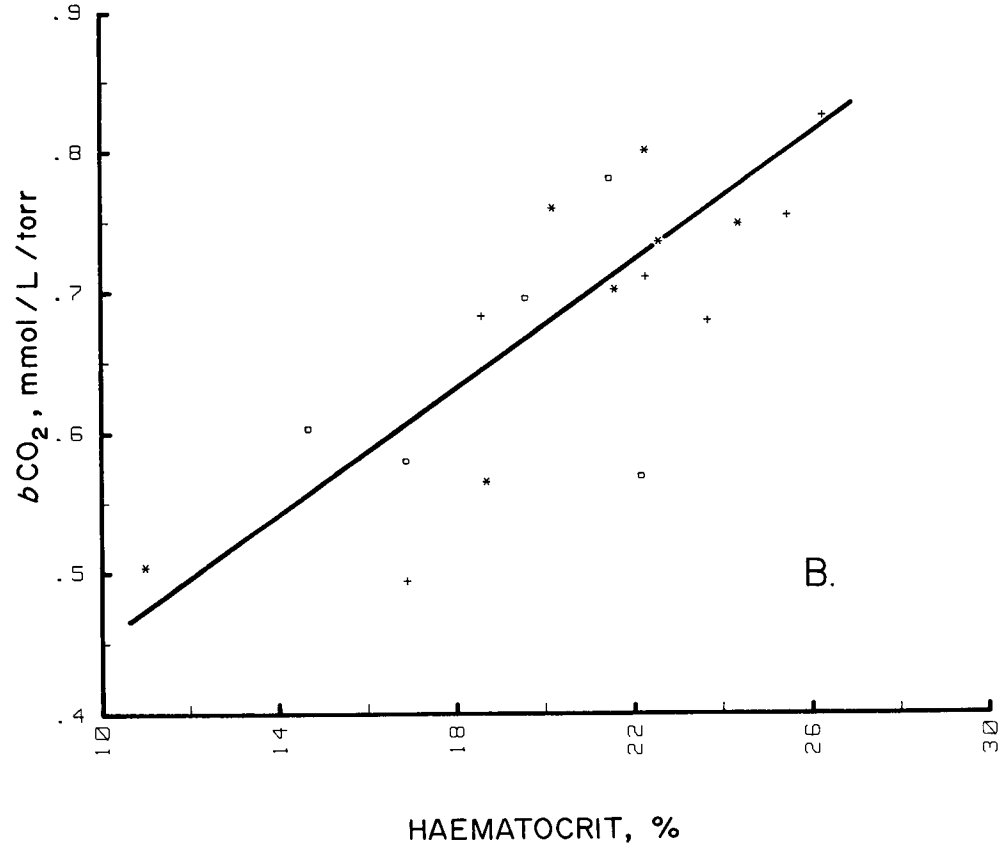

.9
.8
.7
.6
.5
.4
bCO_2, mmol/L/torr
B.
10
14
18
22
26
30
HAEMATOCRIT, %

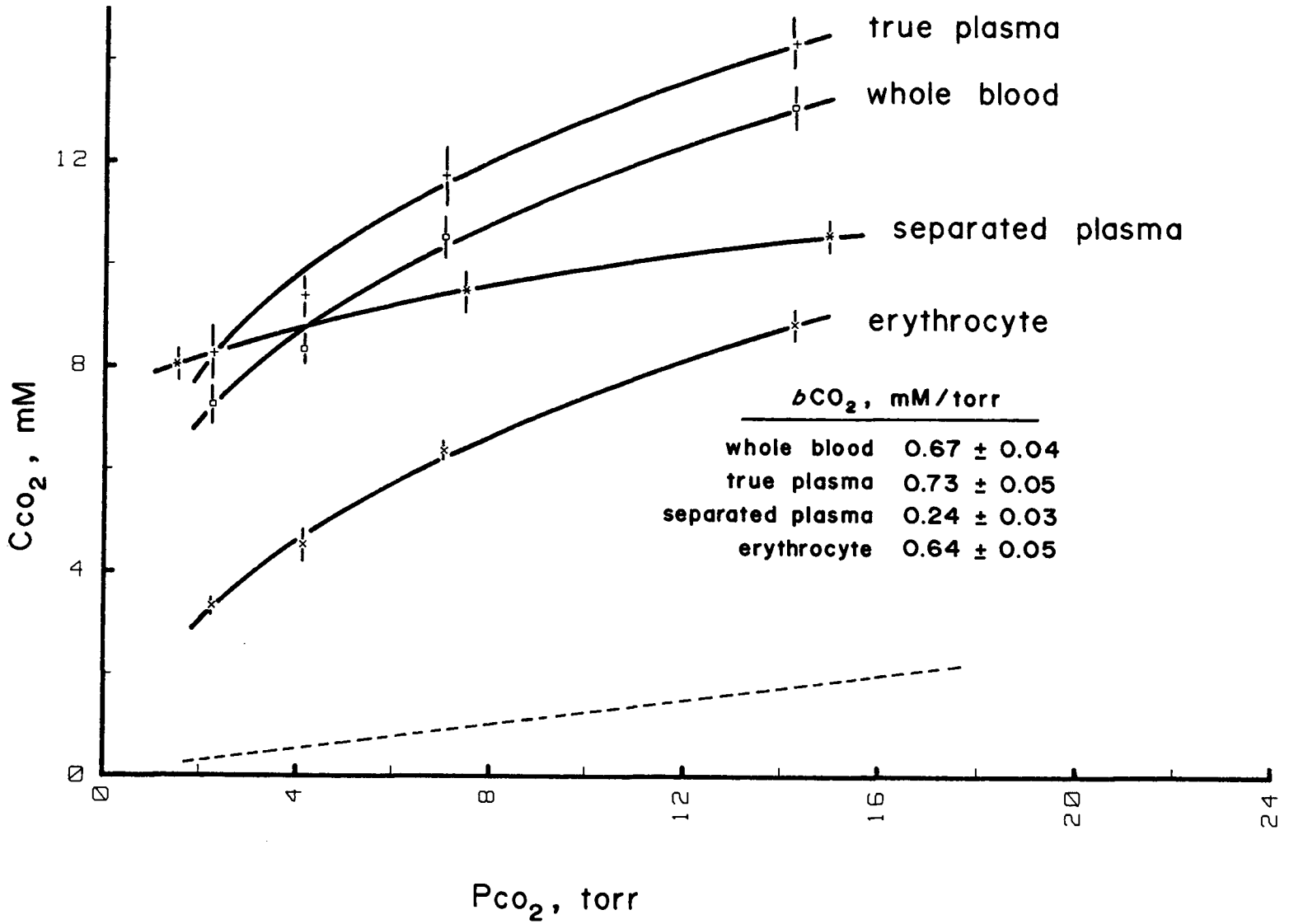
Table 2. *In vivo* respiratory and ionic characteristics of *Salmo gairdneri* plasma at 10 C. Arterial and venous values are arithmetic means ± 1 S.E. (n). 'Arterial minus venous' values are population mean differences ± 95% confidence intervals. Population mean differences denoted with * are different significantly from zero at $P \leq 0.05$ (paired t-test). hct, haematocrit.

	Arterial	Venous	Arterial minus venous
pH	7.93 ± 0.03(8)	7.88 ± 0.03(8)	0.05 ± 0.01*
C_{CO_2}(mM)	8.08 ± 0.87(8)	9.20 ± 0.97(8)	-1.11 ± 0.23*
P_{CO_2}(torr)	2.31 ± 0.17(8)	2.99 ± 0.19(8)	-0.68 ± 0.11*
$bHCO_3^-$(mM)	7.93 ± 0.85(8)	9.01 ± 0.96(8)	-1.08 ± 0.23*
hct(%)	22.6 ± 3.0(8)	23.8 ± 3.1(8)	-1.2 ± 0.8*
Cl^-(mM)	121.61 ± 2.44(18)		
Na^+(mM)	154.04 ± 2.26(18)		
K^+(mM)	4.34 ± 0.26(18)		
Ca^{2+}(mM)	2.96 ± 0.07(18)		
Mg^{2+}(mM)	0.70 ± 0.03(18)		
blood lactate (mM)	0.47 ± 0.08(15)		

CO_2 dissociation curves of whole blood, true plasma (plasma equilibrated in the presence of red cells), separated plasma (plasma equilibrated in the absence of red cells) and erythrocytes at 10 C are compared in Figure 3. Direct comparison of the absolute C_{CO_2} values in Figure 3 was complicated by the fact that separated plasma values were determined using a different blood pool from that used to measure the other values. Nonetheless, such a comparison was deemed valid because the blood pools used were in similar respiratory and acid-base states when the blood was withdrawn *in vivo*. Theoretically, the dissociation curves of separated and true plasma from a single blood source cross at the P_{CO_2} at which centrifugation for separated plasma took place, which under anaerobic conditions should reflect the P_{CO_2} *in vivo*. Separated and true plasma lines in Figure 3 crossed at a P_{CO_2} (2.3 torr) close to the physiological P_{CO_2} of arterial blood (Table 2). Moreover, the measured bCO_2 for separated plasma (0.24) was close to the value predicted for whole blood at zero haematocrit (0.23, Fig. 2).

True plasma had the highest C_{CO_2} at P_{CO_2} above 4 torr followed by, in descending order, whole blood, separated plasma and red cells (Fig. 3). Differences in the CO_2-combining properties of true plasma and separated plasma were attributable to the presence of red cells during CO_2 equilibration, and resulted from movements of H^+ and /or HCO_3^- between red cells and plasma. The amount of HCO_3^- which crossed the red cell membrane was estimated by comparing the changes in $bHCO_3^-$ of true and separated plasma over an identical range of P_{CO_2} (Fig. 3). These estimates indicated that, over a range of P_{CO_2} from 2 to 7 torr,

Figure 3. CO_2 dissociation curves and mean capacitances (βCO_2 = dC_{CO_2} / dP_{CO_2}, between 2 and 7 torr) of oxygenated whole blood (mean haematocrit ± 1 S.E. = 20.1 ± 1.7), true plasma, separated plasma and erythrocytes of *Salmo gairdneri* determined *in vitro* at 10 C. Values are means ± 1 S.E. (n=6-7). Dashed line represents the amount of dissolved CO_2 present.


true plasma
whole blood
separated plasma
erythrocyte
βCO_2, mM/torr
whole blood 0.67 ± 0.04
true plasma 0.73 ± 0.05
separated plasma 0.24 ± 0.03
erythrocyte 0.64 ± 0.05
Cco_2, mM
Pco_2, torr
0
4
8
12
16
20
24

62% of the increase in true plasma C_{CO_2} was due to HCO_3^- added to the plasma from red cells. Since red cells constituted only about 20% of the blood volume under consideration, this indicates that the majority of CO_2 hydration occurred inside the red cell. For the most part, partitioning of C_{CO_2} among the various blood compartments reflected differences in buffering capacity (Fig. 4, Table 3). Effects of temperature on buffering capacity were not evident (Table 3).

Interestingly, red cells had the highest buffering capacity (1.6 times that of true plasma, Table 3), but had the lowest C_{CO_2} at any given P_{CO_2} and an intermediate δCO_2. Since red cells were equilibrated in the presence of plasma, this probably was attributable to the same H^+ and/or HCO_3^- movements between red cells and plasma that were responsible for differences between the CO_2-combining properties of true and separated plasma. Intracellular pH varied linearly with plasma pH over the pH range examined (Table 4). Temperature had little effect on this relationship.

Increases in the P_{CO_2} of trout blood were accompanied by a net influx of Cl^- into the red cell; the Donnan Cl^- ratio (R_{Cl^-} = [erythrocyte] / [plasma]) was related inversely to blood pH (Fig. 5). The amount of Cl^- gained by red cells from plasma during CO_2 equilibrations was stoichiometrically equivalent to the amount of HCO_3^- gained by true plasma from red cells (Fig. 6). This indicates that bicarbonate movements in trout red cells involved a one-for-one counter exchange of bicarbonate and chloride similar to the chloride shift found in mammalian erythrocytes

Figure 4. Buffer lines for oxygenated whole blood (mean haematocrit ± 1 S.E. = 20.1 ± 1.7), true plasma, separated plasma and erythrocytes of *Salmo gairdneri* deterimined *in vitro* at 10 C. Values are means ± 1 S.E. (n=6-7).

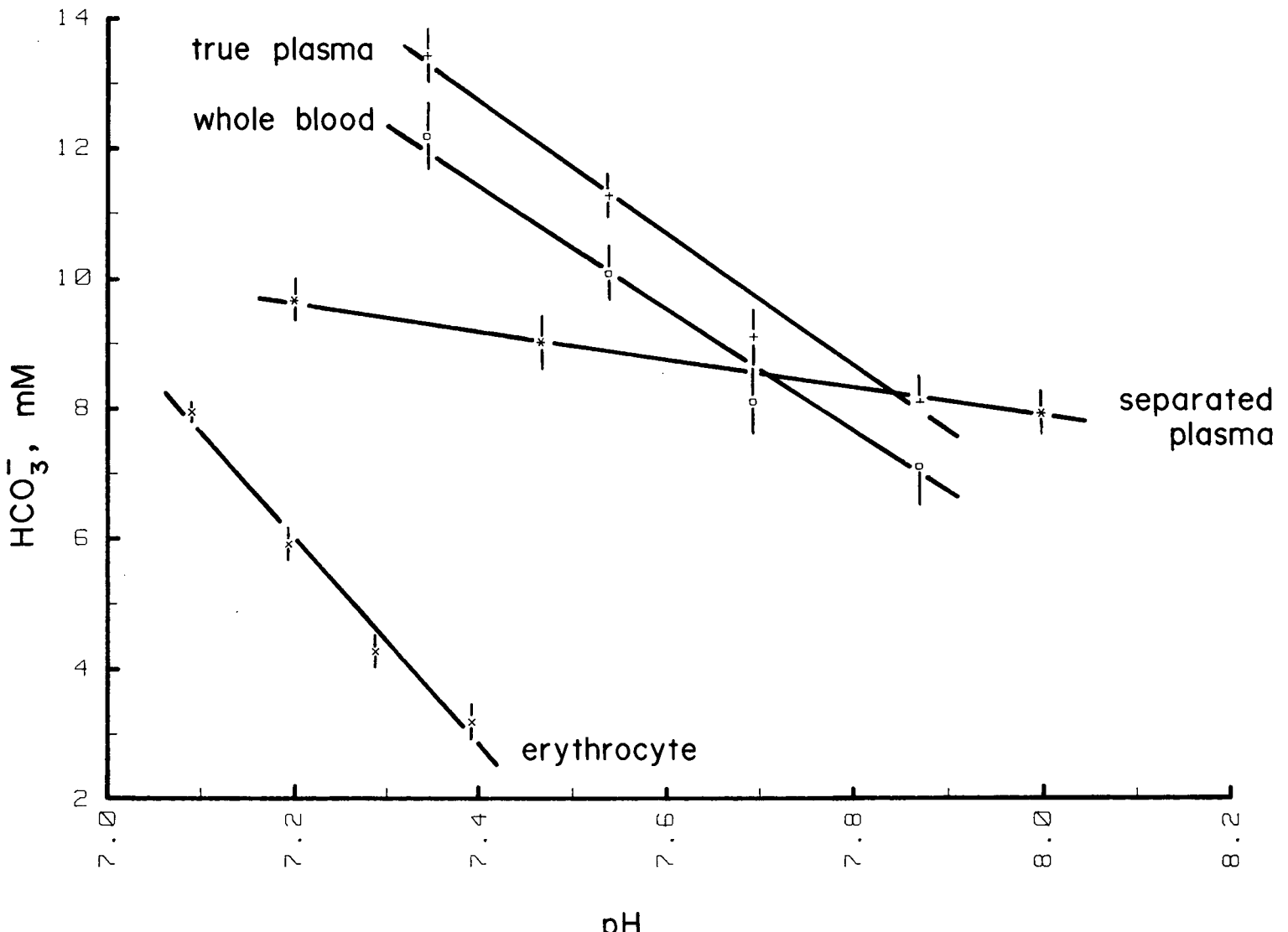

true plasma
whole blood
separated plasma
erythrocyte
HCO_3^-, mM
pH
14
12
10
8
6
4
2
7.0
7.2
7.4
7.6
7.8
8.0
8.2

Table 3. Physiological buffering capacity in slykes (mM bicarbonate·pH unit^{-1}) of oxygenated whole blood, true plasma, separated plasma and erythrocytes of *Salmo gairdneri* determined *in vitro* between 5 and 15 C. Values are means ± 1 S.E. (n=5-7). Temp, temperature. Hct, haematocrit.

Temp (C)	Hct (%)	Buffering capacity			
		whole blood	erythrocyte	true plasma	separated plasma
5	22.2 ± 1.6	9.4 ± 0.3	16.1 ± 2.4	9.7 ± 0.5	
10	20.1 ± 1.7	9.9 ± 0.9	16.5 ± 2.0	10.5 ± 1.0	2.2 ± 0.1
15	19.0 ± 1.4	9.1 ± 0.5	14.1 ± 1.6	9.4 ± 0.6	

Table 4. Least squares regression lines describing the relationship between blood pH (pH_E), and intracellular pH (pH_I), R_{H+} ([plasma]/[erythrocyte]), R_{Cl-} ([erythrocyte]/[plasma]), and R_{HCO_3-} ([erythrocyte]/[plasma]) of *Salmo gairdneri* blood between 5 and 15 C. Also shown is the relationship between carbaminohaemoglobin concentration (carb, mmol·L red cell^{-1}) and blood P_{CO_2} (torr). Regression coefficients are presented ± 95% confidence intervals. Temp, temperature (C).

Relation	Temp	a	b	n	r
$pH_I = a + b\ pH_E$	5	3.476 ± 0.572	0.501 ± 0.074	24	0.949
	10	2.708 ± 0.580	0.595 ± 0.076	27	0.955
	15	3.566 ± 0.631	0.479 ± 0.083	19	0.947
$R_{H+} = a + b\ pH_E$	5	4.254 ± 0.644	−0.494 ± 0.083	24	0.935
	10	3.500 ± 0.604	−0.403 ± 0.079	27	0.903
	15	4.218 ± 0.655	−0.500 ± 0.086	19	0.948
$R_{Cl-} = a + b\ pH_E$	5	4.064 ± 1.684	−0.473 ± 0.217	22	0.713
	10	3.858 ± 1.375	−0.450 ± 0.181	25	0.732
	15	3.764 ± 2.619	−0.429 ± 0.344	18	0.552
$R_{HCO_3-} = a + b\ pH_E$	5	4.499 ± 1.282	−0.493 ± 0.165	22	0.812
	10	4.476 ± 1.325	−0.486 ± 0.174	27	0.754
	15	4.698 ± 1.697	−0.520 ± 0.223	18	0.778
carb = a + b log P_{CO_2}	5	1.305 ± 0.816	1.616 ± 1.045	23	0.575
	10	0.847 ± 0.371	1.574 ± 0.457	27	0.818
	15	0.745 ± 0.544	1.538 ± 0.696	19	0.749

Figure 5. Donnan ratios of H^+ (R_{H+} = [plasma] / [erythrocyte]), Cl^- (R_{Cl-} = [erythrocyte] / [plasma]) and $bHCO_3^-$ (R_{HCO3-} = [erythrocyte] / [plasma]) of *Salmo gairdneri* blood, when blood pH was titrated with CO_2 *in vitro* at 5 (+), 10 (*) and 15 (o) C. Least squares regression lines are given in Table 4.

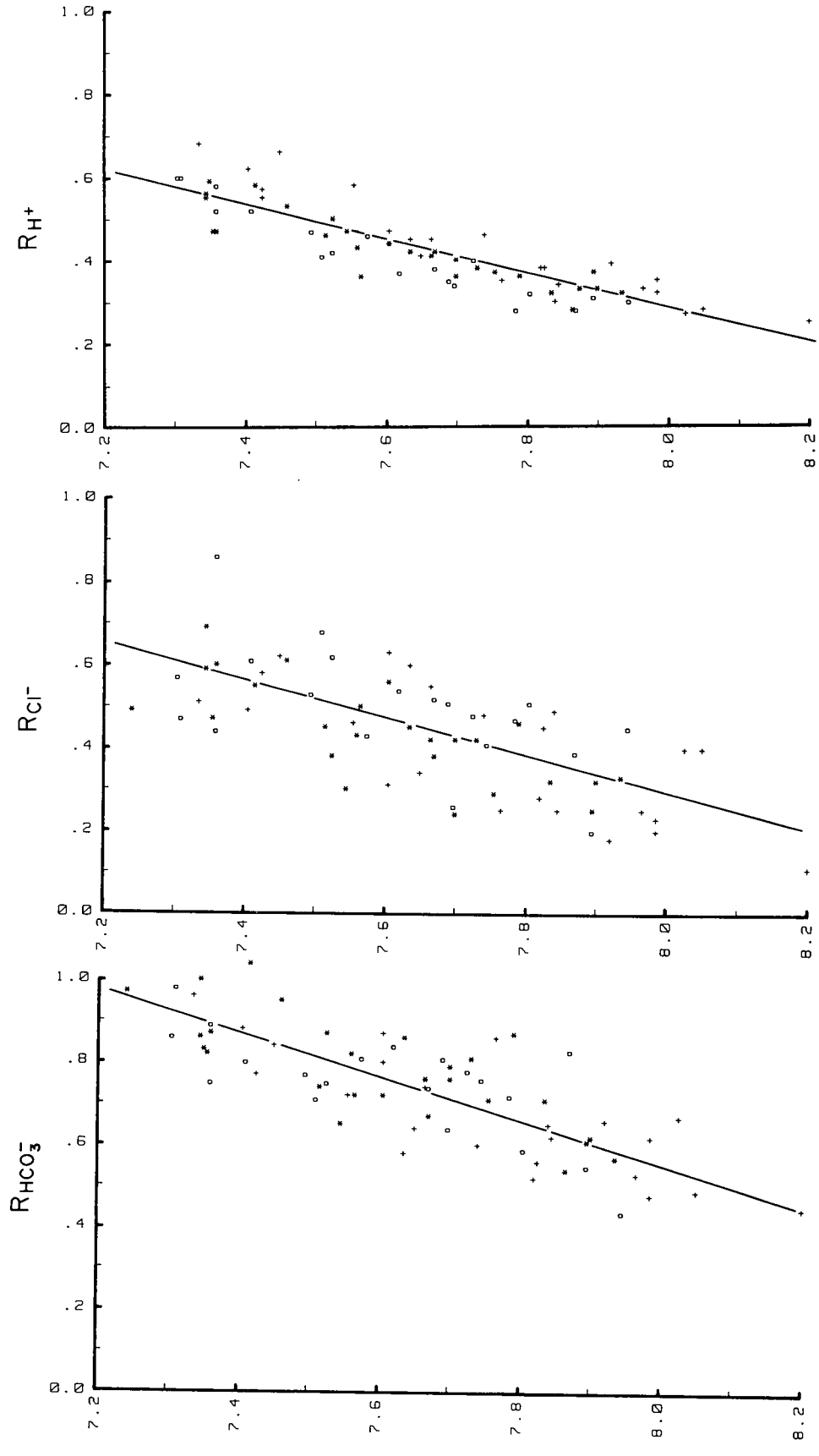
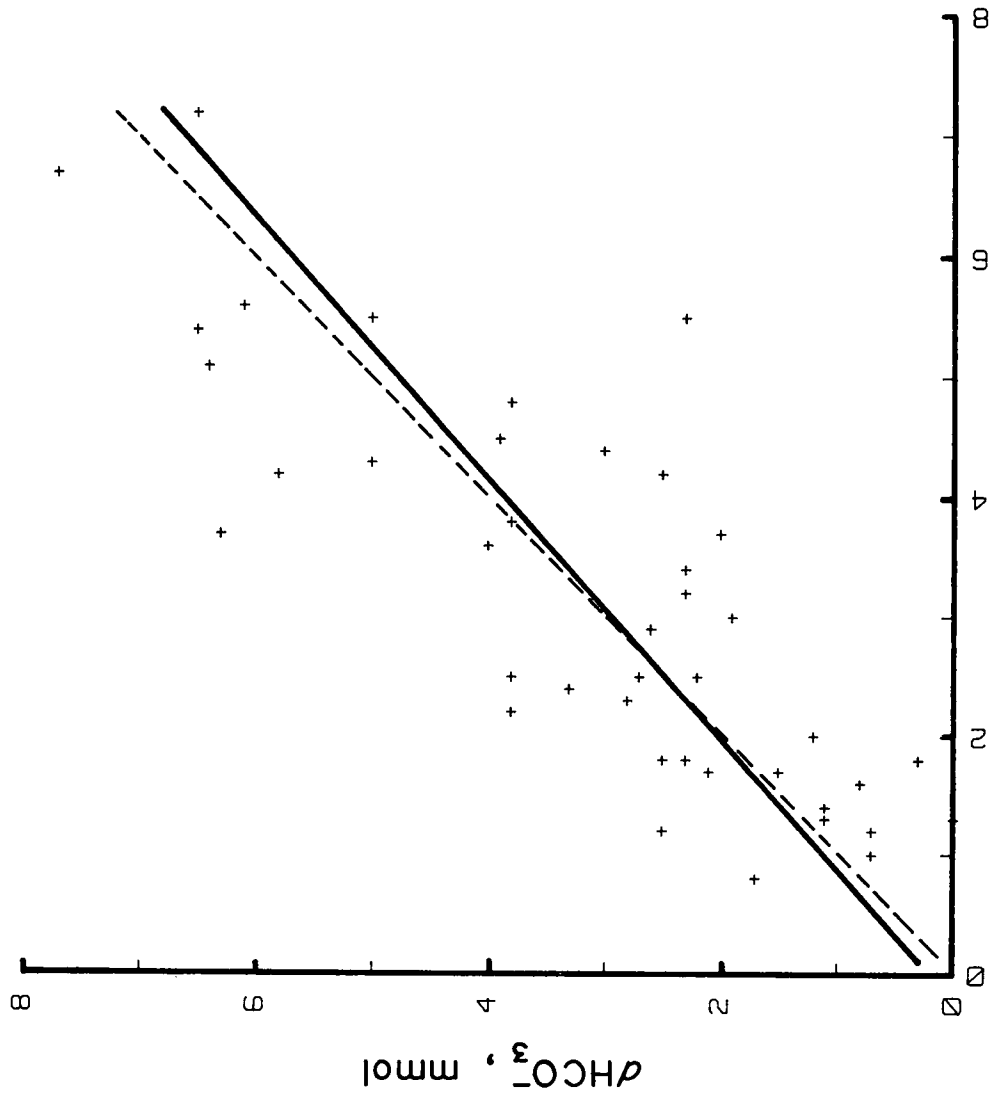

R_{H^+}
R_{Cl^-}
$R_{HCO_3^-}$
1.0
.8
.6
.4
.2
0.0
7.2
7.4
7.6
7.8
8.0
8.2
BLOOD pH

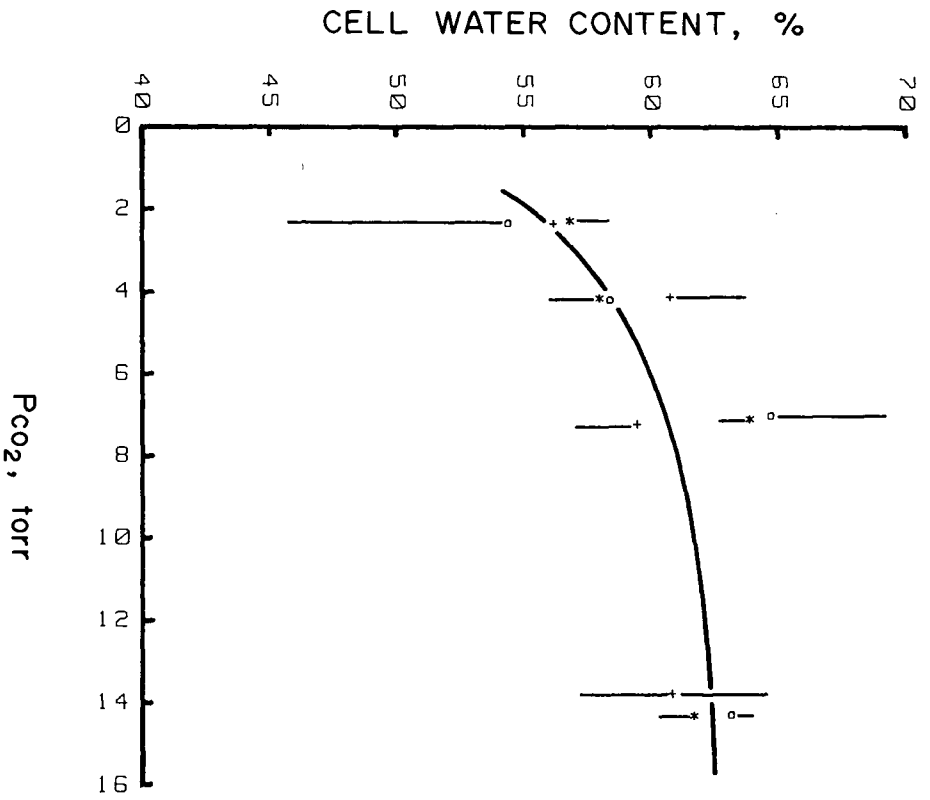
Figure 6. Relationship between the amount of bicarbonate added to plasma from red cells and the amount of chloride added to red cells from plasma during *in vitro* CO_2 equilibrations of *Salmo gairdneri* blood at 10 C. All concentrations were corrected for water content and haematocrit. Least squares regression line is $d[bHCO_3^-] = 0.155 + 0.943\ d[Cl^-]$ ($r=0.802$, $n=42$). Dashed line is $x=y$.


$d\mathrm{HCO_3^-}$, mmol
0
2
4
6
8
$d\mathrm{Cl^-}$, mmol

(Roughton 1964). Formation of intracellular HCO_3^- and the subsequent bicarbonate:chloride exchange resulted in net movement of water into the red cell and cell swelling (Fig. 7). Water content of red cells increased from 55.8 ± 2.9 % to 63.2 ± 1.9 % over the range of P_{CO_2} from 2.3 to 14.1 torr. Over the same range of P_{CO_2}, plasma water content decreased from 94.0 ± 0.5 % to 93.7 ± 0.6 %. Temperature appeared to have no effect on these water movements or on the resultant changes in cell size (Fig. 7).

Changes in the Donnan ratios of both $bHCO_3^-$ ($R_{HCO_3^-}$ = [erythrocyte] / [plasma]) and H^+ (R_{H^+} = [plasma] / [erythrocyte]) paralleled changes in R_{Cl^-} when blood pH was titrated with CO_2 (Fig. 5 and 8). Assuming that Cl^- was distributed passively across the red cell membrane, this indicates that $bHCO_3^-$ and H^+ also were distributed passively. However, $R_{HCO_3^-}$ values were consistently greater than R_{Cl^-} values by 0.30 to 0.35 units (Fig. 8). This indicates that actual intracellular $bHCO_3^-$ values were higher than what could be accounted for on the basis of a Gibbs-Donnan equilibrium for Cl^-. This 'unaccounted-for' intracellular $bHCO_3^-$ varied from 1.30 ± 0.56 mmol·L red cell^{-1} (2.4 torr P_{CO_2}, 15 C) to 3.24 ± 0.88 mmol·L red cell^{-1} (13.8 torr P_{CO_2}, 5 C) (Fig. 9). As such, it represented 33-57% of the measured erythrocytic $bHCO_3^-$. It must be remembered, however, that $bHCO_3^-$ does not distinguish between HCO_3^-, CO_3^{2-} or carbamino compounds. The concentration of 'unaccounted-for' intracellular $bHCO_3^-$ far exceeded the concentration of CO_3^{2-} predicted from measured $bHCO_3^-$ concentrations (6.7-7.0 μmol·L red cell^{-1}) using the true second dissociation constant of carbonic acid (Robinson and

Figure 7. Water content and relative volume of *Salmo gairdneri* erythrocytes in relation to P_{CO_2} at 5 (+), 10 (*) and 15 (o) C. Values are means ± 1 S.E. (n=5-7).


CELL WATER CONTENT, %
40
45
50
55
60
65
70
0
2
4
6
8
10
12
14
16
Pco2, torr

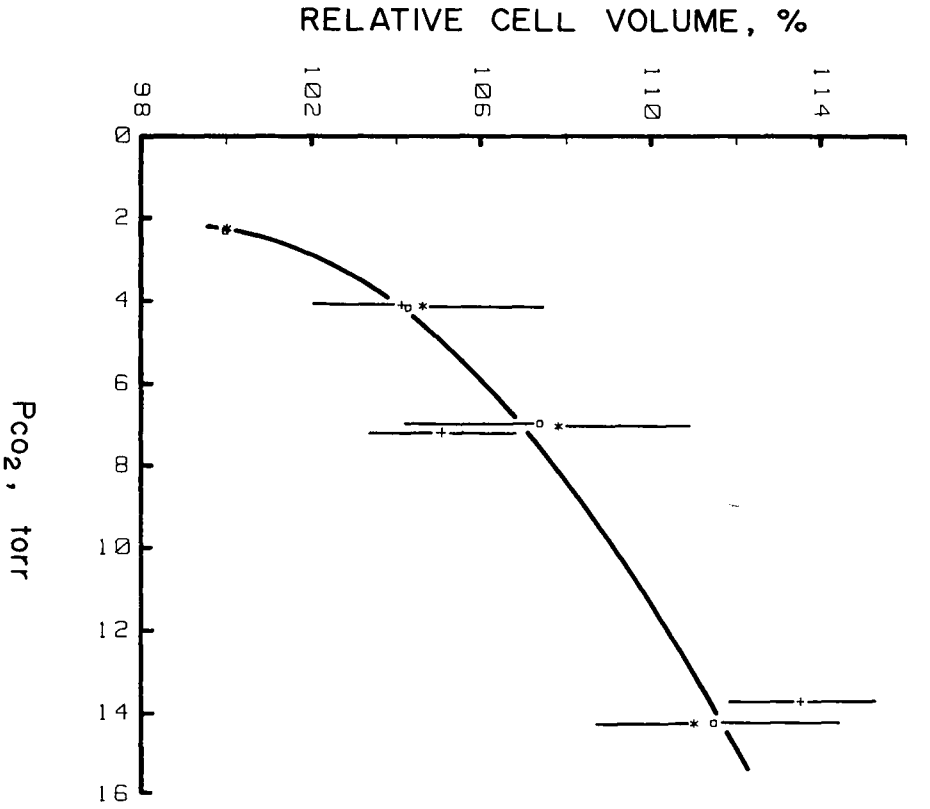

RELATIVE CELL VOLUME, %
98
102
106
110
114
0
2
4
6
8
10
12
14
16
Pco2, torr

Figure 8. Relationship between the Donnan Cl^- ratio (R_{Cl-} = [erythrocyte] / [plasma]), and the Donnan ratios of H^+ (R_{H+} = [plasma] / [erythrocyte]) and $bHCO_3^-$ (R_{HCO3-} = [erythrocyte] / [plasma]) of *Salmo gairdneri* blood at 5 (+), 10 (*) and 15 (o) C. Values are means ± 1 S.E. (n=5-7). Least squares regression of R_{H+} on R_{Cl-} has slope of 0.741 ± 0.350 and y-intercept of 0.094 ± 0.160 (± 95% confidence intervals, n=12, r=0.831). Least squares regression of R_{HCO3-} on R_{Cl-} has slope of 0.878 ± 0.302 and y-intercept of 0.347 ± 0.138 (± 95% confidence intervals, n=12, r=0.898). Dashed lines are x=y.

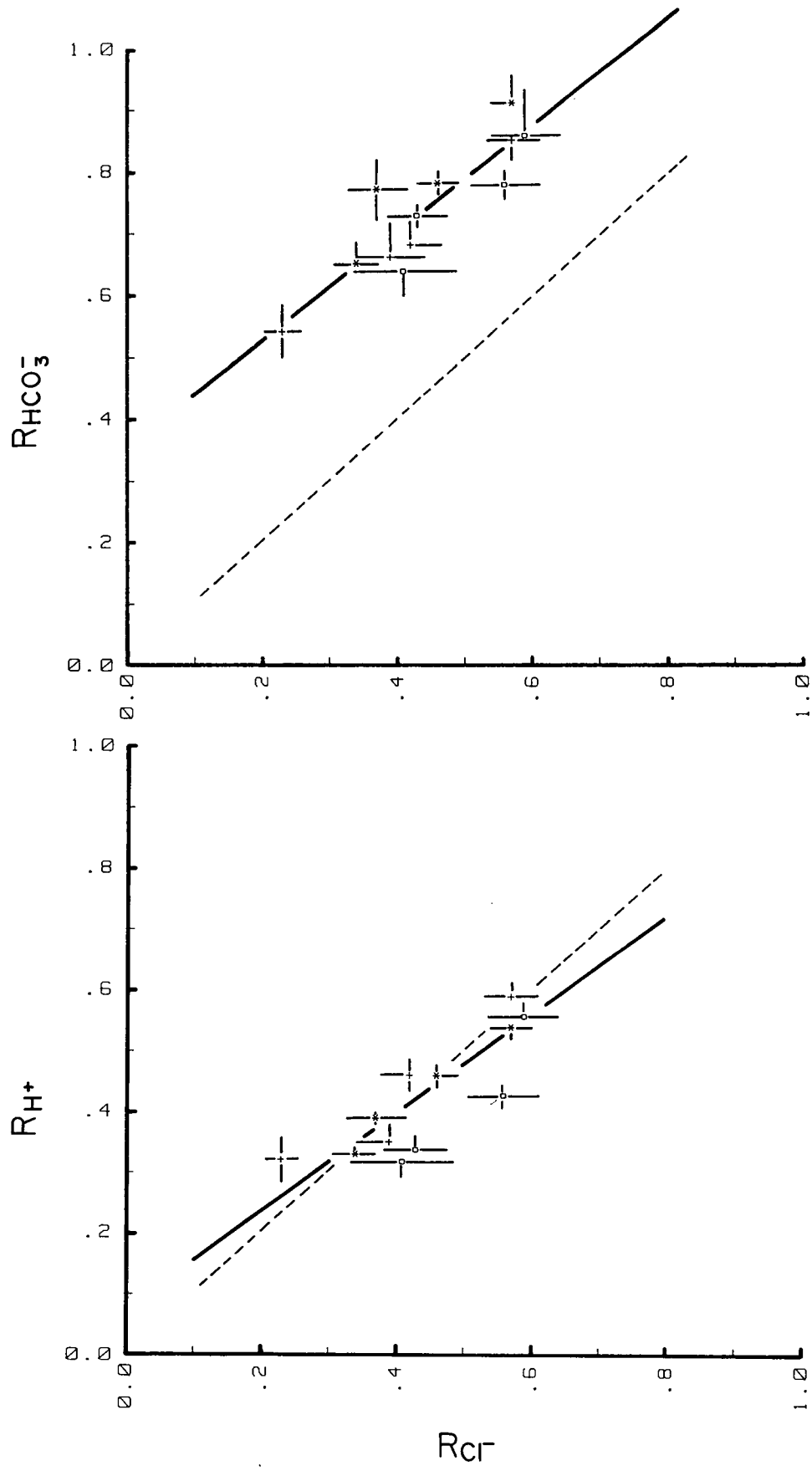
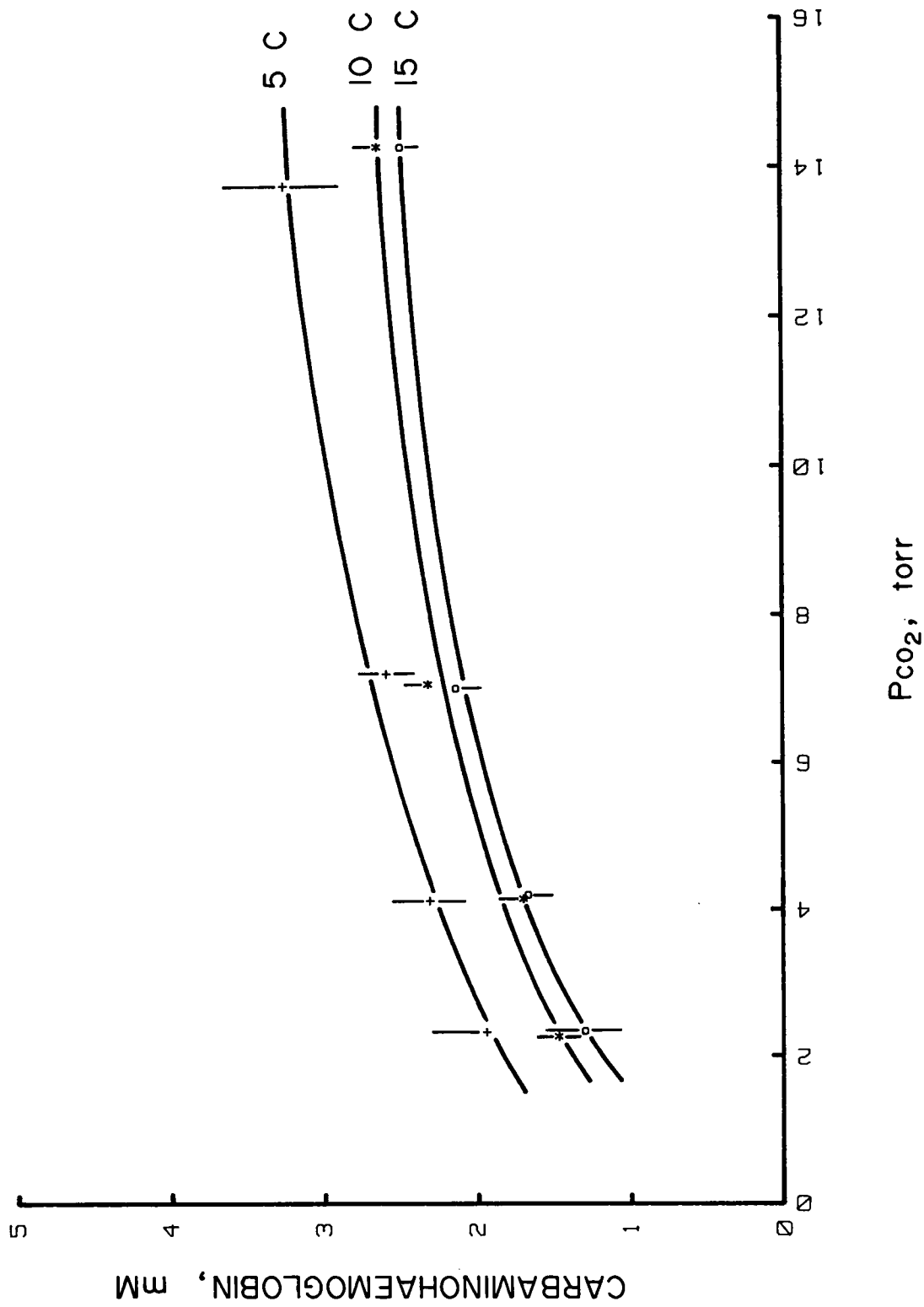

1.0
.8
.6
.4
.2
0.0
$R_{HCO_3^-}$
0.0
.2
.4
.6
.8
1.0
1.0
.8
.6
.4
.2
0.0
R_{H^+}
0.0
.2
.4
.6
.8
1.0
R_{Cl^-}

Figure 9. Carbaminohaemoglobin formation in *Salmo gairdneri* blood showing effects of P_{CO_2} and temperature. Values are means ± 1 S.E. (n=5-7). Least squares regression lines are given in Table 4.


5 C
10 C
15 C
CARBAMINOHAEMOGLOBIN, mM
0
1
2
3
4
5
0
2
4
6
8
10
12
14
16
P_{CO_2}, torr

Stokes 1959). Rather, this 'unaccounted-for' intracellular $bHCO_3^-$ probably represented carbaminohaemoglobin, that is, CO_2 bound directly to haemoglobin. Since rainbow trout blood contains about 4.33 mmol haemoglobin·L red cell^{-1} (Heming, unpublished data), this carbamino formation represented binding of only 0.30-0.75 mol CO_2 per mol haemoglobin. Theoretically, fish haemoglobin should be capable of binding a maximum of 2 mol CO_2 per mol haemoglobin (Farmer 1979). Formation of carbaminohaemoglobin demonstrated saturation kinetics and perhaps was temperature-dependent (Fig. 9). Although the concentration of carbaminohaemoglobin at a given P_{CO_2} tended to increase at lower temperatures, the differences were not significant statistically.

DISCUSSION

CO_2 loading of *Salmo gairdneri* blood *in vitro*, and presumably *in vivo*, was consistent with the classical mammalian model of Roughton (1964). During CO_2 loading, the majority of CO_2 diffusing into the blood hydrated and then dissociated to form HCO_3^- and H^+. Relatively small proportions of CO_2 remained as the dissolved gas or were bound by haemoglobin. Hydration of CO_2 occurred predominantly inside the red cell. However, very little of the resultant HCO_3^- remained inside the erythrocyte. The majority of this HCO_3^- traversed the cell membrane in an one-for-one exchange with Cl^-, reminiscent of the chloride shift in mammalian erythrocytes. Formation of intracellular bicarbonate and the subsequent HCO_3^-:Cl^- exchange was accompanied by influx of water and cell swelling. Although not examined, CO_2 unloading of rainbow trout blood was presumed to involve the same chemical and transport processes, operating in reverse.

The Donnan distributions of $bHCO_3^-$ and H^+ paralleled that of Cl^- as plasma pH was titrated with CO_2, suggesting that the transmembrane distributions of $bHCO_3^-$, H^+ and Cl^- were dependent upon similar mechanisms. Numerous studies of mammalian erythrocytes have indicated that chloride is distributed passively across the red cell membrane. Mammalian erythrocytes are extremely permeable to Cl^- (Gunn *et al.* 1973, Hoffman and Laris 1974, Knauf *et al.* 1977) and the variation in R_{Cl^-} with pH in mammalian blood is consistent with that predicted on the basis

of a passive Cl^- distribution (Harris and Maizels 1952). The most convincing evidence for a passive Cl^- distribution, however, is the excellent agreement between the measured steady-state membrane potential of mammalian erythrocytes and the calculated Nernst chloride equilibrium potential (Lassen 1972, Hoffman and Laris 1974, Ronne and Lassen 1977). Chloride transport in fish erythrocytes is at least as rapid as that in mammalian red cells under similar conditions (Cameron 1978b, Obaid *et al.* 1979). Moreover, Haswell *et al.* (1978) have shown that the relationship between R_{Cl^-} and plasma pH in erythrocytes of the teleost fish, *Tilapia mossambica*, is virtually identical to that in human red cells. These studies strongly suggest that Cl^- is distributed passively in fish erythrocytes. It follows from the present *in vitro* data then that HCO_3^- and H^+ also are distributed passively in red cells of *S. gairdneri*. In itself, a passive HCO_3^- distribution in trout erythrocytes does not contradict the gill model of CO_2 excretion (Haswell and Randall 1976, 1978, Haswell *et al.* 1980), which asserts that fish red cells are functionally impermeable to bicarbonate *in vivo*. Functional impermeability could be attained *in vivo* by plasma inhibition of erythrocytic carbonic anhydrase. However, time course studies of the chloride shift in teleost and elasmobranch blood (Cameron 1978b, Obaid *et al.* 1979) clearly demonstrate that plasma has no effect on the activity of erythrocytic carbonic anhydrase. These latter studies provide strong evidence against the gill model of CO_2 excretion.

The present R_{Cl^-} values in rainbow trout erythrocytes do not agree satisfactorily with observed (Dill *et al.* 1932, Fitzsimmons and Sendroy 1961, Gunn *et al.* 1973, Reeves 1976a, Haswell *et al.*

1978) or theoretically predicted (Jacobs and Stewart 1947, Freedman and Hoffman 1979) ratios in mammalian erythrocytes. Variation in R_{Cl^-} with plasma pH in *S. gairdneri* erythrocytes was such that mammalian and trout R_{Cl^-} values were in good agreement only at about pH 7.4. However, the slope of the relationship between R_{Cl^-} and pH in trout erythrocytes (-0.429 to -0.473) was much steeper than that in mammalian red cells (-0.290 to -0.344: Dill *et al.* 1932, Fitzsimmons and Sendroy 1961, Gunn *et al.* 1973, Reeves 1976a, Haswell *et al.* 1978). This also holds true for the relationships between dR_{H^+} / dpH (-0.403 to -0.500) and $dR_{HCO_3^-}$ / dpH (-0.486 to -0.520) in trout erythrocytes. These values are virtually identical to those obtained by Wood *et al.* (1981) for $dR_{HCO_3^-}$ / dpH (-0.43) in *S. gairdneri* red cells. Thus, within the physiological pH range of trout blood, the measured Donnan ratios of trout red cells are much less than mammalian values.

Differences between the Donnan Cl^- ratios of mammalian and fish erythrocytes probably are a consequence of the presence of a nucleus in non-mammalian red cells. The nucleus of rainbow trout erythrocytes occupies about 18% of the cell volume (Eddy 1977). Direct measurement of the membrane potential of nucleated red cells has shown consistently that the Nernst chloride equilibrium potential underestimates the measured intracellular voltage, and that this discrepancy increases as R_{Cl^-} decreases at higher pH values (Lassen 1977, Stoner and Kregenow 1980). Differences between the measured and calculated membrane potentials of nucleated red cells and hence between the measured and predicted Donnan ratios in such cells appear to be an inherent

characteristic of nucleated erythrocytes. These discrepancies may be due to exclusion of Cl^- from the nucleus (Lassen 1972, Hoffman unpublished observation cited in Stoner and Kregenow 1980). Nuclear exclusion of chloride would effectively reduce the mean ionic activity coefficient of Cl^- in nucleated red cells. In contrast, enucleate mammalian red cells are adequately described as well-mixed single compartments with mean ionic activity coefficients of unity (Freedman and Hoffman 1979). Thus, the present results infer that the nuclear compartment of fish erythrocytes is more acidic and contains less Cl^- and HCO_3^- than the cytosolic compartment. It follows then that measurements of erythrocyte pH made using the freeze-thaw technique, or any other method that yields an average pH for the entire cell, underestimate cytosol pH. The assumption of cytosolic and nuclear discontinuity in nucleated red cells fails to explain the reported similarity in R_{Cl^-} between nucleated *Tilapia* red cells and enucleate human cells (Haswell *et al.* 1978); perhaps *Tilapia* red cells possess a relatively small nuclear compartment when compared to other nucleated erythrocytes.

The significant differences between $R_{HCO_3^-}$ and R_{Cl^-} in the present study were assumed to reflect formation of carbaminohaemoglobin in *S. gairdneri* red cells; the technique used to measure bicarbonate did not distinguish between the various species of chemically-bound CO_2. This assumption is supported by the observed saturation kinetics of carbamino formation with increasing P_{CO_2}. Similar differences between $R_{HCO_3^-}$ and R_{Cl^-} have been noted in erythrocytes of *S. gairdneri*

(Ferguson and Black 1941) and the dogfish, *Mustelus canis* (Ferguson *et al.* 1938). In the carp, *Cyprinus carpio*, the ratios are approximately equivalent (Ferguson and Black 1941). Differences between the present $R_{HCO_3^-}$ and R_{Cl^-} can be accounted for by assuming that at most 37.5% of the available beta-chain terminals of trout haemoglobin had bound CO_2. The terminal amino groups on the alpha-chains of rainbow trout haemoglobin are acetylated (Riggs 1979, Powers 1980) and thus only the terminals of the beta-chains are available to bind CO_2 (Weber and Lykkeboe 1978, Farmer 1979). Since the beta-chain terminals also bind organic phosphates (Riggs 1970, Johansen *et al.* 1976, Greaney and Powers 1977) which are present in fish erythrocytes in higher concentrations than CO_2 (Albers *et al.* 1983), one would expect a low binding efficiency for CO_2. It is interesting to speculate about the possible enhancement of carbaminohaemoglobin formation at lower temperatures. Formation of carbamino compounds is increased at lower temperatures (Stadie and O'Brien 1937, Albers *et al.* 1983). As well, Eddy (1974) has shown that the Haldane effect in *S. gairdneri* blood, which is dependent in part on carbamino formation, is inversely related to temperature. Reasons for a temperature effect on carbamino formation probably include the effects of temperature on blood pH (Eddy 1974), on binding constants of organic phosphates (Powers 1980), and on the intracellular concentration of organic phosphates.

The present $R_{HCO_3^-}$ values are consistently greater than those reported by Wood *et al.* (1981). This difference in $R_{HCO_3^-}$ values can be largely if not wholly attributed to Wood and

coworkers' use of literature values from Eddy (1974) for red cell and plasma water contents of 70% and 95% respectively. Eddy (1974) clearly states that those water content values are assumed values and not direct determinations. Those water contents differ substantially from the values measured by the present author and ignore the known effect of plasma pH on red cell water content (Fig. 7 this study, Gunn *et al.* 1973, Freedman and Hoffman 1979). Recalculation of the $R_{HCO_3^-}$ values of Wood *et al.* (1981), using the present values for red cell (55.8 to 63.2%) and plasma (94.0 to 93.7%) water contents, increases the $R_{HCO_3^-}$ values of Wood and coworkers by some 10 to 25% and brings their values into general agreement with the present $R_{HCO_3^-}$ values.

Effects of temperature and haematocrit on the CO_2 transport properties of fish blood are well described (Albers 1970, Randall 1970a, Riggs 1970). In general, the CO_2 carrying capacity of blood is an inverse function of temperature; CO_2 solubility increases at lower temperatures and the dissociation constants of both protein buffers and carbonic acid decrease (Albers 1970, Boutilier *et al.* 1984a) shifting the CO_2:HCO_3^- equilibrium in the direction of the dissociated moiety. Temperature had no effect on the buffering capacity of trout blood, plasma or erythrocytes. This is consistent with the findings of Eddy (1974). Reeves (1976a, b) has demonstrated that this temperature-independency arises from the virtual absence of significant water and ion movements between red cells and plasma as temperature varies at constant C_{CO_2}. As a result, the net charge state of red cell and plasma protein buffers is unaffected by temperature.

The presence of erythrocytes in blood influenced CO_2

transport properties of rainbow trout blood by serving as a proton sink. Red cells and their complement of haemoglobin enhanced the buffering capacity of blood and thus favoured the formation of bicarbonate during CO_2 loading. Binding of H^+ by haemoglobin influences the oxygen binding affinity of haemoglobin (Bohr effect) and its O_2 carrying capacity (Root effect) (for review see Riggs 1970). This creates a functional link between CO_2 and O_2 transport.

CO_2 transport in arterial and venous blood of rainbow trout was modelled using *in vivo* data (Table 2) and results of the CO_2 equilibration studies. This model is presented in Table 5. The following assumptions were made during construction of Table 5. Carbamino formation in plasma was taken as 0.14% of plasma C_{CO_2} (value for human plasma from Albritton 1952), and CO_2 solubility of red cells was taken as 86% of plasma αCO_2 (value for ox erythrocytes from Van Slyke and Sendroy 1928). Comparable data for fish blood are not available in the literature. CO_3^{2-} levels were calculated using a pK_2 for carbonic acid at 10 C of 10.4906 (Robinson and Stokes 1959). This ignores the known effects of ionic strength on the dissociation constants of weak acids and consequently slightly overestimates CO_3^{2-} levels. The oxygenation state of blood was not considered in calculation of carbamino formation, nor was the level of erythrocyte organic phosphates. As a consequence, the carbamino levels estimated for venous blood in Table 5 probably underestimate the actual levels present.

Lamellar blood transit time in rainbow trout is about 3 s at

Table 5. A model of CO_2 transport in arterial and venous blood of *Salmo gairdneri* at 10 C. See text for details. Values are μmol.

	Venous	Arterial	Venous minus arterial	
			μmol	% of total
WHOLE BLOOD (1000 mL)				
Total CO_2 content	7800	6810	990	100.00
PLASMA (750 mL)				
Total CO_2 content	6900	6060	840	84.85
as HCO_3^-	6732.7	5925.6	807.1	81.53
as CO_3^{2-}	16.6	16.4	0.2	0.02
as CO_2	141.0	109.5	31.5	3.18
as carbamino	9.7	8.5	1.2	0.12
ERYTHROCYTES (250 mL)				
Total CO_2 content	900	750	150	15.15
as HCO_3^-	445.8	357.4	88.4	8.93
as CO_3^{2-}	0.7	0.6	0.1	0.01
as CO_2	40.4	31.4	9.0	0.91
as carbamino	413.2	360.6	52.6	5.31

rest (Randall 1982a). During that transit, blood C_{CO_2} decreases by about 1 mM. The data of Table 5 indicates that approximately 82% of the CO_2 excreted is plasma HCO_3^-. Free CO_2 accounts for only about 4% of CO_2 excretion. The remaining 14% is divided almost equally between red cell bicarbonate and red cell carbamino. The contributions of CO_3^{2-} and plasma carbamino to CO_2 excretion are neglible.

In conclusion, CO_2 loading of *S. gairdneri* blood proceeds in a typically mammalian fashion. Red cells play a predominant role in CO_2:HCO_3^- conversion and display a typical chloride shift. This chloride shift is similar in its time course and its dependence on intracellular carbonic anhydrase to that of mammalian cells (Cameron 1978b, Obaid *et al.* 1979). The activity and plasma inhibition of erythrocytic carbonic anhydrase is dealt with in the following chapter. HCO_3^-, Cl^- and H^+ are distributed passively in fish red cells *in vitro* and, therefore, are freely permeable under those conditions. Fish red cells cannot be considered to be a single homogenous compartment, however. The nucleus of fish erythrocytes may be more acidic and contain less chloride and bicarbonate than the cytosol. During lamellar blood transit, about 1 mM of CO_2 is excreted of which 82% is derived from plasma HCO_3^-. Since the basolateral membrane of trout gills is largely impermeable to HCO_3^- (Perry *et al.* 1982), this reduction in plasma bicarbonate must necessitate its conversion back to CO_2. This HCO_3^-:CO_2 conversion probably involves a simple reversal of the chemical and transport processes that operate during CO_2 loading.

Chapter 2.

ACTIVITY AND INHIBITION OF CARBONIC ANHYDRASE IN FISHES

The interconversion of bicarbonate and carbon dioxide is catalysed by the enzyme carbonic anhydrase (CA) (EC 4.2.1.1.). Since the pioneering work of Meldrum and Roughton (1933) with ox erythrocytes, the distribution and physiological function of CA in a wide variety of animal tissues has been well documented (Maren 1967, Lindskog *et al.* 1971, Carter 1972, Bauer *et al.* 1980). CA has been found in the erythrocytes of all vertebrates examined (Maren 1967), with the exception of the amphibian mudpuppy, *Necturus maculosus*, (Toews *et al.* 1978, Maren and Azar 1981) and perhaps the flounder, *Platichthys flesus* (Mashiter and Morgan 1975). Erythrocyte CA classically has been assigned the predominant role in catalysing the interconversion of plasma HCO_3^- and CO_2 (Roughton 1964). CA also is present in the tissues of most gas exchange organs, including invertebrate gills (Maren 1967, Randall and Wood 1981, Henry and Cameron 1982), vertebrate gills (Maren 1967, Haswell and Randall 1978, Toews *et al.* 1978), and vertebrate lungs (Crandall and O'Brasky 1978, Effros *et al.* 1978, Klocke 1978). Little is known of the enzyme's distribution among the air-breathing organs of fish, although its presence appears to be limited to those organs which are derived embryonically from gill tissue (Burggren and Haswell 1979, Daxboeck and Heming 1982). CA in vertebrate branchial epithelia is involved in ion transport (Maetz 1956a, Maren 1967). The role

of CA in pulmonary epithelia is unclear, but the enzyme has been implicated in secretion of lung fluid in the fetus (Adamson and Waxman 1976), in dampening of CO_2 transients between alveolar gas and arterial blood (Farhi *et al.* 1976), and in attainment of chemical equilibrium in blood during pulmonary capillary transit (Crandall and O'Brasky 1978, Effros *et al.* 1978, Klocke 1978). The epithelial CA of both gills and lungs plays a minor role if any, however, in overall excretion of CO_2 (Crandall and Bidani 1981, Perry *et al.* 1982).

A number of assay techniques have been developed to follow the catalysed CO_2:HCO_3^- conversion (for review see Davis 1963). Each method has its own inherent advantages and disadvantages, and no single technique has emerged as a standard for measuring CA activity. The original technique of Meldrum and Roughton (1933), the modified boat assay, was the method of choice in early studies and has survived to the present with a number of modifications (Giacobini 1962, Hoffert 1966, Haswell and Randall 1976, Giraud 1981). The basic reaction is evolution of CO_2 from a buffer solution containing $NaHCO_3$. This technique is susceptible, however, to problems related to the diffusion of CO_2 out of solution (Heming and Randall 1982).

A vast array of methods have evolved around measurement of the pH changes associated with CO_2:HCO_3^- conversion, including simple indicator changes (Maren 1960), continuous electrometric recording (Davis 1963), and the use of pH-stat titration (Hansen and Magid 1966). These techniques have the advantage of being applicable to continuous and stop-flow systems (Forster and Crandall 1975, Crandall *et al.* 1971).

A recent development in CA measurement techniques has been the discovery that the enzyme catalyses the hydrolysis of many esters, notably p-nitrophenyl acetate, and the hydration of acetaldehyde and related carbonyl compounds (Schneider and Lieflander 1963, Pocker and Meany 1965, Pocker and Stone 1965). These reactions can be followed colourimetrically (Armstrong *et al.* 1966, Houston and McCarty 1978). However, none of these new substrates is physiological. Moreover, certain tissue CA lacks esterase activity (Tashian 1965) and so is undetectable using esterase assay techniques.

The use of these various assay techniques, the results of which are not always interconvertable or physiologically interpretable, has made it difficult to compare results of various CA studies. With that in mind, the present study examined the relative merits and usefulness in physiological studies of three assay techniques: the modified boat assay; the esterase assay; the pH-stat assay. The linearity, sensitivity and detection limits of each assay were determined in order to develop a reliable CA assay that could be used in studies with crude tissue homogenates. Because of the importance of CA in CO_2 excretion and ionic regulation in fish, a distributional study of carbonic anhydrase in fish then was undertaken using both the esterase assay and the pH-stat assay.

MATERIALS AND METHODS

A. Manometric Method: Modified Boat Assay

Evolution of CO_2 from a phosphate buffer containing $NaHCO_3$ was measured manometrically as described by Haswell and Randall (1976). A reaction vessel or 'boat' was constructed from a 50-mL Ehrlenmeyer flask, partitioned along its bottom by a raised glass ridge. Two mL of bicarbonate solution (200 mM $NaHCO_3$ in 20 mM NaOH, pH > 8.00) were placed on one side of the ridge and 2 mL of phosphate buffer (mixture of 200 mM $NaHPO_4$ and 200 mM KH_2PO_4, pH 6.80 at the prevailing assay temperature) were placed on the other side; 0.2 mL of material to be assayed were added to the buffer.

The boat was connected to a pressure transducer (Statham P23Db) by way of polyethylene tubing (PE-160) and a 3-way stopcock, and then was immersed in a shaking temperature bath. The immersed boat was left open to the atmosphere for 3-5 min during which time the temperature of its contents equilibrated with that of the bath. The boat then was sealed, the shaker was turned on, and the internal gas pressure was monitored as the dehydration reaction proceeded. The rate of increase in gas pressure was taken as being directly proportional to the rate of HCO_3^- dehydration. The pressure transducer was calibrated with injections of known volumes of air made at the prevailing assay temperature. Reaction rates (mL CO_2 evolved·min^{-1}) were calculated from changes in pressure equivalent to changes in

volume of from 0.1 to 0.3 mL; the increase in gas pressure was linear over this range of volumes.

B. Colourimetric Method: Esterase Assay

Catalysis of p-nitrophenyl acetate (pNA) hydrolysis by CA was measured spectrophotometrically as described by Houston and McCarty (1978) and Watson *et al.* (1982). Two mL of freshly-prepared pNA (3 mM pNA in 3% acetone) were added to a thermostatted cuvette. This was followed by either 1.8 mL of Tris buffer (125 mM Tris (hydromethyl) methylamine, pH titrated to 7.50 at the prevailing assay temperature with concentrated H_2SO_4) to measure total esterase activity or 1.8 mL of acetazolamide solution (500 mM acetazolamide in an identical Tris buffer) to measure acetazolamide-insensitive activity. Finally, 0.2 mL of material to be assayed were added. The change in absorbance at 348 nm then was followed for at least 3 min using a Pye Unicam spectrophotometer (model SP8-200). The rate of change in absorbance was converted to mmol pNA·min^{-1} using an extinction coefficient of 4.69·mmol^{-1}·cm^{-1} (Watson *et al.* 1982). Acetazolamide-sensitive esterase activity was calculated as the difference between the total esterase activity and the acetazolamide-insensitive activity. Acetazolamide-sensitive esterase activity was assumed to reflect CA activity.

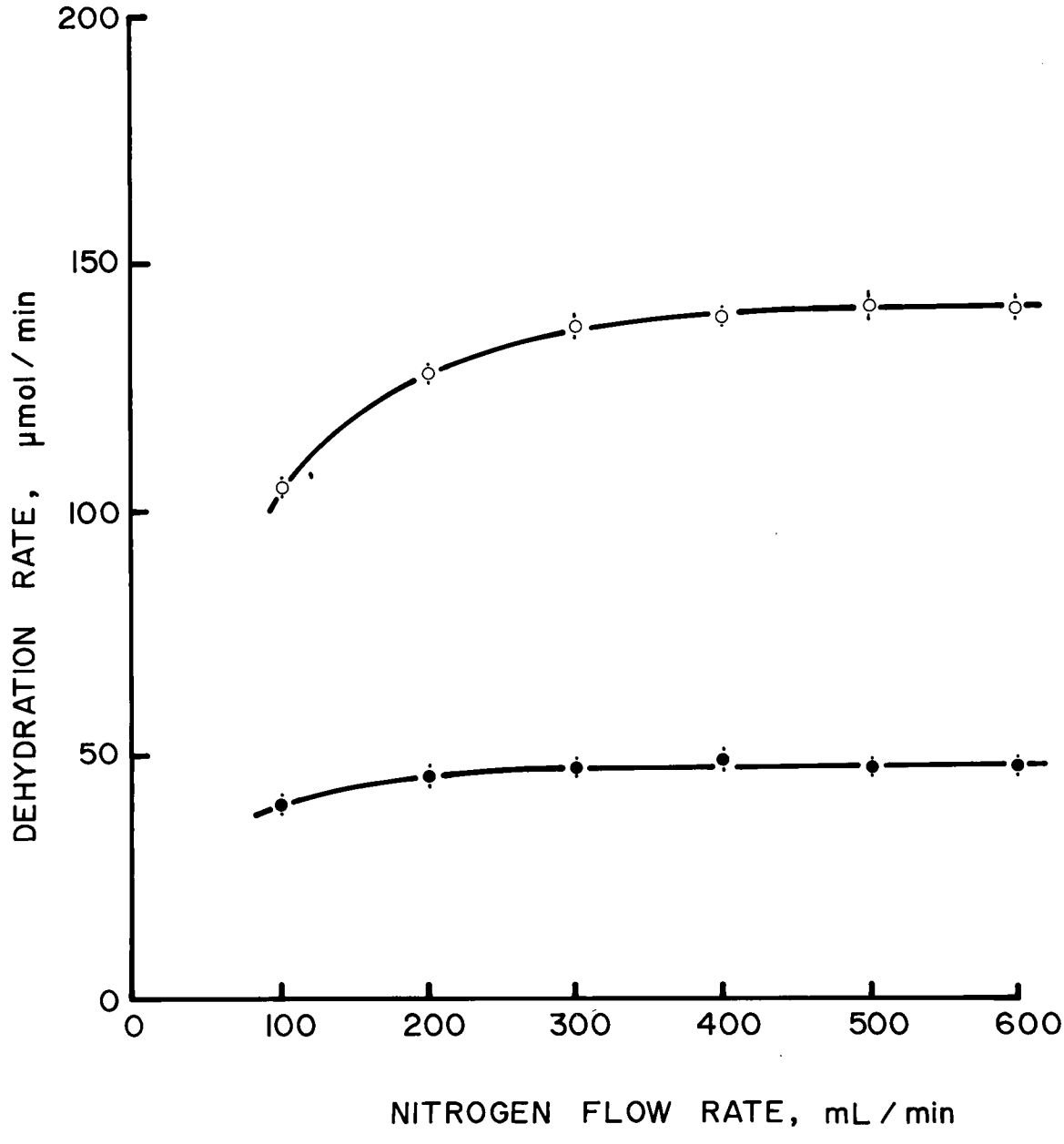
C. Electrometric Method: pH-stat Assay

Bicarbonate dehydration in a phosphate buffer was followed electrometrically with an autotitration assembly (Radiometer,

PHM64 research pH meter, TTT80 titrator, ABU12 autoburette, TTA80 titration assembly) using a modification of the methods of Hansen and Magid (1966), as described by Henry and Cameron (1983). In the present study, a reaction vessel was constructed from a thermostatted 30-mL glass vessel with a 30-mm diameter porous (medium porosity) glass disc embedded in its bottom. Nitrogen gas was passed through a CO_2-trap (saturated KOH solution) and bubbled through the glass disc at a rate exceeding 500 $mL \cdot min^{-1}$ to purge CO_2 from the reaction mixture as CO_2 was formed. The rate of HCO_3^- dehydration was independent of gas flow rate, at flow rates above 400 $mL \cdot min^{-1}$ (Fig. 10). Glass pH and calomel reference electrodes (Radiometer, types G2040C and K4040, respectively) were used to monitor pH of the reaction mixture. A constant pH was maintained by the titrator set to its pH-stat mode; titration was done using 0.125-0.400 N HCl delivered by the autoburette.

The reaction mixture consisted of a phosphate buffer (mixture of 15 mM Na_2HPO_4 and 15 mM KH_2PO_4, pH 0.30 units less than the desired assay pH at the prevailing assay temperature) and a bicarbonate solution (20-60 mM $NaHCO_3$). In some assays, sufficient NaCl was added to the bicarbonate solution to raise the overall ionic strength of the reaction mixture to 0.15 M. Three mL of buffer were added to the reaction vessel first and were allowed to equilibrate with the CO_2-free nitrogen gas until a stable pH was achieved. Three mL of bicarbonate solution then were added together with 0.01-0.10 mL of material to be assayed delivered as 0.10 mL in Cortland saline (Wolf 1963). Titration was initiated when the pH of the mixture reached the desired

Figure 10. Uncatalysed rate of HCO_3^- dehydration (solid circles) and catalysed rate using 1.0 ug of bovine CA (open circles) determined electrometrically at 25 C, pH 7.500 and 50 mM $NaHCO_3$, showing effect of the flow rate of CO_2-free nitrogen gas through the reaction vessel. Values are means $\pm$ 1 S.E. (n=5).


200
150
100
50
0
0
100
200
300
400
500
600
DEHYDRATION RATE, μmol / min
NITROGEN FLOW RATE, mL / min

assay pH. The reaction then was followed for 15-30 sec, after which time the volume of acid added was read from the autoburette to the nearest 1.0 μL. The rate of acid addition (mmol $H^+ \cdot min^{-1}$) was taken as being equivalent to the rate of bicarbonate dehydration (mmol$\cdot min^{-1}$) since the stoichiometry of H^+:HCO_3^- in the dehydration reaction is 1:1.

D. Chemical Studies

The linear range, detection limits and sensitivity of each assay technique were determined using purified bovine carbonic anhydrase (Sigma C-7500).

A lack of definitive data on the kinetics of the uncatalysed CO_2 reactions under the physiological conditions of trout blood prompted their measurement. The uncatalysed dehydration rate constant, k_{HCO_3}, of the reaction $HCO_3^- + H^+ \rightarrow CO_2 + H_2O$ in a buffered NaCl solution (I=0.15) was determined using the pH-stat assay. Bicarbonate dehydration in the pH-stat assay was an unopposed first order reaction since the reaction product, CO_2, was purged continually from the reaction mixture. The dehydration rate constant was calculated from measured rates of HCO_3^- dehydration under specific conditions of known pH (7.000, 7.250, 7.500, 7.750, 8.000), temperature (5, 10, 15 C) and bicarbonate concentration (20, 40, 60 mM) as described by Maren *et al.* (1976).

E. Physiological Studies

CA activity in fish tissues was measured using both the

esterase assay at 25 C, pH 7.500 and 3 mM pNA, and the pH-stat assay at ambient water temperatures (9-11 C), pH 7.500 and 50 mM $NaHCO_3$. Animals used in this study were obtained as follows: rainbow trout (*Salmo gairdneri*) were obtained from Sun Valley Hatchery (Mission, B.C.) and were held as described in the General Materials and Methods; cutthroat trout (*Salmo clarki*), largescale suckers (*Catostomus macrocheilus*) and brown bullheads (*Ictalurus nebulosus*) were captured in Devil's Lake (B.C.) and were held at Simon Fraser University (Burnaby, B.C.) in dechlorinated Burnaby tap water; bowfins (*Amia calva*) were captured in Lake Erie (Ontario) and were airlifted to U.B.C. where they were maintained in facilities identical to those described for rainbow trout; black pricklebacks (*Xiphister atropurpureus*)) were captured near the Bamfield Marine Station (Bamfield, B.C.) and were transported to U.B.C. where they were maintained in recirculated sea-water (salinity 28-33‰).

Erythrocyte samples were obtained by centrifugation of blood withdrawn from the caudal blood vessels of anaesthetized animals (see General Materials and Methods). These red cells were washed twice in Cortland saline and then were lysed using saponin. The resulting lysate was centrifuged at 5,000 *g* and 1 C (Sorval-superspeed RC2-B centrifuge) for 5 min to remove nuclear debris (Watson *et al.* 1982). The supernatent was retained on ice for CA assays. Fish were cleared of remaining red cells by infusing Cortland saline at pressures of between 60 and 80 cm H_2O through a cannula inserted in the ventricle. Tissues to be assayed were excised and homogenized (homogenizing medium: 250 mM sucrose; 40 mM Tris (hydromethyl) methylamine; 5 mM ethylene

diamine tetraacetate (EDTA); pH adjusted to 7.50 with H_2SO_4). These homogenates were centrifuged at 900 *g* and 1 C for 10 min to remove cellular debris and nuclei (Watson *et al.* 1982). The supernatent was retained on ice for CA assays. Aliquots (0.01 mL) of Cortland saline or separated plasma were added to some assays to determine the effects of plasma on CA activity. All measurements were made at least in duplicate.

Where possible, CA activites were converted to arbitrary enzyme units (eu), as

(9) eu = (catalysed reaction rate - uncatalysed rate) / uncatalysed rate.

The reaction rate of Cortland saline was taken to represent the uncatalysed rate in these calculations.

Haemoglobin levels were measured (alkaline haematin method of Anthony 1961) in separated plasma and tissue samples to obtain a measure of the contamination of these samples by erythrocyte CA. No detectable contamination was present in any of the reported studies.

RESULTS

A. Chemical

All three techniques showed a linear relationship between reaction rate and enzyme content over at least some portion of the range of bovine CA concentrations used (Fig. 11, 12 and 13). The esterase assay was linear for all amounts of bovine CA used from 5 μg and above. Although not shown in Figure 11, linearity was preserved up to 1000 μg bovine CA over the entire temperature range used (10-37 C). The lower detection limit of this assay was about 2.5 μg CA; the rate of reactions using less than that amount of enzyme could not be distinguished statistically from the background acetazolamide-insensitive rate. The esterase assay was characterized by very low reaction rates (0.002 to 0.117 μmol pNA$\cdot$min^{-1}) even at the relatively high enzyme levels and high temperatures used (Fig. 11). In addition, this assay had a low sensitivity, as indicated by the slope of the regression of reaction rate against enzyme concentration (0.00095 μmol$\cdot$min$^{-1}\cdot\mu$g CA^{-1} at 10 C to 0.00314 μmol$\cdot$min$^{-1}\cdot\mu$g CA^{-1} at 37 C). It was not possible to convert these sensitivity values to eu$\cdot\mu$g CA^{-1} since by definition the uncatalysed rate of acetazolamide-sensitive pNA hydrolysis is zero.

The modified boat assay gave a linear response from 0 to about 2 μg bovine CA at temperatures from 10 to 25 C (Fig. 12). At 37 C, however, the upper limit of this linear range was reduced to approximately 1 μg bovine CA. The lower detection

Figure 11. Acetazolamide-sensitive esterase activity of purified bovine carbonic anhydrase measured colourimetrically at pH 7.500 and 3 mM p-nitrophenylacetate (pNA), showing effects of reaction temperature and enzyme content. Values are means ± 1 S.E. (n=5).

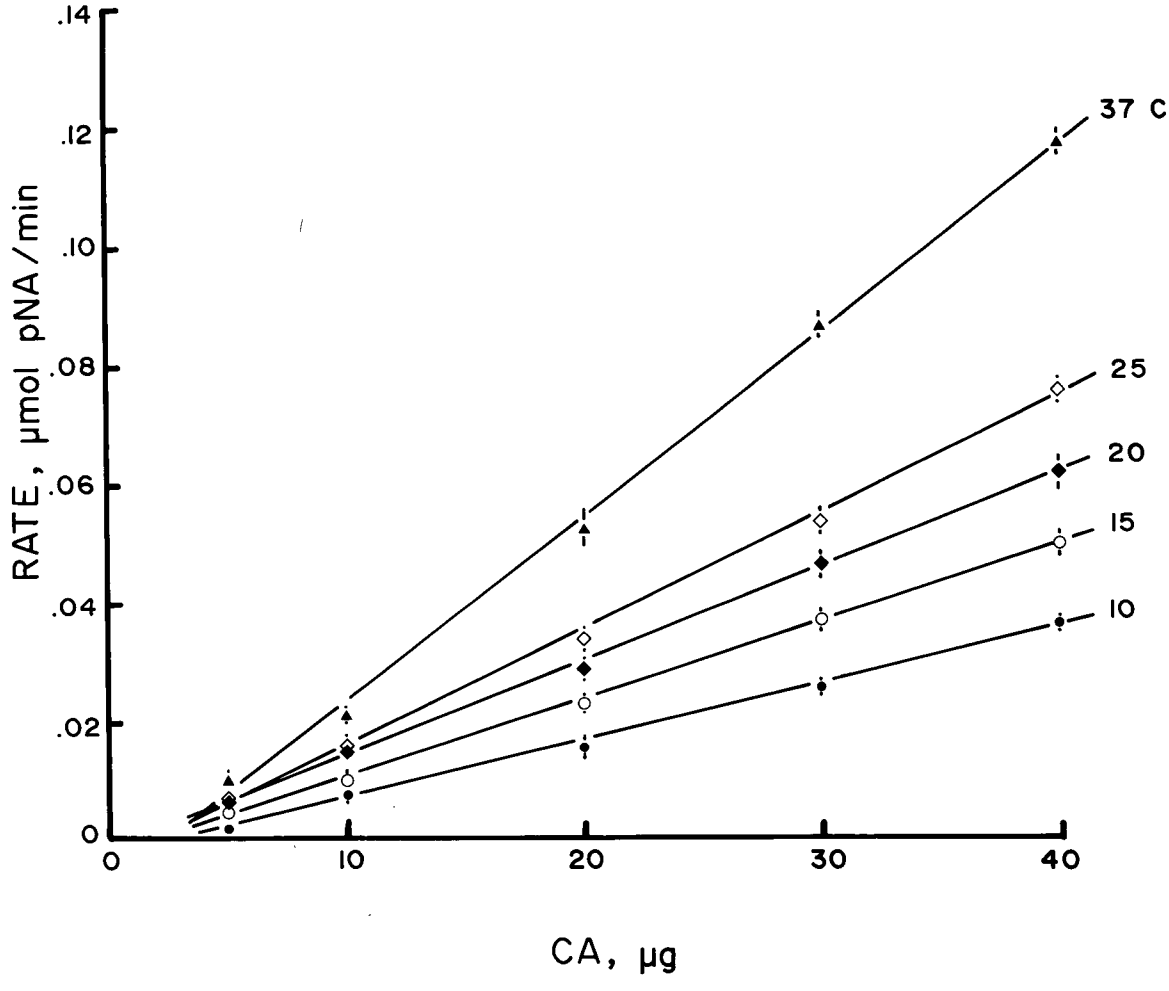

.14
.12
.10
.08
.06
.04
.02
0
RATE, µmol pNA/min
0
10
20
30
40
CA, µg
37 C
25
20
15
10

Figure 12. Catalysis of CO_2 evolution from a buffered $NaHCO_3$ solution by purified bovine carbonic anhydrase measured manometrically at pH 6.800 and 200 mM $NaHCO_3$, showing effects of reaction temperature and enzyme content. Values are means $\pm$ 1 S.E. (n=5).

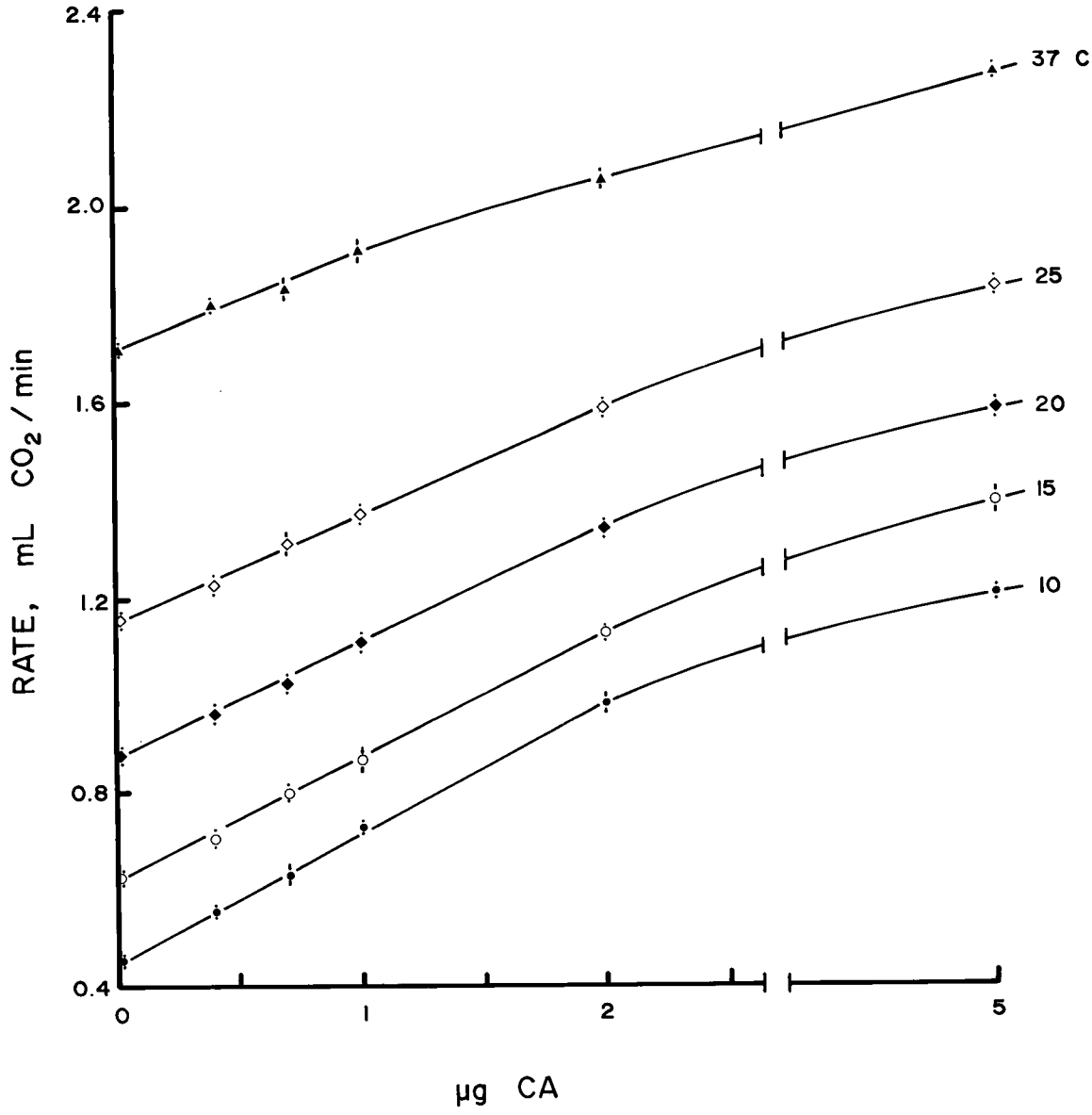
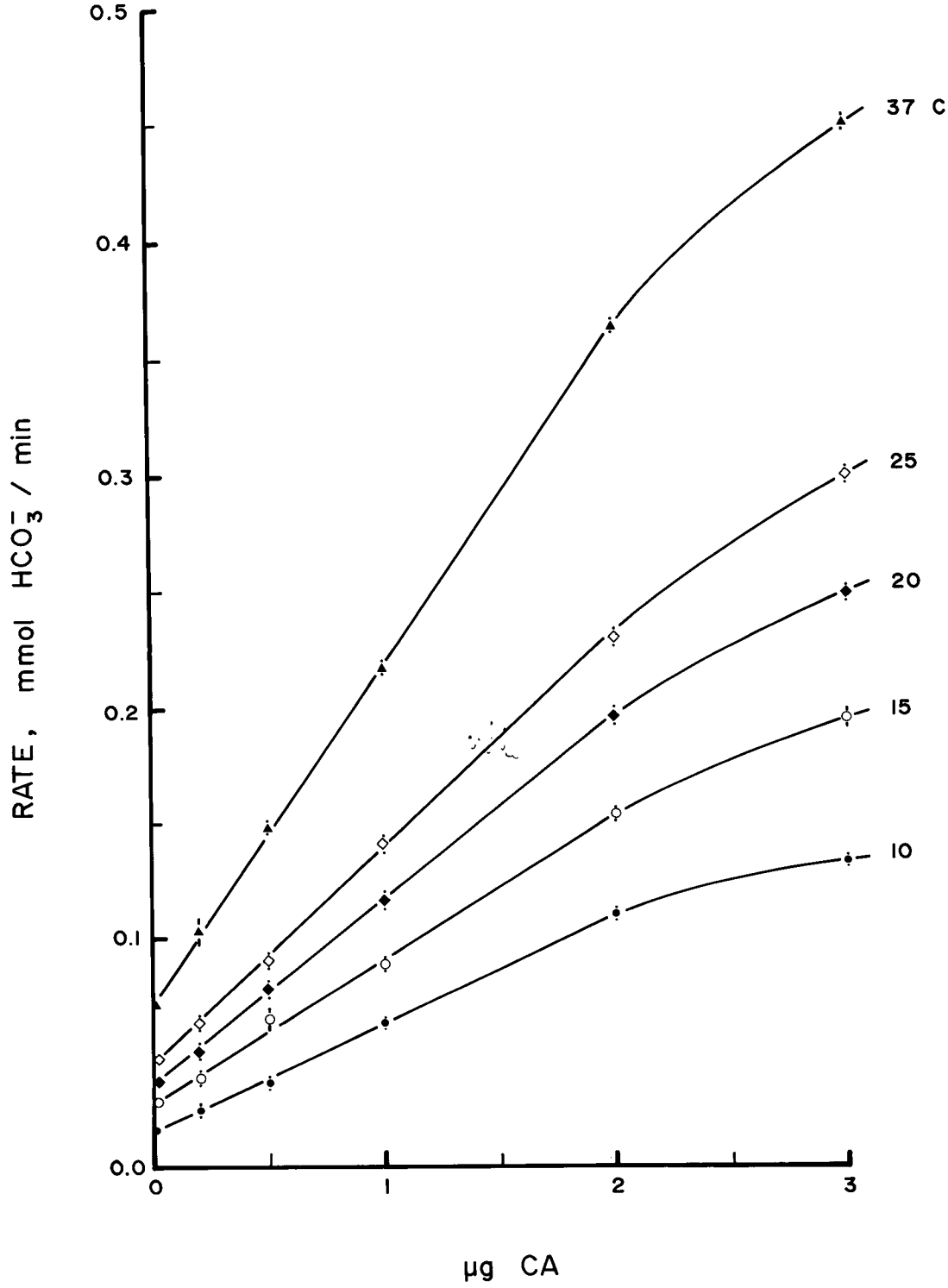

2.4
2.0
1.6
1.2
0.8
0.4
RATE, mL CO_2 / min
0
1
2
5
µg CA
37 C
25
20
15
10

Figure 13. Catalysis of bicarbonate dehydration by purified bovine carbonic anhydrase measured elecrometrically at pH 7.500 and 50 mM $NaHCO_3$, showing effects of reaction temperature and enzyme content. Values are means $\pm$ 1 S.E. (n=5).


0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
RATE, mmol HCO_3^- / min
µg CA
37 C
25
20
15
10

limit of the assay was about 0.1 μg CA in all cases. Sensitivity of the modified boat assay varied inversely with temperature from 0.26 $mL \cdot min^{-1} \cdot \mu g\ CA^{-1}$ at 10 C to 0.17 $mL \cdot min^{-1} \cdot \mu g\ CA^{-1}$ at 37 C, equivalent to 0.6 and 0.1 $eu \cdot \mu g\ CA^{-1}$, respectively.

The most satisfying results in terms of detection and sensitivity were obtained using the pH-stat assay. The pH-stat assay at pH 7.500 and 50 mM $NaHCO_3$ gave a linear response from 0 to 2 μg bovine CA at all temperatures used (10-37 C) (Fig. 13). The lower detection limit of this assay was about 0.02 μg CA when 0.125 N HCl was used as the titrant and could have been reduced still further by using a more dilute titrant. Sensitivity of the pH-stat assay varied from 47.1 $\mu mol \cdot min^{-1} \cdot \mu g\ CA^{-1}$ at 10 C to 146.6 $\mu mol \cdot min^{-1} \cdot \mu g\ CA^{-1}$ at 37 C, equivalent to an average sensitivity of 2.2 $\pm$ 0.2 $eu \cdot \mu g\ CA^{-1}$ (n=5) over the temperature range 10 to 37 C. This represented between a 3.7- to 22-fold improvement in sensitivity over the modified boat assay.

The uncatalysed HCO_3^- dehydration reaction was sensitive to pH and temperature (Fig. 14). The unopposed dehydration rate constant increased when pH decreased or when temperature increased. However, the measured k_{HCO3} values were surprisingly small and indicated half-times for the unopposed dehydration reaction ranging from 3.8 min (15 C, pH 7.000) to 105.0 min (5 C, pH 8.000).

B. Physiological

Table 6 summarizes th CA activity in tissues of several fishes, as determined using the esterase assay at 25 C and the pH-stat assay at ambient water temperatures (9-11 C). Attempts

Figure 14. Dehydration rate constant, k_{HCO_3}, and corresponding half-time for the unopposed reaction $HCO_3^- + H^+ \rightarrow CO_2 + H_2O$ at I=0.15, showing effects of reaction temperature and pH. Values are means $\pm$ 1 S.E. (n=15).

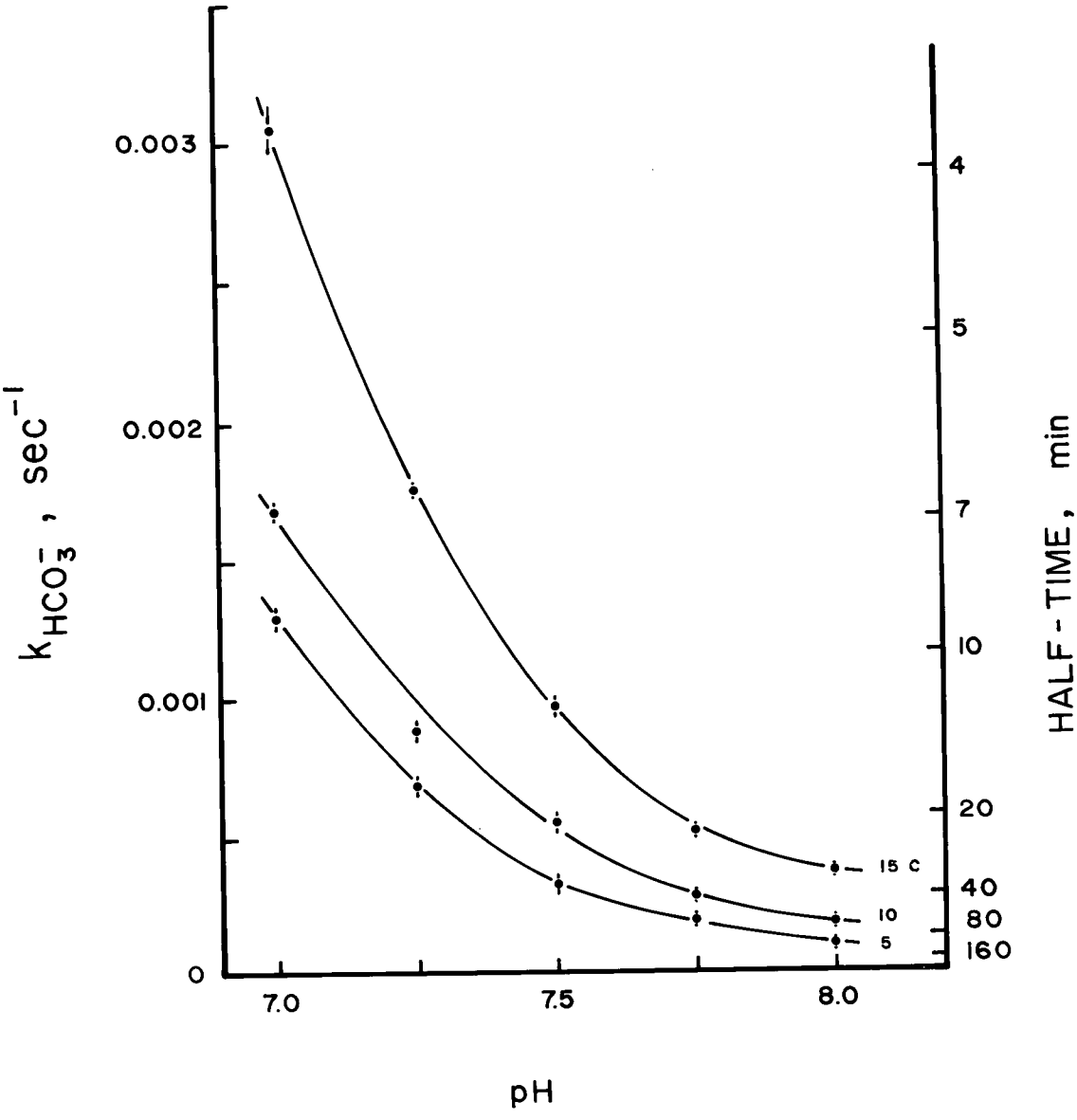

0.003
0.002
0.001
0
$k_{HCO_3^-}$, sec^{-1}
7.0
7.5
8.0
pH
15 C
10
5
4
5
7
10
20
40
80
160
HALF-TIME, min

Table 6. Carbonic anhydrase activity in tissues of several fishes. Measurements were made using the esterase assay at 25 C, pH 7.500 and 3 mM pNA and the pH-stat assay at ambient water temperatures (9-11 C), pH 7.500 and 50 mM $NaHCO_3$. Values are means ± 1 S.E. eu, enzyme unit. ND, none detected.

Tissue	Sample size	Esterase assay ($\mu mol \cdot min^{-1} \cdot g^{-1}$)	pH-stat assay ($eu \cdot g^{-1}$)
Salmo gairdneri			
erythrocyte	16	1.44 ± 0.12	882 ± 154
gill	16	0.72 ± 0.06	773 ± 60
swimbladder	4	0.04 ± 0.02	5 ± 2
skin	6	ND	ND
Salmo clarki			
erythrocyte	7	2.14 ± 0.38	858 ± 148
gill	7	1.28 ± 0.34	478 ± 53
Catostomus macrocheilus			
erythrocyte	6	2.33 ± 0.49	1370 ± 167
gill	6	0.64 ± 0.10	752 ± 80
Ictalurus nebulosus			
erythrocyte	2	1.51 ± 0.25	1262 ± 47
gill	2	0.28 ± 0.10	750 ± 138
Xiphister atropurpureus			
erythrocyte	6	2.41 ± 0.48	1004 ± 200
gill	6	0.50 ± 0.12	396 ± 97
skin	6	ND	ND
Amia calva			
erythrocyte	4	1.31 ± 0.35	210 ± 40
gill	4	0.52 ± 0.08	144 ± 51
swimbladder	4	0.17 ± 0.04	14 ± 2

to conduct the esterase assay at ambient water temperatures proved unsuccessful in my hands, presumably due to the effect of temperature on sensitivity and the inability of this assay to detect small amounts of CA (Fig. 11). The ratio of CA activity as measured using the pH-stat assay to that determined by the esterase assay varied by an order of magnitude among the different tissues (Table 6). Since the two assays were conducted at widely different temperatures, however, this variation in the activity ratio probably reflects different thermal coefficients and/or thermal sensitivities for carbonic anhydrase of different tissues. Nonetheless, the two different techniques yielded similar trends in CA activity among the various tissues. Both techniques demonstrated that, on a wet tissue weight basis, erythrocytes had higher CA activity than gill tissue. Teleostean red cells possessed from 858 to 1370 eu·gm tissue^{-1}. Holoestean erythrocytes, those of *Amia calva*, demonstrated less CA activity (210 eu·gm tissue^{-1}). In general, the results indicate that fish blood contains sufficient carbonic anhydrase per mL to accelerate HCO_3^- dehydration by some 53 to 343 times, assuming an haematocrit of 25% (Cameron and Davis 1970). Swimbladder homogenates had substantially less CA activity than gill tissue, but nonetheless possessed significant activity. Skin homogenates lacked detectable CA activity.

The plasma of most fishes examined contained an endogenous inhibitor of CA which was active against both red cell and gill CA (Table 7). CA activities were reduced from between 14 to 88% by addition of plasma to the assays, independent of the technique

Table 7. Effect of plasma on carbonic anhydrase activity in tissues of several fishes. Values denoted by * are significantly different from zero at $P \leq 0.05$ (paired t-test). Rest of caption as in Table 6.

		Percent change from control activity	
Tissue	Sample size	Esterase assay	pH-stat assay
Salmo gairdneri -- sexually immature			
erythrocyte	6	−53 ± 18*	−30 ± 13*
gill	4	−66 ± 7*	−60 ± 10*
Salmo gairdneri -- sexually mature			
erythrocyte	6	19 ± 16*	24 ± 13*
Salmo clarki -- sexually immature			
erythrocyte	4	−26 ± 13*	−14 ± 6*
Salmo clarki -- sexually mature			
erythrcoyte	4	14 ± 13	15 ± 11
Catostomus macrocheilus			
erythrocyte	6	−19 ± 7*	−17 ± 10*
Ictalurus nebulosus			
erythrocyte	2	−32 ± 8*	−22 ± 18
Xiphister atropurpureus			
erythrocyte	6	−49 ± 17*	−88 ± 8*
Amia calva			
erythrocyte	4	7 ± 3*	5 ± 4
gill	4	9 ± 8	15 ± 6*

used. Since diffusive processes are not important in the esterase assay, such plasma inhibition could not have been the result of diffusion-related artifacts (Heming and Randall 1982). Moreover, plasma of all sexually mature *Salmo* sp. and of the holoestean fish *Amia calva* either had no effect or slightly elevated the measured CA activities of tissue homogenates (Table 7). Plasma of those fish must have lacked a functional inhibitor of CA.

DISCUSSION

Each of the three CA assay techniques tested provided valid measurements of CA activity. The pH-stat assay was judged to be the more favourable for use with tissue homogenates, however, because it exhibited the lowest limit of detection and the highest degree of sensitivity (Fig. 13). It also performed well at physiological levels of pH, temperature and bicarbonate concentration. Moreover, it directly measured the rate of bicarbonate dehydration. On the other hand, the modified boat assay measured the rate of CO_2 evolution in a closed vessel and, consequently, was dependent upon the diffusive equilibrium of CO_2 between an aqueous and a gaseous phase. As well, since CO_2 levels were allowed to increase in the reaction mixture of the modified boat assay, the measured reaction rate was dependent upon the opposed CO_2:HCO_3^- equilibrium, that is, the difference between bicarbonate dehydration and simultaneous CO_2 hydration. In contrast, the pH-stat assay measured the unopposed first order reaction. The decrease in sensitivity of the modified boat assay at higher temperatures (Fig. 12) probably resulted from the inability of the shaking process to ensure rapid diffusive equilibrium of CO_2 at the higher reaction rates, coupled with the differential effect of temperature on dehydration and hydration rate constants (Edsall 1969) which favours CO_2 hydration at higher temperatures. Giraud (1981) was able to increase the sensitivity of the modified boat assay to levels comparable to

those of the present pH-stat assay by maximizing the surface to volume ratio of the reaction mixture through use of small reagent volumes and a small reaction vessel, and by conducting the assay at 0 C. An inherent advantage of the modified boat assay, however, is its potential for use with intact cells (Chapter 3 this study, Booth 1938a, Maren 1967, Haswell and Randall 1976, Haswell *et al.* 1978, Heming and Randall 1982).

The esterase assay possessed a wider usable range of enzyme concentrations than either the modified boat or pH-stat assay (Fig. 11), and was by far the simplest of the three techniques to use. However, it was characterized by an extremely slow reaction rate and as a consequence had poor detection limits and sensitivity, especially at lower temperatures. Armstrong *et al.* (1966) have shown that the CA catalysed rate of pNA hydrolysis proceeds at only 10^{-5} times the rate of CO_2 hydration. The inablility of this assay to detect small quantities of carbonic anhydrase has been cited as a possible explanation for the finding of Mashiter and Morgan (1975) that erythrocytes of flounder (*Platichthys fleus*) lack CA activity (Haswell 1977). Nonetheless, the esterase assay does provide an excellent control technique for the other two assays since, unlike the other two techniques, diffusive processes are not important in the esterase assay.

The uncatalysed dehydration of bicarbonate was surprizingly slow under the conditions tested with half-times for the unopposed reaction in the range of 3.8 to 105.0 min (Fig. 14). Swenson and Maren (1978) report a half-time of 1.6 min (k_{HCO3} = 0.0073 s^{-1}) at 37 C, pH 7.1 and I=0.15. Recalculation of

Knoche's data (1980), using the relation $k_{HCO_3} = k_{H_2CO_3}/10^{pH-pK}$ (Swenson and Maren 1978), yields a half-time of 1.7 min (k_{HCO_3} = 0.0070 s^{-1}) at 25 C, pH 7.1 and I=0. An Arrhenius plot (logarithum of k_{HCO_3} versus reciprocal of absolute temperature) of the present data (Fig. 14) at pH 7.10 predicts half-times of 0.8 min (k_{HCO_3} = 0.0136 s^{-1}) at 37 C and 2.0 min (k_{HCO_3} = 0.0057 s^{-1}) at 25 C. These values are in good agreement with those of Swenson and Maren (1978) and Knoche (1980) considering the length of temperature extrapolation involved and the differences in ionic strength.

The uncatalysed HCO_3^- dehydration reaction occurred far too slowly to account for the observed arterial-venous decrease in plasma bicarbonate across the trout gill (Tables 2 and 5). The half-time for unopposed HCO_3^- dehydration ($HCO_3^- \rightarrow CO_2$) at physiological pH values of trout blood (7.75-8.00) varied from 22 to 105 min (Fig. 14). By way of comparison, the residence time of blood in the gill ranges from a maximum of 3 s at rest to about 0.5 s during exercise (Cameron and Polhemus 1974, Randall 1982a). The complete circulation time in rainbow trout at rest is only about 48 to 96 s (Davis 1970). However, when examining biological systems the opposed reaction ($HCO_3^- \rightleftharpoons CO_2$) must be taken into account because of the appreciable amount of free CO_2 present in blood. The half-time ($t_{1/2}$) for HCO_3^- dehydration in an opposed CO_2:HCO_3^- equilibrium was calculated as

$$(10) \quad t_{1/2} = \ln 2 \,/\, (k_{CO_2} + k_{OH}[OH^-] + a k_{H_2CO_3} + a' k_{HCO_3})$$

where a and a' are the fractions of bound CO_2 present as H_2CO_3 and HCO_3^- respectively (Kern 1960). The variables a and a' were assumed to be 0 and 1 respectively, since the ratio $[HCO_3^-]$ to ([free CO_2] + $[H_2CO_3]$) in blood is about 20:1 (Knoche 1980). Values of k_{CO_2} and k_{OH} at I=0.15 were obtained from Pinsent *et al.* (1956). The biological applicability of $t_{1/2}$ values calculated in this way is open to question. For instance, the calculations ignore the formation of CO_3^{2-} and carbamino compounds, the catalysis of CO_2 hydration by oxyanions (eg. HPO_4^{2-}, $H_2PO_4^-$) (Edsall 1969), and the inverse relationship between k_{CO_2} and buffering capacity (Gray 1971). Nonetheless, given the chemical complexity of fish blood, these $t_{1/2}$ values (Table 8) probably are the best estimates available. Half-times for HCO_3^- dehydration in an opposed carbon dioxide:bicarbonate equilibrium (Table 8) were much smaller than those of the unopposed reaction (Fig. 14) but still exceeded the maximum lamellar blood transit time (3 s at rest, Randall 1982a) by more than 17 fold. Hence, excretion of plasma bicarbonate during gill transit by way of the uncatalysed conversion of bicarbonate to carbon dioxide probably is neglible under normal circumstances.

Red cell, gill and swimbladder invariably contained carbonic anhydrase (Table 6), in keeping with the well documented role of the enzyme in CO_2 excretion, ion transport and gas gland functioning (Maetz 1956a, Fange 1966, Maren 1967, Carter 1972). Fish skin, like that of many amphibians (Maren 1967, Smith 1974, Toews *et al.* 1978), lacked detectable carbonic anhydrase. In terms of distribution and activity levels, the present results are in good agreement with those of Maren (1967) and Houston and

Table 8. Half-time (sec) for HCO_3^- dehydration in an uncatalysed opposed carbon dioxide:bicarbonate equilibrium in aqueous solution, showing the effects of pH and temperature. See text for details. [A]data of Kern (1960) at I=0. [B]data of present study at I=0.15.

	Temperature (C)				
pH	0[A]	5[B]	10[B]	15[B]	25[A]
7.00	240	136	82	48	26
7.50	-	165	91	55	-
8.00	300	165	90	52	25

McCarty (1978). Teleost blood in the present study contained sufficient CA to accelerate bicarbonate dehydration by some 215 to 343 times per mL blood. This exceeds the catalytic potential determined by Haswell and Randall (1976) with the modified boat assay (60-90 fold per mL blood), which is to be expected considering the differences in sensitivity between the modified boat and pH-stat assays. Teleost blood, therefore, has only slightly less catalytic potential than mammalian blood; mammalian blood is capable of accelerating CO_2 reactions by some 440 to 800 fold, assuming an haematocrit of 40% (Maren 1967).

The swimbladder of the bowfin, an organ which is adapted for aerial respiration, contained approximately 2.5 times the CA activity of the nonrespiratory swimbladder of rainbow trout (Table 6). Bowfins differ in this respect from *Calamoichthys calabancus*, the only other bimodally breathing fish in which the CA activity of a respiratory swimbladder has been measured; *C. calabancus* swimbladder lacks detectable carbonic anhydrase activity (Burggren and Haswell 1979). The proposal, therefore, that "the epithelium of the air-breathing organ (of fish) does not contain carbonic anhydrase except in those organs derived from gill tissue" (Randall *et al.* 1981) must be reconsidered. CO_2 excretion across the air-breathing organs of fish appears to be limited during air exposure to the uncatalysed rate of bicarbonate dehydration (Randall *et al.* 1978, Daxboeck and Heming 1982). The present findings indicate that factors other than the presence or absence of epithelial CA must be involved in this response. Epithelial carbonic anhydrase in mammalian tissues has

been implicated in attainment of chemical equilibrium in blood during capillary transit (Crandall and O'Brasky 1978, Effros *et al.* 1978, Klocke 1978) and in facilitation of CO_2 diffusion (Kawashiro and Scheid 1976); perhaps the CA in respiratory swimbladders has a similar role.

The functional presence of plasma inhibitors of carbonic anhydrase was both species-specific and temporally-specific (Table 7). All teleostean fishes examined possessed endogenous CA inhibitors during at least some portion of their life history. The inhibitor was absent in sexually mature individuals, although only sexually mature salmonids were available for study. *Amia calva* also lacked an inhibitor when examined. However, considering the temporal variations found in the teleost inhibitor, one cannot conclude that the absence of an inhibitor in *Amia* plasma is a permanent condition. Plasma inhibitors of CA have been known for some time. Inhibitors have been found in the plasma of a variety of fish and mammals (Booth 1938b, Maetz 1956b, Leiner *et al.* 1962) and more recently in humans (E.D. Crandall, personal communication). Evidence of an inhibitor in other vertebrate phyla has not been found. Plasma inhibitors of CA are believed to be globular proteins (Booth 1938b, Leiner *et al.* 1962, Haswell and Randall 1976), although definative characterization of the inhibitor has not been published. Hence, temporal variations in the teleost inhibitor may have resulted from variations in plasma protein composition. The electrophoretic protein pattern of rainbow trout plasma has been observed to vary qualitatively (Haider 1970, Borchard 1978) and quantitatively (Schlotfeldt 1975) during the course of

development. However, plasma protein patterns also are influenced by stock differences (Langholtz and Dinklage 1970), starvation (Kawatsu 1974), seasonal temperature variations (Schlotfeldt 1975), and disease (Snieszko *et al.* 1966, Lowe-Jinde 1979). The possibility that one or more of these latter factors influenced the present results cannot be ruled out completely, although signs of disease and starvation were not evident.

What possible advantages would be conferred by temporal variation of plasma inhibition of CA? Booth (1938b) originally proposed that plasma inhibitors of CA functioned to immobilize carbonic anhydrase released into the plasma from red cells during the course of the cells' normal destruction or during injury. The present study supports Booths' proposal in as much as the plasma of some individuals not only lacked an inhibitor, but also increased the measured carbonic anhydrase activity of tissue homogenates (Table 7). Since endogenous activators of CA are doubtful (Clark and Perrin 1951), the plasma of those individuals probably contained free CA. This plasma activity could not be attributable to haemolysis during sample preparation since no haemolysis was detected. The advantage of having CA confined inside erythrocytes is that the juxaposition of CO_2:HCO_3^- conversion and haemoglobin facilitates Bohr and Root effects. The presence of CA in plasma conceivably would negate this advantage and so might be disadvantageous. However, under such circumstances, the intracellular reaction still would be expected to dominate because of the greater buffering capacity of red cells over plasma. The presence of extracellular CA during

periods of decreased inhibitor activity would facilitate attainment of chemical equilibrium between red cells and plasma during transit through pulmonary and tissue capillaries (Crandall and O'Brasky 1978, Effros *et al.* 1978, Klocke 1978). Several studies have claimed that the inhibitor is active against carbonic anhydrase inside intact erythrocytes (Maetz 1956b, Haswell and Randall 1976) and thus variations in inhibitor activity might modulate intracellular CA activity. However, more recent studies have been unable to confirm those earlier results (Cameron 1978b, Obaid *et al.* 1979), and the validity of the original technique used has been questioned (Heming and Randall 1982). Another possibility is that the inhibitors modulate the activity of endothelial CA, such as is present in the capillaries of vertebrate lungs (Crandall and O'Brasky 1978, Effros *et al.* 1978, Klocke 1978). The functions of this endothelial CA are unclear at present.

In conclusion, the uncatalysed rate of CO_2:HCO_3^- conversion occurs too slowly under the physiological conditions of fish blood to account for the observed arterial-venous difference in plasma bicarbonate. Instead, the interconversion of plasma HCO_3^- and CO_2 occurs predominantly inside the erythrocytes (Chapter 1), which contain sufficient carbonic anhydrase to accelerate CO_2:HCO_3^- reactions by several orders of magnitude. Movement of HCO_3^- between red cells and plasma is examined in the following chapter. CA also is present in the epithelium of the gills and swimbladder. However, this epithelial CA plays only a minor role in catalysing the dehydration of plasma HCO_3^-, in as much as the basolateral membrane of the gill is largely impermeable to HCO_3^-

(Perry *et al.* 1982). Fish plasma contains an inhibitor of CA which probably functions to suppress CA released from erythrocytes during their normal destruction. The activity of this inhibitor shows temporal variations and perhaps species variations.

Chapter 3.

ION TRANSPORT AND EQUILIBRIA ACROSS THE MEMBRANE OF *SALMO GAIRDNERI* ERYTHROCYTES

The slowest step in capillary CO_2 exchange is HCO_3^-/Cl^- transport across the red cell membrane (Forster and Crandall 1975, Chow *et al.* 1977, Crandall and Bidani 1981, Forster 1982). Even this step, however, is rapid with a half-time in mammalian erythrocytes at 37 C of about 0.1 s (Dalmark 1972, Klocke 1976, Chow *et al.* 1977, Obaid and Crandall 1979). Although the mechanics and kinetics of anion transport in fish erythrocytes have received scant attention, they have been studied extensively in mammalian red cells. Anion exchange in mammalian red cells involves a carrier-mediated transport mechanism (Gunn *et al.* 1973, Dalmark 1976, Chow *et al.* 1977, Crandall *et al.* 1978) which is associated with a transmembrane protein channel, the Band 3 anion exchange pathway (Rothstein *et al.* 1976, 1978, Cabantchik *et al.* 1978). This carrier system is inhibited selectively by disulphonic stilbene derivatives like DIDS and SITS (Cabantchik and Rothstein 1972, Gunn *et al.* 1973, Rothstein *et al.* 1976). For the most part, the transport mechanism is a one-for-one exchange of anions from one side of the cell membrane to the other. The carrier system also is capable of net anion movements (Gunn 1977), albeit at rates approximately 10^5 times slower than those of anion exchange (Knauf *et al.* 1977). There is conflicting evidence as to the applicability of this transport

mechanism to anion movements in fish red cells. Several authors have demonstrated that teleost and elasmobranch erythrocytes possess a HCO_3^-/Cl^- exchanger that is comparable to that in mammalian red cells (Cameron 1978b, Obaid *et al.* 1979). Similarly, Haswell *et al.* (1978) found that Cl^-/acetate exchange in *Tilapia* erythrocytes is comparable to that in human red cells. However, Haswell (1978) has stated that Cl^- self-exchange in rainbow trout erythrocytes is unaffected by SITS-inhibition of the Band 3 anion exchange pathway. Moreover, studies of the CA activity of intact fish red cells have suggested that fish plasma contains a factor which renders fish red cells functionally impermeable to HCO_3^- *in vivo* (Haswell and Randall 1976, Haswell *et al.* 1978).

The role of the red cell in CO_2 exchange ultimately will be influenced by any factor which alters either anion exchange across the cell membrane or the activity of cellular CA. Adrenaline, for example, has been shown to activate the carbonic anhydrase in avian erythrocytes and thus may enhance CO_2 excretion during strenuous flight (Siegmund *et al.* 1974). As well, beta-adrenergic agents have been demonstrated to stimulate ion transport and to induce swelling in erythrocytes of fish (Nikinmaa 1982, 1983), amphibians (Rudolph and Greengard 1980), birds (Riddick *et al.* 1971, Kregenow 1973, Alper *et al.* 1980, Palfrey *et al.* 1980), and mammals (Rasmussen *et al.* 1975, Quist and Roufogalis 1977). Both adrenergic swelling and non-hormonal volume regulatory ion movements are associated with net transport of anions via mechanisms such as $Na^+/K^+/Cl^-$ cotransport (Kregenow 1973, Alper *et al.* 1980) or coupled alkali metal/H^+, Cl^-/HCO_3^-

exchangers (Cala 1980). Adrenergic responses of erythrocytes also include a decrease in cellular organic phosphate levels (Nikinmaa 1983, Nikinmaa *et al.* 1984) and an increase in cellular Ca^{2+} levels (Rasmussen *et al.* 1974). Both depletion of cellular organic phosphates and increased cellular Ca^{2+} have been found to decrease anion permeabilitiy in red cells via structural rearrangement of the membrane (Motais *et al.* 1981). Effects of catecholamines on anion transport and on the cellular CA activity of fish red cells have not been investigated previously.

The objectives of studies in this chapter were to examine anion transport and the cellular CA activity of erythrocytes of *Salmo gairdneri*. Chloride transport was followed using ^{36}chloride as a tracer. Bicarbonate transport was monitored by examining the capacity of intact red cells for catalysing the dehydration of extracellular HCO_3^-. This reaction involved influx of extracellular HCO_3^- into the cell, catalysed HCO_3^- dehydration and diffusion of the resultant CO_2 out of the cell. Bicarbonate transport into the red cell was presumed to be the rate limiting step in this reaction. The sensitivity of chloride and bicarbonate transport to SITS, acetazolamide and blood plasma were eludicated. As well, the response of fish erythrocytes to adrenaline was examined in detail.

MATERIALS AND METHODS

A. Chloride Transport in Red Cells

Red cells were obtained from blood samples withdrawn from the dorsal aortas of chronically cannulated rainbow trout (see General Materials and Methods). These blood samples were diluted to three times their original volume with ice-cold medium A (medium A: 140.0 mM NaCL; 5.0 mM KCl; 1.3 mM $CaCl_2$; 1.2 mM $MgCl_2$; 5.0 mM D-glucose; 27.0 mM glycylglycine; pH titrated on ice to 7.80 with 1.0 N NaOH). The erythrocytes were washed twice in ice-cold medium A and finally were suspended to 25% haematocrit in 5 mL of medium A, B, C or D. Media B, C and D consisted of medium A containing 10^{-3} M acetazolamide, 10^{-4} M SITS (4-acetamido-4'-iso-thiocyanato-stilbene-2,2'-disulphonic acid) and 10 vol% plasma, respectively. Plasma used in medium D had been equilibrated at room temperature with humidified room air (P_{CO_2} = 0.033%, Glueckauf 1951) and had a C_{CO_2} of less than 2 mM (see Chapter 1 for details of measurement technique). Red cell suspensions were loaded with $^{36}Cl^-$ by addition of 5 uL of 118 mM $Na^{36}Cl$ (0.66 mCi/mol, Amersham) and then were allowed to equilibrate on ice for approximately 30 min. Finally, the suspensions were transferred to chilled, plastic tubes (heat-sealed barrels of 1-mL tuberculin syringes) and centrifuged at 12,000 *g* and 1 C for 15 min (Sorval-superspeed RC2-B centrifuge). Approximately 0.2 mL of packed red cells were isolated from each tube by cutting the tube below the cell-medium interface. It was

assumed that this pellet of packed red cells contained 2% trapped $^{36}Cl^-$-laden medium (Gunn *et al.* 1973). Packed red cells, along with a 5 mL rinse of the appropriate $^{36}Cl^-$-free medium, were injected into a shaking tonometer flask containing 4.8 mL of the same $^{36}Cl^-$-free medium, thermostatted at 1 C. The resulting haematocrit was between 1 and 2%. At known time intervals, 0.4 mL samples of this final suspension were withdrawn, layered onto 0.8 mL of ice-cold dibutyl phthalate and immediately centrifuged for 30 sec in a Fisher Micro-centrifuge (model 235). Warming of samples during this centrifugation was minimized by storing the centrifuge rotor in a freezer at -4 C until just prior to its use. $^{36}Cl^-$ activity in the recovered medium was determined using a Nuclear Chicago Isocap liquid scintillation counter. This activity was corrected for trapped activity and then was expressed as a % recovery, where

(11) % recovery = (counts at time t / counts at 30 min) · 100.

Thirty minutes corresponded to a period of time which was about 300 times longer than the expected half-time of the chloride exchange. In studies with SITS (medium C), the 30 min value of the control (medium A) was used in determination of the % recovery.

Extracorpuscular haemoglobin levels were measured (Anthony 1961) to monitor haemolysis; none was detected in any of the reported studies.

B. Bicarbonate Transport in Red Cells

Bicarbonate transport in trout erythrocytes was assessed by measuring the CA activity of intact red cells suspended in a phosphate buffer containing $NaHCO_3$. Catalysis of HCO_3^- dehydration by intact red cells was followed manometrically using the modified boat technique described in Chapter 2. The only difference in the design of assays with intact cells was the inclusion of 50 mM NaCl in the bicarbonate solution to facilitate HCO_3^-:Cl^- exchange across the red cell membrane. Two series of experiments were undertaken: (i) studies of the effect of blood plasma; (ii) studies of the effect of pharmaceutical agents. Red cells used in plasma studies were obtained from blood withdrawn from the caudal blood vessels of anaesthetized rainbow trout (see General Materials and Methods). Haswell and Randall (1976) state that anaesthesia with MS-222 has no effect on HCO_3^- movements in intact trout erthyrocytes. Red cells used in all other studies were obtained from blood withdrawn from the dorsal aortas of unanaesthetized cannulated trout. Regardless of the method of sampling, all blood samples were diluted to three times their original volume with ice-cold Cortland saline. Erythrocytes were washed twice in Cortland saline and finally were suspended to 10% haematocrit in either Cortland saline ('Cortland blood') or plasma ('whole blood'). All samples were kept on ice until used. Assays were conducted at the ambient water temperature (8-11 C).

In studies with plasma, 0.2 mL of either Cortland blood or whole blood were assayed along with 0.1 mL of either Cortland saline, a foaming agent or a defoaming agent. Foaming agents

used were bovine serum albumin (5 mg%, Sigma No. A-9647) and a common colloidal osmotic filler, polyvinyl-pyrrolidinone K-30 (5 mg%). Defoaming agents used were octanol (0.5 mL%) and a commercial silicone-based defoamer, No-foam (0.2 mL%, Argent F-1402-S).

In pharmaceutical studies, 0.2 mL of Cortland blood were assayed along with 0.04 mL of either Cortland saline or a pharmaceutical agent. Agents used were (-)-adrenaline, L-noradrenaline, an alpha-adrenergic antagonist phentolamine (Regitine), and a beta-adrenergic antagonist DL-propranolol HCl. These reagents were prepared in Cortland saline within 30 min of their use to ensure high activity. Effects of the anion transport inhibitor, SITS (10^{-4} M), and the CA inhibitor, acetazolamide (10^{-5} M), also were examined. Red cells used in these studies were pretreated with the appropriate pharmaceutical agent for at least 10 min prior to their use in assays.

CA activity of intact red cells was converted to arbitrary enzyme units (eu) using equation 9 (Chapter 2). The reaction rate of Cortland saline was taken to represent the uncatalysed rate for Cortland blood, whereas the reaction rate of plasma was taken to represent the uncatalysed rate for whole blood. Plasma used in experiments reported in this study did not contain carbonic anhydrase; reaction rates of this plasma were consistently less than that of Cortland saline.

Extracorpuscular haemoglobin levels were measured (Anthony 1961) in all reaction mixtures to monitor haemolysis. No haemolysis was detected in any of the reported assays.

C. Effect of Adrenaline on Ionic Equilibria Across the Red Cell Membrane

Blood samples were withdrawn from the dorsal aortic cannulae of several rainbow trout and were pooled on ice. Two-mL aliquots of pooled blood were equilibrated at 10 C with humidified gas mixtures containing 0.2, 0.5 and 1.0% CO_2 in air (Wostoff pumps). Blood samples were gassed for 30 min and then 0.05 mL of either saline (100 mM perchloric acid diluted to 0.4 vol% with Cortland saline, pH 7.71) or adrenaline (10 $mg \cdot mL^{-1}$ (-)-adrenaline in 100 mM perchloric acid, diluted to 0.4 vol% with Cortland saline, pH 7.69) were added. The adrenaline solution was prepared within 10 min of its use. Samples then were gassed for another 30 min before their respiratory and acid-base states were assessed.

A portion of each equilibrated blood sample was used to measure haematocrit in quadruplicate, blood water content in duplicate and blood C_{CO_2}. A second portion of each sample was centrifuged anaerobically at room temperature for 5 min. The resulting plasma and red cell pellet were used to measure plasma pH, intraerythrocytic pH, plasma water content in duplicate and plasma C_{CO_2}. Water contents and pH were measured as described previously. C_{CO_2} values were measured gasometrically with a Carle analytical gas chromatograph (model 0111) as described by Boutilier *et al.* (1984b). Adrenaline and noradrenaline concentrations were measured on 0.5 mL aliquots of plasma by high pressure liquid chromatography with a Spectra-Physics H.P.L.C. (model SP8700) using the methods of Woodward (1982). Further aliquots of equilibrated blood and plasma were stored frozen for

later analyses of Cl^-, K^+, Na^+ and haemoglobin. Ion concentrations were measured as described in Chapter 1. Haemoglobin concentrations were measured as iron by flame photometry with a Perkin-Elmer atomic absorption spectrophotometer (model 2380) using the methods of Zettner and Mensch (1967). P_{CO_2} values were calculated from those in the gas mixtures as described in the CO_2 solubility study (Chapter 1). Values of $bHCO_3^-$ were calculated using equation 6 (Chapter 1). Intracellular levels of $bHCO_3^-$, Cl^-, Na^+, K^+ and water were calculated using equation 7 (Chapter 1). Ion concentrations were expressed per kilogram of red cell or plasma water in calculations of Donnan ion ratios.

In a second series of experiments, similar blood samples were equilibrated at 0.2 and 1.0 % CO_2 in air in the presence of $5 \cdot 10^{-4}$ M adrenaline and either 10^{-4} M DL-propranolol HCl or 10^{-4} M SITS. Only haematocrit, plasma pH, intraerythrocytic pH and blood haemoglobin content were measured in this series of experiments.

RESULTS

Transport of chloride was extremely rapid across the plasma membrane of *Salmo gairdneri* erythrocytes. Chloride self-exchange between intracellular and extracellular compartments in the control situation was completed within 60 s (Fig. 15). The half-time for chloride self-exchange, calculated from the regression of time against the natural logarithm of [percent recovery + 1] for the first 60 s of the reaction, was 6.1 $\pm$ 0.4 s ($\pm$ 95% confidence interval). Inhibition of carbonic anhydrase with acetazolamide had no effect on chloride movements (Fig. 15). The half-time for Cl^- self-exchange in the presence of acetazolamide was 6.4 $\pm$ 0.4 s. Similarly, addition of plasma (C_{CO_2} less than 2 mM) had no effect on chloride transport (Fig. 15). The half-time for chloride transport in the presence of plasma was 6.2 $\pm$ 0.5 s. Inhibition of the membrane anion exchange pathway with SITS, however, virtually abolished chloride movements across the red cell membrane (Fig. 15).

Rainbow trout erythrocytes were permeable to bicarbonate, as demonstrated by their ability to catalyze the conversion of extracellular HCO_3^- to CO_2. Intact red cells suspended in Cortland saline ('Cortland blood') catalysed HCO_3^- dehydration by 43.12 fold per mL red cells (Table 9). This value is not comparable directly to those measured in Chapter 2 because of differences in sensitivity between the pH-stat and modified boat assays. The carbonic anhydrase activity of intact erythrocytes,

Figure 15. Efflux of ^{36}chloride from erythrocytes of *Salmo gairdneri* at 1 C, showing effects of acetazolamide (10^{-5} M), SITS (10^{-4} M) and plasma (10% by volume). Percent recovery was calculated as activity of $^{36}Cl^-$ in extracellular medium at time *t* divided by activity in medium after 30 min equilibration, multiplied by 100 (see text for details). Points represent data from 3-4 trials.

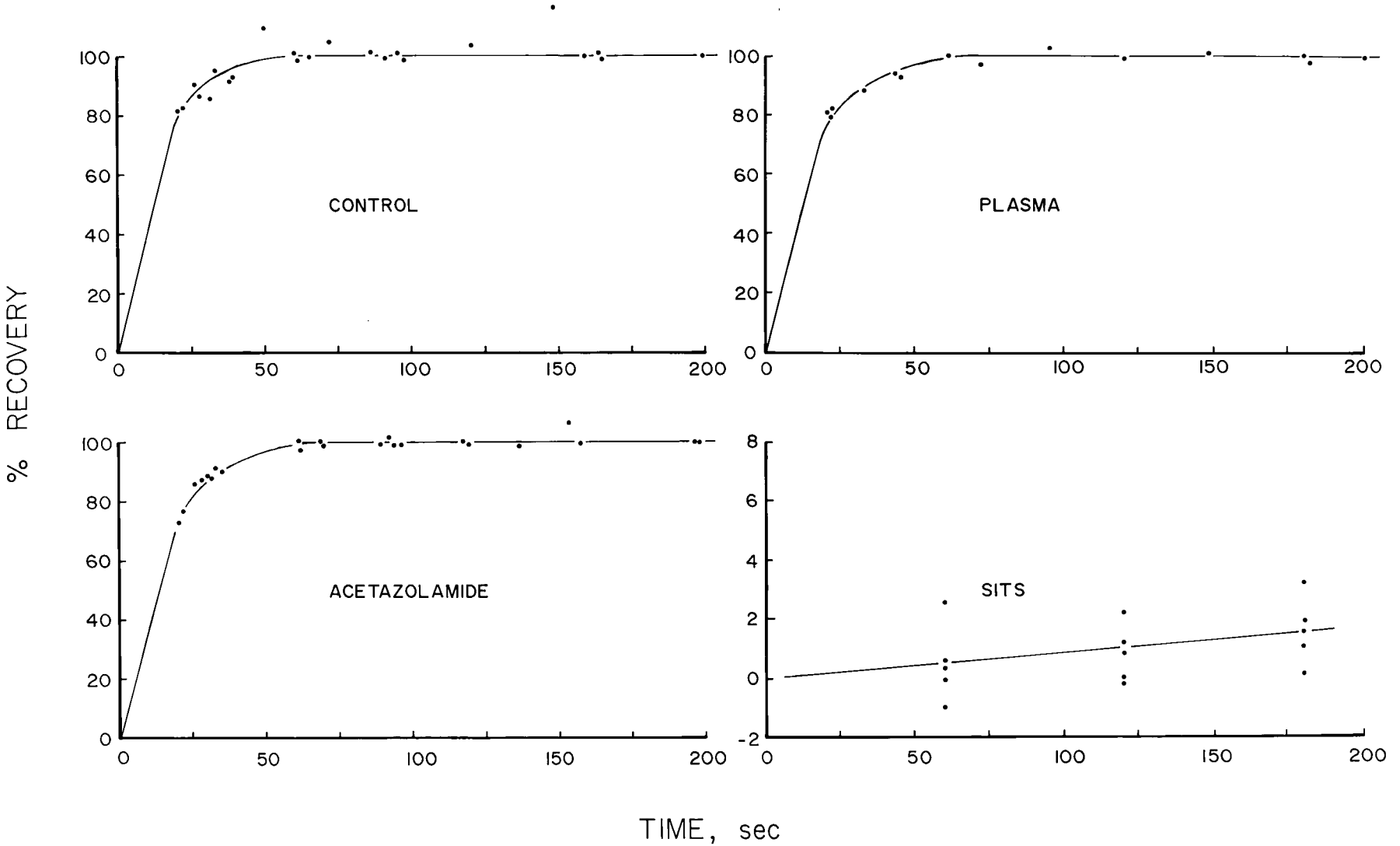

% RECOVERY
CONTROL
PLASMA
ACETAZOLAMIDE
SITS
TIME, sec

Table 9. Net HCO_3^- flux through intact erythrocytes (eu·mL red cell^{-1}) of *Salmo gairdneri*, showing effects of plasma, acetazolamide, SITS, foaming agents, and defoaming agents. Values are means ± 1 S.E. (n=8).

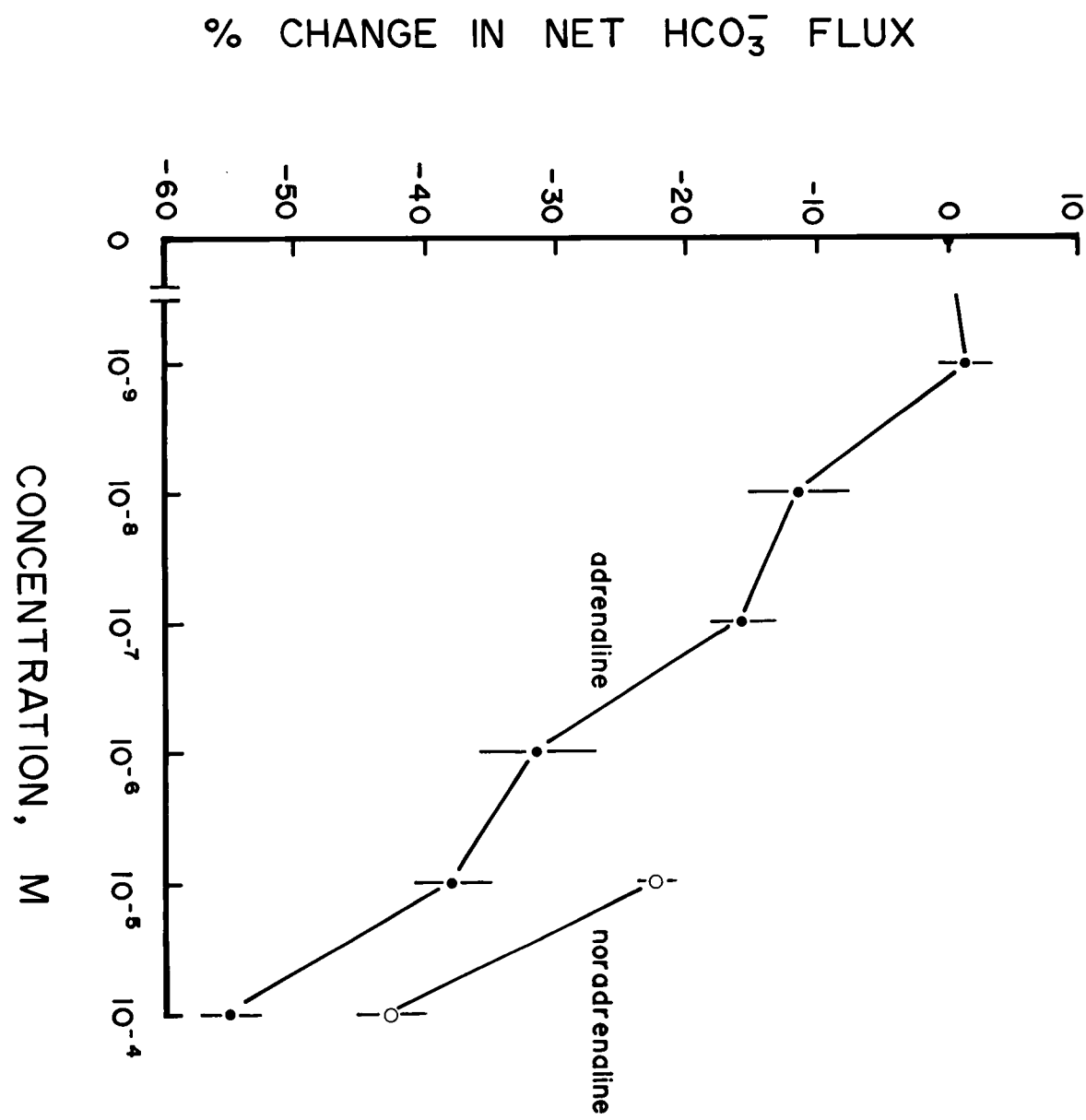
	Whole blood (cells in plasma)	Cortland blood (cells in saline)
Control		
Cortland saline	9.93 ± 0.33	43.12 ± 1.76
Acetazolamide		0.00 ± 0.01
SITS		0.01 ± 0.01
Foaming agents		
bovine serum albumin	1.63 ± 0.03	20.09 ± 1.21
polyvinyl-pyrrolidinone	0.73 ± 0.02	10.09 ± 0.40
Defoaming agents		
octanol	101.46 ± 3.96	108.82 ± 5.83
No-foam	91.50 ± 2.97	99.32 ± 3.45

and hence the net flux of HCO_3^- through intact red cells, was abolished by acetazolamide and by SITS (Table 9).

Plasma significantly influenced the net bicarbonate flux through intact red cells (Table 9). Suspensions of red cells in plasma ('whole blood') demonstrated only 23.0% of the net flux of red cells suspended in saline ('Cortland blood'). However, substantial foaming of whole blood was observed during the assay procedure. Similar foaming was not evident when Cortland blood was assayed. Investigation of the consequences of reagent foaming indicated that foaming could in itself account for the observed inhibitory effects of plasma. Addition of foaming agents (bovine serum albumin, polyvinyl-pyrrolidinone) significantly reduced the measured bicarbonate fluxes in both whole and Cortland blood (Table 9). Foaming agents reduced the HCO_3^- flux in Cortland blood to near the level of control whole blood. On the other hand, addition of defoaming agents (octanol, No-foam) significantly increased the measured net HCO_3^- flux and negated any differences in net flux between whole blood and Cortland blood (Table 9). Thus, defoaming agents countered the effects of plasma on flux of HCO_3^- through red cells.

Net HCO_3^- flux through trout erythrocytes was influenced significantly by adrenergic stimulation. Adrenaline and noradrenaline both inhibited the net bicarbonate flux through intact red cells in a manner which was dose-dependent (Fig. 16). Adrenaline had a greater effect than noradrenaline. Adrenergic inhibition of net HCO_3^- flux was negligible at the physiological levels of adrenaline present in unstressed fish (10^{-9} M, see below), but was responsible for a 31.7% reduction in net

Figure 16. Effects of adrenaline and noradrenaline on net bicarbonate flux through intact erythrocytes of *Salmo gairdneri*. Values are means ± 1 S.E. (n=4–6).


% CHANGE IN NET HCO_3^- FLUX
-60
-50
-40
-30
-20
-10
0
10
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
CONCENTRATION, M
adrenaline
noradrenaline

bicarbonate flux at the physiological levels present in stressed trout (10^{-6} M, Mazeaud and Mazeaud 1981). Adrenergic effects were not an artifact of reagent foaming; use of No-foam in four trials did not alter the inhibitory effect of 10^{-5} M adrenaline. Nor were they due to direct inhibition of the CA enzyme; neither adrenaline or isoproteronol had any signficant effect on the CA activity of trout haemolysates, as measured in four trials with the pH-stat assay (see Chapter 2 for details of assay method). Inhibition of net HCO_3^- flux by adrenaline and noradrenaline was abolished by simultaneous beta-blockade with propranolol (Table 10), indicating that the inhibition was mediated by beta-adrenergic sites. On the other hand, the inhibitory effects of noradrenaline were enhanced by simultaneous alpha-blockade with phentolamine (Table 10). Since noradrenaline contains both alpha- and beta-adrenergic activity, this raises the possibility that alpha-adrenergic agonists enhance HCO_3^- flux and, consequently, antagonize beta-adrenergic effects.

Effects of adrenaline on ion movements across the red cell membrane were investigated further in a series of CO_2 equilibration studies. In these studies, the adrenaline concentration of whole blood was increased from levels present *in vivo* in unstressed fish ($3.1 \pm 0.5 \cdot 10^{-9}$ mol·L plasma^{-1}, n=24) to $4.6 \pm 0.3 \cdot 10^{-6}$ mol·L plasma^{-1} (n=24). Noradrenaline concentrations remained unchanged at $1.2 \pm 0.1 \cdot 10^{-9}$ mol·L plasma^{-1} (n=48). The results, as presented below, demonstrate that adrenaline significantly influenced the equilibrium distributions of H^+, Na^+ and Cl^-, as well as, HCO_3^-.

Table 10. Effects of alpha-adrenergic and beta-adrenergic agonists and antagonists on the net HCO_3^- flux (eu·mL red cell^{-1}) through intact erythrocytes of *Salmo gairdneri*. Values are means ± 1 S.E. (n=6).

	Net bicarbonate flux	% change from control
Control Cortland saline	41.27 ± 2.00	0.0
10^{-5} M adrenaline	25.69 ± 2.37	-37.8
10^{-5} M adrenaline + 10^{-4} M propranolol	42.92 ± 1.65	4.0
10^{-5} M noradrenaline	31.89 ± 1.22	-22.7
10^{-5} M noradrenaline + 10^{-4} M propranolol	43.58 ± 0.41	5.6
10^{-5} M noradrenaline + 10^{-4} M phentolamine	22.08 ± 0.82	-46.5

Adrenergic stimulation resulted in a net loss of H^+ from trout erythrocytes (Fig. 17); adrenaline-treated blood had a higher intracellular pH and a lower plasma pH at each P_{CO_2} level than control blood. The magnitude of the adrenaline effect was related directly to blood P_{CO_2}; adrenaline-induced changes in intracellular H^+ concentrations increased from 2.05 $\pm$ 0.35 nM at 1.8 torr P_{CO_2} to 6.59 $\pm$ 0.62 nM at 7.8 torr P_{CO_2}. These H^+ movements increased the Donnan H^+ ratio, R_{H+}, in adrenaline-treated blood (Fig. 18).

Adrenergic stimulation also resulted in a net cellular gain of Cl^- and Na^+, but had no effect on the distribution of K^+ (Fig. 18). R_{Cl-} ([erythrocyte]/[plasma]) increased with adrenaline, R_{Na+} ([plasma]/[erythrocyte]) decreased, and R_{K+} ([plasma]/[erythrocyte]) remained unchanged. R_{Cl-} values of both control and adrenaline-treated bloods were virtually identical to R_{H+} values, and both R_{Cl-} and R_{H+} were in good agreement with the data of Figure 5 (Chapter 1). This indicates that Cl^- and H^+ remained passively distributed following treatment with adrenaline. In all cases trout erythrocytes maintained a low intracellular concentration of Na^+ and a high intracellular concentration of K^+, relative to plasma levels, with Donnan ratios vastly different from those of H^+, Cl^- and HCO_3^-, and from each other. The distribution of Na^+ and K^+ in these red cells was maintained by the known Na^+/K^+ pump on the red cell membrane.

Adrenaline had a significant effect on the CO_2-carrying capacity of trout blood; adrenaline-treated blood contained less $bHCO_3^-$ at each P_{CO_2} than control blood (Fig. 19). This decrease in blood $bHCO_3^-$ was due primarily to a decrease in cellular

Figure 17. Plasma pH and intraerythrocytic pH of *Salmo gairdneri* blood as a function of P_{CO_2} determined *in vitro* at 10 C, showing effects of adrenaline. Adrenaline concentrations were $3.1 \cdot 10^{-9}$ mol·L plasma^{-1} in control blood and $4.6 \cdot 10^{-6}$ mol·L plasma^{-1} in adrenaline blood. Values are means $\pm$ 1 S.E. (n=8). Adrenaline values denoted with * are different significantly from corresponding control values at $P \leq 0.05$ (paired t-test).

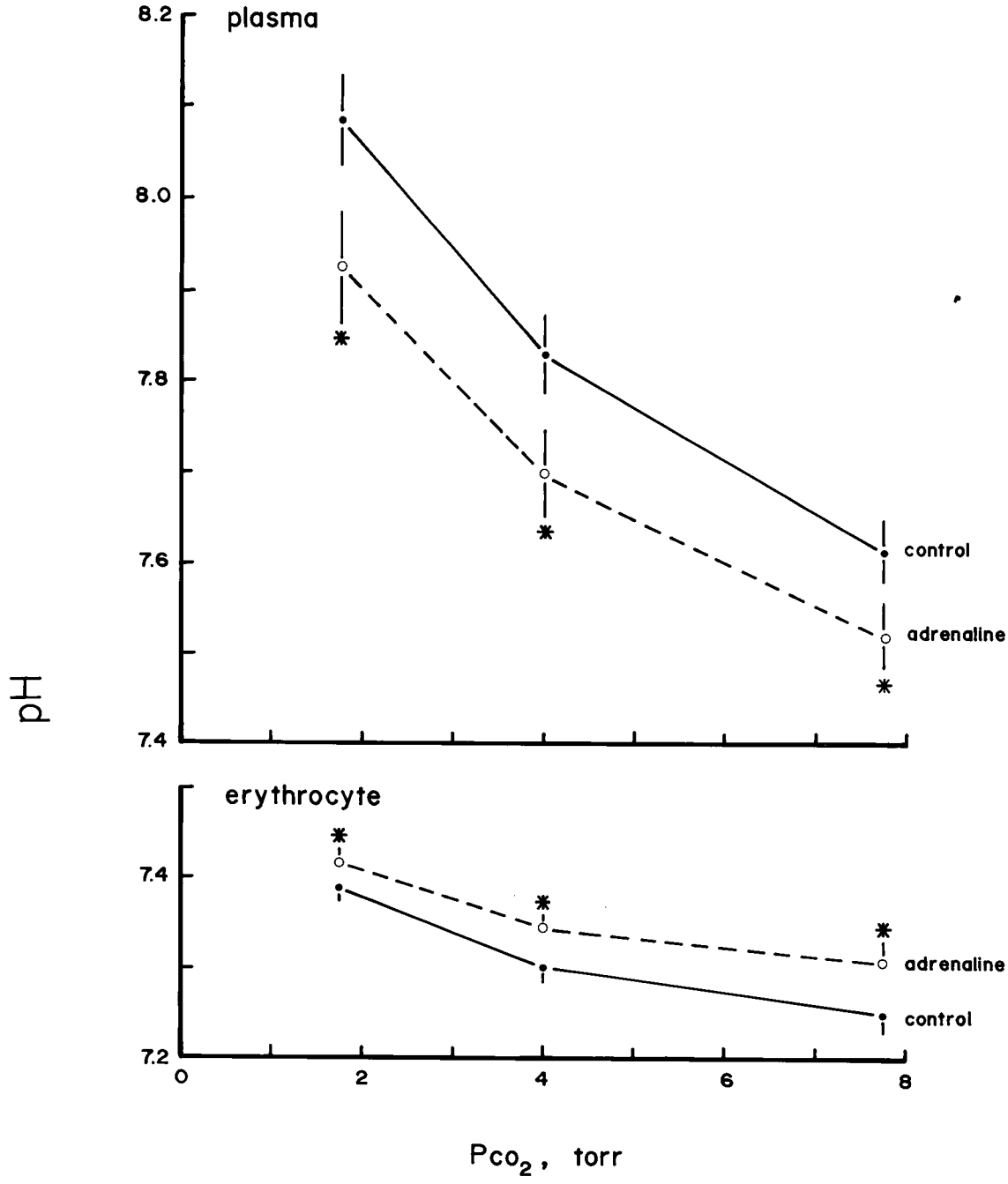

plasma
8.2
8.0
7.8
7.6
7.4
control
adrenaline
erythrocyte
7.4
7.2
adrenaline
control
0
2
4
6
8
pH
Pco2, torr

Figure 18. Donnan ratios of H^+, Na^+, K^+, Cl^-, and $bHCO_3^-$ in *Salmo gairdneri* blood as a function of P_{CO_2} determined *in vitro* at 10 C, showing effects of adrenaline. Anion ratios are [erythrocyte]/[plasma] whilst cation ratios are [plasma]/[erythrocyte]. Rest of caption as in Figure 17.

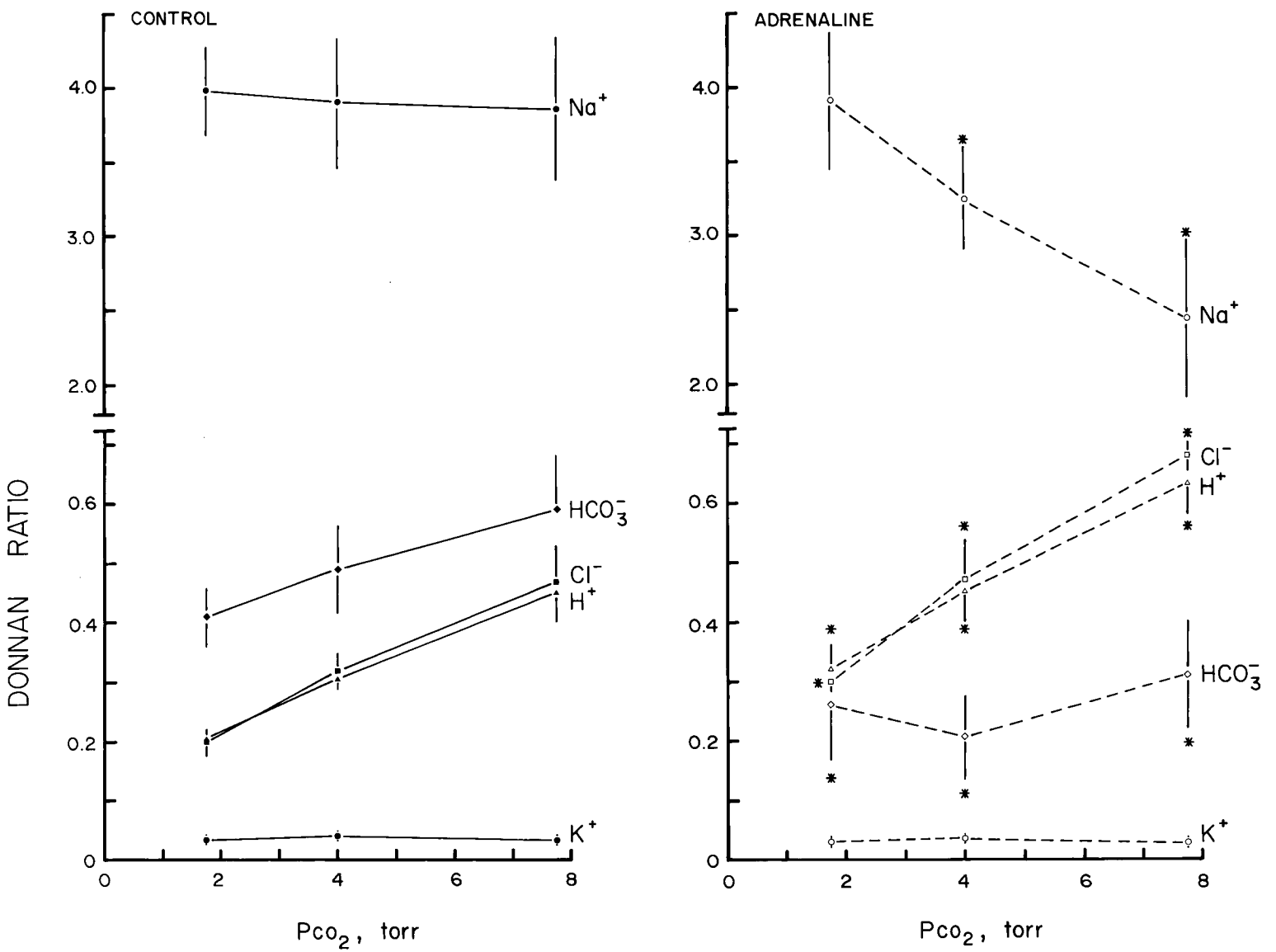
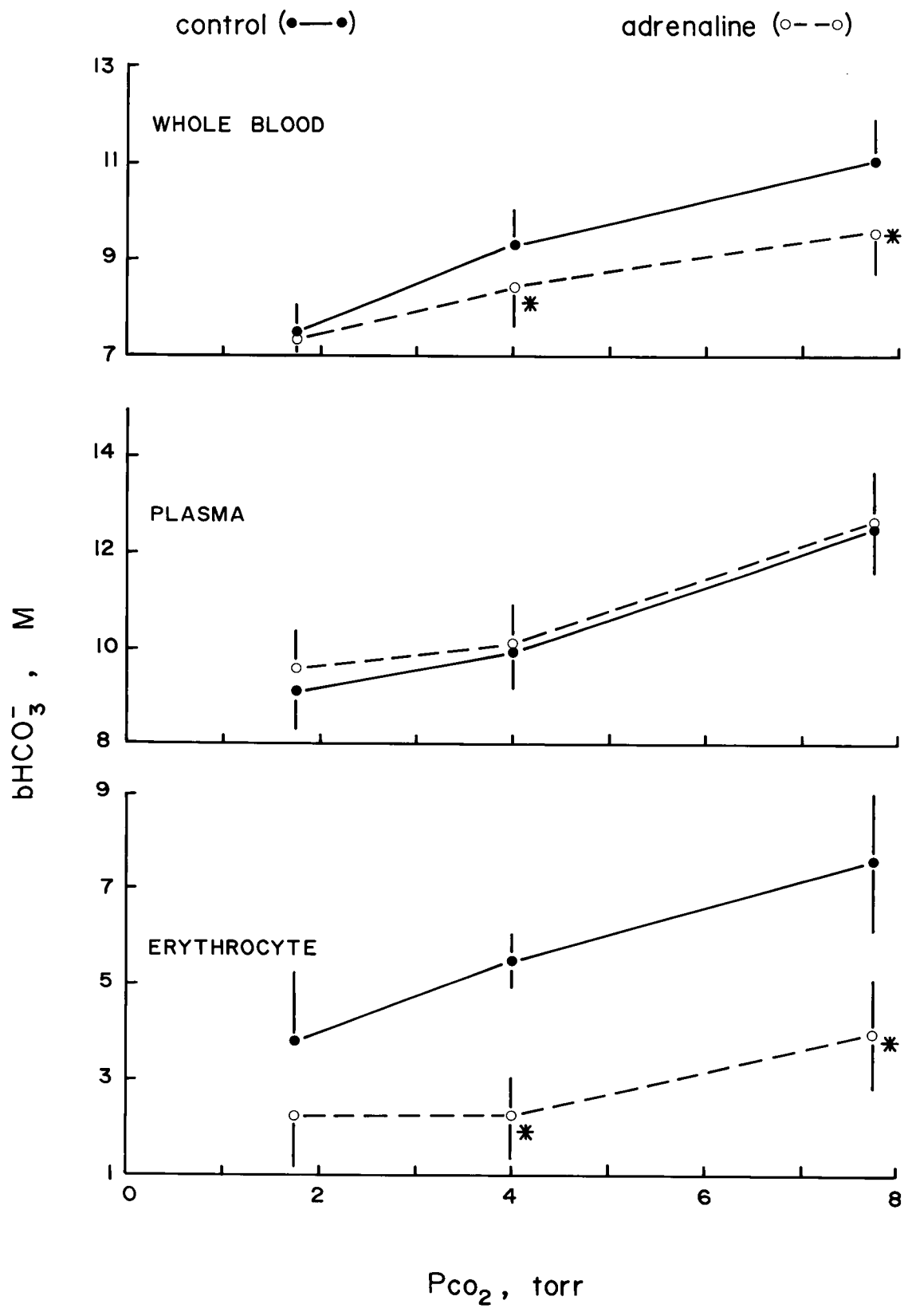

CONTROL
ADRENALINE
DONNAN RATIO
4.0
3.0
2.0
0.6
0.4
0.2
0
Na+
HCO3−
Cl−
H+
K+
0
2
4
6
8
Pco2, torr

Figure 19. $bHCO_3^-$ concentations in oxygenated whole blood, plasma, and erythrocytes of *Salmo gairdneri* as a function of P_{CO_2} determined *in vitro* at 10 C, showing effects of adrenaline. Rest of caption as in Figure 17.


control (•—•)
adrenaline (○- -○)
WHOLE BLOOD
PLASMA
ERYTHROCYTE
13
11
9
7
14
12
10
8
9
7
5
3
1
0
2
4
6
8
bHCO3-, M
Pco2, torr

$bHCO_3^-$; plasma $bHCO_3^-$ was not affected significantly (Fig. 19). As a result, adrenaline produced a significant decrease in the Donnan $bHCO_3^-$ ratio (Fig. 18). This decrease in R_{HCO3-} was in sharp contrast to the adrenaline-induced increase in R_{H+} and R_{Cl-} (Fig. 18).

Adrenaline-induced ion movements were accompanied by a significant increase in the mean cell size of erythrocytes (measured as the quotient of haematocrit (%) and haemoglobin concentration, g·L blood^{-1}; Fig. 20) and by net movement of water into the cell (Fig. 20). These effects were abolished by blockade of beta-adrenergic sites with propranolol and by SITS (Fig. 21). Adrenergic redistribution of H^+ also was sensitive to blockade of beta-adrenergic sites; adrenaline had no effect on R_{H+} when beta-sites were blocked with propranolol (Fig. 21). As well, adrenergic effects on pH were sensitive to inhibition of the membrane anion exchange pathway with SITS (Fig. 21).

Figure 20. Mean cell size (haematocrit / [haemoglobin]) and water content of *Salmo gairdneri* erythrocytes as a function of P_{CO_2} determined *in vitro* at 10 C, showing effects of adrenaline. Rest of caption as in Figure 17.

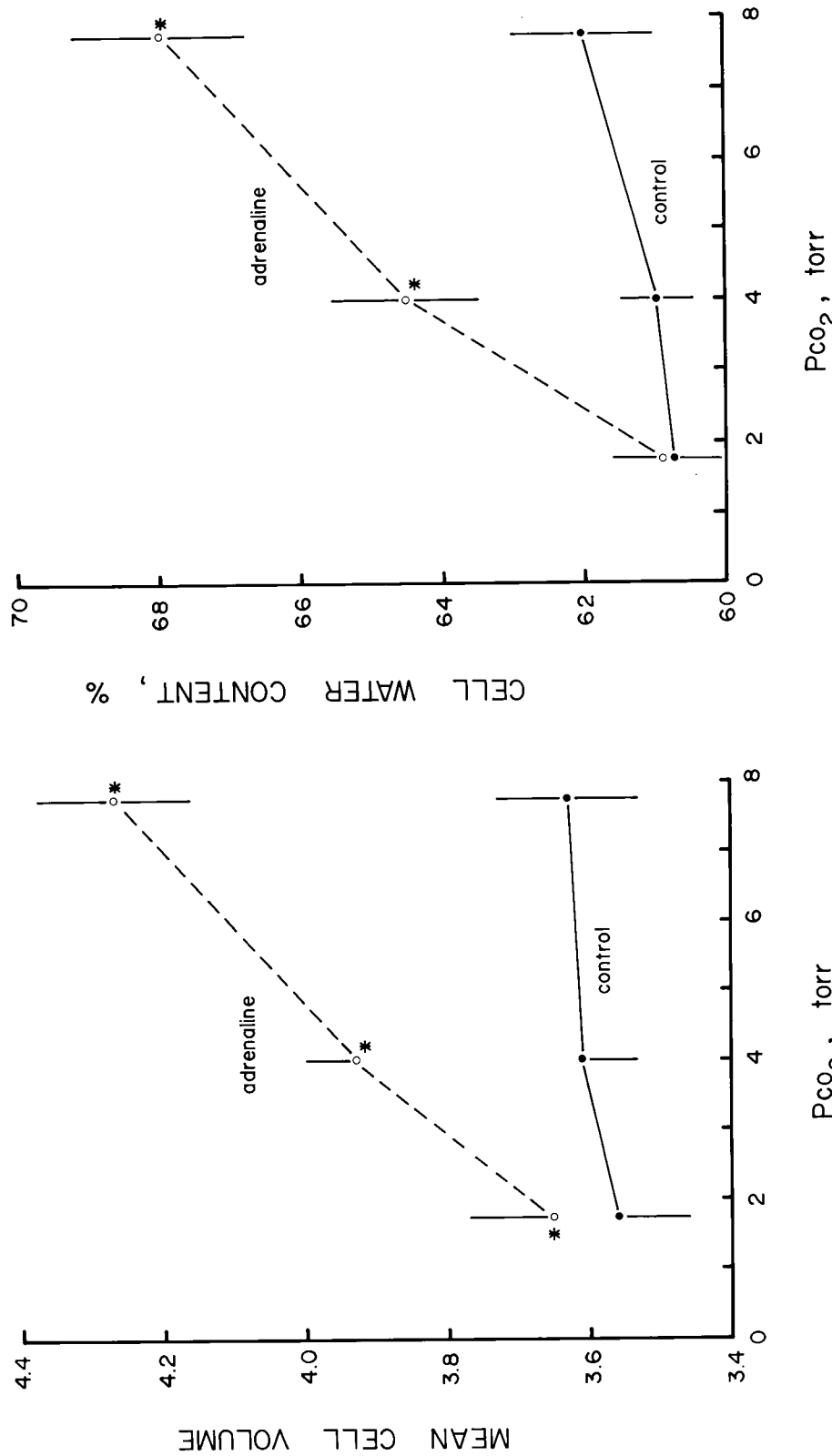
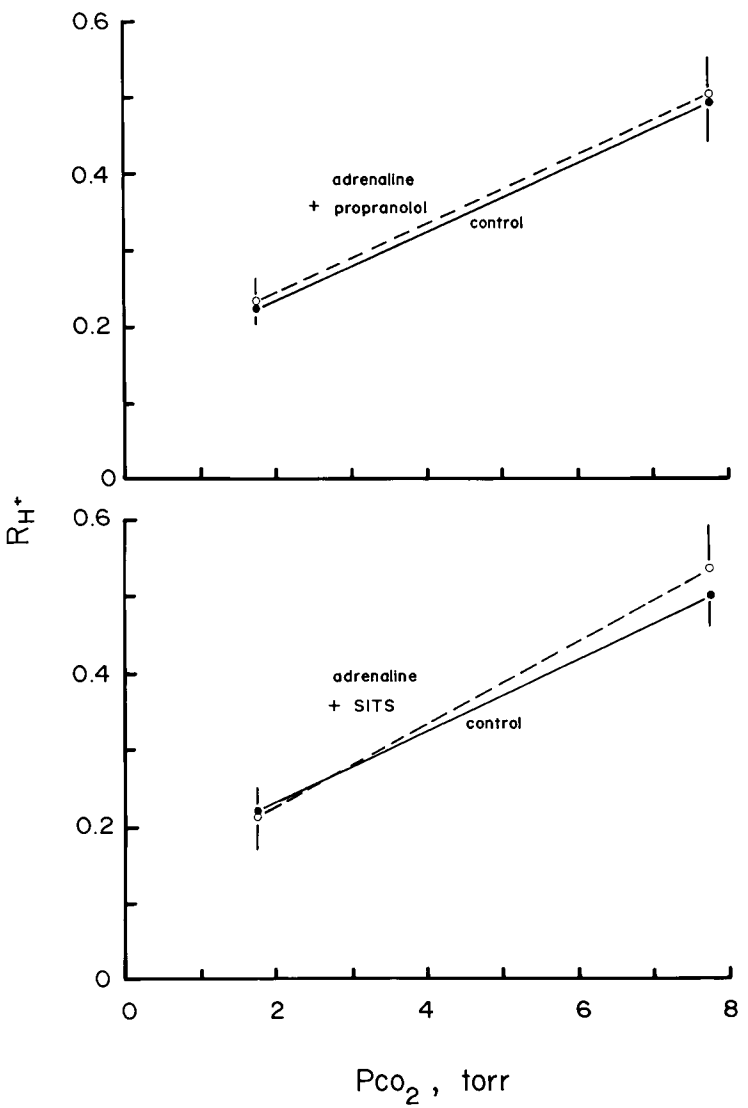
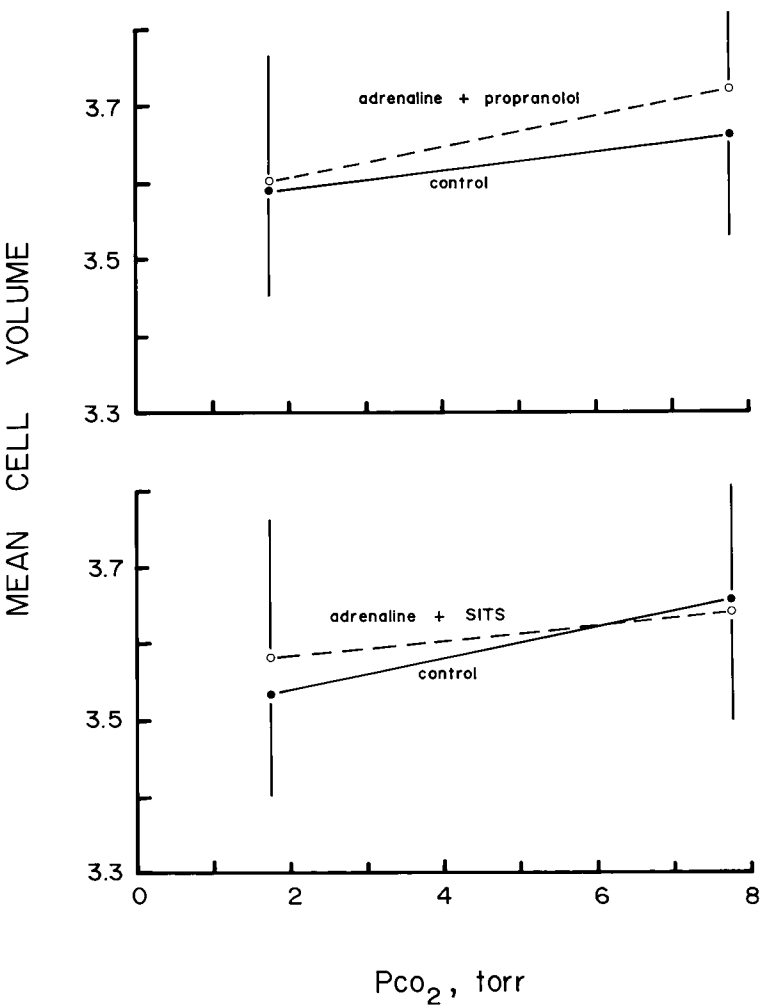

CELL WATER CONTENT, %
70
68
66
64
62
60
adrenaline
control
0
2
4
6
8
Pco_2, torr
MEAN CELL VOLUME
4.4
4.2
4.0
3.8
3.6
3.4
adrenaline
control
0
2
4
6
8
Pco_2, torr

Figure 21. Donnan H^+ ratio ([plasma]/[erythrocyte]) and mean cell size of erythrocytes of *Salmo gairdneri* as a function of P_{CO_2} determined *in vitro* at 10 C, showing effects of addition of adrenaline ($5 \cdot 10^{-6}$ M) and either propranolol (10^{-4} M) or SITS (10^{-4} M). Values are means $\pm$ 1 S.E. (n=4).


0.6
0.4
0.2
0
adrenaline
+ propranolol
control
0.6
0.4
0.2
0
adrenaline
+ SITS
control
0
2
4
6
8
R_{H^+}
Pco_2, torr


3.7
3.5
3.3
adrenaline + propranolol
control
3.7
3.5
3.3
adrenaline + SITS
control
0
2
4
6
8
MEAN CELL VOLUME
Pco_2, torr

DISCUSSION

Trout erythrocytes were readily permeable to chloride, with a half-time for Cl^- self-exchange at 1 C (6.1 s) which was comparable to that of mammalian red blood cells under similar conditions (7-8 s, Wieth and Bjerrum 1982). This large permeability is further evidence for a passive Cl^- distribution. Since HCO_3^- has been shown to be as good an exchange partner for Cl^- as Cl^- itself (Dalmark 1972), the present Cl^- self-exchange data also suggest that fish red cells are readily permeable to bicarbonate. The findings that trout erythrocytes were capable of catalyzing the dehydration of extracellular HCO_3^- was direct evidience of their HCO_3^- permeability. Fluxes of both Cl^- and HCO_3^- through trout red cells were inhibited by SITS, as occurs in mammalian red cells (Cabantchik and Rothstein 1972). SITS specifically inhibits the Band 3 anion exchange pathway responsible for the chloride shift in mammalian erythrocytes (Cabantchik *et al.* 1978) and probably has an identical effect on fish erythrocytes. Membrane transport of anions in trout red cells was independent of erythrocytic CA activity. However, as one might expect, erythrocytic carbonic anhydrase activity was dependent upon anion transport. Inhibition of anion exchange during CO_2 loading effectively isolates intracellular CA from plasma bicarbonate, its major source of substrate, whilst inhibition during CO_2 loading slows CO_2 hydration via the 'Law of Mass Action' as HCO_3^- accumulates inside the cell. In this way, inhibition of anion exchange is functionally identical to

inhibition of erythrocytic CA.

Blood plasma had no specific effect on anion transport or on intracellular CA activity. This is consistent with evidence that teleost and elasmobranch red cells possess a mammalian-like chloride shift which is unaffected by plasma (Cameron 1978b, Obaid *et al.* 1979). Haswell and Randall (1976) and Haswell *et al.* (1978), however, found that fish plasma renders red cells functionally impermeable to bicarbonate using a manometic technique identical to that used in the present study. The present results indicate that this plasma inhibition is a non-specific artifact related to reagent foaming during the modified boat assay, and not the action of a specific transport/enzyme inhibitor. Similar decreases in intracellular CA activity were observed in the presence of bovine serum albumin and polyvinyl-pyrrolidinone. These effects, including that of plasma, were countered by use of defoamers. Hence, CO_2 produced by HCO_3^- dehydration during the modified boat assay is retained in foam. This foaming slowed the diffusive equilibrium of CO_2 between the liquid and gaseous phases of the assay. Care must be taken, therefore, to minimize or to standardize reagent foaming in order to obtain valid results with the modified boat assay. Use of defoamers cannot be recommended, however, unless the effects of their presence on the CA enzyme and on membrane transport properties are understood.

The equilibrium distributions of most major ions in *S. gairdneri* blood were influenced significantly by catecholamines. Beta-adrenergic stimulation of trout red cells resulted in a net cellular gain of Na^+, Cl^- and H_2O, a net cellular loss of H^+ and

HCO_3^-, and a pronounced cell swelling. The equilibrium distribution of K^+ remained virtually unchanged. Beta-adrenergic swelling and an associated H^+ extrusion have been noted previously in erythrocytes of rainbow trout and striped bass, *Morone saxitilis* (Nikinmaa 1982, 1983, Nikinmaa *et al.* 1984). Moreover, Nikinmaa and Huestis (1984) have shown that adrenergic swelling and the associated pH changes in rainbow trout red cells are independent of extracellular K^+ and insensitive to furosemide, but are dependent upon extracellular Na^+ and sensitive to DIDS and amiloride. Adrenergic swelling of fish erythrocytes thus appears to involve a different molecular mechanism than that involved in avian red cells. In avian erythrocytes, adrenergic swelling involves $Na^+/K^+/Cl^-$ cotransport into the cell, requires high, nonphysiological levels of extracellular K^+, and is sensitive to furosemide but insensitive to disulphonic stilbene derivatives (Riddick *et al.* 1971, Kregenow 1973, Palfrey *et al.* 1980). In terms of adrenergic swelling, fish erythrocytes behave more like amphibian red cells, in as much as the regulatory volume increases of amphibian erythrocytes in hypertonic media also do not require extracellular K^+ to maintain uptake of Na^+ and H_2O (Kregenow 1981). Cala (1980) found that amphibian erythrocytes gain sodium, chloride and water during regulatory volume increases in hypertonic media. These ion fluxes were electrically silent, were sensitive to medium bicarbonate levels and DIDS, and were associated with H^+ extrusion. This information led Cala (1980) to propose that volume regulatory ion movements in amphibian red

cells involve obligatorily coupled alkali metal/H^+ and Cl^-/HCO_3^- exchangers. The present data indicate that similar exchangers exist in fish erythrocytes and that they are sensitive to beta-adrenergic stimulation in isotonic media. Accordingly, binding of beta-agonists to membrane receptors on fish erythrocytes results in Na^+ and Cl^- influxes in exchange for H^+ and HCO_3^-; the latter ion pair do not exert osmotic pressure when in equilibrium with CO_2 and H_2O. The net result is an increase in the number of osmotically-active particles in the intracellular compartment and, consequently, uptake of extracellular water and cell swelling. These ion exchangers probably are involved in volume regulation of fish erythrocytes during osmotic perturbations as well. The molecular mechanism involved in swelling of avian red cells is sensitive to both osmotic perturbations and beta-adrenergic stimulation (Kregenow 1973). Moreover, Cala (1977) has shown that regulatory increases in erythroycte volume after osmotic perturbation of flounder (*Pleuronectes flesus*) red cells is accompanied by a net cellular gain of sodium, chloride and water, with little change in potassium.

Alternative explanations of the molecular mechanism underlying adrenergic swelling of fish erythrocytes do not account for the observed results as well as coupled Na^+/H^+, HCO_3^-/Cl^- exchangers. Coupled Na^+/H^+, coupled Na^+/Cl^- and $Na^+/HCO_3^-/Cl^-$ cotransport mechanisms have been found in a number of invertebrate and vertebrate tissues (for review see Roos and Boron 1981, Murer *et al.* 1983, Palfrey and Rao 1983), and would appear to be the most obvious alternatives. However, each of

these mechanisms can be discounted. Na^+/H^+ exchangers are insensitive to disulphonic stilbene derviatives (SITS, DIDS), whilst coupled transport of $Na^+/HCO_3^-/Cl^-$ results in a net cellular gain of Na^+ and HCO_3^-, and a loss of Cl^-. Na^+/Cl^- exchangers are sensitive to furosemide, whereas Nikinmaa and Huestis (1984) have demonstrated that the adrenergic response of fish erythrocytes is not. In human erythrocytes, adrenergic swelling has been proposed to involve a calcium-mediated change in the submembrane cytoskeleton of the red cell (Rasmussen *et al.* 1975). This cytoskeleton, the microfilamentous spectrin complex, is thought to control cell shape and size (Steck 1974). Swelling due to simple structural changes could conceivably increase sodium leakage down its concentration gradient into the cell and would alter the Donnan distribution of passively permeant ions by affecting the net charge state of haemoglobin (Gary-Bobo and Solomon 1971). Under such circumstances, however, one also would expect significant leakage of K^+ out of the cell. Moreover, changes in the net charge state of haemoglobin would affect R_{H+}, R_{Cl-} and R_{HCO3-} all in the same direction, whereas in the present study adrenaline caused an increase in R_{H+} and R_{Cl-} but a decrease in R_{HCO3-}. At this point, however, the dubious nature of the present R_{HCO3-} estimates needs to be considered. It was argued in Chapter 1 that the present R_{HCO3-} values are biased by carbaminohaemoglobin formation. It is conceivable, therefore, that adrenergic-induced decreases in R_{HCO3-} reflect a decline in carbamino formation rather than bicarbonate extrusion. This is an unlikely explanation for the following reason. Adrenergic

swelling of fish erythrocytes is associated with a decrease in cellular nucleoside triphosphate levels (Nikinmaa 1983, Nikinmaa *et al.* 1984) and, since nucleoside triphosphates compete with CO_2 for the beta-chain terminals of trout haemoglobin (Greaney and Powers 1977), one would expect adrenaline to enhance carbamino formation if anything.

The present results demonstrate that the mechanism involved in adrenergic swelling of trout red cells is sensitive to SITS and thus involves the Band 3 anion exchange pathway or an analogous structure. The results also indicate that the cation and anion exchangers are linked tightly in as much as inhibition of the anion pathway also inhibits cation movements. In contrast, Nikinmaa and Huestis (1984) found that, while DIDS inhibited adrenergic swelling in rainbow trout red cells, DIDS had no effect on H^+ extrusion. They concluded from this information that the cation and anion exchangers involved in adrenergic swelling of fish erythrocytes are linked only loosely. Their results, however, were obtained under extremely adverse conditions in which trout red cells were suspended in an air-equilibrated HEPES buffer solution at pH 7.143-7.277 and 23 C, and thus may have been inflenced by degradation of the red cells. Eddy (1977), for example, has demonstrated that the normal metabolic functions of rainbow trout erythrocytes deteriorate above 20 C.

Can H^+, Cl^- and HCO_3^- truly be considered passively distributed across the red cell membrane in light of the present information? The adrenergic responses of fish red cells are minimal at resting catecholamine levels (Nikinmaa 1982, Fig. 16

this study) and under those conditions the equilibrium distributions of H^+, Cl^- and HCO_3^- appear to be adequately described as passive (Chapter 1). Even at elevated catecholamine levels, a passive distribution adequately describes the observed equilibrium distributions of H^+ and Cl^-. The distribution of HCO_3^-, however, remains in question. Whereas, one would have expected an increase in R_{HCO3-} when plasma pH decreased in adrenaline, the observed R_{HCO3-} actually declined. To a certain extent, this decrease in R_{HCO3-} probably reflects changes in chemically-bound species other than HCO_3^- (ie. CO_3^{2-}, carbamino). However, it is doubtful that the magnitude of those changes was significant to produce the observed decline in red cell $bHCO_3^-$. Indeed, a passive distribution for HCO_3^- may not hold when adrenaline levels are elevated. Definitive statements about the transmembrane distribution of HCO_3^-, however, are difficult to make from the present data because of the inclusion of more than one chemical species in the term '$bHCO_3^-$' and because of the possibility of nuclear:cytosolic discontinuities inside the red cell.

A striking feature of the beta-adrenergic response of trout red cells was the concurrent inhibition of net HCO_3^- flux through the red cells, as indicated by the functional decrease in cellular CA activity. This inhibition of net bicarbonate flux did not represent a direct effect on the CA enzyme itself; beta-adrenergic agonists had no effect on the CA activity of trout haemolysates. Nor was it due to a general anion impermeability. In fact, inhibition of anion transport with SITS also inhibited

the adrenergic response, at least as regards cell swelling. Several mechanisms could be involved in this adrenergic inhibition of net bicarbonate flux. Firstly, the K_M of the erythrocyte CA of fish is large (12-300 mM, Maren and Wiley 1966, Girard and Istin 1975, Haswell 1978) relative to the cellular concentrations of bicarbonate at physiological CO_2 tensions (2-5 mM, Fig. 3 and 20, Table 5). Thus, intracellular CO_2:HCO_3^- reactions probably exhibit first order kinetics. As such, decreases in the cellular bicarbonate concentration in response to adrenaline would depress the catalysed rate of HCO_3 dehydration. Secondly, numerous studies have demonstrated that the slowest step during capillary CO_2 exchange is HCO_3^-/Cl^- transport across the red cell membrane (Forster and Crandall 1975, Crandall and Bidani 1981, Forster 1982). Adrenergic extrusion of HCO_3^- from the erythrocyte during CO_2 unloading of blood could conceivably slow the net influx of plasma HCO_3^- to the cell and hence depress cellular CA activity. It is interesting to note that both of these mechanisms would operate maximally during branchial capillary transit when plasma HCO_3^- is the substrate of primary importance, whereas they would have little if any effect during tissue capillary transit when CO_2 is the primary substrate.

What is the possible adaptative significance of the adrenergic response of fish erythrocytes? The resting levels of plasma catecholamines measured in the present study (3.1 nM adrenaline, 1.2 nM noradrenaline) are among the lowest yet reported for fish (Mazeaud and Mazeaud 1981) and are comparable to these noted by Ristori *et al.* (1979)(2.95 nM adrenaline, 2.30

nM noradrenaline) for rainbow trout. A characteristic of the adrenergic response of fish to stressful stimuli, including strenuous exercise, physical disturbance, hypoxia and injury, is a rapid and large elevation in plasma catecholamine levels, commonly to levels exceeding 1000 nM (for review see Mazeaud and Mazeaud 1981). Such stressful stimuli generally are accompanied by lactate acidemia in fish. The adrenergic responses of fish red cells probably enhance regulation of intracellular pH and thus ameliorate the effects of this metabolic acidosis. Nikinmaa (1983), for instance, has demonstrated that the adrenergic responses of rainbow trout erythrocytes enhance O_2-haemoglobin affinitiy, primarily as a result of H^+ extrusion. Moreover, Nikinmaa *et al.* (1984) have shown that the beta-adrenergic responses of striped bass red cells serve to maintain arterial oxygen content in the face of metabolic acidosis following periods of strenuous exercise. Obviously, the disadvantages to O_2 uptake and release conferred by reduction in the surface area to volume ratio of swollen erythrocytes (Jones 1979) are outweighed by the advantages to O_2 affinity conferred by adrenergic H^+ extrusion. Adrenergic HCO_3^- extrusion would do little to alter plasma HCO_3^- levels directly in as much as red cells normally contain very little HCO_3^- and constitute less than half of the blood volume. However, the associated reduction in net bicarbonate flux through erythrocytes would result in retention of plasma HCO_3^- during branchial blood transit, independent of the exact molecular mechanism involved in that reduction. HCO_3^- is the primary non-protein buffer in biological

systems (Albers 1970, Comroe 1974). Numerous studies have demonstrated that myocardial cells, neural cells and CSF uptake HCO_3^- during periods of acidosis and/or adrenergic stimulation (Riegle and Clancy 1975, Boron and DeWeer 1976, Fenton *et al.* 1978, Gonzalez and Clancy 1981, Thomas 1982, Ahmad and Loeschcke 1983), and thus increase their physiological buffering capacities. Moreover, plasma HCO_3^- facilitates lactate release from some skeletal muscle in acidosis (Hirche *et al.* 1975). Thus, adrenergic swelling and the accompanying reduction in net bicarbonate flux through fish erythrocytes can be considered an 'altruistic' behaviour by which the benefits of HCO_3^- buffering are shared with other cells via the milieu interieur. This exemplifies the general priority in vertebrates of intracellular pH regulation over extracellular.

In conclusion, fish erythrocytes are readily permeable to chloride and bicarbonate which traverse the cell membrane via the Band 3 anion exchange pathway or an analogous SITS-sensitive structure. The inhibitors of CA present in fish plasma (see Chapter 2) have no effect on either membrane transport of anions or on intracellular CA. Ion transport in fish red cells is significantly influenced, however, by catecholamines. Beta-adrenergic agonists appear to stimulate coupled Na^+/H^+, HCO_3^-/Cl^- exchangers, similar to those involved in volume regulatory ion movements in amphibian erythrocytes (Cala 1980). Beta-adrenergic stimulation of fish erythrocytes results in a net cellular gain of Na^+, Cl^- and H_2O, a net cellular loss of H^+ and HCO_3^-, a pronounced cell swelling, and a reduction in net bicarbonate flux through the red cell. Under these conditions, the transmembrane

distribution of HCO_3^- may not be passive. The adrenergic responses of fish erythrocytes probably are important in maintaining O_2 transport and in enhancing intracellular buffering in stress. The effects of adrenaline *in vivo* are investigated in the following chapter.

Chapter 4.

EFFECTS OF ADRENALINE ON CO_2 EXCRETION AND ACID-BASE STATUS OF *SALMO GAIRDNERI*, *IN VIVO*

The principal pathway for CO_2 excretion in fish, as in other vertebrates, is via movement of plasma bicarbonate into the red blood cell where the bicarbonate is rapidly dehydrated to form CO_2 in the presence of carbonic anhydrase. This CO_2 then diffuses down its concentration gradient from the red cell into the gas exchange medium (water/air). Catecholamines influence the CO_2 carrying capacity of blood *in vitro*, alter the transmembrane distribution of HCO_3^- in red blood cells, and reduce the activity of erythrocyte CA of rainbow trout blood (Chapter 3). As such, adrenergic stimulation would be expected to decrease exchange of CO_2 in rainbow trout *in vivo*.

The primary response to many forms of stress in fish is a rapid and often extremely large increase in blood catecholamine levels (Nakano and Tomlinson 1967, Mazeaud *et al.* 1977, Butler *et al.* 1978, 1979). The secondary effects of this neuro-hormonal response vary in their duration and pattern according to the fish species under consideration and the nature of the stress (for review see Mazeaud and Mazeaud 1981). In general, however, transport of blood gases is influenced by catecholamines via cardiovascular effects including increased cardiac output (Jones and Randall 1978, Randall 1982a, b), lamellar recruitment (Holbert *et al.* 1979), and increased permeability of gill

epithelium (Isaia, *et al.* 1978a, b). As well, O_2-haemoglobin affinity increases in stress due to adrenergic H^+ extrusion from the red cell and a reduction in erythrocytic nucleoside triphosphate levels (Nikinmaa 1983, Nikinmaa *et al.* 1984). Thus, catecholamines would appear to modulate transport and exchange of both oxygen and carbon dioxide in stress via not only cardiovascular effects, but also via direct modulation of blood respiratory properties. The adrenergic responses of fish erythrocytes would tend to favour O_2 exchange in stress, but to disfavour CO_2 exchange.

The objective of the present study was to examine the effects of adrenaline on the transport and exchange of CO_2 and O_2 in *Salmo gairdneri*. Injection of catecholamines into the circulatory system of fish elicits secondary effects virtually identical to those associated with stress (Peyraud-Waitzenegger 1979, Mazeaud and Mazeaud 1981, Nikinmaa 1982). In the present study, the respiratory and acid-base states of venous and arterial bloods were assessed simultaneously in order to determine the effects of injection of adrenaline on O_2 uptake and CO_2 excretion.

MATERIALS AND METHODS

Rainbow trout (300-400 g) were implanted with both dorsal and ventral aortic cannulae as described in the General Materials and Methods. Fish also were fitted with a buccal cannula. Buccal cannulae were constructed from sections of polyethylene surgical tubing (PE-90) which had been heat-flared at one end. The flared end was implanted in the buccal cavity. Buccal cannulae exited the fish via a flanged section of surgical tubing (PE-200) which passed through the roof of the mouth in front of the nares. All cannulae were connected to pressure transducers (Statham P23Db) by way of 3-way stopcocks to allow continuous recording of blood and buccal pressures. The 3-way stopcocks provided sampling and injection ports.

Some fish were given a 0.5 mL-bolus injection of either Cortland saline or (-)-adrenaline ($5 \cdot 10^{-11}$ mol·g fish^{-1} in Cortland saline) via the dorsal aortic cannula. This injection produced an estimated 10^{-6} M increase in circulating adrenaline levels, assuming the blood volume to be 5% of body weight (Smith 1966). Other fish were injected continuously for a period of 1 h and at a rate of 0.5 mL·h^{-1} with (-)-adrenaline (10^{-4} M in Cortland saline) via the dorsal aortic cannula. This injection produced an estimated 10^{-6} M increase in blood adrenaline levels within 24 min and an estimated $2.5 \cdot 10^{-6}$ M increase after 1 h. Ambient water temperature was 10 C.

At known time intervals, blood samples were withdrawn

simultaneously from both the dorsal and ventral aortic cannulae. These samples were replaced with an equivalent volume of Cortland saline. A portion of each blood sample was used to measure haematocrit in duplicate and total O_2 content (C_{O_2}). C_{O_2} was measured with an oxygen electrode (Radiometer, type E5046) using the methods of Tucker (1967). The remainder of the samples were centrifuged at room temperature for 1-2 min. All subsequent measurements were made on plasma. P_{O_2} and pH values were measured using an Instrumentation Laboratory Micro 13, pH/blood gas analyser with associated electrodes and calibration standards thermostatted at 10 C. C_{CO_2} values were measured using the methods of Cameron (1971) as described previously. P_{CO_2} values were calculated using equation 8 and the values of aCO_2 and pK'_{app} obtained in Chapter 1. Area mean blood pressure, heart rate and ventilation rate were taken from the pressure records.

RESULTS

Injection of adrenaline into the dorsal aorta of *Salmo gairdneri* produced significant changes in the respiratory and acid-base states of the fish (Fig. 22 and 23). Injection of an equivalent volume of saline was without effect (Table 11). Elevation of circulating adrenaline levels was associated with a significant transient decrease in the gas exchange ratio ($R = dC_{CO_2}/dC_{O_2}$)(Fig. 24). It should be noted, however, that since the reported R values were calculated using true plasma C_{CO_2}, the reported values overestimate the actual R values; true plasma C_{CO_2} is necessarily greater than whole blood C_{CO_2} (see Figure 3). Nonetheless, the reported values demonstrate that the rate of CO_2 excretion decreased, relative to the rate of O_2 uptake, following adrenaline injection. The reduction in R was fully compensated for within 60 min of the injection. Reductions in R were accompanied by a significant increase in plasma C_{CO_2} and P_{CO_2}, and a significant decrease in plasma pH (Fig. 22). Changes in C_{CO_2}, P_{CO_2} and pH were fully compensated for within the 120-min time frame of continuous-injection studies, but did not return to pretreatment levels within the shorter time frame (45 min) of bolus-injection studies.

Injection of adrenaline either reversed the direction of or abolished arterial-venous differences in pH and P_{CO_2} (Fig. 22). Prior to injection of adrenaline, arterial plasma was more basic and contained less free CO_2 (P_{CO_2}) than its venous counterpart.

Figure 22. Arterial-venous differences in pH, C_{CO_2} and P_{CO_2} across the gills of *Salmo gairdneri*, showing the effects of injection of adrenaline as a bolus ($5 \cdot 10^{-11}$ mol·g fish^{-1}) and as a continuous stream ($5 \cdot 10^{-8}$ mol·h^{-1}). Arterial values are shown by open circles. Venous values are shown by solid circles. Values are means ± 1 S.E. (n=3). Values denoted with + are different significantly from the time zero value at $P \leq 0.05$ (paired t-test). Arterial values denoted with * are different significantly from the corresponding venous value at $P \leq 0.05$ (paired t-test).

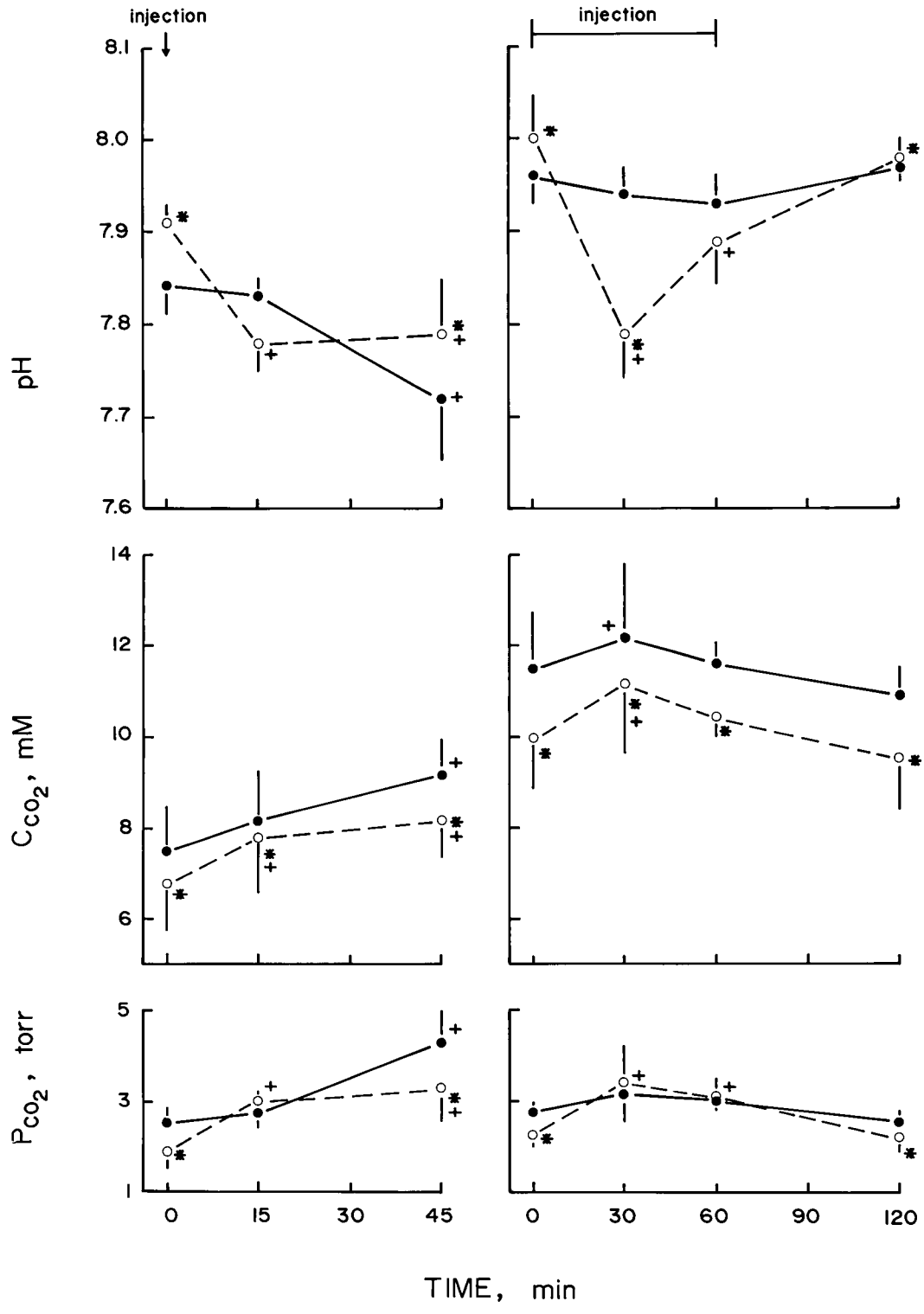

injection
injection
8.1
8.0
7.9
7.8
7.7
7.6
pH
14
12
10
8
6
C_{CO_2}, mM
5
3
1
P_{CO_2}, torr
0
15
30
45
0
30
60
90
120
TIME, min

Figure 23. Arterial-venous differences in C_{O_2}, P_{O_2} and haematocrit across the gills of *Salmo gairdneri*, showing the effects of injection of adrenaline as a bolus ($5 \cdot 10^{-11}$ mol·g fish^{-1}) and as a continuous stream ($5 \cdot 10^{-8}$ mol·h^{-1}). Rest of caption as in Figure 22.

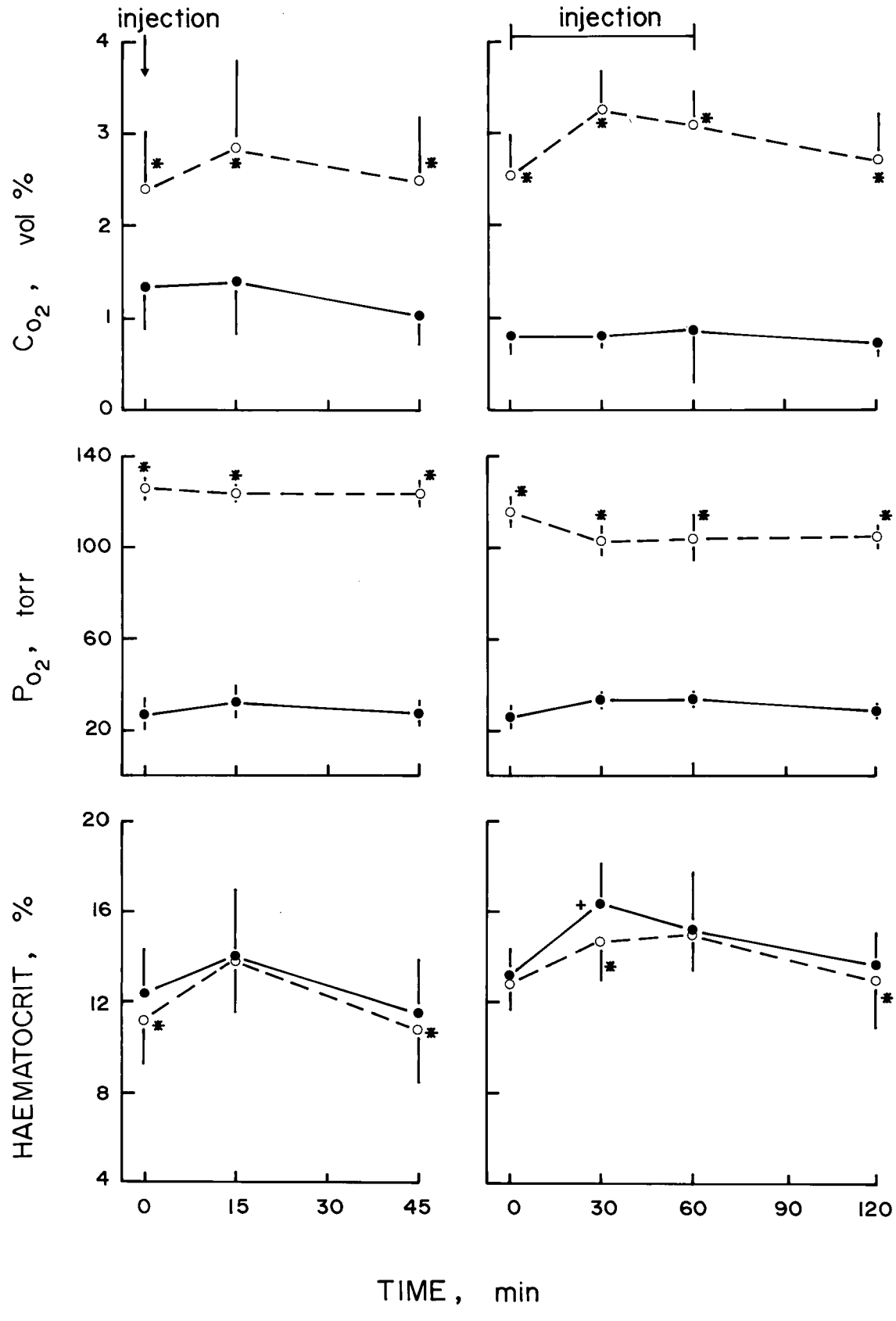
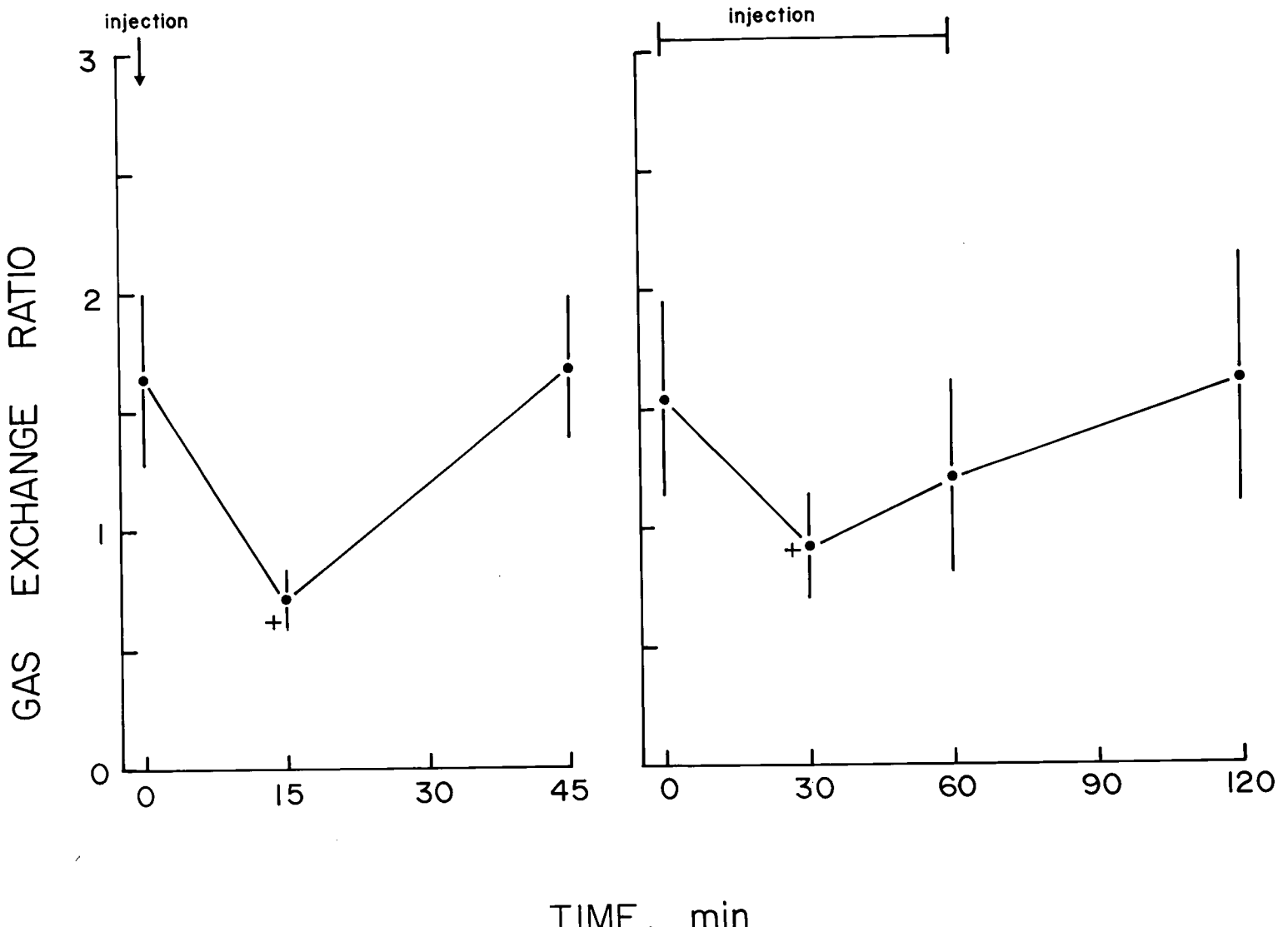

injection
injection
C_{O_2}, vol %
P_{O_2}, torr
HAEMATOCRIT, %
TIME, min
0
15
30
45
60
90
120

Table 11. Respiratory and acid-base characteristics of arterial plasma of *Salmo gairdneri*, showing effects of a bolus injection of 0.5 mL of Cortland saline. Values are means ± 1 S.E. (n=5). Hct, haematocrit.

	Time post-injection (min)		
	0	15	90
pH	8.046 ± 0.019	8.049 ± 0.027	8.057 ± 0.027
C_{CO_2} (mM)	9.21 ± 0.43	9.33 ± 0.45	9.05 ± 0.32
P_{CO_2} (torr)	2.00 ± 0.05	2.02 ± 0.11	1.92 ± 0.07
Hct (%)	17.9 ± 1.6	16.6 ± 1.5	14.7 ± 1.1

Figure 24. Gas exchange ratio ($R = d\ plasmaC_{CO_2}/d\ bloodC_{O_2}$) of *Salmo gairdneri*, showing the effects of injection of adrenaline as a bolus ($5 \cdot 10^{-11}$ mol·g fish^{-1}) and as a continuous stream ($5 \cdot 10^{-8}$ mol·h^{-1}). Values are means ± 1 S.E. (n=3). Values denoted by + are different significantly from the time zero value at $P \leq 0.05$ (paired t-test).

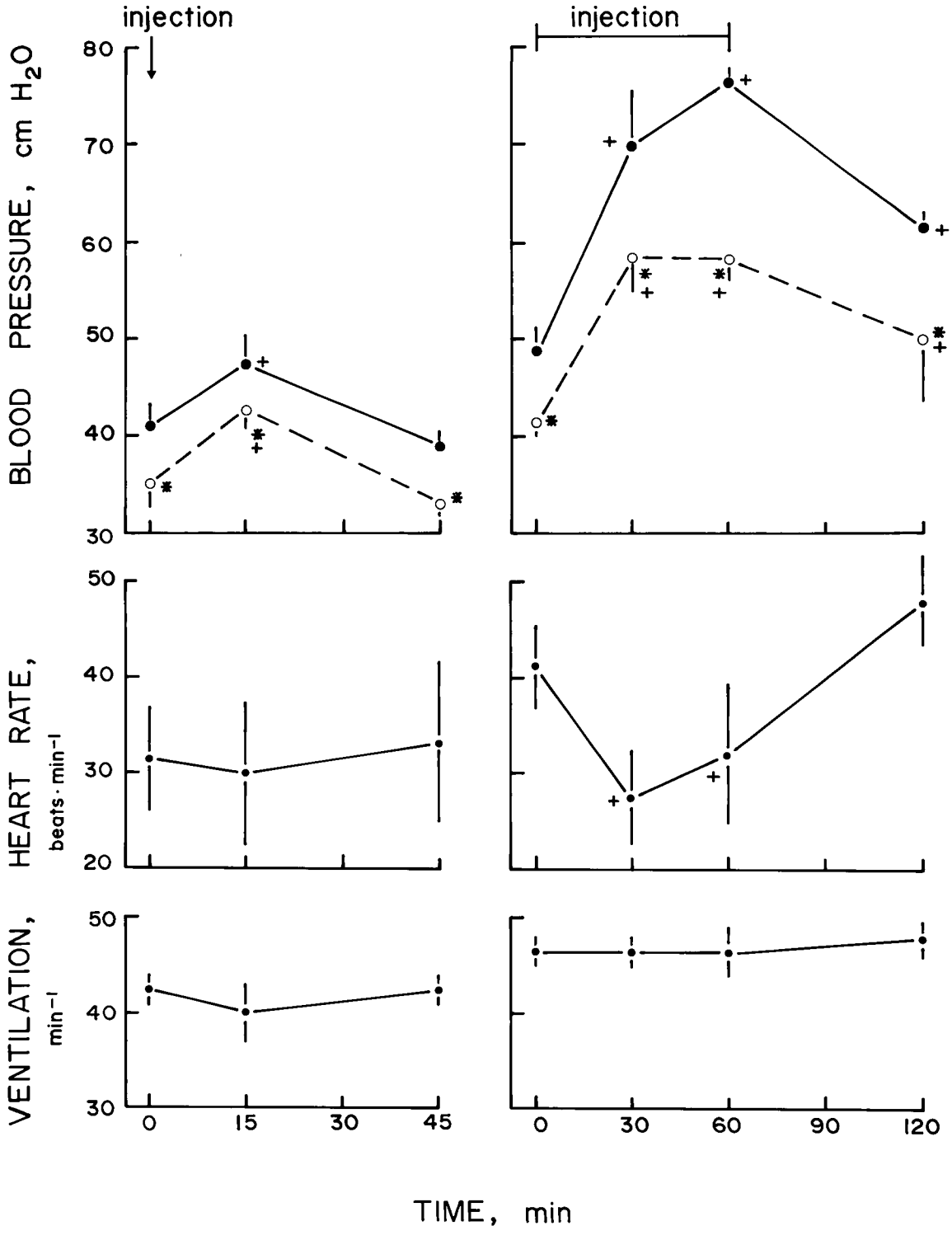

injection
injection
3
2
1
0
GAS EXCHANGE RATIO
0
15
30
45
0
30
60
90
120
TIME, min

After injection of adrenaline, however, arterial plasma tended to be more acidic than and had about the same P_{CO_2} as venous plasma, despite the observation that fish at all times maintained a negative arterial-venous difference in C_{CO_2}, that is, positive excretion of CO_2. Arterial-venous differences in pH and P_{CO_2} returned to pretreatment conditions within 60 min following the injection.

The significant arterial acidosis associated with elevated adrenaline levels was not accompanied by a corresponding decrease in blood C_{O_2} (Fig. 23) which one might have expected due to Bohr and Root effects. Blood oxygen content actually tended to increase as plasma pH fell, although the changes in C_{O_2} were not significant statistically. This absence of a pH effect on C_{O_2} was not attributable to an effect of adrenaline on blood P_{O_2}; arterial and venous P_{O_2} levels remained unchanged throughout the studies (Fig. 23). It is conceiveable that the absence of a pH effect was attributable to increased O_2 affinity of blood (cf. Nikinmaa 1983, Nikinmaa *et al.* 1984). However, recruitment of additional erythrocytes into the circulating blood cannot be ruled out, since haematocrit also tended to increase in concert with the decrease in plasma pH (Fig. 23).

Adrenaline had significant effects on the cardiovascular system of *Salmo gairdneri* (Fig. 25). Bolus injection of adrenaline produced an immediate increase in arterial and venous blood pressures. Blood pressures peaked within 3 min of the bolus injection and then slowly decreased, returning to pretreatment levels within 45 min of the injection. Continuous injection of adrenaline also produced an increase in blood

Figure 25. Effects of adrenaline injection as a bolus ($5 \cdot 10^{-11}$ mol·g fish^{-1}) and as a continuous stream ($5 \cdot 10^{-8}$ mol·h^{-1}) on area mean blood pressure, heart rate and ventilation rate of *Salmo gairdneri*. Rest of caption as in Figure 22.


injection
injection
BLOOD PRESSURE, cm H_2O
80
70
60
50
40
30
HEART RATE, beats · min⁻¹
50
40
30
20
VENTILATION, min⁻¹
50
40
30
0
15
30
45
0
30
60
90
120
TIME, min

pressures, albeit this increase occurred at a much slower rate. In these studies, blood pressures continued to increase slowly throughout the entire 60-min injection period and remained elevated for more than 60 min following that period. Adrenaline had no detectable effect on ventilation rate and only a moderate effect on heart rate producing a mild bradycardia which was compensated for within 60 min of the injection (Fig. 25).

DISCUSSION

Elevation of blood adrenaline levels in *Salmo gairdneri* was associated with a transient reduction in CO_2 clearance and an increase in body CO_2 stores. These results are consistent with an adrenergic reduction in net HCO_3^- flux through the red cell during branchial blood transit. Similar increases in blood C_{CO_2} and P_{CO_2} have been observed following whole-body inhibition of carbonic anhydrase in dogfish (*Squalus acanthias*)(Maren 1962), lake trout (*Salvelinus namaycush*)(Hoffert and Fromm 1966), and rainbow trout (Hoffert and Fromm 1973). Selective inhibition of erythrocyte CA with an aminothiadiazole derivative (CL 11,366), which reportedly lacks access to gill CA, has shown that increases in body CO_2 stores following CA inhibition are due solely to red cell effects (Maren and Maren 1964). Perry *et al.* (1982) have demonstrated with a blood-perfused rainbow trout preparation that SITS-inhibition of the Band 3 anion exchange pathway in erythrocytes also causes a reduction in CO_2 excretion and an increase in body CO_2 stores. This reiterates that inhibition of erythrocyte anion transport has the same functional effect on branchial CO_2 exchange as inhibition of erythrocyte CA (Chapter 3). Adrenergic reductions in CO_2 excretion probably were related to adrenergic HCO_3^- extrusion from the red cell, rather than the result of general anion impermeability or direct inhibition of the CA enzyme (Chapter 3). Adrenergic HCO_3^- extrusion from the red cell would reduce net HCO_3^- influx during

branchial blood transit when plasma HCO_3^- is the substrate of concern for erythrocyte CA, but would have little if any effect on CO_2 exchange during tissue blood transit when CO_2 is the substrate of concern.

Perry *et al.* (1982) noted that inhibition of erythrocyte HCO_3^-/Cl^- exchange significantly reduced O_2 uptake in a blood-perfused rainbow trout preparation. Presumably, inhibition of HCO_3^- influx to the red cell and the resultant reduction in the intracellular HCO_3^- dehydration reaction allowed oxy-labile H^+ to accumulate sufficiently within the red cell to slow Bohr-on and Root-on shifts during branchial blood transit. Under the present experimental conditions, however, adrenergic reduction of net HCO_3^- flux through *S. gairdneri* erythrocytes had no effect on O_2 loading of blood. This emphasizes that the observed reduction in CO_2 excretion was not due simply to an adrenergic response of the cardiovascular system in as much as cardiovascular responses would alter CO_2 excretion and O_2 uptake in the same direction. O_2 uptake in the present *in vivo* study probably was maintained in the face of a reduction in CO_2 excretion by a combination of factors: adrenergic H^+ extrusion from the red cells which would partially offset the reduction in erythrocyte bicarbonate buffering capacity; a decrease in erythrocyte nucleoside triphosphate levels in response to adrenaline which enhances O_2-haemoglobin binding affinity (Nikinmaa 1983, Nikinmaa *et al.* 1984); haemoconcentration due to splenic contraction and the release of additional erythrocytes, and to diuresis (Stevens 1968, Wood and Randall 1973, Nilsson *et al.* 1975); and adrenergic responses of the cardiovascular system (for review see

Jones and Randall 1978, Randall 1982a, b). Maintenance of O_2 loading when catecholamines are released in stress would ensure O_2 transport to the tissues in those situations. At the same time, retention of plasma HCO_3^- would maintain an extracellular HCO_3^- pool which then would be available to enhance the physiological buffering capacity of intracellular compartments. Respiratory H^+ associated with this HCO_3^- retention could remain in the extracellular fluids or be excreted across the gills. Fish gill epithelium is very permeable to H^+ (McWilliams and Potts 1978) and recent evidence (van den Thillart *et al.* 1983) indicates that CO_2 and H^+ excretion across the gill vary independently of each other.

The responses of rainbow trout to exogenous adrenaline included a transient disruption of the arterial-venous differences in plasma pH and P_{CO_2} across the gill. Typically, arterial plasma has a lower CO_2 content, a lower CO_2 tension and is more basic than its venous counterpart. Following injection of adrenaline, however, arterial plasma had a similar P_{CO_2} as and tended to be more acidic than venous plasma, despite the observation that a net loss of CO_2 was still maintained during branchial blood transit. These results could have been obtained only from blood that was far from CO_2:HCO_3^- chemical equilibrium at the time the blood was withdrawn *in vivo*. When in chemical equilibrium, an aqueous solution with a lower C_{CO_2} also has a lower P_{CO_2} and a higher pH than a solution with a higher C_{CO_2}. In the present study, net interconversion of $[CO_2 + H_2O]$ and $[HCO_3^- + H^+]$ must have continued to occur during the blood

sampling and measurement procedures. Thus, the observed pH and P_{CO_2} values did not reflect the actual pH and P_{CO_2} of arterial plasma at the site of withdrawal *in vivo*. A chemical disequilibrium in arterial blood also is consistent with an adrenergic inhibition of net HCO_3^- flux through the red cell during branchial blood transit. Fish gill epithelium is very permeable to CO_2 but relatively impermeable to HCO_3^- (Perry *et al.* 1982), and consequently the bulk of CO_2 excreted during branchial blood transit exits the blood as CO_2 (see Table 5). In the absence of catecholamine effects, CO_2:HCO_3^- chemical equilibrium is maintained during branchial CO_2 exchange via catalysis of CO_2:HCO_3^- reactions by erythrocyte CA. The uncatalysed rate of CO_2:HCO_3^- reactions is at least one order of magnitude too slow to enable chemical equilibrium to be achieved during branchial blood transit (Chapter 2). Following adrenergic stimulation of fish erythrocytes, however, the functional activity of erythrocyte CA is reduced and consequently the rate of HCO_3^- dehydration during branchial blood transit can no longer keep pace with diffusion of dissolved CO_2. As a result, HCO_3^- is retained in the plasma and the blood is no longer able to attain CO_2:HCO_3^- chemical equilibrium during branchial transit. Chemical equilibrium would be attained in vessels downstream of the gill, and would be accompanied theoretically by an increase in both arterial P_{CO_2} and pH. Arterial pH under such circumstances, however, also would be influenced by H^+ movements across the gill (McWilliams and Potts 1978, van den Thillart *et al.* 1983) and by adrenergic H^+ extrusion from red cells (Chapter 3).

The effects of adrenaline were transient in nature. This is to be expected for several reasons. Firstly, a reduction in CO_2 excretion causes the diffusion gradient for dissolved CO_2 at the gas exchange organ to increase. Eventually, the point will be reached at which simple diffusion of CO_2 matches metabolic production of CO_2 and a steady-state is re-established. Swenson and Maren (1978) have examined the effects of total inhibition of erythrocyte CA on CO_2 excretion in mammals. They found that, at rest in the presence of erythrocytic CA activity, 78% of excreted CO_2 was derived from the catalysed dehydration of HCO_3^-, 10% was from dissolved CO_2, and 11% was from carbamino compounds (see Table 5 for fish values). Total inhibition of erythrocyte CA had no effect on the steady-state rate of CO_2 excretion. In the absence of CA, however, 80% of excreted CO_2 was derived from dissolved CO_2, 4% was from uncatalysed HCO_3^- dehydration, and 11% was from carbamino compounds. Secondly, while catecholamines are relatively stable in fish blood (half-times of 65.8 min and 163 min for adrenaline and noradrenaline, respectively, (Mazeaud 1979), catecholamines generally are rapidly deactivated, at least in mammalian blood (Goodman and Gilman 1956). Lastly, adrenaline injections in intact animals cause a multitude of cardiovascular and metabolic effects which then lead to the initiation of reflexes to counteract them.

The cardiovascular responses of fish to catecholamines have been reviewed by Randall (1970b, 1982a, b) and by Jones and Randall (1978). The present results are consistent with the known effects of adrenaline on the cardiovascular system of fish:

a positive inotropic effect of the heart; a dilation of gill vasculature and decrease in gill resistance; and a rise in blood pressure which elicts a baroreceptor reflex causing simultaneous vagal bradycardia.

In conclusion, the adrenergic responses of intact rainbow trout at rest included a transient reduction in CO_2 excretion, an increase in body CO_2 stores, and a disruption of arterial-venous differences in plasma pH and P_{CO_2}. All these results are consistent with an adrenergic reduction in net HCO_3^- flux through the red cell during branchial blood transit. O_2 uptake by blood was unaffected by this retention of CO_2. Nikinmaa *et al.* (1984) have demonstrated that the beta-adrenergic responses of fish erythrocytes serve to maintain arterial O_2 content in stress. The present study indicates that these adrenergic responses also serve to maintain an extracellular pool of HCO_3^- in stress which then can be used to enhance intracellular buffering capacities.

GENERAL DISCUSSION

This thesis has examined the transport and excretion of carbon dioxide in fish, and the role of the red blood cell in that process. The results demonstrate that CO_2 excretion in fish proceeds in a typically mammalian fashion (cf. Roughton 1964). CO_2 is transported in the blood primarily as HCO_3^- (90-95% of C_{CO_2}), which at an haematocrit of 25% is distributed between the plasma water space and the red cell water space in a ratio of about 9:1. Relatively small proportions of the CO_2 content of blood are present as dissolved CO_2 (2-3%) or as carbamino compounds (5-6%). In resting fish, the red cell membrane is readily permeable to HCO_3^-, Cl^- and H^+, all of which are distributed passively across the cell membrane. During branchial CO_2 exchange, plasma HCO_3^- traverses the erythrocyte membrane in an one-for-one exchange with cellular Cl^-. This 'chloride shift' is similar in its time course and its sensitivity to disulphonic stilbene derivatives and acetazolamide as is HCO_3^-/Cl^- exchange in mammalian erythrocytes (Cameron 1978b, Obaid *et al.* 1979), and thus presumably involves a transport mechanism similar to the mammalian Band 3 anion exchange pathway. Fish erythrocytes contain sufficient carbonic anhydrase to catalyse the interconversion of CO_2 and HCO_3^- by several orders of magnitude. Thus, upon entry to the red cell, plasma HCO_3^- is rapidly dehydrated to form CO_2. This CO_2 then diffuses down its concentration gradient out of the red cell and across the gill

epithelium. This pathway for CO_2 excretion is unaffected by endogenous inhibitors of carbonic anhydrase present in fish plasma; these inhibitors lack access to the intracellular enzyme and have no effect on the anion transport mechanism in fish erythrocytes. Hence, the gill model of CO_2 excretion which asserts that fish erythrocytes are functionally impermeable to HCO_3^- *in vivo* and that the bulk of CO_2 crosses the gill epithelium directly as HCO_3^- (Haswell and Randall 1978, Haswell *et al.* 1980) no longer can be considered correct. Indeed, in light of recent evidence that the basolateral membrane of the fish gill is relatively impermeable to HCO_3^- (Perry *et al.* 1982), the bulk of CO_2 leaving the blood during branchial capillary transit must do so as dissolved CO_2, and therefore must exit via the red blood cell. The contribution of uncatalysed dehydration of plasma HCO_3^- to overall CO_2 excretion is negligible; the uncatalysed reaction proceeds far too slowly to be of any importance during lamellar blood transit. The flux of plasma HCO_3^- through the red cell is important in maintaining O_2 uptake during branchial blood transit (Perry *et al.* 1982), presumably due to bicarbonate buffering of oxy-labile H^+ and facilitation of Bohr-on and Root-on shifts.

Carbon dioxide behaves like a weak acid in aqueous solution; increases in CO_2 tension necessarily decrease pH while decreases in CO_2 tension necessarily increase pH. Maintenance of a stable internal pH in biological systems, therefore, requires a balancing of CO_2 production and excretion. In general, vertebrates regulate their internal acid-base status by adjustments to blood CO_2 levels. Fish, and perhaps most other

water-breathing vertebrates, regulate blood pH by modulating blood HCO_3^- levels while allowing blood P_{CO_2} to parallel environmental levels (Cameron and Randall 1972, Randall and Cameron 1973, Janssen and Randall 1975, Eddy *et al.* 1977). Elevation of plasma HCO_3^- levels increases blood pH while decreases in plasma HCO_3^- levels have the opposite effect. This relationship is possible because the fish gill is relatively impermeable to HCO_3^- (Perry *et al.* 1982) but is readily permeable to H^+ (McWilliams and Potts 1978) and CO_2, which are excreted across the gill epithelium independent of one another (van den Thillart *et al.* 1983).

While there is little doubt that the $Na^+/H^+(NH_4^+)$ and $Cl^-/HCO_3^-(OH^-)$ exchangers present on the apical membrane of gill epithelium are involved in acid-base regulation (Cameron 1978a, Heisler 1984), the present studies clearly indicate that extracellular HCO_3^- concentrations in fish are controlled at least in part at the level of the red blood cell. Fish erythrocytes possess Na^+/H^+ and Cl^-/HCO_3^- exchangers that are sensitive to beta-adrenergic agonists and perhaps to osmotic perturbations of the red cell. Adrenergic stimulation of fish red cells results in a net cellular gain of Na^+, Cl^- and H_2O, a net cellular loss of H^+ and HCO_3^-, a pronounced cell swelling, and a reduction in the functional activity of erythrocyte carbonic anhydrase. *In vivo*, these responses slow the rate of CO_2 excretion during branchial blood transit and result in retention of plasma HCO_3^-. Retention of plasma HCO_3^- probably is important during periods of stress in compensating for

cardiovascular effects such as lamellar recruitment (Holbert *et al.* 1979) which conceivably could result in washout of blood CO_2. As well, maintenance of an extracellular pool of HCO_3^- in stress makes plasma HCO_3^- available to enhance the intracellular buffering capacities of other tissues. This pool of extracellular bicarbonate, although small, would have a considerable effect on intracellular pH compensation because of the initially low intracellular bicarbonate levels of water-breathing vertebrates (1-5 mM, see Table 5). The adrenergic responses of fish erythrocytes also serve to maintain O_2 uptake at the gills in the face of the accompanying net reduction in HCO_3^- flux through the red cell. The reduction in intraerythrocytic bicarbonate buffering of oxy-labile H^+ in stress is partially compensated for by H^+ extrusion from the red cell and by reductions in erythrocyte organic phosphate levels which enhance O_2-haemoglobin binding affinity (Nikinmaa 1983, Nikinmaa *et al.* 1984). The adrenergic responses of fish red cells probably are transient in nature and exert their maximal effect on acid-base regulation shortly after a pH disturbance occurs. As such, they can be considered a 'first line of defence' against fluctuations in internal pH. Long-term pH regulation most likely is achieved via gill function.

The adrenergic responses of fish erythrocytes probably explain the absence of functional erythrocyte CA activity in bimodally-breathing fish during air exposure. Several studies (Randall *et al.* 1978, Daxboeck and Heming 1982) have found that, while bimodally-breathing fish are capable of maintaining O_2 uptake during air exposure, CO_2 excretion in air is limited to

the uncatalysed rate of HCO_3^- dehydration. The gas exchange ratios of these fish typically were 0.2–0.4 during air exposure. Whole-body inhibition of CA was without effect on CO_2 excretion during air exposure of these animals, whereas injection of exogenous carbonic anhydrase significantly increased CO_2 excretion in air and returned the gas exchange ratio to about unity. The erythrocytes of bimodally-breathing fish contain significant amounts of carbonic anhydrase (Burggren and Haswell 1979, Daxboeck and Heming 1982) and it is unlikely that these erythrocytes are generally impermeable to anions. More likely, the stress of handling and air exposure in the above studies caused release of catecholamines into the blood which in turn reduced the functional activity of erythrocyte CA via adrenergic stimulation of the red cells.

A valuable and informative technique for quantifying the movements of acid-base relevant ions in fish recently has been developed which involves balancing the uptake and excretion of CO_2, H^+ and counter-exchange ions (Na^+, Cl^-) between water and the extracellular (plasma) compartment of fish (for review see Heisler 1984). Use of this technique has demonstrated that movements of HCO_3^- between the intracellular and extracellular compartments, and between the extracellular compartment and the water occur during pH compensation. However, the possibility of a stress-related redistribution of Na^+, Cl^-, H^+ and HCO_3^- across the red cell membrane seldom has been considered in these ion balancing studies. Since the red cells of fish typically occupy 25–30% of the total blood volume, the adrenergic responses of

fish erythrocytes probably are a significant source of error in ion balancing studies. This is especially true in studies in which ion movements across the gill are inferred solely from measurements of plasma ion levels in the absence of measurements of water ion levels (cf. Wilkes *et al.* 1981).

ABBREVIATIONS AND SYMBOLS

aCO_2 - CO_2 solubility coefficient

bCO_2 - mean CO_2 capacitance or dC_{CO_2}/dP_{CO_2}

$bHCO_3^-$ - apparent bicarbonate or chemically-bound CO_2

CA - carbonic anhydrase

C_{O_2} - total oxygen content

C_{CO_2} - total carbon dioxide content

dX - delta X or change in any variable X, for example, dCl^-

P_{O_2} - partial pressure of oxygen

P_{CO_2} - partial pressure of carbon dioxide

R - gas exchange ratio or dC_{CO_2}/dC_{O_2}

R_x - Donnan ratio of any ion X, for example, R_{Cl^-}

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