CO_2 EXCRETION AND ACID-BASE REGULATION IN THE RAINBOW TROUT, *SALMO GAIRDNERI*

by

MONTY STEPHEN HASWELL

B.S., California State University, Long Beach

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Department of Zoology

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

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ABSTRACT

The role of carbonic anhydrase in carbon dioxide excretion and acid-base regulation in the rainbow trout, Salmo gairdneri has been investigated. While a significant amount of carbonic anhydrase was found in the blood of the trout, calculations based on red cell hemolysates suggest that the probable circulating levels of carbonic anhydrase activity in blood may not be sufficient to account for the observed carbon dioxide excretion. An analysis of carbonic anhydrase activity in whole blood from the trout revealed that intact fish erythrocytes, unlike mammalian erythrocytes totally fail to facilitate the dehydration of extracellular bicarbonate. The possible mechanism of this phenomenon has been examined; however the salient point was that fish red blood cells do not appear capable and therefore by implication apparently not necessary for the excretion of carbon dioxide at the gills of trout.

The observed excretion of carbon dioxide in the trout was found to be accounted for by the gills and their compliment of carbonic anhydrase. This finding was based on the following observations. (1) Depletion of circulating blood carbonic anhydrase levels during severe anemia was without effect on carbon dioxide excretion rates or blood acid-base status.

(2) Introduction of the carbonic anhydrase inhibitor, diamox into anemic fish produced a severe acid-base disturbance associated with a fall in observed carbon dioxide excretion. (3) Isolated perfused gill preparations excrete carbon dioxide at rates comparable to those observed *in vivo* from free swimming fish. (4) Carbon dioxide excretion in isolated gill preparations is abolished by diamox. The excretion of carbon dioxide in fish occurs via the movement of plasma bicarbonate into the branchial epithelium, where it is subsequently dehydrated into molecular carbon dioxide and excreted. A model is proposed and supportive evidence presented to account for the coupling of ionic exchange occurring across the gill with carbon dioxide excretion. The proposed model distinguishes between control of plasma hydrogen ion activity and regulation of plasma total carbon dioxide concentration *per se*.

The functional significance of this pattern of carbon dioxide excretion for aquatic animals is discussed along with the implications for air breathing fish.

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INTRODUCTION

The biological significance of carbon dioxide has long been appreciated and possibly has not been more elcquently stated than by L.J. Henderson in 1913 in "The Fitness of the Environment" (Chapter 4,p. 133): "Two chemical individuals stand alone in importance for the great biological cycle upon the earth. The one is water, the other carbon dioxide. These two simple substances are the common source of every one of the complicated substances which are produced by living beings and they are the common endproducts of the wearing away of all the constituents of protoplasm, and the destruction of these materials which yield energy to the body." Not only is CO2 a major endproduct of all aerobic metabolic pathways, it also forms the building blocks of photosynthesis by plants, which is vital for the replenishment of environmental oxygen.

CO2 in the gaseous state behaves much like any other gas and is found to obey the physical gas laws. When CO2 enters the aquecus medium the equilibrium reaction can be described by equation #1.

Equation #1 $CO_2 + H_2O = H_2CO_3 = HCO_3 + H = CO_3 + H$

Equation #1a $CO_2 + H_2O = HCO_3 + H = CO_3 + H$

Because the ionization of H2C03 is so rapid the net equation can be reduced to equation # 1a. At neutral pH's in buffered solutions, HC03 is the dominant molecular species comprising approximately 95% of the total C02 (TC02) while the remainder is primarily molecular C02 with a small amount of C03. At acidic pH's the dominant species becomes molecular C02 and, conversely at alkaline pH's the C03 becomes significant . Molecular C02 is not an acid strictly speaking; however in a functional sense the

addition or removal of C02 in solutions at physiological pH's is equivalent to adding or removing hydrogen ions . Thus, for any organism, C02 is not merely the metabolic endproduct which must be removed, it is also a highly reactive chemical moiety capable of altering pH and ionic and osmotic strength as well as buffering capacity. Therefore it isn't surprising that most organisms exercise control over the transport and ultimate excretion of this molecule.

Movement of C02 into or out of an aqueous solution involves a change in pH. If C02 is bubbled into a solution the pH will necessarily fall according to equation #1a. Equally true, the wholesale removal of C02 from any solution will drive the pH up, all other factors being equal. In biological systems C02 is continually being added from the respiring tissues; however if that quantity of C02 is removed at an equal rate no net change in pH will occur. However it should be apparent that if C02 output from the tissues is increased without a concomitant increase in the rate of C02 excretion a net acidosis will occur. Conversely, a decrease in C02 production or excessive excretion rate will result in a net alkalosis. If C02 excretion is matched with C02 input from the tissues pH disturbances will be minimized. This system, as it occurs in mammals, is fairly well characterized and provides a useful basis for future comparisons with other animal groups.

In blood the bulk of C02 is carried as bicarbonate in the plasma. When blood reaches the lungs of mammals or birds the plasma bicarbonate is shuttled into the erythrocyte in exchange for a chloride ion (referred to as the Hamburger or chloride

shift), where it is rapidly dehydrated into CO2 via carbonic anhydrase which resides in the erythrocyte. CO2 then diffuses out following its concentration gradient. In addition a portion of the CO2 is carried in direct combination with the hemoglobin, referred to as carbamino CO2. Estimates of the importance of carbamino CO2 vary but it probably does not constitute more than 30% of the total CO2 removed (Bauer ,1972).

In examining CO2 excretion in fish, fundamental differences from their mammalian and avian counterparts are readily apparent. First, fish are characterized by very low CO2 tensions in the blood, typically only 1-2 mm Hg above inspired levels compared to 30-50 mm Hg for mammals and birds. In mammals and birds the CO2 gradients from air to blood can be altered by changes in ventilation. Thus an increased ventilation rate will tend to increase the CO2 gradient across the lung, resulting in an increased CO2 loss. The converse is equally true, whereby a decrease in ventilation will cause a retention of CO2. The net result is that adjustments in pH can be accomplished by regulating arterial CO2 tensions (PaCO2) via changes in ventilation. This is not the case for teleosts, changes in ventilation do not alter the CO2 gradients or pH (Randall & Jones ,1973; Randall ,unpublished observations), and pH is regulated via changes in bicarbonate levels and not PaCO2 (Randall & Cameron ,1973; Janssen & Randall ,1975; Cameron & Randall ,1972). A second point for comparison is that, in fish, at least a portion of expired CO2 can be linked to ionic uptake across the gill , for example HCO3/Cl exchange (Maetz et al ,1976). In addition Randall et al (1976) demonstrated that the

dogfish is capable of taking-up HC03 from seawater across the gill in order to facilitate pH regulation during hypercapnia. Thus in fish it appears that C02 movement across the respiratory epithelium may not be as "passive" as the mammalian/avian system. Fish exercise some degree of control over the form of excreted C02 (HC03 or C02) and even in the direction of movement. However a definitive answer as to how C02 is excreted and the significance of bicarbonate in overall C02 excretion as well as ionic and pH regulation remains obscure.

So far it has been pointed out that pH regulation occurs and that C02 has an effect on pH. Only slight changes in pH can cause large fluctuations in the rates of chemical reactions, some being depressed while others may be accelerated. Most chemical reactions carried out in biological systems are mediated by enzymes. All proteins, including enzymes, are affected by changing hydrogen ion concentrations. The hydrogen ion concentration, among other variables, can alter the functional state of enzymes via dissociation and/or weak bond interactions which usually results in a conformational or structural change in the enzyme. While enzymes may be more or less susceptable to an altered pH, most proteins do have a pH where the charge distribution yields a specific three dimensional conformation where enzyme activity is optimized. When the pH is changed the degree of dissociation may be altered so that this three dimensional configuration is varied, and detrimental or even lethal effects on enzyme activity may occur. However, it should be noted that unless a constant temperature is maintained an increase in hydrogen ion activity may not

necessarily reflect an acidosis *per se*. For example, while at 25 C pH 7.0 may reflect the pH of neutrality, at 10 C pH 7.0 is no longer a neutral solution but rather acidic. In fact it has been demonstrated that poikilothermic animals regulate the relative alkalinity (OH/H ratio), not hydrogen ion levels *per se* (Rahn and Baumgardner ,1972; Howell *et al* , 1970).

Given the situation where an organism needs to maintain the difference between pH and neutrality at a constant level, what general mechanisms are available to it? First, all the body fluids are supplied with acid-base "buffer systems" which act as the first line of defence and are also the most rapid to respond to a pH stress.

As an example and to examine the importance of bicarbonate in the buffering of biologically active systems a brief look at the buffering capacity of human blood may be informative. Several individual buffering systems can be isolated within blood, such as albumin, bicarbonate, globins (HHb and HbO2), inorganic and organic phosphates. All these systems combine to give the blood buffering capacity. This is not to imply these buffering capacities are equally distributed in whole blood, that is plasma and/or erythrocytes. The approximate contribution of each has been computed by Winters & Dell (1965), see table #1. Generally these buffers are classified into two major groups - bicarbonate buffers and non-bicarbonate buffers. Bicarbonate buffers account for approximately 53% of the total buffering capacity in mammalian blood. Clearly, besides the contribution of hemoglobin, almost all the buffering capacity resides in the bicarbonate system. Furthermore almost total regulation of pH

TABLE #1. Approximate contribution of individual buffers to total buffering in whole blood. From Winters & Dell (1965).

INDIVIDUAL BUFFERS	PERCENT BUFFERING IN WHOLE BLOOD
Hemoglobin and Oxyhemoglobin	35
Organic Phosphates	3
Inorganic Phosphates	2
Plasma Proteins	7
Plasma Bicarbonate	35
Erythrocytic Bicarbonate	18

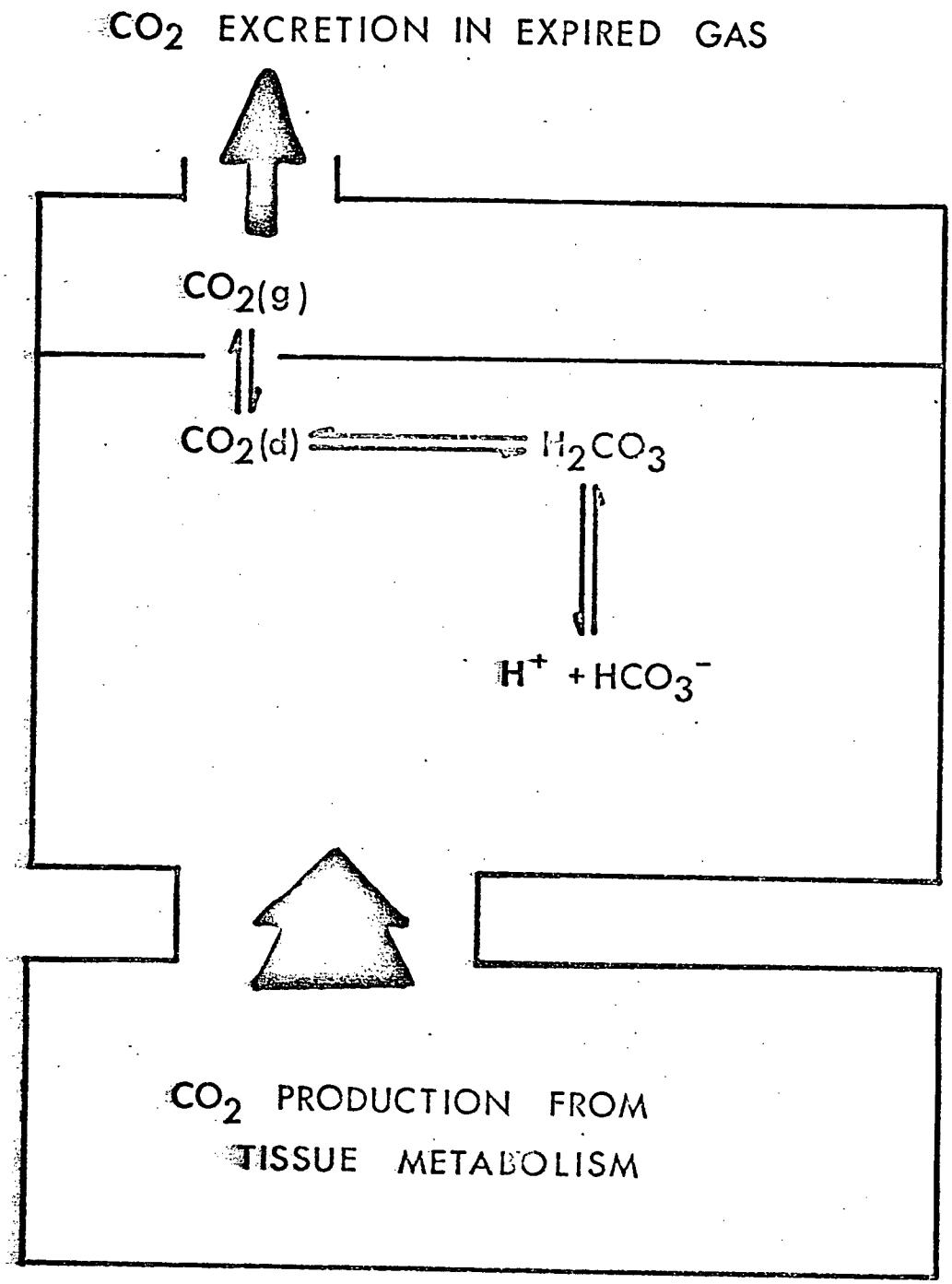
lies within the bicarbonate system when considering the almost limitless supply of C02 to the blood from the body tissues. The functional relationship can perhaps be better appreciated by looking at the Henderson-Hasselbalch equation (equation #2), and by considering the C02 system as depicted in figure #1.

Equation #2 $pH = pK + \log [HC03]/[C02]$

By viewing the diagram and the Henderson-Hasselbalch equation it can be appreciated that three mechanisms of altering pH exists at steady-state C02 production. Namely, either an organism can change dissolved C02 (C02(d)) content of the system, or it can alter the bicarbonate concentration, or both could be regulated. As stated previously it appears that birds and mammals adjust C02(d) via changes in ventilation, while fish appear to regulate bicarbonate levels. The mechanism of this mode of bicarbonate regulation is not clear. Analysis of gas exchange in an aqueous vs aerial medium would suggest this is probably not a chance occurrence but the only possible mechanism available.

Air breathing birds and mammals can regulate their blood pH (within limits) by altering their ventilation; however this is only possible due to the large content of oxygen in air. This enables the organism to greatly alter ventilation for the sake of controlling C02 content, without impairing oxygen uptake and hence delivery to the tissues. In the case of aquatic respiration this simply isn't possible. Fish can ill afford to alter ventilation for the sake of C02 control, in the face of limited and variable amounts of available oxygen in water (Randall & Cameron, 1973).

FIGURE #1. Diagramatic representation of an open CO_2 system. $CO_2(d)$ and $CO_2(g)$ refer to dissolved and gaseous CO_2 respectively.


CO_2 EXCRETION IN EXPIRED GAS
$CO_2(g)$
$CO_2(d)$
H_2CO_3
$H^+ + HCO_3^-$
CO_2 PRODUCTION FROM
TISSUE METABOLISM

In fact it might be argued that C02 excretion in aquatic animals never poses a problem, but indeed the retention of sufficient C02 to prevent loss of buffer reserve and tissue alkalosis may present a more formidable limitation. The capacitance of carbon dioxide and oxygen in air is equal; however the capacitance ratio of carbon dioxide and oxygen in water is around 30 (Dejours, 1975). Additionally, while the conductance of oxygen and carbon dioxide in air is nearly equal the permeation coefficient of carbon dioxide in water is much greater than oxygen. Consequently it has been argued by Rahn (1966) and others that water convection requirements across the gills of fish sufficient for oxygen uptake results in a virtual vacuum for C02. Thus with water acting as a sink for C02 one can readily appreciate why arterial C02 tensions in fish are low. Thus in reanalyzing C02 excretion across the gills of fish one would predict that virtually all C02 would be lost at practically any ventilation rate in normocapnic waters. The fact that arterial C02 tensions do not change in the face of altered ventilation rates, as during hypoxia (Randall & Jones, 1973), is therefore puzzling. Either gills must provide an effective diffusion barrier for C02 but not oxygen or the interconversion of HC03 to C02 may be a rate limiting process in fish blood. The rate of the reversible hydration of C02 as it occurs in these animals appears to be of interest .

The enzyme carbonic anhydrase catalyzes the reaction depicted by equation #1a. This enzyme has one of the fastest turnover rates of any enzyme yet characterized (Maren,1967). Consequently, where sufficient carbonic anhydrase exists the

interconversion of C02 and HC03 is never rate limiting. It is therefore essential to investigate the kinetics of this reaction in fish and particularily important to evaluate how carbonic anhydrase affects these rates in fish .

Carbonic anhydrase has been defined by Maren (1967) as any substance that catalyzes the reversible reaction of H20 + C02 = H2C03 (equation #1a) in the presence of suitable buffers. It seems appropriate that such an enzyme be defined loosely, for its forms and functions are incredibly varied, seemingly even more so than the globin molecules. Carbonic anhydrase is found in plants as well as all groups of the animal kingdom and has even been found in certain groups of bacteria. Given this phyletic distribution, equally impressive is the varied biological rcles to which carbonic anhydrase has been linked . For example carbonic anhydrase has been found in the following organs or organ systems: blood cells; kidney; eye; cerebrospinal fluid formation; stomach; pancreas; liver and the bilary system; salivary glands; sweat glands; taste; reproductive system; avian salt gland; rectal gland; alkaline gland; gills; swimbladder; intestine; inner ear; sickling in red cells; adrenal gland and also the thyroid gland. Since this enzyme's initial discovery by Meldrum and Roughton (1933) a large amount of literature has accumulated ; however only recently have significant strides been made concerning the actual biochemistry of this enzyme which are directly applicable to the physiological aspects.

Most of the early investigations centered around the presence or absence of carbonic anhydrase in various systems or organisms, and reaction rates and some inhibitor studies, as

clinical applications were quickly realized. To illustrate how elusive progress has been with carbonic anhydrase it is interesting to note that the actual product of the hydration reaction (HCO3 or H2CO3) remains in doubt. Most carbonic anhydrases are characterized by very high turnover rates, in fact the high turnover numbers of some mammalian carbonic anhydrases cannot be accounted for with existing catalytic models. Khalifah (1973) treats this fascinating subject in greater detail.

As generally found in human erythrocytes, carbonic anhydrase exists as one of two isozymes (isoenzymes). There is a highly active "C" form, which represents approximately 15-20% of the total carbonic anhydrase present, while a less active "B" form comprises the remainder. It should be noted that "less active" in this instance is a relative term since the "C" form has one of the highest turnover rates of any known enzyme (approximately one million per second), the "B" form is less active but still significant at 100,000 per second (Edsall & Khalifah, 1972). Due to the relative abundance of the "B" form it was purified in workable amounts first, and consequently was the first carbonic anhydrase isozyme with its complete amino acid sequence determined. Recently, several groups have reported the completion of the sequencing of the "C" form and a general picture of the enzyme can be presented. Carbonic anhydrase (in animals) exists as a single polypeptide chain with a zinc ligand bound by histadyl residues in the approximate geometric center of the active enzyme (Waara et al, 1972). The "C" form has 260 amino acid residues while the "B" form has one less at 259;

furthermore Lin & Deutsch (1974) claim that over 60% of the residues in human "B" and "C" are identical in homologous positions. These individuals also claim that most of the differences can be accounted for by a single base substitution in the responsible codon. As mentioned, carbonic anhydrase is functionally and phyletically varied; however in the future discussion only its role in C02 transport and acid-base regulation will be discussed.

In the late 1920's and early 1930's there were two main theories as to the mode of C02 transport in the blood. One, C02 was carried from organs to the lungs in the form of bicarbonate, and at the lungs the proteins of the blood, acting as weak acids, converted this bicarbonate to carbonic acid which in turn was dehydrated to C02 plus water , and owing to the volatility of C02, diffused into the gas space of the lung. The second, stated that in addition, part, and possibly all the physiologically important C02 is carried in direct reversible combination with the blood proteins. From 1917 to 1921 the problems were worked on intensly by numerous British physiologists with the result that the bicarbonate theory , in the view of most writers, was favored. Up until about 1925, attention had been given solely to equilibrium states of the process; however in this year Hartridge and Roughton, working on the combination and dissociation of oxygen with hemoglobin, pointed out the desirability of studying the kinetics of the carbon dioxide process in blood. In 1926 Henriques tackled this problem with a startling outcome. According to the bicarbonate theory the detailed chemical reactions which lead to C02

evolution in the lungs was as follows:

(1) HP (protein acid) + NaHC03= NaP (protein salt) + H2C03

(2) H2C03 = C02 + H20

Reaction (1) is purely ionic and could proceed quite rapidly, but (2) was known to be a rather slow step. So in the bicarbonate theory, reaction (2) would be the rate limiting step. Using the appropriate velocity constants, Henriques (1926) calculated the rate at which C02 could be liberated under physiological conditions, and found it to be far less than those values actually observed in vivo .

Henriques concluded that either a catalyst must exist or that the physiological transport of carbon dioxide in the blood must take place by some mechanism other than the bicarbonate one. However , Henriques had the misfortune of having the only known case of a hemolyzed blood solution lacking carbonic anhydrase and was prevented from discovering this enzyme (Maren, 1972). Finally in 1932 Meldrum & Roughton isolated from ox blood a white substance of which 1 part in 10 million was active in accelerating the reaction. So it was that work on C02 movement led to the hypothesis of this enzyme's existence and eventually to its discovery.

Given the huge catalytic potential of carbonic anhydrase and the importance of controlling bicarbonate ions, it seems essential to take a closer look at the tissues where carbonic anhydrase could play a role in fish respiration and acid-base homeostasis.

CHAPTER 1 - SOME PROPERTIES OF CARBONIC ANHYDRASE IN THE TROUT.

INTRODUCTION

The presence of carbonic anhydrase in fish, indeed in all aquatic animals, has been known for some time, for details see the review by Maren (1967). Early investigations were concerned with documenting its mere presence; however only since the discovery of potent and specific inhibitors of carbonic anhydrase, for example acetazolamide, has it been possible to investigate specific effects. It has been repeatedly shown that inhibition of branchial carbonic anhydrase interferes with ionic movements occuring at the gill. For example sodium uptake across the gill is blocked during inhibition of carbonic anhydrase in trout (Kerstetter & Kirschner, 1972); goldfish (Maetz & Garcia-Romeau, 1964) and dogfish (Payan & Maetz, 1973). Thus branchial carbonic anhydrase is generally thought to be essential for in ionic regulation in fish (Maetz , 1973; Maetz, 1971).

The role of red cell carbonic anhydrase in the excretion of respiratory C02 in mammals is well documented . All vertebrate red blood cells, including those of fish, contain appreciable amounts of carbonic anhydrase. Consequently when a C02 retention (and resultant acidosis) develops in fish during acetazolamide treatment it has been attributed to inhibition of red cell carbonic anhydrase (Hoffert, 1966 ; Hodler et al , 1955; Maren & Maren, 1964). Indeed Maren & Maren (1964) stated categorically that branchial carbonic anhydrase was not involved in the excretion of respiratory C02 in the dogfish. However two observations cause problems with analysis of branchial versus red cell carbonic anhydrase function. Table #2 depicts pooled data from the literature giving ratios of branchial to

erythrocytic carbonic anhydrase present in several species of fish. Due to variations in the methods it is not possible to express absolute levels for comparison between fish; however it is clear that on a per gram wet weight basis fish possess as much carbonic anhydrase in the branchial tissue as in blood. Cameron (1976) demonstrated that in the arctic grayling only 2-4% of the total expired C02 is utilized to provide the gill bicarbonate ions (presumibly via the hydration of C02 within the gill) for chloride uptake. Kerstetter & Kirschner (1972) argue that sufficient bicarbonate is available from plasma to ensure chloride uptake at the gill epithelium, as inhibition of carbonic anhydrase was without effect on chloride uptake in the trout. Why then should the gill require an equal or larger amount of enzyme for the hydration of C02 produced by an equal or smaller amount of carbonic anhydrase via the dehydration reaction in red cells? Biological systems presumably don't operate by such an overkill mechanism. Secondly, attempts to duplicate the experimental approach of Maren & Maren (1964) on trout have been inconclusive (D.J. Randall, personal communication). Therefore attempts to classify branchial carbonic anhydrase as distinctly ionic or respiratory in a functional sense are probably premature. However based on C02 conductance rates and the capacitance of C02 in water it can be argued that carbonic anhydrase in aquatic animals was probably initially related to ionic transport and not the movement of C02 per se. Furthermore under these conditions C02 excretion would be coupled to ionic regulation in such a fashion that while the loss of molecular C02 may be uncontrollable, the translocation

of ions can be tightly regulated.

Krogh (1941) estimated that animals smaller than 1mm in diameter could satisfy their oxygen requirements purely on the basis of diffusion. Given the huge C02 sink that water provides it is clear that cytoplasmic anoxia would result long before retention of C02 (and the associated acidosis) could ever develop. Given the relatively slow rates of the uncatalyzed hydration of C02 (results section #2) the loss of molecular C02 may not be controllable. Therefore in order for these cells and/or organisms to establish a buffer reserve it would be an advantage to hydrate metabolic C02 to HC03 plus the proton. This reaction would of necessity be catalyzed by carbonic anhydrase. The proton could be eliminated by ionic exchange , e.g. Na/H exchange on the membrane (Sachs, 1977), with a similar exchanger (Cl/HC03) controlling anion levels. Thus the uncontrolled loss of dissolved C02 would be effectively prevented by coupling C02 loss to ionic exchange. Of course an obvious advantage to this system is that counterions are now available for the electrically silent exchange of environmental sodium and chloride. That this scheme may be functional at the cellular level is indicated by the demonstration that isolated frog oxynetic cells placed in C02 free media maintain constant cytoplasmic total C02 levels with time while maintaining a higher intracellular pH (Michelangeli, 1978). If this is an accurate description thus far and the loss of C02 is indeed coupled to ionic exchange, then these aquatic organisms would effectively regulate intracellular pH independent of absclute C02 levels. Thus so long as intracellular pH is at the "set

TABLE #2. Ratio of blood versus gill carbonic anhydrase activities. Enzyme activities expressed per gram tissue (wet weight).

ORGANISM	BLOOD/GILL CARBONIC ANHYDRASE
Lake Trout[1]	0.86
Perch[2]	1.32
Sea Bass[3]	0.96
Parrot-fish[3]	1.06
Dogfish[4]	0.36

1= Hoffert (1965)

2= Maetz (1956)

3= Smith & Paulson (1975)

4= Hodler et al (1955)

point" total CO2 may fall where it may, with pHi adjustments regulated via either anionic or cationic membrane exchange processes. Thus if pHi falls hydrogen ions or their equivalent would be excreted. Conversely an elevation in pHi may produce a reduction in proton pumping. This appears to be the situation in isolated single cells (Boron, 1977; Thomas, 1976; Aiken & Thomas, 1975; ; Roos, 1975; Russell & Boron, 1976; Boron & De Weer, 1976a,b,). It can be demonstrated that cells subjected to pH perturbations respond with an appropriate increase or decrease in translocation of hydrogen ion equivalents. It is therefore interesting to note the response of the multicelled aquatic fish to an acid load. If one considers the gill surface area as delineating the water membrane or site of cationic and anionic exchange, and if one views plasma as a very large cytoplasmic pool, the systems are at least superficially very similar. However instead of utilizing either the anionic or the caticnic exchangers, it would appear both are involved. For example during hypercapnic acidosis the arctic grayling increases hydrogen ion pumping, as evidenced by increased sodium uptake rates (Cameron, 1976). Sodium uptake has been shown to correlate well with hydrogen ion excretion (Kirschner et al , 1973; Maetz, 1973; Payan & Maetz, 1973). It has already been stated that hypercapnic acidosis resulted in the uptake of bicarbonate from seawater in the dogfish (Randall et al , 1976). Also it has been demonstrated that the movement of HCO3/Cl across the fish gill is an electrically silent 1:1 coupled exchange (Maetz et al , 1976). Obviously comparing an intact fish with a well defined single cell system is pushing the

analogy, and very real complicating factors have been ignored; however it is interesting that the response of aquatic gill breathers may be more closely aligned to aquatic single cell systems rather than to the system employed by air breathing mammals and birds.

Consequently although the current evidence would suggest plasma bicarbonate is probably dehydrated within the fish red cell (catalyzed by erythrocytic carbonic anhydrase) there is no convincing evidence to exclude the possibility that plasma HCO_3 may move directly into the epithelium where it may be acted upon by branchial carbonic anhydrase.

To further investigate this possibility it is first essential to localize and characterize the carbonic anhydrase available for this purpose.

METHODS

CARBONIC ANHYDRASE ASSAY:

Carbonic anhydrase activity was measured manometrically using a modified boat technique as described by Meldrum & Roughton (1933); Roughton & Booth (1946) and as later modified by Hoffert (1966). Basically the method is as follows: a slightly alkaline bicarbonate solution is allowed to mix with a buffered solution of approximately pH 6.8, whereupon CO2 is evolved. The rate of the CO2 evolution can be measured with and without carbonic anhydrase present and thus provides the basis of the assay. The reaction vessel or "boat" consists of a modified 50 ml Erlenmeyer flask. The bottom of the "boat" has been partitioned such that the two solutions (bicarbonate and buffer) remain separated until shaking is commenced, whereupon the solutions mix and the reaction proceeds. The upper portion of the flask is provided with a ground glass joint for attachment to a Gilson Differential Respirometer. The Gilson Respirometer provides the shaking motor and temperature bath, while CO2 evolution is measured by Hewlett-Packard 267BC differential pressure transducers, via HP 350-1100C Carrier Pre-Amplifiers and displayed by a 2-channel Beckman Type RS Dyncgraph. Two such "boats" and associated HP transducers and HP Pre-Amps are utilized such that two assays can be run simultaneously.

Phosphate Buffers

The principle buffer utilized has been a 0.2 M phosphate solution with a pH of 6.8. The buffer is composed of 0.2 M Na2HPO4 and 0.2 M KH2PO4, the relative proportions being

determined by the required pH.

Bicarbonate Solutions:

For bicarbonate solutions the sodium salt was made by dissolving NaHC03 in 0.02 M NaOH. All results presented were obtained utilizing a bicarbonate solution of 200 mM unless otherwise indicated.

Physiological Saline

Whenever a physiological saline was required Cortland saline (Wolf, 1963) was utilized.

Tissue Preparations:

Blood: Blood hemolysates were prepared in the following manner: whole blood was centrifuged, the plasma removed and the packed cells washed 3X in Cortland saline. An aliquot of cells was then hemolyzed in known volumes of distilled water. A small quantity of either saponin or Triton X-100 was found to greatly facilitate hemolysis and was routinely utilized for this purpose.

Gill homogenates:

Fish were immobilized by a blow to the head and a ventral incision made to expose the pericardium . The gills were subsequently perfused via the ventral aorta with heparinized Cortland saline to remove trapped erythrocytes. When the gills appeared devoid of erythrocytes the arches were removed and placed in ice cold Cortland saline. The arches were separated from the filaments or utilized whole. Gill tissues in known volumes of 300 mM sucrose were homogenized by hand over ice utilizing a glass homogenizing tube. This suspension was then centrifuged to remove the cellular debris.

Subcellular Distribution

The subcellular distribution of branchial carbonic anhydrase was estimated utilizing differential centrifugation. After homogenization the crude homogenate was centrifuged at 20,000 X G for 35 minutes at 4 C using a Sorvall refrigerated centrifuge. The pellet represented the nuclear and mitochondrial debris plus the heavy microsomes. The supernatant was centrifuged for 20 minutes at 4 C at 100,000 X G using a Beckman Ultracentrifuge to obtain the microsomal pellet.

General procedure:

The transducers were calibrated by altering the volume of the closed system (including the Gilson manometer, the reaction vessel and pressure transducer) and recording the change in pressure. To perform an assay 2 mls of bicarbonate solution were placed into one chamber of the "boat" while the other was filled with 2 mls of phosphate buffer plus the material to be tested. If the test material contained carbonic anhydrase, the uncatalyzed control value was obtained by substituting an equal volume of Cortland saline. The "boats" were then attached to the Gilson, submerged in the water bath and allowed to temperature equilibrate (usually 2-3 minutes) to the bath temperature of 5 C. When it was apparent no pressure changes were occurring the shaking motor was turned on and the reaction allowed to proceed. After full deflection of the pens the valves were opened and the shaking motor and chart recorder turned off. The reaction vessels were then removed, washed and air dried and ready for the next assay.

Calculations:

The rates of the reaction are expressed as microliters of C02 evolved per second. These values are easily obtained from the chart recorder tracing. Carbonic anhydrase activity can then be expressed according to the following formula:

$$E = Kc - Ko / Ko$$

where, E is equal to enzyme units of carbonic anhydrase activity, Kc is equal to the rate of the catalyzed reaction, and Ko is equal to the rate of the uncatalyzed reaction. "E" values are arbitrary units such that when the catalyzed rate is exactly twice the uncatalyzed rate "E" equals 1. The effects of acetazolamide (Diamox, Lederle) and chloride on carbonic anhydrase dehydration activity were also investigated. In these instances inhibition of enzyme activity was calculated as follows:

$$\% \text{ Inhibition} = Kc - Kt/Kc \times 100$$

where, Kc is the same as above and Kt is the catalytic rate in the presence of the appropriate test solution.

Estimation of Km bicarbonate:

Carbonic anhydrase affinity for the dehydration substrate HC03 was determined as follows: a constant amount of crude hemolysate or gill homoginate was added to sodium bicarbonate concentrations of 25, 37 and 71 mM. The reaction rates were plotted graphically and the Km fitted by eye.

Protein determination:

Total protein was determined using a modified biuret method (Accu-Stat, Clay Adams, Parsippany, N.J.) with albumin as a standard.

Localization of branchial carbonic anhydrase

Histochemical localization:

Intracellular localization of carbonic anhydrase was demonstrated using a modification of the Hansson (1967, 1968) method as described by Ridderstrale (1976)

Autoradiographic localization:

Carbonic anhydrase has been localized in the gill using the labeled inhibitor acetazolamide as described by Gay & Mueller (1973). 5.0 MCi of acetazolamide-3H, representing 6.3 mg of dry powder, was obtained by special order from New England Nuclear (Boston, Mass.). As Gay & Mueller (1973) found *in vitro* labeling unsatisfactory, the following *in vivo* exposure was utilized: the acetazolamide-3H was suspended in Cortland saline and 3mCi introduced via a chronic indwelling catheter residing in the dorsal aorta, see Methods Chap. III. After six hours exposure the animal was sacrificed and the gills removed for autoradiographic analysis. The sections were fixed in glutaraldehyde and critical point dried. The critical point dried sections were mounted in Paraplast (Sherwood Ind., St. Louis, Mo.) for sectioning. 3 micron sections were mounted on clean dry slides and dipped in NTB-2 tracking gel (Eastman Kodak, Rochester, n.y.). The dry slides were placed in light and air-tight boxes containing drierite and stored at 4 C. The autoradiographs were developed for 2 minutes in Kodak Dektol developer (1:2 dilution with water), fixed for 10 minutes and rinsed with water for 20 minutes. After drying the slides were counterstained using Nuclear Fast Red. This staining procedure has been found not to induce a differential shrinking or shifting of the photographic emulsion which could otherwise

distort its relationship with underlying tissue sections (C. Sloneker, Anatomy Dept. Univ. Of B.C., personal communication).

Carbonic Anhydrase Isozymes:

The presence of multiple enzyme forms (isozymes) was determined by cellulose acetate electrophoresis utilizing a Beckman Microzone electrophoresis chamber and associated power pack. Electrophoresis was carried out in the cold using barbital buffer (0.06 M) at pH 8.6. The voltage was held constant at 250 V which usually resulted in a current of between 8 and 10 milliamps.

The location of carbonic anhydrase activity after electrophoresis was determined utilizing bromolthymol blue as an indicator as described by Tashian (1969).

RESULTS

Significant quantities of carbonic anhydrase are present in both blood and gill tissue. Table #3 presents the enzyme activities based on a per gram of tissue and per gram of protein basis. As a comparison, using the manometric assay at 5 C, the enzyme activity of rat blood was approximately 450 units per gram tissue, while purified bovine carbonic anhydrase (Sigma, St Louis, Mo.) possessed aprroximately 2,000 Eu per gram of purified enzyme.

Carbonic Anhydrase Isozymes:

Figure #2 shows that carbonic anhydrase found in gill and blood is a combination of two electrophoretically distinct forms. Utilizing crude gill homogenates two distinct staining regions are evident, a densely staining slow migrating front and a lighter staining fast migrating band. This staining pattern contrasted with purified bovine carbonic anhydrase , which contains only one enzyme form, also run as a marker. No further attempt to quantify or characterize the respective isozymes was attempted.

Substrate Affinity:

The apparent Km bicarbonate for branchial carbonic anhydrase obtained from three trout ranged from 22-25 mM with a mean of 23.3 mM. The erythrocytic carbonic anhydrase from 5 rainbow trout ranged from 30-34 mM with a mean of 31.4 mM. At HCO3 concentrations in excess of 100 mM the enzyme activity (both branchial and erythrocytic) was depressed. This was not evident in either bovine or rat carbonic anhydrases at the bicarbonate concentration utilized.

FIGURE #2. Diagramatic representation of carbonic anhydrase isozymes as revealed by cellulose acetate electrophoresis. Bovine carbonic anhydrase was run as a marker.

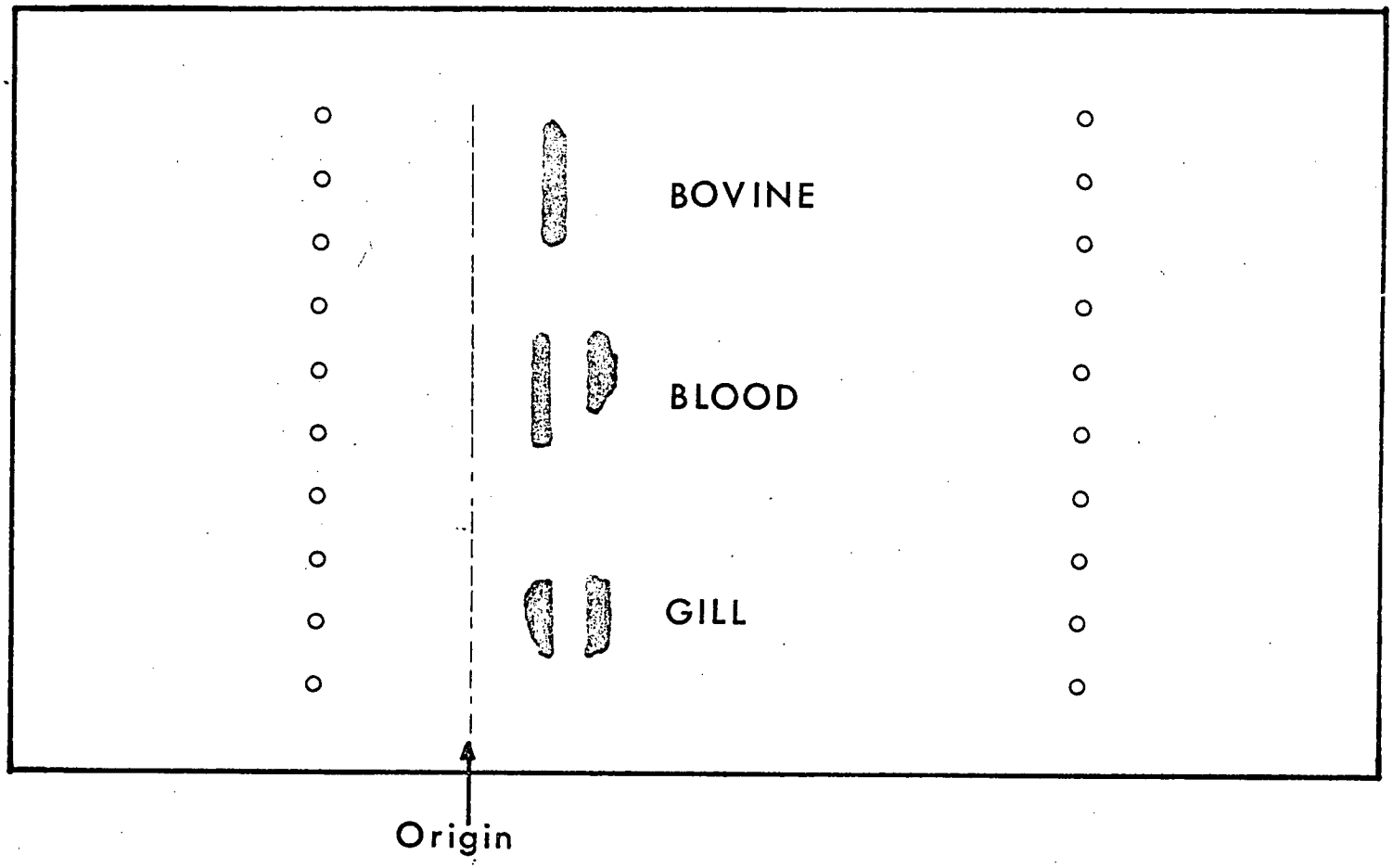

BOVINE
BLOOD
GILL
Origin

TABLE #3. Branchial and erythrocytic carbonic anhydrase activity in the rainbow trout. (mean ± S.D.)

	g. protein/100 ml	Eu/g tissue	Eu/g protein
BLOOD (n=5)	21 ± 6	68 ± 23	319 ± 47
GILL (n=5)	6 ± 1	62 ± 8	972 ± 186

Carbonic Anhydrase Inhibition by Diamox:

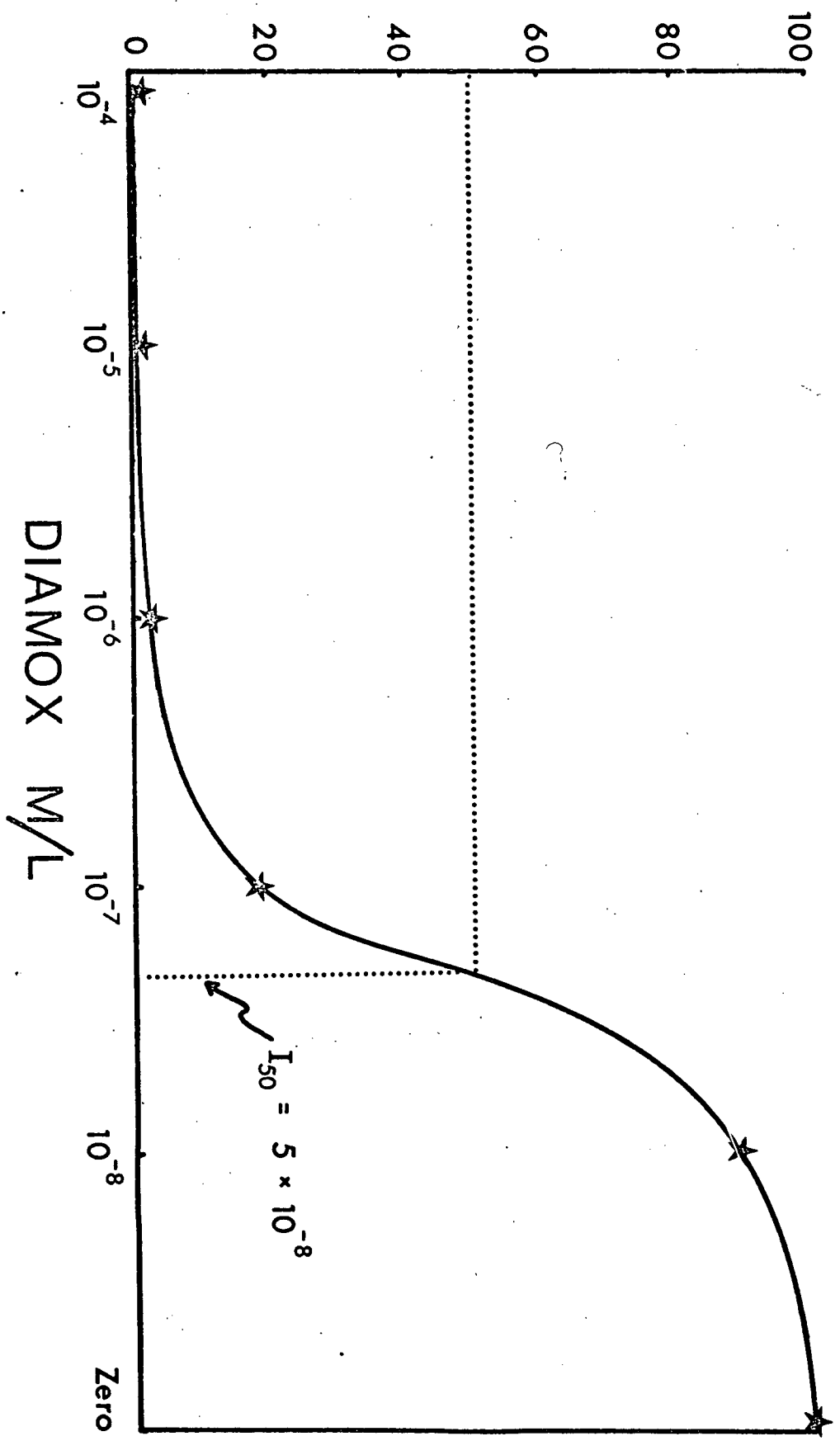
Diamox (Acetazolamide, Lederle) was found to strongly inhibit branchial and erythrocytic carbonic anhydrase. Figure #3 is a plot of branchial enzyme activity versus increasing concentrations of Diamox. The apparent I50 (concentration of inhibitor to reduce the enzyme activity by half) is 4×10^{-8} M.

Localization of Branchial Carbonic Anhydrase:

The histochemical staining technique and acetazolamide-3H autoradiography revealed several features concerning the localization of carbonic anhydrase in this tissue. First, most of the enzyme appeared to be localized rather generally in the cytoplasm. Carbonic anhydrase did not appear to be sequestered in any specific cells, e.g. Chloride cells, but was associated principally with respiratory cells. Secondly, in addition there appeared to be some enzyme activity associated with the plasma membrane. In most instances it appeared the enzyme was associated with the apical border; however in some sections it appeared the enzyme was associated with the basal laminal membrane as well. It was not possible to resolve the specific location using light microscopy in all cases.

In an attempt to further test the possibility that a fraction of branchial carbonic anhydrase could be associated with the membrane, differential centrifugation was utilized to isolate the microsomal fraction of the branchial tissue. This subcellular fraction in addition to containing the ribosomes contains the plasma membranes. While the bulk of branchial carbonic anhydrase was found in the cytoplasm, carbonic anhydrase was also found in the microsomal fraction of the two

FIGURE #3. Inhibition of branchial carbonic anhydrase by Diamox.


C.A. ACTIVITY
(=Percent of Control)
0
20
40
60
80
100
DIAMOX M/L
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
Zero
$I_{50} = 5 \times 10^{-8}$

fish which were examined. In the second analysis sufficient microsomes were collected to measure protein and carbonic anhydrase. The crude homogenate possessed 1,220 Eu/g protein compared with 1,473 and 441 Eu/g protein for the supernatant and microsomal fraction respectively. Carbonic anhydrase activity of gill arches 1 thru 4 from a single trout was 52.9, 52.4, 57.7 and 56.3 Eu/g tissue respectively, indicating that the enzyme is probably distributed equally among the respective arches.

Distribution of Carbonic Anhydrase in Blood

No evidence to suggest the presence of carbonic anhydrase in plasma was ever obtained . Consequently it must be concluded that all blood carbonic anhydrase activity resides in the erythrocytes.

Initially hemolysates were obtained using only distilled water. This crude hemolysate was then centrifuged and carbonic anhydrase measurement based on the supernatant fraction; however analysis of the pellet revealed significant carbonic anhydrse activity. When hemolysis was carried out with the addition of saponin or Triton X-100 (both agents capable of disrupting membrane integrity) a homogenous mixture with increased enzyme activity was invariably obtained.

DISCUSSION

Significant quantities of carbonic anhydrase are present in both blood and gills of the rainbow trout. While the activity of blood and gill are nearly equal when expressed on a per gram tissue basis it can be seen that the gill is particularily active when expressed on a per gram protein basis.

At least some erythrocytic carbonic anhydrase appears to be associated with the membrane, in contrast to mammalian red blood cells where it is thought to be exclusively localized in the cytoplasm (Maren, 1967). This conclusion was initially based on the finding that a sizable fraction of carbonic anhydrase activity was lost upon centrifugation and recovered in the pellet. Subsequently, utilization of membrane detergents such as Saponin or Triton X-100 to achieve hemolysis further increased carbonic anhydrase activity and implied a fraction of erythrocytic carbonic anhydrase was associated with the membrane. This conclusion has subsequently been substantiated (Houston & McCarty, 1978). The significance of a membrane bound carbonic anhydrase remains obscure.

The bulk of carbonic anhydrase found in the gill is soluble and located in the cytoplasm. A small fraction has however been demonstrated to be present in the plasma membrane as demonstrated by autoradiographic and histochemical analysis. Additionally the presence of significant carbonic anhydrase activity in the microsomal fraction lends further support to this conclusion. It is noteworthy that Ridderstralle (1976) and Wistrand & Kinne , (1977) also found evidence for a membrane bound carbonic anhydrase in the kidney tubules of the frog and

rabbit. It would appear that with improved methods and current techniques, Maren's (1967) contention that all carbonic anhydrase is cytoplasmic may be subject to some qualifications. Unfortunately it wasn't always possible utilizing light microscopy to determine whether the membrane bound carbonic anhydrase was associated with the apical or basolateral membrane or both. A further investigation at the electron microscope level might be expected to resolve this question.

Enzyme Kinetics

Due to the presence of multiple carbonic anhydrase isozymes it is not rigorously correct to assign a Km value (Segel, 1975); however it does reflect the affinity of the tissue in question for the dehydration substrate. The finding that branchial carbonic anhydrase has a greater affinity for bicarbonate than the erythrocytic carbonic anhydrase might imply that, all factors being equal, principally enzyme/substrate accessability factors, plasma bicarbonate pools might be more easily dehydrated within the gill epithelium. Multiple isozymes have also been demonstrated in eel gill and blood (Girard & Istin, 1975). Girard & Istin also found eel gill carbonic anhydrase possessed a greater affinity for bicarbonate than the erythrocytic form. The absolute Km for bicarbonate by fish carbonic anhydrase would appear to be somewhat high. For example the Km for bovine carbonic anhydrase is around 10 mM or approxiamtely half the bicarbonate concentrations found in plasma (20-30 mM). On the other hand plasma bicarbonate levels in the trout are between 6-10 mM while the red cell carbonic anhydrase Km for bicarbonate is between 30-35 mM. This value is

approximately 3X the plasma bicarbonate levels. Trout red cell bicarbonate levels can exceed plasma concentrations (Giles & Haswell, unpublished); however bicarbonate levels are still far below the observed Km for bicarbonate. The situation for the branchial carbonic anhydrase is seemingly more favorable with a Km between 20-25 mM. No good estimate of epithelial cell bicarbonate levels is currently available; however they would not be expected to greatly exceed plasma levels. Two seperate determinations on bovine carbonic anhydrase (Sigma, St. Louis) gave a mean Km bicarbonate of 13 mM. De Voe & Kistiakowski (1961) working under similar conditions found a Km of 9.6 mM for bovine carbonic anhydrase . Thus although experimentally derived Km's for gill and blood carbonic anhydrase appear high they are probably reliable. Girard & Istin (1975) found apparent Km's in excess of 100 mM. It is not clear why eel carbonic anhydrase Km's should be so high. However in this study it was found that bicarbonate concentrations in excess of 100 mM depressed apparent carbonic anhydrase activity in the trout and it may be possible eel carbonic anhydrase is likewise affected. If this were the case carbonic anhydrase activity at the higher bicarbonate concentrations would skew a Lineweaver-Burke plot (Girard & Istin utilized this method for estimating their Km values) such as to generate higher than actual values. In this study Km's were fitted by eye and bicarbonate concentrations kept well below 100 mM.

Both gill and erythrocytic carbonic anhydrase were strongly inhibited by Diamox. The I50 of 10^{-8} M for Diamox is in good agreement with the mammalian fast forms, e.g. Human "C" where

the I50 for Diamox is also 10^{-8} M (Maren et al , 1976). Chloride concentrations up to 200 mM are without effect on apparent carbonic anhydrase activity. Human "B" (slow form) is fully 50% inhibited at chloride concentrations of 100 mM. Human "C" requires a chloride concentration of 600 mM to achieve this same inhibition.

Tashian et al (1977) claims the appearance of the carbonic anhydrase slow form is a fairly recent evolutionary occurence, probably the result of a gene duplication in early mammalian evolution. Thus far the slow form has only been demonstrated in mammals. It is possible that the slow form carbonic anhydrase would be masked in a solution or tissue preparation containing both forms. Indeed in human blood the fast form or "C" isozyme represents approximately 15% of the molar concentration of carbonic anhydrase present and yet accounts for over 90% of the enzyme activity (Maren, 1967). Consequently the isolation and further purification of fish carbonic anhydrase will be required before the existence or absence of kinetically distinct isozymes and their possible functional significance can be assessed. However it is worth mentioning that while distinct isozymes have been found in trout and eel, both euryhaline species, no evidence of multiple enzymes was found in two species of shark (Maynard, 1971).

In summary significant amounts of carbonic anhydrase exist in the gills and blood of the rainbow trout. This carbonic anhydrase appears as two electrophoretically distinct bands, suggesting the presence of functional isozymes. The bulk of branchial carbonic anhydrase is soluble and present in the

cytoplasm, although a smaller fraction appears to be localized in the plasma membranes. In blood a larger portion, possibly over 50%, may be incorporated into, or at least associated with, the membrane while the remainder is cytoplasmic. On a per gram protein basis the gills possess much higher enzyme activities than erythrocytes; this fact coupled with the greater bicarbonate affinity demonstrated by branchial carbonic anhydrase compared with erythrocytic carbonic anhydrase suggests the gill tissue may be predisposed to the dehydration of plasma bicarbonate.

CHAPTER II - CARBONIC ANHYDRASE ACTIVITY IN THE TROUT RED BLOOD CELL.

INTRODUCTION

In mammalian blood the interconversion of plasma bicarbonate and molecular C02 is greatly facilitated by red blood cells and their compliment of carbonic anhydrase. It has long been known that sufficient carbonic anhydrase is present in mammalian red blood cells to catalyze the observed C02 excretion. Kernohan & Roughton (1966) calculated 13,000X more carbonic anhydrase was present in red blood cells than catalytically necessary. Forster and Crandall (1975) have since revised this estimate to a 6,000X excess. Clearly, the interconversion of HC03-C02 within the red blood cell should not normally be limiting. As blood passes through the lung capillaries molecular C02 will diffuse from blood into the lung space due to the partial pressure gradient existing between blood and alveolar gas phases. As approximately 90% of the total C02 in blood is resident in plasma as bicarbonate, this loss of C02 could create an apparent disequilibrium situation should the production of C02 from plasma bicarbonate lag behind. However, due to the rapid exchange of plasma bicarbonate for red cell chloride, plasma bicarbonate is rapidly dehydrated to molecular C02 within the red cells by carbonic anhydrase, thus keeping the reaction in apparent equilibrium. Klocke (1976) has demonstrated that the translocation of plasma bicarbonate into the red cell is never normally the rate limitation in the production of C02. Thus these two processes (red cell carbonic anhydrase activity and the chloride shift) ensure arterial C02 tensions equal alveolar C02 tensions even during exercise when blood capillary residence time is shortest (Chinard *et al*, 1960). Thus, in

mammals, at any fixed lung blood perfusion, increasing the arterial-alveolar C02 partial pressure gradient will result in a larger conversion of plasma bicarbonate to C02 within blood.

Although sufficient carbonic anhydrase exists in mammalian red blood cells, this may not be the case in the trout red cell. Figure #4 is a plot of the calculated half-time for the dehydration of bicarbonate at various temperatures. Although this is a simplification of the uncatalyzed reaction, and factors such as pH, ionic strength, non-bicarbonate buffer strength, and the back reaction have been ignored , it does give an estimate, as well as demonstrating the effect of temperature on this reaction. With these limitations in mind and assuming a half-time of 5 minutes at 15 C, and further assuming a gill residence time of 1-3 seconds (Haswell & Randall, 1978), the amount of enzyme required can be calculated as follows:

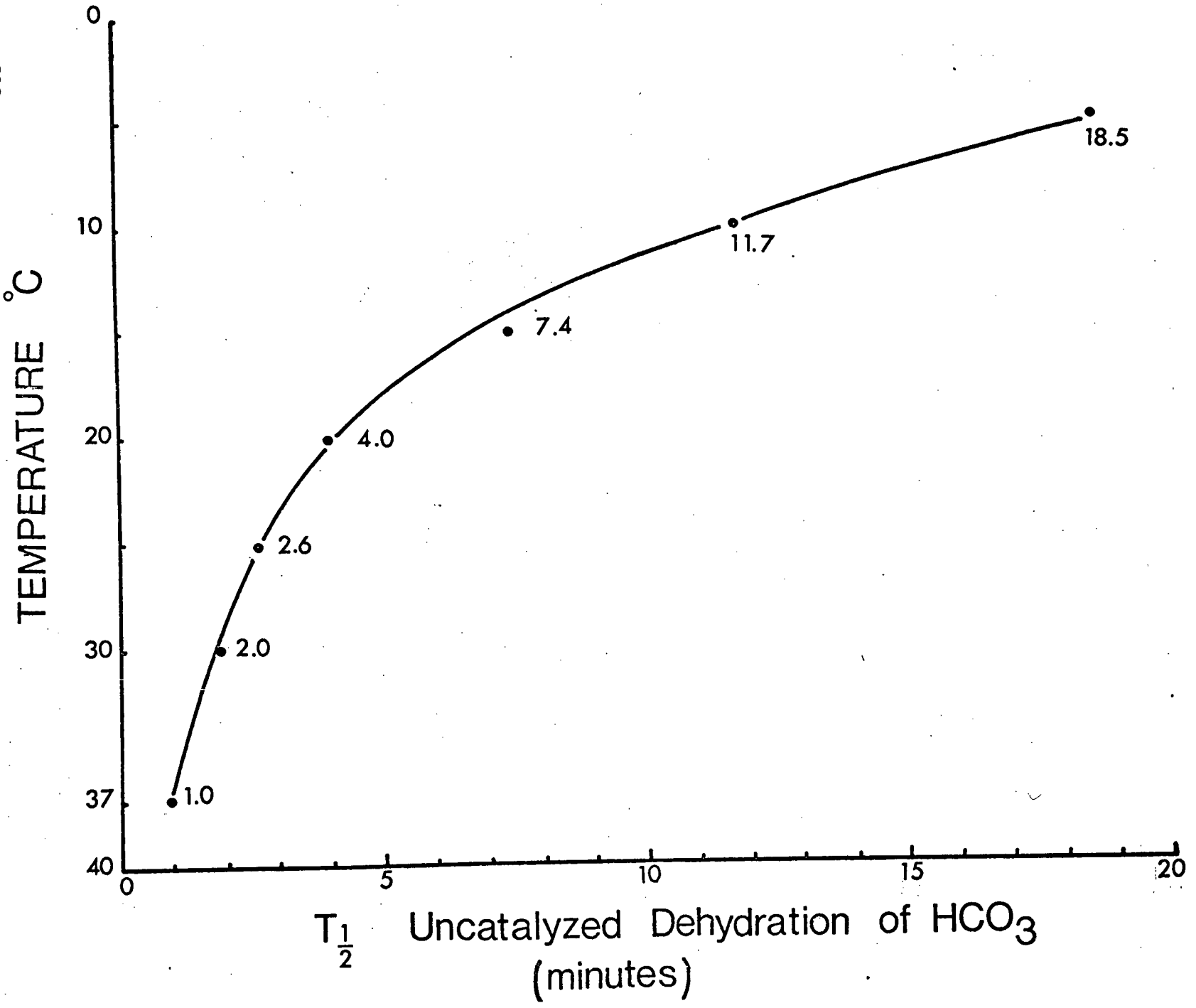
Catalytic factor = Uncatalyzed rate/ Gill residence time

Utilizing the above values a catalytic factor of 200 is required. Thus for each ml of blood passing through the gills the reaction would have to be accelerated some 200 times. From table #3 it can be seen that trout blood only possesses around 70 enzyme units or less than half the predicted requirement. Since Roughton's(1935) initial estimate of the uncatalyzed reaction velocity (approximately 1 minute at 37 C) recent investigations have found the apparent *in situ* half-time to be approximately 10 seconds (Hill *et al*, 1977; Forster & Crandall, 1975). Thus Roughton's original estimate was too high by a

factor of nearly 10. If the calculations presented in figure #5 are likewise too high by a factor of 10 then the uncatalyzed rate at 10 C would be closer to 60 seconds instead of 11.8 minutes. It is interesting therefore that introduction of aliquots of sodium bicarbonate into trout plasma generated a rise in PCO2 with an apparent half-time of between 60-70 seconds at 10 C . If 60 seconds is a better estimate of the uncatalyzed reaction time the catalytic requirement can now be reduced to 30-60 enzyme units for blood at 10 C. Thus by minimizing uncatalyzed rates it would appear the requirement might possibly be met by the trout. However it is also worth noting that the enzyme activity expressed in table #3 was obtained when assay bicarbonate levels were well in excess of the bicarbonate Km (35 mM). Thus while maximally 70 units/ml of carbonic anhydrase activity may exist in trout blood, at <u>in vivo</u> bicarbonate concentrations of 6-10 mM the <u>in situ</u> carbonic anhydrase activity is probably far less than the catalytic requirement. Additionally lower temperatures and exercise, among other variables, might be expected to impose severe limitations on CO2 excretion, and certainly the huge catalytic reserve as evident in mammals is not present in the trout. Thus it may be possible that the interconversion of HCO3-CO2 is a rate limitation in the production of CO2 in fish blood, and this would provide a possible explanation as to why a greater washout of CO2 does not occur.

It thus became of interest to investigate carbonic anhydrase activity as might be found in fish erythrocytes <u>in situ</u> . It was therefore essential to evaluate the dehydration of

FIGURE #4. Effect of temperature on the calculated half-times for the uncatalyzed production of CO_2 from HCO_3. Adapted from Roughton (1964); rate constants from Edsall (1969).


TEMPERATURE °C
0
10
20
30
37
40
1.0
2.0
2.6
4.0
7.4
11.7
18.5
0
5
10
15
20
$T_{\frac{1}{2}}$ Uncatalyzed Dehydration of HCO_3
(minutes)

bicarbonate by intact fish red blood cells.

Materials and Methods

Carbonic Anhydrase Activity: in order to clarify what contribution, if any, the erythrocytic carbonic anhydrase was making to overall C02 excretion, the rapid mixing manometric assay as previously described in Chapter I has been utilized. Fish blood was obtained via dorsal aortic puncture on MS-222 anesthetized fish or from dorsal aortic catheters from free swimming fish, utilizing heparinized syringes. The presence or absence of MS-222 did not affect the results obtained. Some whole blood experiments were carried out utilizing rat blood, this blood was obtained via cardiac puncture on animals anesthetized with ether. All blood was kept on ice until required and usually was analyzed within 10-15 minutes of removal from the animal . The water bath was maintained at 5 C unless otherwise indicated.

Cl/HC03 Exchange:

As this exchange process forms such an integral part of the C02 excretion mechanism in mammalian red blood cells it was of interest to demonstrate its presence or absence in fish red blood cells.

Red cell alkalinization

This is an indirect method recently employed by Zeidler & Kim (1977) on calf red blood cells. When red blood cells are placed in isotonic sucrose internal chloride is lost in exchange for external bicarbonate. The progress of this reaction can be followed by measuring intracellular pH. Thus as bicarbonate replaces chloride within the red cell pHi will tend to rise. In this study intracellular pH's were determined on ethanol/dry ice

freeze thawed cells as described by Zeidler & Kim (1977). Sucrose solutions were 300 mM + 5 mM HCO3, with pH adjusted using PO4 buffers.

Carbon Dioxide Partitioning in Blood

The partitioning of CO2 between plasma and erythrocytes was determined as follows: Blood suspensions were tonometered against known gas concentrations for at least one hour. Gas mixtures were obtained from Wostoff Gas Mixing pumps (Bochum, Germany). A blood sample was withdrawn and analyzed for total CO2 (see methods Chap III) and hematocrit. A second sample was centrifuged and plasma total CO2 measured. Erythrocyte total CO2 was determined by differences between plasma and whole blood total CO2, corrected for hematocrit.

RESULTS

Rat Blood:

Figure #5 demonstrates the change in dehydration reaction velocity of a noncatalyzed control (100 ul Cortland saline) and the same reaction using 100 ul of whole rat blood. A decrease in velocity occcurred if the reaction was followed to completion (not depicted in figure #5); however, unless very small volumes of whole blood were utilized the rate of C02 evolved always remained linear through the first 200 microliters of C02 evolved. Figure #6 shows the change in enzyme activity by increasing the volume of rat blood utilized per assay. It is apparent that an increase in blood volume is accompanied by an increased enzyme activity. All experiments with rat blood were performed at 5 C.

Whole Fish Blood:

Repeated assays using up to one ml of whole blood (unwashed) from the rainbow trout failed to demonstrate any significant dehydration activity in excess of the uncatalyzed controls. Assays were routinely performed at 5-6 C, but the fish were maintained at water temperatures near 8-10 C, therefore several assays were performed at 10 C and 15 C. The change in temperature failed to demonstrate any increased dehydration activity over the uncatalyzed control rates.

Most assays were performed with the phosphate buffer at or near pH 6.8; however to assess the effects of pH on the dehydration activity of the intact erythrocyte, phosphate buffers with varying pH's were also utilized. There was no

FIGURE #5. Typical tracing of uncatalyzed (100 ul Cortland saline) and the catalyzed (100 ul of whole rat blood, upper trace) dehydration reaction.

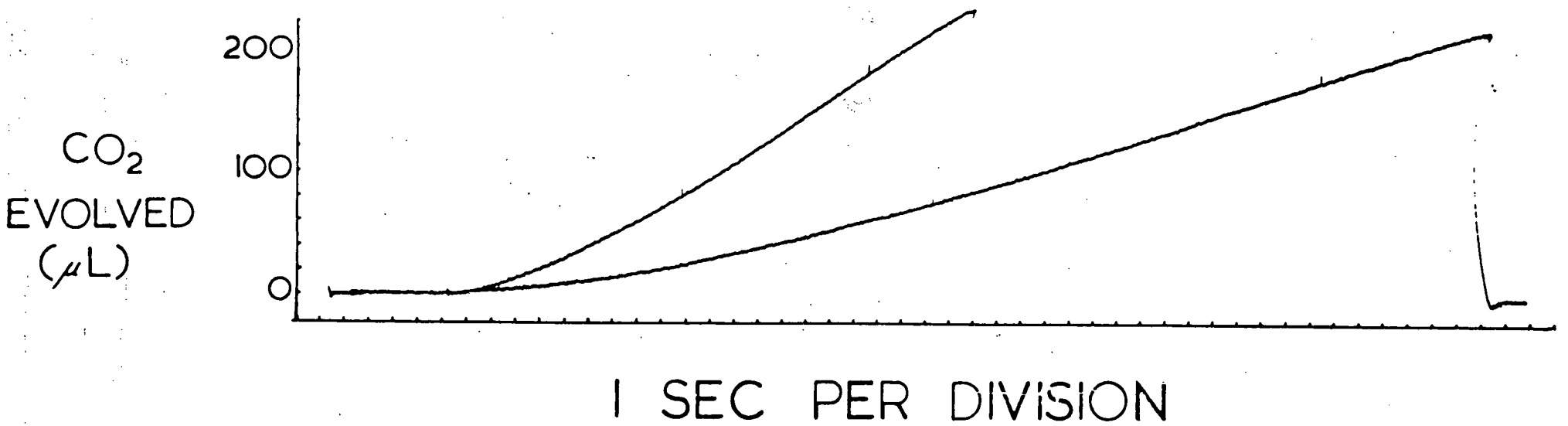
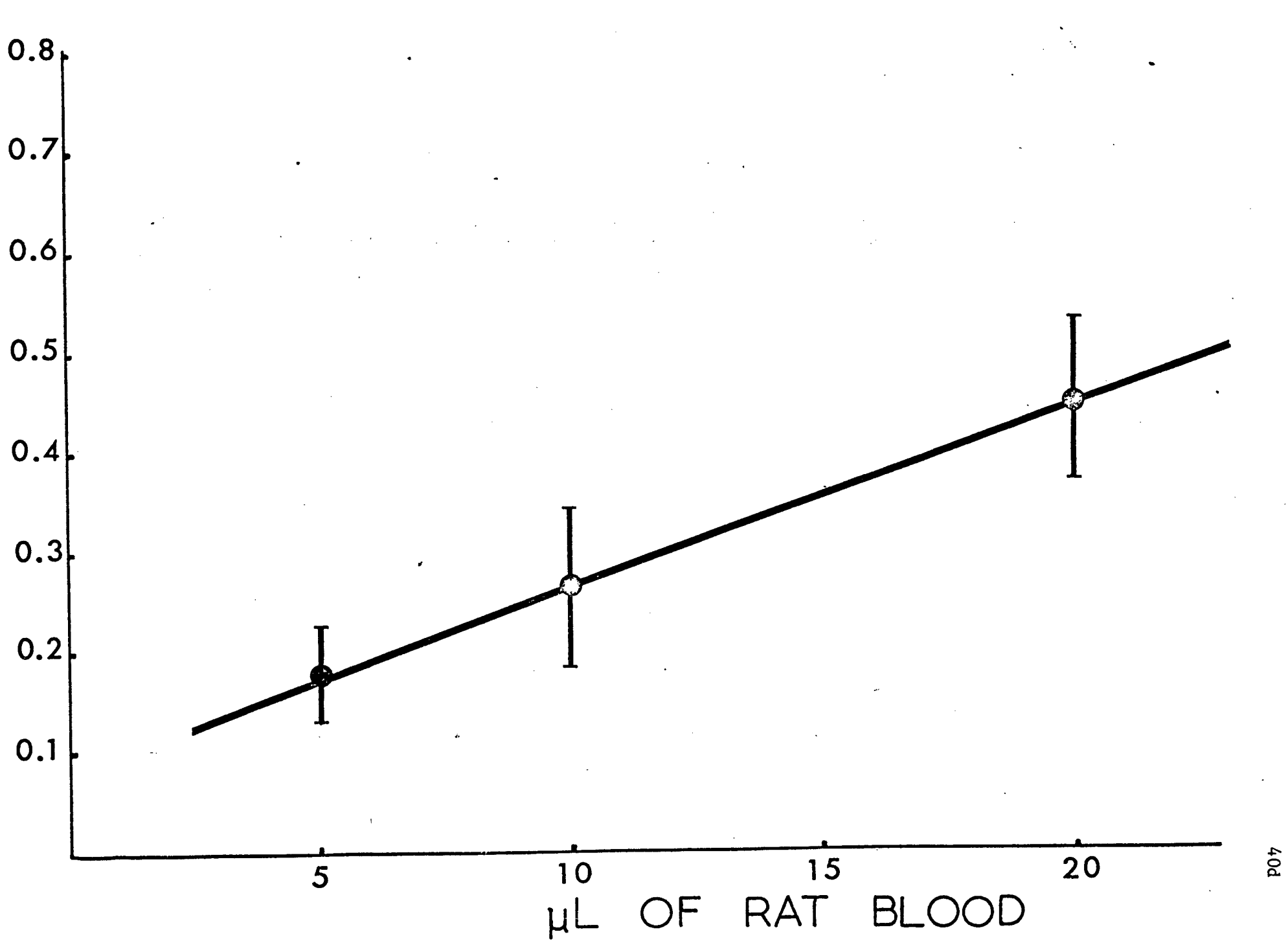

200
100
0
CO_2
EVOLVED
(μL)
1 SEC PER DIVISION

FIGURE #6. Effect of increasing volumes of whole rat blood on enzyme activity, demonstrating linear kinetics (± S.D., where n=6).


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
5
10
15
20
µL OF RAT BLOOD
404

effect of increasing the pH on enzyme activity.

Occasionally small amounts of activity were noticed. Unlike rat blood , increasing volumes of whole fish blood were not accompanied by increasing enzyme activity , and enzyme activity was always associated with some red cell hemolysis, presumably releasing carbonic anhydrase into the plasma.

Assays using whole blood from the following fish also failed to demonstrate any dehydration activity: carp (Cyprinus), lingcod (Ophiodon), skilfish (Erilepis) , flounder (Platichthys),and the mouthbreeder (Tilapia).

Carbonic anhydrase activity of hemolyzed trout blood:

Rainbow trout contain appreciable quantitites of carbonic anhydrase as demonstrated by assays on erythrocytes hemolyzed with saponin (Chapter #1). One ml of blood from the average rainbow trout contains approximately 60-90 enzyme units (table #3). Thus each ml of rainbow trout blood with intact red cells contains sufficient carbonic anhydrase to potentially generate a 60-90 fold increase in dehydration activity, but it does not. Therefore, either carbonic anhydrase is inhibited in intact cells or bicarbonate does not enter the red cells at appreciable rates.

Washed Trout Erythrocytes:

Plasma was replaced by Cortland saline (three washes) and then this red blood cell suspension analyzed for carbonic anhydrase activity. The red cell suspension was found to have pronounced enzyme activity which was lost if Cortland saline was replaced by plasma (see below). The rate was equivalent to 3-8 enzyme units/ml, less than that for hemolyzed blood , but much faster

than ever found using the unwashed blood. Cortland saline washed blood, termed "Cortland Blood", showed linear kinetics (figure #7) , unlike the occasional activity found in unwashed blood. The supernatant of Cortland blood assays (obtained by centrifugation) was analyzed for activity; it never showed any carbonic anhydrase activity, indicating that hemolysis did not occur in these experiments.

At this point the existence of some modifier in the plasma seemed possible . Evidence for a plasma inhibitor was confirmed in the following manner. The remaining blood was centrifuged, the plasma removed and kept on ice for further use. The erythrocytes were then washed three times and resuspended with Cortland saline as outlined above. The Cortland blood was then assayed. At this point the remaining Cortland blood was then centrifuged to remove the Cortland saline. The supernatant was discarded and the cells resuspended to the initial hematocrit using the original plasma. This reconstituted blood showed zero activity. Hemolysis of the remaining blood and subsequent assay of the hemolysate clearly demonstrated that the remaining blood still possessed carbonic anhydrase activity. This approach was repeated three more times with trout blood with the same results. Figure #8 represents the results of one of the investigations.

It is interesting to note that experiments on the blood of freshwater carp , mouthbreeder , the marine lingcod, and the euryhaline flounder, Platichthys flesus gave similar results, indicating a wide distribution of the plasma inhibitor in teleosts. This approach with rat blood was attempted only once;

FIGURE #7. Effect of increasing volumes of "Cortland Blood" on enzyme activity, demonstrating linear kinetics. (± S.D., where n=6).

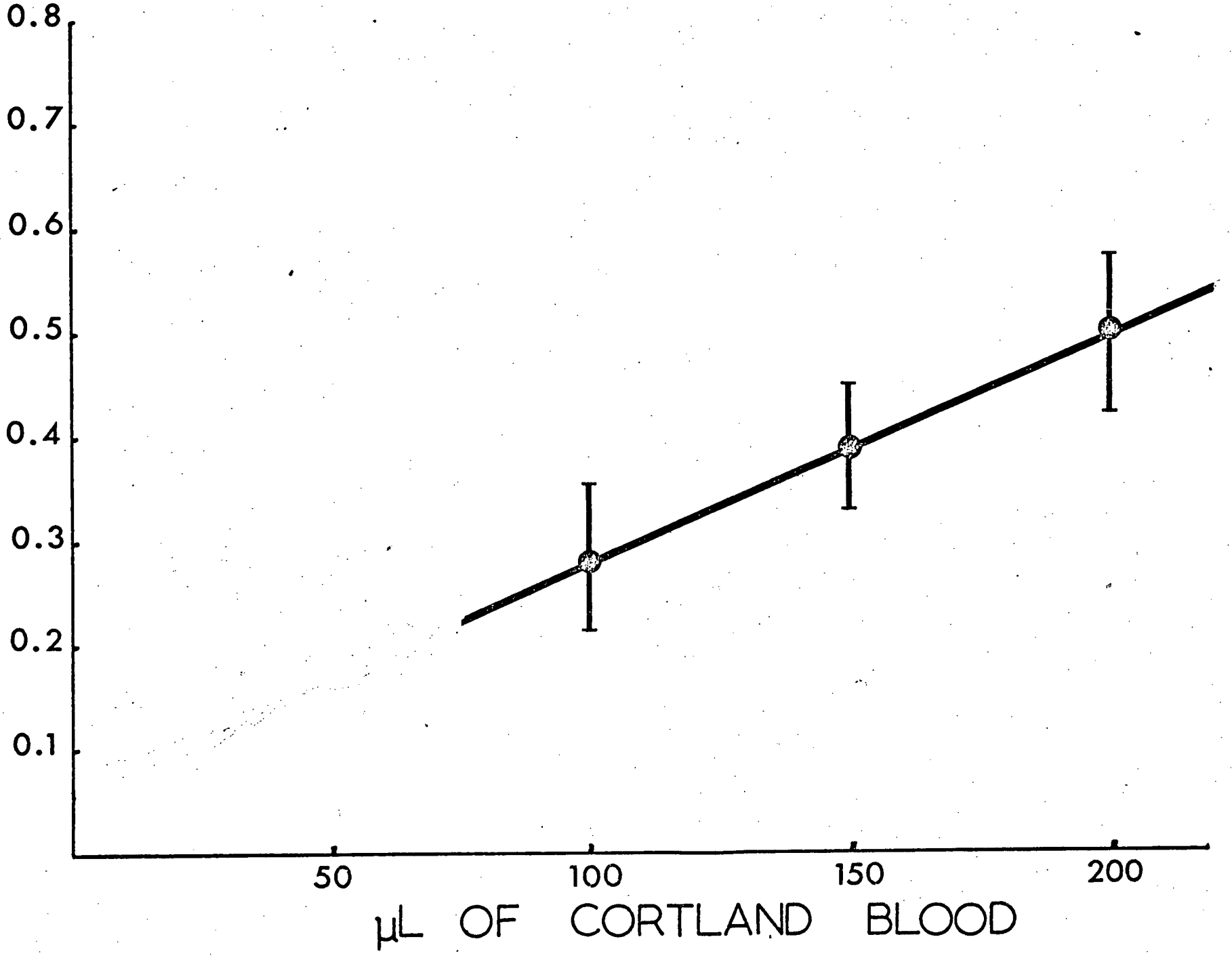

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
50
100
150
200
µL OF CORTLAND BLOOD

FIGURE #8. Effect of removing original plasma on dehydration activity of trout blood.

however, it appeared that washing the cells was without effect, and the presence of an effective inhibitor at the whole cell level was not evident.

Chloride Exchange in Fish Red Cells

The finding that trout red cells failed to dehydrate extracellular bicarbonate implies that either chloride/bicarbonate exchange mechanism does not exist in trout red cells or alternately carbonic anhydrase is inhibited *in situ* preventing the catalyzed dehydration reaction.

Rates of Red Cell Alkalinization

Under these experimental conditions the net efflux of chloride is directly related to the presence and/or ability of red cells to take-up bicarbonate as the cells attempt to maintain Donnan equilibrium. Under these conditions the uptake of bicarbonate *per se* is not measured but rather the resultant change in red cell pH.

Washed trout red cells suspended in 300 mM sucrose rapidly take-up bicarbonate as reflected by the rapid and large rise in red cell pH, see figure #9. This rise in pH continues at a slower rate until the red cells ultimately hemolyse. At 5 C all washed cells were completely hemolyzed after several hours. On the contrary unwashed cells suspended in sucrose show a small initial rise in pHi of approximately .2-.4 pH units. However the red cell pH rises only very slowly or not at all after the first minute. Blood from two separate fish was subjected to sucrose + 50 mM bicarbonate. Even under these elevated plasma bicarbonate levels red cell pH remained stable, table #4. In fact even after approximately 24 hours pHi was elevated only .2 units from the

FIGURE #9. Effect of suspending trout erythrocytes in 300 mM sucrose + 5 mM $NaHCO_3$ on intracellular pH. Closed circles represent erythrocytes from whole blood while all other cells were obtained from washed cells. See text for details. Water temperature was maintained at 5 C.

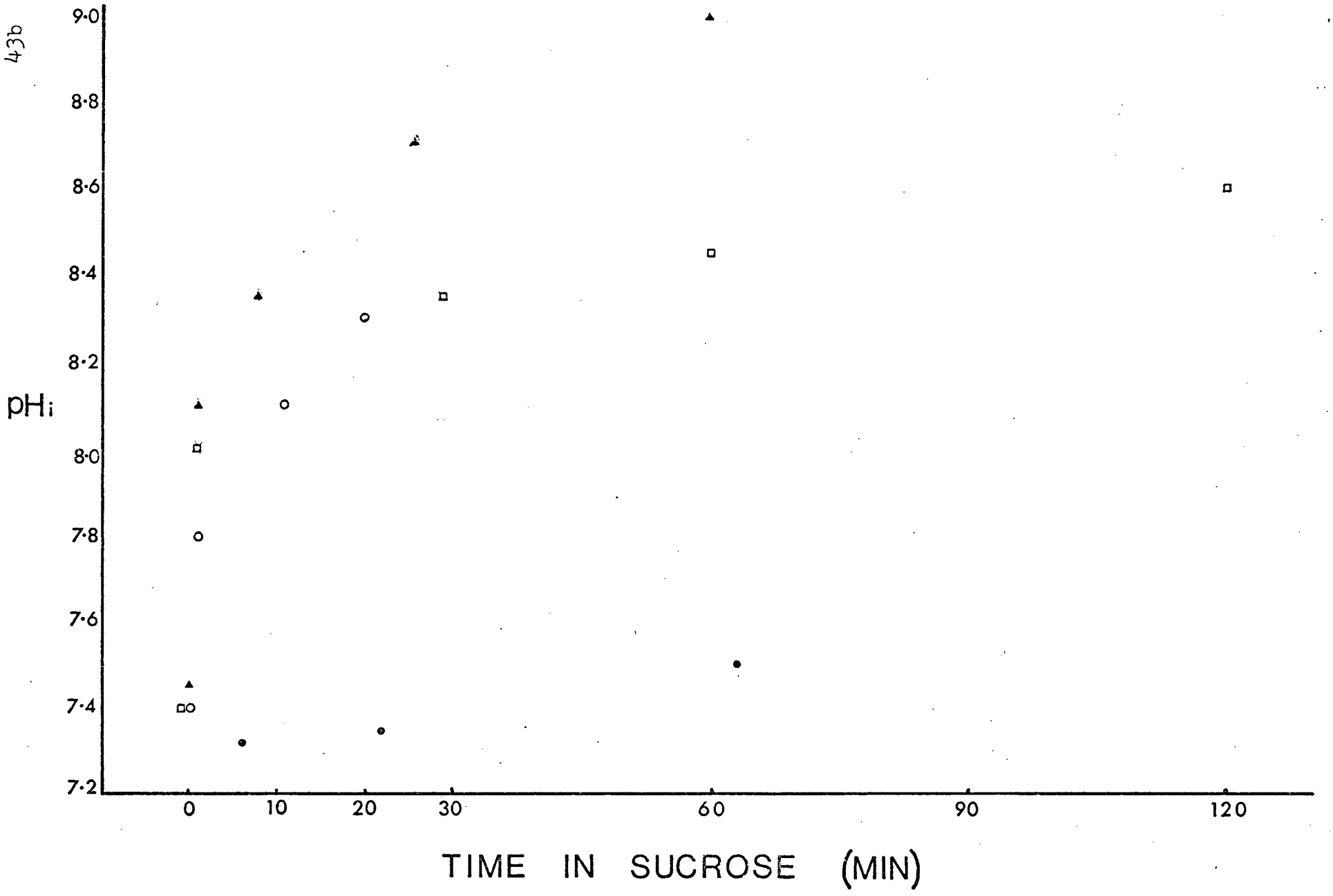

9·0
8·8
8·6
8·4
8·2
pHi
8·0
7·8
7·6
7·4
7·2
0
10
20
30
60
90
120
TIME IN SUCROSE (MIN)

TABLE #4. Effect of suspending red cells from whole trout blood in 300 mM sucrose + 50 mM $NaHCO_3$ on intracellular pH.

		TIME IN SUCROSE + 50 mM $NaHCO_3$					
	Initial pHi	1 min.	8 min.	16 min.	30 min.	60 min.	24 hours
Blood Sample "A"	7.6	7.9	7.9	7.95	-	8.05	8.0
Blood Sample "B"	7.6	8.1	8.05	8.0	8.0	-	8.3

reading obtained after 1 min and hemolysis was never evident.

When trout blood was tonometered for one hour with 1% CO2 in air, approximately 90% of the total CO2 was found to be present in the plasma.

Partial Characterization of Plasma Factor

Preliminary attempts to isolate and characterize the plasma inhibitor in trout plasma have revealed the following. The plasma factor is heat and acid labile. The factor is nondialyzable against either deionized water or balanced salt solutions. The greatest fraction of activity is removed by salting out the proteins with a 50-75% ammonium sulfate solution. Thus it would appear the plasma inhibitor is probably protein in nature and sufficiently large as to be unable to pass through dialysis tubing.

DISCUSSION

Booth (1938a) concluded that it was impossible to measure carbonic anhydrase activity of intact erythrocytes utilizing manometric techniques. Booth, using rat blood, found that only the initial few seconds of the reaction reflected true carbonic anhydrase activity, after which the rate fell to the uncatalyzed rate. He stated that this was due to enzyme substrate accessibility factors. He argued that maximal carbonic anhydrase activity was only possible during the initial phase of the reaction when intracellular chloride would be available to exchange with extracellular bicarbonate.

In my hands, whole rat blood and washed fish blood consistently gave linear activity during the evolution of the first 200 microliters of carbon dioxide evolved. Booth also stated that some methods such as the "blob technique" (a term coined by Booth whereby the red cells remain aggregated rather than dispersing randomly) and minimizing temperature equilibration times helped maintain intracellular chloride levels, so that the rate of CO2 evolution would be maintained longer. While the times for temperature equilibration were kept constant, it was not possible to demonstrate any difference between the "blob technique" and "normal" pipetting. The reason for the disparity in Booth's results and mine is not clear.

Booth's conclusion that enzyme substrate activity is crucial to carbonic anhydrase activity in intact erythrocytes seems unquestionably true. If a small quantity of saponified rat blood is analyzed for carbonic anhydrase activity it has approximately a 7-10 fold greater activity than the unlysed

equivalent. This finding is in agreement with Meldrum and Roughton (1933), who found that lysed blood from the ox and the goat had a 4 and 40 fold greater activity than the unlysed equivalents.

The salient feature concerning the present assay of rat blood, in contrast to Booth's data, is that the reaction rates were measurable, reproducible and obeyed linear kinetics. It is also interesting to note that Meldrum & Roughton make no mention of difficulty in assaying carbonic anhydrase activity from whole blood . Thus it seems that the assay methods used could accurately portray any catalysis of bicarbonate to C02 occuring within the intact erythrocytes, the rate being determined by carbonic anhydrase activity and accessibility of substrate.

Original attempts to estimate trout carbonic anhydrase activity were difficult to evaluate. While it certainly appeared that trout blood lacked any dehydration activity, Booth's arguments seemed compelling. Initially it was felt that due to the methods used any dehydration activity was simply going undetected. However when even 10-20 microliter volumes of rat blood gave obvious dehydration activity it became harder to understand why milliliter volumes of trout blood lacked activity. The chance finding of a plasma inhibitor generated new confidence in the method and certainly helped explain the difference between fish blood and rat blood.

That an inhibitor of erythrocytic dehydration activity exists is demonstrated by the results of trout assays included in figure #8. The occasional activity found in some of the earlier preparations is probably a reflection of a combination

of liberated carbonic anhydrase from hemolyzed cells and/or a dilution of the inhibitor.

A plasma inhibitor of carbonic anhydrase is not a new finding . Booth (1938b) found inhibitors in the plasma of sheep,pigs, and rats among others. Maetz (1956b), working with fish, alsc found a plasma inhibitor. Leiner et al (1962) partially purified the plasma inhibitor of sheep and evaluated its effects on carbonic anhydrase from a variety of organisms, including fish. However all plasma inhibitors thus far established have been assayed for their inhibition of either hemolysates or some form of the purified enzyme. Any effects which these inhibitors may have had on intact erythrocytes has not been investigated. It is interesting to note, however, that washing rat blood had no effect on the dehydration activity (Booth found a plasma inhibitor in the blood of the rat).

Booth, in discussing his plasma inhibitor (he claimed a globular protein of some sort, later confirmed by Leiner's group), suggested its possible function was in immobilizing carbcnic anhydrase liberated by erythrocytes during the course of their normal destruction or during their injury. This originally attractive proposal was undermined when Booth found that human blood apparently lacked a plasma inhibitor.

Preliminary attempts to characterize the trout inhibitor do not exclude the possibility that it is also a globular protein. The plasma inhibitor is heat and acid labile and presumably is a large molecule as judged by its inability to pass through dialysis tubing. Also the finding that the inhibitor can be salted out with ammonium sulfate is consistent with the

suggestion that the plasma inhibitor is protinacious. The plasma inhibitor could seemingly operate in one of two fashions. The inhibitor could act at the level of the enzyme per se ,or the same effect could be achieved by inhibiting the influx of bicarbonate ions. A complete explanation of how the trout inhibitor functions is not possible; however the following tentative explanation can be offered at this time.

If trout blood was hemolyzed, dehydration activity was always observed, even if the original plasma was present. While this type of experiment does not conclusively demonstrate how the inhibitor functions it does establish that the integrity of the erythrocytic membrane is essential for the carbonic anhydrase inactivation to occur. Cabantchik and Rothstein (1974a,b), working with human erythrocytes, have demonstrated the presence of anion channels in the membrane. Furthermore, with the use of diethyl stilbene derivatives they found it possible to inhibit (in excess of 98%) anion fluxes, while cation fluxes remain unaffected. If this type of channel exists in trout erythrocytes a plasma inhibitor could functionally inhibit the enzyme in this fashion. Haswell et al (1978) using the red blood cells of the mouthbreeder , were unable to demonstrate any effect of plasma on the rates of chloride/chloride self-exchange. Preliminary investigations suggest plasma is also without effect on chloride/chloride exchange in the rainbow trout red blood cells. Thus it would appear that the presence or absence of plasma does not confer a generalized anion impermeability to fish red cells. In these investigations (present study and Haswell et al , 1978) it was

not possible to study in detail the effects of plasma on net chloride flux. In mammalian red cells the rate of chloride self-exchange proceeds at a rate some 1,000 times faster than net movements of chloride (Knauf et al , 1977). Thus the rates of ^{36}chloride equilibrium between plasma and cells proceeds with a half-time of only seconds during conditions of self-exchange. The analogous reaction, for example Cl/Acetate or Cl/lactate, takes of the order of minutes or longer . There is some evidence that these two processes (self-exchange versus net exchange) may utilize different transport mechanisms (Gunn et al ,1973). SITS and DIDS (both stilbonic acid derivatives) appear to inhibit both net and self-exchange in human erythrocytes. However the effects of SITS is variable in its response, for example, in the cow SITS produces only 80-90% inhibition of chloride efflux versus 98-99% in human red cells (Zeidler, personal communication). Additionally SITS was without effect on Cl/Cl exchange in trout red cells, although effective on red cells of Tilapia (Haswell et al, 1978). Thus it is possible that a negative effect of plasma on Cl/Cl exchange is not sufficient evidence to rule out the possible direct effect of plasma on Cl/HC03 exchange per se. Indeed the rates of red cell alkalinization would appear to confirm this suggestion.

The adoption of the cell alkalinization protocol provides a method of following the actual Cl/HC03 exchange, albeit under artificial conditions. When red blood cells are suddenly suspended in a chloride free medium a large chemical concentration gradient is established. However the movement of anions into erythrocytes is electrically silent via an exchange

diffusion process (Gunn et al, 1973), and the eventual efflux of chloride is only possible if counter ions of like charge are available on the outside. Thus , as chloride leaves, another anion must enter to maintain electroneutrality. In this study all solutions were made of analytical grade or better chemicals and the added bicarbonate was essentially the only available counterion. Under these conditions, as chloride leaves bicarbonate enters, maintaining Donnan equilibrium. The net consequence is an increase in red cell pHi. Washed red cells, when suspended in sucrose, rapidly gained bicarbonate as evidenced by the increased pHi. Hydrogen ion concentrations continued to fall although at a slower rate throughout the measurement period. This exchange process would be expected to continue until chloride equilibrium is eventually reached. After approximately two hours hemolysis was severe and further pHi determinations were impossible due to insufficient packed red cells. These results using trout erythrocytes are qualitatively in agreement with those of Zeidler & Kim (1977) working with calf red cells. In calf red cells hemolysis occurs before final equilibrium due to the loss of membrane structural integrity. These authors found that alkaline pH caused the loss of integrity of a membrane protein, the band 3 protein, which was responsible for the hemolysis. Hemolysis was not associated with red cell volume changes . In contrast to results obtained with washed erythrocytes, utilizing cells from whole blood resulted in quantitative and qualitative differences . Although an initial rise in pHi of between .2-.4 units was observed upon exposure to sucrose, the pHi thereafter remained constant or

rose only slightly. In the extreme situation where extracellular bicarbonate was elevated to 50 mM the pH change after 24 hours was only .2 units higher. Additionally no signs of hemolysis were apparent even after 24 hours. Thus it would appear that trout cells taken from whole blood are susceptible to elevations in pHi as compared to those cells previously washed and devoid of plasma. It may be the small initial rise in pHi is the result of outward diffusion of molecular C02 from the cell into the extracellular medium and/or associated with a changing red cell volume. There can be little doubt that trout red cell functioning is affected by the presence or absence of plasma. This is not the only instance where plasma born factors are known to affect red cell activity. For example, Seider & Kim (1978) demonstrated a factor resident in cow plasma which is capable of modulating red blood cell glycolytic rates.

The apparent nonpermeation of bicarbonate is significant from the standpoint of C02 excretion, but it can readily be appreciated the ability to rapidly alkalinize red cell pH will affect oxygen loading and thus may be important in oxygen transport as well.

Indeed this seems to be the situation in fish blood. Berg & Steen (1968) found that the rate of oxygen binding by blood leaving the rete structure of the eel swimbladder was much slower than the rate of oxygen unloading within the rete. This phenomenon has important consequences in swimbladder functioning (Fange, 1973; Steen, 1970). Forster & Steen (1969) took a closer look at oxygen binding kinetics of fish blood, using eel blood and clearly demonstrated the asymmetrical nature of the "Root"

shift. Forster & Steen found that at 10 C the half-time of oxygen release in the face of elevated PC02 and hydrogen ion concentration was around 0.02 seconds. Under the reverse conditions of decreased hydrogen ion concentration and elevated bicarbonate the rate of oxygen rebinding was fully 9 seconds or over 100X slower. Under conditions which should enhance oxygen binding (decreased hydrogen ion concentration and elevated bicarbonate levels) it would be predicted that plasma bicarbonate would shuttle into the red cell, combine with a cytoplasmic proton and form C02 and H20. This C02 molecule would then diffuse out of the cell following the C02 gradient. As previously stated carbonic anhydrase catalyzes this reaction in mammalian red cells and also the movement of bicarbonate is very rapid, therefore it is not surprising that the rates of oxygen loading and unloading in mammalian red cells are essentially equal (Forster & Steen, 1968). The asymmetrical nature of oxygen loading and unloading in eel red blood cells is consistent with the present analysis of trout red cell alkalinization . Clearly the rates of oxygen loading may be affected by the plasma factor . Another explanation of the data however does exist. As demonstrated in Chapter #1 a large portion of red cell carbonic anhydrase is bound to or at least associated with the trout red cell membrane. This also appears to be the situation in flounder erythrocytes (Haswell, 1977) and probably in all fish. Thus if this carbonic anhydrase is accessible to the extracellular environment a plasma born factor could functionally inhibit the enzyme <u>per se</u> . However the hydration reaction does occur in intact erythrocytes (Forster & Steen, 1968). Thus a plasma

factor would only inhibit the dehydration reaction and not the hydration reaction. Currently, no known carbonic anhydrase inhibitor is capable of inhibiting one reaction, either dehydration or hydration, without also inhibiting the back reaction. It seems reasonable at present to assume the enzyme can catalyze either reaction but substrate availability is tightly controlled.

In summary, it can be concluded that while enough carbonic anhydrase may possibly reside in fish red cells to catalyze the dehydration of plasma bicarbonate to C02, it is not possible to demonstrate carbonic anhydrase dehydration activity in intact erythrccytes. This inability of fish red cells to dehydrate plasma bicarbonate is apparently the result of the inability of this bicarbonate to gain access to red cell carbonic anhydrase. A factor in the plasma, probably protein in nature, is responsible for this inactivation. Over 90% of the total C02 found in trout blood resides in the plasma(table #10)and the ability to generate molecular C02 within the blood must surely limit the role of the erythrocytes and erythrocytic carbonic anhydrase in the excretion of C02 at the gills.

CHAPTER III: CO_2 EXCRETION IN THE TROUT: A ROLE FOR BRANCHIAL CARBONIC ANHYDRASE

INTRODUCTION

Over 90% of the total C02 in fish blood resides in the plasma and the bulk of excreted C02 originates as plasma bicarbonate. Furthermore, it can be demonstrated that the formation of molecular C02 is a slow process, and carbonic anhydrase is probably necessary. The significance of this slow uncatalyzed rate would be compounded if gill residence times are relatively short as is true of blood in mammalian lung capillaries (West, 1974). Hoffert and Fromm (1973); Hodler et al ,(1955); Maren & Maren, (1964) have repeatedly demonstrated the requirement of carbonic anhydrase for C02 excretion in fish. However this study has indicated that (1) fish red cells may not possess sufficient carbonic anhydrase to catalyze the dehydration reaction, based solely on theoretical grounds, and (2) trout red cells in vitro do not appear to dehydrate extracellular bicarbonate at all. Given the high levels of carbonic anhydrase activity in the gill it may be possible this tissue is the source of carbonic anhydrase responsible for the excretion of C02 at the gills. Certainly the finding that branchial carbonic anhydrase has a greater affinity for bicarbonate than the erythrocytic carbonic anhydrase provides further support for this possibility. The following experiments were designed to directly test this hypothesis.

MATERIAL & METHODS

All experiments were performed on rainbow trout (Salmo gairdneri) weighing between 200-400 grams. These fish were maintained in large circular tanks provided with oxygenated dechlorinated Vancouver tap water. The water temperature during the course of these experiments was between 8-10 C.

All fish were implanted with chronic indwelling catheters for blood sampling. Cannulas were implanted in the dorsal aorta according to Smith & Bell (1964) with the following modifications. Instead of utilizing a needle lodged in the dorsal aorta a length of P.E. 50 tubing (Clay-Adams) is advanced directly into the dorsal aorta utilizing a Sovereign Indwelling Catheter and needle assembly (Sherwood Medical, St. Louis, Mo.). The catheter assembly consists of a 20 gauge needle which fits snugly into the tip of a flared 2 inch long catheter. Upon successful location and penetration of the dorsal aorta the needle is withdrawn leaving the catheter still in the aorta. Once the needle is removed a length of P.E. 50 tubing can be advanced into the dorsal aorta. After insertion of the P.E. 50 tubing in the dorsal aorta, the Sovereign catheter is withdrawn from the aorta and removed, the P.E. 50 tubing is then secured with a silk ligature and pulled through a polyethylene nosecone (Smith & Bell ,1964). A modification of this technique is also utilized to cannulate the ventral aorta. In this case the P.E. 50 tubing was secured with two silk ligatures in the tongue, with the P.E. 50 tubing exiting through a small perforation in the soft tissue in the jaw. This technique is superior to the original method utilizing needles in that the cannulas remain

patent for much longer periods of time. All operative procedures were performed under M.S. 222 anesthesia. Fish utilized for gill perfusion studies were immobilized by a blow to the head subsequent to cannulation, the pericardinal cavity was exposed by a ventral incision, and a length of P.E. 190 tubing was secured with a ligature in the bulbus arteriosus. All other fish were allowed to recover in individual darkened lucite chambers for at least 24 hours before the experiments were initiated.

Blood Measurements

pH determinations were made utilizing a Radiometer PHM-71 Acid/Base Analyzer and associated micro pH electrode. In an attempt to improve *in vitro* measurements the use of a medical mass spectrometer, the "Medspect" (Searle Instr., Balt. MD.), has been employed. The Medspect is actually designed for *in situ* blood gas measurements in clinical practice. Gas analysis is obtained by utilizing either silastic or teflon covered hollow stainless catheters. The terminal segment of the catheters (approximately 1 inch in the silastic catheter) are perforated with a series of holes over which the silastic coating provides a diffusion barrier. The catheters (2) are fastened to the "Medspect" and the entire system operates under vacuum (approximately 10^{-6} torr.). Gases flow into the catheters from the membrane covered tips (air or liquid phase) to the mass spectrometer for subsequent analysis. Oxygen and carbon dioxide partial pressures (mm Hg) are displayed on digital meters. CO2 tensions can be expressed in 0.01 of a mm Hg while oxygen is read to the nearest mm Hg. The response times are essentially independent of temperature and the CO2 analysis is sufficiently

sensitive to detect differences between inspired and expired water PCO2's from a trout. While the system is designed for insertion in human arteries, due to the size and flow dependence of the silastic catheter it is not possible to obtain in situ gas analysis on trout. Consequently an in vitro system was constructed. One of the two catheters rests in a small thermostatted cuvette (0.3 ml volume) for determination of oxygen and carbon dioxide partial pressure. Although the response times of silastic catheters are faster than the teflon catheters (1-2 minutes versus 3-5 minutes) they are flow dependent. Consequently the cuvette is provided with a small teflon stirring bar and the whole apparatus placed on a magnetic stirrer. The second catheter in a second cuvette is utilized to determine oxygen and carbon dioxide contents. A degassed solution of acidified potassium ferricyanide (Van Slyke, 1927) is placed into the content cuvette (approximate volume 2 mls) and the initial PO2 and PCO2 noted. After introduction of a blood sample the final PO2/PCO2 are recorded (approximately 3 minutes). The total O2 is determined in accordance with the method of Tucker (1967), while total CO2 is calculated by utilizing NaHCO3 standards (Cameron, 1971). Gas mixtures provided by Wostoff gas mixing pumps (Bochum, W. Germ.) are utilized for calibration. Although this system still has a response time of 60-90 seconds it is a considerable improvement over CO2 electrodes and the increased stability and sensitivity over the temperature and CO2 range utilized is without comparison.

Experimental Protocol

A) Anemic Fish. After recovery initial blood samples were obtained to establish control levels for pH, PC02, total C02 content (TC02) and hematocrit (hct.). Severe anemia was then induced either by intraperitoneal injections of phenylhydrazine (Cameron & Davis, 1970) or by repeated bleeding, the blood lost being replaced by returning the plasma plus Cortland saline to the fish. It was difficult to remove all erythrocytes by either method and the anemic fish group had hematocrits of less than 4 percent compared with the control group with hematocrits of 18-25%. Dorsal aortic blood was sampled and pHa, PaC02 and TC02 were measured 24 hours after anemia had been established. Diamox dissolved in saline was injected (10 mg/kg body weight) into the dorsal aorta of anemic fish. Six hours later pHa, PaC02 and TC02 were measured in blood sampled from the dorsal aorta. Thus pHa, PaC02 and TC02 of arterial blood were measured in normal, anemic, and anemic plus Diamox injected fish. The same fish made up the anemic and anemic Diamox injected groups of fish.

C02 Excretion Rates

The effect of anemia on C02 excretion rates was measured by sealing a rainbow trout in a lucite chamber closed except for a water inlet and outlet. The water flow rate through the box and the C02 content of inflowing and outflowing water were determined. Anemia was then induced by intraperitoneal injections of phenylhydrazine and 24 hours later C02 excretion rates were again determined. This same procedure was also utilized to assess the effects of Diamox on C02 excretion.

Perfused Gills:

These experiments were carried out on 14 rainbow trout. A fish

was secured ventral side up in a lucite chamber and the gills were perfused via the ventral aorta with heparinized (10 I.U./ml) Cortland saline using a Harvard Apparatus motor driven syringe pump and a 100 ml glass syringe. The saline was equilibrated with 1% C02 mixed with air and held at water temperature. The saline passed through the gills, around the body and out through the cut ventricle at a rate of 4.5 ml/min. The first 100 mls of perfusate was used to wash out erythrocytes. Measurements were made on the second 100 mls of perfusate before and after flowing through the gills. The postbranchial sample was obtained through the indwelling dorsal aortic catheter. Gill ventilation was maintained at 1,000 ml/min from a constant head reservoir thrcugh a rubber tube inserted in the mouth. This rate of water flow should have been adequate to ensure C02 removal (Davis & Cameron, 1970). Diamox (10 mg/kg body weight) dissclved in saline was injected intraperitoneally into eight of the fourteen fish, the remaining six acting as a ccntrcl group. Diamox was injected six hours before any surgery was initiated.

The Henderson-Hasselbalch equation was used to calculate bicarbonate concentrations. In the perfusicn experiments TC02 content was also calculated utilizing the following equation.

TC02 = (alpha x PC02) + (alpha x PC02 x antilog pH - pK)

where, alpha is the solubility coeffecient of C02 in saline at 10 C and pK values are from Albers (1970) for human plasma.

RESULTS

A) Anemia

Anemia did not result in any change in pHa, PaC02, or the C02 content of arterial blood (table #5). The addition of Diamox, however, caused a marked drop in pHa and a near tripling of PaC02. The injection of Diamox into anemic fish was often lethal whereas injection of the same dose into controls was rarely so. Presumably the difference in effect is due to the buffering power of hemoglobin. All anemic fish survived the first six hours and the values were recorded at this time. Although no further change in pH or PaC02 was apparent after six hours TC02 frequently continued to rise, as evident in those fish surviving for longer periods of time.

Arterial blood pH and PaC02 was unaffected by hematocrit (Figures #10 and #11). C02 excretion rates were also unaffected by anemia (table #6). Anemia was correlated with a decrease in blood oxygen capacity as expected.

B) Gill Perfusion:

Perfusion of the gills with saline equilibrated with 1% C02 in air resulted in the removal of 12% of the total C02 content of the perfusate (table #7). Only 5% of the total C02 present in the inflowing perfusate was molecular C02, the remainder was bicarbonate. Transit time for saline flow through the gills was 1-3 seconds as judged by the appearance of methylene-blue in the dorsal aortic catheter. The half-time for the uncatalyzed reaction velocity for bicarbonate at 10 C is around one minute

(see Chapter #2). Treatment of rainbow trout with Diamox before saline perfusion reduced C02 excretion to zero in saline-perfused gills (table #7). The excretion rates in untreated gill preparations are quite similar to those calculated from arterial-venous TC02 content differences found in free swimming fish (mean = 11.4% plus or minus 4.3%, when n=5 plus or minus S.D.).

TABLE #5. Effect of anemia and subsequent carbonic anhydrase inhibition (Diamox) on dorsal aortic pH and PCO_2 in the rainbow trout. Water temperature was 9 C.

	pH	pCO_2
Control period	7.82 ± .08	2.64 ± .5
Anemic period	7.86 ± .08	2.55 ± .35
Diamox + 6 hrs.	7.44 ± .16	6.85 ± 1.7

[n=11, mean ± s. dev.]

FIGURE #10. Effect of hematocrit on arterial pH in the rainbow trout.

62d

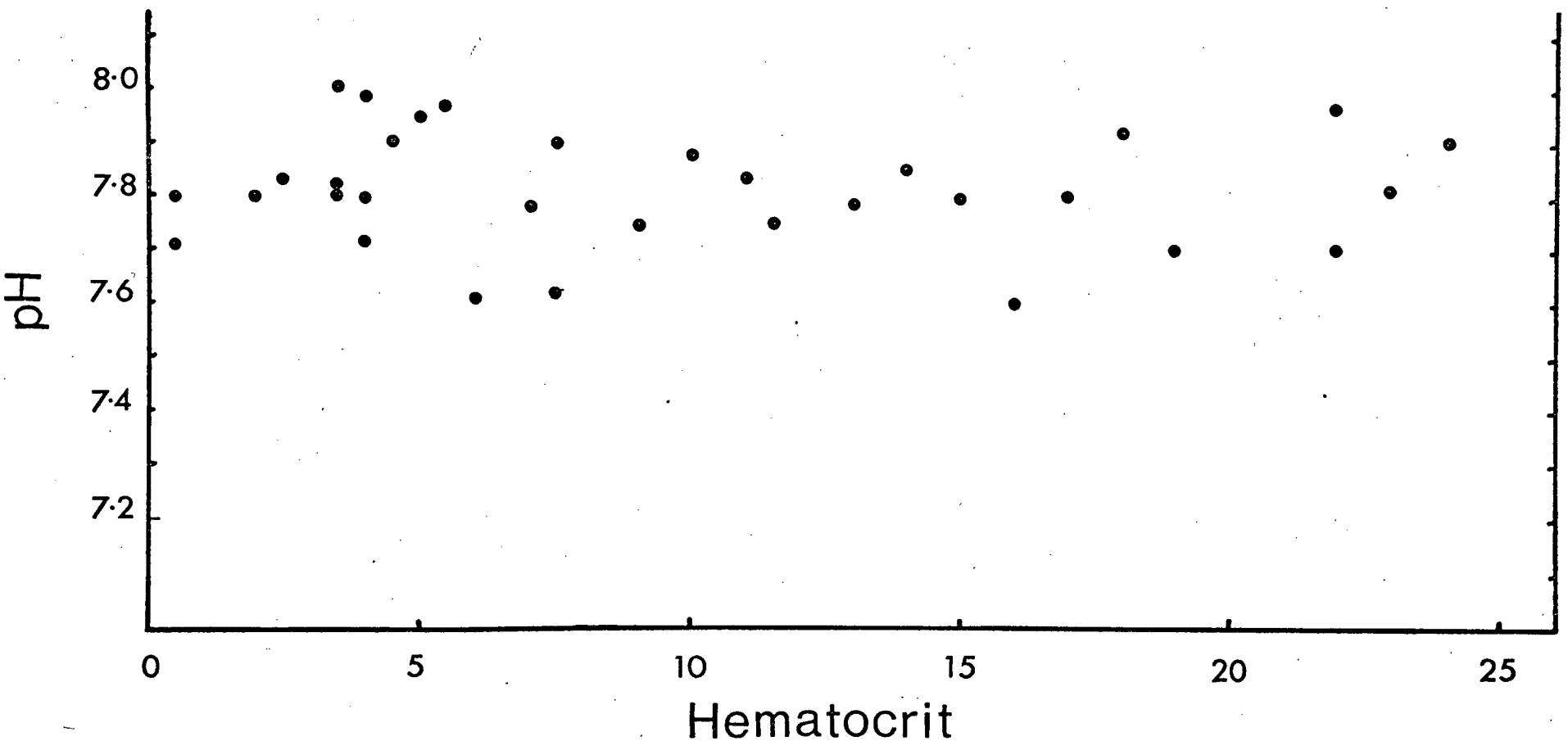


FIGURE #11. Effect of hematocrit on arterial PCO_2 in the rainbow trout.

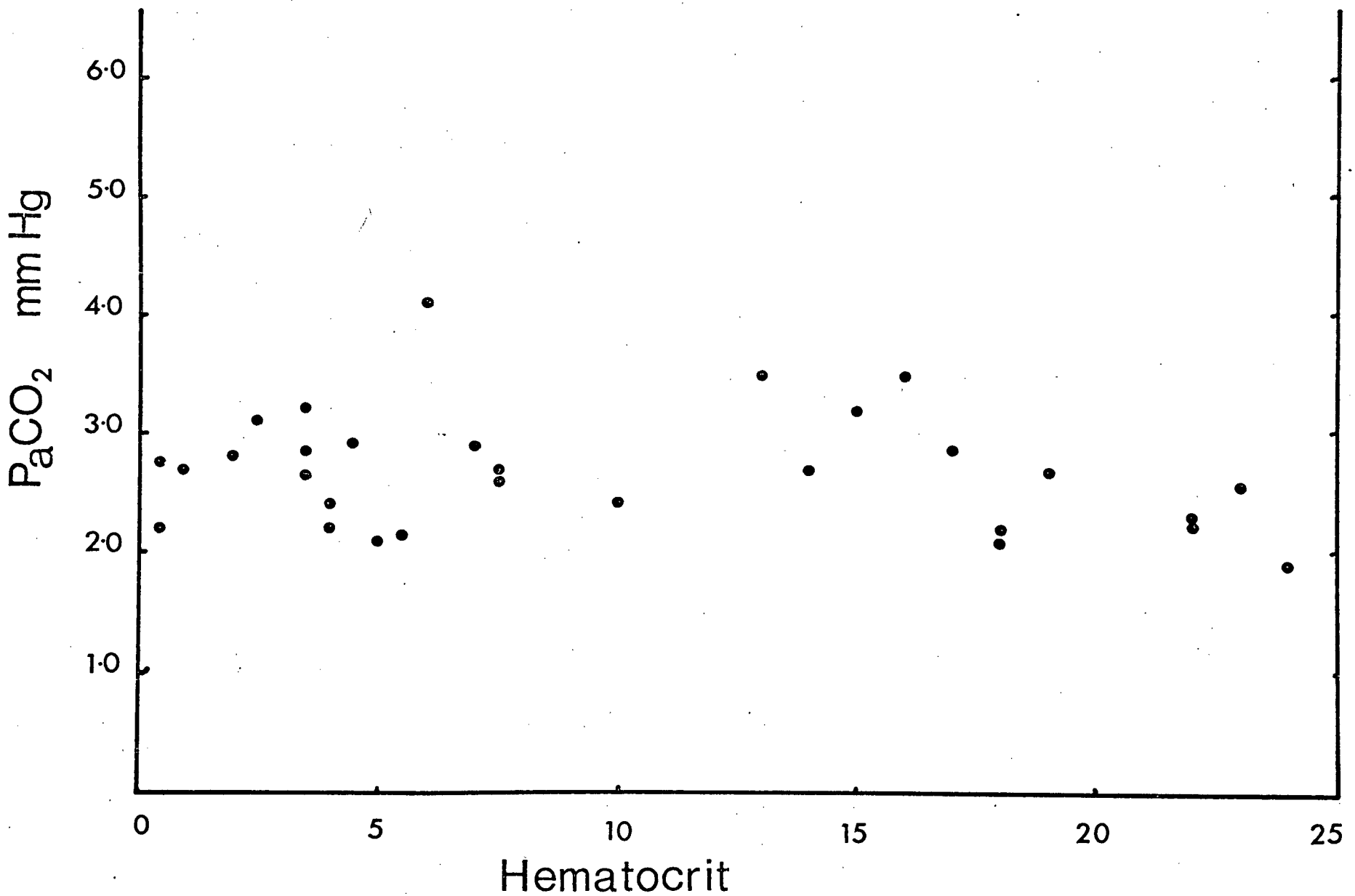

6·0
5·0
4·0
3·0
2·0
1·0
P_aCO_2 mm Hg
0
5
10
15
20
25
Hematocrit

TABLE #6. CO_2 excretion following phenylhydrazine treatment in rainbow trout (see text for details). Values not significantly different.

	CONTROL	ANEMIC
CO_2 Excretion Rate (mM/hr) (± S.D., where n=6)	4.83 ± 1.36	3.99 ± 1.65

TABLE #7. Effect of diamox on CO_2 excretion in perfused trout gills. All values depicted for control fish are significantly different at the 95% confidence level using the students "t" test. Experimental values not significantly different.

	CONTROL FISH (n=6 mean ± S.D.)		DIAMOX-INJECTED FISH (n=8 mean ± S.D.)	
	Perfusate	Dorsal aorta sample	Perfusate	Dorsal aorta sample
pH	7.495 ± 0.008	7.586 ± 0.051	7.469 ± 0.017	7.419 ± 0.085
PCO_2	7.5	5.36 ± 0.69	7.5	8.68 ± 2.29
CO_2 (g) (mM)	0.51	0.37 ± 0.05	0.51	0.59 ± 0.16
HCO_3 (mM)	9.39 ± 0.18	8.4 ± 0.67	8.86 ± 0.35	8.81 ± 0.65
Total CO_2 (mM)	9.9 ± 0.18	8.77 ± 0.67	9.37 ± 0.35	9.4 ± 0.75
EXCRETION (%)	11.5		Zero	

DISCUSSION

In intact rainbow trout the dehydration of plasma bicarbonate provides the majority of excreted CO_2, and plasma bicarbonate concentrations are reduced by between 10-30% as the blood passes through the gills. Diamox injected into trout results in a marked increase in $PaCO_2$ and a reduction in pHa, indicating that carbonic anhydrase is important in CO_2 excretion (Hoffert & Fromm, 1973). Plasma bicarbonate is excreted as CO_2 and the dehydration reaction is catalyzed by carbonic anhydrase because, firstly, in the present study, CO_2 excretion was reduced to zero in the saline-perfused gill following application of Diamox and, secondly, the transit time for blood flow through the gills is of the order of a second, much less than the half-time for the dehydration reaction.

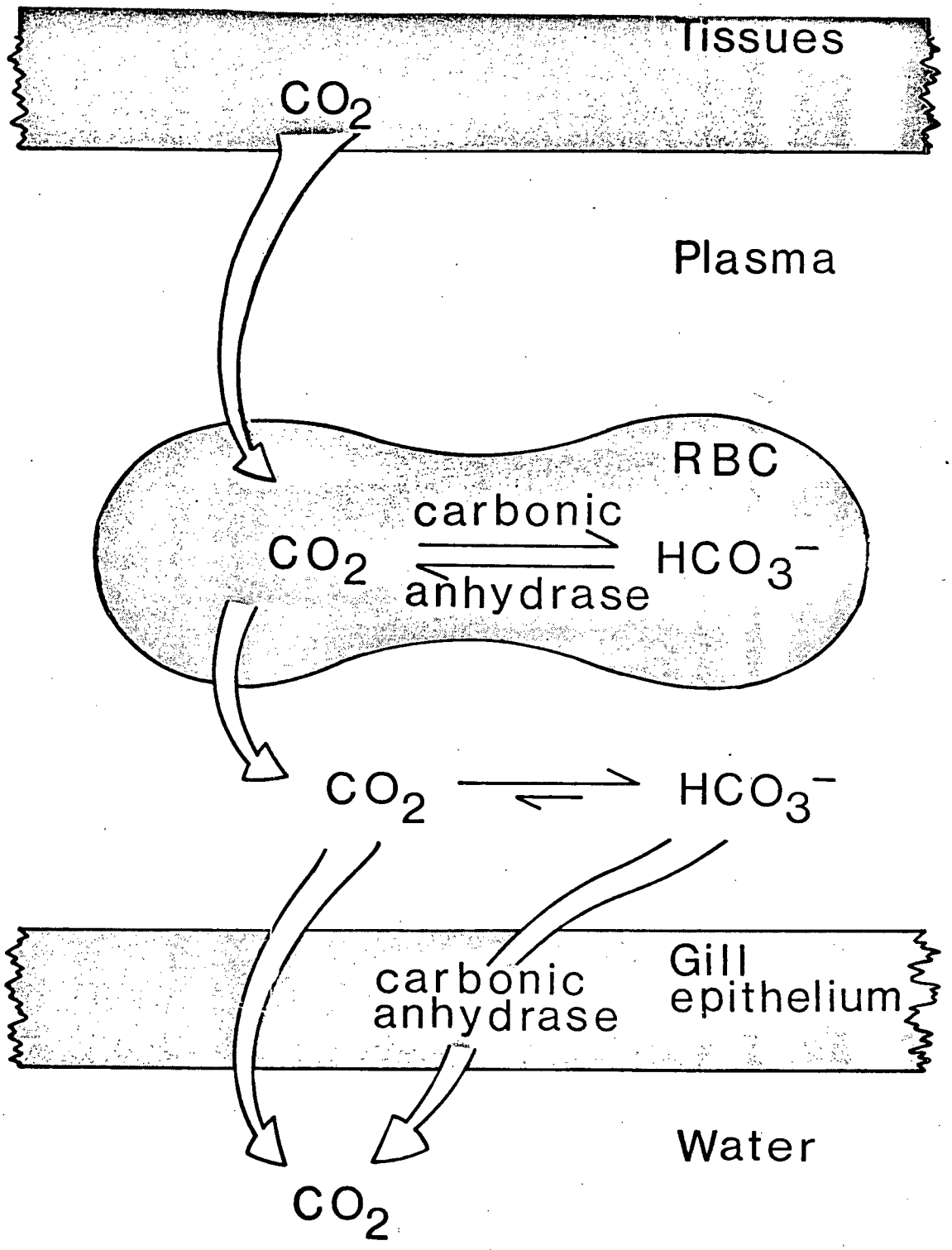
The results of the anemia experiments indicate that erythrocytes are unnecessary for plasma bicarbonate dehydration, a conclusion consistent with the observation that fish erythrocytes *in vitro* do not catalyze plasma bicarbonate dehydration. Normal rates of CO_2 excretion are maintained during anemia and the saline-perfused gill is able to excrete bicarbonate ions in the absence of erythrocytes.

The ratio of gill epithelial to erythrocytic carbonic anhydrase is around 1 (table #3). Carbonic anhydrase from the gill has a higher substrate affinity than that from erythrocytes (this study and Girard & Istin, 1975) and it is concluded that plasma bicarbonate is dehydrated within the gill epithelium.

What then is the function of erythrocytic carbonic anhydrase in fish? Although fish red cells may be impermeable to

plasma bicarbonate they are permeable to C02 and so bicarbonate will be formed in the erythrocytes as C02 enters the blood in the tissues, causing a Bohr shift augmenting oxygen transfer to the tissues. The slow Root-on shift observed by Berg & Steen (1968) is consistent with the hypothesis that the fish erythrocytes are impermeable to the efflux as well as influx of bicarbonate across the red cell membrane. If this is the case then as blood leaves the tissues and enters the veins , plasma bicarbonate will be formed at the uncatalyzed rate following the rise in plasma C02 levels in the tissues. The blood in the veins is a closed system, hence, as plasma bicarbonate levels increase, PC02 falls. C02 will diffuse from the erythrocytes into the plasma and red cell bicarbonate will be dehydrated. The rate limiting step will be the uncatalyzed hydration reaction velocity in the plasma. Thus some of the bicarbonate formed in the red cells while blood is in the tissue capillaries will be dehydrated to C02 before blood reaches the gills. Plasma bicarbonate enters the gill epithelium and is dehydrated to C02 before diffusing into water flowing over the gills (figure #12). Blood transit times are around a minute or two in fish (Davis, 1971) and this plus the experimentally derived half-time of around one minute give support for this hypothesis. Calculations based on C02 excretion rates and changes in plasma bicarbonate before and after the gills indicate that the majority of C02 excreted originates as plasma bicarbonate. Thus the inclusion of carbonic anhydrase in fish red blood cells is probably <u>a priori</u> to produce a rapid Bohr and/or "Root- off" shift. Thus erythrocytic carbonic anhydrase is utilized to facilitate the

FIGURE #12. Summary of carbon dioxide excretion in rainbow trout.


Tissues
CO_2
Plasma
RBC
carbonic
anhydrase
CO_2
HCO_3^-
CO_2
HCO_3^-
carbonic
anhydrase
Gill
epithelium
Water
CO_2

loading and unloading of oxygen within red blood cells with little direct effect on C02 transport. Red blood cells are not required to effect C02 excretion and acid-base regulation in fish. This becomes even more apparent when one considers that the icefish (an antartic fish characterized by being totally devoid of either hemoglobin or erythrocytes and hence probably carbonic anhydrase as this enzyme has never been found extracellularly) , is characterized by normal rates of oxygen uptake and therefore presumably C02 excretion rates (Holeton , 1970).

As previously stated trout, like all other aquatic fish live in a medium relatively poor in oxygen, and must continually face the problem of extracting sufficient environmental oxygen to supply tissue needs. Fish cannot utilize changes in ventilation to achieve pH regulation, as do mammals and birds, without compromising oxygen delivery (Randall & Cameron, 1973). In mammals and birds the dehydration of plasma bicarbonate is never the rate limiting step in the production of dissolved C02. In fish, water/blood diffusion distances and ventilation:perfusion ratios are optimized to ensure oxygen transfer. If the dehydration reaction occurring in the blood was <u>not</u> the rate-limiting step, then the control of C02 excretion would not be possible, as the loss of molecular C02 would be uncontrollable. Therefore in fish the production of molecular C02, as it occurs in whole blood, is possible only at the uncatalyzed rate, since red cell carbonic anhydrase is unavailable to plasma bicarbonate. Due to the long uncatalyzed reaction times (especially at lower ambient temperatures) and

short residence times for blood in the gill very little CO2 will be formed from plasma bicarbonate as blood moves through the gill. This is supported by the observation that no excretion of CO2 occurred in the isolated perfused gills previously treated with Diamox. The observed CO2 excretion in fish is the result of the movement of plasma bicarbonate into the gill epithelium. Unlike molecular CO2 the movement of bicarbonate can be, and is, regulated to achieve control of overall CO2 excretion.

The movement of bicarbonate across the gill epithelium is likely to be complex, for instance, Randall et al (1976) have shown that bicarbonate flux can be reversed in dogfish. This observed reversal of bicarbonate flux across the gills modulated the acidosis caused by elevated CO2 levels in the blood. When bicarbonate enters the gills from plasma and forms CO2 there must be a co-transport of hydrogen ions into the epithelium . This problem has yet to be resolved.

The functional significance of this pattern of CO2 excretion compared with that seen in mammals and birds is the following. Firstly, the formation of plasma bicarbonate by hydration in the plasma, rather than bicarbonate diffusion from the erythrocytes, results in an elevation in erythrocytic pH and a binding of oxygen to hemoglobin in the veins, lowering PvO2 as blood flows from the tissues to the gills and augmenting oxygen gradients across the gills. Secondly, the excretion of a significant proportion of total CO2 as plasma bicarbonate via the gill epithelium allows for the modulation of CO2 excretion, and therefore blood pH, independent of oxygen-mediated ventilatory adjustments.

It is therefore possible tc further stress the analoqy of acid-base regulation in fish as compared to that in isolated single cell systems. However the exact relationship between ionic coupling and C02 excretion across the qill remains obscure.

CHAPTER IV - ACID/BASE REGULATION IN THE RAINBOW TROUT : A MODEL.

INTRODUCTION

There can now be little doubt that the gill of the aquatic telecst is the site of plasma bicarbonate dehydration. Furthermore the involvement of branchial carbonic anhydrase in this reaction is now firmly established. What remains obscure is the underlying mechanism which ultimately provides the desired alkalinity in the plasma of the free swimming fish. The isolated perfused gill preparation should provide a means of further characterizing the movement of bicarbonate into the gill; however it is not clear what determines the actual loss of C02 frcm this tissue. Although the rate of formation of molecular C02 (or the reverse reaction) will not be rate limiting due to the presence of branchial carbonic anhydrase, the relative loss of C02 may well be limited by the epithelial cell cytoplasmic pH. For example an alkalinization of the epithelial cell would shift equation #1a to the right reducing the formation of C02. Alternately the relative acidification of the epithelial cell would favor the formaticn of C02. It is assumed that a sufficient gradient from cell to water would exist so that molecular C02 once formed would be rapidly lost to the water. As previously mentioned single cells regulate intracellular pH by utilizing either cationic or anionic exchange mechanisms operating at the membrane level. Ionic exchange processes are of course known to be present in the gills of fish (Maetz, 1971; Maetz et al , 1976) and indeed these exchange processes may be modulated to achieve pH adjustments in fish (Cameron, 1976; De Renzis, 1975; Bornancin et al , 1977). Thus it may be possible that gill cationic and anionic exchange processes are modulated

in such a fashion as to achieve either a loss or gain of bicarbonate from plasma by controlling epithelial cell pH. Therefore, in addition to following bicarbonate movements from plasma into gill, it is of interest to follow plasma total C02 and pH as a function of salt movements into the gill.

MATERIALS AND METHODS

Isolated Perfused Gill Preparations

CO2 excretion as a function of perfusate bicarbonate levels:

The effect of increasing perfusate bicarbonate concentrations was analyzed using the perfused gill preparation as described in Chapter III. A total of 15 fish were perfused with varying HCO3 concentrations, sodium bicarbonate being added to Cortland saline to bring the final concentration of CO2 to the desired level. After the addition of bicarbonate pH was adjusted to approximately 7.48-7.5 with either HCL or NaOH.

Effects of SITS and Amiloride on CO2 excretion:

During the previously described perfused gill experiments it was found that the gill preparation was subject to a rapid degradation. Using this preparation as described the initial CO2 excretion rates invariably fell to levels approaching zero, or even negative values, after the first 30-60 minutes of perfusion. Due to this problem it was only possible to utilize one bicarbonate level per gill preparation and to analyze CO2 excretion only through the first 100 ml's of perfusion. As a result the variability among different preparations obscured any trends. An attempt was therefore made to increase their stability, and the following alterations were found to greatly increase the useful lifespan of the gill preparation. To Cortland saline 4% PVP (polyvinylpyrrolidine, MW = 40,000) was added as an osmotic filler after which the solution was filtered (0.45 micron, Millipore Corp). The filtered perfusate was then bubbled with nitrogen to lower the PO2 to approximate *in vivo*

PO2's (20-50 mm Hg). To this solution NaHCO3 was added to bring the final TCO2 concentration to the desired level. These alterations were found to greatly improve the preparation and stable CO2 excretion rates could be maintained for 4-5 hours. All subsequent perfused gill experiments incorporated these changes.

The effects of 0.1 mM concentrations of SITS and Amiloride in the perfusate on CO2 excretion rates were determined as follows. After initial CO2 excretion rates were determined either SITS or Amiloride was added to the perfusate. CO2 excretion rates were again determined after perfusion of the first 100 mls of perfusate or approximately 20-25 minutes after introduction of the drug.

Whole Animal Experiments

Chloride Uptake Rates

Apparent chloride influxes were determined by following the disappearance of radiochloride from the bathing medium in a small volume recirculating system of approximately 1.5 L. 36-Chloride was purchased as Na-^{36}Cl from New England Nuclear. Counting was done in a Nuclear Chicago Isocap liquid scintillation counter.

Effect of Na and Cl uptake inhibition on blood acid-base balance:

At least 24 hours after operative procedures duplicate blood samples were withdrawn for initial determinations of TCO2, PCO2 and plasma Cl. After sampling, the system, as described for the chloride flux rates above, was closed and the appropriate drugs were added to the bathing solution. NaSCN was added to a

final concentration of 10 mM. SITS (British Drug House) was added to a final concentration of .1 mM. Amiloride, a generous gift of Dr Dorian of Merck Frosst Laboratories, also was added to bring the final concentration to .1 mM. All values cited represent one hour of exposure to the drugs, unless otherwise stated, whereupon the animals were returned to freshwater. Experimental values were measured in duplicate.

Effect of SCN on Branchial Carbonic Anhydrase Activity.

Due to the significance of branchial carbonic anhydrase in the excretion of CO2, and the ability of SCN to inhibit this enzyme (Maren, 1967), the effect of SCN on branchial carbonic anhydrase was evaluated (see Chapter I, methods). Values at any given SCN concentration were measured in triplicate.

Analytical Procedures:

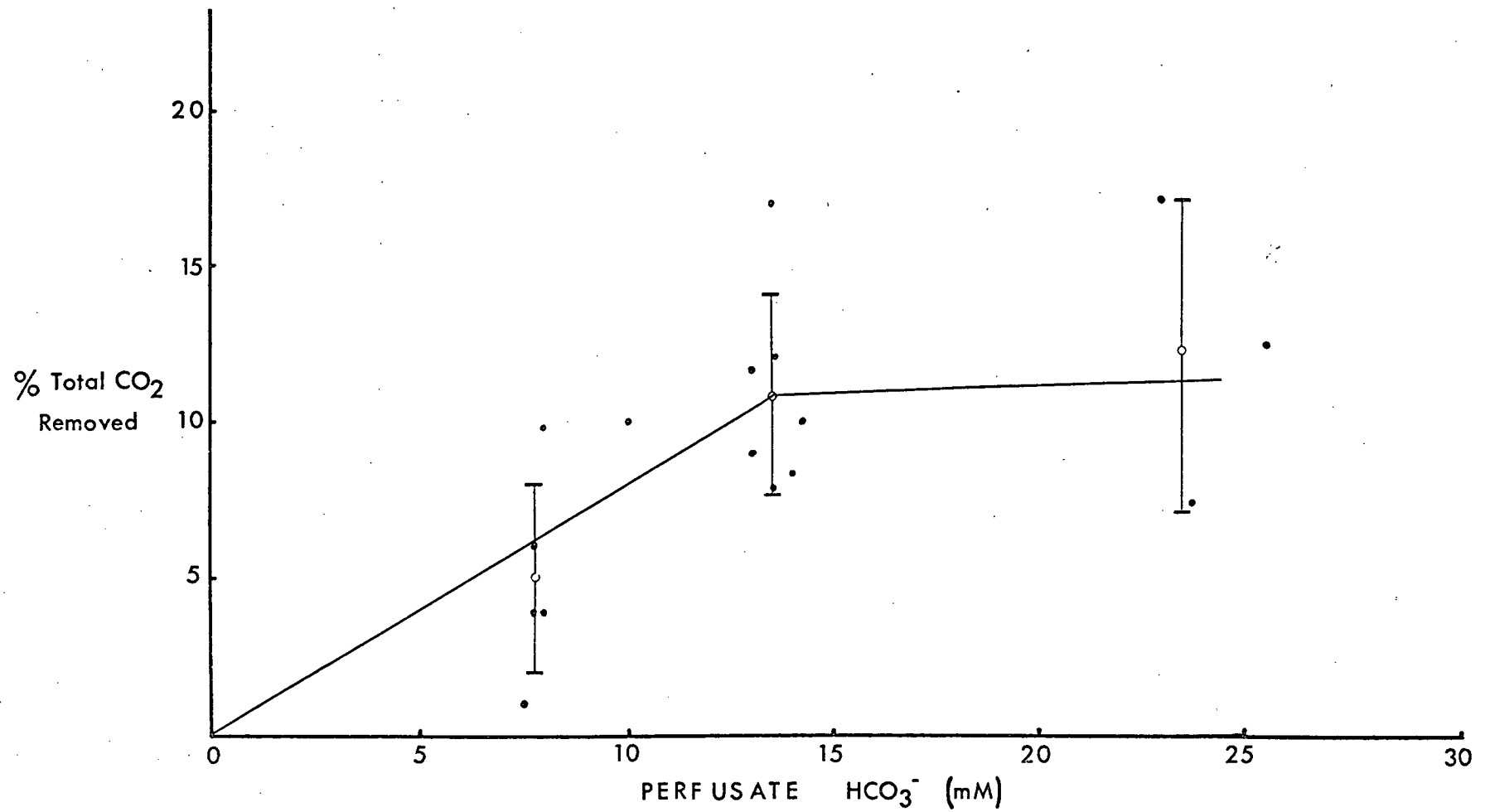
Due to technical difficulties with the Mass Spectrometer it wasn't operational during much of the investigative period reported in this section. Therefore during the perfused gill experiments TCO2 was determined utilizing the method of Cameron (1971). During amiloride and SITS experiments on whole animals plasma TCO2 was determined using a Harleco-micro CO2 analyzer. Chloride concentrations were measured in plasma samples utilizing a Radiometer CMT-10 chloride titrator.

RESULTS

Perfused Gill Experiments:

A total of fifteen fish, representing three experimental groups were perfused with either 8,14 or 24 mM bicarbonate. CO2 excretion rates with low bicarbonate levels (8 mM) were approximately 5%. This was lower than the CO2 excretion rates of approximately 10% obtained with medium bicarbonate levels (14 mM) or the highest bicarbonate concentrations employed (24 mM). These results are summarized in figure # 13. When these experiments were performed it was only possible to obtain one experimental point per fish, and consequently there is a large amount of variability in the data. However it appears the movement of bicarbonate into the epithelium is dependent on the perfusate bicarbonate concentration at any fixed pH. The apparent saturation observed at the highest bicarbonate concentration utilized may reflect a rate limiting step in the dehydration reaction, for example hydrogen ion availability, or alternately it may reflect saturation of the anion transport process. Introduction of .1 mM SITS into the perfusate totally abolished CO2 excretion in 4 of 6 fish tested. SITS cut CO2 excretion in another fish by 50% but was without effect in one fish. Thus inhibition of anion transport totally abolished or greatly reduced CO2 excretion in 5 of 6 fish examined. The reason for the lack of effect in the one fish is not clear; however it may be possible that the SITS was not of sufficient concentration or alternately may not have blocked anion transport in this particular animal.

FIGURE #13. Effect of increasing perfusate bicarbonate levels at constant pH on CO_2 excretion from the perfused gill preparation. (mean $\pm$ S.D.)


% Total CO_2 Removed
20
15
10
5
0
5
10
15
20
25
30
PERFUSATE HCO_3^- (mM)

That the principle source of protons for the dehydration reaction is from the perfusate, is demonstrated with the cation transport inhibitor amiloride. Amiloride in the perfusate completely abolished CO2 excretion in all three gill preparations exposed to amiloride.

Whole Animal Experiments:

When trout were exposed to 10 mM NaSCN in the bathing medium, chloride uptake was completely inhibited in the three fish where fluxes were determined. Figure #14 is typical of the results obtained and shows the change in bath 36-chloride counts/min versus time in control and thiocyanate exposure. After one hours exposure all fish (n=6) had a pronounced alkalosis, correlated with an increase in TCO2. Arterial CO2 tensions were unchanged while plasma Cl fell (Table #8). In one animal it was possible to determine VCO2 by following inspired and expired water PCO2's as described in Chapter III. Upon exposure to SCN, VCO2 rapidly fell by 22% (Table 9). After removal of SCN from the bathing solution introduction of Diamox (10 mg/kg IV) produced a reduction in VCO2 of approximately 60%. As branchial carbonic anhydrase plays a most vital role in CO2 excretion the effect of increasing SCN concentrations on branchial carbonic anhydrase was investigated, figure #15. At a concentration of 10 mM SCN the enzyme is fully 80% inhibited, with an apparent I50 of 0.8 mM.

SITS Treatment

In the trout, chloride uptake is inhibited by SITS in a dose dependent fashion . Figure #16 shows the typical response

FIGURE #14. Effect of 10 mM SCN on chloride influx in a single fish.

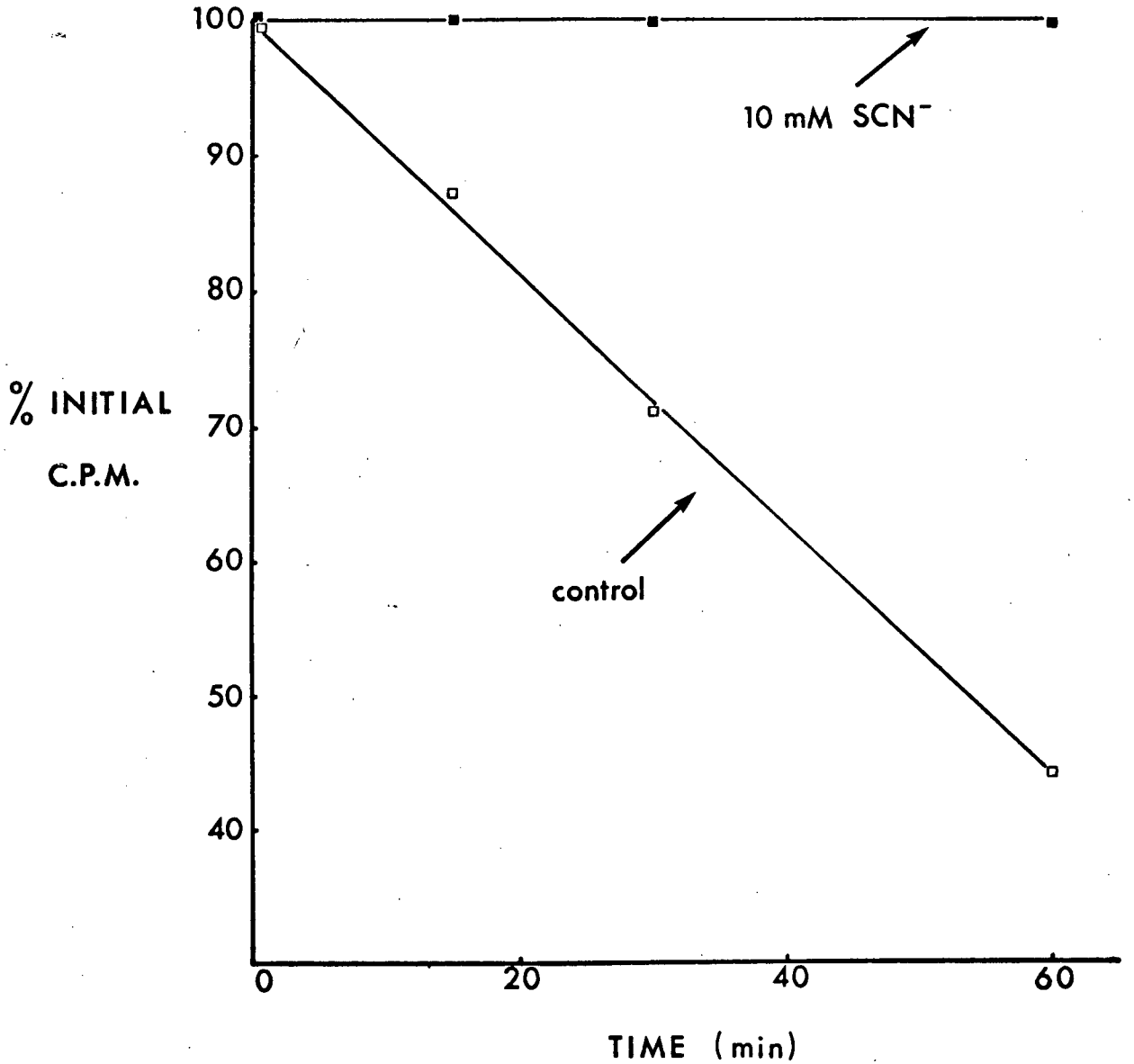

100
90
80
70
60
50
40
% INITIAL
C.P.M.
10 mM SCN⁻
control
0
20
40
60
TIME (min)

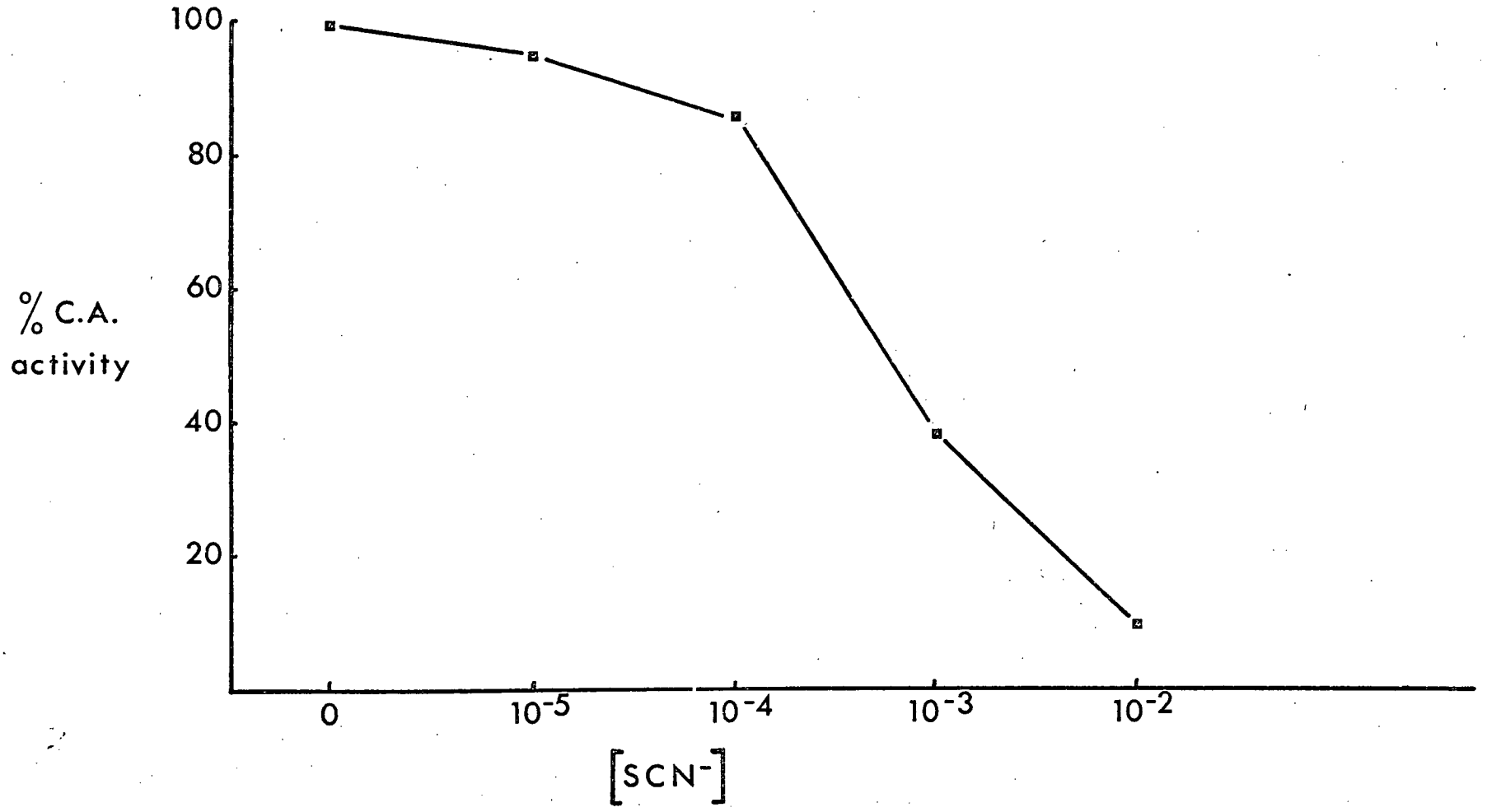
TABLE #8. Effect of 10 mM SCN on blood acid-base and chloride status. ($\pm$ S.D., where n=6)

	H^+	PCO_2	TCO_2	Cl^-
CONTROL	13.3 ± 1.77	2.41 ± 0.47	8.64 ± 1.37	121 ± 8.6
SCN	10.7 ± 1.1	2.41 ± 0.69	10.35 ± 0.82	112 ± 6.9
% Change	-19	N.C.	+24.6	-9.2

TABLE #9. Effect of 10 mM SCN and Diamox on CO_2 excretion rates in trout #5.

	Inspired Water PCO_2	Expired Water PCO_2	delta PCO_2	% Change
CONTROL	1.72	2.86	1.14	100%
10 mM SCN	1.71	2.60	0.89	22%
DIAMOX	1.80	2.30	0.50	56%

FIGURE #15. Branchial carbonic anhydrase activity with increasing SCN concentrations.


100
80
60
40
20
% C.A. activity
0
10-5
10-4
10-3
10-2
[SCN-]

of increasing concentrations of SITS on chloride uptake in a single fish. Inhibition was complete at .1 mM in all four fish where chloride influx was measured. Unlike its action in mammalian erythrocytes the inhibitory action of SITS appeared to be reversible in the trout gill. The effect of external SITS at .1 millimolar on blood acid-base status and chloride are depicted in table #10. No significant difference was obvious after one hour of SITS treatment. However if the SITS treatment was continued for 3 hours there was a significant rise in arterial pH and TCO2, table #11. This trend was evident in two fish where the measurements were continued past the first hour of exposure. SITS up to 1 millimolar was without effect on branchial carbonic anhydrase.

Amiloride Treatment

Kirschner et al (1973) demonstrated in the trout, that amiloride at 0.1 mM in the bathing water almost completely abolished sodium uptake. This reduction in sodium uptake was correlated with a 56% fall in titratable acid excreted. In this study amiloride at 0.1 mM resulted in a significant fall in pHa. In four of five fish tested TCO2 fell after one hour of exposure, although the means are not statistically different due to the large individual variation among animals. Chloride fell in a non-significant manner, table #12.

FIGURE #16. Effect of increasing concentrations of SITS in inspired water on chloride influx in a representative rainbow trout.

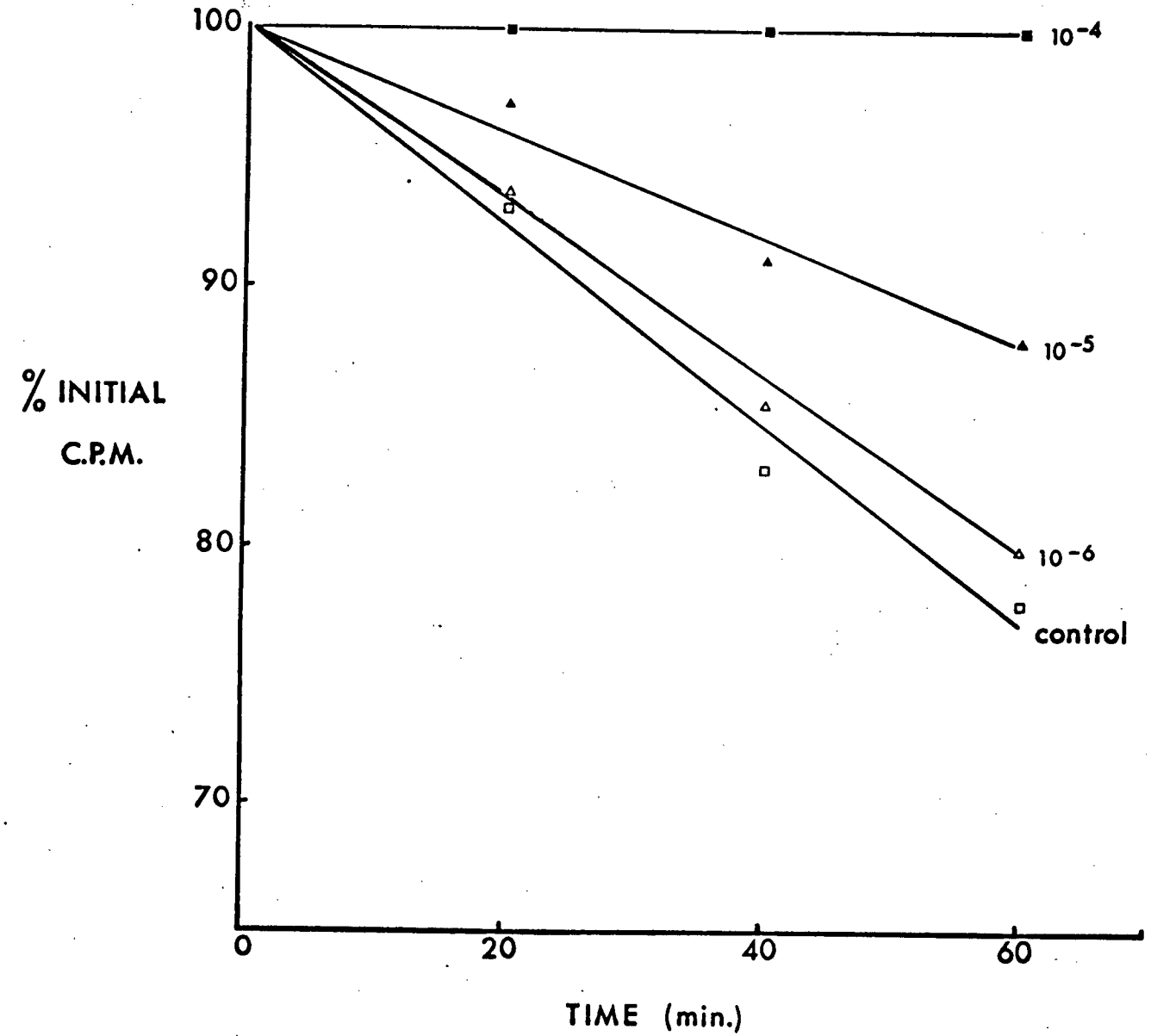

100
90
80
70
% INITIAL
C.P.M.
0
20
40
60
TIME (min.)
10-4
10-5
10-6
control

TABLE #10. Effect of one hour exposure to 10^{-4} M. SITS on blood acid-base and chloride levels. Values are not significantly different.

	pH	T_{CO_2} mM	Cl^- meq
Control	7.77 ± 0.07	10.3 ± 2.3	115.2 ± 2.9
10^{-4} SITS	7.72 ± 0.11	10.8 ± 2.5	113.9 ± 3.5

N = 6 , ± S.D.

TABLE #11. Effect of 3 hours exposure to SITS on blood acid-base and chloride status in an individual rainbow trout.

	Control	SITS + 1	SITS + 2	SITS + 3
pH	7.79	7.81	7.82	7.85
T_{CO_2}	7.5	7.74	7.82	7.85
Cl^-	113	112	110	108

TABLE #12. Effect of amiloride (10^{-4} M.) on blood acid-base and chloride levels in trout.

	pH	T_{CO_2}	Cl^-
Control	7.78 ± 0.05	11.55 ± 3.3	114.8 ± 2.6
Amiloride 10^{-4} M	7.67 ± 0.09	10.45 ± 2.4	113.6 ± 4.2

N = 5 ± S.D.

DISCUSSION

C02 movements through the teleost gill can now be characterized as follows. The ultimate dehydration of plasma bicarbonate occurs within the gill epithelium, this dehydration reaction being greatly dependent upon branchial carbonic anhydrase. The movement of the bicarbonate into the epithelium is a passive process being governed by the gradient between plasma and epithelial cell. The actual translocation of bicarbonate into the epithelial cells possess many of the characteristics of an exchange diffusion process similar to the anion transport system found in the red blood cell (Gunn et al ,1973). For example the transport of bicarbonate into the epithelium is probably a saturatable process (fig #13). The finding that SITS, a potent anion transport inhibitor, inhibited C02 excretion is also consistent with this conclusion. The maximum C02 excretion rates may be a function of the number of available anionic transporting sites available for bicarbonate transport. Thus for any given blood distribution pattern the C02 excretion rates attainable will be proportional to the entry of bicarbonate.

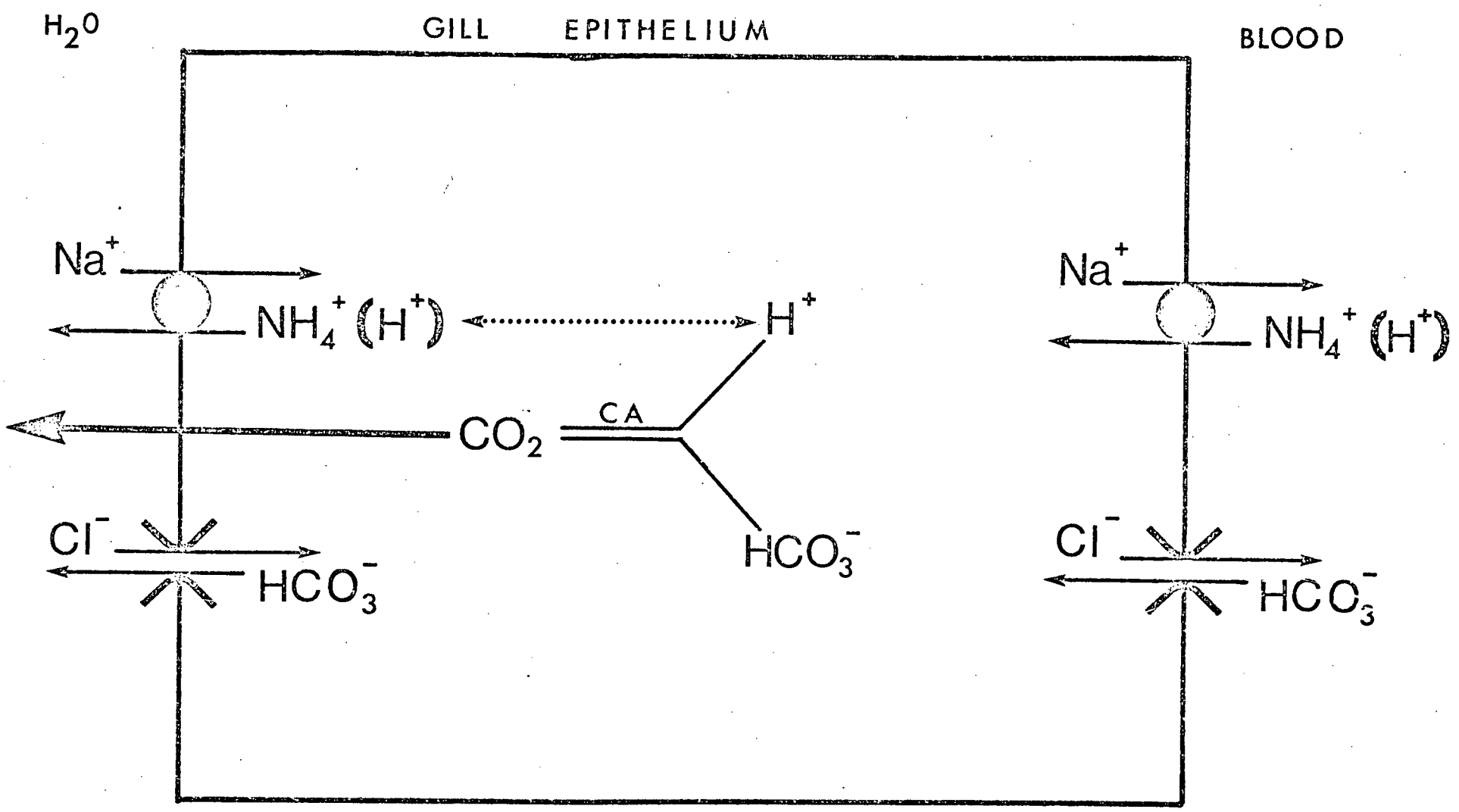
The ultimate source of protons for the bicarbonate dehydration reaction comes principally from plasma. This conclusion is based on the following observations. (1) Introduction of acid into either the blood or the gut pass through the plasma/gill membrane as judged by the stimulation of sodium uptake on the apical membrane (Payan & Maetz ,1973; Maetz ,1973). (2) In this study amiloride in the perfusate resulted in the total loss of C02 excretion from the isolated

perfused gill preparation. Kirschner et al (1973) have already demonstrated that amiloride potentiated inhibition of sodium uptake from water and also blocked the excretion of hydrogen ion efflux. Thus ,if amiloride also blocks hydrogen ion entrance into the gill from the perfusate, the fall in C02 excretion can be interpreteted as probably a proton limitation for the dehydration reaction occurring within the epithelium.

NaCl movements across the Gill: A Model for Acid-Base Regulation in Aquatic Fish.

In the trout, inhibition of chloride uptake resulted in an alkalosis and retention of C02. On the contrary, inhibition of sodium uptake resulted in a blood acidosis and lowering of plasma TC02. These results now make it possible to formulate a simple model capable of explaining acid-base regulation as well as predicting salt movements. In this model the excretion of C02 is not controlled per se ,but rather is a necessary consequence of the active regulation of hydrogen ion levels, or more precisely a constant relative alkalinity (Howell et al , 1970). Thus TC02 in blood may rise or fall, but this rise and fall will be tightly correlated to the appropriate bicarbonate levels necessary to achieve any fixed hydrogen ion concentration. Foremost to the effective regulation of plasma acid-base status is the ability of fish to control hydrogen ion levels within the cytoplasm of epithelial cells. The following scheme can now be constructed to explain C02 excretion through the gill epithelium, see figure #17. The placement of anionic and cationic exchange sites on the apical membrane is well

FIGURE #17. Pattern of CO_2 and salt movement through the teleost gill presented diagramatically.


H_2O
GILL EPITHELIUM
BLOOD
Na^+
$NH_4^+ (H^+)$
H^+
CO_2
CA
HCO_3^-
Cl^-
HCO_3^-
Na^+
$NH_4^+ (H^+)$
Cl^-
HCO_3^-

established (Maetz et al , 1976; Maetz, 1971). At least part of this cationic exchanger is ouabain sensitive and requires ATP (Payan & Maetz, 1973). The Cl/HC03 exchange is prcbably an exchange diffusicn system. The existence of a HC03-dependent ATPase in fish gills has been well documented (De Renzis & Bornancin , 1977; Kirschner & Kerstetter, 1974; Van Amelsvoort et al ,1977); however its functional involvement in ionic exchange has yet to be established uneguivocally. The location of the ionic uptake sites on the basolateral border have already been discussed.

During steady-state conditions the fate of bicarbonate will depend on several factors, the foremost being cytoplasmic hydrogen ion levels. If cytoplasmic hydrogen ion levels rise equation #1 would be driven to the left, thus favoring the formation of C02, the presence of carbonic anhydrase ensuring the reaction velccity is not rate limiting. Once formed the half-life of C02 in the cytoplasm would be extremely short as it is lost via diffusion to expired water. Conversely, should hydrogen ion levels fall in the cytoplasm the reaction wculd be shifted to the right, resulting in a retention of HC03 as protons become limiting. Amiloride in the perfusate totally abolished C02 excretion in the isolated perfused qill preparation and is an extreme example. Thus under steady-state conditions (constant metabolic rate and movement of bicarbonate and protons into the epithelial cells) it is clear that the availability of protons for the dehydration reaction will determine the volume of C02 produced from the dehydration of bicarbonate, albeit catalyzed. Under these steady-state

conditions, altering the exchange rates on the water/epithelial membrane will produce predictable effects on the cytoplasmic pH. When chloride uptake is inhibited at the outer membrane (apical), as during SITS exposure, bicarbonate (normally excreted in exchange for chloride) accumulates and results in an accumulation of bicarbonate within the blood (table #17). The failure of inhibition of Cl/HC03 exchange tc produce a more obvicus effect may be related to its relatively small capacity. Cameron (1976) calculated that maximally only 2-4% of the TC02 excreted was linked to the uptake of chloride in the arctic grayling. A change of 2-4% in the plasma TC02 is probably within the experimental error of the TC02 measurements, so that it may go undetected during the first hour. When sodium uptake is inhibited the retained hydrogen icns produce a fall in blood pH and TC02.

De Renzis (1975) allowed goldfish to acclimate (6-9 weeks) in water devoid of either sodium or chloride. He found that fish placed in chcline chloride had lower plasma pH values and TC02 levels when compared to untreated controls. When fish were placed in sodium sulfate they became alkaline and had elevated TC02 levels. However it is worth noting that these fish were allowed to acclimate for long periods of time. The small changes in acid-base status observed during this study with SITS treatment are probably indicative of the small time allowed for trout to manifest these changes. It is predicted trout exposed for longer lengths of time would also show a more pronounced acid-base shift as was found in acclimated goldfish.

The overall regulation of pH in aquatic fish appears quite

similar to the recently described single cell systems such as in squid giant axon (Russell and Boron, 1976) and the isolated barnacle muscle (Boron, 1977). In the squid giant axon, regulation occurs via an exchange of chloride and bicarbonate, while the sodium/hydrogen exchanger does not appear to be involved. On the contrary Aikin & Thomas (1977) has shown that in the mouse soleus muscle cell, regulation is predominently via a cationic exchange process with little involvement of anion exchange. In the trout inhibition of cationic exchange at the gill is capable of producing significant changes in hydrogen ion levels and it is tempting to draw the conclusion that this exchanger is the principle mechanism utilized in fish. While the effects of chloride inhibition on plasma pH were not as dramatic as during sodium transport inhibition, a slight alkalosis did result, along with an increase in TCO2. Thus it would appear the activity of this exchange system may also be effective in controlling hydrogen ion levels. Therefore regulation of both anionic and cationic flux rates may be utilized to regulate plasma hydrogen ion levels in the fish. The difficulty in the current model arises when trying to assess changes in the flux rates in the face of imposed acid-base disturbances. While the result of switching pumps off completely on acid-base status is now clear, the model does not yet allow one to predict the magnitude or even the specific exchanger to be utilized. For example, fish subjected to an acidosis compensate by increasing plasma bicarbonate levels (Janssen & Randall, 1975; Randall *et al*, 1976; Bornancin *et al*, 1977), and inhibition of chloride uptake produces the appropriate response, e.g. an alkalosis

achieved via increased bicarbonate levels. Thus it would seem that fish subjected to an acidosis would necessarily reduce chloride/bicarbonate exchange activity to achieve this end. However this could also be achieved by increasing sodium/hydrogen exchange. Cameron (1976) induced acid-base disturbances in the arctic grayling via hypercapnia and rapid thermal changes and followed sodium and chloride fluxes. Cameron found that during hypercapnia pH regulation was associated with increased sodium uptake rates. During an alkalosis induced via an acute increase in temperature, there was an increase in chloride/bicarbonate exchange and in sodium exchange. Thus <u>in situ</u> the animal may decrease or increase ionic exchange rates to adjust epithelial pH and ultimately plasma pH. Despite these difficulties it seems that this model accurately depicts the appropriate direction of cytoplasmic hydrogen ion movement within the gill epithelium in order to ensure constancy of pH in the plasma of fish.

SCN Treatment & Branchial Carbonic Anhydrase:

The initial experiments utilizing SCN resulted in an obvious blood alkalosis after only one hour, as predicted; however the rapid and large fall in VCO2 as evident in fish #5 when treated with SCN was disconcerting . This fall in VCO2 was much larger than expected based on Cameron's (1976) calculations for grayling, and a decrease of only a few percent was anticipated. The inhibition of chloride uptake could conceivably generate a larger reduction in VCO2 but this would only be possible after the gill epithelium was sufficiently alkaline to shift equation #1 to the right. Clearly from the SITS

experiments this is a slow process and the rapid fall in VCO2 cannot be accounted for in this fashion. Unfortunately it was not possible to follow changes in VCO2 with SITS treatment due to technical difficulties. A simple and more satisfactory explanation is to assume carbonic anhydrase activity was also inhibited during SCN exposure. At 10 mM, 80% of the enzyme's activity is inhibited. SCN is only poorly taken-up from freshwater (Epstein et al , 1975), even so a SCN concentration of 0.8 mM inhibits the enzyme 50%. It thus seems probable the bulk of the large fall in VCO2, as evident in fish #5, is due to the inhibition of branchial carbonic anhydrase by SCN.

It is of interest to compare the effects of carbonic anhydrase inhibition on acid-base status when inhibiton is brought about by SCN and Diamox. As can be seen in table #14, SCN results in a rise in arterial TCO2, HCO3 and pH, PCO2 does not change, while VCO2 falls. Chloride uptake is completely inhibited, while sodium fluxes are unaffected (Kerstetter & Kirschner, 1972). The results of Diamox are strikingly similar, TCO2 and HCO3 rise and VCO2 falls; however, unlike SCN treatment, PaCO2 and pH fall. Diamox inhibits sodium uptake, while its effects on chloride is somewhat variable. In the goldfish, Diamox inhibits chloride uptake (Maetz & Garcia-Romeau, 1964); however in the trout Diamox is apparently without effect on chloride fluxes (Kerstetter & Kirschner, 1972). Thus the effect of both these carbonic anhydrase inhibitors is similar except for their differential action on sodium transport. The chloride transport inhibition brought about by SCN treatment is probably independent of its carbonic anhydrase

effect in light of SCN's demonstrated effect on HC03-dependent ATPase (Bornancin & de Renzis, 1977). Given the present model and data the differences between SCN and Diamox mediated carbonic anhydrase inhibition can be fully explained in terms of the sodium response, such that inhibiton of sodium uptake during SCN exposure would produce the blood acidosis and consequent rise in arterial C02 tensions evident during Diamox exposure.

DISCUSSION

Teleosts represent one of the larger and more successful groups of organisms to inhabit the aquatic environment. Probably the success as evidenced by teleosts can be largely attributed to the efficiency of the gills. In teleosts, salt balance, water movements and ammonia excretion occur at the gill; and they also provide the necessary surface area for the diffusion of respiratory gases. In aquatic teleosts the overall design as well as the ventilation and perfusion of the gill results in an efficient means of extracting environmental oxygen to meet tissue demands. Many fish are capable of extracting oxygen at efficiencies matching or even exceeding those of mammalian lungs (Randall, 1970). The efficiency of oxygen extraction is further attested to by the ability of some tunas to maintain metabolic rates equal to or even exceeding those of comparably sized mammals (Stevens , 1972). Consequently in normoxic waters oxygen delivery to the tissues probably never poses a problem; however as efficient as gills may be, most fish are extremely sensitive and vulnerable to decreased environmental oxygen levels (Shelton, 1970). For example a nominal decrease in inspired water oxygen tensions (PiO2) from 150 - 110 mm Hg is sufficient to elicit cardiovascular responses from free swimming active rainbow trout , and as PiO2 decreased much below half-saturation, standard metabolic rates can no longer be maintained in the trout (Holeton & Randall, 1967). Thus although fish gills are efficient at extracting oxygen, their ventilation and perfusion must be responsive to changes in environmental oxygen levels to ensure adequate rates of oxygen delivery to the tissues. If fish were presented with the added task of

controlling CO2 levels by ventilatory adjustments, oxygen delivery would surely be compromised. This idea as originally proposed by Randall & Cameron (1973) makes good biological sense for an aquatic teleost. While gills facilitate gas transfer by greatly increasing the available surface area and decreasing diffusion distances between blood and water, the gill cannot be considered simply as a thin sheet of blood covered by epithelial cells. The gill is a very complex and metabolically active tissue and combines the functions of the mammalian lung with some of the functions of the mammalian kidney. Therefore it really is no more remarkable that acid-base status is unaffected by changes in Vg (mediated to achieve constant metabolic rates) than the fact that salt and ammonia homeostasis is likewise unaffected. Clearly changes in ventilation and perfusion of the teleost gill are primarily for the purpose of maintaining adequate oxygen uptake at energetically favorable rates. That the pattern of CO2 excretion and acid-base regulation in fish is distinctly unlike the system as exemplified by birds and mammals there can now be little doubt and in some respects the notion that fish red cells contain carbonic anhydrase and hence must function just like mammalian red cells has hitherto only served to confuse the situation of acid-base regulation in fish.

As the diffusion of CO2 in an aquatic medium exceeds that for oxygen, it can be appreciated that any organism capable of procuring sufficient oxygen via diffusion would not face a problem excreting molecular CO2. Thus the excretion of CO2 in small aquatic organisms never presented a problem. Of much greater importance would be the maintenance of salt and water

balance along with control of hydrogen ion levels. In these small aquatic organisms a premium would be on ionic exchange mechanisms capable of effectively modulating the internal environment with respect to solute and water balance. At ambient aquatic temperatures, little bicarbonate or hydrogen ions would be formed via the uncatalyzed hydration of C02. Consequently the production of endogenous counterions for exchange of sodium and chloride would be small as the bulk of C02 would be rapidly lost via diffusion into the surrounding environment . The inclusion of carbonic anhydrase in these cells would thus greatly facilitate the hydration reaction and hence an energetically favorable mechanism to provide the endogenously required counterions. In addition to providing counterions for the apical exchange process, the coupling of C02 excretion to ionic exchange would provide a means of controlling cellular buffer reserve. Note that the inclusion of carbonic anhydrase in these cells is to facilitate ionic exchange processes at the expense of metabolic molecular C02 and not to facilitate the production of molecular C02 (via the dehydration reaction). As organisms grow in size and transit times and/or distances increase tissue C02 stores will build and the bulk of extracellular C02 will now be as bicarbonate as the reaction moves toward equilibrium. Thus at the exchange site either bicarbonate (and hydrogen ions) must be excreted directly or alternately C02 must be dehydrated from bicarbonate and hydrogen ions at the exchange site to avoid a build-up of C02. If normal gill functioning is severely impaired in fish such as by inactivation of the gills, as when fish are exposed to air or inactivation of branchial carbonic anhydrase

as during diamox treatment, a retention of CO2 develops along with the associated acidosis. However it now appears that these cases are not really physiological and the gills are actually a hydrogen ion excreting and regulating tissue, rather than a CO2 excreting and regulation pathway. Obviously CO2 excretion does occur at the gill; however this is a consequence of the active regulation of hydrogen ion activity within plasma. Only when the input of metabolic CO2 surpasses the capabilities of the "proton pumping" mechanism at the gill would excretion of CO2 per se become significant. Thus so long as plasma hydrogen ion activity falls near some defined "set point" total CO2 would not be expected to be controlled. This appears to be the situation in fish, as Randall & Cameron (1973) found that during temperature induced acid-base disturbances arterial CO2 tensions remained constant while total CO2 rose and fell appropriately. Again during hypercapnia hydrogen ion activity is regulated independently from arterial CO2 tensions (which remained approximately 2.0 mm Hg above inspired levels) by increasing total CO2. As stated previously these changes in hydrogen ion activity and TCO2 are achieved independent of ventilatory changes. Thus while the ability to excrete CO2 and/or controlling their absolute levels in aquatic teleosts probably rarely poses a problem, hydrogen ion activity is tightly regulated. This regulation of hydrogen ion activity is facilitated by coupling expired CO2 to salt movements across the water/gill membrane (Chapter IV). The mechanisms utilized to control plasma hydrogen ion levels are remarkably similar to the responses of single cell systems exposed to acid-base

challenges. Thus either cationic, anionic or both exchange processes are utilized to move hydrogen ions or their equivalents in the appropriate direction from the internal mileau. Cationic and anionic exchange processes are also utilized in the trout when faced with an acid-base challenge and the total inhibition of these exchange systems results in acid-base disturbances in the trout (Chapter IV).

The exact nature of proton pumping in the teleost gill and the mechanism of regulation remains obscure. Hydrogen ion pumping is well documented in numerous tissues and organisms and possibly two of the better understood systems are the amphibian and reptilian urinary bladder and acid secretion in vertebrate gastric mucosa. The urinary bladder is characterized by the ability to acidify the luminal side solution both *in vivo* and *in vitro* . Because this tissue is morphologically a sheet like structure it is possible to mount tissue preparations in Ussing type chambers and has proved most useful in assessing proton pumping in biological systems. Much of the present understanding of this tissue is based on the work of Steinmetz,Schwartz and their co-workers and the following account is based primarily on their work (Steinmetz, 1967,1969,1974; Steinmetz & Lawson , 1971; Schwartz, 1976; Schwartz & Steinmetz, 1971; Schwartz *et al* , 1972; Leslie *et al*, 1973; Al-Awqati *et al* , 1976; Al-Awqati *et al* , 1977). At the luminal border of the bladder a molecule of water is cleaved in some manner producing a proton plus a hydroxyl ion. The proton is excreted with the associated inward movement of a sodium ion maintaining electroneutrality. It's not certain if this sodium/hydrogen translocation is an obligatory

exchange; however removal of luminal side sodium or exposure to amilcride decreases acid secretion. Alternately decreasing rates of acid secretion affect the rates of sodium influx at the luminal border. The hydroxyl remaining from the photolysis of water would now be expected to drastically elevate cellular pH unless buffered or excreted. In fact it can be demonstrated that this hydroxyl ion is buffered by the hydration of CO2 + OH to HCO3, with the bicarbonate ion having little direct effect on cellular pHi. This buffering is crucial to the ability of the bladder to excrete protons. This conclusion is based on the following. The production of metabolic CO2 isn't sufficient to maintain maximal proton pumping rates. If CO2 however is increased in a stepwise fashion on the serosal side of the bladder the increase in titratable acid on the luminal side increases until a maximal rate of pumping is achieved whereupon further increases in CO2 are without effect (Schwartz, 1976). The buffering action via the hydration of CO2 is carbonic anhydrase dependent, as diamox treatment produces a fall in proton pumping. Carbonic anhydrase in the toad bladder is found in the cytoplasm but also seems to be incorporated into the luminal membrane. Diamox in the luminal bath produces a rapid fall in proton pumping while diamox in the serosal bath requires higher concentrations and is characterized by a definite time lag before proton pumping falls (Schwartz, 1976). This type of evidence is interpreted to mean the bound carbonic anhydrase in the luminal membrane is probably responsible for the fall in proton pumping during diamox inhibition. Interestingly when proton pumping is inhibited in the turtle bladder with diamox,

sodium influx also falls. In the trout, carbonic anhydrase is also in the apical (='s luminal membrane of the urinary bladder) membrane and diamox also inhibits sodium influx. The cellular bicarbonate formed from the hydration of C02 in the bladder is excreted ionicly. The bulk of the bicarbonate moves through the serosal membrane into the bathing solution. This step can be blocked by SITS. Some bicarbonate also leaves via the luminal membrane as well. The movement of this bicarbonate is dependent on external chloride and appears to be a tight 1:1 coupling. In the urinary bladder the magnitude of this exchange is small and diamox doesn't appear to alter chloride influx greatly. It may be possible that epithelia characterized by a greater chloride/bicarbonate exchange capacity may be more or less susceptable to inhibition of anion movements during carbonic anhydrase inhibition. Thus if one fish had a high capacity to take-up environmental chloride (in exchange for bicarbonate) diamox might appreciably inhibit that flux by inhibiting the hydration reaction and hence the supply of bicarbonate free to exchange. If the magnitude of the pump was small it might not be affected to any great extent. If this is true then the differing effects of diamox on chloride uptake in fresh water fish might be explainable. Maetz and Garcia-Romeau found chloride uptake was inhibited with diamox in the goldfish while diamox was apparently without effect in the trout (Kerstetter & Kirschner, 1972). Acid secretion in the urinary bladder and the fish gill are similar in several other respects. Amiloride blocks sodium uptake across the gill and blocks hydrogen ion excretion in trout (Kirschner et al , 1973). Amiloride has the same effect in

the urinary bladder . As just mentioned acid secretion is strongly correlated to nutrient (serosal) side C02 levels. When the only source of C02 for the hydration reaction is from endogenous metabolic C02 production, proton pumping is greatly reduced. Payan and Matty (1975) working with perfused trout gills measured the acidification of the bathing solution with time when the gills were perfused with 5% C02 or zero C02 gas equilibrated solutions. When the perfusate lacked C02 the apparent rate of acidification of the environmental water was approximately half that found during perfusion with 5% C02. The increase in acidification was not the result of diffusion of molecular C02 and represented hydrogen ions . This observation is consistent with the data for the turtle bladder where hydrogen ion secretion is dependent on serosal C02 levels. Unfortunately while the urinary bladder is easily tested experimentally the teleost gill provides much more formidable technical difficulties. Presently it can only be suggested that the gill appears remarkably similar to acid secretion in the well defined amphibian and reptilian urinary bladder. Clearly this area requires further work but also appears to be a fruitful area for future research.

That fish and many single cell systems regulate internal hydrogen ion levels when faced with an acid-base challenge is clear; however the ultimate controlling mechanisms remains obscure. In examining acid secretion in the vertebrate gastric mucosa Sachs (1978) has suggested that at least two levels of control exist in this tissue. First, blood flow or perfusion can be utilized. In the stomach acid secretion only occurs when the

acid secreting membranes are perfused. Thus many neural and humoral agents known to stimulate acid secretion in the stomach do so by their ability to increase perfusion of the appropriate acid secreting membranes. The second level of control resides at the cellular or biochemical level. For example in the turtle bladder aldosterone increases acid secretion by stimulating sodium uptake at the luminal membrane (Al-Awquati et al , 1976). Cyclic-AMP is likewise effective (Aceves, 1977) . Thus many possible controlling mechanisms exist at the cellular level in fish. Regulation of acid secretion in the fish is probably normally never perfusion limited with regulation most likely occuring at the cellular level possibly humorally mediated. If aquatic teleosts never faced the problem of retaining CO2 and the gill is utilized to adjust plasma hydrogen ion levels via ionic exchange mechanisms across the water/gill membrane what restrictions are placed on aerial respiration?

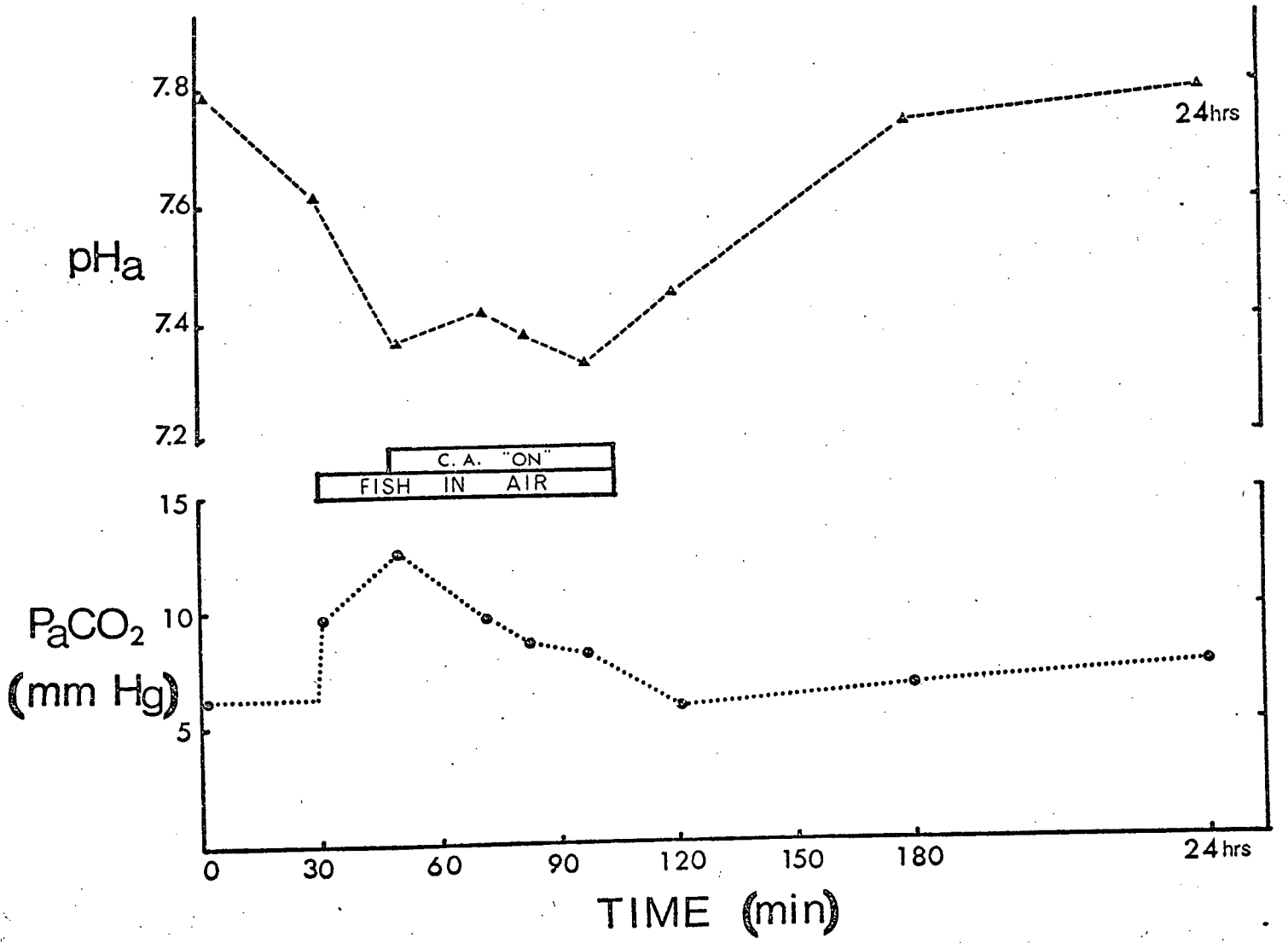
In most fish totally removed from water the ability of the gills to function is greatly reduced. The principal site of environmental exchange in the teleost gill is the leaf-like secondary lamellae . These structures are easily supported in water; however in the less dense medium of air the secondary lamellae collapse under their own weight. The collapse of the secondary lamellae creates a huge diffusion dead space and undoubtedly an increase in gill vascular resistance. Thus typically, gills in air are diffusion and possibly perfusion limited with respect to oxygen and carbon dioxide transfer. However in addition to limiting gas transfer ionic processes occurring in the presence of water will also be affected. Thus

ammonia excretion and ionic exchange would also be limited in fish denied the ability to ventilate their gills with water. Therefore a rainbow trout placed in air would be limited with respect to oxygen uptake and carbon dioxide excretion but also the ability to regulate arterial hydrogen ion levels as well as ammonia and salt levels. Thus for the trout in air a build-up of C02 would occur and this retention of C02 would clearly not be compensated for as proton pumping is not now possible. However it is possible that in the case of a rainbow trout the limiting factor for survival may be oxygen procurement and tissue anoxia would prove fatal before the animal encountered a hypercapnic acidosis of sufficient magnitude to be lethal. Many fish have developed alternate or accessory gas exchange areas to utilize atmospheric oxygen and do not face anoxia during-air exposure.

Accessory gas exchange organs have evolved in numerous groups of fish in order to obtain oxygen from air. These air breathing organs may be modified swimbladders, pharyngeal cavities and even stomach and intestine (see Johansen, 1970; Munshi, 1976; Singh, 1976 for reviews). These organs are utilized for oxygen uptake, as indicated by their typical respiratory quotients of 0.1 - 0.4. The gills are thus retained as the major route for carbon dioxide excretion with the method presumably identical to that in truly aquatic fish such as the trout. When air breathing fish such as the bladder breather Hoplerythrinus unitaeniatus are air exposed arterial oxygen content remains high (Haswell, unpublished observations). Johansen (1966) found that in Synbranchus blood oxygen carrying capacity actually increased from a maximal of 50-60% saturation,

while in water, to 100% saturation during air exposure. During this same air exposure C02 content actually rose and did not fall until aquatic ventilation was initiated. Thus although in Symbranchnus air exposure and utilization of an accessory gas exchange pathway actually enhanced oxygen uptake a retention of C02 develops. As fish red cells fail to dehydrate plasma bicarbonate (this study) and the accessory exchange organs do not possess carbonic anhydrase (Burggren & Haswell, 1978) these animals can excrete C02 at these exchange sites only at the uncatalyzed rates. That this is true is further demonstrated by Randall et al (1978). These authors found that infusion of bovine carbonic anhydrase into air-exposed Hoplerythrinus resulted in a doubling of the bladder respiratory quotient which greatly alleviated the rise in blood PaC02 and fall in pHa normally evident during air exposure in this fish. Figure #18 demonstrates the effect of air exposure and subsequent infusion of bovine carbonic anhydrase on blood acid-base status in a single fish. Thus in air breathing fish exposed to air the inability of red cells to participate in the dehydration of plasma HC03 is for the first time no longer an advantage but rather a liability. Many fish such as lungfish and some eels have utilized increased vascularization of the skin to help excrete C02. This increased capillary density in close proximity with a moist skin would thus enhance the loss of molecular C02. It is anticipated that increased temperatures would also enhance the facilitation of C02 production from plasma bicarbonate via the uncatalyzed reaction. Increased capillary distances may result in long skin capillary residence times and would thus

FIGURE #18. Changes in dorsal aortic pH (pHa) and PCO_2 ($PaCO_2$) during air exposure and the effect of infusion of carbonic anhydrase (C.A.) into the dorsal aorta of an individual Hoplerythrinus.


7.8
7.6
7.4
7.2
pHa
24hrs
C. A. "ON"
FISH IN AIR
15
10
5
PaCO2
(mm Hg)
0
30
60
90
120
150
180
24 hrs
TIME (min)

also facilitate the loss of C02; however no relevant data exists to draw any reliable conclusions concerning this possibility. Although the vascularization of the skin and increasing lung volumes may help facilitate the loss of C02 the gills invariably are retained and provide a major pathway for the release of C02 and presumibly are still utilized via ionic exchange processes occuring in the gill to regulate plasma hydrogen ion activity. Ultimately metabolic rates in airbreathing fish may be limited by their ability to excrete C02 without ventilating their gill structures with water. Those air breathing fish which have become the most "terrestial" may be forced to pay for this freedom by either enduring periods of acidosis and/or reducing metabolic C02 production. Consequently estivation by lungfish may provide a means of reducing C02 production and avoiding what would otherwise result in a lethal rise in blood C02 during periods when drought conditions may limit excretion of C02 across the skin (Delaney et al , 1977).

In amphibians well developed lungs are utilized to procure oxygen from air; however their skin provides the major site of C02 excretion (Vinegar & Hutchison, 1965; Hutchison et al , 1968; Emilo & Shelton, 1974). In more terrestial vertebrates such as reptiles as well as birds and mammals the lungs provide the site for oxygen uptake and C02 excretion. It is therefore interesting that intact red blood cells from Xenopus , Amphiuma , Bufo and Rana pipiens also appear to lack carbonic anhydrase dehydration activity while the red blood cells of the turtle Crysemes possess obvious carbonic anhydrase activity. Clearly any mutation enabling the red cell to participate in the

dehydration of plasma bicarbonate would be expected to greatly facilitate the movement of animals from aquatic to fully terrestial air breathers. These possibilities should provide a rewarding area for future research.

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