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A ROLE FOR EPINEPHRINE IN ACID-BASE AND GAS TRANSFER REGULATION IN RAINBOW TROUT

by

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ABSTRACT

Increases in the blood levels of catecholamines have been noted during or following stresses in fish; regulation of blood acid-base status and blood oxygen transport have both been postulated as roles for these catecholamines. Responses of freshwater rainbow trout, Salmo gairdneri, to adrenergic stimulation were studied, using elevations of either exogenous epinephrine via prolonged infusions or endogenous epinephrine during hypercapnia.

Monitoring of blood acid-base, respiratory and ionic variables during prolonged epinephrine infusion revealed a respiratory acidosis caused by retention of carbon dioxide. Although whole blood pH was significantly depressed, red blood cell pH either remained constant or increased. Pre-treatment of fish with propranolol (a beta-adrenergic antagonist) or phentolamine (an alpha-adrenergic antagonist) prior to epinephrine infusion revealed this red blood cell pH regulation to be beta-adrenergically mediated, presumably due to a putative Na^+/H^+ exchange, located in the red blood cell membrane. Increases in hematocrit, due to red blood cell swelling, diuresis and splenic release of red blood cells were also noted during epinephrine infusion. Alpha-antagonism blocked the increases in hematocrit and hemoglobin concentration. Increased arterial oxygen tension was observed during epinephrine infusion, and this was related to increases in gill ventilation, perfusion and diffusive conductance.

Branchial and renal parameters were also monitored during epinephrine infusion. Increased branchial acid excretion during epinephrine infusion was observed to be the result of a complex

modulation of both ionic influxes and effluxes: a significant correlation was found between the arithmetic difference of the net branchial fluxes of sodium and chloride and the amount of acid excreted. Increases in the buffering of the urine permitted increased excretion of acid through the kidney. Partitioning of the acid excretion showed that the gills handled about 85% of the excreted acid, while the kidney handled the remaining 15%. Although increased renal and branchial acid excretion were observed, it was not possible to distinguish whether the modulation of this acid excretion was in response to adrenergic stimulation, or to the acidosis accompanying epinephrine infusion.

Exposure of trout to hypercapnia revealed that they are able to regulate blood acid-base status and maintain blood oxygen content during this stress. Alpha- or beta-adrenergic blockade during hypercapnia did not interfere with the ability of the fish to regulate the extracellular acidosis. Blood oxygen content regulation was partially blocked by pre-treatment of the fish with either phentolamine or propranolol. Pre-treatment with both phentolamine and propranolol, simultaneously, caused death of the fish, presumably due to the severe hypoxemia. Thus, both alpha- and beta-adrenergic stimulation are important to their ability to regulate blood oxygen content, but their mediation of blood acid-base status is questionable.

Although blood respiratory responses indicated that epinephrine played a direct role in mediating responses associated with to oxygen carrying capacity, evidence for a role in acid-base regulation was not as persuasive. A correlation between increased epinephrine levels and increased branchial and renal acid excretion did exist, but the role of

epinephrine in acid-base regulation is thought to be minor. Therefore, it is speculated that the primary role of epinephrine mobilization during stresses is the immediate regulation of blood oxygen carrying capacity.

RESUME

Des augmentations dans les concentrations sanguines de catécholamines ont été notées durant ou suivant des périodes de contraintes physiologiques chez les poissons; la régulation de l'état acido-basique et du transport d'oxygène sanguin sont des rôles postulés pour ces catécholamines. Les réponses de la truite arc-en-ciel d'eau douce, Salmo gairdneri, à des apports adrénergiques ont été étudiées, en utilisant des élevations d'adrénaline, soit exogène (par infusion prolongée) ou endogène (durant l'hypercapnie).

Des observations des variables acido-basiques, respiratoires et ioniques durant une infusion ont révélées une acidose respiratoire entrainée par la rétention du dioxide de carbone. Bien que le pH sanguin était abaissé, le pH des globules rouges était constant ou a augmenté. Le pré-traitement des poissons avec le propranolol (un antagoniste beta-adrénergique) ou le phentolamine (un antagoniste alpha-adrénergique) avant l'infusion d'adrénaline a révélé que la régulation du pH des globules rouges était de nature beta-adrénergique, due à un échange Na^+/H^+ au niveau de la membrane cellulaire. Des augmentations dans l'hématocrite, due au gonflement des globules rouges, à l'augmentation de la production d'urine et aussi au relachement des globules rouges de la rate, ont aussi été notées durant l'infusion d'adrénaline. L'antagonisme-alpha bloqua les augmentations d'hématocrité et de concentration d'hémoglobine. Un gain dans la tension artérielle d'oxygène fut observé durant l'infusion d'adrénaline, et ceci fut relié aux augmentations dans la ventilation et la perfusion branchiale et aussi une augmentation dans la diffusion trans-branchiale.

Les paramètres branchials et rénals ont aussi été enregistrés durant l'infusion d'adrénaline. Des augmentations dans l'excrétion branchiale d'acide durant l'infusion d'adrénaline furent le résultat de changements complexes dans les fluxs ioniques: une relation signifiante fut trouvée entre la différence mathématique entre les fluxs nets de sodium et de chlore et le montant d'acide excrété. Un accroissement du pouvoir tampon de l'urine permit une augmentation de l'excrétion d'acide par l'entremise du rein. La répartition de l'excrétion d'acide démontra que les branchies ont passées 85% de l'acide excrétée, tandis que les reins en passèrent 15%. Bien que des augmentations dans l'excrétion rénal et branchial furent notés, il ne fut pas possible de distinguer si le changement dans le montant d'acide excrété était en réponse à une stimulation adrénergique ou a l'acidose qui accompagnait l'infusion d'adrénaline.

L'exposition des truites à l'hypercapnie révéla qu'elles sont capables de régler l'état acido-basique du sang et aussi de maintenir le niveau d'oxygène face à cette imposition. Le blockage alpha- ou beta-adrénergique durant l'hypercapnie n'a pas enlevé l'abilité des poissons de régler l'acidose extracellulaire. Le contenu sanguin d'oxygène était partiellement bloqué par pré-traitement des poissons avec soit la phentolamine ou le propranolol. Le pré-traitement simultané avec la phentolamine et le propranolol causa la mort des poissons, présumément due à l'hypoxémie. Ainsi, les stimulations alpha- et beta-adrénergiques sont importantes aux abilités des poissons de régler le contenu sanguin d'oxygène, mais leur role dans le règlement de l'état acido-basique reste en doute.

Même si les réponses respiratoires indique un role direct pour

l'adrénaline dans la modulation des réponses oxygéniques du sang, un role pour celle-ci dans la régulation du pH n'est pas aussi defini. Une correlation entre des niveaux d'adrénaline élevés et une augmentation dans l'excrétion rénale et branchiale d'acide existe, mais le role de l'adrénaline dans la régulation du pH sanguin est probablement mineur. Néanmoins, il est avancé que le role primordiale de la mobilisation adrénergique durant les perturbations est le règlement immédiat du montant d'oxygène sanguin.

ABBREVIATIONS

C_{CO2}.....total carbon dioxide

C_{O2}.....total oxygen

Hct.....hematocrit

rbc.....red blood cell (erythrocyte)

Hb.....hemoglobin

PO_2.....oxygen tension

PCO_2.....carbon dioxide tension

pH_e.....whole blood pH

rbc pH....red blood cell pH

UFR.....urine flow rate

J_{net}.....net flux

J_{in}.....influx

J_{out}.....efflux

J_{TA}.....net flux of titratable alkalinity

J_{amm}.....net flux of ammonia

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CHAPTER 1

GENERAL INTRODUCTION

Regulation of both blood oxygen content and blood pH is intimately linked in fishes. Through modifications in ventilatory rates, oxygen uptake and carbon dioxide excretion may be varied. Carbon dioxide, in aqueous solutions (e.g. blood), behaves as a weak acid; therefore, changes in CO_2 levels caused by differential rates of CO_2 excretion will affect blood pH. A large quantity of work has dealt with the effects of stress on blood oxygen carrying capacity and acid-base status. Although a wealth of information on the effects of stressful perturbations on the internal status in fishes has been accumulated, less is known about the hormonal and humoral responses elicited by these perturbations. Catecholamines are rapidly mobilized in response to stress; therefore, a role for their involvement in blood oxygen transport and acid-base regulation is plausible and this possible involvement is outlined subsequently.

Catecholamines

Variations of blood pH have been described following various stress in fish. Decreases in blood pH (acidoses) have been shown after exhaustive exercise (Black, Chiu, Forbes and Hanslip, 1959; Piiper, Meyer and Drees, 1972), environmental temperature increases (and consequentially of the blood; Rahn and Baumgardner, 1972; Randall and Cameron, 1973; Heisler, Weitz and Weitz, 1976; Walsh and Moon, 1982), environmental hyperoxia (Dejours, 1972, 1973; Wood and Jackson, 1980; Wilkes, Walker, McDonald and Wood, 1981), hypercapnia (Lloyd and White, 1967) and hypoxia (Holeton and Randall, 1967). Exposure to acidic waters may (Packer and Dunson, 1970; Janssen and Randall, 1975) or may not (Diveley, Mudge, Neff and Antony, 1977) cause a

corresponding drop in blood pH, depending on the acidity and the hardness of the water. Decreases in blood pH are accompanied by a concomitant increase in plasma epinephrine levels; these increases have been characterized during acidosis caused by intravascular acid infusion (Boutilier, Iwama and Randall, 1986), exposure to low environmental Ca^{2+} concentrations (Perry, Verbost, Vermette and Flik, 1986), exhaustive exercise (Primmett, Randall, Mazeaud and Boutilier, 1986), violent swimming activity instituted by chasing, prodding or tail-grasping (Nakano and Tomlinson, 1967; Opdyke, Carrol and Keller, 1982; Ristori and Laureht, 1985) and hypercapnia (Perry, 1986; Perry, Malone and Ewing, 1986a) (see also Table 1). Epinephrine also increases in concentration during acute hypoxia, although it is as yet unclear whether this is due to the acidosis incurred by hypoxia or the accompanying drop in C_{O2} (Butler, Taylor, Capra and Davison, 1978; Fievet, Motais and Thomas, 1986).

In fishes, most of the circulating catecholamines are released from the chromaffin tissue of the head kidney (Nilsson, 1983). This chromaffin tissue is analogous in function to the adrenal medulla of the higher vertebrates.

Physiological Consequences of Epinephrine Release

1) Effects on red blood cells:

During a blood acidosis, decreasing intracellular rbc pH would lead to a detrimental reduction in blood oxygen carrying capacity due to the Bohr and Root effects (Root and Irving, 1943). An adrenergic activation of the transmembrane sodium/proton antiporter decreases the

Table 1 . Peak plasma epinephrine concentrations in various fish species during or following a variety of stresses or physical activities.

Type of stress or physical activity.	Resting value (nM)	Stress value (nM)	Species	Source
[1]Exhaustive exercise	1.0	3.7	*S. gairdneri*	Primmett *et al.*, 1986
[2]Exhaustive exercise	1.4	14.4	*S. gairdneri*	Butler *et al.*, 1986
Spontaneous swimming	5.9	19.3	*Scyliorhinus canicula*	Butler *et al.*, 1986
Spontaneous swimming	1.2	1.9	*S. gairdneri*	Ristori and Laurent, 1985
[3]Violent exercise	1.5	7.9	*S. gairdneri*	Ristori and Laurent, 1985
[3]Violent exercise	3.3	25.0	*Squalus acanthias*	Opdyke *et al.*, 1982
[4]Violent exercise	2.2	29.7	*S. gairdneri*	C.L. Milligan and C.M. Wood (pers. comm.)
[4]Violent exercise	19.1	33.0	*Plathichthys stellatus*	C.L. Milligan and C.M. Wood (pers. comm.)
External hypercapnia	1.5	24.0	*S. gairdneri*	Perry *et al.*, 1986a
[5]Blood acidosis	1.9	5.5	*S. gairdneri*	Boutilier *et al.*, 1986
Low external Ca^{2+} (0.025 mM)	2.9	80.0	*S. gairdneri*	Perry *et al.*, 1986b
Air-exposure (10 min)	[6]29.8	292.5	*Gadus morhua*	Wahlquist and Nilsson, 1980
External hypoxia (24 h) (PO_2 = 30 torr)	0.4	8.8	*S. gairdneri*	R.G. Boutilier and D.J. Randall (pers. comm.)

[1] Fish were swum to exhaustion at 120% critical swimming velocity in a Brett-type (Brett, 1964) swimming respirometer.

[2] Fish were swum to exhaustion at a swimming speed of approximately 2 body-lengths·sec^{-1} in a water channel (Johnston and Moon, 1980).

[3] Violent physical activity was initiated by firmly grasping the tail and holding on for a 3 min. period.

[4] Fish were exercised by chasing them around a tank for 6 min. (trout) or 10 min. (flounder).

[5] Blood acidosis was induced by infusing fish with 5 ml·kg^{-1} body weight of 0.05 M HCl dissolved in saline.

[6] This value is probably not representative of epinephrine levels in truly resting fish since animals were allowed to recover from the effects of surgery and anaesthesia for only 1 h.

acidosis of the intracellular rbc milieu, thereby protecting the hemoglobin from the extracellular pH change (Nikinmaa and Huestis, 1984; Cossins and Richardson, 1985; Mahe, Garcia-Romeu and Motais, 1985).

Increases in the intracellular concentrations of osmotically active ions, due to variations in proton excretion may bring about increases in the volume of rbcs. Swelling of rbcs may cause dilution of intracellular nucleoside triphosphates (NTP: ATP and GTP) and hemoglobin, thereby leading to decreased binding of NTP to hemoglobin and increasing oxygen affinity of the hemoglobin (NTPs are negative modulators of hemoglobin oxygen affinity; Weber, 1982) (Nikinmaa, 1983; Jensen, 1986). Swelling also alters the transmembrane distribution of chloride by diluting impermeable ions within the rbc: the Donnan chloride distribution ratio will increase and along with it, intracellular rbc pH will increase (Hladky and Rink, 1977).

An adrenergic stimulation of splenic release of rbcs will cause a Hct increase (Yamamoto, Itazawa and Kobayashi, 1980; Nilsson, 1983). This increasing Hct causes increased total circulating hemoglobin levels, thereby increasing oxygen transport capacity during stress (Nilsson, 1983). Other possible causes of increased Hct in the blood may be the reduction of blood volume due to increased diuresis (Wood and Randall, 1973; Tetens and Lykkeboe, 1985) and/or plasma shifts between various internal compartments (Yamamoto et al., 1980). These may also be under adrenergic control.

Thus, the possibility of adrenergic effects on rbc pH, rbc volume, rbc release from the spleen and reduction of plasma volume could serve

to maintain hemoglobin oxygen affinity and/or oxygen transport capacity during a stress-induced extracellular acidosis. This possible involvement is a primary subject of this thesis.

2) Circulatory effects of epinephrine

The effects of epinephrine have been characterized in systemic, branchial and coronary circulatory systems in teleosts. Both alpha-constrictory and beta-dilatory mechanisms are demonstrable in each system, although dominance varies with the tissue selected. Alpha-adrenoceptor mediated vasoconstriction dominates in the systemic vasculature, though overall systemic resistance may decrease during exercise (Jones and Randall, 1978; Wood and Perry, 1985). Changes in microcirculation to and within various muscle or organ systems may be under local control: flow to white and red muscle and kidney increases during exercise, while flow to "deep" organs (liver, stomach and spleen) decreases (Wood and Perry, 1985). Furthermore, coronary vascular vasodilation in response to epinephrine has been shown in Pacific blue marlin heart (Davie and Daxboeck, 1984). Both alpha-adrenoceptor mediated vasoconstriction of arterio-venous anastomoses and efferent lamellar arterioles and beta-adrenoceptor vasodilation of afferent lamellar arterioles are observed in rainbow trout gill (Pettersson and Johansen, 1982; Pettersson, 1983; Perry, Daxboeck and Dobson, 1985). These combined effects cause a net decrease in branchial vascular resistance, an increase in lamellar recruitment and an increase in blood flow through the gills (Pettersson, 1983; Perry et al., 1985).

3) Branchial and renal effects of epinephrine

Increased water influx across the gills has been demonstrated during exercise in rainbow trout (Wood and Randall, 1973). This could be related to increased epinephrine levels, as epinephrine increases the permeability of the gill to small hydrophilic molecules such as water and urea and the lipophilic molecule butanol (Isaia, Maetz and Haywood, 1978). This increase in gill permeability might also apply to oxygen (Haywood, Isaia and Maetz, 1977; Isaia et al., 1978; Isaia, 1979, 1984). Increased pulsatility, such as that induced by the positive chronotropic action of epinephrine (Wood and Shelton, 1980), also increases the permeability of the gill epithelium to small lipophilic molecules (i.e. ethanol; Davie and Daxboeck, 1982). Gill diffusive conductance may also be increased by increasing the functional surface area of the gill via hemodynamic effects (see above; Pettersson, 1983; Perry et al., 1985). Epinephrine induced modulation of branchial ionic fluxes ($Na^+/NH_4^+(H^+)$ and/or $Cl^-/HCO_3^-(OH^-)$ exchanges) have also been postulated as an acid-base regulatory role of epinephrine (Perry, Payan and Girard, 1984a). Finally, changes in ventilatory rates have also been hypothesized as a response to epinephrine (Wood and Perry, 1985): hyperventilation in response to epinephrine injections has been observed in the eel (Anguilla anguilla) (Peyraud-Waitzenegger, 1979).

Increased diuresis during exercise may also be adrenergically modulated in response to increased water influx across the gills (Wood and Randall, 1973; Tetens and Lykkeboe, 1985). Changes in renal handling of ionic components of the urine, as well as excretion of acid-base equivalents, have also been postulated as roles for the

kidneys (Wood and Caldwell, 1978; Cameron, 1980; Kobayashi and Wood, 1980; McDonald and Wood, 1981; Cameron and Kormanik, 1982; Evans, 1982; Holeton and Heisler, 1983; McDonald, Walker, Wilkes and Wood, 1982; Hobe, Wilkes, Walker, Wood and McMahon, 1983; Wheatly, Hobe and Wood, 1984). The most important role of the kidney during acid-base disturbances is the retention of blood HCO_3^-: by linking acid secretion into the urine to the reabsorption of HCO_3^-, loss of HCO_3^- into the urine may be prevented (Pitts, 1974; Wood and Jackson, 1980; Wheatly et al., 1984), and blood pH may be regulated by adjustments of HCO_3^- concentration.

Although much work has been accomplished in an attempt to elucidate the role of epinephrine during stress, many of these roles remain speculative, due to the indirect nature of the experiments involved (i.e. increases in epinephrine concentrations by an outside stimulus). Direct evidence as to the role of epinephrine during stress was needed; therefore, the objectives of this thesis were to determine the direct effects of epinephrine on blood acid-base status, blood respiratory status and the branchial and renal consequences of epinephrine release. In order to accomplish this, prolonged intra-arterial infusion of epinephrine in order to simulate blood levels during stress was carried out (chapters 2, 3, 4 and 5). A second method utilized in this study was to once again simulate increases in blood epinephrine concentrations; this time, specific agonists were infused, in order to monitor specific alpha- and beta-adrenergic effects directly. The third and final method employed to obtain evidence for

the implications of epinephrine release during stress was to simulate increased levels of epinephrine via a physiological stress (hypercapnia) and block the effects of epinephrine. By selectively blocking either alpha- or beta-adrenoceptors or both, it was possible to indirectly observe specific alpha- or beta-adrenergic responses to this stress.

It must be stated that the following chapters (2 through 7) constitute five papers which have been submitted for publication or are in press at the time of submission of this thesis.

CHAPTER 2

THE EFFECTS OF PROLONGED EPINEPHRINE INFUSION ON THE PHYSIOLOGY OF THE RAINBOW TROUT, *SALMO GAIRDNERI*

I. BLOOD RESPIRATORY, ACID-BASE AND IONIC STATES.

INTRODUCTION

Increases in the circulating levels of catecholamines have been noted during periods of stress in fishes (see review by Mazeaud and Mazeaud, 1981; see also Table 1). Although it is known that epinephrine is released in response to stress, little is known about the involvement of catecholamines in acid-base regulation in fish.

Catecholamines are important modulators of cardiovascular function (see reviews by Nilsson, 1984a,b), gas transfer and blood gas transport (Peyraud-Waitzenegger, 1979; Pettersson, 1983; Perry, et al., 1985). They also have a putative role in modulating branchial permeabilities (Isaia, 1984) to small lipophilic molecules (butanol, ethanol and maybe oxygen). Recently, the involvement of catecholamines in rbc pH regulation has also been elucidated (see review by Nikinmaa, 1986).

In this chapter, the hypothesis is advanced that epinephrine mobilization during periods of acute extracellular acidosis operates to prevent pH-related disturbances of blood O_2 transport by stabilizing rbc pH. The experimental protocol for this and subsequent chapters (3,4,5) involves simulating the elevated circulating levels of epinephrine, measured during acid-base disturbances, by continuous intra-arterial infusion. Data presented here and in chapters 4 and 5 were obtained from the same experimental series of fish.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout (Salmo gairdneri) of either sex weighing between 171 and 442 g (mean weight = 272.4 ± 11.4 g(SEM); n = 42) were obtained from Thistle Springs Trout Farm (Ashton, Ontario) and transported to the University of Ottawa. Fish were held indoors in large rectangular fiberglass tanks (Frigid Units, Inc., Toledo, Ohio) supplied with flowing, aerated and dechlorinated Ottawa City tapwater ($[Na^+]$ = 0.10 mM; $[Cl^-]$ = 0.10 mM; $[Ca^{2+}]$ = 0.35 mM; $[K^+]$ = 0.03 mM; pH = 7.0-8.0). The temperature of the holding and experimental water varied between 8 and 14°C during the four month span of the experiments (chapters 2, 4 and 5; June-Sept., 1985). Fish were fed a daily diet of dried commercial trout pellets (Purina Trout Chow) but were not fed for 48 h before experimentation.

Animal Preparation

Fish were anesthetized in a 1:10000 (w/v) solution of ethyl m-aminobenzoate (MS 222, Sigma) buffered to approximately pH 7.0 with $NaHCO_3$ and placed onto an operating table (Smith and Bell, 1967) that permitted continuous irrigation of the gills with the same solution. To permit blood withdrawal and epinephrine infusion, indwelling cannulae were implanted into the dorsal aorta (Smith and Bell, 1964) using flexible polyethylene tubing (Clay Adams PE 50, ID = 0.58 mm, OD = 0.965 mm). The trout were also fitted with urinary catheters (Wood and Randall, 1973) by inserting a heat-flared flexible polyethylene tube (Clay Adams PE 60, ID = 0.76 mm, OD = 1.22 mm) through the urinary papilla and advancing it so that the catheter tip was located within the

urinary bladder. The catheter was secured by a ligature around the papilla and was sutured to the anal fin.

To determine plasma catecholamine levels during dorsal aortic epinephrine infusion, a second cannula was implanted in the caudal artery/vein of four fish according to the method of Wood, McMahon and McDonald (1977), using PE 50 tubing. Urinary bladder catheterizations were not performed on these fish.

Following surgery, fish were placed into opaque acrylic boxes (volume = 3 L), similar to those of McDonald (1983), supplied with flowing water and allowed to recover for at least 48 h prior to experimentation. The flux boxes were partially immersed in a cooling bath to maintain constant temperature and vigorously aerated to provide adequate water convection. The urinary catheters drained continuously into plastic vials that were secured outside of the fish boxes approximately 7 cm below water level. Dorsal aortic and caudal artery/vein cannulae were flushed at least once daily with approximately 0.3 ml of freshwater teleost saline solution (modified from Wolf, 1963) containing 113 mM NaCl, 13 mM $NaHCO_3$, 4.2 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.0 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 0.1 mM $(NH_4)_2SO_4$ and 10 units$\cdot ml^{-1}$ heparin (ammonium salt, Sigma).

In Vivo Protocol

Initially, fish were infused via the dorsal aorta cannulae with saline (further modified so that $[HCO_3^-]$ = 4 mM, pH = 7.8-8.0) for a 4 h period using syringe pumps (Sage Model 352) at a flow rate of 0.6 $ml\cdot h^{-1}$. This 4 h preliminary period was considered essential to re-establish steady-state conditions with respect to urine flow rate

(UFR) and other sensitive variables. Fish then were subjected to a further 3 h of saline infusion at the same rate, followed by 24 h of L-epinephrine (experimental group) or saline (control group) infusion and finally an additional 12 h interval of saline infusion (experimentals and controls). L-epinephrine (bitartrate salt, Sigma) was added to saline immediately prior to infusion to yield a final concentration of 2×10^{-5} M (pH = 7.8-8.0). Based on approximations of internal epinephrine distribution volume (assumed to be equivalent to extracellular fluid volume; about 300 $ml \cdot kg^{-1}$ body weight), biological half-life (15 minutes; from unpublished personal observations of blood pressure changes in trout following bolus injections of epinephrine (S.F. Perry); see also Nekvasil and Olson, 1986a, b) and an infusion rate of 0.6 $ml \cdot h^{-1}$, plasma epinephrine levels were estimated to be 5.0×10^{-8} M throughout the experimental infusion period. These estimated levels were confirmed after 4 h of infusion (see below) by high pressure liquid chromatography (HPLC) measurements. In order to impede the oxidation of epinephrine during infusion, syringes and cannula tubing were blackened to prevent light penetration.

Urine was collected continuously over 3, 6 or 9 h intervals and analyzed to provide measurements of UFR, pH, total CO_2 (C_{CO2}), $[Na^+]$, $[Cl^-]$, $[K^+]$, $[Ca^{2+}]$, inorganic phosphate (P_i), $[NH_4^+]$, flux rates (excretion) of these solutes and net acid excretion (see materials and methods for chapter 5 for details). Water samples were removed at appropriate times for determination of branchial solute exchanges (see materials and methods for chapter 4 for details). In this study, 0.7 ml blood samples were withdrawn from the dorsal aortic cannula midway

through the first 3 h saline infusion period, then at 1.5 h, 4.5 h, 10.5 h, 21.5 h of the 24 h epinephrine or saline infusion period, and finally at 1.5 h, 4.5 h and 10.5 h of the final 12 h terminal saline infusion interval. Measurements were made of hematocrit (hct), C_{CO2}, oxygen partial pressure (PO_2), whole blood pH (pH_e) and rbc pH as described below. Remaining blood was centrifuged and the plasma removed; 10% (v/v) of the anti-oxidant, sodium bisulphite (5 mM), was added to the plasma samples destined for catecholamine analysis. Samples were then quickly frozen in liquid nitrogen and stored at -70° C for subsequent ion and/or catecholamine determinations (see analytical procedures below). Remaining blood cells (approximately 0.2 ml) were resuspended in saline and reinjected into fish via the dorsal aortic cannulae.

In Vitro Protocol

Approximately 3 ml of blood was withdrawn slowly (1 $ml \cdot min^{-1}$) from the dorsal aortic cannula of fish that had recovered from surgery for at least 48 h. Blood from each fish (n = 12) was added to individual 50 ml tonometer flasks containing ammonium heparin (100 units) that were immersed in a temperature controlled, shaking water bath set at ambient water temperature (10° ± 2° C). Flasks were shaken continuously and gassed for approximately 1 h with 0.3% CO_2 in O_2 (PCO_2 = 2.3 torr) before the experiments commenced. Experiments consisted of monitoring pH_e, rbc pH, and hct at various PCO_2s, and epinephrine levels using a paired experimental design. Gas mixtures of 0.3%, 0.5% and 1% CO_2 in O_2 were provided by mixing CO_2 and O_2 using a gas mixing pump (Wosthoff Model M301 A-F). 25 ul of L-epinephrine (in saline) was added directly to the tonometer flasks

from freshly prepared stock solutions (pH = 7.8-8.0) to yield estimated final concentrations of 5×10^{-8}, 5×10^{-7} and 5×10^{-6} M. Again, actual epinephrine levels were later determined by HPLC (see below). Blood samples were withdrawn and analyzed 30 min. after the addition of epinephrine or the switching of the gasses.

Analytical Procedures

pH_e and PO_2 were determined by using a micro capillary pH electrode (Radiometer G299A) and PO_2 electrode (E5046) maintained at ambient temperature in conjunction with a Radiometer PHM 71 acid-base analyser and BMS3 Mk2 blood micro system. Rbc pH was determined with the same apparatus, utilizing the fast freeze-thaw technique of Zeidler and Kim (1977). Blood C_{CO2} was determined on 100 ul samples using a total CO_2 analyzer (Corning Model 905). Hct was determined by centrifuging 80 ul of blood in a heparinized capillary tube for 8 min. at 5000 g. PCO_2 and bicarbonate concentration ($[HCO_3^-]$) were calculated using a reorganization of the Henderson-Hasselbalch equation. The operational pK values of carbonic acid (pK') at various temperatures and pH's and the solubility coefficients of CO_2 (alpha CO_2) for trout plasma were obtained from Boutilier, Heming and Iwama (1984).

Plasma Na^+ and K^+ levels were determined on diluted plasma (200x) using flame photometry (EEL Flame Photometer), plasma Cl^- by coulometric titration on 100 ul of undiluted sample using a chloridometer (Buchler-Cotlove), plasma Ca^{2+} and P_i by spectrophotometry using commercial assay kits (Sigma), and plasma ammonia by a micro-modification of the salicylate-hypochlorite reaction (McDonald and

Wood, 1981). P_i and ammonia determinations were performed on samples de-proteinized with 12% TCA.

Plasma catecholamines were determined using HPLC (Waters Chromatograph) in conjunction with electrochemical detection (Bioanalytical Systems LC-4B Electrochemical Detector) following the extraction procedures of Woodward (1982). A reverse phase column (Waters, Nova Pak C_{18}) was used with a 0.15 M monochloroacetate buffer (pH = 3.0; containing 2 $mM \cdot l^{-1}$ Na_2EDTA, 200 mg sodium octyl sulfate) mobile phase at a flow rate of 1.0 $ml \cdot min^{-1}$. The applied potential to the LC-4B oxidative flowcell was +0.65 volts versus the Ag/AgCl reference electrode.

Statistical Analysis:

In Figures and Tables, variability of the data is indicated by ± 1 SEM. Results have been statistically analysed using paired and unpaired Student's t-test where appropriate between sample means; 5% was taken as the fiducial limit of significance.

RESULTS

Maintaining the patency of dorsal aortic cannulae throughout the course of experimentation was a major problem in the present study. As a consequence of cannula failure, n numbers declined during the latter stages of infusion. In order to offset the attrition-related lack of values at 22.5 h of epinephrine infusion and during post-epinephrine infusion, results from two separate groups of fish were pooled. In one group, blood was sampled throughout the entire experiment while in the other, blood sampling commenced after 10.5 h of epinephrine infusion. These are the reasons for variable n numbers in the Figures and Tables. As variability in both groups was similar, we believe such a change in the experimental design did not alter the results. Cannula failure was not a problem in control animals.

Continuous intra-arterial infusion of saline was without significant effect on the circulating levels of epinephrine or norepinephrine (Table 2) thus any differences between control and experimental groups presumably reflects physiological effects of exogenous rather than endogenous epinephrine. Infusion of 2×10^{-5} M epinephrine for 4.5 h at a flow rate of 0.6 $ml \cdot h^{-1}$ caused plasma epinephrine levels to rise by approximately 16X to 6×10^{-8} M (Table 3). This value is similar to the estimated and desired value of 5×10^{-8} M. It is assumed that plasma epinephrine remained elevated at this level throughout the remainder of the infusion period.

Epinephrine infusion induced a pronounced but transient blood acidosis that had disappeared by 10.5 h of infusion and thereafter blood pH remained constant. The acidosis was respiratory in origin as

Table 2: Plasma levels of epinephrine and norepinephrine during continuous intra-arterial infusion of teleost saline. Infusion rate = 0.6 $ml \cdot h^{-1}$. Means ± SE; values of n are shown in parentheses.

Time (h)	[Epinephrine] (nM)	[Norepinephrine] (nM)
Pre-infusion (6)	4.65 ± 1.24	7.93 ± 3.01
4.5 (6)	6.90 ± 1.26	3.10 ± 0.25
7.5 (6)	7.31 ± 2.55	8.48 ± 1.89
10.5 (6)	5.11 ± 0.99	6.11 ± 0.17
16.5 (6)	5.38 ± 0.99	4.83 ± 0.50
28.5 (5)	5.74 ± 0.85	4.92 ± 0.51
31.5 (5)	3.79 ± 1.58	5.75 ± 2.36
34.5 (5)	2.55 ± 0.68	5.86 ± 5.86
40.5 (5)	2.54 ± 0.45	6.22 ± 0.99

Table 3: Plasma catecholamine levels after 4h of continuous intra-arterial infusion of 2×10^{-5} M epinephrine. Asterisk denotes significant difference ($P<0.05$) from saline infusion value. Means $\pm$ SE (4).

Condition	[Epinephrine] (nM)	[Norepinephrine] (nM)
Saline infusion	3.87 ± 2.2	3.38 ± 1.2
Epinephrine infusion	62.09 ± 16.7*	2.21 ± 0.7

Figure 1: The effects of prolonged intra-arterial epinephrine infusion on measured (whole blood pH (pH_e); oxygen tension (PO_2)) and calculated (carbon dioxide tension (PCO_2); bicarbonate concentration, [HCO_3^-]) blood respiratory and acid-base variables. Control fish (open circles) were infused with saline continuously for 42 h while the epinephrine-treated fish (solid circles) were infused initially for 7 h with saline (pre-epi) followed by 24 h of epinephrine infusion and finally 12 h of saline infusion (post-epi). Asterisks denote significant differences from pre-epinephrine values; daggers denote significant differences from corresponding values in the control group. n values are indicated at each data point.

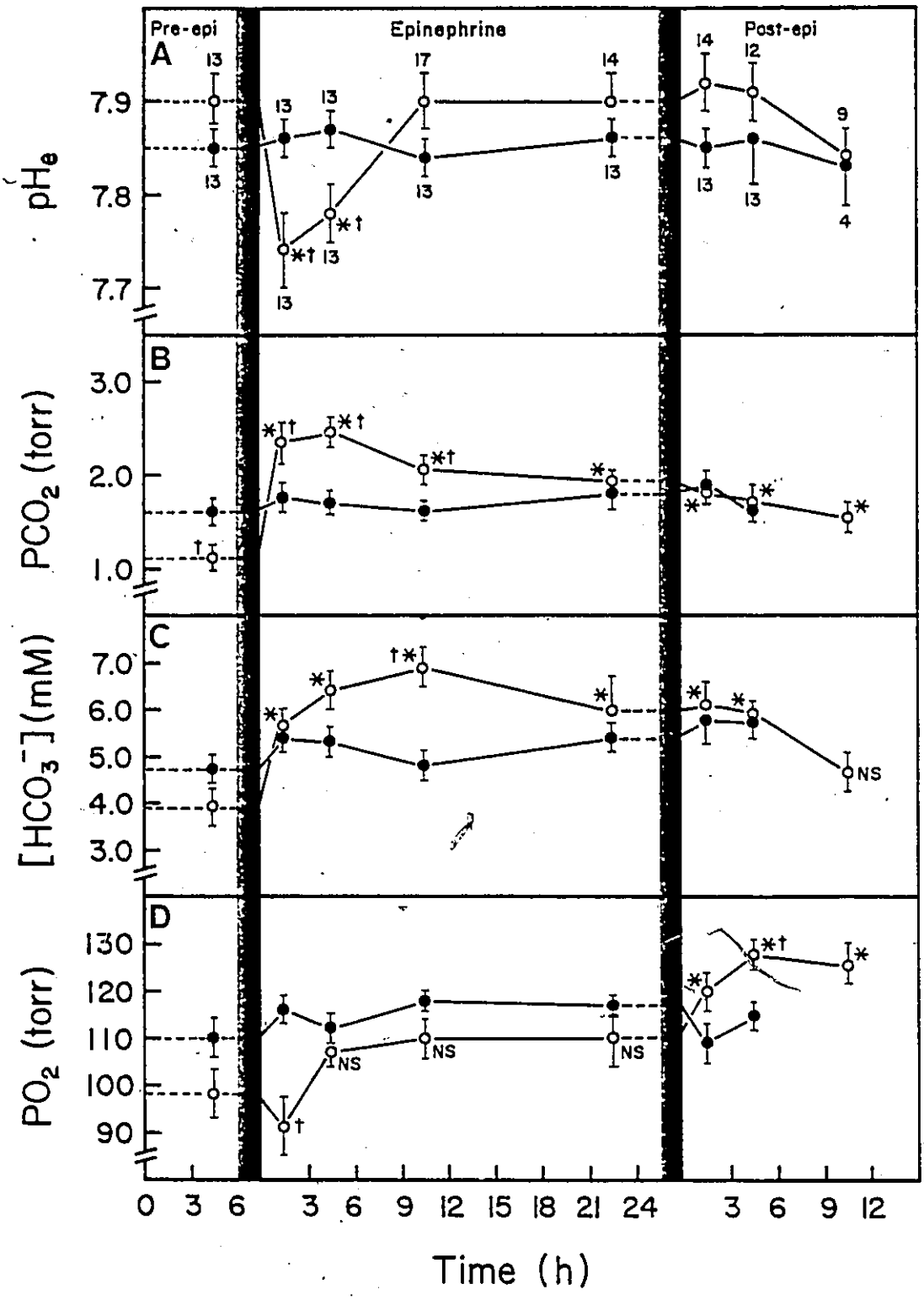
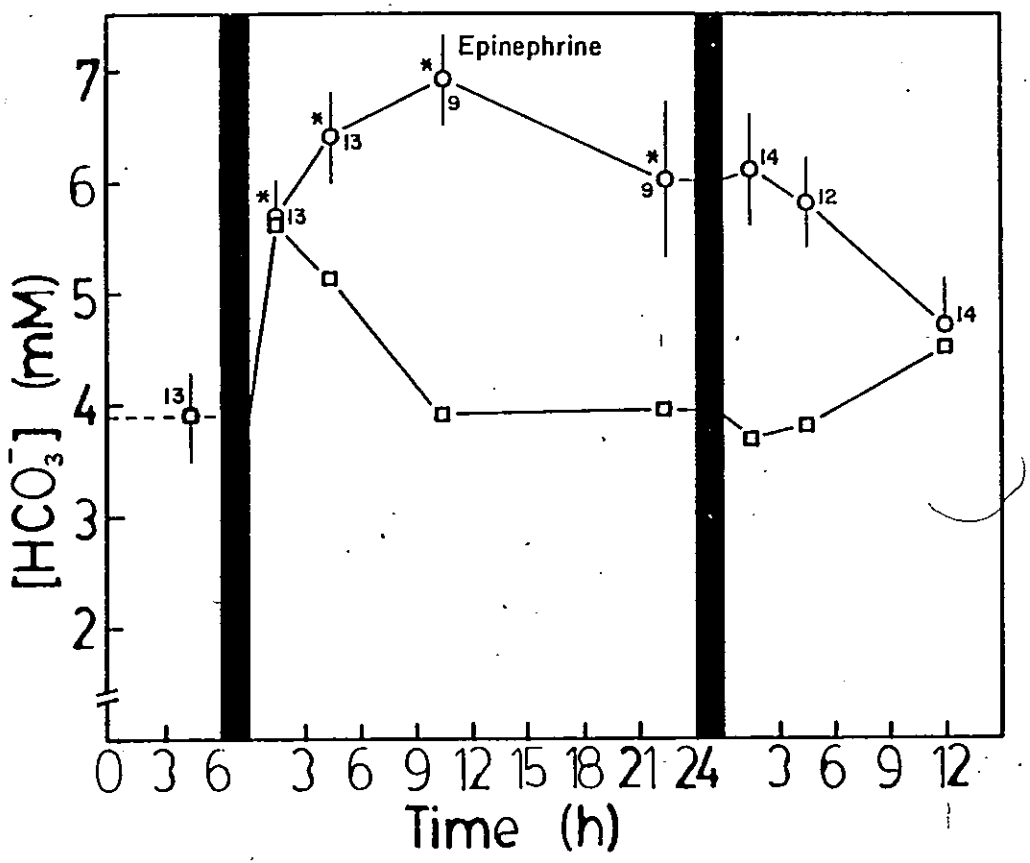

Pre-epi
Epinephrine
Post-epi
A
B
C
D
pH_e
PCO_2 (torr)
$[HCO_3^-]$ (mM)
PO_2 (torr)
7.9
7.8
7.7
3.0
2.0
1.0
7.0
6.0
5.0
4.0
3.0
130
120
110
100
90
NS
0 3 6 3 6 9 12 15 18 21 24 3 6 9 12
Time (h)

Figure 2: Measured (open circles) and predicted (open squares) changes in blood bicarbonate concentration ($[HCO_3^-]$) during prolonged intra-arterial epinephrine infusion. Predicted $[HCO_3^-]$ has been calculated using the experimentally determined buffer value (beta) of -9.81 ± 0.9 $mM \cdot l^{-1}$ whole blood (n = 6) according to the relationship $[HCO_3^-]$ = (-beta) $\cdot$ (dpH). Asterisks denote significant differences from predicted values. n values are indicated at each data point.


Epinephrine
[HCO3-] (mM)
7
6
5
4
3
2
0 3 6 3 6 9 12 15 18 21 24 3 6 9 12
Time (h)
13
13
13
9
9
14
12
14
*

indicated by the elevation of arterial PCO_2 that persisted throughout the experimental period (Fig. 1b). The apparent elevation of PCO_2 during the post-epinephrine saline infusion may be artifactual and related to the unusually low PCO_2 during the initial saline infusion. Indeed, experimental and control PCO_2's were not significantly different during the final 12 h of infusion. Plasma $[HCO_3^-]$ levels increased as a consequence of epinephrine infusion (Fig. 1c) and correlated well with the restoration of blood pH to pre-epinephrine values. Plasma $[HCO_3^-]$ gradually returned toward initial and control levels during the final 24 h of infusion. The changes in $[HCO_3^-]$ induced by epinephrine treatment were over and above those predicted by non-bicarbonate carbon dioxide buffering (Fig. 2). PO_2 was depressed temporarily by epinephrine and became significantly elevated during the 12 h post-epinephrine period (Fig. 1b). Epinephrine elicited an immediate rise in hct that returned to control values by 4.5 h (Fig. 3a). Serial blood sampling led to gradual but significant reductions in hct in both experimental and control groups (Fig. 3a) even though a considerable quantity of removed rbcs were resuspended and reinjected into fish after each sampling period. Mean hct never decreased below 12% in either group.

The reduction of whole blood pH (pH_e) induced by epinephrine, in vivo, was not accompanied by a parallel reduction of red blood cell pH (Fig. 3b). In fact, rbc pH actually increased slightly during epinephrine infusion although the changes were not statistically significant. The dissimilar patterns of pH_e and rbc pH changes during the course of epinephrine infusion caused the pH gradient across the

Figure 3: The effects of prolonged intra-arterial epinephrine (open circles) infusion on A) hematocrit (hct) and B) whole blood pH (ph_e), red blood cell pH (rbc pH) and the pH gradient across the rbc membrane (ph_e - rbc pH). Asterisks denote significant differences from pre-epinephrine values; daggers denote significant differences from corresponding values in the control group (solid circles). n values are indicated at each data point. All other details are as in Fig. 1.

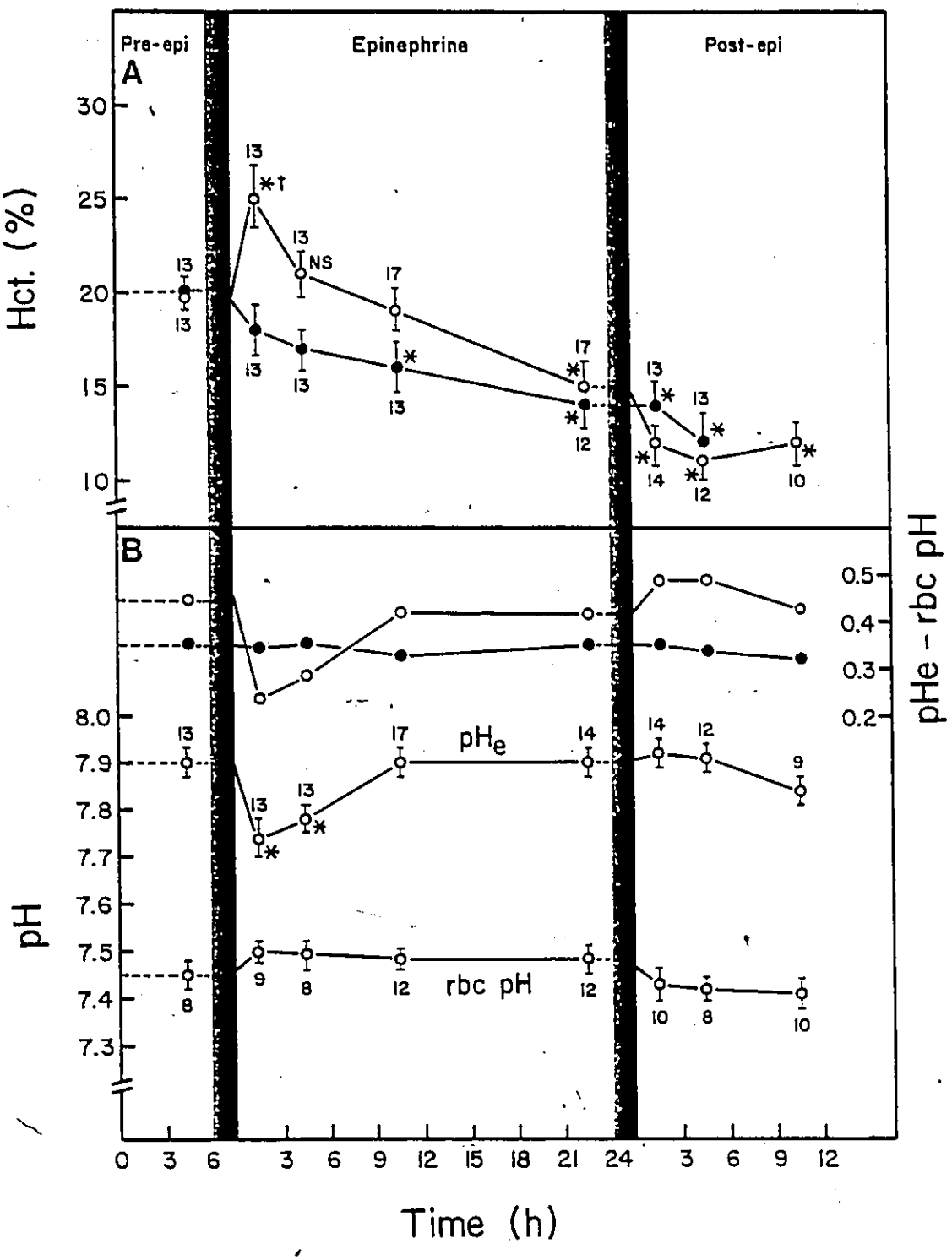
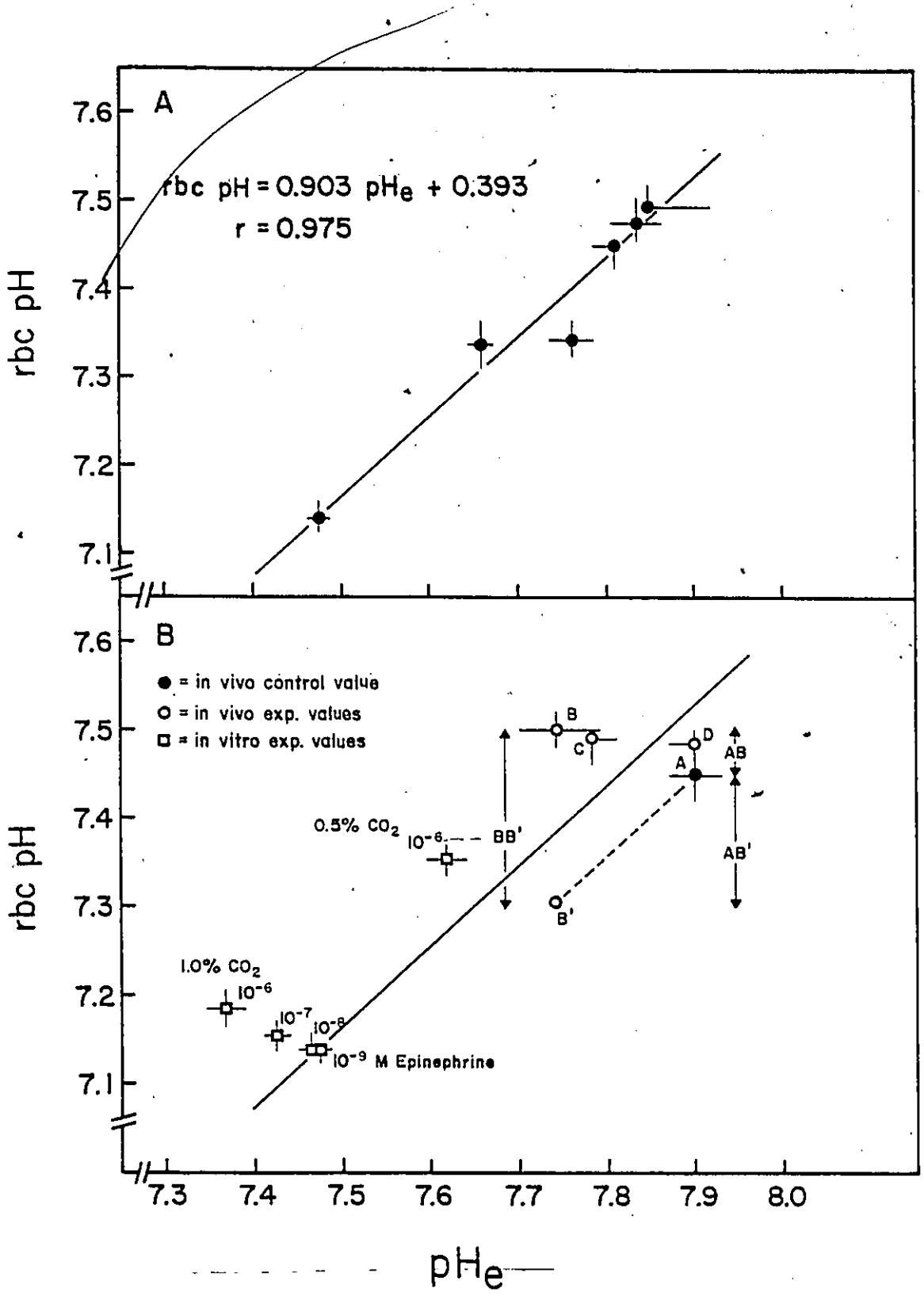

Pre-epi
Epinephrine
Post-epi
A
B
Hct. (%)
pH
pHe - rbc pH
pHe
rbc pH
Time (h)
0 3 6 3 6 9 12 15 18 21 24 3 6 9 12

Figure 4:-The relationships between whole blood pH (pH_e) and red blood cell pH (rbc pH) and the interactive effects of epinephrine both A) *in vitro* and B) *in vivo*. The pH_e *versus* rbc pH relationship was determined *in vitro* by equilibrating blood obtained from 6 chronically catheterized fish, in separate tonometer flasks with 3 different CO_2 tensions (0.3, 0.5 and 1.0 % CO_2). The average epinephrine concentration in the *in vitro* experiment was $9.0 \pm 2.5 \times 10^{-9}$ M. The *in vitro* pH_e *versus* rbc pH relationship has been reproduced as the solid line in Fig. 6B with various *in vivo* and *in vitro* values superimposed. Point A represents the values during initial saline infusion while points B, C and D represent the pH values after 1.5, 4.5 and 10.5 h of epinephrine infusion, respectively. Point B' signifies the predicted rbc pH value at 1.5 h, given the same change in pH_e as in point B, according to the *in vitro* relationship. Thus the vertical distance AB' is the predicted decrease in rbc pH at 1.5 h, AB is the actual measured increase in rbc pH at 1.5 h, and BB' is the deviation from the *in vitro* relationship attributed to elevated epinephrine. For comparative purposes, the *in vitro* effects of epinephrine on pH_e *versus* rbc pH have been illustrated (open squares). See text for further details.


A
rbc pH = 0.903 pHe + 0.393
r = 0.975
B
● = in vivo control value
○ = in vivo exp. values
□ = in vitro exp. values
0.5% CO2
10-6
BB'
1.0% CO2
10-6
10-7
10-8
10-9 M Epinephrine
A
B
C
D
AB
AB'
B'
rbc pH
pHe
7.1
7.2
7.3
7.4
7.5
7.6
7.3
7.4
7.5
7.6
7.7
7.8
7.9
8.0

red cell membrane (pH_e - rbc pH) to become significantly reduced (Fig. 3c), especially during the first 4.5 h of infusion. The relationship between pH_e and rbc pH observed *in vivo*, during epinephrine infusion, was also much different than the relationship quantified *in vitro* as shown in Fig. 4a. *In vitro* levels of epinephrine were 9.0 $\pm$ 2.5 nM (n = 6), approximately an order of magnitude lower than the level measured during epinephrine infusion. The strikingly different relationships between pH_e and rbc pH *in vitro* and during epinephrine infusion are illustrated in Fig. 4b. Point A represents values from fish during the initial saline infusion period; point B' represents the predicted values of pH_e and rbc pH, based on the *in vitro* relationship (solid line), following 1.5 h of epinephrine infusion. Thus, the vertical distance AB' is the predicted reduction of rbc pH during the epinephrine induced extracellular acidosis. Point B and the vertical distance AB represent the actual values of pH_e and rbc pH following 1.5 h of epinephrine infusion and the actual change in rbc pH during the extracellular acidosis, respectively. It is clear that epinephrine caused rbc pH to deviate far from the *in vitro* pH_e *versus* rbc pH relationship and this deviation can be quantified as the vertical distance BB' (0.20 pH units). Following 4.5 h of epinephrine infusion (Point C, Fig. 4b), rbc pH still was significantly elevated above the predicted value by 0.14 pH units but had returned to the pre-epinephrine value by 10.5 h (Point D). Deviation from the "normal" pH_e *versus* rbc pH relationship also was observed *in vitro* when epinephrine levels were progressively elevated to 1.2×10^{-6} M during equilibration with either 0.5% or 1.0% CO_2 (Fig. 4c, Fig. 5). Significant reductions in the rbc

Figure 5: In vitro effects of epinephrine on A) hematocrit (hct), B) whole blood pH (pH_e) and red blood cell pH (rbc pH) and C) the pH gradient across the red blood cell membrane (pH_e - rbc pH). Blood was collected from cannulated trout (n = 6) and equilibrated in separate flasks with 1% CO_2. C = control blood (9.0×10^{-9} M epinephrine); where not indicated, SEM's lie within the mean. Asterisks denote significant differences from control values.

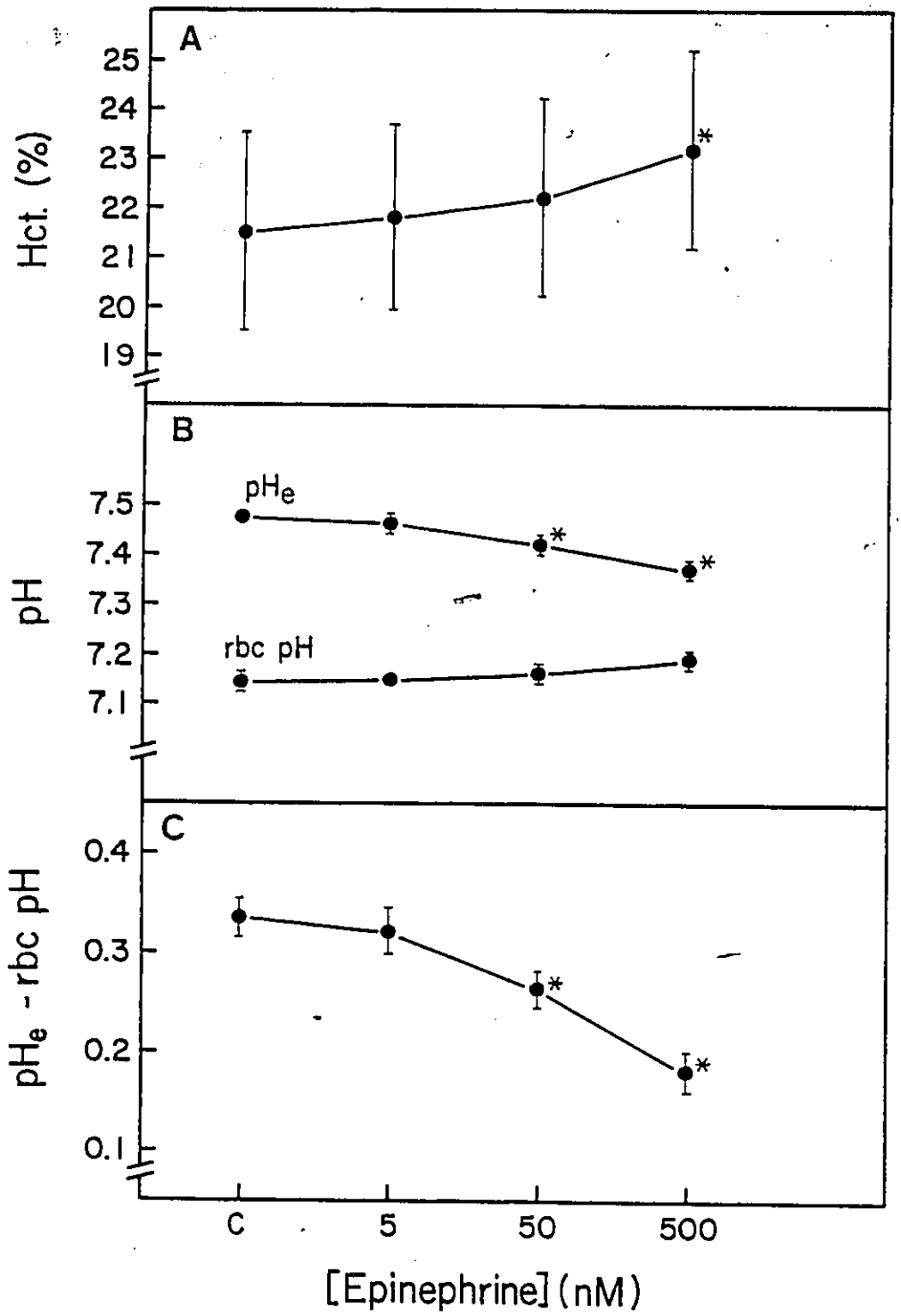
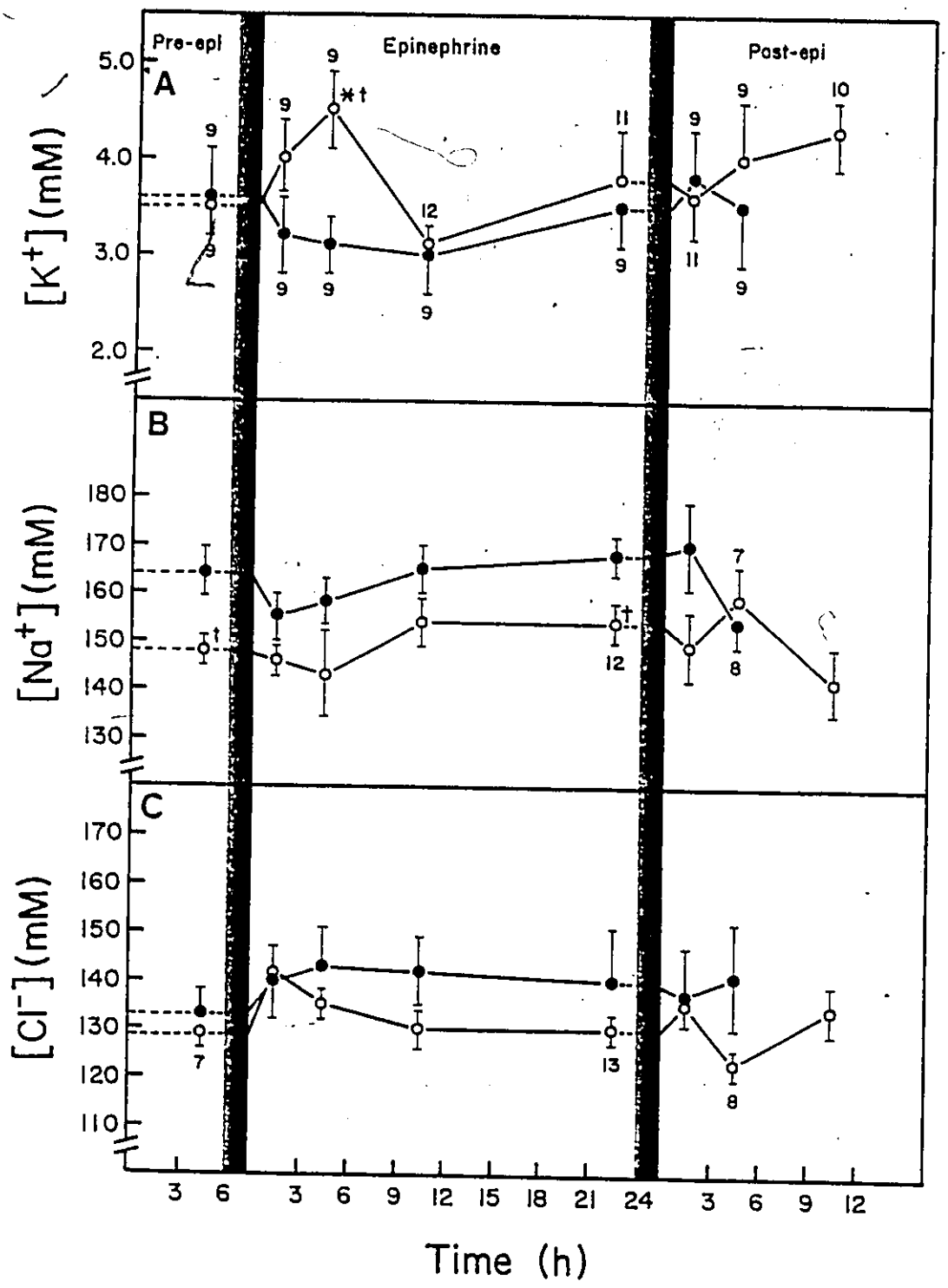

A
Hct. (%)
25
24
23
22
21
20
19
B
pH
pHe
rbc pH
7.5
7.4
7.3
7.2
7.1
C
pHe - rbc pH
0.4
0.3
0.2
0.1
C
5
50
500
[Epinephrine] (nM)

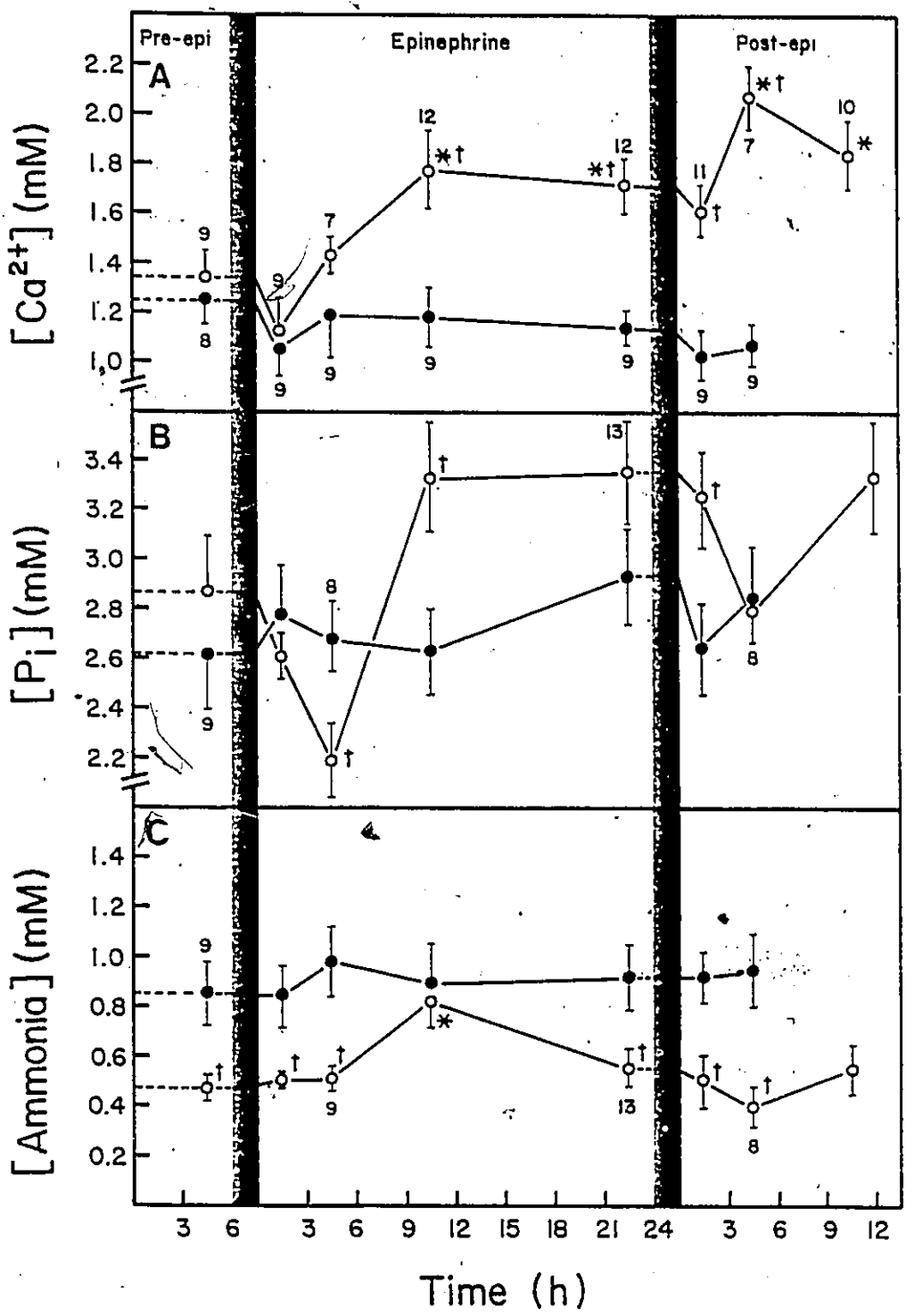
Figure 6: The effects of prolonged intra-arterial epinephrine (open circles) infusion on plasma A) potassium levels, B) sodium levels and C) chloride levels. Asterisks denote significant differences from pre-epinephrine values; daggers denote significant differences from corresponding values in the control group (solid circles). n = 9 for both groups except where otherwise indicated. All other details are as in Figure 1.


Pre-epi
Epinephrine
Post-epi
A
B
C
[K+](mM)
[Na+](mM)
[Cl-](mM)
5.0
4.0
3.0
2.0
180
170
160
150
140
130
120
110
3
6
9
12
15
18
21
24
Time (h)

transmembrane pH gradient were noted only at epinephrine levels of 2.0 x 10^{-7} M or higher (Fig. 5c) and were due to reciprocal changes in both pH_e and rbc pH, although only the changes in pH_e were statistically significant. The epinephrine-induced reductions of pH_e - rbc pH, in vitro, were similar in magnitude to those noted in vivo although the effect, in vivo, occurred at a lower epinephrine level (10X lower). Epinephrine, in vitro, caused rbcs to swell as indicated by increasing hct (Fig. 5a). This effect was minor compared to the elevation of hct observed in vivo (Fig. 3a) and was only statistically significant at an epinephrine concentration of 1.2 x 10^{-6} M (20X greater than during epinephrine infusion).

Epinephrine infusion had no apparent effect on plasma levels of Na^+ or Cl^-, but did induce significant elevations of plasma $[K^+]$ during the first 4.5 h of epinephrine infusion (Fig. 6). Plasma Ca^{2+} levels increased significantly throughout the epinephrine infusion period and remained elevated during the first 12 h of saline infusion (Fig. 7a). The effect of epinephrine on plasma $[P_i]$ and [ammonia] were somewhat variable and difficult to interpret although plasma $[P_i]$ tended to decrease during the initial 4.5 h of epinephrine treatment and rise during the latter stages of infusion (Fig. 7b). It is unclear why plasma ammonia levels were significantly elevated in the control group.

Figure 7: The effects of prolonged intra-arterial epinephrine (open circles) infusion on plasma A) calcium levels, B) inorganic phosphate (P_i) levels and C) ammonia levels. Asterisks denote significant differences from pre-epinephrine values; daggers denote significant differences from corresponding values in the control group (solid circles). n = 9 for both groups except where otherwise indicated. All other details are as in Fig. 1.


Pre-epi
Epinephrine
Post-epi
A
B
C
[Ca2+] (mM)
[Pi] (mM)
[Ammonia] (mM)
Time (h)

DISCUSSION

Previous studies examining the physiological effects of exogenous catecholamines in fishes have utilized single, rapid intravascular injections (e.g. Peyraud-Waitzenegger, 1979; Peyraud-Waitzenegger, Barthelemy and Peyraud, 1980; Nikinmaa, 1982a,b; Hughes, Peyraud, Peyraud-Waitzenegger and Soulier, 1982; Epple and Nibbio, 1985; Epple and Kahn, 1985) in order to simulate circulating plasma levels as measured during a variety of stressful conditions. In nearly all instances, plasma catecholamine levels were not measured but were calculated based on the concentration of the solution injected and estimates of biological half-life and internal catecholamine distribution volume. Based on the recent catecholamine measurements of Epple and Nibbio (1985), following single injections of epinephrine into American eels (Anguilla rostrata) (1-8 $ug \cdot kg^{-1}$), it is likely that the actual circulating epinephrine levels in previous studies using such estimations (see above) ranged between 10^{-6}-10^{-7} M. These levels are considerably higher than the recently reported values in trout during or following a variety of stresses (see Table 1). In the present study, "resting" values of epinephrine and norepinephrine were 4.7 and 7.9 x 10^{-9} M, respectively and are similar to the values reported by Ristori, Rehm and Laurent (1979), Woodward (1982) and Heming, Randall and Mazeaud (in preparation) for intact, catheterized rainbow trout. These recently measured values are significantly lower than in earlier reports ([epinephrine] = 2.7 x 10^{-8} M; Nakano and Tomlinson, 1967; [norepinephrine] = 1.2 x 10^{-8} M; Rich, 1979) presumably due to refinements in blood sampling techniques and catecholamine measuring

methodology. During continuous infusion of epinephrine, plasma levels increased to 6.2×10^{-8} M (Table 3), which are similar to the levels measured during physiological stress (see above). Due to the rapid degradation of epinephrine following a single bolus injection, it is believed that continuous infusion permits long-term evaluation of elevated epinephrine levels, thereby simulating more closely the prolonged elevation of plasma epinephrine induced by some stressors (LeBras, 1982 and above). Nikinmaa (1982b) reported that the effect of epinephrine on increasing hct was dissipating within only 10 min. of a single injection. The technique of intra-arterial infusion used in the present study ($0.6\ ml \cdot h^{-1}$) did not appear to unduly stress the fish as indicated by stable catecholamine levels (Table 2) and other physiological variables. The only parameter to be affected by infusion of saline was urine flow rate (see chapter 5) which increased presumably as a result of vascular hypervolemia or a related elevation of blood pressure. It is recommended, however, that a significant period (4-6 h) be allowed for fish to adjust to the process of infusion itself, before experimentation commences. The limitations of prolonged intra-arterial infusion of epinephrine include possible desensitization of the cAMP response and subsequent blunting of physiological responses.

Desensitization and/or loss of the cyclic AMP response is a complex matter. Increased levels of epinephrine may cause complete saturation of the receptors, but this is unlikely (Kunos, 1976). Maximal response to epinephrine may be achieved by stimulating only 50% of the available receptors (Atlas, Stear and Levitzki, 1976). This may be due to the fact that "spare" receptors are not bound to

adenylyl cyclase and are therefore not functionnal. Continued occupancy of a receptor by epinephrine due either to continued presence of epinephrine or to the slow dissociation of the epinephrine-receptor (E-R) complex does not result in continued activation of the adenylyl cyclase system. This reduction in sensitivity is due to a two-step process: i) the E-R complex conformation is altered and becomes uncoupled from the adenylyl cyclase and ii) the E-R complex or the receptor alone is removed from the cell surface and moved into lysosomes for degradation and/or storage (Kirchik, Codina, Hildebrandt, Iyengar, Roja, Abramowitz, Hunzicker-Dunn and Birmbauer, 1984). The uncoupling of the E-R complex from the adenylyl cyclase has a half-life of 10 min in incubated mouse cells and the reduction in absolute number of E-R complexes occurs within 2-6 h (50-60% loss) and peaks at 24 h (80-95% loss) (Su, Kendall and Perkins, 1980). This removal of receptors and reduction in response sensitivity could conceivably be the reason for a reduction in the effects of epinephrine infusion during the final hours of experimentation.

Two additional problems associated with studying the effects of catecholamines in fishes that are unrelated to the method of administration (i.e. injection versus continuous infusion) are seasonnality (see Peyraud-Waitzenegger et al., 1980) and the possibility of catecholaminotropic effects (Epple and Nibbio, 1985). In the present study, experiments were conducted between June 13 and Sept. 18 1985, thus it is unlikely that the results were affected significantly by changing seasons. Norepinephrine levels remained constant during infusion of epinephrine but the possibility of catecholaminotropic effects

cannot be dismissed since dopamine levels were not monitored. Epple and Nibbio (1985) reported significant elevations of dopamine following injections of 2-8 $ug \cdot kg^{-1}$ of epinephrine in American eels.

In this study, evidence has been presented showing that infusion of epinephrine induces a condition of respiratory acidosis along with an elevation of plasma [HCO_3^-], which ultimately corrects the extracellular acidosis. The rise in plasma [HCO_3^-] initially is due to non-bicarbonate H^+ buffering but ultimately results from enhanced net acid excretion at the gills and kidney (see chapters 4 and 5). There are two possible causes for retention of CO_2 during epinephrine infusion; these are hypoventilation and inhibition of erythrocytic bicarbonate dehydration. Hypoventilation has been shown to induce hypercapnic acidosis by inhibiting CO_2 excretion (Boutilier et al., 1986; Iwama, Boutilier, Heming, Wright and Randall, manuscript submitted) and may result from epinephrine administration (Peyraud-Waitzenegger et al., 1980) via stimulation of alpha-adrenergic receptors. In European eels (Anguilla anguilla), the hypoventilatory effect of epinephrine has been observed only during winter months whereas identical treatment during summer causes hyperventilation, a result of beta-adrenergic receptor stimulation (Peyraud-Waitzenegger et al., 1980). Thus, based on seasonality, one would predict hyperventilation and elevation of PO_2 (Peyraud-Waitzenegger, 1979) in the present investigation, in the absence of species or dose-related differences. Since ventilation volumes were not measured in the present study, the ventilatory effects of epinephrine in trout remain unclear. However, the results are not consistent with hyperventilation. Indeed, the initial

reduction of PO_2 and the pronounced elevation of PO_2 following the removal of epinephrine support the hypothesis of epinephrine-induced hypoventilation. Restoration and maintenance of "normal" PO_2 values during the final 22.5 h of epinephrine infusion may be related to adrenergic enhancement of gill diffusing capacity as demonstrated by Pettersson (1983) and Perry et al. (1985) which would tend to offset the effects of hypoventilation on arterial oxygenation. However, any factor which enhances gill O_2 diffusing capacity should have a similar effect on CO_2 diffusing capacity yet PCO_2 did not change in a reciprocal fashion as PO_2, except at 1.5 h of infusion (Fig. 1). For similar reasons, the prolonged elevation of PCO_2 cannot be attributed to epinephrine induced increases in metabolism (Larsson, 1973).

The prolonged elevation of PCO_2 in the face of unaltered PO_2 provides evidence for a reduction in the rate of rbc HCO_3^- dehydration, perhaps due to inhibition of rbc Cl^-/HCO_3^- exchange, a process which has been postulated as the rate limiting step in piscine CO_2 excretion (Perry, Davie, Daxboeck and Randall, 1982; see also review by Perry, 1986). This hypothesis is supported by in vitro data on rainbow trout blood showing inhibition of Cl^-/HCO_3^- exchange (Heming, 1984), an increase in the Donnan Cl^- ratio (Cl_i/Cl_e) and a concomitant increase in plasma [HCO_3^-] (Heming et al., manuscript submitted) following epinephrine addition (10^{-6} M). It must be emphasized that a direct comparison between the results of the present study with those of Peyraud-Waitzenegger (1979) are probably not justified given the likelihood of higher circulating levels of epinephrine (estimated to be 10^{-7} M), the short duration of the experiments

(measurements were made within 5 min. of epinephrine injection), and markedly lower initial levels of PO_2 (30-50 torr versus 100-110 torr).

The results of this study demonstrate that epinephrine may play an important role in stabilizing rbc pH during conditions of extracellular acidosis. Indeed, reductions of plasma pH, induced by intra-arterial acid infusion (Boutilier et al., 1986) or external hypercapnia (Perry et al., 1986) have been shown to initiate the release of epinephrine into the circulation. We have shown, here, that circulating levels as low as 6×10^{-8} M, in vivo, are sufficient to prevent a reduction in rbc pH during a simultaneous lowering of pH_e by 0.16 units. An identical effect of epinephrine was observed in vitro (Figs. 4,5), although significantly greater quantities of epinephrine were required to elicit similar changes in the transmembrane proton gradient (a significant reduction of pH_e - rbc pH was observed only at 2.0×10^{-7} M epinephrine). Similar in vitro changes in the proton distribution across the rbc have been reported by Nikinmaa (1982a) and Heming et al., (manuscript submitted), although at considerably higher levels of epinephrine (5×10^{-6} M). It is unclear why higher levels of epinephrine are required to initiate effects on the rbc in vitro, but possibilities include the absence of catecholaminotropic effects and interactions with other humoral/neurohumoral systems. Alternatively, the rbcs in vitro may be gradually deteriorating with respect to physiological function. The mechanism responsible for epinephrine-induced rbc alkalinization apparently involves beta-receptor mediated stimulation of Na^+/H^+ exchange (Baroin, Garcia-Romeu, Lamarre and Motais, 1984; Cossins and Richardson, 1985; Nikinmaa,

1986). Stimulation of Na^+/H^+ exchange also is consistent with red cell swelling which is known to accompany rbc alkalinization (Nikinmaa, 1982a; Nikinmaa and Huestis, 1984). Recently, Bennett and Rankin (1985) have identified beta receptors on eel rbc membranes which provides further evidence for their involvement in stabilizing rbc acid-base status. The maintenance of rbc pH during extracellular acidosis is of physiological significance because it allows arterial oxygen content to remain relatively constant by preventing deleterious shifts in the hemoglobin oxygen dissociation curve (Bohr and Root effects) (Root and Irving, 1943). The importance of epinephrine in the maintenance of arterial oxygen content is indicated by the observations of constant CaO_2 following exhaustive exercise in trout (Primmett et al., 1985) and striped bass (Morone saxatilis; Nikinmaa, Cech and McEnroe, 1984) and HCl infusion (Boutilier et al., 1986). In these experiments, pH_e decreased by 0.16-0.30 units, epinephrine levels were elevated (not measured in the study of Nikinmaa et al., 1984), and the changes in rbc pH were significantly less than those predicted based on a passive transmembrane distribution of protons (from in vitro studies). Pre-treatment of the exercising fish with the beta receptor antagonist, propranolol, resulted in significant reductions of CaO_2 which were associated with reduced rbc pH, when compared to "control" exercising fish.

No changes in the plasma levels of Na^+ or Cl^- were observed during epinephrine infusion even though significant changes in branchial net fluxes and renal excretion of these ions were noted (see chapters 4 and 5). These results are in agreement with those of Epple

and Nibbio (1985) following single injections of epinephrine into eels and are not surprising given the large extracellular pool size of NaCl in relation to the low rates of the branchial and renal net fluxes (no greater than 200 $uM \cdot kg^{-1} \cdot h^{-1}$ in the present investigations). The increase of plasma $[K^+]$ during the early stages of epinephrine infusion may reflect K^+/H^+ exchange between intracellular and extracellular compartments (Lade and Brown, 1963) and might be a significant mechanism in the regulation of blood pH during acute extracellular acidosis. A similar, although more prolonged, elevation of plasma $[K^+]$ was reported by Wheatly et al., (1984) during the regulation of hyperoxic acidosis in rainbow trout. The significant elevation of plasma $[Ca^{2+}]$ was not due to increased renal reabsorption (chapter 5) but may have been related to Ca^{2+}/H^+ exchange between extracellular fluid and bone and/or mobilization of bone calcium carbonate. Whether or not such mechanisms operate during regulation of acid-base disturbances in rainbow trout has not been examined, although Cameron (1985) concluded that the bone compartment of the Channel catfish (Ictalurus punctatus) does not contribute to the compensation of hypercapnic acidosis.

The results presented here and in the following three chapters have been attributed to elevated circulating levels of epinephrine. It must be emphasized, however, that the interpretation of these results is somewhat complicated by simultaneous reductions of plasma pH that were induced by epinephrine. While the possibility that some results are due to depressed pH and not epinephrine cannot be ignored, we consider this unlikely as many of these effects persist even after plasma pH is

restored. The problem of separating the effects of epinephrine from the effects of pH is discussed in greater detail in chapter 4.

Based on the results of this study and others, we speculate that the release of epinephrine into the circulation during periods of acute extracellular acidosis initiates a series of adrenergic responses that include stabilization of rbc pH and accumulation of plasma bicarbonate. Rbc pH stabilization prevents deleterious reductions of arterial oxygen content whereas plasma HCO_3^- accumulation is necessary for restoring extracellular pH. The branchial and renal mechanisms involved in plasma HCO_3^- accumulation are discussed subsequently.

CHAPTER 3

A PHARMACOLOGICAL STUDY OF THE EFFECTS OF PROLONGED EPINEPHRINE INFUSION ON BLOOD RESPIRATORY AND ACID-BASE STATES IN THE RAINBOW TROUT SALMO GAIRDNERI

INTRODUCTION

In recent years, the involvement of catecholamines in the control of branchial gas transfer and blood gas transport in fishes has been the focus of intensive research (see reviews by Nilsson, 1984a,b; Randall and Daxboeck, 1984; Wood and Perry, 1985; Nikinmaa, 1986). Experiments primarily have involved the elevation of epinephrine levels in vivo (e.g. Peyraud-Waitzenegger et al., 1980), in vitro (e.g. Nikinmaa, 1982a,b) or in perfused gill preparations (e.g. Pettersson, 1983) and subsequent analysis of various adrenergic responses. The results of these studies and the numerous observations of increased circulating catecholamine levels during or following stress in fishes (see Table 1) have formed the basis of a model in which epinephrine is thought to play a crucial role in the control of oxygen transfer and transport during stress (e.g. Wood and Perry, 1985). However, the importance of adaptive adrenergic responses, especially during physiologically relevant stresses such as exercise, has been questioned (Butler et al., 1986) because the concentrations of circulating catecholamines achieved during such stresses are substantially lower than the levels normally used experimentally to elicit compensatory responses in gas transfer and transport.

In the present study, a procedure of continuous intravascular infusion has been utilised; this procedure was shown to adequately simulate the increased circulating epinephrine levels associated with stress (chapter 2). In chapter 2, epinephrine infusion was used as a means of simulating stress in fish. This chapter was designed as a follow-up to chapter 2; the infusion process was shortened in order to

more accurately reflect the actual effects of epinephrine *in vivo* (the effects of epinephrine infusion were diminishing after 12 h in the previous chapter) and blood oxygen content and hemoglobin concentrations were measured. The primary objective was to demonstrate that a realistically elevated concentration of epinephrine (approximately 6×10^{-8} M) is indeed capable of initiating important responses to enhance O_2 transfer and transport, and secondarily, to characterize the adrenoceptor types involved.

MATERIALS AND METHODS

Experimental Animals:

Rainbow trout (Salmo gairdneri) of either sex, weighing between 117 and 326 g (mean weight= 210 ± 9.6 g (SEM); N= 30) were obtained from Thistle Springs Trout Farm (Ashton, Ontario) and transported in oxygenated water to the University of Ottawa. Fish were maintained according to the holding procedures outlined in chapter 2.

Experimental Protocol:

Dorsal aortic cannulae were implanted into the rainbow trout according to the preparation methods outlined in chapter 2. Maintenance of the animals prior to and during experimentation was also as outlined in Chapter 2.

Experiments consisted of monitoring blood acid-base and respiratory variables during epinephrine or adrenergic agonist infusions, in the presence or absence of selective adrenoceptor blockade. Fish initially were injected either with a bolus injection of 0.5 ml Cortland saline (Wolf, 1963) (saline control group), 2 $mg \cdot kg^{-1}$ phentolamine (alpha-adrenoceptor antagonist; Rogitine, Ciba-Geigy), 2 $mg \cdot kg^{-1}$ propranolol (beta-adrenoceptor antagonist; Sigma), or both phentolamine and propranolol. In all cases, the bolus injection was followed immediately by infusion of saline for 1 h at a rate of 0.6 $ml \cdot h^{-1}$. In the control groups, saline infusion was continued at this rate for a further 12 h whereas in the experimental groups, fish were infused for 12 h with 2×10^{-5} M epinephrine (L-epinephrine bitartrate; Sigma). Actual concentration of epinephrine was not measured for this study, but was assumed to be the same as in chapter 2, due to the

identical protocol. Control injections of antagonists were carried out; no variations in blood acid-base or respiratory variables were observed, and the results of these injections are presented in chapter 6.

Intravascular infusions of selective or non-selective adrenergic agonists were performed in a similar manner as described above for epinephrine. Briefly, groups of fish were infused with Cortland saline for 1 h, followed by a 12 h period of infusion with either $2x10^{-5}$ M phenylephrine ($alpha_1$ agonist; L-phenylephrine hydrochloride; Sigma), $2x10^{-3}$ M clonidine ($alpha_2$ agonist; clonidine hydrochloride; Sigma) or $2x10^{-5}$ M isoprenaline (non-selective beta agonist; isoproterenol bitartrate; Sigma).

All drugs were dissolved in Cortland saline immediately before use and the resultant solutions were adjusted to pH 7.8-8.0 with 1 N NaOH or HCl. In order to impede the oxidation of epinephrine and isoprenaline during infusion, syringes and cannulae tubing were blackened to prevent light penetration.

Arterial blood samples were withdrawn after 1 h of saline infusion (pre-infusion sample) and after 1, 4, 8 and 12 h of either saline, epinephrine or agonist infusion. A final sample was taken 12 h following the conclusion of infusion (post-infusion period).

Blood samples were analysed immediately for Hct, pH_e, rbc pH, C_{O2}, C_{CO2} and PO_2. The remaining blood (approximately 100 ul) was stored at -20^{o} C for subsequent determination of hemoglobin levels. Approximately 0.6ml of saline was reinjected slowly into the dorsal aorta after each sample, to partially replace lost blood volume.

Analytical Procedure:

Analysis of samples for pH_e, rbc pH, $C_{CO2}^{\wedge}$, PO_2, Hct and C_{O2} was done immediately using the protocol outlined in chapters 2 and 6. Hemoglobin measurements were performed on duplicate samples using a commercially available hemoglobin assay kit (Sigma). Blood was hemolyzed and stored by freezing; 0.05 ml of blood was added to 1.0 ml of the reagent and measured spectrophotometrically versus the supplied standard.

Statistical Analysis

Statistical analysis of all data in Figures and Tables was performed as described in chapter 2.

RESULTS

Intravascular infusion of epinephrine, in the absence of adrenoceptor antagonism, caused a transient acidosis of arterial blood (Fig. 11). The acidosis was respiratory in origin, as indicated by a pronounced elevation of PCO_2 from 1.2 to 1.8 torr and an increase in C_{CO2} of 0.9 mM which is similar to the increase expected from non-bicarbonate buffering of CO_2 (1.1 mM). This predicted change in C_{CO2} was calculated using the experimentally determined buffer value (beta) of -9.81 ± 0.9 mmol·L^{-1} whole blood (chapter 2) according to the relationship $C_{CO2} = -9.81 \times pH$.

Although whole blood pH was transiently lowered by epinephrine, rbc pH actually increased significantly by as much as 0.2 pH units and remained elevated throughout the entire period of epinephrine infusion (Fig. 11). Rbc pH was no longer significantly elevated above the pre-infusion value at the post-infusion sampling time.

Serial blood sampling and the resultant anemia caused arterial C_{O2} to decrease during all treatments. For this reason, corrected values of C_{O2} are presented in the figures. The corrected C_{O2} values were obtained by adjusting the absolute C_{O2} according to the inverse relationship between C_{O2} and Hct that was established in saline-injected fish ($C_{O2} = 0.15 \cdot Hct + 2.40$; from chapter 6, Fig. 31). Due to the variability of the initial Hcts among treatment groups, all initial C_{O2}'s were standardized to a starting Hct of 25%, once again using the relationship $C_{O2} = 0.15 \cdot Hct + 2.40$.

The effects of epinephrine on the oxygen transporting properties of trout blood are illustrated in Fig. 9. Arterial oxygen content was

Figure 8: The effects of prolonged intravascular epinephrine infusion on selected blood acid-base variables in the rainbow trout in the absence of adrenergic blockade (epinephrine control; solid circles; N = 6) or following pre-treatment with the alpha-adrenergic antagonist, phentolamine (solid triangles; N = 6). Control fish (open circles; N = 6) were infused with saline throughout the experimental period. The pre-infusion (Pre) blood sample was withdrawn 1 h after saline or antagonist injection and the post-infusion (Post) blood sample was taken 12 h after terminating the infusion. Asterisks denote significant differences from the pre-infusion values. See text for further details.

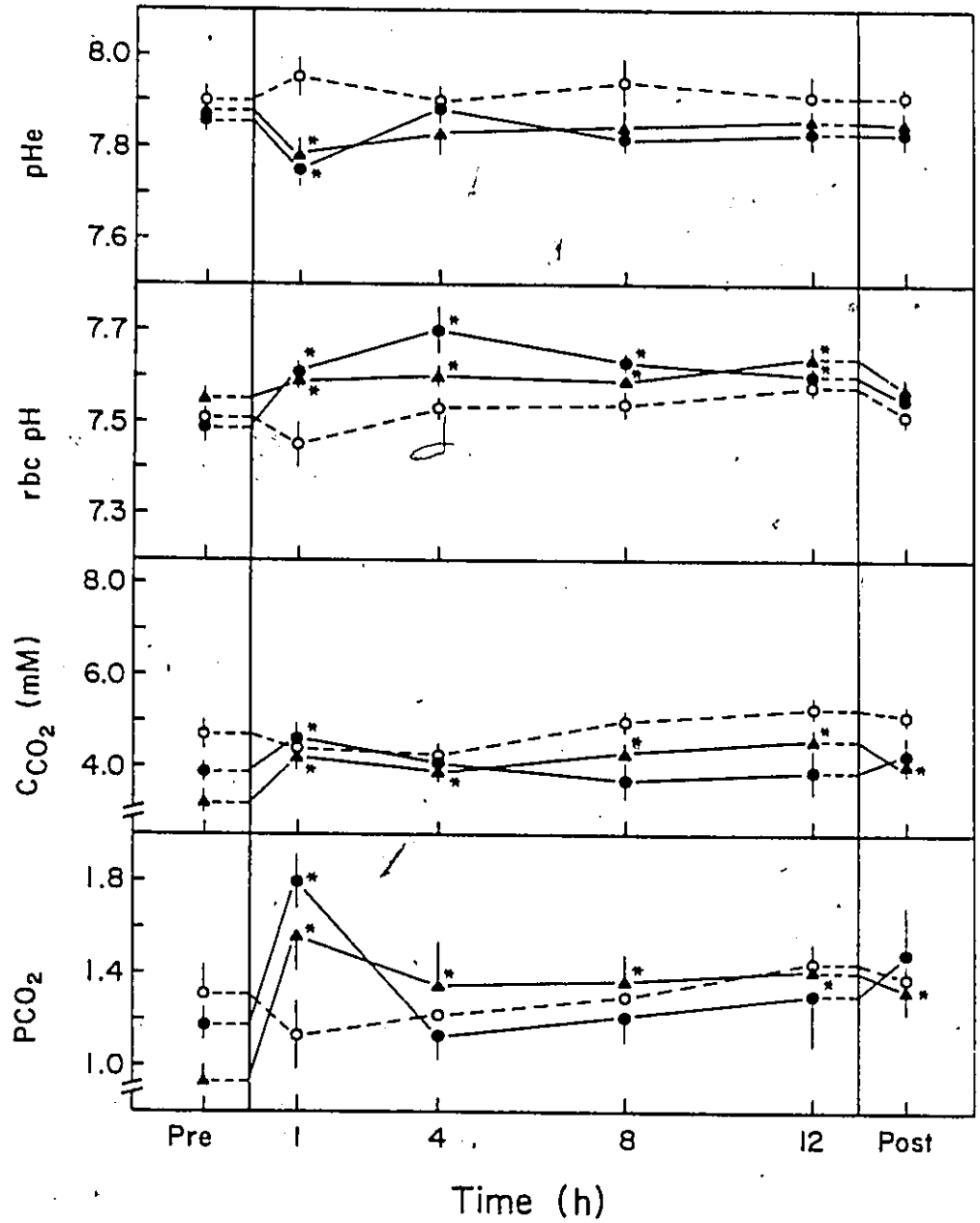
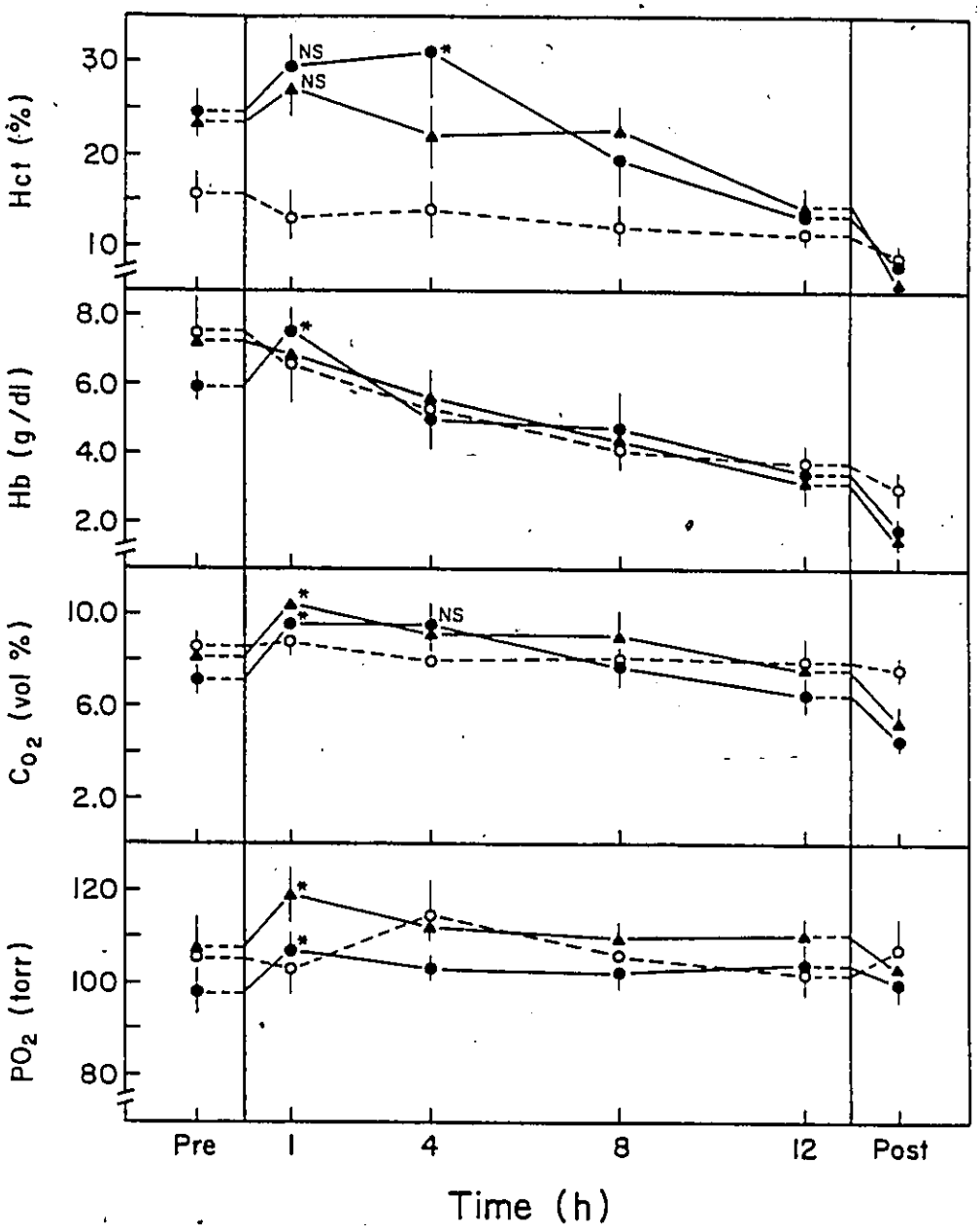

pHe
8.0
7.8
7.6
rbc pH
7.7
7.5
7.3
C_{CO_2} (mM)
8.0
6.0
4.0
PCO_2
1.8
1.4
1.0
Pre
1
4
8
12
Post
Time (h)

Figure 9: The effects of prolonged intravascular epinephrine infusion on selected blood respiratory variables in the rainbow trout in the absence of adrenergic blockade (epinephrine control; solid circles; N = 6) or following pre-treatment with the alpha-adrenergic antagonist, phentolamine (solid triangles; N = 6). Control fish (open circles; N = 6) were infused with saline throughout the experimental period. Asterisks denote significant differences from the pre-infusion values; NS = non-significant. All other details as in Fig. 8.


Hct (%)
30
20
10
NS
NS
*
Hb (g/dl)
8.0
6.0
4.0
2.0
*
C_{O_2} (vol %)
10.0
6.0
2.0
*
*
NS
PO_2 (torr)
120
100
80
*
*
Pre
1
4
8
12
Post
Time (h)

elevated transiently due to temporary elevations in blood oxygen carrying capacity and PO_2. The elevation in blood oxygen carrying capacity was reflected by increases in Hct and hemoglobin levels. In experimental groups (Figs. 9,11,13), there was a tendency for Hct and [Hb] to decline throughout the infusion period due to dilution of blood caused by serial sampling.

Pre-treatment of fish with the alpha-adrenergic antagonist, phentolamine, did not affect the immediate changes in blood acid-base status caused by epinephrine, although there were a few subtle differences in the latter stages of infusion (Fig. 8). Most notably, PCO_2 and C_{CO2} remained elevated above the pre-infusion values. However, the values of PCO_2 and C_{CO2} in the phentolamine-treated and untreated groups were not significantly different at any sampling period during epinephrine infusion (Fig. 9). Thus, it is possible that the PCO_2 and C_{CO2} values in the phentolamine-treated fish were abnormally low during the pre-infusion period, for unknown reasons. Pre-treatment with phentolamine abolished the increases in Hct and [Hb] that were characteristic of the epinephrine infusion (Fig. 9). Arterial oxygen content remained elevated, however, due to a pronounced rise in PO_2.

Beta-adrenergic blockade, with propranolol, caused no immediate effects on the changes in whole blood acid-base status during epinephrine infusion but did completely eliminate the marked alkalinization of the rbc (Fig. 10). Additionally, the increases in PCO_2 and C_{CO2} were sustained through 8 h of epinephrine infusion in contrast to the shorter duration (less than 4 h) of these changes in the

Figure 10: The effects of prolonged intravascular epinephrine infusion on selected blood acid-base variables in the rainbow trout in the absence of adrenergic blockade (epinephrine control; solid circles; N = 6) or following pre-treatment with the beta-adrenergic antagonist, propranolol (solid squares; N = 5). Control fish (open circles; N = 6) were infused with saline throughout the experimental period. Asterisks denote significant differences from the pre-infusion value; daggers denote significant differences from epinephrine control value at corresponding time; NS = non-significant. All other details as in Fig. 8.

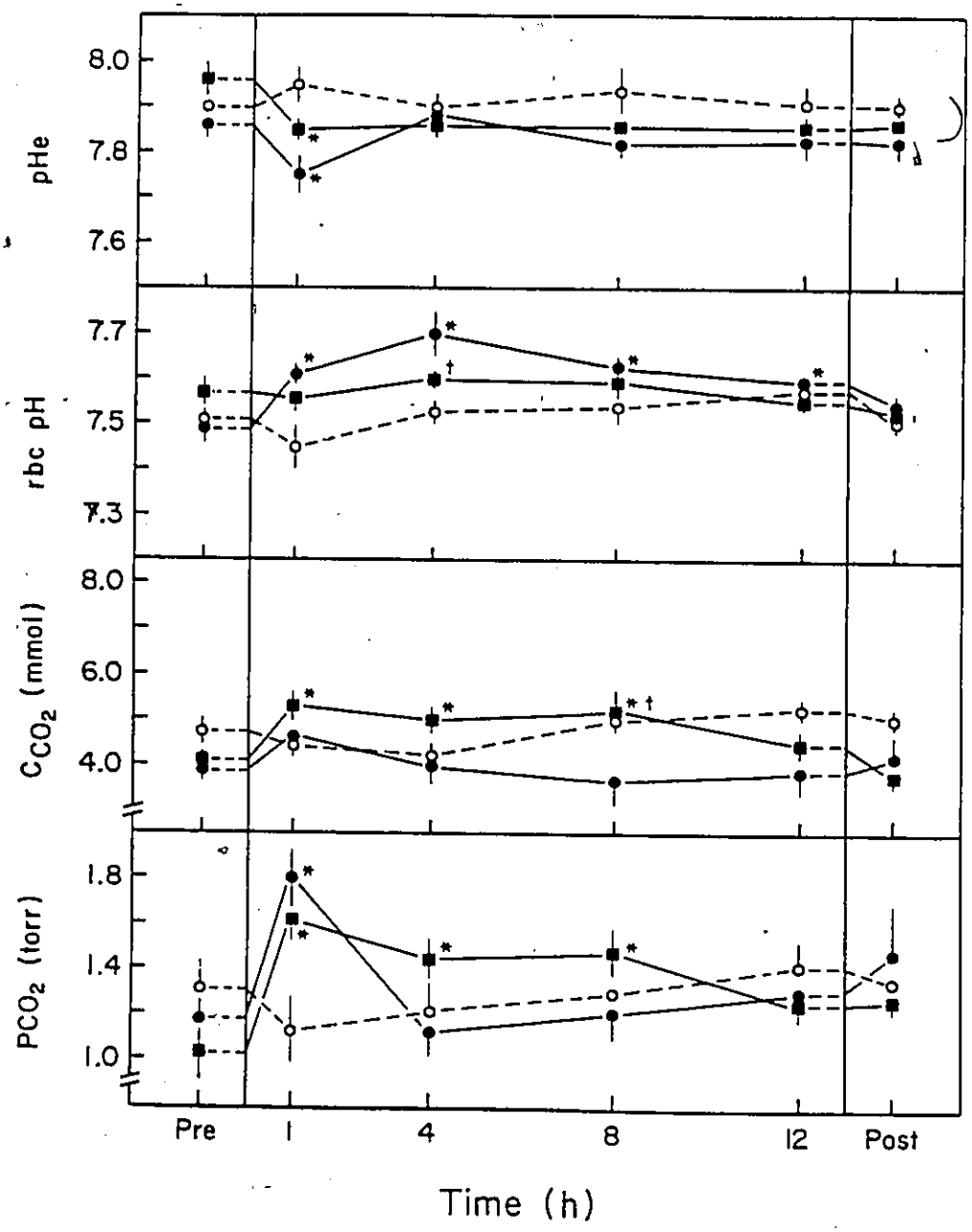

8.0
7.8
7.6
pHe
7.7
7.5
7.3
rbc pH
8.0
6.0
4.0
C_{CO_2} (mmol)
1.8
1.4
1.0
PCO_2 (torr)
Pre
1
4
8
12
Post
Time (h)

Figure 11: The effects of prolonged intravascular epinephrine infusion on selected blood respiratory variables in the rainbow trout in the absence of adrenergic blockade (epinephrine control;solid circles; N = 6) or following pre-treatment with the beta-adrenergic antagonist, propranolol (solid squares; N = 5). Control fish (open squares; N = 6) were infused with saline throughout the experimental period. Asterisks denote significant differences from the pre-infusion values; NS = non-significant. All other details as in Fig. 8.

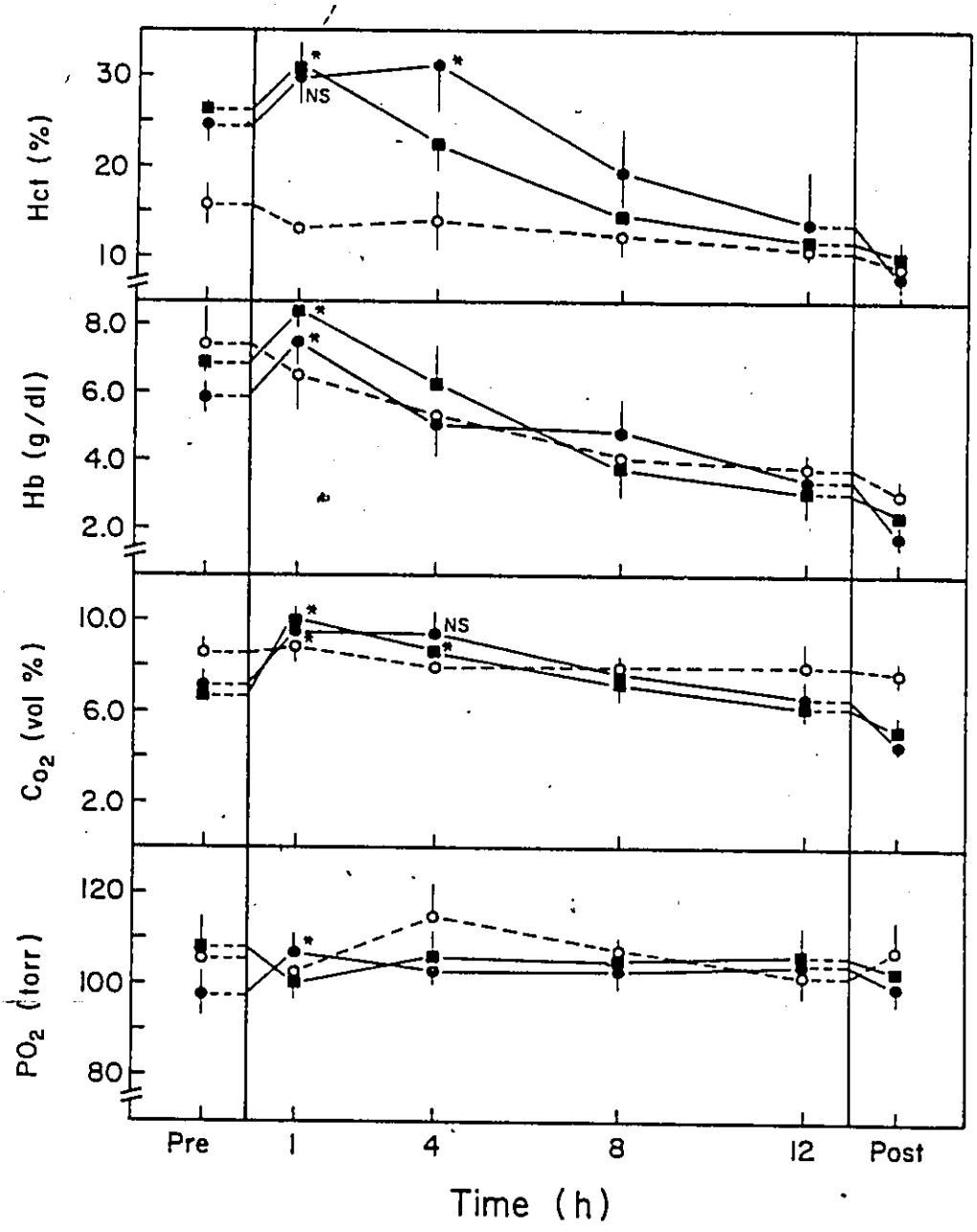
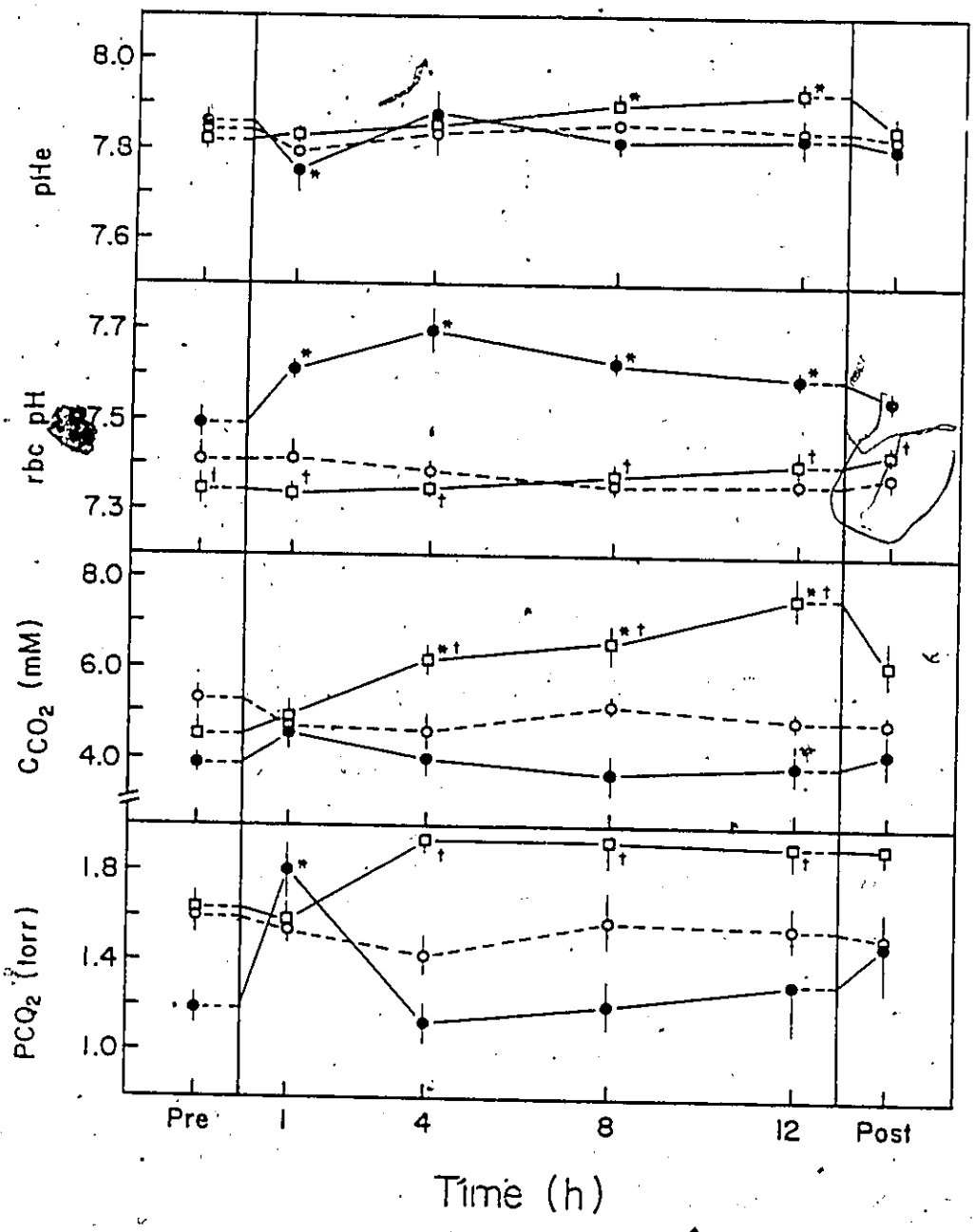

Hct (%)
30
20
10
Hb (g/dl)
8.0
6.0
4.0
2.0
C_{O_2} (vol %)
10.0
6.0
2.0
P_{O_2} (torr)
120
100
80
NS
NS
Pre
1
4
8
12
Post
Time (h)

Figure 12: The effects of prolonged intravascular epinephrine infusion on selected blood acid-base variables in the rainbow trout in the absence of adrenergic blockade (epinephrine control; solid circles; N = 6) or following complete adrenergic blockade using both alpha- and beta-adrenergic antagonists, phentolamine and propranolol (open squares; N = 6). Control fish (open circles; N = 6) were infused with saline throughout the experimental period after double antagonist injection. Asterisks denote significant differences from the pre-infusion values; daggers denote significant differences from epinephrine control values at corresponding time; NS = non-significant. All other details as in Fig. 8.


8.0
7.8
7.6
pHe
7.7
7.5
7.3
rbc pH
8.0
6.0
4.0
C_{CO_2} (mM)
1.8
1.4
1.0
PCO_2 (torr)
Pre
1
4
8
12
Post
Time (h)

absence of propranolol. The epinephrine-induced increases in Hct, [Hb] and C_{CO2} were unaffected by propranolol treatment, although the increase in PO_2 was prevented (Fig. 11).

Infusion of epinephrine after injection of both propranolol and phentolamine did not affect any measured blood acid-base or respiratory variable (Figs. 12,13) other than causing delayed elevations of PCO_2, C_{CO2} and PO_2 (non-significant).

Infusions of the alpha-adrenergic agonists, phenylephrine ($alpha_1$ agonist) or clonidine ($alpha_2$ agonist) caused significant depressions of whole blood pH (Table 4). Phenylephrine infusion was associated with a pronounced increase of rbc pH in contrast to the stable rbc pH measured during infusion of clonidine (Table 4). The reductions in pH_e caused by alpha-adrenergic stimulation were respiratory in origin, as indicated by the increases in PCO_2 (Table 4). The infusion of clonidine or phenylephrine caused a pronounced reduction in PO_2 during the initial 4 h of infusion although the decrease at 1 h in the phenylephrine-infused fish was not statistically significant due to the variability of the data (Table 4).

Infusion of the non-selective beta-adrenergic agonist, isoprenaline, caused variable and apparently time-dependent changes in blood acid-base and respiratory parameters (Tables 4,5). During the initial 4 h of infusion, pH_e was elevated and PCO_2 reduced, suggesting a condition of respiratory alkalosis. However, during the final 8 h of infusion, pH_e was decreased (not significantly) compared to the pre-infusion value and PCO_2 was elevated, indicating respiratory acidosis. Rbc pH was significantly elevated throughout the entire

Figure 13: The effects of prolonged intravascular epinephrine infusion on selected blood respiratory variables in the rainbow trout in the absence of adrenergic blockade (epinephrine control; solid circles; N = 6) or following complete adrenergic blockade using both alpha- and beta-adrenergic antagonists, phentolamine and propranolol (open squares; N = 6). Control fish (open circles; N = 6) were infused with saline throughout the experimental period after double antagonist injection. Asterisks denote significant differences from the pre-infusion values; daggers denote significant differences from epinephrine control values at corresponding time; NS = non-significant. All other details as in Fig. 8.

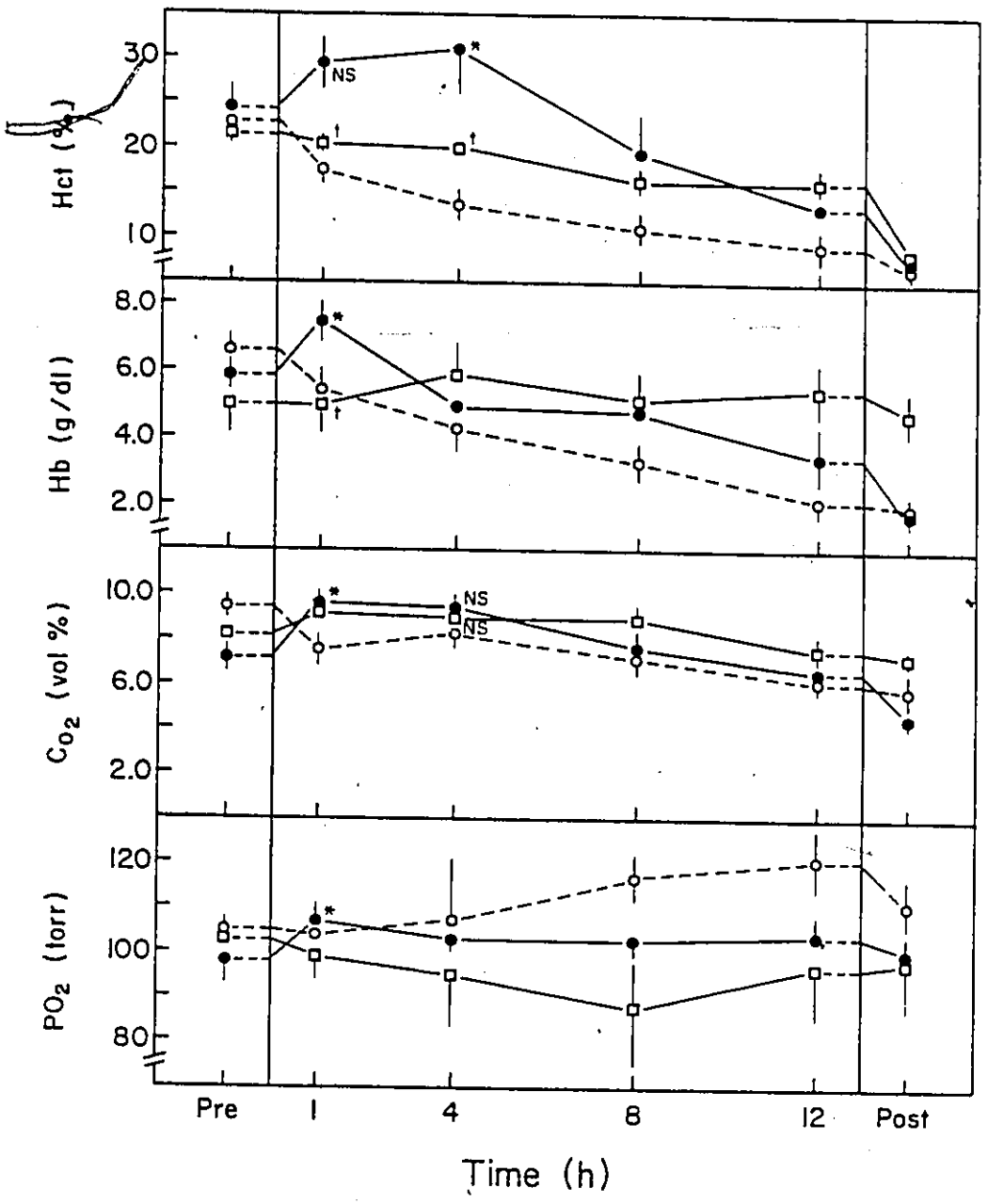

Hct (%)
30
20
10
NS
*
†
Hb (g/dl)
8.0
6.0
4.0
2.0
C_{O_2} (vol %)
10.0
6.0
2.0
NS
NS
PO_2 (torr)
120
100
80
Pre
1
4
8
12
Post
Time (h)

Table 4. Whole blood pH (pH_e) and red blood cell pH (rbc pH) before and during intra-arterial infusion (0.5 $ml \cdot h^{-1}$) of saline, phenylephrine (2×10^{-5} M), isoprenaline (2×10^{-5} M) or clonidine (2×10^{-3} M). N numbers are given in parentheses; means±SEM; * denotes significant difference from the pre-infusion value.

Time (h)	Saline (Control)		Phenylephrine		Isoprenaline		Clonidine	
	pH_e	rbc pH	pH_e	rbc pH	pH_e	rbc pH	pH_e	rbc pH
Pre-infusion	7.90±0.03(6)	7.51±0.02(6)	7.88±0.02(5)	7.48±0.02(5)	7.97±0.05(6)	7.45±0.03(6)	7.96±0.03(6)	7.52±0.02(6)
1	7.95±0.04(6)	7.45±0.05(6)	7.85±0.01(5)	7.58±0.02(5)*	8.05±0.06(6)	7.64±0.03(6)*	7.83±0.04(6)*	7.52±0.02(6)
4	7.89±0.04(6)	7.53±0.02(6)	7.80±0.05(5)	7.61±0.02(5)*	8.09±0.07(6)*	7.72±0.02(6)*	7.78±0.03(6)*	7.50±0.02(6)
8	7.94±0.05(6)	7.54±0.03(6)	7.76±0.04(5)*	7.61±0.02(5)*	7.94±0.07(6)	7.71±0.05(6)*	-	-
12	7.91±0.04(6)	7.58±0.03(6)	7.81±0.01(5)*	7.64±0.01(5)*	7.90±0.06(6)	7.64±0.02(6)*	-	-

Table 5. Blood PCO_2 and PO_2 before and during intra-arterial infusion (0.6 ml·h^{-1}) of saline, phenylephrine (2×10^{-5} M), isoprenaline (2×10^{-5} M) or clonidine (2×10^{-3} M). N numbers are given in parentheses: means ± SEM. * denotes significant difference from the pre-infusion value.

Time (h)	Saline (Control)		Phenylephrine		Isoprenaline		Clonidine	
	PCO_2 (torr)	PO_2 (torr)	PCO_2 (torr)	PO_2 (torr)	PCO_2 (torr)	PO_2 (torr)	PCO_2 (torr)	PO_2 (torr)
Pre-infusion	1.31±0.13(6)	106.3±5.6(6)	1.81±0.24(5)	105.3±7.3(5)	1.11±0.21(6)	94.3±9.1(6)	1.06±0.10(6)	98.5±10.4(6)
1	1.13±0.15(6)	103.3±6.2(6)	2.09±0.27(5)	86.8±11.3(5)	0.84±0.13(6)*	86.3±5.2(6)	1.58±0.14(6)*	73.5±5.9(6)*
4	1.22±0.12(6)	115.2±7.5(6)	2.50±0.39(5)*	94.3±4.8(5)*	1.01±0.21(6)	110.7±6.9(6)*	2.09±0.11(6)*	85.3±8.5(6)*
8	1.30±0.15(6)	106.5±2.7(6)	2.40±0.32(5)*	112.5±2.9(5)	1.43±0.30(6)*	104.3±4.3(6)*	-	-
12	1.42±0.11(6)	102.7±5.5(6)	2.12±0.20(5)	108.9±3.3(5)	1.49±0.27(6)*	123.0±5.7(6)*	-	-

period of isoprenaline infusion (Table 4).

DISCUSSION.

Methodology

In the present study, a procedure of prolonged intravascular infusion of epinephrine has been utilized as in chapters 2, 4 and 5, rather than the more conventional procedure of single bolus injections (e.g. Peyraud-Waitzenegger, 1979; Peyraud-Waitzenegger et al., 1980; Nikinmaa, 1982a,b; Epple and Nibbio, 1985; Epple and Kahn, 1985) in order to simulate elevated plasma epinephrine levels associated with stress, in fishes. Clearly, there are advantages and disadvantages with both methods of epinephrine administration (i.e. single injection versus continuous infusion) yet it is believed that continuous infusion is the preferred method for evaluating the long-term physiological effects of elevated epinephrine levels and may simulate more closely the extended elevation of plasma [epinephrine] accompanying many stresses (up to 12 h in duration; e.g. Perry et al., 1986a). Recently, Nekvasil and Olson (1986a) have estimated that the residence time of biologically active epinephrine following a bolus arterial injection in rainbow trout is approximately 10 min due to the combined actions of plasma clearance and metabolic inactivation (see also Nekvasil and Olson, 1986b). Thus, it is not surprising that Nikinmaa (1982b) reported that the stimulatory effect of epinephrine on arterial Hct was dissipating within only 10 min of a single injection.

Control of Arterial PO_2

The results of this study suggest a similar adrenergic involvement in the control of ventilation and arterial PO_2 in the rainbow trout as has been shown previously in the eel (Anguilla anguilla)

(Peyraud-Waitzenegger, 1979; Peyraud-Waitzenegger et al., 1980). It must be emphasized, however, that gill ventilation was not actually monitored in our study but that adjustments in ventilation volume have been inferred from changes in arterial gas tensions. The elevation of PO_2 by epinephrine or isoprenaline infusion, the abolishment of the epinephrine-induced increase by pre-treatment with propranolol but not phentolamine, and the lowering of PO_2 by phenylephrine or clonidine infusion, are consistent with beta-adrenergic hyperventilation and alpha-adrenergic hypoventilation as in the eel (Peyraud-Waitzenegger, 1979; Peyraud-Waitzenegger et al., 1980).

Additionally, adrenergic adjustments of PO_2 may have been elicited by alterations of gill O_2 diffusive conductance caused by lamellar recruitment (Booth, 1979; Holbert, Boland and Olson, 1979) and/or changes in gill epithelial permeability to oxygen (Haywood et al., 1977). Indeed, epinephrine has been shown to raise arterial PO_2 in the absence of ventilatory adjustments both in vivo (in curarized eels; Peyraud-Waitzenegger, 1979) and in perfused head preparations (Pettersson, 1983; Perry et al., 1985) thereby indicating the importance of changes in O_2 diffusive conductance in the control of arterial PO_2. It is difficult to assess the roles of adrenoceptors in controlling gill epithelial permeability to oxygen because of accompanying hemodynamic effects (Payan and Girard, 1977; Farrel, Daxboeck and Randall, 1979; Pettersson, 1983; Nilsson, 1984). Nonetheless, a model has been formulated (see Isaia, 1984) in which stimulation of beta-adrenoceptors increases gill permeability to small lipophilic molecules by increasing the fluidity of the lipid plasmalemma.

Epinephrine is known to increase cardiac output in teleosts by beta-adrenoceptor mediated positive inotropic and chronotropic effects (see review by Nilsson, 1983) and hence will reduce the transit time of blood flowing within lamellae. A reduction in blood transit time within the gill may tend to reduce arterial PO_2 as has been demonstrated in perfused gill preparations (Perry et al., 1985). Wood and Perry (1985) have proposed that increases in ventilatory and diffusive conductances induced by epinephrine during such periods of elevated cardiac output serve to offset gill transit time limitations and thereby maintain or actually increase arterial PO_2, as in the present study.

Although unable to distinguish between the ventilatory, cardiovascular, hemodynamic and permeability effects of epinephrine on PO_2, we have demonstrated nevertheless that the elevation of epinephrine to levels similar to those measured during periods of stress can increase arterial PO_2 and thereby raise arterial oxygen content. Because the experiments were performed during summer months (July - September, 1986), we presume that beta-receptor mediated responses predominated (Peyraud-Waitzenegger, et al., 1980) and were solely responsible for promoting the rise in PCO_2 during epinephrine infusion. The results presented in this study contradict those of the preceding chapter in which blood PO_2 decreased with epinephrine infusion. I am not able to explain this discrepancy. Similarly, it is worth noting that Nikinmaa (1982b) reported no change in PO_2 following intra-arterial injection of epinephrine in rainbow trout. It is unclear whether the discrepancy between these studies is related to seasonal differences because Nikinmaa (1982b) did not report the time of year his

experiments were performed. During winter months, we have observed pronounced reductions in arterial PO_2 after epinephrine injection (unpublished observations) presumably due to the predominance of alpha-adrenoceptor-mediated hypoventilation (Peyraud-Waitzenegger et al., 1980).

At least two additional adrenergic mechanisms contributed to the increase in arterial oxygen content during epinephrine infusion: elevation of blood oxygen carrying capacity and an increase in hemoglobin oxygen affinity.

Control of oxygen carrying capacity

The elevation of blood oxygen carrying capacity, as reflected by increased hemoglobin levels, was mediated solely by alpha-adrenoceptors. It is speculated that the principal reponse was contraction of the spleen and concomitant release of rbcs into the circulation. Previous studies on a variety of species have shown that spleen contraction, in fish, is controlled by alpha-adrenoceptors (see Nilsson, 1984). Other factors that may have contributed to the elevation of blood oxygen carrying capacity are fluid shifts between vascular and interstitial compartments (Yamamoto et al., 1980), diuresis (chapter 5) and "plasma skimming" at the gill (Nikinmaa, 1982a). Indeed, Nikinmaa (1982a) concluded that "plasma skimming" at efferent arterio-venous anastomoses was the major cause of the increased arterial [Hb] after an injection of epinephrine because venous [Hb] did not change. It is unlikely, however, that "plasma skimming" contributed to the alpha-adrenergic increase in [Hb] reported in the present study because stimulation of alpha-adrenoceptors is thought to cause

constriction of efferent arterio-venous anastomoses (Payan and Girard, 1977; see also reviews by Nilsson, 1983, 1984). Such a response presumably would decrease the shunting of low Hct blood into central filamental sinuses and promote a decrease in dorsal aortic [Hb] and Hct rather than the converse, as we have shown. Furthermore, Olson (1984) demonstrated no consistent effects of epinephrine on "plasma skimming" in blood-perfused gills of three teleosts.

Control of HbO_2 affinity

The pronounced adrenergic increase in rbc pH was mediated entirely by beta-adrenoceptors that are known to be associated with piscine rbc membranes (Bennett and Rankin, 1985). This observation, at physiological epinephrine levels, supports the numerous reports of beta-adrenoceptor mediated rbc alkalinization during catecholamine administration in vitro (Nikinmaa, 1982b; Nikinmaa and Huestis, 1984; Cossins and Richardson, 1985; chapter 2) or in vivo (chapter 2) and during or following stress (Nikinmaa, et al., 1984; Primmett et al., 1986; Perry et al., 1986a; chapter 6). The various cellular mechanisms promoting the adrenergic increase in rbc pH are reviewed by Nikinmaa (1986) and clearly the most important effect is the stimulation of rbc transmembrane Na^+/H^+ exchange (Baroin et al., 1984). The stimulation of this Na^+/H^+ exchange, by epinephrine, not only raises rbc pH but also promotes swelling (see Nikinmaa, 1986). Both of these events contribute to the elevation of arterial oxygen content by increasing HbO_2 affinity via the Bohr effect (leftward shift of the oxygen dissociation curve due to increased pH) and decreased cellular NTP concentrations (due to swelling).

A curious result in the present study was the pronounced elevation of rbc pH caused by the infusion of phenylephrine (Table 4). It is unlikely that this was a direct consequence of rbc alpha-adrenoceptor stimulation because clonidine or epinephrine in the presence of propranolol did not affect rbc pH. A possible explanation is that phenylephrine (an $alpha_1$ agonist) infusion initiated the release of epinephrine from chromaffin tissue whereas clonidine (an $alpha_2$ agonist) infusion did not ($alpha_1$ catecholaminotropic response).

Control of arterial PCO_2

The retention of carbon dioxide and associated extracellular acidosis caused by epinephrine infusion confirm the results of chapter 2 that demonstrated a similar, although more persistent, respiratory acidosis during epinephrine treatment. There are two possible hypotheses for the retention of carbon dioxide during epinephrine infusion: i) hypoventilation and/or decreased gill CO_2 conductance, and ii) inhibition of erythrocytic bicarbonate dehydration. Reductions in ventilation and/or gill diffusive conductance should produce reciprocal changes in PCO_2 and PO_2 (see Discussion, chapter 2). It is clear, however, that the infusion of epinephrine in the absence of adrenergic blockade did not cause reciprocal changes in PCO_2 and PO_2 and that CO_2 retention was occurring during conditions of optimized branchial gas transfer as indicated by increased PO_2. Moreover, alpha-adrenergic antagonism did not prevent the epinephrine-induced rise in PCO_2. Thus, the retention of carbon dioxide caused by epinephrine surely was not due to hypoventilation and/or decreased gill CO_2 diffusive conductance but presumably due to beta-adrenergic

inhibition of rbc bicarbonate dehydration. Possible mechanisms promoting the putative decrease in bicarbonate dehydration are inhibition of rbc Cl^-/HCO_3^- exchange (the chloride shift) which is thought to be the rate-limiting step in piscine CO_2 excretion (Perry et al., 1982; see also review by Perry, 1986) or inhibition of erythrocytic carbonic anhydrase. Although definitive data are lacking, I believe that inhibition of Cl^-/HCO_3^- exchange is more likely and is supported by in vitro data on rainbow trout blood demonstrating an increase in the Donnan Cl^- ratio (rbc $[Cl^-]$ / plasma $[Cl^-]$) and a concomitant increase in plasma $[HCO_3^-]$ after the addition of epinephrine (T.A. Heming, D.J. Randall and M.M. Mazeaud, in preparation).

Regardless of the precise mechanism(s) involved, the beta-adrenergic retention of CO_2 reported here may explain the pronounced elevation of arterial PCO_2 that has been observed consistently following exhaustive exercise in numerous fish species (see review by Wood and Perry, 1985). Indeed, the state of the fish following exhaustive exercise is essentially analogous to the condition of the animal during epinephrine infusion because circulating epinephrine levels are elevated (see Table 1) and branchial ventilatory and diffusive conductances are enhanced (Wood and Perry, 1985). Hence, the increase in PCO_2 after exhaustive exercise is likely due to beta-adrenergic inhibition of rbc HCO_3^- dehydration.

The simulation of physiologically relevant levels of circulating epinephrine promoted a series of adrenergic responses consistent with enhancement of branchial O_2 transfer and blood oxygen transport

capacity. These responses include alkalinization of the rbc (beta-adrenergic effect) thereby promoting an increase in HbO_2 affinity, increased blood oxygen carrying capacity (alpha-adrenergic effect) presumably due to spleen contraction, and an elevation of arterial PO_2 (beta-adrenergic effect) owing to gill ventilatory and/or diffusive conductance adjustments. Thus, it is considered that the release of epinephrine into the circulation during periods of stress is an important adaptive response in the maintenance of arterial oxygen content.

CHAPTER 4

THE EFFECTS OF PROLONGED EPINEPHRINE INFUSION ON THE PHYSIOLOGY OF THE RAINBOW TROUT, SALMO GAIRDNERI

II. BRANCHIAL SOLUTE FLUXES.

INTRODUCTION

In freshwater fishes, internal ionic regulation is dependent upon the active branchial uptake of Na^+ and Cl^- ions from the external environment. The branchial ionic uptake mechanisms involved have been studied in great detail (see reviews by Maetz, 1971; Evans, 1982, 1984; Payan, Girard and Mayer-Gostan, 1984) and there is strong evidence that Na^+ uptake is linked to the excretion of acidic equivalents (H^+ and/or NH_4^+) while Cl^- uptake is coupled to the excretion of basic equivalents (HCO_3^- and/or OH^-). For these reasons, ionic regulation and acid-base regulation are intimately related. Few studies have addressed the problem of how such adjustments of branchial ionic fluxes are achieved. In this study, the hypothesis that epinephrine is involved in the control of gill ionic exchange mechanisms for the purposes of acid-base regulation is studied. This hypothesis is based upon observations of elevated plasma epinephrine levels during extracellular acidosis (see Table 1) as well as the results of gill perfusion experiments that indicate adrenergic control of branchial ionic uptake (Payan, Matty and Maetz, 1975; Payan, 1978; Perry et al., 1984a). As in the chapter 2, experiments were performed in vivo during continuous infusion of epinephrine to elevate its level to that measured during periods of extracellular acidosis.

MATERIALS AND METHODS

Experimental conditions and the treatment of animals prior to and during experimentation were as outlined in chapter 2. Branchial solute fluxes were determined on fish fitted with indwelling dorsal aortic cannulae and urinary catheters. Continuous urine collection permitted separation of branchial and renal fluxes. Skin and gut contributions were considered to be negligible.

Protocol

Fish were subjected to the infusion regimen described in Materials and Methods for chapter 2, in order to achieve an estimated blood epinephrine concentration of 5×10^{-8} M. Actual epinephrine levels were determined by high pressure liquid chromatography, as described in chapter 2.

Ammonia accumulation, water acid-base disturbances and isotopic backflux problems were minimized by determining solute fluxes over 3 h intervals only. Additionally, the boxes were flushed for at least 10 min (0.7-0.8 l $H_2O \cdot min.^{-1}$) between successive flux periods.

Branchial unidirectional Na^+ and Cl^- fluxes were determined on separate groups of fish by monitoring the disappearance of ^{22}Na (as NaCl; Amersham) or ^{36}Cl (as HCl; ICN). Approximately 1-1.25 uCi of isotope (the amount added was increased gradually at each flux period in an attempt to maintain a constant ratio of external to internal specific activities) was added to each box and allowed to mix for 15 min. An initial 10 ml water sample was removed following the mixing period and another after 3 h. Activity of ^{22}Na or ^{36}Cl was determined immediately on 5.0 ml samples while the remaining sample was frozen for subsequent

ionic analysis (Na^+, Cl^-, K^+ and ammonia). Eight time periods were identified as being most interesting (3-6 h saline; 0-3 h, 3-6 h, 9-12 h and 21-24 h epinephrine; 0-3 h, 3-6 h and 9-12 h final saline) and were selected for flux determinations. Otherwise, fish were supplied with continuous water flow.

Branchial net acid fluxes were determined on a separate group of infused fish (n = 5) from measurements of titratable alkalinity and ammonia concentrations (see below) on initial and final water samples.

Sample analysis and calculations

^{22}Na and ^{36}Cl activities were determined on 5 ml water samples by liquid scintillation counting (LKB 1215 Rackbeta). Water concentrations of Na^+ and K^+ were determined by flame photometry (EEL Flame Photometer); concentrations of Cl^- were measured by amperometric titration (Buchler-Cotlove Chloridometer). Total ammonia concentrations were determined using the method of Verdouw, vanEchteld and Dekkers (1978). Net fluxes (J_{net}) for these electrolytes and unidirectionnal fluxes (i.e. J_{in}, J_{out}) for Na^+ and Cl^- were determined according to Maetz (1956). Accordingly, influxes (J_{in}) and net gains by the animal have positive signs while effluxes (J_{out}) and net losses have negative signs.

Titratable alkalinity was determined on 10 ml water samples by titrating to pH 4.00 with 0.02 M HCl as described by McDonald and Wood (1981). The net branchial acid flux ($J_{net}H^+$) was calculated as the sum of the titratable acidity flux (J_{TA}; i.e. negative titratable alkalinity) and ammonia flux (J_{amm}).

RESULTS

Occasionally, significant differences were noted between the control and experimental groups during the initial period of saline infusion (Figs. 14b, 15a and 16a). As a consequence, the results have been statistically analyzed in 2 ways: 1) by using a paired approach in which all values were compared to the initial saline infusion values, and 2) by comparing control and experimental values at identical time periods using a non-paired design. Saline infusion (control group) had no effect on any measured variable.

Infusion of epinephrine caused a reduction in the branchial net fluxes of Na^+ and Cl^- (i.e. becoming more negative) indicating an increase in net NaCl loss from the animal into the external environment. However, there were significant differences between the pattern of $J_{net}Na^+$ and $J_{net}Cl^-$ adjustment during epinephrine infusion. Most importantly, the changes in $J_{net}Cl^-$ were more rapid and of a greater magnitude than the changes in $J_{net}Na^+$. The reductions in $J_{net}Na^+$ and $J_{net}Cl^-$ both tended to persist upon return to saline infusion (Fig. 14a,b). Branchial $J_{net}K^+$ was slightly negative in both groups and was unaffected by epinephrine infusion (Fig. 14c).

The reduction in $J_{net}Na^+$ was due solely to an inhibition of sodium influx ($J_{in}Na^+$, Fig. 15a) whereas the larger reduction in $J_{net}Cl^-$ was a result of Cl^- influx ($J_{in}Cl^-$) inhibition (Fig. 15b) combined with increased Cl^- efflux ($J_{out}Cl^-$, Fig. 16b). Changes in $J_{in}Na^+$ and $J_{in}Cl^-$ were most pronounced following 9-12 h of epinephrine infusion. $J_{in}Cl^-$ had been restored to pre-epinephrine levels following 21-24 h while $J_{in}Na^+$ remained slightly depressed (Fig. 15). Both $J_{in}Na^+$ and $J_{in}Cl^-$

Figure 14. The effects of prolonged intra-arterial epinephrine infusion on branchial net fluxes of A) sodium, B) chloride and C) potassium. Control fish (open bars) were infused with saline continuously for 42 h while the epinephrine-treated fish (hatched bars) were infused initially with saline for 7 h (pre-epi) followed by 24 h of epinephrine infusion and finally 12 h of saline infusion (post-epi). Asterisks denote significant differences from pre-epinephrine values; daggers denote significant differences from corresponding values in control group. n = 14 except where otherwise indicated.

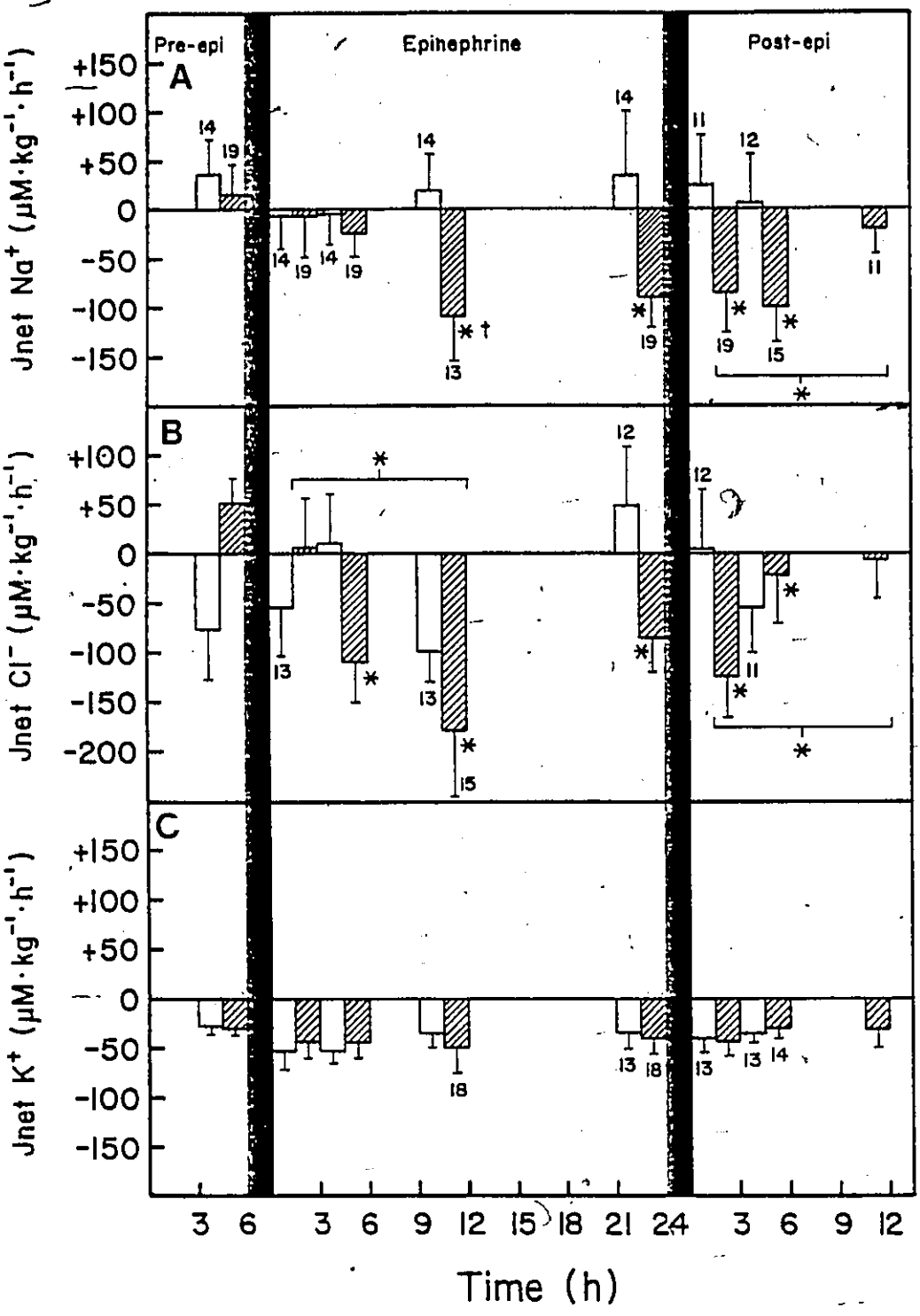

Pre-epi
Epinephrine
Post-epi
A
B
C
Jnet Na+ (μM·kg-1·h-1)
Jnet Cl- (μM·kg-1·h-1)
Jnet K+ (μM·kg-1·h-1)
+150
+100
+50
0
-50
-100
-150
-200
3 6 3 6 9 12 15 18 21 24 3 6 9 12
Time (h)

Figure 15. The effects of prolonged intra-arterial epinephrine infusion (hatched bars) on branchial influxes of A) sodium and B) chloride. Asterisk's denote significant differences from pre-epinephrine values; daggers denote significant differences from corresponding values in the control group (open bars). n numbers as indicated. All other details are as in Fig. 14.

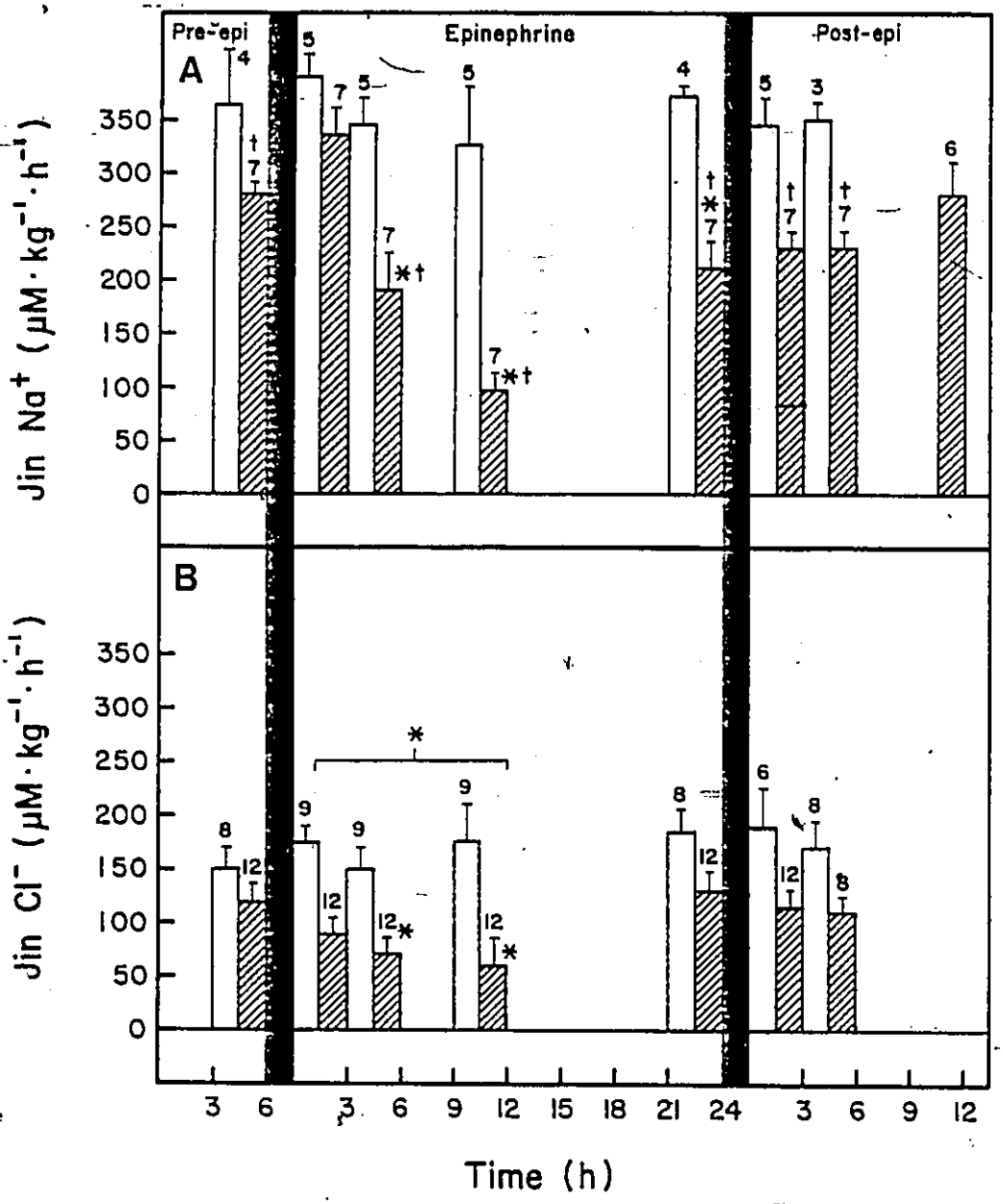
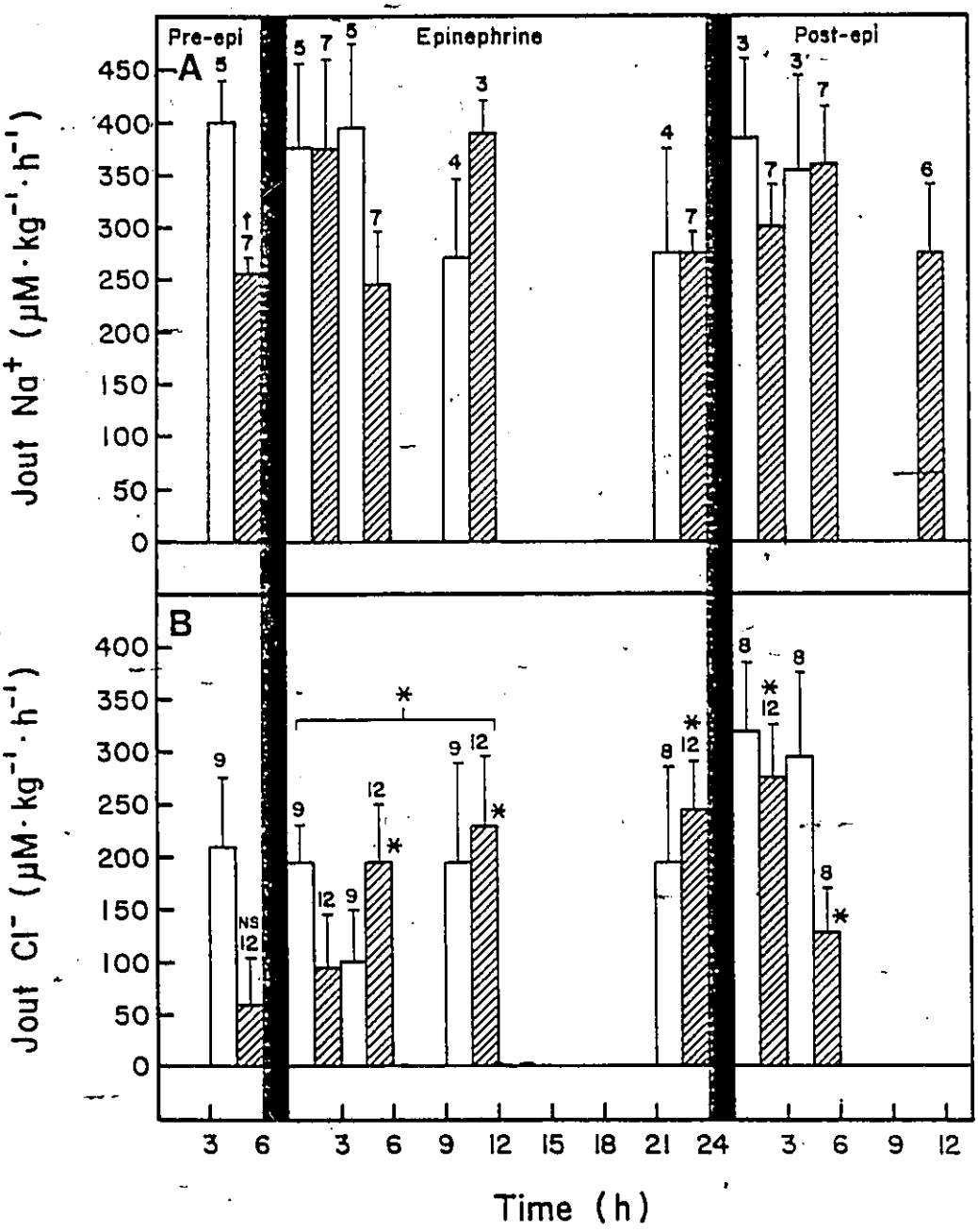

Pre-epi
Epinephrine
Post-epi
A
B
Jin Na+ (μM·kg-1·h-1)
Jin Cl- (μM·kg-1·h-1)
Time (h)

Figure 16. The effects of prolonged intra-arterial epinephrine infusion (hatched bars) on branchial effluxes of A) sodium and B) chloride. Asterisks denote significant differences from pre-epinephrine values; daggers denote significant differences from corresponding value in the control group (open bars). n numbers as indicated. All other details are as in Fig. 14.


Pre-epi
Epinephrine
Post-epi
A
B
Jout Na+ (μM·kg-1·h-1)
Jout Cl- (μM·kg-1·h-1)
NS
Time (h)

were not significantly different from pre-epinephrine values during the final saline infusion period. $J_{out}Cl^-$ remained elevated until 3-6 h of the final saline infusion (Fig. 16).

As a consequence of the differential effects of epinephrine on the branchial unidirectional fluxes of Na^+ and Cl^- (see above), a physiologically significant pattern emerged with respect to the difference between $J_{net}Na^+$ and $J_{net}Cl^-$ ($J_{net}Na^+ - J_{net}Cl^-$; Fig. 17). $J_{net}Na^+ - J_{net}Cl^-$ was elevated rapidly during epinephrine infusion, peaked between 3 and 12 h of infusion and was returning toward pre-epinephrine levels by 21-24 h (Fig. 17). $J_{net}Na^+ - J_{net}Cl^-$ was unaffected by further saline infusion. Based on empirical evidence (Wood, Wheatly and Hobe, 1984), the magnitude of $J_{net}Na^+ - J_{net}Cl^-$ is the prime determinant of branchial net acid excretion ($J_{net}H^+$). Indeed, in the present study, a positive relationship was observed between $J_{net}Na^+ - J_{net}Cl^-$ and net acid excretion (Fig. 18). Although the relationship was somewhat obscured by a high degree of variability, it is clear that branchial extrusion of acidic equivalents was greatest (i.e. $J_{net}H^+$ becoming less positive) when $J_{net}Na^+ - J_{net}Cl^-$ also was elevated, i.e. during the first 12 h of epinephrine infusion (Fig. 18). Two variables contribute to the calculation of $J_{net}H^+$: these are titratable alkalinity flux (J_{TA}) and ammonia flux (J_{amm}). In the present investigation, the elevation of net acid extrusion (Fig. 19c) was due entirely to a reduction in J_{TA} (Fig. 19a). The minor changes in J_{TA} during the final saline infusion were offset by similar non-significant changes in J_{amm} such that $J_{net}H^+$ remained constant.

Figure 17. The effects of prolonged intra-arterial epinephrine infusion on the arithmetic difference between sodium and chloride net flux ($J_{net}Na^{+} - J_{net}Cl^{-}$). Asterisks denote significant differences from pre-epinephrine values. n numbers as indicated.

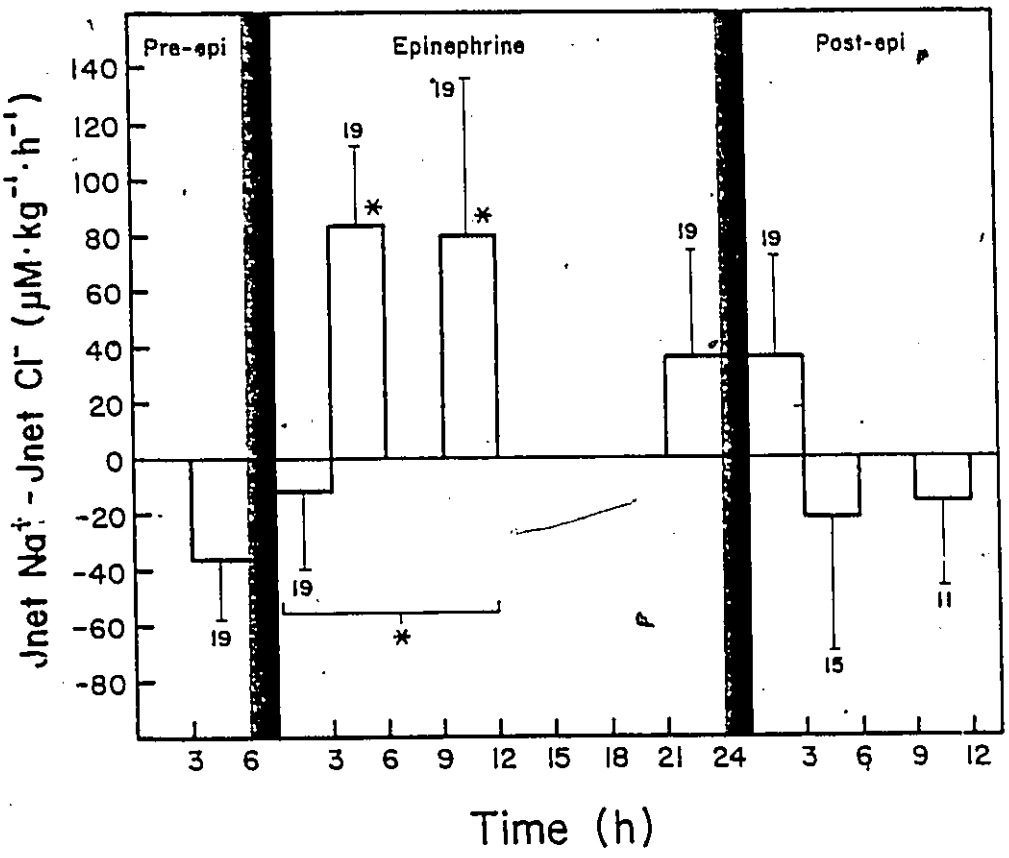

Pre-epi
Epinephrine
Post-epi
Jnet Na+ - Jnet Cl- (μM·kg-1·h-1)
140
120
100
80
60
40
20
0
-20
-40
-60
-80
19
19
19
19
19
19
19
15
11
*
*
*
3
6
3
6
9
12
15
18
21
24
3
6
9
12
Time (h)

Figure 18. The relationship between $J_{net}Na^+ - J_{net}Cl^-$ and branchial net acid excretion in the trout, Salmo gairdneri. The data were collected during prolonged intra-arterial infusion of saline or epinephrine; n = 6. See text for further details.

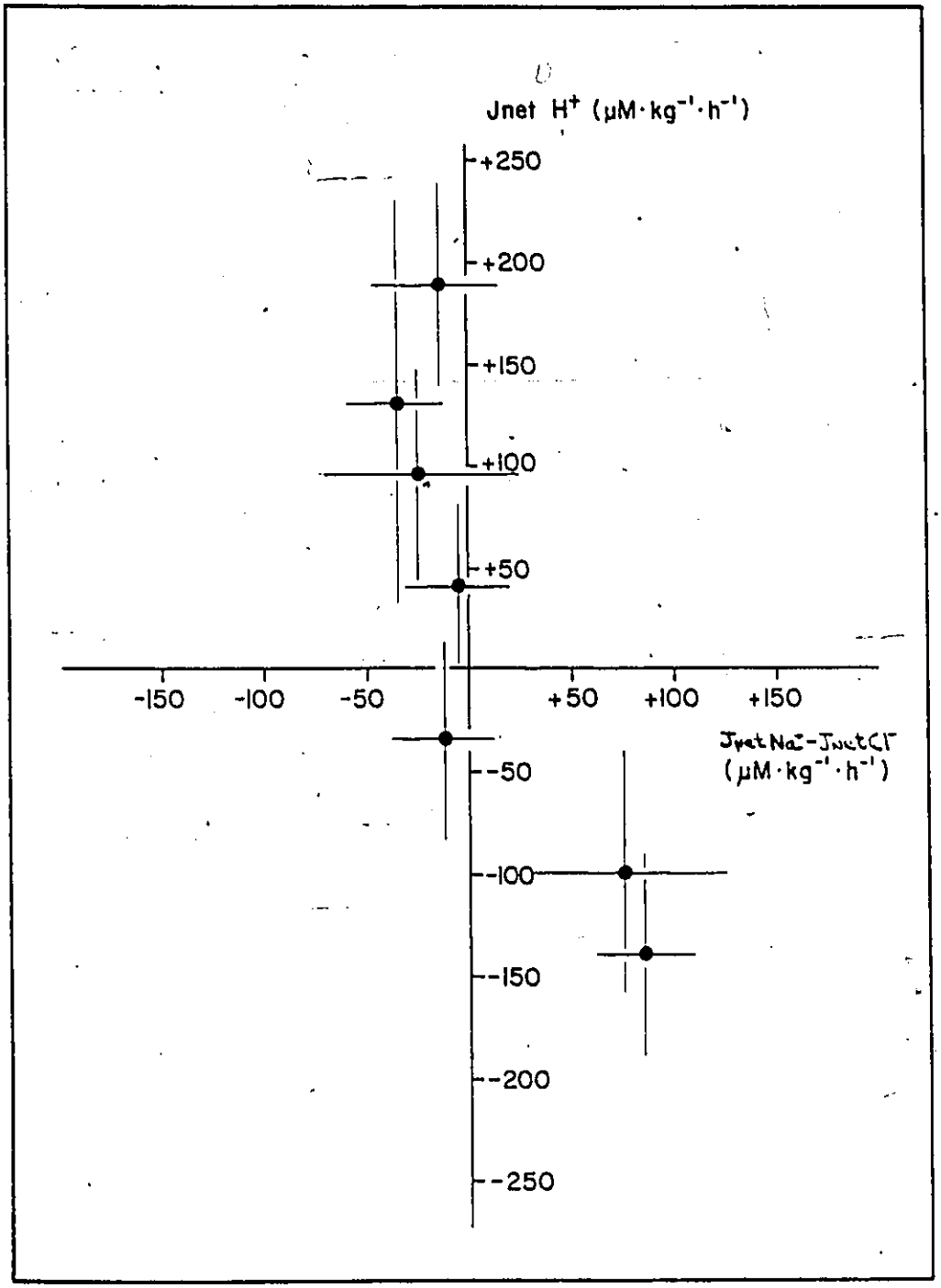
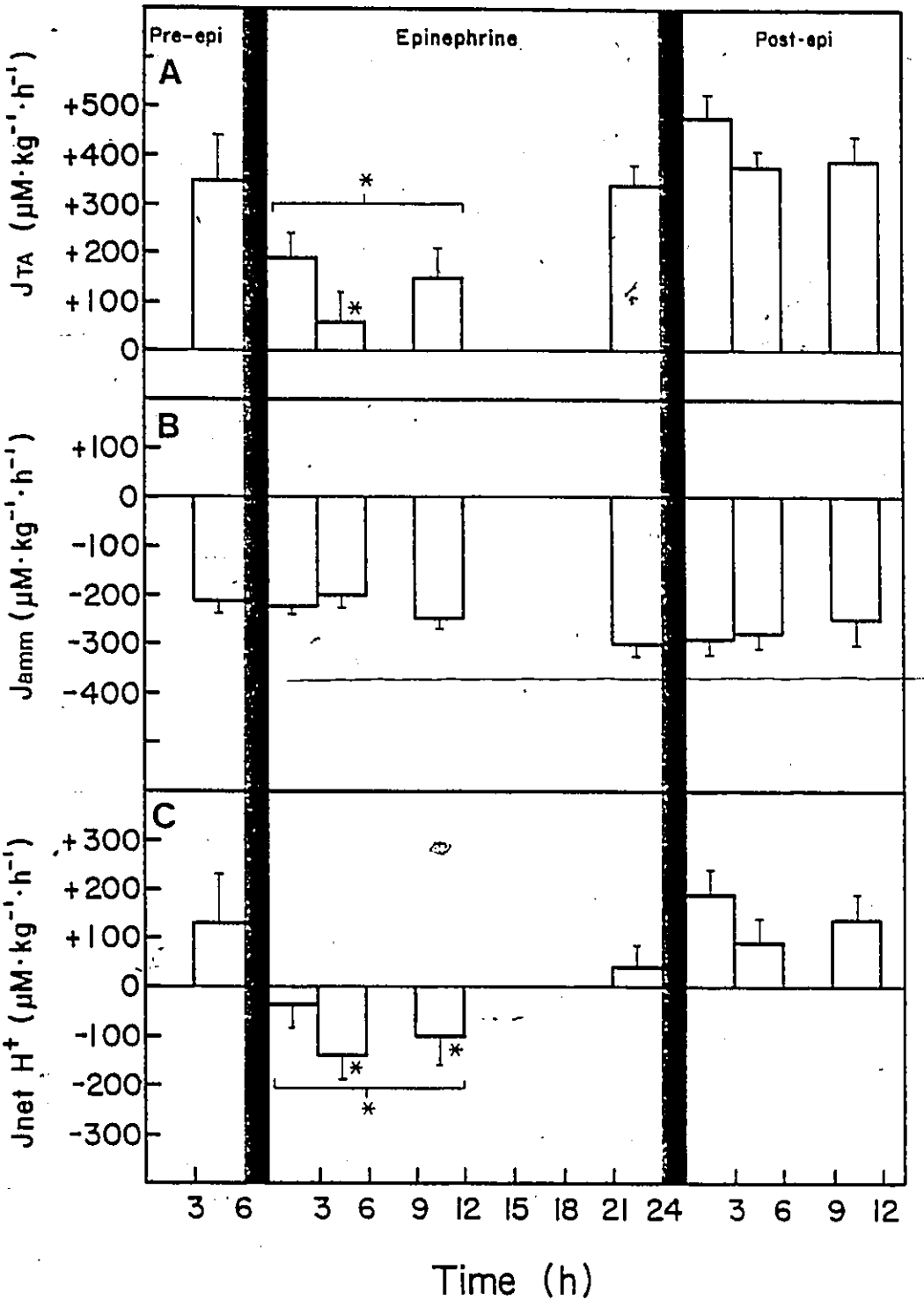

Jnet H+ (μM·kg⁻¹·h⁻¹)
+250
+200
+150
+100
+50
-150
-100
-50
+50
+100
+150
JnetNa⁺-JnetCl⁻
(μM·kg⁻¹·h⁻¹)
-50
-100
-150
-200
-250

Figure 19. The effects of prolonged intra-arterial infusion of epinephrine on branchial net fluxes of A) titratable acidity (J_{TA}), B) ammonium (J_{amm}) and C) acidic equivalents ($J_{net}H^+$). Asterisks denote significant differences from pre-epinephrine values. n = 6.


Pre-epi
Epinephrine
Post-epi
A
B
C
J_{TA} ($\mu M \cdot kg^{-1} \cdot h^{-1}$)
J_{amm} ($\mu M \cdot kg^{-1} \cdot h^{-1}$)
J_{net} H^+ ($\mu M \cdot kg^{-1} \cdot h^{-1}$)
+500
+400
+300
+200
+100
0
-100
-200
-300
-400
3 6 3 6 9 12 15 18 21 24 3 6 9 12
Time (h)

DISCUSSION

The effects of catecholamines on branchial solute fluxes in freshwater fishes have been investigated previously in some detail using isolated, saline-perfused gill preparations (Payan et al., 1975; Payan, 1978; Payan, Mayer-Gostan and Pang, 1981; Girard and Payan, 1977, 1980; Perry et al., 1984a). The conclusions based on such perfusion experiments have been subjected to criticism for a variety of reasons including the use of abnormally high levels of catecholamines in order to elicit effects, difficulty in distinguishing between specific effects of catecholamines and secondary effects caused by haemodynamic adjustments, and non-physiological conditions of perfusion (e.g. lack of dorsal aortic pressure). In the present investigation, we have evaluated the effects of epinephrine on branchial solute fluxes in the intact fish using levels of this catecholamine consistent with those measured during a variety of stressful conditions (6.2×10^{-8} M after four hours of continuous intra-arterial infusion; see also Table 1). A comparison between the present results and those of previous perfusion studies reveal some common features but also considerable differences. Unlike the results of perfused trout head experiments which show a marked stimulatory effect of epinephrine on Na^+/NH_4^+ exchange (Payan et al., 1975; Payan, 1978; Girard and Payan, 1977), in vivo infusion actually caused significant inhibition of Na^+ uptake that was not associated with lowered ammonia efflux. On the other hand, inhibition of Cl^- uptake (Fig. 15) is consistent with the beta-receptor mediated inhibition of branchial $J_{in}Cl^-$ in the perfused trout head preparation (Perry et al., 1984a). Reasons for these discrepancies are unclear

although we speculate that branchial ionic uptake in some earlier perfusion studies may have been limited by the diffusive properties of the gill epithelium such as surface area and thickness of the diffusion barrier. If this were so, epinephrine may have stimulated $J_{in}Na^+$ in previous studies simply by increasing the diffusive conductance of the gill via lamellar recruitment (Booth, 1979) in a similar manner as for oxygen transfer (Pettersson, 1983; Perry et al., 1985). It is unlikely that active ionic uptake is diffusion limited in the intact animal.

The results presented here suggest an important role for epinephrine in acid-base regulation and are consistent with observations of elevated levels of circulating catecholamines during conditions of internal acidosis induced by intravascular acid infusion (Boutilier et al., 1986), external hypercapnia (Perry et al., 1986a), exhaustive exercise (Opdyke et al., 1982; Primmett et al., 1986; see also Wood and Perry, 1986) or acute hypoxia (Fievet et al., 1986). Based on those observations and the stimulatory effect of elevated plasma [epinephrine] on branchial excretion of acidic equivalents reported here, it is tempting to assign a role to epinephrine in the compensation of extracellular acidoses as speculated by Perry et al. (1986a) during regulation of hypercapnic acidosis. Certainly a role for epinephrine in acute erythrocyte acid-base balance has already been established (Nikinmaa et al., 1984; Boutilier et al., 1986; Primmett et al., 1986; see also chapter 2). However, the elevations of plasma epinephrine levels during persistent acid-base disturbances have been shown to be transient (Boutilier et al., 1986; Perry et al., 1986) so other factors likely are involved in the regulation of chronic acid-base disturbances.

Based on strong ion difference theory (i.e. blood pH is determined by the difference between strong cations and anions; Stewart, 1981) and empirical data (Wood et al., 1984) it is apparent that the magnitude of net branchial acid excretion ultimately depends upon the arithmetic difference between $J_{net}Na^+$ and $J_{net}Cl^-$ across the gill. Typically, the regulation of depressed extracellular pH caused by respiratory disturbances is characterized by an increase of branchial $J_{net}Na^+ - J_{net}Cl^-$ (Cameron, 1976; Wood et al., 1984; Claiborne and Heisler, 1984; Perry et al., 1986a) that is believed to result from dynamic manipulation of the influx and/or efflux rates of these ions. The relationship between $J_{net}Na^+ - J_{net}Cl^-$ and gill acid excretion apparently stems from the relationship between the exchanges of Na^+ and Cl^- for acidic and basic equivalents, respectively. Epinephrine infusion, in the absence of any external acid-base disturbance, caused similar increases in $J_{net}Na^+ - J_{net}Cl^-$ and net H^+ excretion as has been observed during actual acid-base perturbations. It is clear, however, that the changes in $J_{net}Na^+ - J_{net}Cl^-$ did not result from simple modifications in the rates of Na^+ and Cl^- influx but to complex and differential adjustments to both influx and efflux. Indeed, the apparent stimulation of Cl^- efflux in the absence of any effect on Na^+ efflux or K^+ net flux suggests independent control of the passive outward movement of these ions. Such a scheme, however, is difficult to explain given that salt loss in freshwater fishes is thought to be governed by gill diffusive properties. Regardless of the mechanisms involved, there is little doubt that epinephrine enhances the extrusion of acid equivalents into the external environment which should be reflected by elevated levels of

bicarbonate in the plasma. In chapter 2, it was demonstrated that plasma [HCO_3^-] increased by approximately 1.9 mM between 1.5 and 10.5 h of epinephrine infusion over and above the increase attributable to non-bicarbonate buffering. Assuming that plasma HCO_3^- is in equilibrium with the extracellular fluid (ECF) compartment and an ECF volume of 274 ml·kg^{-1} body weight (Hobe, Wood and Wheatly, 1984), approximately 525 umol·kg^{-1} of additional acidic equivalents must have been excreted across the gill. Our calculations indicate that additional branchial H^+ excretion over this particular period could account for the removal of about 1800 umol·kg^{-1}, an amount well in excess of the calculated increase in ECF [HCO_3^-]. Such a discrepancy may have resulted from the transfer of HCO_3^- between extracellular and intracellular compartments.

Although the results obtained in the present study suggest a distinct role for epinephrine in the regulation of branchial acid excretion, the possibility of interactive factors being involved cannot be ruled out. One such factor is the acidosis induced by epinephrine infusion. To our knowledge, the direct effects of reduced blood pH on branchial acid excretion have not been evaluated; however, the transient and relatively minor extent of the acidification indicate that direct pH effects probably cannot account for all of the various physiological responses observed in our study. Certainly, branchial net H^+ excretion remained elevated and other branchial effects also persisted even after plasma pH had been restored to pre-infusion values. Specific effects of reduced pH on branchial ionic fluxes rarely have been investigated, although Payan (1978) reported that Na^+ uptake

was inhibited in the perfused trout head preparation following acidification of the perfusion fluid. In a similar preparation, Perry et al., (1984b) noted that hypercapnic acidosis of the internal perfusate caused a stimulation of Cl^- uptake. It is difficult to reach a conclusion on the possibility of direct pH modulation of branchial ion uptake processes given the paucity of available data. Other experiments designed to investigate the relationships between internal acid-base status and gill ionic uptake have been performed on intact animals in which secondary events such as catecholamine mobilization could not be controlled. Certainly, this is an area of research that warrants further investigation.

CHAPTER 5

THE EFFECTS OF PROLONGED EPINEPHRINE INFUSION ON THE PHYSIOLOGY OF THE RAINBOW TROUT, *SALMO GAIRDNERI*

III. RENAL IONIC FLUXES.

INTRODUCTION

Dynamic manipulations of both branchial and renal acid excretory mechanisms have been postulated as important means of acid-base regulation in fish (see reviews by Cameron, 1978; Heisler, 1984). Although direct modulation of these pathways during acid-base disturbances was demonstrated, the magnitude of the branchial/renal partitioning of acid or base excretion during correction of acid-base disturbances has not been clearly established and may be related to species differences as well as to the nature of the acid-base disturbance. The variable nature of the involvement of the fish kidney in acid-base regulation is indicated by reports of complete renal compensation (Wood and Caldwell, 1978), minor renal compensation (Wheatly et al., 1984), or complete lack of renal compensation (Evans, 1982) following imposed internal acid loads. Regardless of the contribution of the kidney to whole body acid excretion, it is likely to play an important role in preventing filtered HCO_3^- from being excreted in the urine by linking acid secretion to HCO_3^- reabsorption (Wood and Jackson, 1980; Wheatly et al., 1984) in a similar manner as in mammals (Pitts, 1974). The prevention of urinary HCO_3^- loss is crucial for successful acid-base regulation in fishes because of the reliance on plasma [HCO_3^-] adjustments to correct pH disturbances (Heisler, 1984).

Perturbations of blood acid-base status have been shown to cause elevated plasma levels of epinephrine. Such elevations appear to interact with branchial acid extrusion mechanisms to enhance net acid excretion (chapter 4) thereby elevating plasma pH. The interactions of

epinephrine with renal ionic and acid-base excretory mechanisms, however, have not been evaluated. In this chapter, a detailed examination is conducted on the effects of continuous intra-arterial epinephrine infusion on renal excretion of electrolytes and acid-base equivalents in order to assess the role of epinephrine in controlling renal compensation of acid-base disturbances.

MATERIALS AND METHODS

The experimental conditions and treatment of animals before and during experimentation were as described in chapter 2. Fish were fitted with dorsal aortic cannulae and urinary catheters and only the fish in which both cannula and catheter were patent were used for experimentation. Continuous urine collection for the duration of the experiments was accomplished by allowing the urinary catheters to drain, by gravity, into plastic vials located below the acrylic holding boxes.

Urine was collected during a preliminary 3 h saline infusion period; during a 24 h epinephrine or saline infusion period (between 0-3 h, 3-6 h, 6-9 h, 9-21 h and 21-24 h) and during a final 12 h period of post-epinephrine saline infusion (0-3 h, 3-6 h, 6-9 h and 9-12 h). Urine samples were analyzed immediately upon collection for pH, total CO_2 (C_{CO2}) and volume and were then diluted 10X, acidified (1% v/v) with 1 N nitric acid and left frozen at -20° C until further assays could be performed. Samples then were thawed and total ammonia, phosphate, Na^+, Cl^-, K^+ and Ca^{2+} concentrations were determined.

Urine from a separate group of five infused fish was titrated immediately upon collection to determine the titratable component (TA - HCO_3^-) and the non-titratable component (ammonia) of urinary acid excretion. Total urinary acid excretion was calculated as the sum of urinary [TA - HCO_3^-] and total [ammonia] multiplied by urine flow rate (UFR).

Electrolyte Analysis

Urine pH was measured using a micro capillary pH electrode

(Radiometer G299A) in conjunction with a Radiometer PHM 71 acid-base analyzer and BMS3 MK2 blood micro system. CO_2 was measured by injecting evolved gas into a gas chromatograph (Carle AGC Series 100) following acidification of 100 ul of urine with 2 ml of HCl (1 N) in a gas-tight syringe (Hamilton) that had previously been purged with N_2 (ultra-high purity). The total ammonia concentration of urine was determined using a micro-modification of the salicylate-hypochlorite technique (Verdouw, vanEchteld and Dekkers, 1978). Urine phosphate and calcium levels were measured using commercially available kits (Sigma). Potassium and sodium concentrations were determined by flame photometry (EEL Flame Photometer) and chloride concentrations were measured by amperometric titration (Buchler-Cotlove Chloridometer).

Urinary [TA - HCO_3^-] was measured by adding a known volume of 0.2 N HCl to 200 ul of urine in order to lower the final pH below 5.0, then vigourously aerating and agitating the sample for 15 min to remove CO_2. NaOH was then gradually added using a microburet (Gilmont) to restore urine pH to the blood pH representative of the particular sampling period (see chapter 2). The difference between the quantities of acid and base added to the urine yielded the titratable component of urinary acid excretion.

Urinary effluxes for ammonia, phosphate, Na^+, Cl^-, K^+ and Ca^{2+} were taken as the product of urinary concentrations of the electrolytes multiplied by UFR. Renal clearance ratios for these electrolytes (C_X) were calculated using the following equation:

$$C_X = \frac{[X]_u \cdot UFR}{[X]_p \cdot GFR} \qquad (2)$$

where $[X]_u$ and $[X]_p$ represent the concentrations of the particular electrolyte in the urine and plasma, respectively (for plasma concentrations, see chapter 2) and GFR is assumed to be 1.77 x UFR (Holmes and Stainer, 1966). A linear relationship between UFR and GFR has been demonstrated (Hoffmann and Butler, 1979) thus the conversion factor of 1.77 is assumed to be constant over the range of experimental urine flow rates.

Statistical analysis

Due to variability between the control and experimental groups (although not statistically different), much of the data have been presented as absolute changes from the initial control or experimental value during the initial period of saline infusion. For simplicity and clarity, only the absolute values of the experimental group have been indicated. The results were statistically analyzed by comparing the sample means of control and experimental animals at identical time periods using two-tailed paired and unpaired Student's t-tests. 5% was accepted as the fiducial limit of significance.

RESULTS

Considerable differences were observed between control and experimental values during the initial period of saline infusion for many of the measured renal variables. Such differences, although never statistically significant, tended to obscure actual changes during epinephrine infusion. Thus, for reasons of clarity, much of the data have been presented as absolute changes from the initial saline infusion values. For simplicity, in such instances, only the absolute pre-epinephrine infusion values from the experimental group have been reported.

An immediate and pronounced effect of epinephrine infusion was a transient increase in urine flow rate (UFR) that returned to normal levels following 9 h of infusion (Fig. 20). Upon return to saline infusion, there was a significant reduction of UFR. In a similar manner, epinephrine caused a significant depression of urine pH during the first 9 h of administration whereas return to saline infusion caused urine pH to increase (Fig. 20). Associated with the reduction of urine pH was a short-lived elevation of urinary acid excretion primarily due to a stimulation of the titratable component, ($J_{TA} - HCO_3^-$) (Fig. 21), although J_{amm} also increased in a non-significant fashion. Similarly, renal acid excretion was reduced during the post-epinephrine period as a consequence of both altered ($J_{TA} - HCO_3^-$) and J_{amm} (Fig. 21). Urinary C_{CO2} was constant throughout the epinephrine infusion period, but was consistently elevated during the 12 h post-epinephrine period (Fig. 21).

Large and persistent increases in urinary concentrations of Na^+

Figure 20. The effects of prolonged intra-arterial infusion of epinephrine on A) urine pH and B) urine flow rate (UFR). Control fish (open bars) were infused continuously with saline for 42 h while the epinephrine-treated (hatched bars) fish were infused initially for 7 h with saline (pre-epi) followed by 24 h of epinephrine infusion and finally 12 h of saline infusion (post-epi). Asterisks denote significant differences from pre-epinephrine values; daggers denote significant differences from corresponding values in control group. n numbers are as indicated.

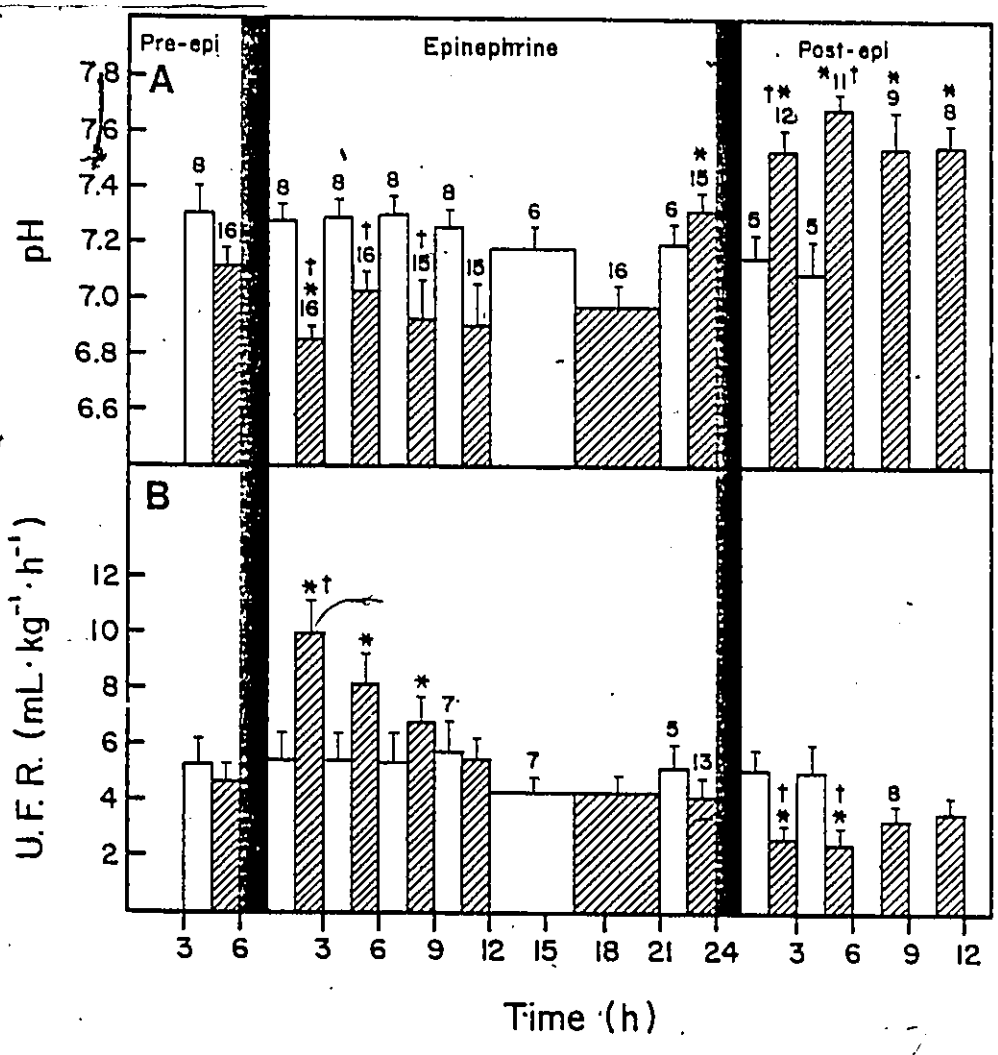

Pre-epi
Epinephrine
Post-epi
A
B
pH
U.F.R. (mL·kg⁻¹·h⁻¹)
Time (h)

Figure 21. The effects of prolonged intra-arterial infusion of epinephrine on the renal efflux rates of A) titratable acid minus bicarbonate ($J_{TA} - HCO_3^-$; measured as a single component), B) ammonium (JNH_4^+), C) acidic equivalents ($J_{net}H^+$) and D) total urinary CO_2 levels (C_{CO2}). Asterisks denote * significant differences from pre-epinephrine values; n = 6 throughout.

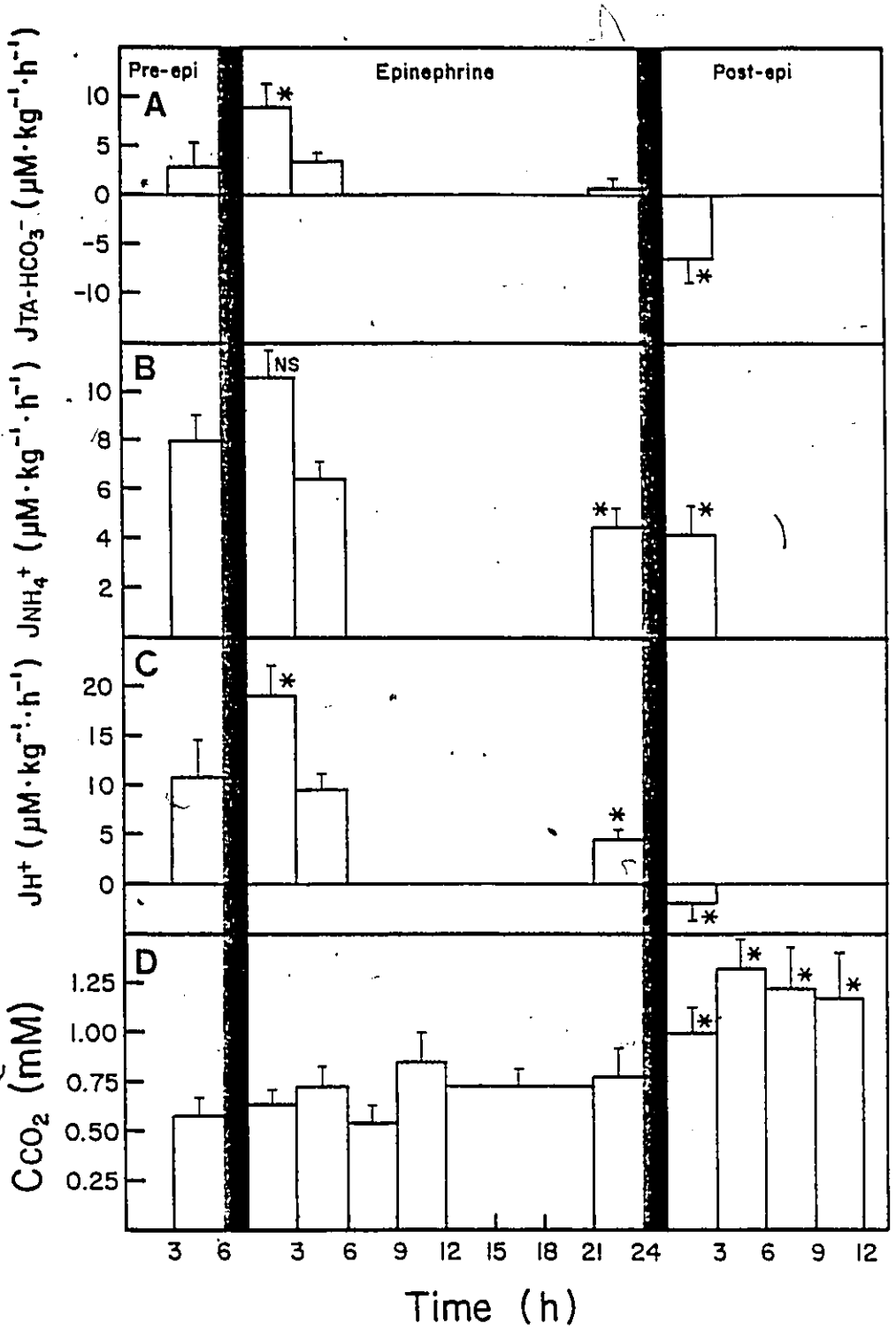
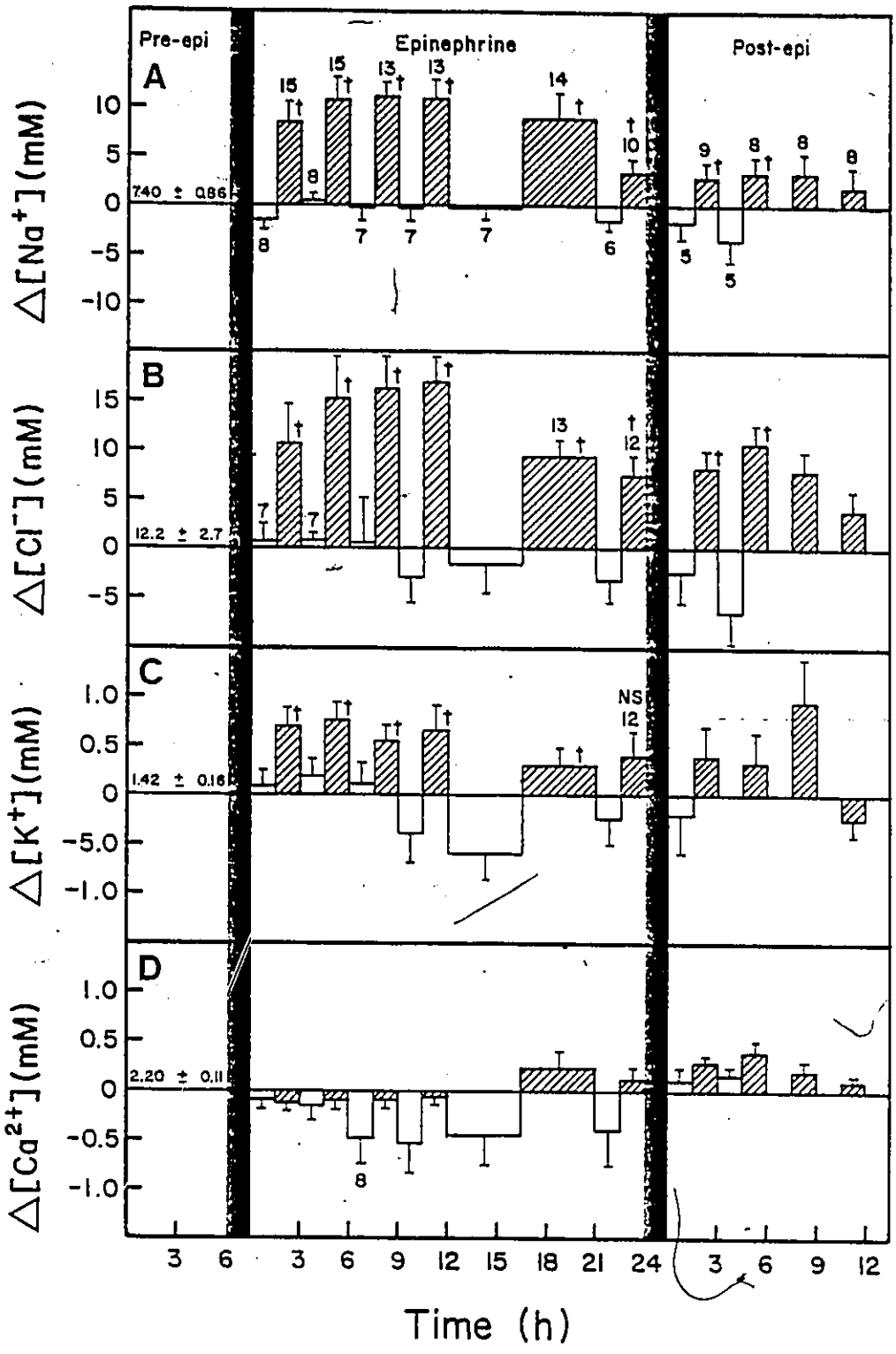

Pre-epi
Epinephrine
Post-epi
A
B
C
D
NS
$J_{TA-HCO_3^-}$ ($\mu M \cdot kg^{-1} \cdot h^{-1}$)
$J_{NH_4^+}$ ($\mu M \cdot kg^{-1} \cdot h^{-1}$)
J_{H^+} ($\mu M \cdot kg^{-1} \cdot h^{-1}$)
C_{CO_2} (mM)
Time (h)

Figure 22. The effects of prolonged intra-arterial infusion of epinephrine (hatched bars) on urinary concentrations of A) sodium, B) chloride, C) potassium and D) calcium. Data have been plotted as absolute changes from the values during an initial period of saline infusion (pre-epi). Daggers denote significant differences from corresponding values in control group (open bars). n numbers are as indicated. All other details are as in Fig. 20.


Pre-epi
Epinephrine
Post-epi
A
B
C
D
Δ[Na+](mM)
Δ[Cl-](mM)
Δ[K+](mM)
Δ[Ca2+](mM)
7.40 ± 0.86
12.2 ± 2.7
1.42 ± 0.16
2.20 ± 0.11
NS
Time (h)

and Cl^- were observed during epinephrine treatment with $[Cl^-]$ increasing to a greater extent than $[Na^+]$ (Fig. 22). The changes in urine $[Na^+]$ and $[Cl^-]$ were due to decreased tubular reabsorption as indicated by increased renal clearance ratios (Fig. 23). Urine K^+ concentration also was increased (Fig. 22), but the renal clearance ratio for K^+ was not significantly elevated except at one time period (between 9-12 h; Fig. 23) suggesting that the increase of urine $[K^+]$ probably was not due to altered tubular functioning, but simply due to the increased K^+ levels in the blood (chapter 2). Urine Ca^{2+} levels were unaffected by epinephrine infusion although renal Ca^{2+} efflux was markedly elevated, primarily due to the increase in UFR (Fig. 24). Renal efflux rates of Na^+, Cl^- and K^+ (Fig. 24) were greatly stimulated due to the combined effects of decreased tubular reabsorption (Na^+ and Cl^- only) and increased UFR. The effects of epinephrine on urinary ionic excretion were most evident during the first 12 h of infusion (Fig. 24).

The effects of epinephrine on the renal handling of the two most prevalent urinary buffers, inorganic phosphate (P_i) and ammonia, are shown in Figs. 25 and 26. Urine P_i levels increased throughout epinephrine infusion (Fig. 25A) but due to the large variability of the data, no individual value could be shown to be significantly different from control values. A statistical comparison between the overall mean value of the epinephrine treated group and the overall mean value of the control group during this 24 h period, however, proved to be highly significant. During the post-epinephrine period, when urine pH was significantly elevated, urine P_i was greatly

Figure 23. The effects of prolonged intra-arterial infusion of epinephrine on the apparent renal clearance ratios for major urinary electrolytes. Renal clearance ratios were calculated using an estimated GFR of 1.77 x UFR and have been plotted on a logarithmic scale. Asterisks denote significant differences from pre-epinephrine values. n numbers as indicated for NH_4^+ (top). See text for further details.

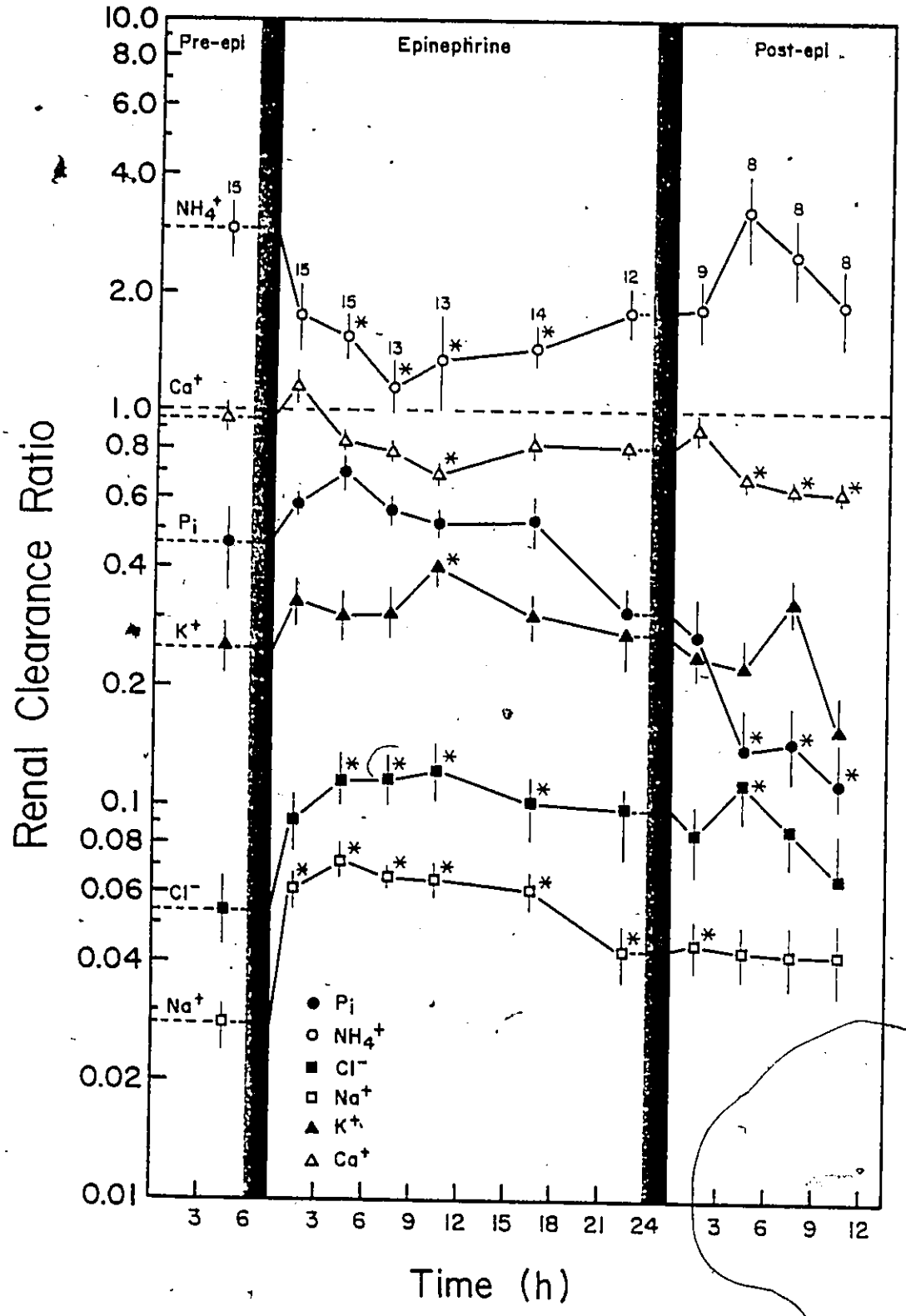

Pre-epi
Epinephrine
Post-epi
Renal Clearance Ratio
Time (h)
10.0
8.0
6.0
4.0
2.0
1.0
0.8
0.6
0.4
0.2
0.1
0.08
0.06
0.04
0.02
0.01
NH4+
Ca+
Pi
K+
Cl-
Na+
3 6 3 6 9 12 15 18 21 24 3 6 9 12
Pi
NH4+
Cl-
Na+
K+
Ca+

Figure 24. The effects of prolonged intra-arterial infusion of epinephrine (hatched bars) on renal efflux rates of A) sodium (JNa^+), B) chloride (JCl^-), C) potassium (JK^+) and D) calcium (JCa^{2+}). Data have been plotted as absolute changes from the values during the period of initial saline infusion (pre-epi). For clarity, only the absolute values of the experimental group are shown. Daggers denote significant differences from corresponding values in control group (open bars). n numbers are as indicated. All other details are as in Fig. 20.

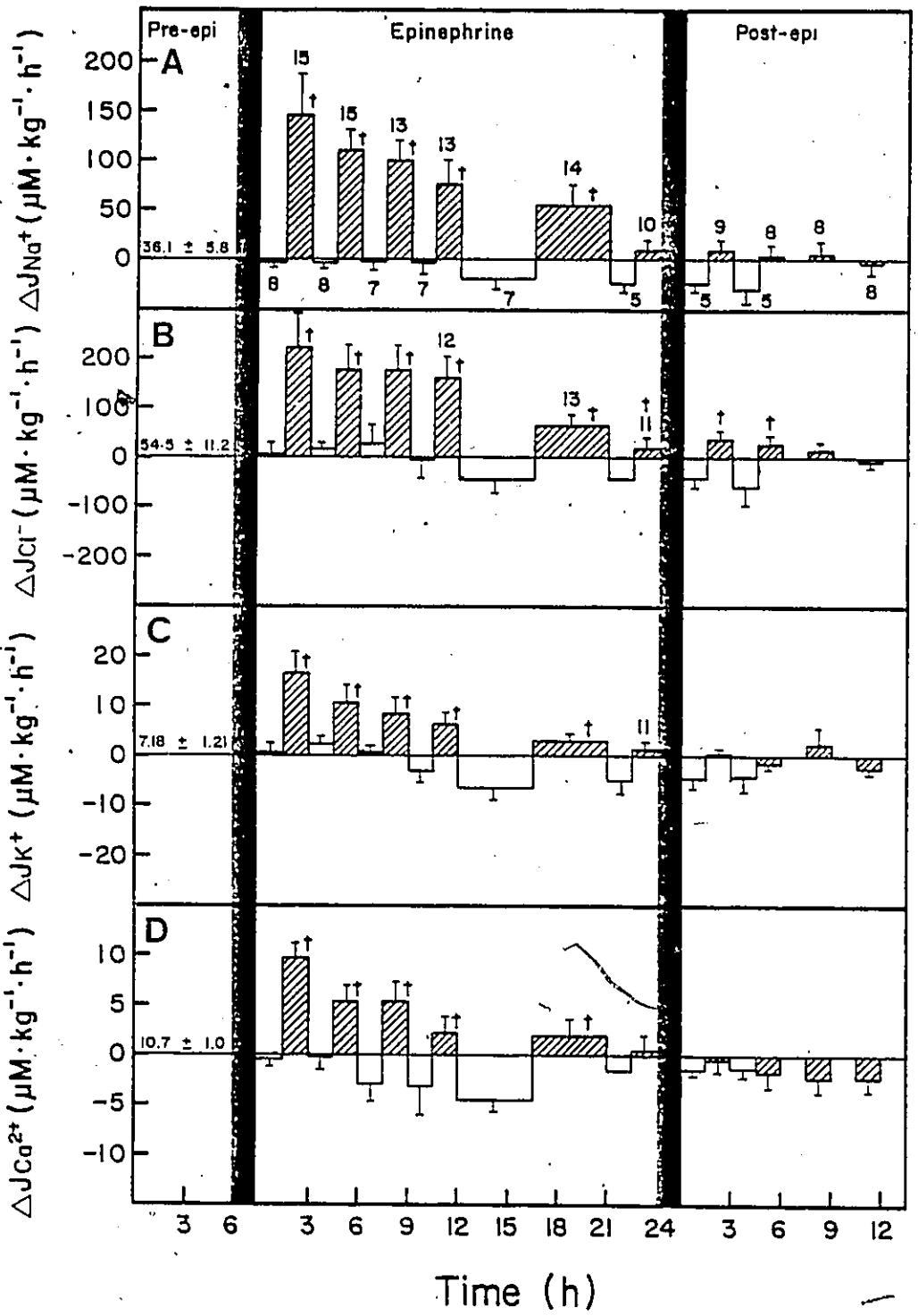

Pre-epi
Epinephrine
Post-epi
A
B
C
D
36.1 ± 5.8
54.5 ± 11.2
7.18 ± 1.21
10.7 ± 1.0
ΔJNa+ (μM · kg-1 · h-1)
ΔJCl- (μM · kg-1 · h-1)
ΔJK+ (μM · kg-1 · h-1)
ΔJCa2+ (μM · kg-1 · h-1)
Time (h)

Figure 25. The effects of continuous intra-arterial infusion of epinephrine (hatched bars) on urinary concentrations of A) inorganic phosphate (P_i) and B) ammonia. Data have been plotted as absolute changes from the values of the experimental group during the initial saline infusion (pre-epi). For clarity, only the absolute values of the experimental group during the initial saline infusion are shown. Daggers denote significant differences from corresponding values in control group (open bars). n numbers as indicated.

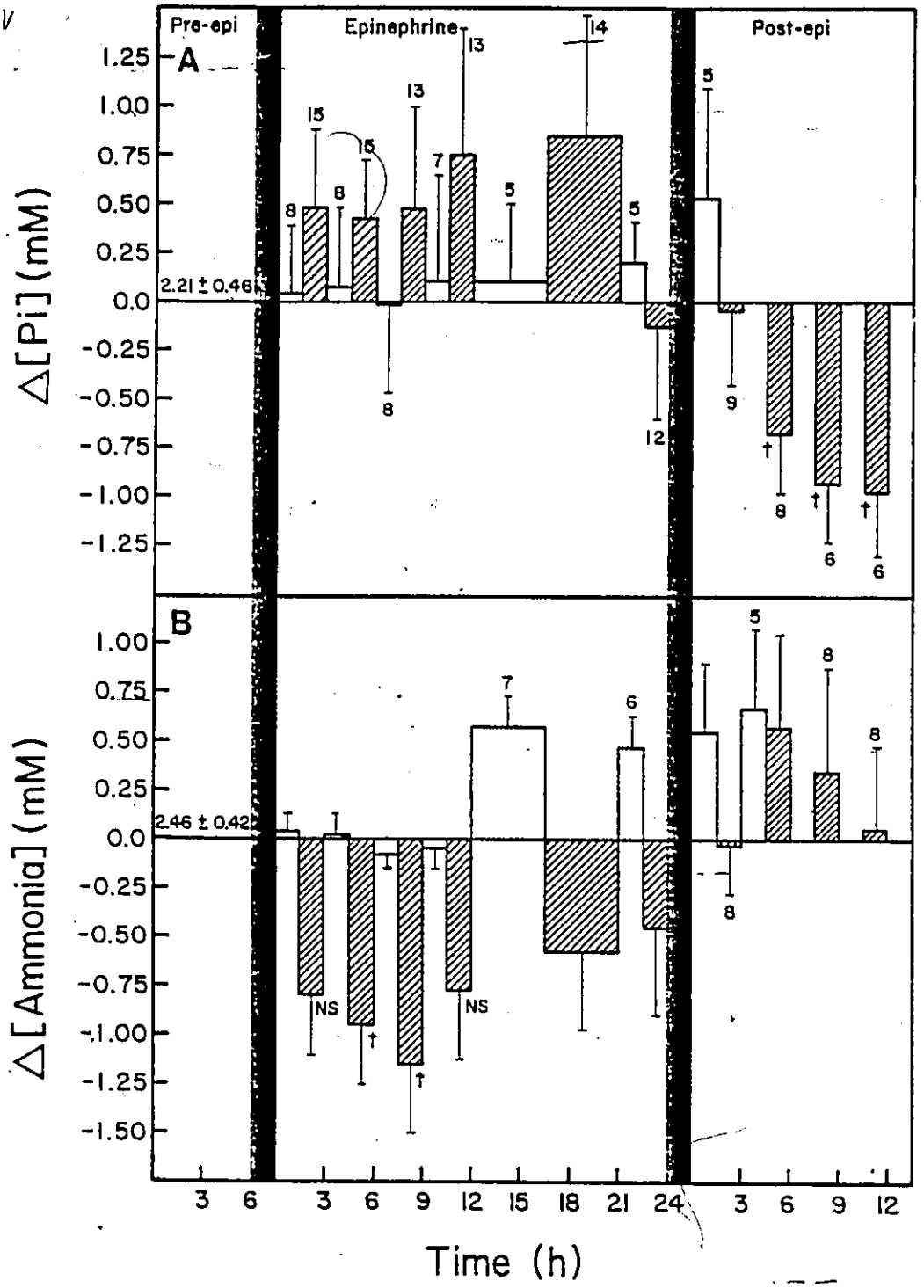

Pre-epi
Epinephrine
Post-epi
A
B
Δ[Pi] (mM)
Δ[Ammonia] (mM)
2.21 ± 0.46
2.46 ± 0.42
NS
Time (h)

Figure 26. The effects of prolonged intra-arterial infusion of epinephrine (hatched bars) on renal efflux rates of A) inorganic phosphate (J_{Pi}) and B) ammonia ($J_{ammonia}$). Data have been plotted as absolute changes from the values during the period of initial saline infusion (pre-epi). For clarity, only the absolute values of the experimental group during the initial saline infusion are shown. Daggers denote significant differences from corresponding values in control group (open bars). n numbers are as indicated.

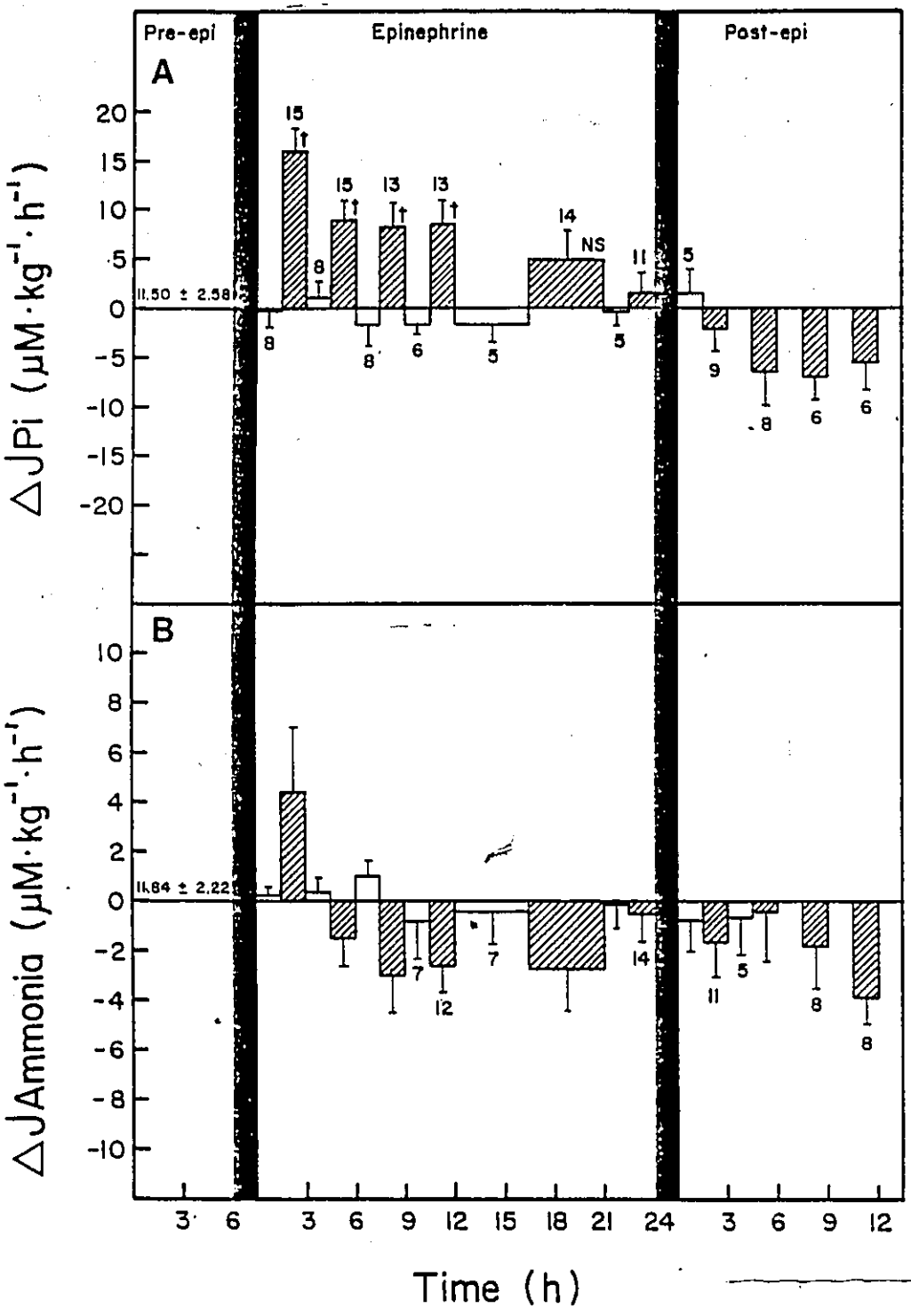
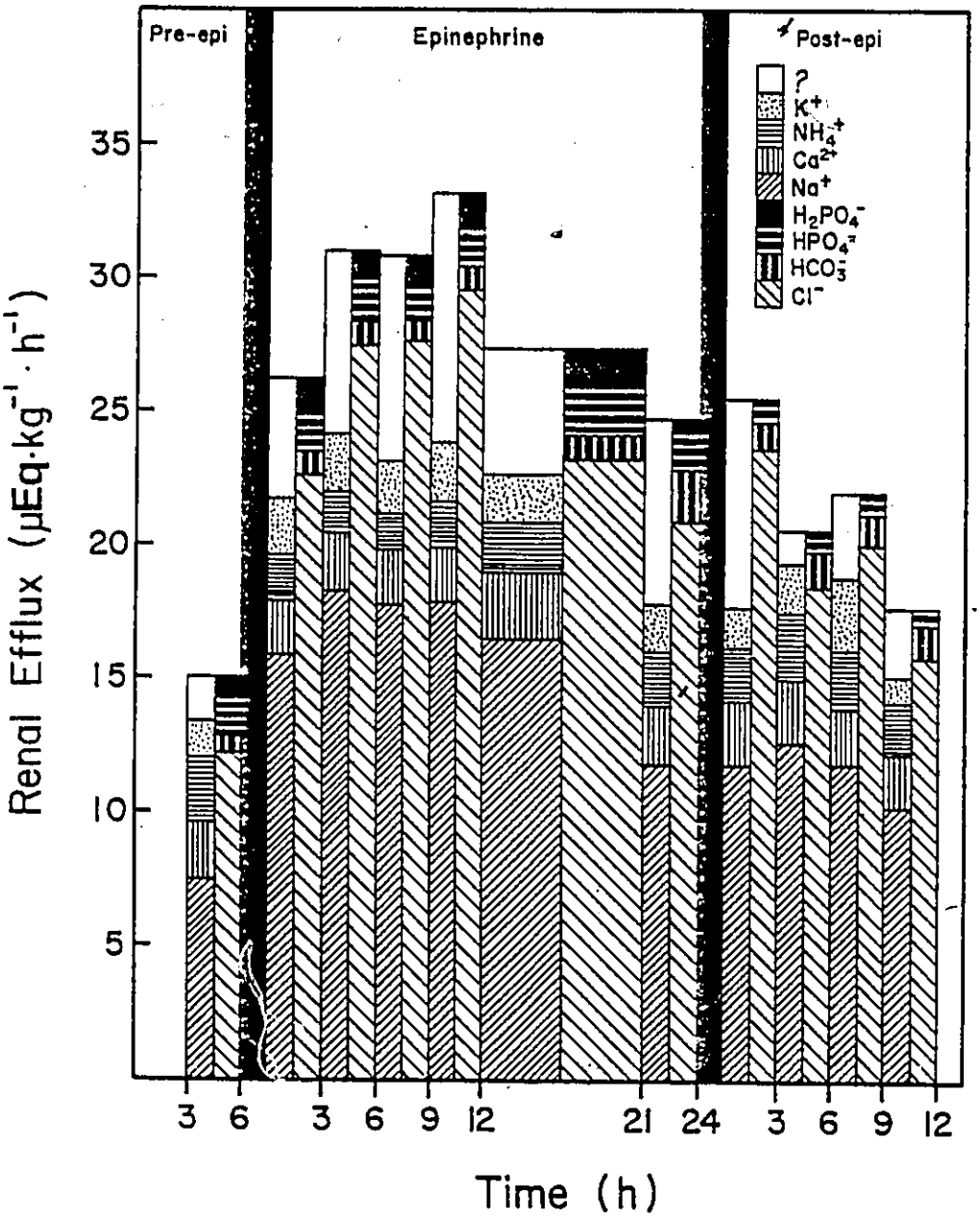

Pre-epi
Epinephrine
Post-epi
A
B
ΔJPi (μM·kg⁻¹·h⁻¹)
ΔJAmmonia (μM·kg⁻¹·h⁻¹)
11.50 ± 2.58
11.84 ± 2.22
NS
Time (h)

Figure 27. The effects of prolonged intra-arterial infusion of epinephrine on renal excretion charge balance. Bicarbonate concentration ($[HCO_3^-]$) was calculated from the concentration of titratable acidity minus bicarbonate ($[TA - HCO_3^-]$) assuming that $H_2PO_4^-$ accounted for all TA. HPO_4^{2-} and $H_2PO_4^-$ concentrations were calculated from the Henderson-Hasselbalch equation using the measured levels of total inorganic phosphate, urine pH and a pK value of 6.86. Urine anions and cations were sampled at identical times; note that concentrations are given in $uEq \cdot l^{-1}$. See text for further details.


Pre-epi
Epinephrine
Post-epi
?
K+
NH4+
Ca2+
Na+
H2PO4-
HPO4=
HCO3-
Cl-
Renal Efflux (μEq·kg-1·h-1)
35
30
25
20
15
10
5
3
6
3
6
9
12
21
24
3
6
9
12
Time (h)

reduced due to an increase of tubular P_i reabsorption by a factor of 3X (Fig. 23). Renal excretion of P_i changed in a similar fashion as urine P_i concentration. During epinephrine infusion, the urinary concentration of ammonia decreased (Fig. 25), but due to the increase of urine flow rate, total ammonia efflux remained relatively constant (Fig. 26) even though renal tubular secretion of ammonia decreased significantly (Fig. 23).

Fig. 27 summarizes the effects of epinephrine on renal electrolyte excretion. It is evident that prior to epinephrine infusion, a state of charge balance existed in the urine with only a minor cationic component of the urine unaccountable (<8%). Epinephrine infusion caused large increases in overall electrolyte excretion; however, the excretion of measured anions increased to a greater extent than measured cations such that charge balance was not possible without the addition of an unknown cation. It is speculated that this cation is magnesium.

DISCUSSION

The values noted in the present study for renal electrolyte and acid excretion in control fish are considerably greater (approximately 1.5-2X) than those associated with resting and non-stressed rainbow trout (e.g. Wood and Jackson, 1980; McDonald and Wood, 1981; McDonald, 1983; Wheatly et al., 1984). These differences were not related to altered tubular reabsorption or secretory mechanisms because urine electrolyte concentrations and renal clearance ratios were similar to values reported in earlier studies. Instead, it is likely that elevated renal excretion rates resulted from abnormally high urine flow rates (approximately 1.5-2X greater than usually reported; e.g. Wood and Jackson, 1980; Wheatly et al., 1984; Fig. 20) that in turn were a consequence of the saline infusion process. Previous studies (Wood and Caldwell, 1978; Kobayashi and Wood, 1980) as well as our own unpublished observations, have indicated that urine flow rate is increased rapidly by plasma volume loading following either single intravascular injections or continuous infusion of saline. In the present study, an attempt was made to minimize plasma volume changes by infusing at a low rate (0.6 $ml \cdot h^{-1}$) but clearly, even this low rate of infusion was capable of elevating glomerular filtration rate. Thus, no claim is made that renal excretion rates reported here are indicative of true resting values, but nonetheless the methodology employed did allow a valid assessment of the relative effects of elevated plasma levels of epinephrine on renal function.

Further problems associated with analyzing urine collected continuously from gas-permeable urinary bladder catheters have been

discussed in detail by Cameron and Kormanik (1982). Briefly, these include the inability i) to collect urine under anaerobic conditions thereby leading to CO_2 loss from the urine and concomitant pH fluctuations and ii) to assess the role of the urinary bladder in acid-base/ionic regulation since the catheterization technique only enables the collection of continuously voided tubular urine. Of course, under normal conditions, urine resides in the bladder for variable periods of time before being excreted.

Epinephrine induced numerous and pronounced changes in renal function, many of which suggest a role for epinephrine in the control of renal compensation of acid-base disturbances. The epinephrine-induced effects on renal variables that are consistent with compensation of internal acidosis included transient elevations of urine flow rate, phosphate excretion and net acid excretion, as well as depressed urine pH. On the other hand, the persistent reductions of urine ammonia levels and ammonia excretion rate that were observed during epinephrine infusion certainly are counterproductive with respect to compensation of internal acidotic conditions because ammonia as well as contributing to net acid excretion, is an important urine buffer. These changes in the renal handling of ammonia were related to pronounced reductions in tubular ammonia secretion and may serve to explain why marked reductions in urine pH during the first 9 h of epinephrine infusion caused only minor and very short-lived increases of net acid excretion. The functional significance of decreased ammonia secretion during conditions of elevated epinephrine is unclear but may be related to the constraints of urine charge balance or an attempt to minimize

changes in urine buffering capacity during the period of enhanced phosphate excretion (Fig. 26). The increase in renal phosphate excretion was due to the combined effects of elevated urine flow rate, non-significant decreases in tubular reabsorption, and increased renal filtration in the latter stages of epinephrine infusion due to elevated plasma phosphate levels (chapter 2).

Although renal net acid excretion was not greatly stimulated by epinephrine infusion, this does not preclude a role for epinephrine in renal compensation of acid-base disturbances. Indeed, our results suggest that epinephrine may be important in reducing the amount of filtered HCO_3^- that actually is excreted in the urine. This suggestion is based on the fact that plasma HCO_3^- levels increased significantly during epinephrine infusion (chapters 2,3) yet urine HCO_3^- levels remained constant and subsequently increased during the post-epinephrine infusion period (Fig. 21). Thus, it is likely that tubular H^+ secretion did increase during epinephrine administration, without major impact on net acid excretion, in order to reabsorb the additional HCO_3^- that must have appeared in the glomerular filtrate. Similarly, we speculate that tubular H^+ secretion was diminished upon return to saline infusion thereby causing urine HCO_3^- concentration to increase and net acid excretion to decrease significantly (Fig. 21). It is important to point out that the primary role of the freshwater teleost kidney in the compensation of respiratory acidosis is considered to be the prevention of excess filtered HCO_3^- from being excreted (Wood and Jackson, 1980; Wheatly et al., 1984; Perry et al., 1986) thereby allowing plasma HCO_3^- levels to rise until pH is restored. This scheme

also necessitates increased tubular H^+ secretion that may, based on our results, be dependent upon elevated epinephrine levels.

The marked increase in urine flow observed during the initial 9 h of epinephrine infusion was probably not due to plasma volume changes as all fish were pre-infused at identical infusion rates with saline. However, the possibility of water movements from interstitial to vascular compartments cannot be discounted. Other possible causes of elevated UFR are decreased tubular reabsorption of water and increased systemic blood pressure. We consider the latter to be the most likely possibility given the known effect of single epinephrine injections in raising systemic blood pressure (Wood and Shelton, 1975; Pettersson and Nilsson, 1980; Farrell, 1981) due to alpha-adrenergic stimulation (see review by Nilsson, 1984). It is interesting that UFR returned to control values during the latter stages of epinephrine infusion and then decreased significantly below control values upon return to saline infusion (Fig. 20). Various other renal variables (pH, $J_{net}H^+$, $[P_i]$, J_{Pi}) responded in a similar manner. These results suggest that it may not be the absolute levels of plasma epinephrine that ultimately affect physiological function but sudden changes in epinephrine levels. Epple and Nibbio (1985) reached a similar conclusion with respect to the catecholaminotropic and glycemic effects of catecholamines in the eel (Anguilla rostrata).

Due to the constraints of charge balance, urine equivalents of anions and cations (valencies considered) must be equal. Given the number of measurements performed and the errors associated with each, I am satisfied that such a balance did exist during the pre-epinephrine,

and throughout most of the post-epinephrine infusion periods (Fig. 27). The minor "cation gap" that did exist (i.e. $[Cl^-] + [HCO_3^-] + [H_2PO_4^-] + 2[HPO_4^{2-}] - [Na^+] - 2[Ca^{2+}] - [K^+] - [NH_4^+]$) presumably reflected a ratio of unmeasured cations to unmeasured anions that was greater than unity and relatively constant during the saline infusion periods. The principle unmeasured ions likely were magnesium and sulphate. The mechanisms involved in the renal excretion of sulphate and magnesium in fresh water fishes have not been studied in detail, but recently Oikari and Rankin (1985) have demonstrated that trout are capable of either net secretion or net reabsorption of magnesium depending on plasma Mg^{2+} status. Thus, the pronounced increase in the magnitude of the "cation gap" during epinephrine infusion may have been due to altered renal handling of magnesium.

It has been proposed that circulating catecholamines are important for mediating osmoregulatory adjustments following transfer between freshwater and seawater (Mazeaud and Mazeaud, 1981; Eddy, 1981). Our results suggest that epinephrine may be important in this regard by decreasing the reabsorption of Na^+ and Cl^- ions thereby stimulating renal excretion of NaCl. Such a response might be beneficial immediately upon acute transfer to seawater when salt loading is maximal and branchial ion extrusion mechanisms have not yet become activated.

In the last three chapters (2, 3 and 4), continuous infusion of epinephrine was used as a method to increase the circulating levels of epinephrine in rainbow trout, without submitting the animals to other stressors that are normally associated with elevated epinephrine levels

(e.g. exercise or hypercapnia). Although distinguishing the effects of epinephrine from the effects of the acid-base disturbance which it incurs is not possible given the present experimental design, it is apparent that epinephrine can play an important role in the regulation of acid-base disturbances. The epinephrine-induced maintenance of rbc pH in the face of whole blood acidosis, serves to ensure adequate arterial oxygenation by preventing the deleterious effects of reduced pH on haemoglobin function (Bohr and Root effects) (chapter 2,3). Increased branchial acid efflux (chapter 4) and renal acid secretion, that occur during epinephrine infusion, correct internal acidosis by allowing the accumulation of HCO_3^- (branchial acid efflux accounts for approximately 85% of total acid efflux, with the kidney accounting for the remaining 15%). It is obvious from this series of experiments that additional work is required to separate the effects of epinephrine from possible pH effects, and to further clarify its role in piscine acid-base regulation.

CHAPTER 6

ADRENERGIC INVOLVEMENT IN BLOOD OXYGEN TRANSPORT AND ACID-BASE BALANCE DURING HYPERCAPNIC ACIDOSIS IN THE RAINBOW TROUT, SALMO GAIRDNERI

INTRODUCTION

Elevated levels of circulating catecholamines (primarily epinephrine) have been observed in several fish species during or following periods of acute internal acidosis. Investigators have utilized a variety of experimental protocols to induce acute acidosis (Nakano and Tomlinson, 1967; Opdyke, et al., 1982; Perry, et al., 1986a,b,c; Boutilier, et al., 1986; Primmett, et al., 1986; Butler, et al., 1986; Ristori and Laurent, 1986). During such stressful conditions, fish must cope with the detrimental effects of the acidosis, per se, and also the deleterious consequences of reduced pH on branchial oxygen uptake and blood oxygen transport due to Bohr and Root effects (e.g. Root and Irving, 1943). Consequently, it has been suggested that the increased plasma levels of catecholamines asssociated with acidosis in fishes may be important for optimizing oxygen transfer and transport as well as initiating a series of acid-base regulatory responses (see review by Wood and Perry, 1985). However, with few exceptions, the evidence supporting a role for catecholamines in acid-base balance or control of oxygen transport during physiological acid-base disturbances is circumstantial and has arisen primarily from studies (in vivo and in vitro) examining the effects of abnormally high exogenous catecholamine levels on selected physiological systems (for relevant reviews, see Nikinmaa, 1986; Wood and Perry, 1985; Randall and Daxboeck, 1984). In the preceding four chapters (2, 3, 4 and 5), it was reported that prolonged intravascular infusion of epinephrine in rainbow trout simulated the plasma levels of this hormone measured during the initial few hours of hypercapnic acidosis ($5x10^{-8}$ M; Perry et al., 1986a) and

promoted numerous branchial, renal, and erythrocytic responses consistent with the suggested involvement of epinephrine in controlling blood acid-base status and oxygen content. These results, obtained at physiological epinephrine levels, demonstrate the potential for epinephrine to regulate acid-base disturbances, but direct evidence is still lacking.

In this chapter, a direct assessment of the involvement of catecholamines in controlling arterial oxygen content and acid-base status during hypercapnia will be attempted by monitoring selected blood respiratory and acid-base variables following selective alpha- or beta-adrenergic blockade.

MATERIALS AND METHODS

Experimental Animals:

Rainbow trout (Salmo gairdneri) of either sex, weighing between 110 and 287g (mean weight= 202 ± 5.7 g(SEM); N= 70) were obtained from Thistle Springs Trout Farm, (Ashton, Ontario) and transported in oxygenated water to the University of Ottawa. Fish were maintained according to the procedures outlined in chapter 2.

In Vivo Protocol

Dorsal aortic cannulae were implanted into the rainbow trout according to the animal preparation methods outlined in chapter 2. Maintenance of the animals was also as previously described in chapter 2.

Experiments consisted of monitoring blood acid-base status and respiratory variables during 12 h of normocapnia or external hypercapnia (1% CO_2) following pre-treatment of the fish with alpha (phentolamine; Ciba-Geigy) and/or beta (propranolol; Sigma) adrenergic antagonists. Propranolol and phentolamine were dissolved in saline immediately before use. The antagonist solution was adjusted to a pH of 7.8 and injected slowly into the dorsal aorta (approximately 0.5 ml) at a dose of 2 $mg \cdot kg^{-1}$ body weight. Normocapnic and hypercapnic control fish were injected in a similar manner with 0.5 ml Cortland saline (Wolf, 1963).

In normocapnic experiments, a blood sample (0.6 ml) was withdrawn slowly 1 h prior to injection of the antagonist(s) and at 1, 4, 8 and 12 h following the injection. In hypercapnic experiments, fish were first injected with the antagonist(s) and a blood sample was taken

1 h after this injection; hypercapnia was initiated and additionnal blood samples were taken at 1, 4, 8 and 12 h.

Hct, pH_e, rbc pH, total oxygen content (C_{O2}), C_{CO2} and PO_2 were determined immediately on each blood sample as described below. Approximately 0.6 ml of saline was reinjected slowly into the dorsal aorta after each sample, to partially restore blood volume.

External hypercapnia was achieved by equilibrating the inflowing water with approximately 1% CO_2 (PCO_2 = 7.5 torr). This was accomplished by allowing water and 7% CO_2 in air to flow in opposite directions through a large surface area gas exchange column. Surface area was increased by filling the column with marbles (diameter = 1 cm). By carefully adjusting the water flow rate through the column, the PCO_2 of the inflowing water could be kept constant for extended periods of time. Water flow was approximately 1200 $ml \cdot min^{-1}$ and was sufficient to supply inflowing water to 3 fish simultaneously. A Wosthoff gas mixing pump (Model M301 A-F) was used to supply the 7% CO_2 in air.

In Vitro Protocol:

Blood was withdrawn slowly from cannulated trout that had been allowed to recover from surgery for 48 h (total number of fish used for in vitro experiments = 20; mean weight = 177 ± 14g). The blood was pooled, centrifuged, and some plasma was removed to increase hematocrit to approximately 25%; rbc's were resuspended in the plasma and the blood was divided into two equal portions. One portion was for immediate use while the other was placed on ice for use within 2 h. The blood for immediate use was then divided into 6 round-bottom

flasks (containing 100 units ammonium heparin) and placed in a temperature controlled, shaking water bath set at ambient temperature ($8^{o}C \pm 2^{o}C$). The blood was gassed with 0.2% carbon dioxide in oxygen for 45 min, after which a 0.7 ml sample was removed. Blood was then gassed with 1 or 2% CO_2 in O_2 following the addition of either 20 ul of $2x10^{-4}$ M epinephrine (dissolved in saline before use and pH adjusted to 7.8-8.0 with NaOH) or 20 ul of saline. The estimated final concentration of epinephrine was $2x10^{-6}$ M. Samples were taken after 45 min of gassing. This procedure was then repeated with the blood which had been placed on ice. Commercial gas mixtures (Air Products Inc.) and/or a Wosthoff gas-mixing pump were used.

Hct, pH_e, rbc pH, PO_2, CO_2 and C_{CO2} were determined immediately upon blood sampling. Analysis of these variables was as previously described in chapter 2. CO_2 was measured utilizing the method of Tucker (1967) using a Radiometer E5046 PO_2 electrode in a chamber thermostatted to ambient water temperature.

RESULTS

In Vitro Experiments:

The relationship between pH_e and rbc pH, *in vitro*, is illustrated in Fig. 28 and is given by the equation: rbc pH = 2.82 + 0.586 pH_e. This relationship was obtained by titrating blood with a range of carbon dioxide-oxygen gas mixtures between 0 and 2% CO_2. The extent of the Root effect in trout blood was estimated using a similar protocol. The data in Fig. 29 demonstrate that trout blood displays a pronounced CO_2- induced Root effect. A decrease in pH_e from 7.85 to 7.40 (the pH_e reduction observed during hypercapnic acidosis, *in vivo*) caused C_{O2} to decrease by 29%. The Root effect ultimately is a function of the rbc pH and because the slope of the regression between pH_e and rbc pH was less than unity, the magnitude of the Root effect as a function of pH_e was considerably less than as a function of rbc pH (Fig. 29A,B).

The addition of 10^{-7} M epinephrine to trout blood did not abolish the Root effect (Fig. 30) although it did reduce the extent of the oxygen content depression. The data have not been statistically analysed because the experiments were performed on blood pooled from 6 animals. The means by which epinephrine reduces the magnitude of the Root effect may involve mechanisms other than preferential rbc pH regulation because in this particular experiment, rbc pH was not markedly increased by epinephrine treatment although the pH gradient across the rbc membrane (pH_e - rbc pH) did decrease substantially.

In Vivo Experiments:

Serial blood sampling and resultant anemia caused C_{O2} to

Figure 28: The *in vitro* relationship between pH_e and rbc pH at 10°C. The relationship was obtained by equilibrating pooled rainbow trout blood obtained from chronically catheterized fish (N=20) with a range of CO_2 tensions from 0 to 2% CO_2 in air.

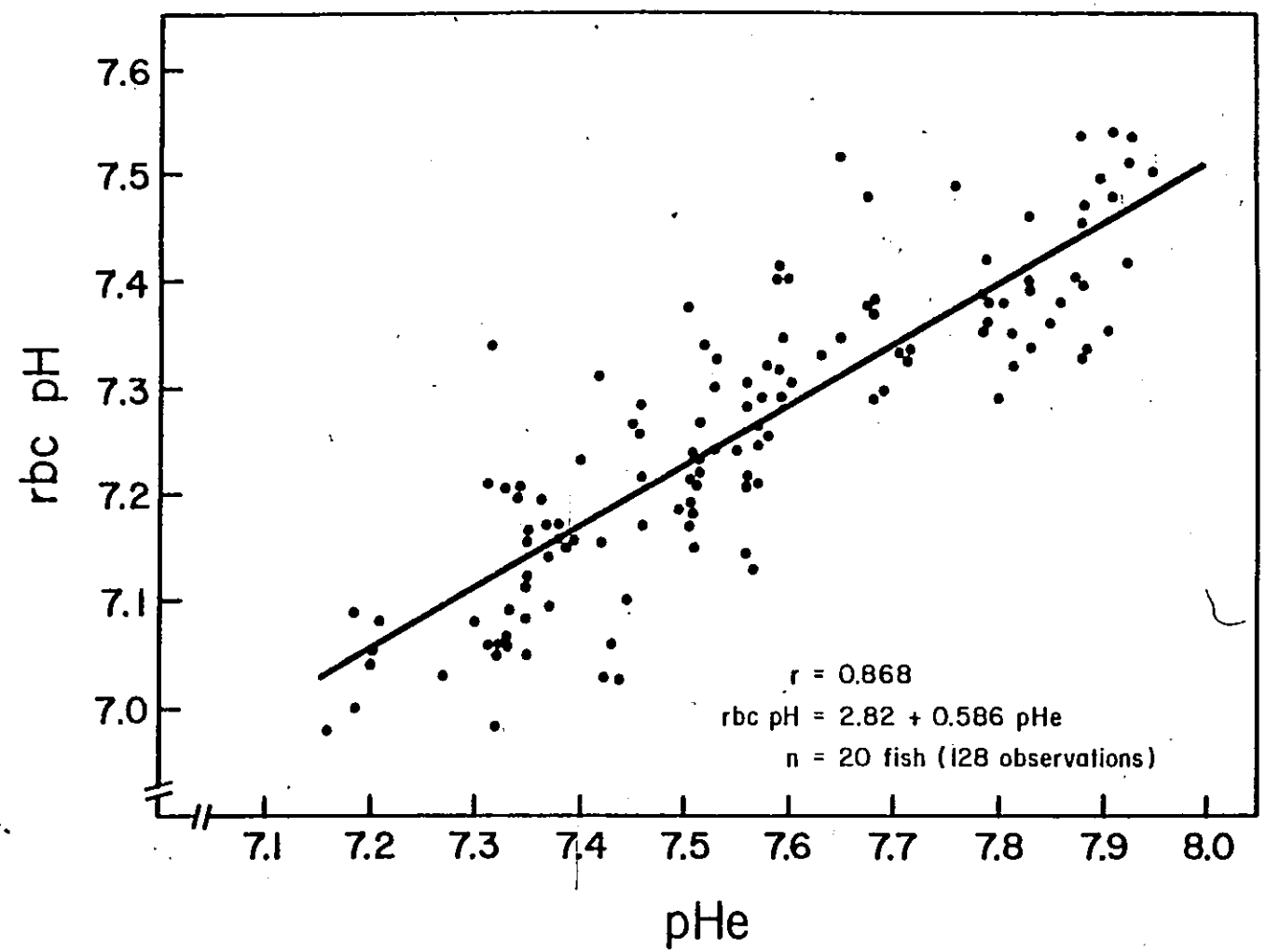

7.6
7.5
7.4
7.3
7.2
7.1
7.0
rbc pH
7.1
7.2
7.3
7.4
7.5
7.6
7.7
7.8
7.9
8.0
pHe
r = 0.868
rbc pH = 2.82 + 0.586 pHe
n = 20 fish (128 observations)

Figure 29: The CO_2 induced Root effect in rainbow trout blood at $10^{\circ}C$ *in vitro* expressed as a function of A) rbc pH or B) pH_e. Values of r for both relationships are statistically significant ($P<0.05$).

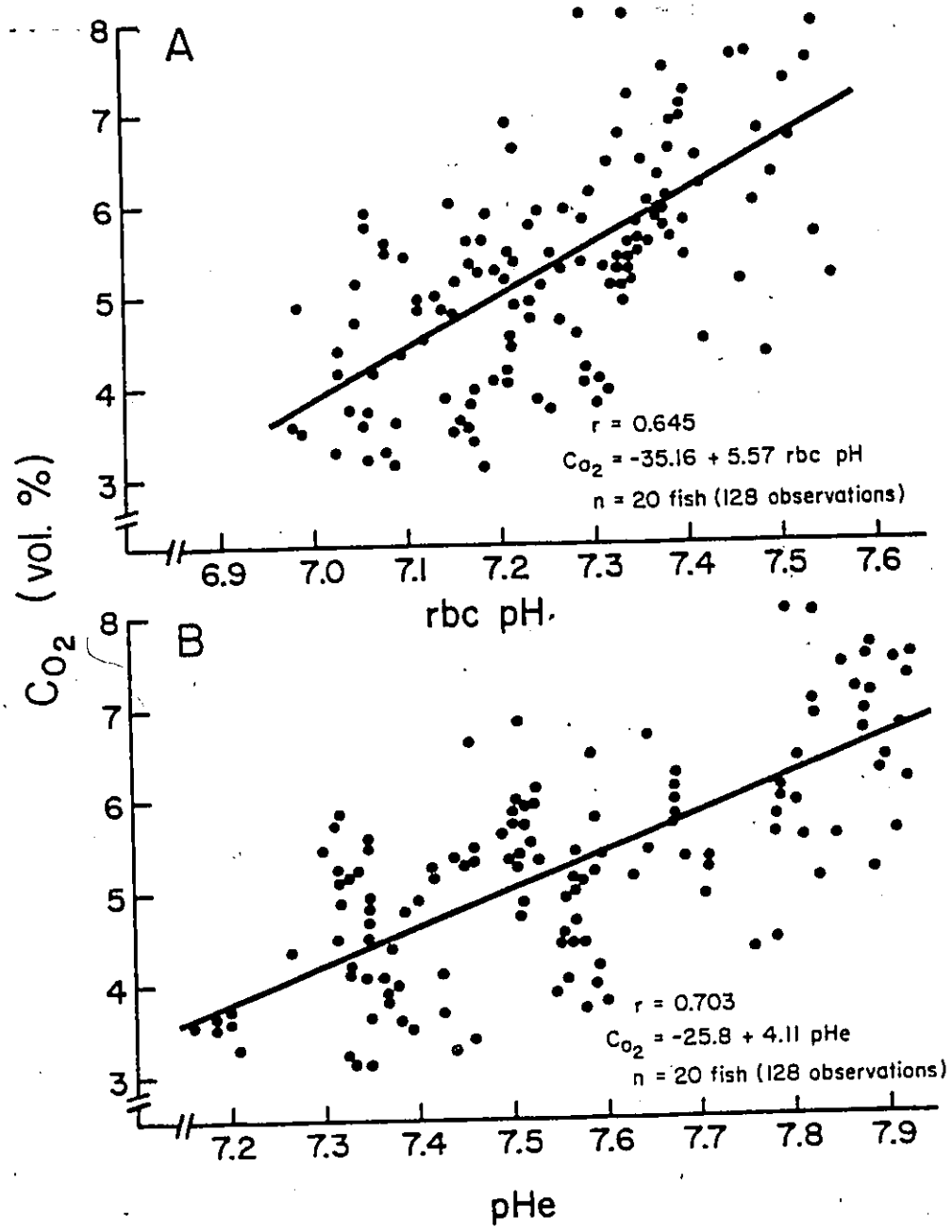

A
8
7
6
5
4
3
6.9
7.0
7.1
7.2
7.3
7.4
7.5
7.6
rbc pH
C_{O_2} (vol. %)
r = 0.645
C_{O_2} = -35.16 + 5.57 rbc pH
n = 20 fish (128 observations)
B
8
7
6
5
4
3
7.2
7.3
7.4
7.5
7.6
7.7
7.8
7.9
pHe
C_{O_2}
r = 0.703
C_{O_2} = -25.8 + 4.11 pHe
n = 20 fish (128 observations)

Figure 30: The *in vitro* effects of increased blood PCO_2, in the absence or presence of elevated epinephrine (approximately $2x10^{-6}$ M; stipled bars), on A) blood oxygen content (C_{O2}), B) whole blood pH (pH_e), C) red blood cell pH (rbc pH) and D) the rbc transmembrane pH gradient (pH_e - rbc pH). Values were obtained from blood pooled from 6 chronically catheterized rainbow trout, hence standard error bars have not been included. See text for further details.

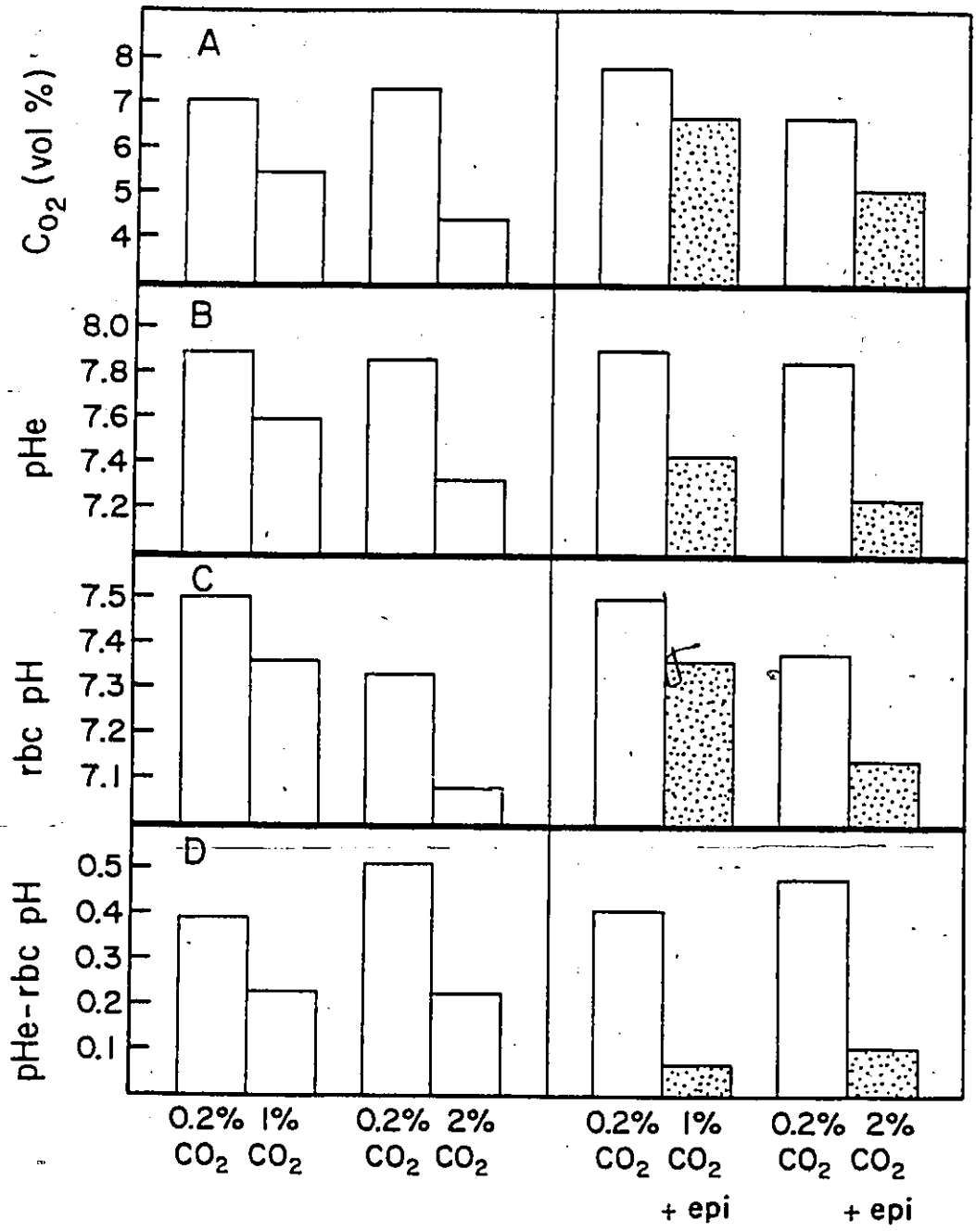
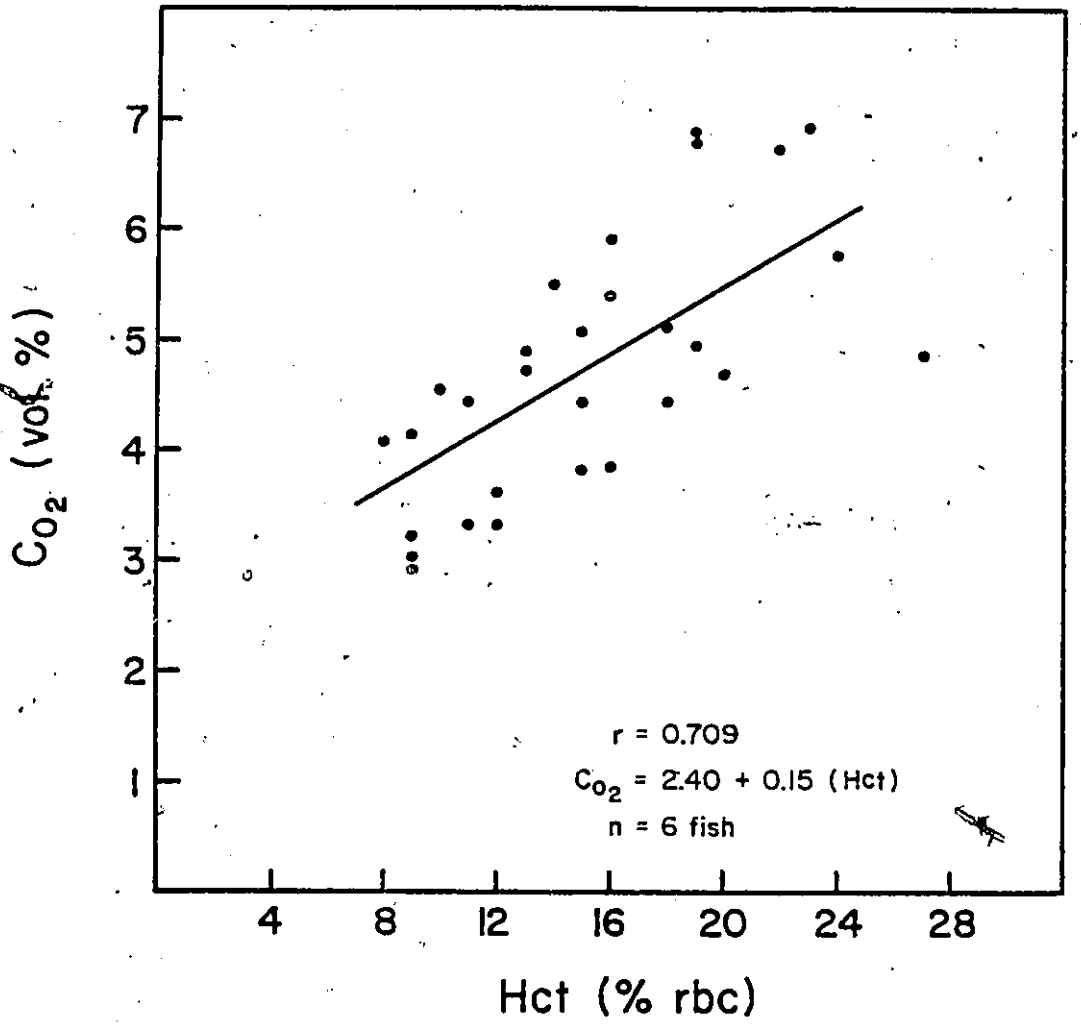

A
B
C
D
C_{O_2} (vol %)
8
7
6
5
4
pHe
8.0
7.8
7.6
7.4
7.2
rbc pH
7.5
7.4
7.3
7.2
7.1
pHe-rbc pH
0.5
0.4
0.3
0.2
0.1
0.2% 1% CO_2 CO_2
0.2% 2% CO_2 CO_2
0.2% 1% CO_2 CO_2 + epi
0.2% 2% CO_2 CO_2 + epi

Figure 31. The in vivo relationship between arterial total oxygen content (C_{O2}) and hct. This relationship was obtained from 6 chronically cannulated fish from which five consecutive blood samples were removed, according to the sampling protocol for experimental fish. This relationship was used to account for sampling dilution in experimental fish. Value of r is significant ($P<0.05$).


C_{O_2} (vol %)
7
6
5
4
3
2
1
4
8
12
16
20
24
28
Hct (% rbc)
r = 0.709
C_{O_2} = 2.40 + 0.15 (Hct)
n = 6 fish

decrease regardless of the treatment. For this reason, both absolute and corrected C_{O2} values are presented in the figures. The corrected C_{O2} values were obtained by adjusting the absolute C_{O2} according to the inverse relationship between C_{O2} and hct that was established in saline-injected normocapnic fish (C_{O2} = 0.15*hct + 2.40; from Fig. 31). Due to the variability of the initial hcts among treatment groups, all initial C_{O2}'s were standardized to a starting hct of 25%, once again using the relationship C_{O2} = 0.15*hct + 2.40. These two corrections allowed effective comparisons to be made between different experimental groups as well as between different periods for the same group.

NORMOCAPNIA

Injection of normocaphic fish with the adrenergic antagonists, phentolamine or propranolol, caused no significant changes in any measured blood parameter (Figs. 32, 33). Occasionally, significant differences were observed between the various treatment groups (Fig. 32A,D) but these differences were unrelated to antagonist injection. Most notably, rbc pH was depressed in the propranolol and phentolamine-treated fish (Fig. 33C) and P_aO_2 elevated in the propranolol-injected fish (Fig. 32B).

HYPERCAPNIA

Blood Acid-Base Variables

External hypercapnia, under control conditions, caused typical changes in blood acid-base status (Fig. 34). Arterial PCO_2 was elevated immediately upon exposure to increased ambient PCO_2 and remained elevated throughout the hypercapnic period. As a result of increased PCO_2, whole blood pH (pH_e) and rbc were decreased.

Figure 32: The effects of intravascular bolus injections ($2mg \cdot kg^{-1}$) of the adrenergic antagonists phentolamine (alpha-adrenoceptor antagonist; solid triangles; N=6) or propranolol (beta-adrenoceptor antagonist; solid squares; N=6) under normocapnic conditions on arterial A) hematocrit (hct), B) blood oxygen content (C_{O2}), C) blood oxygen content corrected for sampling dilutions and normalized to starting hct of 25% (corrected C_{O2}, and D) partial pressure of oxygen (PO_2). Control fish (solid circles) were injected with 0.6 ml Cortland saline. Asterisks denote significant differences from control values at corresponding time. PI = Pre-injection.

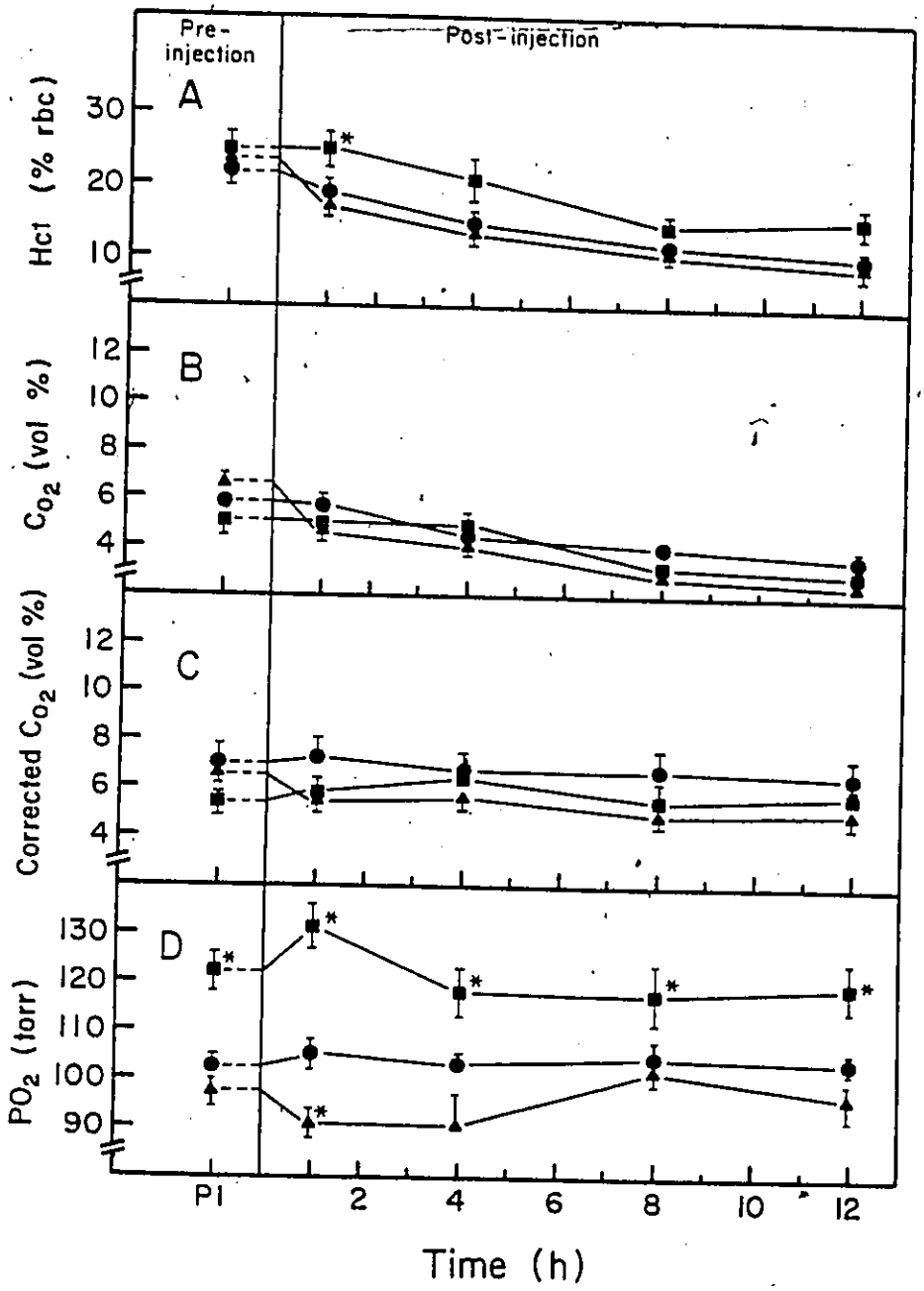

Pre-injection
Post-injection
A
Hct (% rbc)
30
20
10
B
C_{O_2} (vol %)
12
10
8
6
4
C
Corrected C_{O_2} (vol %)
12
10
8
6
4
D
P_{O_2} (torr)
130
120
110
100
90
PI
2
4
6
8
10
12
Time (h)

Figure 33: The effects of intravascular bolus injections ($2 mg \cdot kg^{-1}$) of the adrenergic antagonist phentolamine (alpha-adrenoceptor antagonist; solid triangles; N=6) or propranolol (beta-adrenoceptor antagonist; solid squares; N=6) under normocapnic conditions on arterial A) carbon dioxide tension, B) whole blood pH (pH_e), C) red blood cell pH (rbc pH), D) the rbc transmembrane pH gradient (pH_e - rbc pH) and E) bicarbonate concentration ($[HCO_3^-]$). Control fish (solid circles; N=6) were injected with 0.6 ml of Cortland saline. Asterisks denote significant differences from control values at corresponding time. PI = Pre-injection.

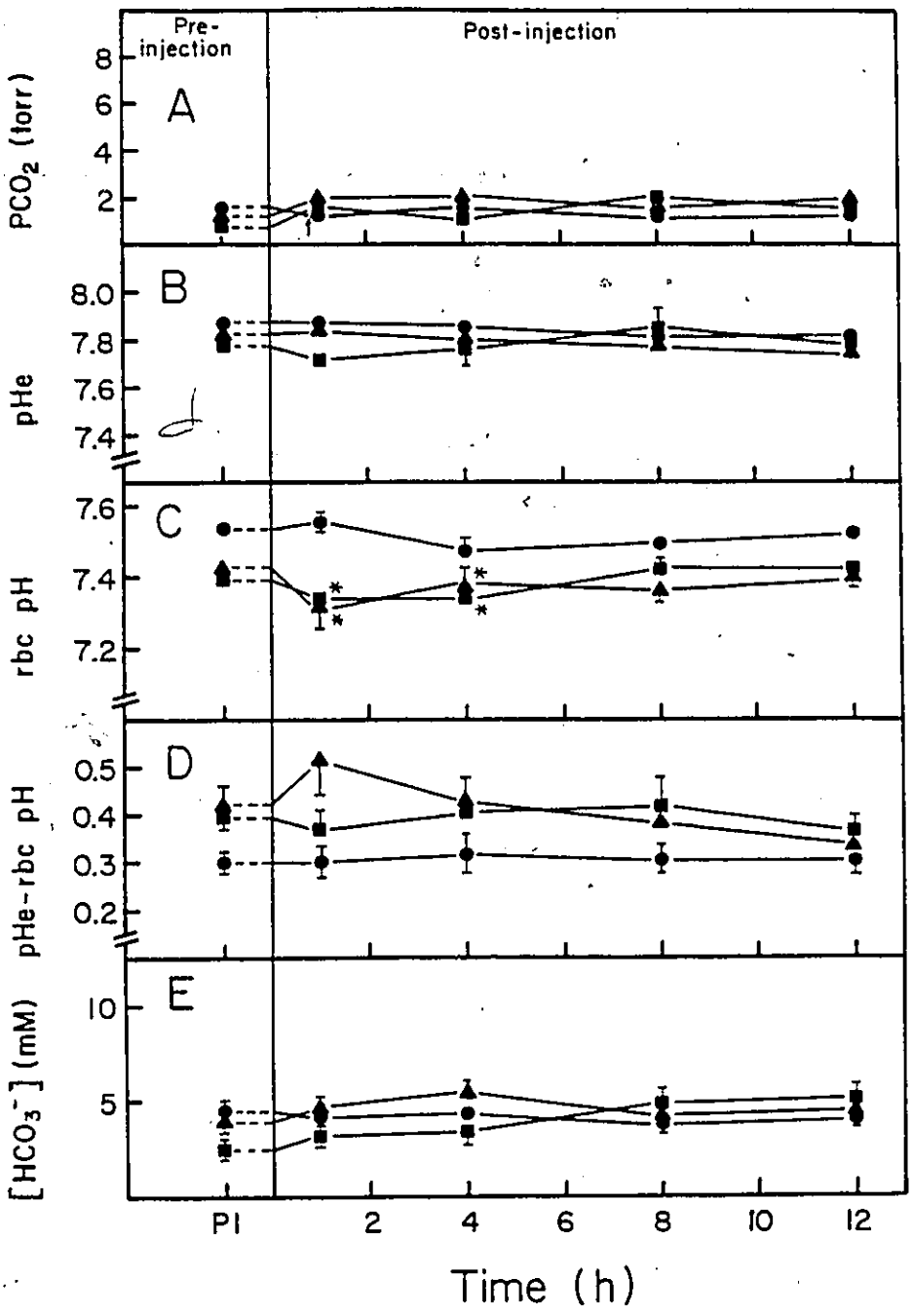
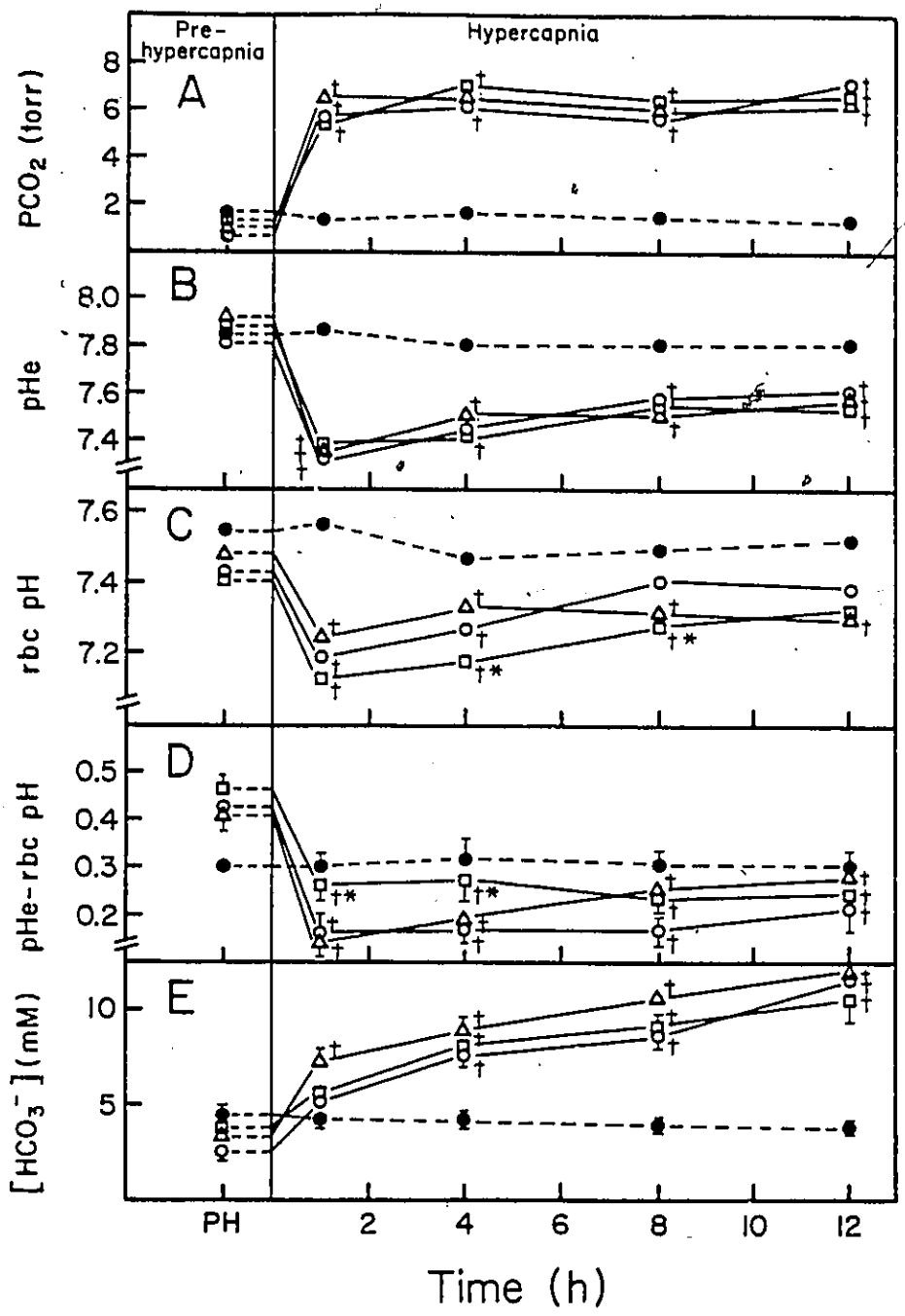

Pre-injection
Post-injection
A
B
C
D
E
PCO_2 (torr)
pHe
rbc pH
pHe-rbc pH
$[HCO_3^-]$ (mM)
PI
2
4
6
8
10
12
Time (h)

Figure 34: The effects of alpha- or beta-adrenergic blockade on selected blood acid-base variables during acute hypercapnia in rainbow trout. Fish were given an intravascular bolus injection ($2mg \cdot kg^{-1}$) of the particular antagonist or saline (0.6 ml; control hypercapnia fish) 1 h before withdrawing an initial pre-hypercapnic (PH) blood sample and the onset of hypercapnia. Solid circles = normocapnic control fish (N=6); open circles = hypercapnic control fish (N=6); open triangles = phentolamine (alpha-adrenoceptor antagonist) injected hypercapnic fish (N=6); open squares = propranolol (beta adrenoceptor blocker) injected hypercapnic fish (N=6). A) arterial carbon dioxide tension (PCO_2), B) whole blood pH (pH_e), C) red blood cell pH (rbc pH), D) the transmembrane pH gradient (pH_e - rbc pH) and E) bicarbonate concentration ($[HCO_3^-]$). Daggers denote significant differences from pre-hypercapnic (PH) values; asterisks denote significant differences from hypercapnic control values at corresponding time. See text for further details.


Pre-hypercapnia
Hypercapnia
A
B
C
D
E
PCO_2 (torr)
pHe
rbc pH
pHe-rbc pH
$[HCO_3^-]$ (mM)
8
6
4
2
8.0
7.8
7.6
7.4
7.6
7.4
7.2
0.5
0.4
0.3
0.2
10
5
PH
2
4
6
8
10
12
Time (h)

pH_e was reduced to a greater extent than rbc pH thereby causing the pH gradient across the rbc membrane (pH_e - rbc pH) to decrease significantly (Fig. 34D). Both pH_e and rbc pH gradually increased toward pre-hypercapnic values throughout the 12 h hypercapnic period due to gradual elevation of plasma HCO_3^- levels (Fig. 34E).

Pre-treatment of fish with adrenergic antagonists did not alter the pattern of extracellular acid-base adjustments during hypercapnia, including the gradual elevation of plasma HCO_3^-. However, pre-treatment with the beta-antagonist propranolol, did cause rbc pH to decrease and pH_e - rbc pH to increase significantly compared to controls.

A comparison between the in vivo and in vitro pH_e versus rbc pH relationship is shown in Fig. 35. Under control conditions, rbc pH, as measured in vivo during hypercapnia, was significantly and constantly elevated above the values predicted from the in vitro relationship (rbc pH = 2.82 + 0.586 pH_e; Fig. 35). Propranolol completely abolished the ability of fish to raise rbc pH above the in vitro predicted values during the final 8 h of hypercapnia (Fig. 35B). Pre-treatment with the alpha-antagonist phentolamine had no effect on the in vivo pH_e versus rbc pH relationship (Fig. 35C).

Blood Respiratory Variables:

Under control conditions, arterial oxygen content remained constant during hypercapnia (Fig. 36C) despite the severe extracellular acidosis and the presence of a marked Root effect in vitro. The maintenance of C_{O2} was due primarily to elevated hct and PO_2 (Fig. 36A,D) and to a lesser extent the partial regulation of rbc pH.

Figure 35: The relationships between whole blood pH (pH_e) and red blood cell pH (rbc pH) both *in vitro* and *in vivo*, prior to and during 12 h of hypercapnia in rainbow trout under A) control conditions (solid circles; N=6); B) following beta-adrenoceptor blockade (open squares ; N=6) and C) following alpha-adrenoceptor blockade (open triangles; N=6). The *in vitro* regression between pH_e and rbc pH was obtained from Fig. 28 and the shaded area represents the 95% confidence interval about this regression line. P = pre-hypercapnic value. See Figs. 28 and 33 for further details.

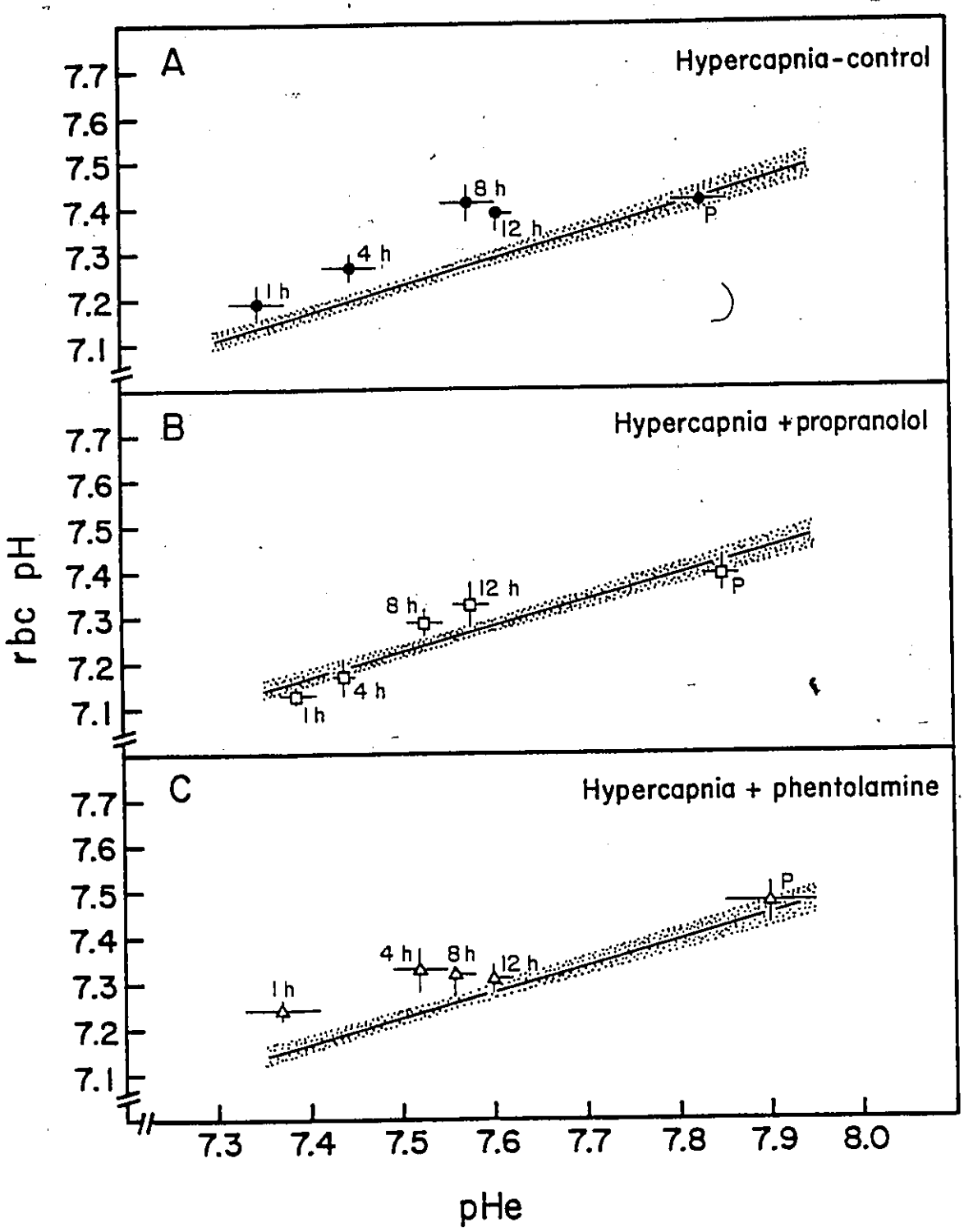
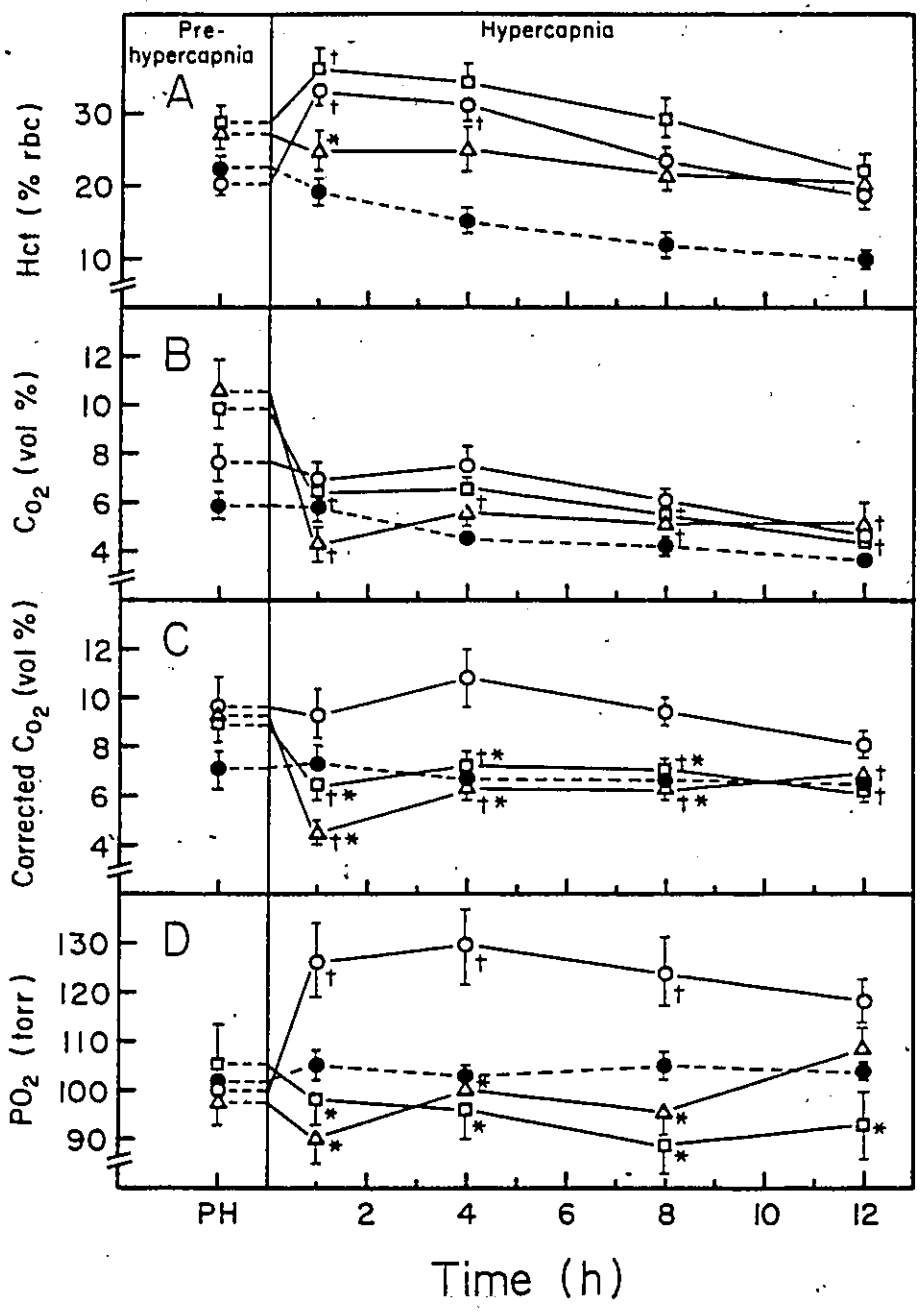

A
Hypercapnia-control
B
Hypercapnia +propranolol
C
Hypercapnia + phentolamine
1 h
4 h
8 h
12 h
P
rbc pH
pHe
7.1
7.2
7.3
7.4
7.5
7.6
7.7
7.3
7.4
7.5
7.6
7.7
7.8
7.9
8.0

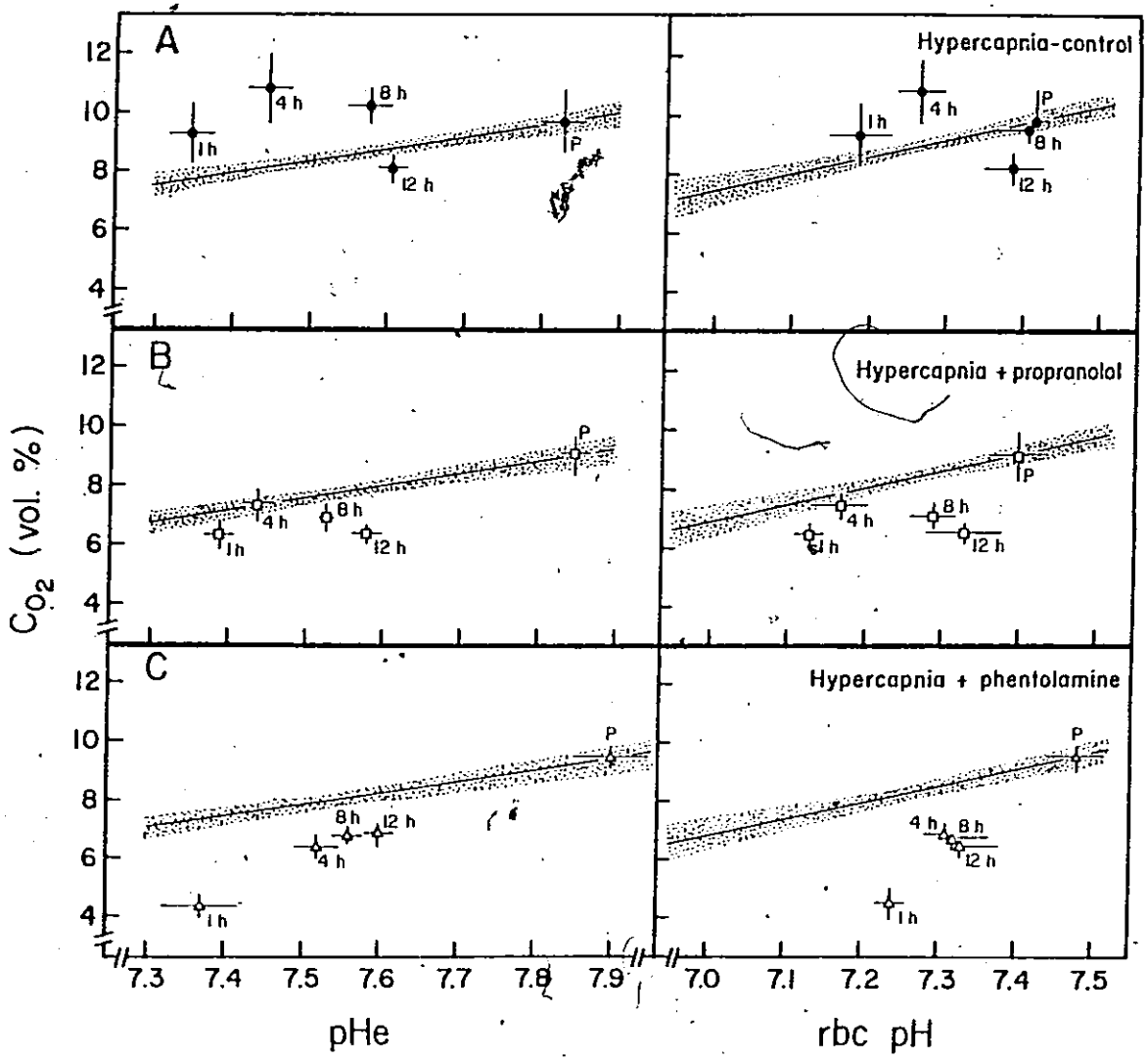
Figure 36: The effects of alpha- or beta-adrenergic blockade on selected respiratory variables during acute hypercapnia in rainbow trout. Fish were given an intravascular bolus injection ($2 mg \cdot kg^{-1}$) of the particular antagonist or saline (0.6 ml; control hypercapnia fish) 1 h before withdrawing an initial pre-hypercapnic (PH) blood sample and the onset of hypercapnia. Solid circles = normocapnic control fish (N=6); open circles = hypercapnic control fish (N=6); open triangles = phentolamine (alpha-adrenoceptor antagonist) injected hypercapnic fish (N=6); open squares = propranolol (beta-adrenoceptor blocker) injected hypercapnic fish (N=6). A) arterial hematocrit (hct), B) arterial oxygen content (C_{O2}), C) arterial oxygen content corrected for sampling dilutions and normalized to an initial hct of 25%, D) partial pressure of oxygen in arterial blood (PO_2). Daggers denote significant differences from pre-hypercapnic (PH) values; asterisks denote significant differences from hypercapnic control value at corresponding time. See text for further details.


Pre-
hypercapnia
Hypercapnia
A
B
C
D
Hct (% rbc)
C_{O_2} (vol %)
Corrected C_{O_2} (vol %)
P_{O_2} (torr)
30
20
10
12
10
8
6
4
12
10
8
6
4
130
120
110
100
90
PH
2
4
6
8
10
12
Time (h)

Pre-treatment of fish with either propranolol or phentolamine caused C_{O2} to decrease during hypercapnia (Fig. 36C). Phentolamine lowered C_{O2} by reducing PO_2 (Fig. 36D) and rbc pH (Fig. 34C).

The extent of the blood oxygen content regulation during hypercapnic acidosis, despite a marked Root effect in trout blood, is illustrated in Fig. 37. Under control conditions, C_{O2} was significantly elevated above the values predicted from the *in vitro* relationship between pH_e and C_{O2} during the initial 8 h of hypercapnia. C_{O2} also was increased above the values predicted from the rbc-C_{O2} relationship but to a lesser extent and for a shorter duration. This lesser deviation of *in vivo* C_{O2} values from predicted *in vitro* values is a reflection of preferential rbc pH regulation. Pre-treatment of fish with adrenergic antagonists caused C_{O2} to decrease below values predicted from the *in vitro* relationship between pH and C_{O2} indicating that additional factors other than the Root effect in hypercapnic trout contribute to the depression of arterial oxygen content.

Figure 37: The relationships between whole blood pH (pH_e) or red blood cell pH (rbc pH) and blood oxygen content (C_{O2}) both *in vitro* and *in vivo*, prior to and during 12 h of hypercapnia in rainbow trout under A) control conditions (solid circles; N=6); B) following beta-adrenoceptor blockade (open squares; N=6) and C) following alpha-adrenoceptor blockade (open triangles; N=6). The *in vitro* regressions between pH_e and C_{O2} and rbc pH and C_{O2} were obtained from Fig. 29 and the shaded area represents the 95% confidence interval about this regression line. P = pre-hypercapnic value. See Figs. 28 and 27 for further details.


A
B
C
Hypercapnia-control
Hypercapnia + propranolol
Hypercapnia + phentolamine
1 h
4 h
8 h
12 h
P
C_{O_2} (vol. %)
pHe
rbc pH
12
10
8
6
4
7.3
7.4
7.5
7.6
7.7
7.8
7.9
7.0
7.1
7.2
7.3
7.4
7.5

DISCUSSION

The results of the present investigation clearly demonstrate an essential role for catecholamines in regulating arterial blood oxygen content during hypercapnic acidosis but on the other hand do not support an important involvement of catecholamines in extracellular acid-base balance. The various mechanisms involved in controlling blood oxygen content and acid-base status during hypercapnia and the importance and nature of the adrenergic interactions with these mechanisms are discussed subsequently.

Blood Oxygen Transport

Despite the presence of a marked Root effect *in vitro* and a pronounced extracellular acidosis *in vivo*, arterial blood oxygen content remained constant during hypercapnia. This result differs from other studies in which slight reductions of blood O_2 content during hypercapnia were reported (Eddy, 1976; Eddy, Lomholt, Weber and Johansen, 1977; Smith and Jones, 1982). Comparisons between the various studies, however, may not be valid due to the differences in sampling times, ambient CO_2 tensions and other relevant environmental conditions (e.g. water temperature and season). Furthermore, it is unclear whether blood O_2 contents were corrected for serial sampling dilutions in the previous studies.

At least three factors, under adrenergic control, contributed to the maintenance of arterial oxygen content in hypercapnic fish; i) an increase in PO_2, ii) elevation of blood oxygen carrying capacity, and iii) preferential regulation of rbc pH.

The elevation of arterial PO_2 during hypercapnia was abolished

during selective alpha- or beta-adrenoceptor antagonism indicating the importance of both types of adrenergic receptors in controlling PO_2. In fishes, arterial PO_2 is sensitive to changes in gill ventilation and perfusion, as well as the diffusive properties of the gill epithelium. Branchial ventilation was not measured in these experiments but the results of previous studies (Janssen and Randall, 1975; Smith and Jones, 1982) clearly demonstrated a hyperventilatory response in rainbow trout during environmental hypercapnia. The involvement of catecholamines in controlling ventilation in rainbow trout is unknown but in the eel, Anguilla anguilla, stimulation of beta-adrenoceptors causes hyperventilation whereas stimulation of alpha-adrenoceptors causes hypoventilation (Peyraud-Waitzenegger, 1979). During summer months, the beta-adrenoceptor mediated response apparently is dominant (Peyraud-Waitzenegger, Barthelemy and Peyraud, 1980) and the net effect of intravascular epinephrine injection in the eel is hyperventilation and elevation of PO_2 (Peyraud-Waitzenegger, 1979). The converse situation apparently exists during the winter season. Applying these conclusions to our results that were obtained during the summer, it is suggested that beta-adrenoceptor mediated stimulation of ventilation was an important factor contributing to the elevation of PO_2 during hypercapnic acidosis. Clearly, the adrenergic control of ventilation during acid-base disturbances is an area that warrants further research.

The involvement of catecholamines in modulating branchial diffusive conductance of oxygen has been examined in perfused gill preparations in which the convective processes (ventilation and perfusion) are

eliminated as dependent variables. In this manner, Pettersson (1983) and Perry et al., (1985) demonstrated that epinephrine enhanced gill O_2 diffusion in saline-perfused cod (Gadus morhua) and rainbow trout heads, respectively. Both studies suggested a role for beta-adrenoceptors in controlling O_2 diffusion by increasing lamellar suface area (Booth, 1979) and/or epithelial O_2 permeability (Haywood, Isaia and Maetz, 1977). A significant difference between the two perfusion studies was alpha-adrenoceptor mediated stimulation of O_2 diffusion in the cod head but inhibition in the trout head. Reasons for this discrepancy are unclear although Perry et al., (1985) suggested alpha-adrenoceptor mediated inhibiton of gill O_2 permeability was the predominant response in the perfused trout head while Pettersson (1983) argued that lamellar recruitment was predominant in the cod head. On the basis of our results and previous studies performed on trout (Booth, 1979; Perry et al., 1985), we speculate that beta-adrenoceptor mediated stimulation of gill O_2 diffusive conductance is an important factor promoting the increase in arterial PO_2 during hypercapnia. Additionally, we cannot exclude the possibility of alpha-adrenoceptor mediated enhancement of diffusive conductance given the results of Pettersson (1983) that suggested alpha-adrenergic stimulation may cause lamellar recruitment by constricting efferent lamellar arterioles. One additional process that might contribute to the increased arterial oxygen tension during hypercapnia is an increase in non-respiratory intralamellar blood shunting (Part, Tuurala, Nikinmaa and Kiessling, 1984) as discussed by Nikinmaa et al., (1984). Briefly, an increase in the proportion of blood flow within non-respiratory basal

portions of lamellae and subsequent mixting of this acidic blood with arterial blood would cause hemoglobin to release some oxygen into solution.

A second factor contributing to maintenance of arterial oxygen content during hypercapnia was a significant elevation of blood oxygen carrying capacity as evidenced by a pronounced increase in haematocrit (from 20 to 33%; Fig. 36A). Although hemoglobin levels were not measured in the present study, it is believed that the increase in hematocrit during hypercapnia was due primarily to an increased number of circulating rbcs and to a lesser extent, rbc swelling (a consequence of increased rbc PCO_2 and adrenergic stimulation of rbc Na^+/H^+ exchange, see below). This view is supported by the observation that hypercapnic acidosis, in vitro, in the presence of absence of elevated epinephrine levels, causes relatively minor increases in hematocrit (Perry et al., 1986a). The increase in blood oxygen carrying capacity reported here was mediated exclusively by alpha-adrenoceptors and presumably resulted from contraction of the spleen and concomitant release of rbcs into the circulation (Nilsson and Grove, 1974; Yamamoto et al., 1980) as well as reductions in plasma volume caused by diuresis (Tetens and Lykkeboe, 1985; see also chapter 5), fluid shifts between various internal compartments (Yamamoto et al., 1980) and plasma skimming (Nikinmaa, 1982a; Olson, 1984). In two recent studies, blood hemoglobin levels were shown to increase following exhausting exercise in striped bass (Nikinmaa, et al., 1984) and rainbow trout (Primmett et al., 1986). In striped bass, the increase in hemoglobin concentration was not prevented by

pre-treatment with the beta-adrenoceptor antagonist, propranolol, and this is consistent with the results presented here, demonstrating alpha-adrenoceptor regulation of arterial oxygen carrying capacity. On the other hand, Nikinmaa (1982b) reported that the circulating number of rbcs was increased by both alpha- and beta-adrenergic mechanisms following a bolus injection of epinephrine in rainbow trout.

The third factor contributing to the maintenance of arterial O_2 content during hypercapnia was preferential regulation of rbc pH as shown in Fig. 34. The elevation of rbc pH at any given pH_e above those values predicted from the in vitro (pH_e - rbc pH) relationship was entirely dependent upon beta-adrenoceptor stimulation. As such, our results agree with the results of numerous in vivo and in vitro studies that have shown beta-adrenoceptor mediated alkalinization of the fish rbc (Nikinmaa, 1982b; Nikinmaa et al., 1984; Nikinmaa and Huestis, 1984; Primmett et al., 1986; Perry et al., 1986a). The mechanism responsible for adrenergic elevation of rbc pH is stimulation of Na^+/H^+ exchange (Baroin, Garcia-Romeu, Lamarre and Motais, 1984; Cossins and Richardson, 1985). The partial regulation of rbc pH caused by the increased rate of rbc transmembrane Na^+/H^+ exchange not only is important for minimizing the Root effect during extracellular acidosis (see also Boutilier et al., 1986; Primmett, et al., 1986) but also promotes rbc swelling which in turn may cause an increase in HbO_2 affinity due to decreased cellular levels of nucleoside triphosphates (NTP) (see review by Nikinmaa, 1986).

It is difficult to judge the relative importances of the various adrenergic mechanisms that control arterial O_2 content during

hypercapnia. However, the results of the in vitro hypercapnic experiments (Fig. 32) suggest that beta-adrenoceptor mediated stimulation of rbc Na^+/H^+ exchange may be less important than the increases in gill diffusive and ventilatory O_2 conductances and the elevation of blood oxygen carrying capacity. It is worth noting that blood oxygen content was depressed to the greatest extent during hypercapnia following alpha-adrenergic blockade. This suggests that the alpha-adrenoceptor mediated increase in blood O_2 carrying capacity may be the most significant response. However, it was only following complete adrenergic blockade (after pre-treatment with both propranolol and phentolamine) that fish actually succumbed, presumably due to severe blood hypoxemia.

Acid-Base Balance

The changes in blood acid-base status reported in the present study are similar to those reported previously for a variety of freshwater fishes exposed to hypercapnic water (Cameron and Randall, 1972; Janssen and Randall, 1975; Eddy, 1976; Eddy et al., 1977; Cameron, 1980; Thomas and LeRuz, 1982; Thomas, Fievet, Barthelemy and Peyraud, 1983; Perry, Haswell, Randall and Farrell, 1981; Claiborne and Heisler, 1984, 1986; Perry et al., 1986a). These studies have clearly demonstrated that fish regulate hypercapnic acidosis by gradually accumulating bicarbonate in the various internal compartments at constant blood PCO_2 (see review by Heisler, 1984). In trout, the predominant mechanism causing plasma bicarbonate levels to increase during periods of hypercapnic acidosis is sustained inhibition of branchial Cl^-/HCO_3^- exchange (Wood et al., 1984; Perry et al., 1986a)

and an associated stimulation of branchial acid efflux (base influx). Additionally, increased renal acid excretion also contributes to bicarbonate accumulation although to a much lesser extent (Wheatly et al., 1984; Perry et al., 1986b).

The control of branchial and renal acid-excretion is poorly understood although recent studies have indicated that catecholamines might play an important role (Perry et al., 1984a; chapters 4 and 5). Indeed, simulation of elevated circulating epinephrine levels by prolonged intravascular infusion of epinephrine promoted numerous branchial and renal responses that have been observed during periods of respiratory acidosis (e.g. inhibition of branchial Cl^-/HCO_3^- exchange, stimulation of branchial and renal acid excretion, and increased urinary buffering capacity (chapters 4 and 5). The results of the present study, however, do not support an essential role for epinephrine in regulating extracellular acid-base disturbances although the possibility of partial involvement cannot be excluded. In other words, the observation that the pattern of plasma HCO_3^- accumulation and pH_e regulation were unaffected by alpha- or beta-adrenoceptor blockade could either reflect the complete absence of an adrenergic role in extracellular acid-base balance or alternatively indicate that there are additional neural or humoral factors interacting with acid-base regulatory mechanisms. Presumably, epinephrine levels were elevated in the present experiments as shown previously in hypercapnic trout (Perry et al., 1986a) so the potential certainly did exist for epinephrine to interact with branchial and renal acid extrusion processes.

The release of epinephrine from chromaffin tissue and its concomitant entry into the circulatory system during acute hypercapnic acidosis promotes a series of adrenergic responses that serve to stabilize arterial oxygen content by increasing PO_2, elevating blood oxygen carrying capacity, minimizing the reduction in rbc pH, and enhancing the affinity of hemoglobin for oxygen. It is speculated that the pronounced increase in PO_2 results from hyperventilation (a beta-adrenoceptor response) and enhanced diffusive conductance (due to beta- and/or alpha-adrenoceptor mediated lamellar recruitment and beta-adrenoceptor mediated increases in gill epithelial permeability to oxygen). The elevation of blood oxygen carrying capacity is primarily due to contraction of the spleen (an alpha-adrenoceptor response) and release of rbcs into the circulation but additional factors such as diuresis, fluid shifts between internal compartments and plasma skimming may also be involved. The partial and preferential regulation of rbc pH is a consequence of stimulated acid extrusion from the rbc via Na^+/H^+ exchange (a beta-adrenoceptor response). The stimulation of Na^+/H^+ exchange causes rbc swelling which, in turn, lowers the concentration of cellular NTP, thereby also increasing the affinity of hemoglobin for oxygen.

CHAPTER 7

GENERAL DISCUSSION

Regulation of internal acid-base status is of primary importance in living organisms: detrimental metabolic effects caused by sudden and rapid variations of pH could be disastrous to the organism. Indeed, pH regulation is of primordial importance for survival. During recent years, much work has been accomplished in attempting to understand the responses of fish to natural stressors (i.e. hypoxia, hyperoxia, hypercapnia, exercise, temperature fluctuations, changes in environmental osmolarity and ionic content, etc.). It has become apparent through this research that an important response to many of these stress is the release of epinephrine from chromaffin tissue (response also elicited by the direct reduction in blood pH caused by infusion of HCl). The role played by epinephrine in mediating responses to stress in fish has been the object of intensive study, not only in vivo and in vitro but also in perfused preparations (gill, head, whole body perfusions). One of the most outstanding problems of this work has been the increase of catecholamine levels during experimentation to levels which are representative of circulating levels during normal stress. Addition of epinephrine to perfusates or incubations (such as rbc incubations) and single bolus injections into fish have posed problems either due to the very high epinephrine levels used or the rapid metabolism and clearance of epinephrine from the system. It is known that epinephrine levels may remain elevated for as long as 12 h following the onset of a stress; therefore, bolus injections whose effects may dissipate after only 10 min (Nikinmaa, 1982a) are not a solution to the problem of studying epinephrine effects. "Stressed" fish have blood levels of epinephrine around 50 nM (see Table 1): many

of the previous studies either reported epinephrine levels of between 100 and 1000 nM (Nikinmaa, 1982a) or did not report levels as they were not measured. Using the estimation method demonstrated in chapter 2 and also the confirming measurements of Epple and Nibbio (1985), it is likely that the actual levels of epinephrine following a bolus injection were much higher (10-100X) than those measured during actual stress. Thus, in order to more closely simulate elevated epinephrine levels, I have used continuous infusions of epinephrine over either 12 or 24 h periods. This sustained increase simulates more closely both the quantity of epinephrine measured during stress and the duration of these elevated levels. Although this method was thought to be the most satisfactory for elevating epinephrine levels, it also posed some problems: i) infusion of fluid increased urine flow rate (UFR) by a small amount, ii) possible catecholaminotropic effects of elevated catecholamine levels and iii) epinephrine receptor desensitization and/or loss of the cyclic AMP response are known effects of the epinephrine infusion.

The increase in UFR was thought not to be a problem as it was elicited by saline infusion and treating control fish exactly the same as experimental fish (as pertains to the infusion rate) eliminated this variable. Catecholaminotropic (i.e. the elevation of epinephrine levels may cause release of other catecholamines) effects, such as elevated dopamine levels following bolus injections of epinephrine into eels (Epple and Nibbio, 1985) are another problem directly related to epinephrine infusion. Although monitoring of dopamine levels was not done in this study, levels of norepinephrine did not vary with epinephrine infusion,

and this is taken to be an indication that there were no catecholaminotropic effects of epinephrine, although this possibility remains. Desensitization, as discussed in chapter 2, might have been a problem, especially in chapters 2, 4 and 5, where elevated epinephrine levels were maintained for 24 h. Regulation of the acidosis induced by hypercapnia may take up to 72 h (Perry et al., 1986a), by which time most of the epinephrine response is desensitized; therefore, a secondary mechanism must come into play over a longer time period. Thus, the reductions in sensitivity and catecholaminotropic effects of epinephrine may have played a part in the results of these studies, but their effect has been deemed as minimal.

Two possible roles of circulating epinephrine are those of: i) regulating the acid-base disturbance and ii) maintaining the oxygen carrying capacity of the blood. This thesis has attempted to elucidate these roles: i) by directly assessing the effects of increased epinephrine levels by increasing them through infusion, ii) by indirectly assessing the effects of increased levels of epinephrine by blocking these effects during an externally applied stress (hypercapnia), iii) by monitoring the effects of blocking epinephrine responses during epinephrine infusion, and iv) by mimicking the effects of elevated epinephrine levels through the infusion of specific adrenergic antagonists.

Certainly, a role for epinephrine in extracellular acid-base regulation has not been discounted by the results presented here, although as stated in chapter 6, other mechanisms or factors may interact in regulating blood acid-base. Increased blood HCO_3^- levels

were rendered possible by epinephrine induced inhibition of branchial Cl^-/HCO_3^- exchange (chapter 4), renal retention of HCO_3^- (chapter 5) and inhibiton of the rbc Cl^-/HCO_3^- exchange (chapter 3). Associated with epinephrine increases were increases in acid excretion at both the gill and the kidney. Branchial Na^+/H^+ exchange was stimulated, while increases in the buffering capacity of the urine (via increased P_i and NH_4^+ concentrations) aided to increase H^+ excretion.

Although the relative importance of the involvement of epinephrine in acid-base regulation remains questionable, it certainly does play a role in maintaining blood oxygen content during stress. Maintenance of carrying capacity, preferential regulation of rbc pH and elevation of arterial oxygen tension are all mediated by epinephrine release. Indirect evidence has been presented here for an involvement of epinephrine in splenic rbc release (i.e. increased [Hb]; chapter 6) and reduction of plasma volume by diuresis (chapter 5). These effects, along with plasma skimming at the gills and fluid shifts between compartments could serve to increase blood hemoglobin levels. Increases in rbc pH following beta-adrenoceptor stimulation (chapters 2,3,6) show a definitive involvement of epinephrine in preferentially regulating rbc pH following acidification of the extracellular milieu. Accompanying this effect are swelling of the rbc (chapters 2 and 6) and inhibition of transmembrane Cl^-/HCO_3^- shift (which increases pH by changes in intracellular HCO_3^- and Cl^- levels). All of these effects serve to protect the intracellular hemoglobin from the nefarious effects of blood pH changes. Indirect evidence for adrenergic involvement in increasing PO_2 via effects on ventilation and perfusion rates and on gill diffusive

conductance was also presented (chapters 2, 3 and 6): this increased PO_2 is an adaptive response to increased requirements for O_2 during stress.

This short study was an attempt to clarify the role of epinephrine during acid-base imbalances following stress in rainbow trout. Although much is now known about adrenergic responses to stress as a result of this and other recent studies, it is apparent that this is an area that warrants further research. The work presented here supports a primary role for epinephrine in regulating blood oxygen carrying capacity; evidence for a direct role in acid-base regulation was not as strong, but a correlation did exist between epinephrine and acid excretion. Certainly, the methods by which regulation of blood oxygen and acid-base balance in fish remains incompletely understood.

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