# THERMOACCLIMATORY VARIATIONS IN THE ACTIVITIES OF ENZYMES IMPLICATED IN ION TRANSPORT IN THE RAINBOW TROUT, *Salmo gairdneri*.

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# ABSTRACT

Two groups of rainbow trout were acclimated to 2°, 10°, and 18°C. Plasma sodium, potassium, and chloride levels were determined for both. One group was employed in the estimation of branchial and renal ($Na^+$-$K^+$)-stimulated, ($HCO_3^-$)-stimulated, and ($Mg^{++}$)-dependent ATPase activities, while the other was used in the measurement of carbonic anhydrase activity in the blood, gill and kidney. Assays were conducted using two incubation temperature schemes. One provided for incubation of all preparations at a common temperature of 25°C, a value equivalent to the upper incipient lethal level for this species. In the other procedure the preparations were incubated at the appropriate acclimation temperature of the sampled fish.

Trout were able to maintain plasma sodium and chloride levels essentially constant over the temperature range employed. The different incubation temperature protocols produced different levels of activity, and, in some cases, contrary trends with respect to acclimation temperature. This information was discussed in relation to previous work on gill and kidney. The standing-gradient flow hypothesis was discussed with reference to the structure of the chloride cell, known thermally-induced changes in ion uptake, and the enzyme activities obtained in this study. Modifications of the model of gill ion uptake suggested by Maetz (1971) were proposed; high and low temperature models resulting. In short, ion transport at the gill at low temperatures appears to involve sodium and chloride uptake by heteroionic exchange mechanisms working in association with carbonic anhydrase. Gill ($Na^+$-$K^+$)-ATPase and erythrocyte

carbonic anhydrase seem to provide the supplemental uptake required at higher temperatures. It appears that the kidney is prominent in ion transport at low temperatures while the gill is more important at high temperatures.

Linear regression analyses involving weight, plasma ion levels, and enzyme activities indicated several trends, the most significant being the interrelationship observed between plasma sodium and chloride. This, and other data obtained in the study was considered in light of the theory that a link exists between plasma sodium and chloride regulatory mechanisms.

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INTRODUCTION:

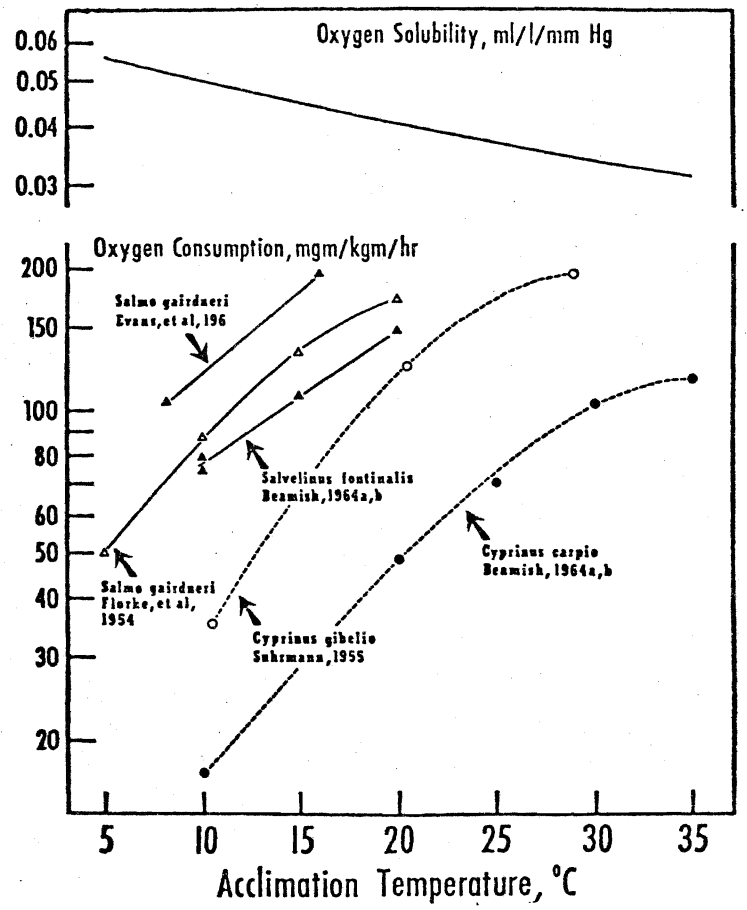
As is the case with most living organisms, teleost fish are challenged with the problem of maintaining relatively precise control over the concentrations of a wide variety of materials within the body fluid system. Inorganic ions, as major extra- and intracellular components, fall in this category. Many essential processes, including membrane permeability, establishment of transmembrane electrical and/or chemical gradients, and enzyme activity and function, are highly sensitive to changes in water-electrolyte status. A potential problem is created by this requirement for "tight" control, for the mechanisms associated with the regulation of hydromineral balance are often subject to perturbation by environmental variations (creating alterations in organism-environment relationships).

In the case of freshwater or freshwater-adapted teleost fishes, two fundamental problems are discernable. Since the medium is hypo-osmotic, often with several orders of magnitude difference between body and environment levels, these animals are subject to the double dilemma of water-loading and electrolyte loss. The gill, in its' role as a respiratory organ, is the major site of external contact. It is also the location of considerable osmoregulatory stress potential because of the relatively high permeabilities required for efficient gas exchange. A positive correlation exists between respiratory activity and the extent of hydromineral regulatory demands as any factor altering the former inevitably imposes changes on the latter.

In company with all other aquatic poikilotherms, a major determinant of metabolic rate in fishes is environmental temperature. Figure 1 shows variations in a common indicator of metabolic activity, oxygen consumption, as a function of temperature, for several species of freshwater fishes. The substantial increase in routine oxygen requirements, as a consequence of increased temperature, is immediately obvious, as is the inverse character of the relationship between oxygen solubility and water temperature. The rainbow trout, Salmo gairdneri (Flörke et al., 1954, cited in Houston, 1973), for example, requires about 3.5 times as much oxygen at 18°C as it does at 5°C, and it must satisfy this increase in oxygen demand in the face of a 30% reduction in oxygen availability. A variety of responses are, in theory, available for resolution of this predicament. One obvious response, compensatory adjustments in blood parameters, does not appear to operate. Reported variations are generally too small to play other than an auxillary role in resolution of the temperature-oxygen demand problem (Houston et al., 1976). The primary means for augmenting oxygen uptake involves increased branchial exchange activity. This is achieved by increasing cardiac output and ventilatory flow, while reducing the number of intralamellar blood and water "shunts". The latter action increases effective exchange area, and reduces mean diffusion path length. In so doing, however, the effective permeability of the gill surface is elevated, and sharply increases ion efflux and water influx.

Fig. 1 Upper section. Change in oxygen solubility with temperature elevation.
Lower section. Changes in routine oxygen consumption estimates for several varieties of fish as a function of acclimation temperature.

# FIGURE I OXYGEN AVAILAILITY AND DEMAND




(FROM HOUSTON, 1973)

Presently available information indicates that the potential water-electrolyte imbalance induced by thermal stress is alleviated in the following manner. The additional water influx is eliminated by means of increased glomerular filtration and urine flow. Although the ionic concentration of urine is generally reduced at higher temperatures by increased reabsorption from the primary urine, the elevation of urine flow produces substantial increases in ion loss. The potential imbalance resulting from this response, together with that resulting from increases in ionic efflux from the gill, is corrected by increased branchial absorption of electrolytes from the surrounding medium. Some economy in metabolic energy expenditure may, however, be achieved by reductions in branchial electrolyte permeability.

Despite disturbances created by thermal stress, water-electrolyte balance can be maintained over wide temperature ranges, even by relatively stenothermal species, such as the trout. Although this control is not absolute, deviations, when present, are generally minor. In short, elevated branchial absorption and renal recovery compensate for thermally-induced changes in ion efflux, and thus maintain electrolyte balance. Since these processes have been associated with specific enzyme systems, the aim of this study lay in investigation of the effect of thermoacclimation on the activity of enzymes implicated in ion transport.

In conducting this investigation the following points have been considered:

1.) Although stabilization of major plasma electrolytes over wide temperature ranges is a commonly observed phenomenon, verification was, of course, necessary. Therefore, determinations of plasma sodium, potassium, and chloride were carried out.

2.) In previous studies the actual body temperatures of poikilotherms have been ignored in establishment of enzyme assay protocol. Temperatures of 30° to 37°C., which are well in excess of those tolerated by salmonids, have been routinely used in determinations of, for example, $(Na^+-K^+)$-ATPase. In the present study, therefore, all determinations were carried out at 25°C, a temperature comparable to the upper incipient lethal level for this species, (Houston et al., 1976) and also at the actual acclimation temperature of the animals. It was hoped that this would give a more physiologically realistic indication of maximum activity, as well as the relation between this and activity at temperatures at which the enzyme systems actually function.

3.) Three enzyme systems, (Na-K)-stimulated ATPase, an anion-stimulated ATPase, and carbonic anhydrase are presently considered to be involved in ion regulation. Accordingly, determinations of the activity of these three systems were carried out.

4.) No evidence is presently available concerning the relative activities of these enzyme systems in the two principal osmoregulatory organs, gill and kidney. This, of course, precludes any estimation of the functional significance of each enzyme within, and between these organs.

In addition, data concerning relative changes in activity during thermal acclimation do not exist. These aspects have also been examined, assuming that the measured activity represents some measure of involvement in transport.

5.) Finally, correlation analysis was used in an attempt to link temperature-related variations in ionic status with a variety of parameters including, branchial and renal enzyme activities, other electrolyte levels, and the physical characteristics of the specimens employed.

# II REVIEW OF LITERATURE

Literature pertinent to this study is reviewed under four main headings: (1) General aspects of Water-Electrolyte Regulation in Freshwater Fishes, (2) Structure and Function of Gill and Kidney in Relation to Hydromineral Maintenance, (3) Characterisation of Enzyme Systems Investigated and (4) Comments Upon the Physiological Relevance of *In Vitro* Enzyme Assays.

## 1. General Aspects of Water-Electrolyte Regulation in Freshwater Fishes.

The fact that freshwater-adapted teleosts, the rainbow trout, *Salmo gairdneri*, being a typical example, are able to regulate water-electrolyte status is well established (Houston, 1973). The classic model of Krogh (1939, cited in Maetz, 1971) defines the essential problems encountered. Water influx through the gill is compensated for by the production of abundant dilute urine. Hickman and Trump (1969) provide data to illustrate this point. A typical freshwater-adapted, euryhaline teleost produces urine at a rate of about $3ml.hr^{-1}.Kg^{-1}$ with a concentration of $50mOsm.l^{-1}$. By contrast, when in the saltwater-adapted state, urine flow is only about $0.2ml.hr^{-1}.Kg^{-1}$ at 300 $mOsm.l^{-1}$ The bulk of electrolyte loss is through the gill, although the kidney contribution is not insignificant, being approximately 25% of the total ion efflux of $20\mu Eq.hr^{-1}.100g^{-1}$ in the case of the trout (Holmes and Stainer, 1966; Kerstetter *et al*, 1970; Kerstetter and Keeler, 1976). This potential deficit is corrected by active electrolyte absorption at the gill balancing efflux losses (Motais *et al.*, 1969;

Kerstetter *et al.*, 1970; Maetz, 1971; Kerstetter and Kirschner, 1972). As the freshwater fish drinks little, or as in the case of the trout, not at all (Shehadeh and Gordon, 1969), and starvation has little immediate effect on hydromineral balance (Holmes and Stainer, 1966), the gut is considered to play a minor role. This is, of course, a major contrast to the saltwater-adapted form where drinking rates have been measured at 13% of the body weight per day, with the gut serving as an important site for water uptake (Shehadeh and Gordon, 1969; Maetz, 1971). The remaining non-branchial components of the body surface are rendered essentially impermeable by skin, scales and mucus, and are not considered to be significantly involved in ion regulation (Black, 1957; Fromm, 1968). The "tightness" of concentration control varies from species to species. The rainbow trout may be considered, in view of presently available data, a relatively precise controller. Maximal differences of 6.7 and 8.8% in plasma sodium and chloride levels, respectively, have been reported over a temperature range of $3^{\circ}$ to $21^{\circ}$C. Elimination of a minor seasonal difference reduces the actual deviation to insignificance (about 1%) (Houston *et al.*, 1968). Although many factors, including photoperiod, season, exercise level, ontological state, salinity, and handling, are also known to affect hydromineral balance (Holmes and Donaldson, 1969; Houston, 1973; Murphy and Houston, 1977), the major environmental challenge to the aquatic poikilotherm is generated by variations in ambient temperature. Despite the fact that heat and cold stress produce similar results, with

respect to loss of osmoregulatory capacity, the reasons for this appear to be quite different (Holmes and Donaldson, 1969; Houston, 1973; Hazel and Prosser, 1974). Nevertheless, in the present study it is apparent that the principal goal lay in definition of the responses of salmonid fish to increasing acclimation temperature.

The fact that fish are able to maintain a constant hydromineral status when confronted by the multifaceted dilemma of thermal stress is well established,although the reasons for this are poorly understood. Therefore, the remainder of the literature survey has been directed to elucidating those processes and procedures which have been implicated in the creation of the observed responses.

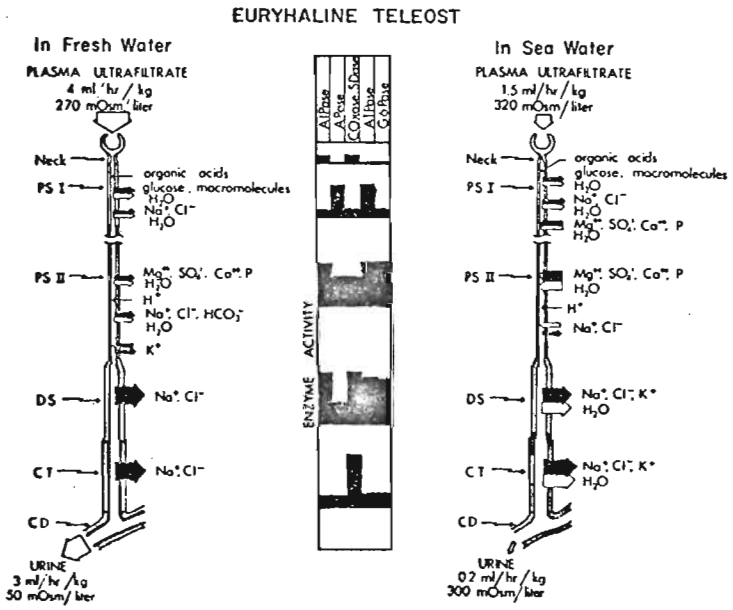
2. Structure and Function of Gill and Kidney in Relation to Hydromineral Maintenance.

(a) Kidney

In the rainbow trout this organ is fused for its' entire length with no clear distinction between the head and trunk portions being obvious; a typical type I kidney in the classification of Hickman and Trump (1969). It is located in the dorsal portion of the coelomic cavity along the backbone, and is tightly adherent to it. Nephrons are of the glomerular type with six tubular segments present (Hickman and Trump, 1969). Figure 2 shows a proposed structure-function relationship for euryhaline fish. A common function attributed to the kidney of many other vertebrates, elimination of nitrogenous wastes, is of minor importance, as most of this material is lost as ammonia ($NH_3$) and

Fig. 2. A model of the fish nephron illustrating the component structures and the role of each in fresh and salt water. Transport into the kidney is represented by a solid arrow while transport into the urine is represented by an open arrow. Rate of flow and concentration of the plasma ultrafiltrate and urine under these conditions are indicated at the top and bottom, respectively.

FIGURE 2 A MODEL OF THE KIDNEY NEPHRON SHOWING STRUCTURE - FUNCTION RELATIONSHIPS




(FROM HICKMAN AND TRUMP, 1969)

ammonium ($NH_4^+$) through the gills (Walton and Cowey, 1977). Only a small amount, in the area of 3% of the body load, is excreted through the trout kidney (Fromm, 1963).

The primary function of this organ in freshwater-adapted animals is the elimination of excess water. This, in conjunction with conservation of filtered electrolytes, results in the production of an abundant dilute urine, as previously noted. The effectiveness of this function is illustrated by the fact that, while water is recovered from the primary urine at a rate of 50% or less of the presented load, ion reabsorption is usually 95% or better, for primary electrolytes (Holmes and McBean, 1963; Holmes and Stainer, 1966). This is achieved by low tubular permeability to filtered water operating concurrently with a monovalent ion reabsorption system, which will be discussed later on.

As water influx rates vary with temperature and other factors (Evans, 1969; Wood and Randall, 1973), some means must be available to provide "fine" control of glomerular filtration rate (G.F.R.). Selective perfusion of the nephrons appears to be the principal means employed in control of G.F.R., with two possible mechanisms having been proposed. Nephrons may be in either an "on" or "off" state - the intermittent flow hypothesis - with increases in capacity being generated by bringing more nephrons to the "on" state. Alternatively, all nephrons may normally operate at less than full perfusion, with increases to full capacity occurring in response to greater demand - the graded activity hypothesis.

The two, of course, are not mutually exclusive (Hickman, 1965; Mackay and Beatty, 1968; Hickman and Trump, 1969). The involvement of other regulatory procedures is not well understood at present, although it appears that systemic blood pressure change is not a normal regulatory mechanism (Hickman and Trump, 1969). It does appear that the hormone prolactin, secreted by the pituitary gland, may be directly involved in decreasing renal water permeability and/or increasing renal (Na-K)-ATPase activity. However, the involvement of prolatin in osmoregulation, although its' means of action is not completely clear, is firmly established, as it is the only known vertebrate hormone that can restore osmoregulatory capacity to hypophysectomized fish. This suggests that, perhaps, it should be considered as a family of hormones, having different functions in different vertebrate groups (Ensor and Ball, 1972; Johnson, 1973).

As temperature increases there is a concomitant increase in G.F.R. and urine flow. This is not regarded as a direct response to temperature, but rather a secondary response to the increased water influx generated by elevated respiratory activity at higher temperatures. This is supported by the fact that water influx and urine flow rate display similar thermal dependency, with $Q_{10}$'s of approximately 2.0 to 2.4 (Mackay and Beatty, 1968; Evans, 1969; Lloyd and Orr, 1969; Isaia, 1972). On the other hand, reduced urine electrolyte concentrations at higher temperatures suggest that ion recovery is a variably-active process, since the concentration of the primary urine (considered an ultrafiltrate of the plasma) should be stable (Houston, 1973; Mackay, 1974).

As G.F.R. and urine flow have been observed to increase up to 10 times with temperature elevation, it is obvious that much larger metabolic expenditures are required to enable the active reabsorption system to generate reduced urine electrolyte concentrations (Pora and Precup, 1960, cited in Houston, 1973). Despite reduced concentrations at elevated flow rates, net depletion increases. This is evident in studies which note that net renal electrolyte losses are sharply elevated with rises in temperature (Houston, 1973; Mackay, 1974). Control of this function by means other than the effects of prolactin, mentioned earlier, is poorly understood.

It is evident that the renal response to elevated temperatures involves increases in kidney perfusion in combination with higher transport-associated activity. While regulation of body water levels is accomplished through kidney action, it is apparent that, although conservation of filtered ions at the kidney is significant and controllable, the bulk of electrolyte recruitment demand generated by increasing temperature must be satisfied elsewhere - at the gill.

(b) Gill

The rainbow trout gill is of a typically teleostian type, consisting of five cartilagenous branchial arches per side, one of which is devoid of gill filaments. These are located in cavities positioned on either side of the pharynx, and covered by the opercula. Each branchial arch bears, perpendicular to it, two hemibranchs; each consisting of a row of primary gill filaments. An interbranchial septum,

orignating at the arch and containing muscles capable of controlling primary filament position, joins the hemibranchs for about 1/2 of their length. Secondary or respiratory lamellae, considered to be the major respiratory portion of the gill, are located transversely along the length of the primary filaments (Fry, 1957; Randall, 1970; Conte, 1971; Morgan and Tovell, 1973; Hughes and Morgan, 1973). The extensive folding created by this arrangement generates a total surface area (generally referred to as the anatomical gill area) substantially greater than the rest of the external body surface. Parry (1966), for example, estimates that anatomical gill area is from 10 to 60 times that of the rest of the body surface. Gill area per unit body weight also decreases with increasing weight, in all species so far examined (Randall, 1970; Hughes and Morgan, 1973).

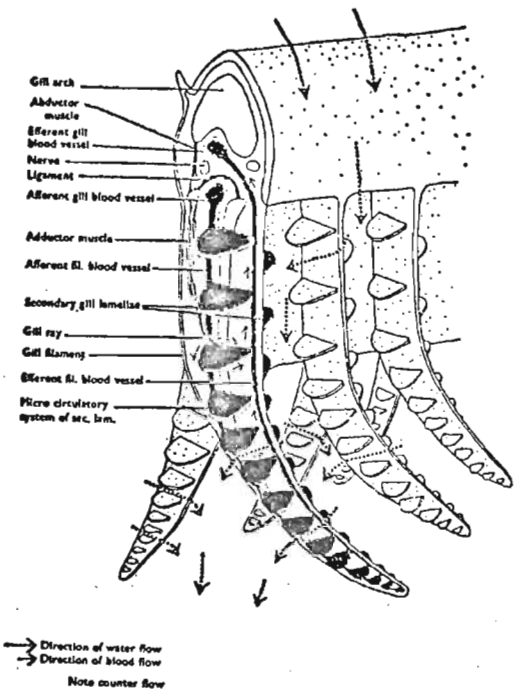
The primary function of the gills, respiratory gas exchange, is a passive diffusion process optimized by the countercurrent arrangement of blood and water flows through and over the respiratory lamellae (Figure 3(a)). Blood: water flow ratios are in the order of 1:80, and are presumably related to the fact that, due to the relatively low solubility of oxygen in water, large volumes must be passed over the gills to ensure extraction of sufficient amounts of oxygen. In addition, other important functions, such as nitrogenous waste clearance, acid-base control, and hydromineral regulation, are carried out by the gill (Maetz, 1971). The complications which result from this multipurpose function of the gill have been alluded to and will be discussed in more detail subsequently.

Fig. 3 A. Diagram outlining the structure of a portion of a gill arch in a typical teleost gill. The countercurrent flow of blood (solid arrows) and water (broken arrows) is indicated.

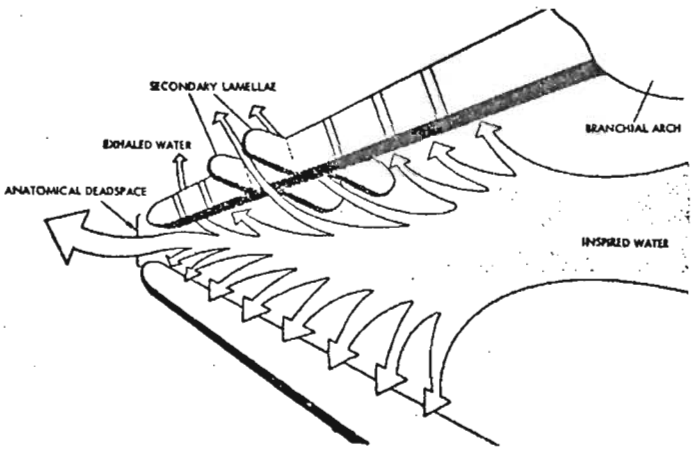
B. Illustration showing how the position of the primary gill filaments can control the anatomical deadspace thus regulating the proportion of inspired water that is shunted through the gills.

FIGURE 3 BLOOD AND WATER FLOW IN THE GILL

A.




B.




(A FROM HUGHES AND MORGAN, 1973)
(B FROM WATERMAN, 1971)

Since variability in respiratory gas exchange demand is expected and observed, some means of adjustment must exist (Randall, 1970). The obvious response-increased blood and water flow rates - is seen, but it is also possible to regulate oxygen uptake by other means. Specifically, regulation of the amount of gill area actually perfused, and therefore the effective gill area, has been reported (Taylor et al., 1968). The resting trout, for example, utilizes only about 20% of the potential effective gill area, and amplifies this in response to increased oxygen demand (Stevens and Randall, 1967). Several mechanisms which may permit this have been proposed. These are considered below.

Although evidence is scanty, and the postulated involvement somewhat speculative, it is possible that the diffusion distance between water and blood can be adjusted by regulation of the thickness of the gill mucous layer (Randall, 1970). The other procedures have more definite substantiation. Blood may be preferentially shunted through the gill in such a way as to preclude any significant participation in exchange. Steen and Kruysse (1964) and Randall (1970), for example, have suggested several possible pathways, which bypass the exchange area of the secondary lamellae. This is based on essentially anatomical grounds. Morgan and Tovell (1973) and Cameron (1974) found no evidence of functional shunt pathways and proposed instead that selective and progressive perfusion of distal secondary lamellae constituted the basic mechanism for increasing effective exchange area. It is, of course, possible that both operate. There is, in addition, evidence that the catecholamines (adrenaline and noradrenaline),

and acetycholine, which are known to affect gill resistance to blood flow and therefore lamellar blood flow rates, may be involved in blood flow control (Randall, 1970). In the case of ventilation, definite shunts are known to operate. Gill flow is composed of three components; residual flow, respiratory flow and shunt flow. Anatomical dead space, a component of shunt flow, is the only portion of this flow subject to control, and is the means by which the inverse relationship between respiratory and shunt flows is generated. Adjustment of the position of the primary gill filaments is controlled by the interbranchial septum muscle sets, referred to earlier, so that only water passing through the pores in the "screen" formed by the secondary lamellae can be involved in exchange. As indicated in Figure 3(b), that channelled between the filaments is effectively "shunted" from the respiratory flow (Randall, 1970; Hughes and Morgan, 1973).

As temperature increases, oxygen availability decreases while oxygen requirements rise. The additional requirement generated in this way is largely satisfied by enhancement of the effective exchange area by increased blood flow, greater branchial irrigation, and enlargement of the active or functional gill surface. It is this which induces the major problem with respect to hydromineral balance, for increasing the effective area induces substantial elevation of ion and water fluxes (Randall *et al.*, 1972). As noted earlier, increases in G.F.R. and urine flow, resulting from increases in the body water load, are the means by which constant water

status is achieved. This process produces increased urinary salt losses despite increased tubular electrolyte reabsorption. These, plus losses due to enhanced efflux of electrolytes from the gill, must be compensated for by recruitment from the medium. Absorption processes are located at the lamellar epithelium and will be discussed under the next heading. Control of this aspect of gill function is not well understood, although again, prolactin is involved, perhaps by controling water permeability and sodium efflux (Ensor and Ball, 1972; Johnson, 1973). The presence of compensatory variations in branchial ion permeability is suggested by the fact that electrolyte efflux increases more slowly, as temperatures rise, than oxygen consumption branchial ventilation and water influx. $Q_{10}$ values for the former range from 1.1 to 1.2 while in the latter cases the range is from 1.5 to 2.0 (Evans, 1969; Houston, 1973; Cameron, 1976). As mentioned previously, changes in branchial water permeability apparently do not occur, as influx is reported to change in proportion to the aforementioned parameters.

The site of transport activity seems to be one of the four cell types in the gill - the "Keys-Willmer" or "chloride" cell. In a variety of studies it has been implicated as a site high in ion transport activity and rich in transport enzymes (Dendy et al., 1973; Olson and Fromm, 1973; Maetz, 1974; Karnaky et al., 1976). Although chloride cells are generally present in both fresh- and saltwater-adapted euryhaline fish, the structure and function appear to differ (Shirai and Utida, 1970; Shirai, 1972). This, and

the differences in transport enzyme activity between salt- and freshwater-adapted forms, is probably a reflection of the substantially different requirements for transport in the two different media.

In the light of the foregoing considerations the expected response to temperature increases should be elevation in the branchial activity of ion transport-associated mechanisms, in accordance with the requirement for increased ion uptake.

(c) Characteristics of the Absorption Process

Reabsorption of electrolytes from the primary urine and absorption at the gill of the two major plasma electrolytes, sodium and chloride, is considered to be an "active" process. Transport of sodium and chloride is characterized by the movement of these solutes against electrochemical gradients by means of an expenditure of metabolic energy which cannot be less than the product of the rate of net transport and the electrochemical potential across the membrane. Active uptake was hypothesized by Kro gh (1939, cited in Maetz, 1974) and has been verified for a wide variety of freshwater organisms (see Maetz, 1974, for a review). An illustration is provided by the fact that fish, having body fluids at slightly less than 300 mOsm.$l^{-1}$, absorb ions from the surrounding water, at 1 to 20 mOsm.$l^{-1}$, and also recover ions from the primary urine at a rate sufficient to produce urine at about 50 mOsm.$l^{-1}$. Although the overall process shows characteristics of "active" transport, in a multi-step process not all of the steps must be active and this may be the case here (Hickman and Trump,

1969; Maetz, 1971).

It is evident that sodium and chloride are regulated by separate mechanisms as fluxes of each may occur, simultaneously, in opposite directions and/or at different rates (Kerstetter et al.,1970; Kerstetter and Kirschner, 1972; Motais and Garcia-Romeu, 1972), while inhibitors may selectively reduce or eliminate one flux without a major disturbance of the other (Maetz, 1974). Also supporting this contention is the fact that, although the maximal influx rates of sodium and chloride are similar, at about $35 \mu Eq.hr^{-1}.100g^{-1}$, the $K_m$ for each process is considerably different, 450 $\mu Eq.l^{-1}$ versus 250 $\mu Eq.l^{-1}$ respectively (Kerstetter et al., 1970; Kerstetter and Kirschner, 1972, DeRenzis and Maetz, 1973). Despite the separate means of regulation, it is now felt that the two are controlled interdependently, which would seem to require at least one common component. This requirement is suggested by the precise absolute, and relative control of the two ions which has been demonstrated, and by the observation that sodium and chloride uptake procedes optimally from sodium chloride solutions. Alternate anions or cations reduce the efficiency of sodium or chloride uptake, respectively (Maetz, 1974).

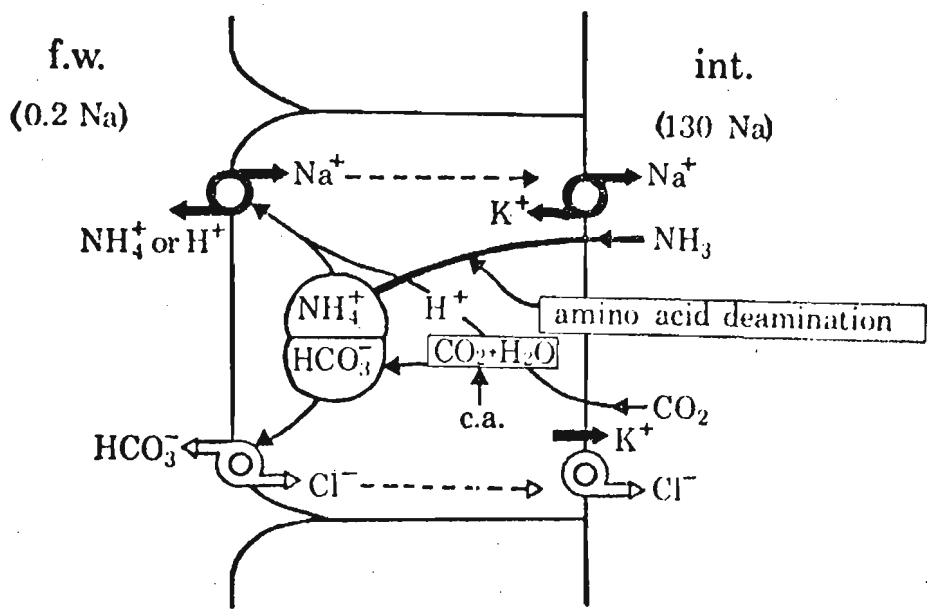
In order to preserve electroneutrality in the body fluid system, the influx of a specific ion must be coupled with elimination of an ion of the same charge or uptake of an ion of the opposite charge. For sodium the endogenous counter-ion could be two, ammonium ($NH_4^+$) or hydronium ($H^+$).

The relative merits and evidence pro and con for both have been extensively examined, the present consensus being that both are involved, although the exact relationship is not firm. It has been proposed that ammonium efflux provides for a relatively constant exchange uptake of sodium, which can be supplemented by hydronium: sodium exchange. Thus sodium uptake is proportional to the sum of ammonium and hydronium efflux. Under certain conditions where sodium uptake is less than ammonium excretion, ammonia ($NH_3$), the free base form, may diffuse out passively. The stoichiometry of this transfer, whether 1:1 or otherwise, has yet to be firmly established (Maetz, 1974; Kerstetter and Keeler, 1976). The fact that bicarbonate ($HCO_3^-$) is exchanged for chloride has also been demonstrated, although the involvement of hydroxide ($OH^-$) has not been entirely excluded (Kerstetter and Kirschner, 1972; DeRenzis and Maetz, 1973). Again the stoichiometry has not been confirmed.

The chloride cell model of Maetz (1971), which is presented in Figure 4, outlines gill involvement in ion uptake and has been the basis of most recent experimental designs. Sodium and chloride are exchanged at the external boundary for their respective counterions, and are then actively transported into the blood by ($Na^+$-$K^+$)-stimulated ATPase and an anion-stimulated ATPase, respectively. Hydronium and bicarbonate are produced from carbon dioxide and water by carbonic anhydrase. Ammonia is produced in the liver and kidney, primarily through deamination by glutamate dehydrogenase (Walton and Cowey, 1977).

Fig. 4 A conceptual model of chloride cell function by Maetz. The location and direction of the various processes is noted, $Na^+$ transport in the upper portion and $Cl^-$ transport in the lower. The contribution of carbonic anhydrase is noted in the middle of the figure.

FIGURE 4 A MODEL OF CHLORIDE CELL FUNCTION




(FROM MAETZ, 1971)

The procedures involved in ion transport in the kidney are not as well understood as those at the gill, but the result, reabsorption of most of the filtered electrolytes, is certainly well established. Carbonic anhydrase and $(Na^+-K^+)$-ATPase are known to be present and essential for ion reabsorption (Hickman and Trump, 1969; Johnson, 1973; McCartney, 1976; Trump and Jones, 1977). Carbonic anhydrase generates hydronium which appears to be excreted here, while $(Na^+-K^+)$-ATPase is considered to be involved in the absorption of sodium. Minor involvement of the kidney in nitrogenous waste excretion, as mentioned earlier, precludes any major involvement of ammonium in heteroionic exchange. Uptake of chloride is thought to be passive in nature, a consequence of active sodium transport. If this is the case, the differences in sodium and chloride levels in the urine and the different $Q_{10}$ values for urine depletion of the two ions could be attributed to membrane permeability differences (Houston, 1973). However, these differences could also be a result of the presence of an active chloride absorption system. This is a distinct possibility, as the enzyme purported to be involved, $(HCO_3^-)$-ATPase, has been reported in the eel kidney (Morisawa and Utida, 1976).

3. Characterization of the Enzyme Systems Investigated

(a) $(Na^+-K^+)$-stimulated Adenosine Triphosphotase (ATP Phosphohydrolase E.C. #3.6.1.3)

This enzyme appears to be the active transport system for sodium and potassium. The actual enzyme molecule, which is imbedded in the cell membrane, is thought to consist of two functional, 100,000 molecular weight protein subunits

and a 55,000 molecular weight glycoprotein. Tight association with approximately 30 phospholipids (termed the phospholipid annulus), whose fluidity appears to have a significant effect on activity, is required for the system to function, as are ATP. magnesium, sodium and potassium. The cardiac glycoside, ouabain, is a specific inhibitor of this enzyme. This provides a basis for its' postulated role in ionoregulation, as sodium transport in the intact cell is also sensitive to ouabain (Skou, 1972, 1975; Grisham and Barnett, 1973; Warren et al., 1974; Warren et al., 1975; Hokin, 1976). Since the general description above is based on works using enzymes from a wide variety of tissues and animals, it may not be entirely representative of trout ($Na^+$-$K^+$)-ATPase, although, on the basis of present evidence, it seems to be quite similar.

Since appreciable amounts of sodium are transported, and the presence of a specific transport enzyme, ($Na^+$-$K^+$)ATPase, has been demonstrated in many tissues, including those of the gill and kidney, this enzyme is considered to be involved in sodium transport (Jampol and Epstein, 1970; Maetz, 1974; McCartney, 1976). As well as producing sodium-potassium exchange, it can provide sodium-sodium and potassium-potassium exchange, and may be the cause of this type of exchange in fish (Maetz, 1974). Further evidence for its' involvement is the fact that differences in activity observed between the gills and kidneys of freshwater-adapted animals, as contrasted to those adapted to seawater, appears to reflect the appropriate loads borne by these organs under the respective conditions (Kamiya and Utida, 1968, 1969;

Epstein et al., 1969; Jampol and Epstein, 1970; Pfeiler and Kirschner, 1972; Sargent et al., 1975; McCartney, 1976).

However, the involvement of this enzyme should not be taken as firmly established, as some presently available data tend to suggest otherwise. One problem inherent to the salt-water-freshwater loading argument is the fact that the directions of pumping are opposite, reflecting the substantially different osmoregulatory problems in each case. Also, the enzymes extracted from salt- and freshwater-adapted organisms appear to be substantially different in character (Motais, 1970, Pfeiler and Kirschner, 1972). The $(Na^+-K^+)$-ATPase specific inhibitor, ouabain, shows no appreciable effect on sodium influx in the isolated gill of freshwater-adapted trout (Kerstetter and Keeler, 1976), or at least, a substantially smaller effect than acetazolamide (Payan et al., 1975). This raises the question of whether or not the enzyme is actually involved in ion transport here. An alternative explanation can be found in work noting that the $(Na^+-K^+)$-ATPase from freshwater-adapted trout gills is rather insensitive to ouabain, unlike other $(Na^+-K^+)$-ATPases (Davis and Wedemeyer, 1971; Pfeiler and Kirschner, 1972). Also, the enzyme demonstrates a very significant thermal dependence (high $Q_{10}$), showing very substantial activity reductions at low temperature. This is probably a function of its' intimate membrane association (Charnock et al., 1975; Russell and Chambers, 1976), and may be the reason Pfeiler and Kirschner (1972) observed very low activity, from a trout gill preparation, when the assay temperature was 13°C, the acclimation temperature of the fish. This was

in contrast to the substantial activity noted at 37°C. Finally, a study of sodium transport and ($Na^+$-$K^+$)-ATPase activity in the intestine of goldfish has shown that the temperature adaption of the two parameters occurs in a significantly different manner (Smith and Ellory, 1971).

(b) ($HCO_3^-$)-stimulated Adenosive Triphosphotase (ATP Phosphohydrolase E.C.#3.6.1.3.)

Maetz (1974) suggested the presence of an active chloride pump in the freshwater adapted gill, and also noted the presence of an obligatory chloride: bicarbonate exchange. The presence of a membrane-associated ATPase not requiring sodium or potassium, and not inhibited by ouabain, has been reported in the gills of trout and eels (Kerstetter and Kirschner, 1974; Solomon et al., 1975). Although direct evidence linking it to the chloride pump mechanism is not readily available, ($HCO_3^-$)-ATPase has recently been reported in eel gill, kidney, and intestine, where an adaptive change, relative to chloride transport in fresh- and saltwater was observed (Morisawa and Utida, 1976). The obvious analogy to the ($Na^+$-$K^+$)-ATPase system is attractive. The location of this carrier is a problem. It has been suggested that the active component of chloride transport is located at the mucosal border, contrary to the model of Maetz (1971). This is supported by the fact that inhibition of transport occurs from the external surface (Kerstetter and Kirschner, 1972; De Renzis, 1975). However, a major problem, with respect to the involvement of this enzyme in active chloride transport, is the fact that thiocyanide ($SCN^-$), a specific inhibitor of both the enzyme and active chloride

uptake, produces different responses in each case (Kerstetter and Kirschner, 1974). Also, the activity of ($HCO_3^-$)-ATPase appears to show no dependence on the level of chloride present (Kerstetter and Kirschner, 1974; DeRenzis, 1975; Solomon et al., 1975).

(c) ($Mg^{++}$)-dependent ATPase (E.C.#3.6.1.3.)

The presence of a ($Mg^{++}$)-dependent ATPase, often termed basal or ouabain-insensitive ATPase, has been reported in association with membrane preparations of ($Na^+$-$K^+$)-ATPase, ($HCO_3^-$)-ATPase, and ($Ca^{++}$-$Mg^{++}$)-ATPase from fish (Pfeiler and Kirschner, 1972; Kerstetter and Kirschner, 1974; Price, 1976), similar to its appearance in other organisms. Its' presence requires that the activity of these enzymes be estimated as the difference between total and ($Mg^{++}$)-ATPase activity. Although the significance of this concurrent distribution is unknown at present, it has been reported, in one case, that ($Na^+$-$K^+$)-and ($Mg^+$+)-ATPase were separable by both kinetic and physical procedures. This suggests that they are, in fact, different enzymes (Hoffman, 1973).

Little information is available on the structure and/or function of this enzyme. If the ($Mg^{++}$)-ATPase isolated by White and Ralston (1976) is indeed the basal ($Mg^{++}$)-ATPase, and this bears some relation to the enzyme measured herein, then the structure is probably a hexamer of six similar 100,000 molecular weight subunits. Unfortunately, a possible function was not reported. As the three enzymes found in association with ($Mg^{++}$)-ATPase are implicated in transport of various ions, speculation regarding a role for this enzyme in magnesium transport is attractive, especially in the light

of the fact that certain cell functions are sensitive to magnesium levels (Wacker and Vallee, 1964; Dunn, 1974). This possibility is interesting as, although magnesium levels in plasma and erythrocytes show no thermal dependence, increases in cardiac muscle and decreases in skeletal muscle and liver levels, related to acclimation temperature, have been observed (Houston *et al.*, 1968; Murphy and Houston, 1977; Smeda and Houston, unpublished data). Unfortunately, it is not known whether active magnesium transport occurs in the tissues mentioned. In the case of erythrocytes, where some information is available, there is no evidence of active magnesium transport (Dunn, 1974).

One final point, significant due to its' ommission from other studies of fish ATPases, is that the $(Mg^{++})$-ATPase activity measured may not be entirely basal in origin. Two enzymes, a calcium-dependent and a calcium-independent $(Mg^{++})$-ATPase, are known to exist. The latter appears to be the source of basal activity, as it requires only magnesium and ATP for activation, while the former demonstrates an absolute requirement for calcium. However, the level of calcium needed for stimulation - 10 $\mu$M or less - is sufficiently low enough to be considered as contamination in normally prepared solutions. A special buffer is required to maintain levels low enough for dependence studies. The $(Ca^{++}$-$Mg^{++})$-ATPase has been reported to form up to 28% of the basal $(Mg^{++})$-ATPase activity under certain conditions (Hasselbach, 1975; Robinson, 1976).

In the light of the previous discussion with regard to this enzyme, the results concerning it will be presented, but

any further discussion will not be undertaken.

(d) Carbonic Anhydrase (Carbonate hydro-lyase E.C.#4.2.1.1.)

Mammalian carbonic anhydrase is composed of one zinc ion and a single polypeptide chain, of about 260 amino acid residues, having a molecular weight of about 30,000. Although only limited evidence is available, it appears that fish carbonic anhydrase is similar, even to the point of specific inhibition by the sulfonamide acetazolamide. The enzyme catalyses, reversibly, the hydration of carbon dioxide. It is thermally insensitive ($Q_{10}$=1.2 to 1.3) and produces some of the highest turnover numbers known for any enzyme (Davis, 1961; Maren, 1967; Lindskog *et al.*, 1971).

The presence of carbonic anhydrase in the erythrocytes, gills, and kidneys of freshwater fishes is firmly established (Maren, 1967). Maetz (1971) incorporates it into his model of gill ion transport, where it fuels the salt exchange process by generating hydronium and bicarbonate. Support for this postulated role is provided by the fact that acetazolamide causes a significant reduction in sodium efflux (Kerstetter *et al.*,1970; Kerstetter and Keeler, 1976). In trout, unlike goldfish, chloride influx is not greatly affected by acetazolamide injections, suggesting that it may not be involved in this species. However, this apparent anomoly can be explained by having the blood as the primary bicarbonate source and/or locating the chloride/bicarbonate exchange on the serosal boundary and the chloride pump on the mucosal boundary, reversing the organization suggested by Maetz (1971) (Kerstetter and Kirschner, 1972). That the enzyme participates in ion transport in the kidney is supported by data showing

that acetazolamide inhibits recovery of salt from the urine (Maren, 1967).

Unfortunately, although the involvement of carbonic anhydrase is firmly established, little data on activity and relative distribution are available. Without this, the relative importance and degree of involvement is difficult to assess.

4. Comments Upon the Physiological Relevance of *In Vitro* Enzyme Assays

The maintenance of hydromineral balance in the trout has been previously established. The possible mechanisms that are involved have been outlined in earlier discussion. It would seem to be a relatively simple process to predict the responses required in the implicated transport systems to maintain constancy when stress is induced by, for example, an increase in ambient temperature. If, after extraction and assay, the changes in enzyme activity reflect the appropriate response, then evidence for the validity of the model on which the experimentation was based would be provided. If no correlations were apparent, then either an explanation must be given or a new hypothesis and model developed. However, the very nature of results from *in vitro* enzyme assays creates difficulties when *in vivo* responses are to be explained. Many potential pitfalls exist and not all can be eliminated.

Most *in vitro* assays are conducted with the enzyme saturated i.e., operating at $V_{max}$, while this condition is rarely found *in vivo*. Changes in temperature affect enzyme-substrate affinity -$K_m$- so that activity could be regulated in this manner, rather than changes in the absolute amount of

enzyme. In fact, $K_m$ can be positively modulated by temperature producing actual increases in rate at lower temperatures, under non-saturation conditions. As well, conformational changes, generated by binding and/or release of substrates or co-factors, whose availability may or may not be correlated to temperature or other factors, can change activity. In fact, temperature can produce a major change in the kinetic properties of what appears to be the same protein. The extreme case of this, the production of different isozymes, has often been observed. In addition, the degree of fluidity of the membrane can have a very great influence on the activity of membrane-associated enzymes. This may be another important site for control. Finally, the consideration of enzyme activity alone ignores the possibility that special structural organization may be significantly involved in the process in question (Fry and Hochachka, 1970; Hochachka and Sumero, 1971; Hochachka, 1973; Hazel, 1973). In short, the presence or absence of *in vitro* - *in vivo* correlations, although perhaps indicative of actual trends and producing valuable information, must be interpreted with great caution.

# III MATERIALS AND METHODS

## 1. Source and Maintenance of Test Organisms

Rainbow trout used in this study were obtained from a local supplier, Goosen's Trout Farm, Otterville, Ontario. The animals were fed daily, ad libertum, with a commercially-prepared pelleted trout food (Purina Trout Chow) and were judged, on the basis of appearance, feeding activity, and the lack of any overt disease symptoms, to have been in good health. Groups were acclimated to 2, 10 and 18°C. on a twelve hour darkness photoperiod regime for at least three weeks prior to sampling. Illumination ranged from 11 to 18 foot-candles at the water surface. Dechlorinated St. Catharines tap water, typically 140 $mg.l^{-1}$ total hardness, 94 $mg.l^{-1}$ total alkalinity, pH 7.6 was used. Dissolved oxygen levels were 80% or better of the saturation capacity. Water analysis determinations were conduced in accordance with American Public Health Association - approved methods (A.P.H.A., 1971)

## 2. Holding Conditions

Three fibreglass Frigid Unit MT-700 tanks of 500l capacity, served as acclimation chambers. Photoperiod control was achieved by means of plywood hoods equiped with light bulbs and automatic timers. Temperature control in each tank was effected by a 1/12 horsepower "Minnow-Cool" circulator/cooler working concurrently with a 1000 watt stainless steel heating coil. The latter component was regulated by a heat control unit, constructed by the Brock University Technical Services Department, accurate to 0.1°C. All temperatures were verified by means of a Fisher certified thermometer. Water was continuously filtered through

polyurethane foam filter pads, which were cleaned, along with removal of excess food and feces, on a regular basis. In addition, a continuous supplementary flow of dechlorinated water, sufficient to provide a complete turnover one to three times daily, was provided for each tank.

Although research was conducted in different years, the ATPase study in the fall (October to December) of 1975 and the carbonic anhydrase in the late summer-fall (September to October) of 1976, holding conditions were kept as constant as possible.

3. Sampling and Electrolyte Determinations

Animals were netted, stunned by a blow to the head, and blood samples quickly removed, by caudal puncture, into syringes previously rinsed with ammonium heparin (Sigma Chemical Co.: 50,000 units.ml$^{-1}$). A Fisher Model 59 blood centrifuge was used to separate the plasma, which was then immediately decanted into plastic vials, capped and stored at -80$^{o}$C until analyzed. Gill and kidney tissue samples were used immediately, as noted in the enzyme assay procedures that follow.

Plasma chloride levels were estimated by means of a Buchler-Cotlove chloridometer using Versatol (Warner-Lambert Co.) as a standard. Plasma sodium and potassium were estimated with a Unicam S.P.90 Atomic Absorption Spectrophotometer operating in the emission mode and using standards produced by British Drug House (B.D.H.).

4. Enzyme Assay Procedures

Deionized, glass distilled water and reagent grade chemicals were used throughout this study. Also, all enzyme preparation was conducted with equipment held on ice, in a cold room held

at 4°C.

(a) ($Na^+$-$K^+$)-stimulated, ($HCO_3^-$)-stimulated, and ($Mg^{++}$)-dependent ATPase Assay Procedure.

The technique that follows is based on those reported by Kamiya and Utida (1968), Kerstetter and Kirschner (1974), and Murphy and Houston (1974)

Gill arches from the right side of the fish and the whole kidney were placed in an ice-cold, 250 mM sucrose, 40 mM Tris-acetate, 5 mM E.D.T.A. (Ethylenediaminetetra-Acetic Acid), pH 7.5, solution until completion of sampling. Two fish per day were sampled. Gill filaments (1.2 to 3.5 g, estimated by volume displacement) were cleared, with forceps, of associated mucus and blood, removed from the arches, and placed with 9 volumes (less 3 to 5 ml of solution for a rinse that was subsequently added) of fresh solution in a 30 ml glass homogenizing vessel. Kidney tissue (0.8 to 2.0g) was treated similarly. Each sample was homogenized using 7 strokes of a motor-driven teflon pestle. The homogenate was then centrifuged at about 0°C for 30 minutes at 10,000 G. The supernatant was decanted and recentrifuged at about 0°C for 40 minutes at 100,000G. The final pellet was respended in 2.5 ml of an ice-cold 40 mM Tris-acetate, 5 mM E.D.T.A.m 0.1% sodium deoxycholate, pH 7.5 solution using an all-glass hand homogenizer. Enzyme preparations were held on ice for the duration of the procedure. The protein content of these final enzyme preparations (0.5 to 1.5 mg) were determined by a modified Lowry technique (Albro, 1975, procedure D) using bovine serum albumin (fraction IV) as the reference standard. Table 1 indicates the final

concentrations of the components of the assay media present in the 2 ml final volume.

TABLE I - Media composition for ATPase Estimations

| Assay | $(Mg^{++})$-ATPase in relation to $(Na^+/K^+)$ ATPase | $(Na^+/K^+)$ ATPase | $(Mg^{++})$-ATPase in relation to $(HCO_3^-)$ ATPase | $(HCO_3^-)$ ATPase |
|---|---|---|---|---|
| TRIS-acetate, mM | 40 | 40 | 40 | 40 |
| pH | 7.5 | 7.5 | 7.5 | 7.5 |
| $MgCl_2$, mM | 5 | 5 | 5 | 5 |
| ouabain, mM | 0.5 | - | 0.5 | 0.5 |
| KSCN, mM | 5 | 5 | 5 | - |
| NaCl, mM | 120 | 100 | - | - |
| KCl, mM | - | 20 | - | - |
| $NaHCO_3$, mM | - | - | - | 5 |

Although potassium and bicarbonate were removed from the solutions used in estimation of $(Mg^{++}_{Na-K})$-and $(Mg^{++}_{HCO_3})$-ATPases, respectively, which should have eliminated any $(Na^+-K^+)$-or $(HCO_3^-)$+ATPase activity, ouabain and thiocyanate, specific inhibitors of the respective enzymes, were added as indicated in Table I, to ensure that no spurious activity occured. These inhibitors were also added to the assay solutions for $(HCO_3^-)$- and $(Na^+-K^+)$-ATPase, as shown in Table I, for the same reason. Also, it should be noted that the $HCO_3^-$ concentration employed is well below the optimum found by Kerstetter and Kirschner (1974). However, it is comparable to estimated cellular levels (Kerstetter and Kirschner, 1972), and preliminary

studies with the procedure used in this study indicated similar activities with either concentration, contrary to the earlier evidence.

Aliquots of enzyme preparation (0.2 ml) were preincubated for 15 minutes before initiation of the reaction through addition of 0.2 ml of 30 mM ATP (Adenosine 5' Triphosphate Disodium Dihydrogen Salt, B.D.H.). Assay incubations were conducted at the appropriate acclimation temperature and also at a common temperature (25°C). The reaction was stopped after 15 minutes by the addition of 0.5 ml of ice-cold aqueous trichloroacetic acid. The test tubes were centrifuged, in a clinical centrifuge at 4°C for 10 minutes at about 500 G to remove the denatured protein, and aliquots were then removed for duplicate determinations of inorganic phosphate by a modified Fiske and Subba Row (1925) procedure. $(Na^+-K^+)$ATPase activity was taken to be the difference between the $(Na^+-K^+)$-and $(Mg^{++})$-ATPase activities. A similar procedure was employed in determinations of $(HCO_3^-)$-ATPase activity. Activities are reported in $\mu$Moles Pi liberated.15 $min^{-1}$.mg $protein^{-1}$.

(b) Carbonic Anhydrase Assay Procedure

This assay technique is based on procedures reported by Armstrong *et al.* (1966), Duff and Coleman (1966), and Verpoorte *et al.* (1967) which take advantage of the fact that this enzyme can function as an esterase on certain artificial substrates.

Since the red blood cells contain large amounts of carbonic anhydrase, and substantial quantitites of blood are found in the gill and kidney, it was necessary to compensate for contamination from this source in gill and kidney carbonic

anhydrase activity estimations. This is the reason for the various activity and protein estimates, and the complex specific activity calculations that appear in this procedure.

Gill arches from the right side of the fish and the whole kidney were placed in an ice-cold 250 mM sucrose, 40 mM Tris-$H_2SO_4$,pH 7.5 solution until completion of sampling. Two to three fish were sampled per day. Gill filaments (0.9 to 2.0g) were cleared as in the ATPase determination, stripped from the arches, and placed with 19 volumes of fresh solution (less 6 ml for a rinse that was subsequently added) in a 30 ml glass homogenizing vessel. Kidney samples (1.0 to 2.0 g) were similarly treated. 1.0 ml of fresh whole blood was placed in a well heparanized homogenizing vessel, diluted 1:20 with homogenizing solution (with provision for a rinse), and vortexed to ensure adequate mixing. All samples were homogenized using 7 strokes of a motor-driven teflon pestle, and then centrifuged, at about $0^{o}C$ for 30 minutes at 500 G, to remove debris. Preliminary investigation indicated that this centrifugation procedure provided a reasonable compromise between precision, activity, and reproducibility. The supernatants were held on ice for the rest of the procedure. Total protein (290 to 840$\mu$g) was estimated by the procedure previously noted. Hemoglobin content (28 to 488 $\mu$g) of blood and tissue preparations was determined by the alkaline hematin method (Anthony, 1961) using Hematrol (Clinton Laboratories) as a standard.

The buffer for the assay reaction was a 125 mM Tris-$H_2SO_4$, pH 7.5 solution. 50 $\mu$M of the carbonic anhydrase specific inhibitor acetazolamide was also prepared in this buffer. Preliminary work indicated that, in the final reaction mixture,

inhibition appeared to be essentially constant at acetazolamide concentrations in excess of 0.2 μM. The concentration noted above, giving a final level of 20 μM, was used throughout. The substrate for the reaction, 3 mM para-nitrophenol-acetate (54.4 mg + 3 ml acetone to 100 ml with water), was prepared fresh daily and cooled to reduce spontaneous degradation. The substrate concentration was monitored, spectrophotometrically, throughout the day to check for any changes. Precipitation and subsequent reduction in concentration occasionally occured, necessitating the preparation of fresh solution.

0.1 ml of enzyme preparation and 0.1 ml of homogenizing medium was added to a small test tube containing 0.8 ml of buffer. Preincubation, at the appropriate temperature, was carried out for 2 minutes in a temperature-controlled water bath. 1.0 ml of substrate solution was added to the tube, swirled to mix, and this was introduced into the thermoregulated, microflowthrough cell of a Baush and Lomb Spectronic 700 spectrophotometer. The change in optical density at 348 mu, the isosbestic point for the products of the reaction, was recorded on a Fisher Omniscribe chart recorder for 2 to 3 minutes. This was then repeated, and the mean value obtained was considered to represent the total esterase activity. The assay was repeated, in duplicate, using the buffer containing acetazolamide to generate the non-carbonic anhydrase esterase activity. The difference was taken as the acetazolamide-inhibited or acetazolamide-sensitive carbonic anhydrase activity and was recorded as μMoles of p-nitrophenol + p-nitrophenolate produced per minute. This method of estimation was necessary as other esterases were present in the preparations. Specific activities were calculated

as follows: BLOOD

$$\frac{(\text{blood } \mu\text{Moles.min}^{-1})}{(\text{g total blood protein})-(\text{g blood hemoglobin})} = \mu\text{Moles.min}^{-1}.\text{g protein}^{-1}$$

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$$\frac{(\text{tissue } \mu\text{Moles.min}^{-1})-(\text{blood } \mu\text{Moles.min}^{-1}.\frac{\text{g tissue hemoglobin}}{\text{g blood hemoglobin}})}{(\text{g total tissue protein})-(\text{g tissue hemoglobin})} = \mu\text{Moles.min}^{-1}.\text{g protein}^{-1}$$

5. Statistical Analysis

Descriptive and inferential statistical procedures were carried out using a Wang 2200 calculator with programs provided by the Wang Corporation. The descriptive statistical program was modified to produce 95% confidence intervals (Alder and Roesslar, 1968). The one-way analysis of variance program was modified to perform tests on logarithmically-transformed (log e) data. This procedure improves approximation to normal distribution in small samples (Alder and Roesslar, 1968). Linear regression analysis was conducted in an attempt to detect any potentially significant relationships between selected variables, with emphasis upon those between weight and electrolyte levels and weight and enzyme activities. Relations between different electrolytes, as well as those between electrolyte concentration and enzyme activity, were also considered. In the latter cases, where weight was not involved, only enzyme activities determined at in vivo temperatures, and ion level-enzyme relations having previous substantiation were considered.

6. Effects of Temperature on Enzyme Activity

In order to examine the effects of temperature on the enzyme activities that were measured, $Q_{10}$ values were calculated, using the following formula and the appropriate mean activity estimates.

$$\log Q_{10} = \frac{10\ (\log K_1 - \log K_2)}{T_1 - T_2}$$

$K_1$ and $K_2$ are enzyme rates

$T_1$ and $T_2$ are temperatures in $^{o}C$

$Q_{10}$ values calculated from within a group (ie. $2^{o}$fish assayed at $2^{o}C$ amd $25^{o}C$) are actual $Q_{10}$ values, while those generated from tests between groups (ie. $10^{o}C$ fish at $10^{o}C$ and $18^{o}C$ fish at $18^{o}C$) are more correctly described as thermal coefficients. The $Q_{10}$ values generated reflect the effect of temperature on enzyme activity while the thermal coefficients indicate relative changes in activity between fish acclimated to different temperatures. To be more precise, these values reflect the amount of stimulation on the rate of a reaction that is induced by a $10C^{o}$ rise in temperature. A $Q_{10}$ of 2 indicates that the reaction doubles with every 10 $C^{o}$ rise, while a value of 3 indicates a tripling, and so on. Although a $Q_{10}$ value for a particular enzyme is directly related to the temperature range and substrate levels under which it was estimated, and, under similar conditions, different processes can demonstrate widely varying values, depending on the system in question, a value of 2 is often considered as typical of biological processes (Keeton, 1967; Hochachka, 1973; Hazel and Prosser, 1974).

# IV. RESULTS

In the figures presented in this section the mean is represented by a horizontal line, the 95% confidence interval of the mean by a rectangle about the mean, and the range by a vertical line. Significant difference, or the lack of it is generally obvious upon examination of the 95% confidence intervals. The results of the extensive analysis of variance investigation are referred to only in the text. Raw data are tabulated in the appendix.

## 1. Physical Characteristics and Plasma Ion Levels

The specimens used in the ATPase and carbonic anhydrase sections of the study did not differ in terms of weight and length (Figure 5). This was also true with respect to electrolyte levels. Acclimation temperature had no significant effect upon the regulation of plasma sodium concentration, and in general, this was true of chloride as well. It should be noted, however, that chloride levels in fish used in the carbonic anhydrase study tended to decline, although not significantly, at higher acclimation temperatures. In both groups plasma potassium was significantly ($P < 0.05$) elevated at higher temperatures. These findings are similar to earlier studies (Houston, 1973), and confirm that the rainbow trout has the ability to maintain the level of major plasma electrolytes constant, despite the thermal stresses upon ionic regulation previously considered. Elevation of plasma potassium at higher temperatures is also a common observation in studies upon salmonids, although the basis for this is not yet known. Analysis of variance comparing each

Fig. 5 Length (upper) and weight (lower) for the two batches of fish, ATP (fish used in ATPase estimations) and CA (fish used in carbonic anhydrase estimations) acclimated to 2°, 10° and 18°C. Mean (horizontal dash), 95% confidence interval of the mean (block about the mean) and range (vertical line) are indicated.

FIGURE 5 PHYSICAL CHARACTERISTICS

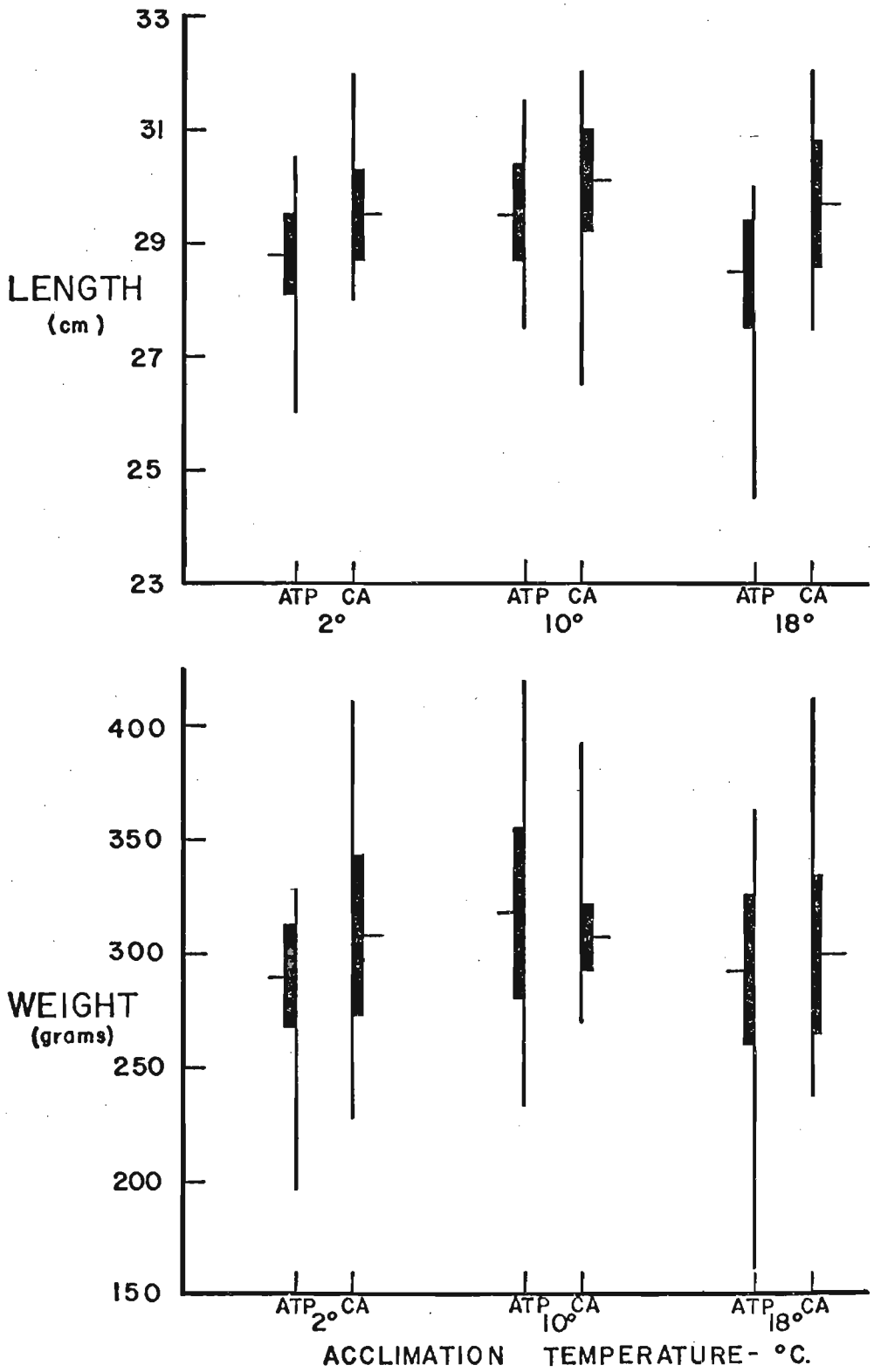
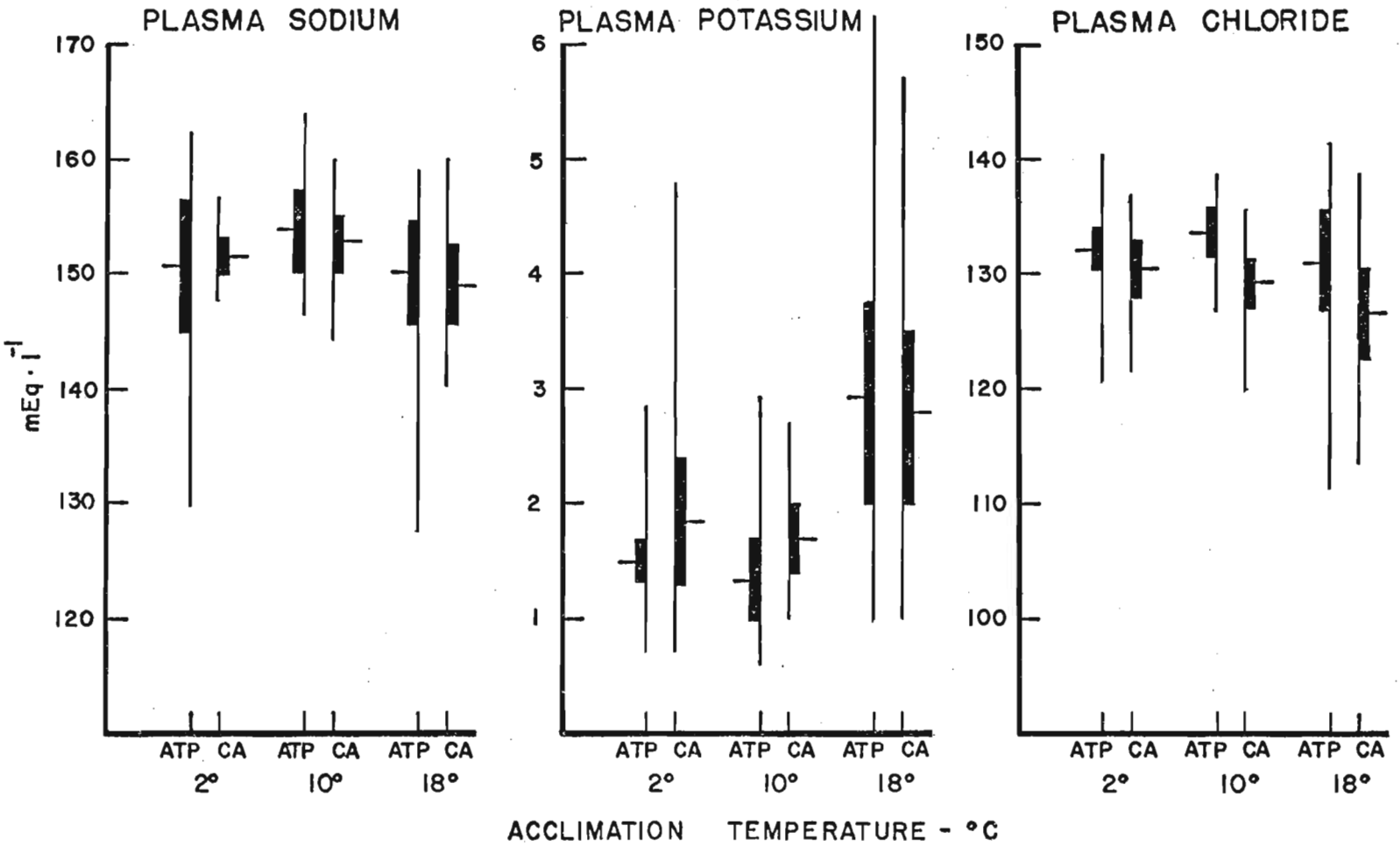


Fig. 6 Plasma $Na^+$ (left), plasma $K^+$ (center) and plasma $Cl^-$ (right) for the two batches of fish, ATP (fish used in ATPase estimations) and CA (fish used in carbonic anhydrase estimations) acclimated to $2^{\circ}$, $10^{\circ}$, and $18^{\circ}C$. Concentrations are in $mEq.l^{-1}$.

FIGURE 6 PLASMA ELECTROLYTE LEVELS



parameter from the ATPase group with its counterpart in the carbonic anhydrase group, at each acclimation temperature, indicated only one significant difference. At 10°C the mean plasma chloride level of the ATPase fish was significantly higher ($P < 0.01$). The absolute difference (4.5 $mEq.l^{-1}$) was, however, only 3.5% of the total chloride.

The plasma sodium/chloride ratio was examined for evidence, with regard to the hypothesized interrelationship between sodium and chloride regulatory mechanisms. Mean plasma sodium and chloride values were employed in the calculation of the ratios, which appear in Table 2. It is evident that, in both groups, the ratio was essentially constant.

TABLE 2 Plasma Sodium/Chloride Ratio

| | ATPase Group | Carbonic Anhydrase Group |
|---|---|---|
| 2°C | 1.14 | 1.16 |
| 10°C | 1.15 | 1.18 |
| 18°C | 1.14 | 1.18 |

On the basis of these results it was concluded that the two groups of test organisms were sufficiently similar to justify subsequent comparisons.

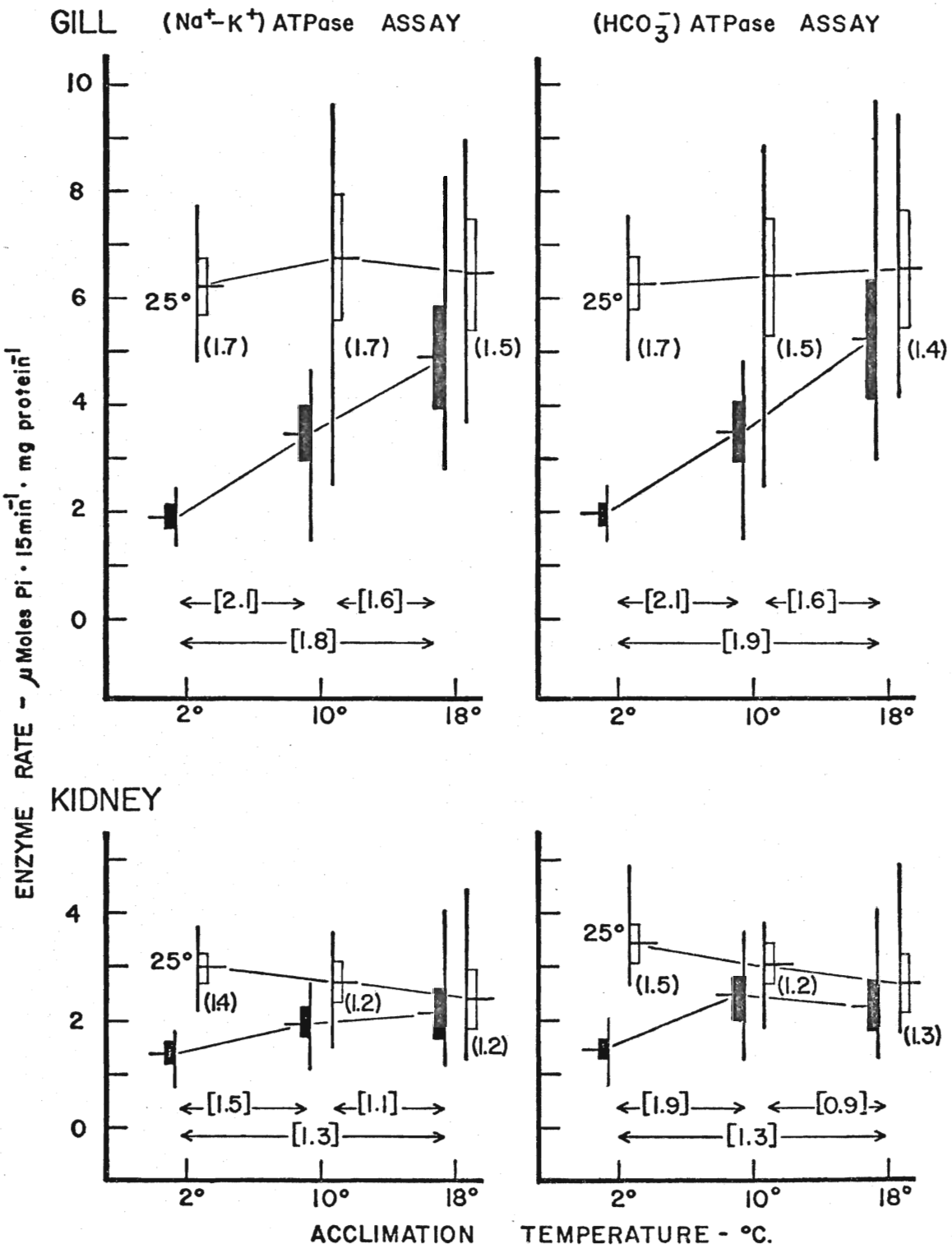
2. Enzyme Activities

(a) ($Mg^{++}$)-dependent ATPase

There appears to be little difference between ($Mg^{++}$)-ATPase activities determined in association with ($Na^{+}$-$K^{+}$)-ATPase and $HCO_3^-$-ATPase (Figure 7), despite the differences in medium composition. At common temperature,

Fig. 7 ($Mg^{++}$)-ATPase activity of gill and kidney extracts for fish acclimated to 2°, 10° and 18°C. The enzyme assays were done at 25°C (open bars) and at the respective acclimation temperatures (closed bars). The ($Mg^{++}$)-ATPase activity associated with the ($Na^{+}$-$K^{+}$)-ATPase estimation is on the left side while that associated with the ($HCO_3$)-ATPase estimation appears on the right. $Q_{10}$'s ( ) and thermal coefficients [] are given for the changes in enzyme activity measured.

FIGURE 7 ($Mg^{++}$) ATPase ACTIVITY



gill preparations were not significantly different, while kidney preparations were characterized by a significant ($P < 0.05$) decline in activity with acclimation temperature. Both gill and kidney preparation activities increased significantly ($P < 0.01$) with acclimation temperature, when assays were conducted at in vivo temperatures. Gill $Q_{10}$ values were in the medium range (1.4 to 1.7), while those of kidney were relatively lower (1.2 to 1.5).

(b) $(Na^+-K^+)$-stimulated ATPase

The activities of both gill and kidney preparations decreased significantly (Gill, $P < 0.05$; Kidney, $P < 0.01$) with respect to acclimation temperature, when assayed at 25°C(Figure 8). At in vivo assay temperatures, however, both preparations showed significant activity increases ($P < 0.01$) with elevated acclimation temperatures. Gill $Q_{10}$ values were, for the most part, in the range of 2.1 to 2.2. A particularly high value (5.3) was obtained in the case of the 2°C fish. Undoubtably, this resulted from the very low activity encountered in gill preparations from 2°C fish assayed at in vivo temperature. Kidney $Q_{10}$ values were also relatively high, ranging from 2.3 to 3.0.

(c) $(HCO_3^-)$-stimulated ATPase

Gill and kidney preparations did not differ significantly in activity when incubated at the common temperature (Figure 9). The 10° and 18°C fish also showed no significant differences in activity from either gill and kidney at in vivo temperatures. A significant difference ($P < 0.01$) was observed, in both activities, at 2°C. $Q_{10}$ values were low in gill (1.1 to 1.3) and kidney (1.1 to 1.2).

Fig. 8 $(Na^+-K^+)$-ATPase activity of gill and kidney extracts for fish acclimated at $2^{o}$, $10^{o}$ and $18^{o}C$. The enzyme assays were done at $25^{o}C$. (open bars) and at the respective acclimation temperatures (closed bars). $Q_{10}$'s ( ) and thermal coefficients [] are given for the changes in enzyme activity measured.

FIGURE 8 $(Na^+ - K^+)$ ATPase ACTIVITY

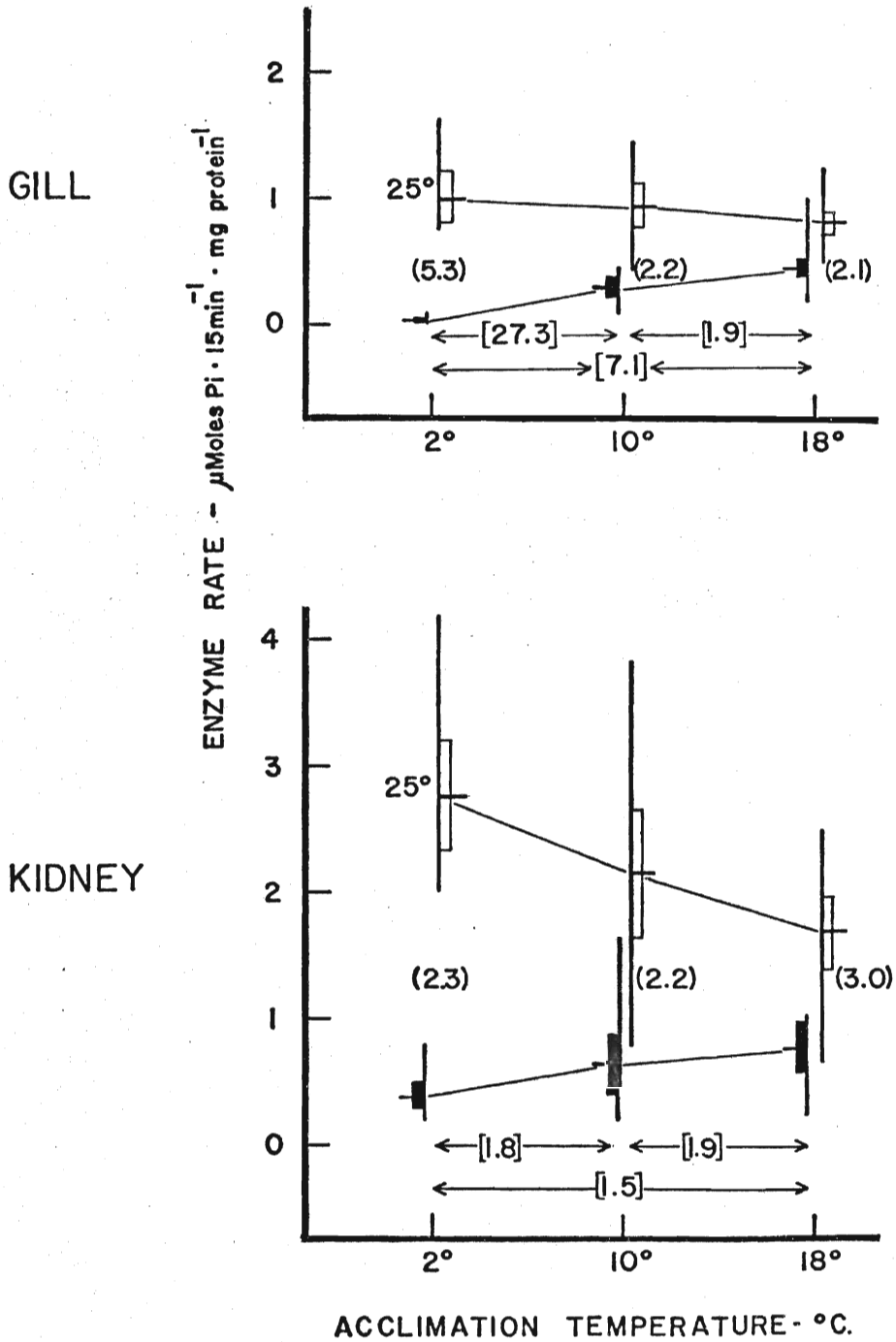
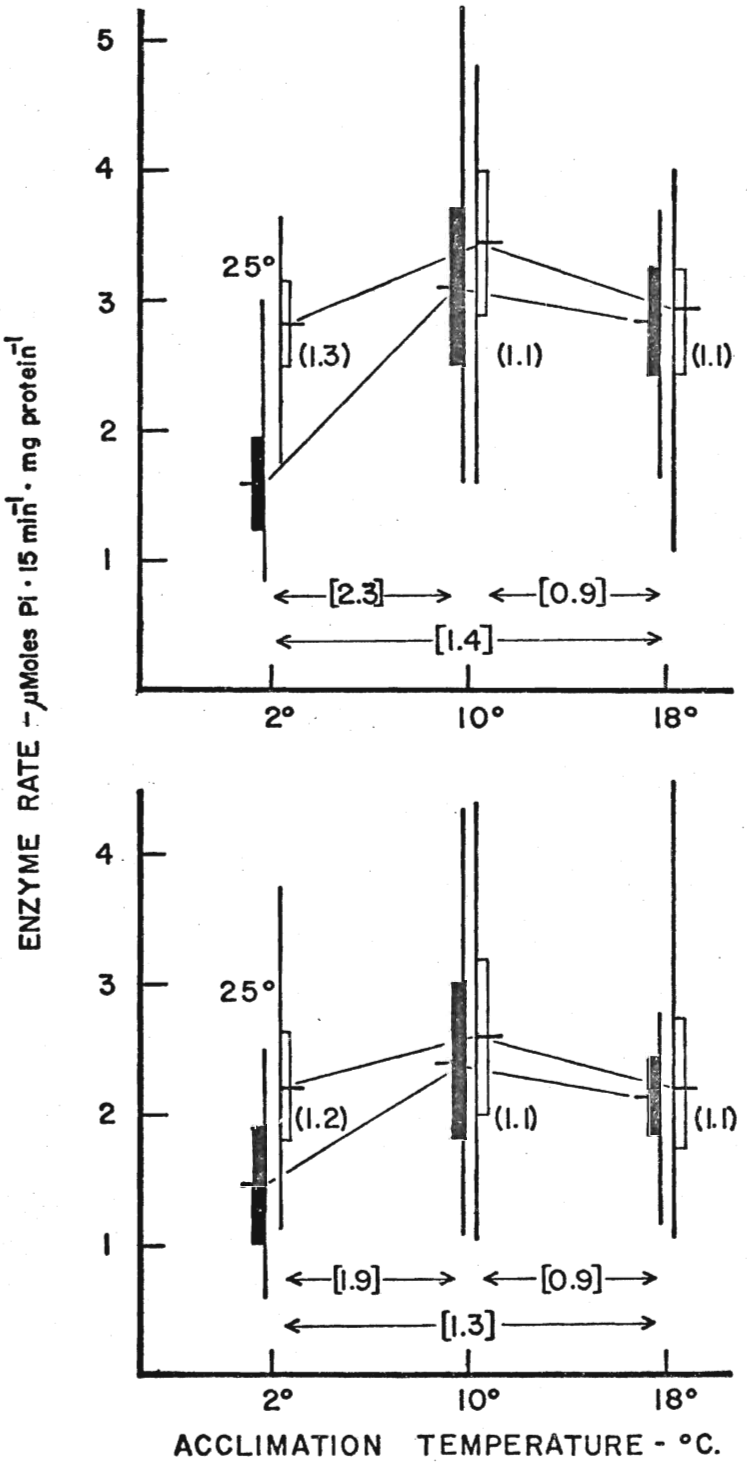


Fig. 9 ($HCO^-_3$)-ATPase activity of gill and kidney extracts for fish acclimated at 2°, 10° and 18°C. The enzyme assays were done at 25°C. (open bars) and at the respective acclimation temperatures (closed bars). $Q_{10}$'s ( ) and thermal coefficients [] are given for the changes in enzyme activity measured.

FIGURE 9 ($HCO_3^-$) ATPase ACTIVITY



(d) Carbonic Anhydrase

No significant differences in the carbonic anhydrase activity of blood were observed at 25°C (Figure 11). On the other hand, significant increases ($P < 0.01$) were apparent when assays were conducted under in vivo temperature conditions. A significant decrease ($P < 0.01$) in activity of gill preparations from 18°C fish was noted both at 25°C and ambient temperature (Figure 10). A significant increase ($P < 0.05$) in kidney preparation activity, with respect to acclimation temperature, was noted under both incubation temperature regimes. The $Q_{10}$ values for blood (1.2 to 1.3), gill (1.2 to 1.3) and kidney (1.1 to 1.3) were consistently low, as is typical of values reported for this enzyme.

Although the values reported here have been corrected, as previously outlined, it should be noted that from 5 to 45% of total branchial and 30 to 135% of total renal carbonic anhydrase activity appeared to be associated with blood.

3. Gill/Kidney Enzyme Activity Ratios

This data is presented in figure 12 and was obtained using the mean activity values of the various enzymes.

(a) ($Mg^{++}$)-dependent ATPase

The values obtained from ($Mg^{++}$-$_{Na-K}$)- and ($Mg^{++}_{HCO_3}$) ATPase estimations showed similar trends. Both 25°C and ambient assay temperature regimes showed increases in the ratio with elevated acclimation temperature. However, the increase at the common temperature was caused primarily by a decrease in kidney activity, with temperature, while at in vivo temperatures the increase results mainly from an increase in gill activity.

Fig. 10 Carbonic anhydrase activity of gill and kidney extracts for fish acclimated at 2°, 10° and 18°C. The enzyme assays were done at 25°C. (open bars) and at the respective acclimation temperatures (closed bars). $Q_{10}$'s ( ) and thermal coefficients [ ] are given for the changes in enzyme activity measured.

FIGURE 10 CARBONIC ANHYDRASE ACTIVITY I

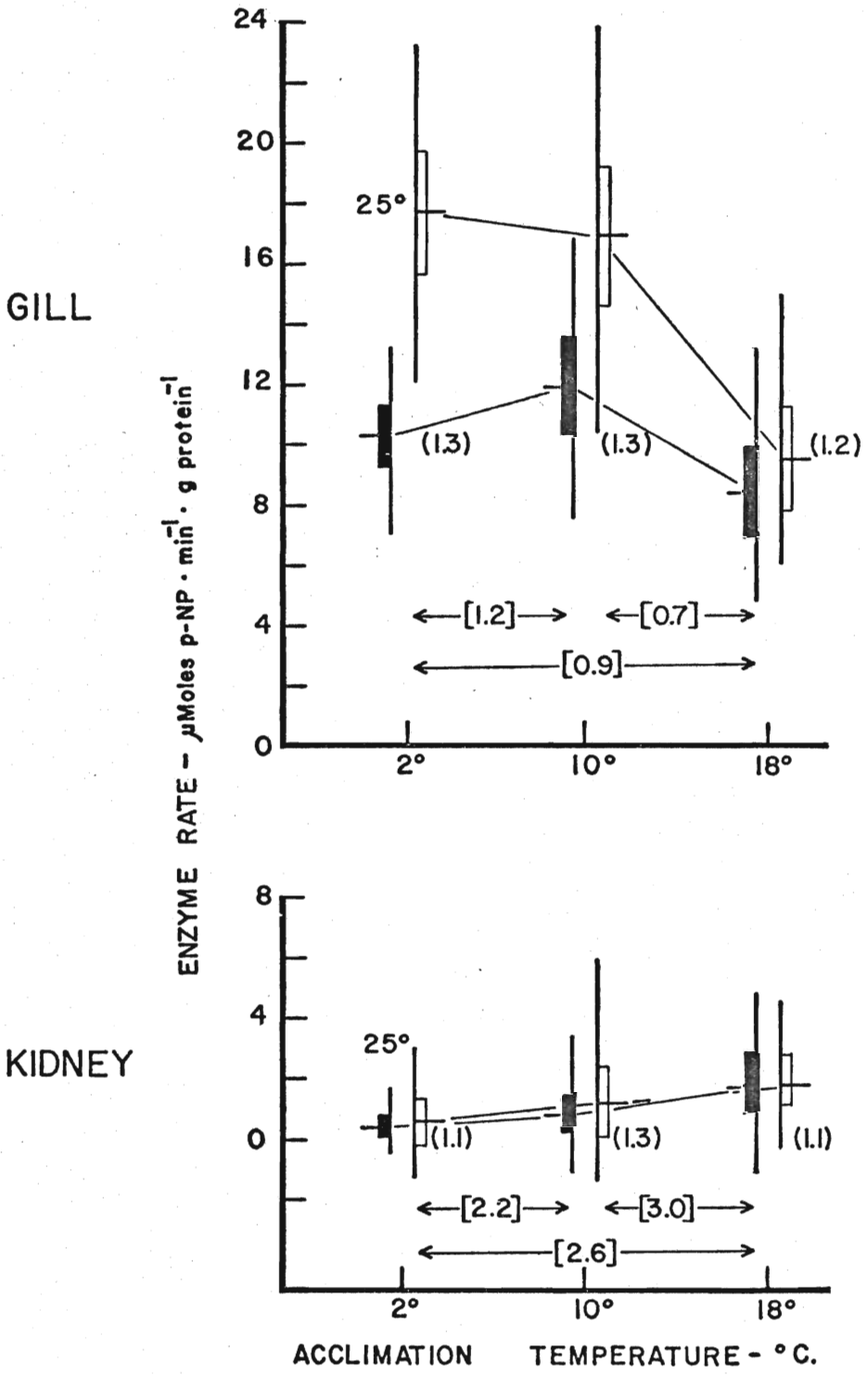


Fig. 11 Carbonic anhydrase activity of blood extract for fish acclimated at 2°, 10° and 18°C. The enzyme assays were done at 25°C. (open bars) and at the respective acclimation temperatures (closed bars). $Q_{10}$'s ( ) and thermal coefficients [ ] are given for the changes in enzyme activity measured.

FIGURE II CARBONIC ANHYDRASE III

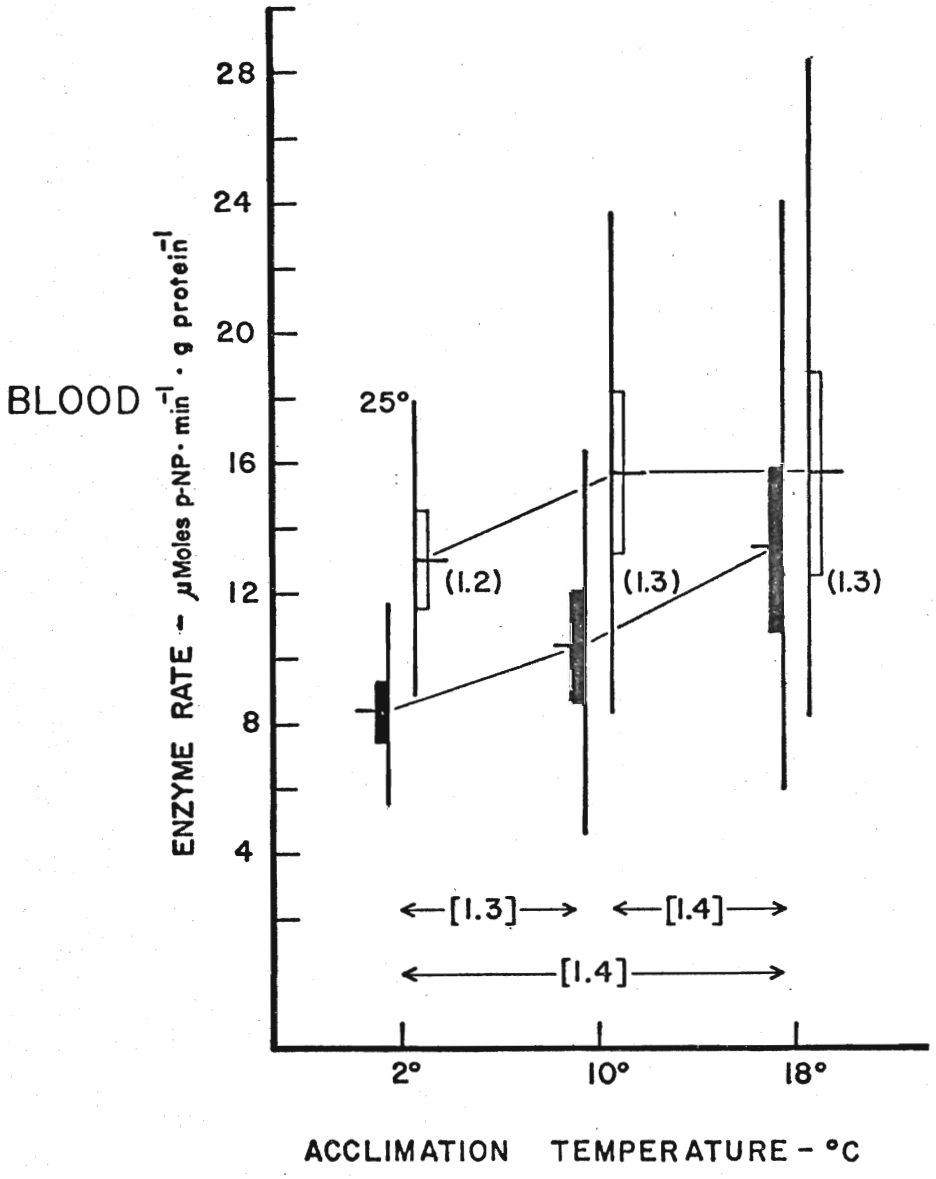
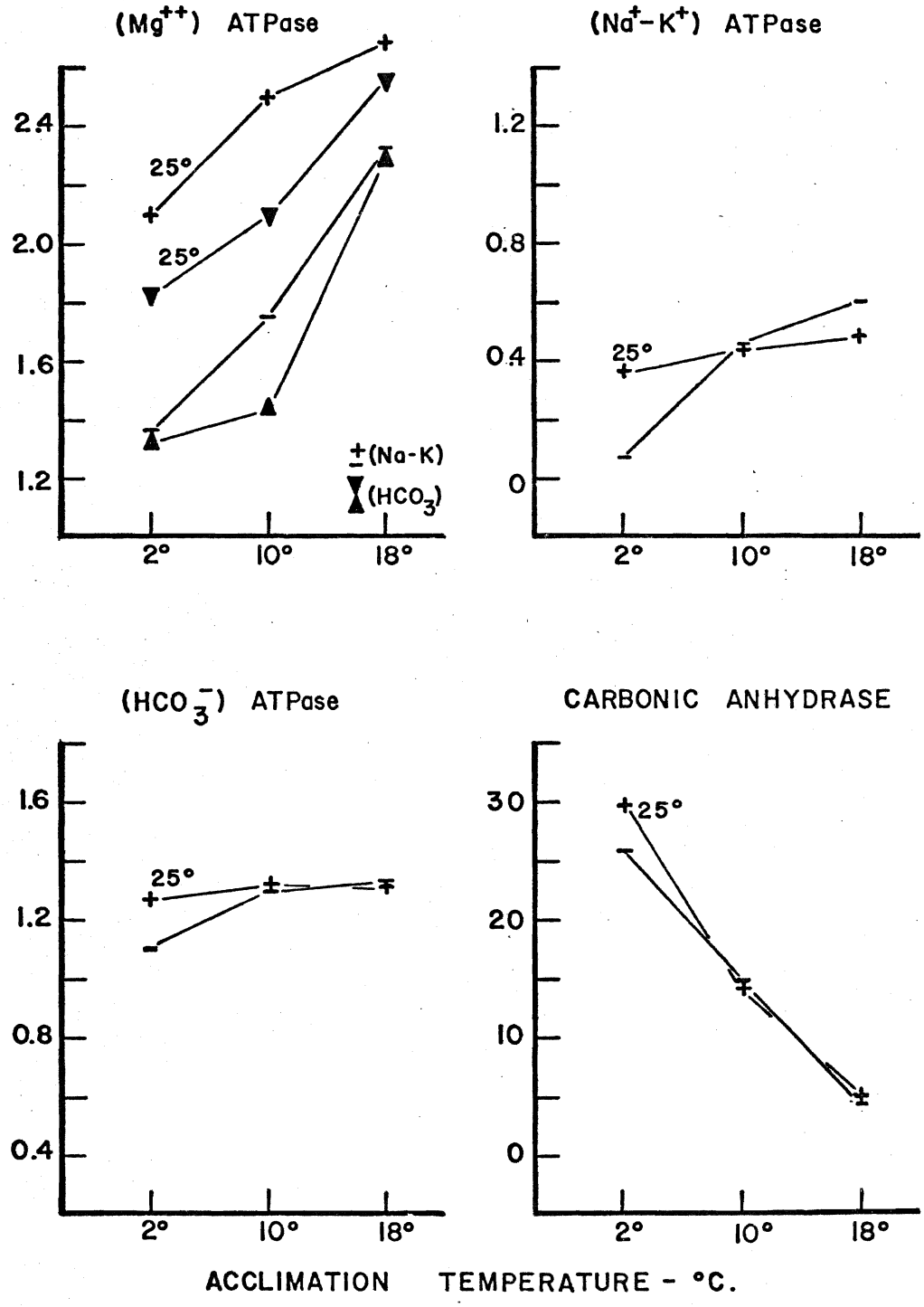


Fig. 12 Gill/kidney activity ratios of the enzymes ($Mg^{++}$)-ATPase, ($Na^{+}$-$K^{+}$)-ATPase, ($HCO^{-}_{3}$)-ATPase, and carbonic anhydrase for fish acclimated to $2^{o}$, $10^{o}$ and $18^{o}C$. Values presented were calculated from mean activity estimates. The results based on the common temperature assay procedure are represented by a cross (+) whereas those generated by the in vivo procedure are represented by a dash (-). For the case of ($Mg^{++}$)-ATPase the symbols presented on the graph indicate which results are based on the ($Na^{+}$-$K^{+}$)-associated estimation and which on the ($HCO^{-}_{3}$)-associated estimation.

FIGURE 12 GILL/KIDNEY ACTIVITY RATIOS



(b) $(Na^+-K^+)$-stimulated ATPase

The increase in the ratio noted here, with respect to temperature, is caused primarily by a decrease, under common temperature incubation conditions, of kidney activity. In the case of incubations at ambient temperatures, the increase seems to be related to an elevation, with increasing acclimation temperature, in gill activity.

(c) $(HCO_3^-)$-stimulated ATPase

The ratio is relatively constant, with respect to acclimation temperature, at the 25°C incubations. In contrast to this, under in vivo conditions a steady increase, predominantly due to increased gill activity at higher temperatures, is noted.

(d) Carbonic Anhydrase

The decrease in the ratio, with respect to acclimation temperature, noted here, in data from both incubation temperature regimes, is the result of increasing kidney activity coupled with a drop in gill activity at 18°C.

4. $(Na^+-K^+)$-ATPase/Carbonic Anhydrase Ratio in Gill and Kidney

Although it is appreciated that the problems inherent in comparison of in vitro estimates of different enzymes assayed by different assay procedures are many, it is, nevertheless, of interest to examine, in the gill and kidney, variations, with acclimation temperature, in the activity ratio of $(Na^+-K^+)$-ATPase to carbonic anhydrase. In doing this the mean activities of $(Na^+-K^+)$-ATPase and carbonic anhydrase, in the gill and kidney, were first converted to a common expression of specific activity - $\mu M.g\ protein^{-1}.15\ min^{-1}$. The ratios were subsequently calculated and appear in

Table 3. With the in vivo incubation temperature regime, the gill shows a dramatic increase in this ratio, especially between 2 and 10°C., while the kidney demonstrates a modest decline. At 25°C the gill ratio appears relatively constant, while the kidney again shows a decline.

TABLE 3 Ratio of ($Na^+$-$K^+$)ATPase/Carbonic Anhydrase in Gill and Kidney

| | Ambient | | 25°C | |
|---|---|---|---|---|
| Acclimation Temperature | Gill | Kidney | Gill | Kidney |
| 2° | 0.2 | 65.3 | 6.5 | 306.8 |
| 10° | 1.7 | 53.3 | 5.3 | 119.2 |
| 18° | 3.7 | 27.7 | 6.4 | 58.9 |

5. Correlation Analysis

The results of the correlation analysis referred to earlier appear in Table 4. Since a large number of tests (about 150) were performed, it was recognized that, at the 0.05 level, at least 8 spurious significant findings might occur by chance alone. Furthermore, with the relatively small sample size employed (N=14) significant correlations might well be missed. Therefore, in addition to significant correlations at the 0.05 level, which were noted as such, correlations having significance between 0.1 and 0.05 were also included. These values, in conjunction with their sign, positive or negative, which is indicative of a direct or inverse relationship, respectively, were considered as suggestive. In the table any correlations common to the two groups were placed opposite each other.

It is of interest that, of the four correlations common

TABLE 4 (a): Weight versus Ion Levels and Weight versus Enzyme Activities: Results of Correlation Analysis

| | ATPase Group | | Carbonic Anhydrase Group | |
|---|---|---|---|---|
| 2°C | P.$Na^+$ | ⊕106.3+0.15 x* | $Na^+$ | 144.4 + 0.1 x |
| | G.($Mg^{++}$ Na-K)-ATPase | 3.15 - 0.0004 x | | |
| | K.($Mg^{++}$ Na-K)-ATPase | 0.04 + 0.005 x | | |
| | K.($HCO_3^-$)-ATPase | -1.32 + 0.01 x | | |
| | K.($HCO_3^-$)(25°C)-ATPase | -0.51 + 0.01 x | | |
| | K.($Mg^{++}$ $HCO_3$)(25°C)-ATPase | 0.26 + 0.0004 x | | |
| 10°C | P.$K^+$ | 2.91 - 0.005 x | | |
| | G.($Na^+$-$K^+$)(25°C)-ATPase | 1.69 - 0.002 x | | |
| | G.($HCO_3^-$)(25°)-ATPase | 5.83 - 0.01 x | | |
| | K.($Mg^{++}$ $HCO_3$)-ATPase | 4.12 - 0.005 x | | |
| 18°C | P.$Na^+$ | 126.5 + 0.08 x* | | |
| | P.$K^+$ | 7.41 - 0.015 x* | $P.K^+$ | 6.2 - 0.01 x |
| | P.$Cl^-$ | 106.6 + 0.08 x* | | |
| | K.($HCO_3^-$)(25°C)-ATPase | 4.12 - 0.007 | | |
| | | | B.C.Aase (25°C) | -0.22+0.05x* |
| | | | G.C.Aase | 14.7 - 0.02x |

B - Blood
G - Gill
K - Kidney
⊕ - regression equation Y = A+Bx
* - $P < 0.05$
NOT MARKED $0.1 > p < 0.05$

TABLE 4 (b):Ion Levels versus Ion Levels and Ion Levels versus Selected Enzyme Activities: Results of Correlation Analysis

| | ATPase Group | | Carbonic Anhydrase Group | |
|---|---|---|---|---|
| $2^0C$ | $P.Na^+$ vs. $P.Cl^-$ | $79.0 + 0.35\ x^*$ | $P.Na^+$ vs. $P.Cl^-$ | $26.1 + 0.69\ x$ |
| | $P.Na^+$ vs. $P.K^+$ | $-3.0 + 0.03\ x$ | $P.K^+$ vs. $P.Cl^-$ | $126.3 + 2.21\ x$ |
| | | | $P.Na^+$ vs. K.C.Aase | $0.16 - 0.001x^*$ |
| $10^0C$ | $P.Na^+$ vs. $P.\ Cl^-$ | $11.1 - 0.06\ x$ | | |
| | $P.Cl^-$ vs. $K.(HCO_3^-)$ -ATPase | $-18.9 + 0.16\ x^*$ | | |
| $18^0C$ | $P.Na^+$ vs. $P.Cl^-$ | $30.5 + 0.6\ x^*$ | $P.Na^+$ vs. $P.Cl^-$ | $-11.3 + 0.93\ x^*$ |
| | P.K. vs. $P.Cl^-$ | $-139.0 - 2.67\ x$ | $P.Na^+$ vs. K.C.Aase | $24.8 - 0.15\ x^*$ |
| | $P.K^+$ vs. $G.(Na^+-K^+)$ -ATPase | $0.22 + 0.08\ x^*$ | | |
| | | | $P.Cl^-$ vs. K.C.Aase | $16.6 - 0.12\ x$ |

B - Blood
G - Gill
K - Kidney
⊕ - regression equation $Y = A+BX$
* - $P < 0.05$
NOT MARKED $0.1 > p < 0.05$

to both groups, only one is significant ($P < 0.05$) in both groups. However, in these cases the signs of correlation were identical and the slopes of the regression lines were generally similar. This supports the use of the above procedure in situations of this kind where suggestive trends are of interest. Several trends are discernible in the data. About twice as many correlations occur in the ATPase group of fish than in the carbonic anhydrase group. It also appears that more correlations occur at $2^{o}$ and $18^{o}C$ than at $10^{o}C$. These two are probably spurious trends resulting from the fact that relatively few correlations were detected in the data. However, the latter trend, where the least number of correlations occured at $10^{o}C$, may result from the extremes of the temperature tolerance range, especially $18^{o}C$, producing more stress on the animal.

Correlations between weight and ion concentrations, when present, show the following relationships, without exception. Sodium and chloride vary directly with weight, while potassium varies inversely. Four of the seven correlations were significant at the 0.05 level. On the other hand, of the eleven correlations occuring between weight and enzyme levels, only one, blood carbonic anhydrase, was significant at the 0.05 level. These trends often demonstrated very minor slopes of regression, a relationship with an enzyme whose function is unknown ($(Mg^{++})$-ATPase), and/or correlation with enzyme activities at $25^{o}C$. It is apparent that little conclusive information is available from this latter analysis.

The most useful information comes from the examination of the interrelationships between electrolyte concentrations. Chloride correlated with sodium significantly ($P < 0.05$),three of the five times a relationship was observed. The only anomoly was that, while the relationship was positive in both groups at $2^{o}$ and $18^{o}C$, the one occurrance noted at $10^{o}C$ was negative. Plasma sodium to potassium and plasma potassium to chloride correlations appeared but these trends were neither consistant nor significant. Although only a few correlations between plasma ion concentration and enzyme activities were present, those observed were interesting. At $2^{o}$ and $18^{o}C$ plasma sodium demonstrated a significant negative relationship with kidney carbonic anhydrase, while at $18^{o}C$ plasma chloride also showed a negative relationship with this enzyme. In addition, plasma chloride and kidney ($HCO_3^-$)-ATPase showed a significant positive relationship and plasma potassium demonstrated a significant positive relationship with gill ($Na^+$-$K^+$)-ATPase.

6. Summary of Results

1. Plasma sodium and chloride levels are stable with respect to acclimation temperature, in contrast to plasma potassium which rises with higher acclimation temperatures.
2. For the most part, assays done at a common temperature yielded substantially different activities and apparent trends, with respect to acclimation temperature, than assays done at <u>in vivo</u> temperatures. In the latter case all of the enzymes examined, with the exception of gill carbonic anhydrase, showed elevation of activity

as acclimation temperature increased, although the amount and degree varied.

3. When the results of the assays conducted at in vivo temperatures are considered, all of the enzymes, with the exception of carbonic anhydrase, demonstrated an increase in the gill/kidney activity ratio as acclimation temperature increased.
4. The ($Na^+$-$K^+$)-ATPase/carbonic anhydrase ratio, when the results of the assays conducted at ambient temperatures were considered, increased dramatically in the gill and decreased in the kidney, with respect to elevated acclimation temperature.
5. Although the results of the correlation analysis were far from conclusive, some interesting trends appeared. They are as follows (a) The least number of correlations occured at 10°C. (b) It appears that plasma sodium and chloride vary directly and potassium inversely, with weight. (c) Plasma sodium and chloride showed a consistant, significant relationship, especially at 2° and 18°C. (d) Correlations between plasma sodium and chloride and carbonic anhydrase, plasma chloride and $HCO_3^-$)-ATPase and plasma potassium and ($Na^+$-$K^+$)-ATPase, appeared.

# V. DISCUSSION

Discussion of the results obtained in this study has been divided into three broad areas to facilitate interpretation. The first, Procedural Techniques, involves examination of the effects of the incubation temperature regimes employed, an interesting observation which has derived from the procedure used, the effect of assay medium ionic strength upon ($Mg^{++}$)-ATPase activity, and some comments regarding the carbonic anhydrase assay protocol. The subsequent section on Ion Regulation is concerned with plasma electrolyte levels, and the results of the correlation analysis. Enzymes and Ion Transport, the third area, considers the relative abundance, importance, and distribution of the enzymes studied, in relation to generally-accepted models of ionic regulation in freshwater fishes. Some modifications, consistant with the results of this study, are suggested. As well, a model relating chloride cell structure, enzyme distribution and sodium uptake, is presented.

## 1. Procedural Techniques

### (a) Effect of Incubation Temperature Scheme

The high common and ambient temperature incubation procedures produced significantly different estimates of activity, in relation to increasing acclimation temperature. In the case of ($Na^{+}$-$K^{+}$)- and ($Mg^{++}$)-ATPases the activities actually indicate opposite trends with respect to acclimation temperature. ($HCO_3^{-}$)-ATPase and carbonic anhydrase, on the other hand, showed similar trends, although differences in absolute activity were present. Recent studies upon membrane lipids may have some bearing upon this.

It is generally conceded that most poikilotherms, including fish, change the fluidity of their membranes by manipulating the degree of unsaturation of component phospholipids - greater unsaturation as temperature decreases - in response to environmental temperature (see Hazel, 1973, for a review). Since membrane lipid composition and conformation have a definite and well-defined influence upon the activities of membrane-associated enzymes (Grisham and Barnett, 1973; Hazel, 1973), such changes may account, in part, for the activity observed. For example, incubation of, preparations from 2°C-acclimated fish at 25°C may generate spuriously high activity in membrane-associated enzymes due to the creation of an abnormally fluid membrane-lipid environment. Incubation at 2°C produces fluidity similar to that in vivo and presumably, more representative enzyme activity.

It has been shown that many metabolic functions are stabilized, over wide temperature ranges by the adaptive effects of isozyme systems and/or temperature-dependent modulation of existing enzymes (Hochachka, 1973; Hazel and Prosser, 1974). These, in conjunction with changes in membrane-lipid fluidity, may contribute to acclimation. This aspect of the study has clearly indicated that incubation temperature conditions for enzymes of poikilothermic origin must be carefully considered in the light of possible adaptive responses. Failure to take such possibilities into account may well have lead to misinterpretation of findings in past studies. For example, salmonid gill ATPase activities have been reported to vary with season, salinity, photoperiod, and, most notably, during the parr-smolt transformation which precedes movement

of juvenile migratory salmon into seawater (Zaugg and McLean, 1970; Zaugg and Wagner, 1973). Increases in rearing temperatures are known to reduce seawater tolerance. Several studies upon transport enzyme activities, in animals reared at different temperatures, have lead to inverse activity-acclimation temperature relationship. Zaugg and his co-workers have therefore concluded that loss of seawater tolerance is related to decreased enzyme activity (Zaugg *et al.*, 1972; Adams *et al.*, 1975; Zaugg and McLean, 1976). $(Na^+-K^+)$-ATPase has been reported to be highly sensitive to heavy metals and pesticides (Davis and Wedemeyer, 1971; Kuhnert and Kuhnert, 1976). In all of these cases however, high common temperature assays were employed. The conclusions of these studies, can be seriously questioned on the basis of the information obtained in this study for it seems highly unlikely that the results obtained are physiologically-relevant.

It is evident that the incubation temperature protocol employed in this study has some definite advantages. The high common temperature employed ($25^{\circ}C$) corresponds to the upper incipient lethal level for this species and provides an indication of enzyme activity at a realistic maximum temperature. Furthermore, activities obtained in this way can presumably be compared with earlier work. The ambient temperature incubation procedure provides an indication of activity at the actual *in vivo* temperature. Thermal effects on these systems can be easily derived from the two.

(b) Heat Death

Although heat death is still a poorly understood phenomenon, it is thought to involve hydromineral regulation; at least in cases where protein denaturation is not a problem. Studies upon euryhaline fish species have, for example, demonstrated that survival under heat stress is substantially increased in solutions of salt approaching isotonicity. Presumably regulatory requirements are reduced (Garside and Jordan, 1968; Jordan and Garside, 1972). In addition, abnormal patterns of electrolyte distribution are sometimes observed at the extremes of temperature tolerance (Houston, 1973).

The thermal characteristics of renal and branchial $(Na^{+}-K^{+})$- and $(Mg^{++})$-ATPase and also of blood and gill carbonic anhydrases may provide insights regarding the basis of heat death near the incipient lethal level. Activities at $25^{o}C$, and at each ambient temperature are assumed to be functions of maximum and actual transport capacities, respectively. Linear extrapolations of $25^{o}C$- and acclimation temperature- activity relationships leads to intersections with a mean of $25^{o} \pm 2^{o}C$. This is close to the upper incipient lethal level of rainbow trout. At temperatures above this, the requirements for ion transport may well exceed absorption capacity, and the resulting net loss of electrolytes may be a significant factor contributing to the death of the organism. $(HCO_3^{-})$-ATPase and renal carbonic anhydrase do not exhibit these characteristics. In the case of $(HCO_3^{-})$-ATPase, intersection occurs at about $20^{o}C$ for both gill and kidney. Renal carbonic anhydrase activities are too low and too similar to provide a reasonable estimate of intersection temperature.

(c) Ionic Strength and ($Mg^{++}$)-ATPase Activity

($Na^+$-$K^+$)-ATPase activity is normally estimated as the difference between the activity in an assay medium containing sodium, potassium, and magnesium, and that in a sodium and potassium-free medium. Large differences in ionic strength - 120 mM- are, therefore, involved. Dr. D. Stewart of the University of Toronto informed the author that ionic strength affects ATPase activity. This fact was taken into account in the development of the assay protocol (Table I). The medium for ($Mg^{++}_{Na-K}$)-ATPase estimation included 120 mM sodium chloride, and this eliminated differences in ionic strength between it and the ($Na^+$-$K^+$)-ATPase assay medium. Lack of potassium and the use of ouabain ensured no ($Na^+$-$K^+$) ATPase activity occured. The ($Mg^{++}_{HCO_3}$)-ATPase medium is similar to that ordinarily employed in ($Na^+$-$K^+$)-ATPase estimations. Thus an examination of the effects of compensation for ionic strength was made possible. Although no significant differences in mean ($Mg^{++}$)-ATPase activity occured (Figure 7), further examination is not without benefit. In kidney preparations mean ($Mg^{++}_{HCO_3}$)-ATPase activities are higher in all six cases, while in gill preparations in one case only was ($Mg^{++}_{Na-K}$)-ATPase higher. Despite the fact that these differences in activity were small, it is apparent that the ($Mg^{++}_{HCO_3}$)-ATPase activity estimation is consistantly higher. However, the small differences observed could be a major source of error, as evidenced by the two examples that follow. In the case of gill tissue from 18°C-acclimated fish incubated at 18°C, ($Mg^{++}_{HCO_3}$)-ATPase is 0.33 $\mu$M.15 $min^{-1}.mg^{-1}$ higher than the ($Mg^{++}_{Na-K}$)-ATPase value. Substituting the former for the ($Mg^{++}_{Na-K}$)-ATPase

estimation, thus simulating the activity estimate that would be obtained in a procedure where the assay medium is not compensated for ionic strength, produces an ($Na^+$-$K^+$)-ATPase activity estimate that is only 28% of the value obtained with the ionic strength-compensated protocol. Similarly, in the kidney, a decrease to 80% is noted. It appears that ($Mg^{++}$)-ATPase is stimulated by decreased ionic strength. As this can have a significant effect on the outcome of the assay, it is certainly deserving of further consideration.

(d) Comments Upon Carbonic Anhydrase Assays

Haswell (1977) has recently questioned the validity of the type of assay used in this study. His objection is based on the fact that the p-nitrophenol acetate procedure, as employed by Mashiter and Morgan (1975), did not consistantly demonstrate carbonic anhydrase activity in flounder erythrocytes, while two subsequent publications, employing carbon dioxide as the substrate, reported significant levels of activity (Carter et al., 1976; Haswell, 1977). It was implied that the p-nitrophenol acetate procedure was insufficiently sensitive. In the present study, substantial blood carbonic anhydrase activity was detected, and further studies (Smeda, unpublished observations) indicate that this is largely associated with the erythrocyte membrane. It appears that no blood carbonic anhydrase activity was detected by Mashiter and Morgan (1975) because cytosol rather than the membrane fraction of the blood preparation was assayed. Lack of sensitivity need not be involked as an explanation for their results. In fact, conversion of the trout blood carbonic anhydrase activities reported by Haswell (1977), on the basis of a manometric

technique, and data from this study to common activity units, gives values of the same order of magnitude. The fact that carbonic anhydrase has been reported to be $10^{-5}$ times less active with p-nitrophenol acetate as the substrate (Armstrong et al., 1966), suggests that Haswell's assay procedure was itself insensitive.

2. Ion Regulation

(a) Plasma Electrolyte Levels

It is clear that the rainbow trout is capable of maintaining sodium and chloride levels virtually constant over a wide temperature range. This is in accordance with previously reported results (Houston et al., 1968; Murphy and Houston, 1977). The ratio of the two is constant despite some fluctuation in plasma chloride levels as noted earlier. These points provide additional support for the contention that some interrelationship exists between the apparently separate control mechanisms of these two ions.

Plasma potassium, however, is not as tightly regulated, showing a significant increast in concentration at 18°C. This response to increased acclimation temperature has been observed elsewhere (Houston et al., 1968; Murphy and Houston, 1977), although, as in those cases, the significance of this response is unknown.

(b) Correlation Analysis

That correlations between weight and certain parameters might exist was suggested by the observation that gill area per unit body weight decreases with increased weight in individuals of the same species (Randall, 1970; Hughes and Morgan, 1973). This variation is the primary cause of reductions in water

flux with increasing weight (Evans, 1969). Since a similar response with respect to weight and branchial ion fluxes would not be unexpected, an examination, for possible relationships between weight and ion levels, and weight and transport enzyme activities, was conducted. Even though the weight range was relatively narrow, and no significant differences in mean weight was observed, some trends were apparent.

Plasma sodium was directly related to specimen weight, while plasma potassium varied inversely. This is in agreement with a previous study (Murphy, unpublished observations) unlike the direct correlation between weight and plasma chloride, which is not. Although the functional significance of such relationships is not known, it is interesting to note that certain photoperiod-temperature schemes significantly reduce such correlations (Murphy, unpublished observations). Although some correlations between weight and enzyme activity occur, the character of these relations, as previously mentioned, precludes any rigorous interpretation.

Plasma sodium and chloride levels are ordinarily well correlated (Maetz, 1974). Accordingly, this and other possible plasma ion relations were examined. Possible relationships between the plasma level of the various ions, and the activities of the enzymes associated with their transport were also tested. The correlation between plasma sodium and chloride was the most notable, with three of the five occurances being significant ($P < 0.05$). Again, support for the hypothesized linkage between sodium and chloride regulatory mechanisms is indicated. Although significant correlations between ion and enzyme levels were not common, those between plasma sodium and

chloride and kidney carbonic anhydrase, plasma potassium and gill ($Na^+$-$K^+$)-ATPase, and, plasma chloride and kidney ($HCO_3^-$)-ATPase could be considered as further support for the involvement those enzymes in the regulation of the respective ions.

It is apparent that the results of the correlation analysis are far from conclusive, yet some interesting trends are suggested. Further study employing a wider temperature and weight range might generate much useful information.

3. Enzymes and Ion Transport

(a) Gill

Sodium uptake at the gill seems to be a biphasic process. It appears that a coupled exchange of sodium for ammonium and/or hydronium occurs at the mucosal border of the chloride cell (Maetz, 1971). The character of this heteroionic exchange is largely unknown. It is apparently obligatory in nature as injections of ammonium and hydronium ions stimulate sodium uptake while increased ammonium in the external medium inhibits uptake (Cameron, 1976; Kerstetter and Keeler, 1976), suggesting that the process may be carrier-mediated. Involvement of carbonic anhydrase in the exchange mechanism is suggested by the fact that acetazolamide reduces sodium influx (Kerstetter *et al.*, 1970; Payan *et al.*, 1975; Kerstetter and Keeler, 1976).

The next step, movement of sodium into the plasma, is thought to result from an ($Na^+$-$K^+$)-ATPase - related transfer (Maetz, 1971). However, the low activity of the gill enzyme at reduced temperatures, which has been observed in the present

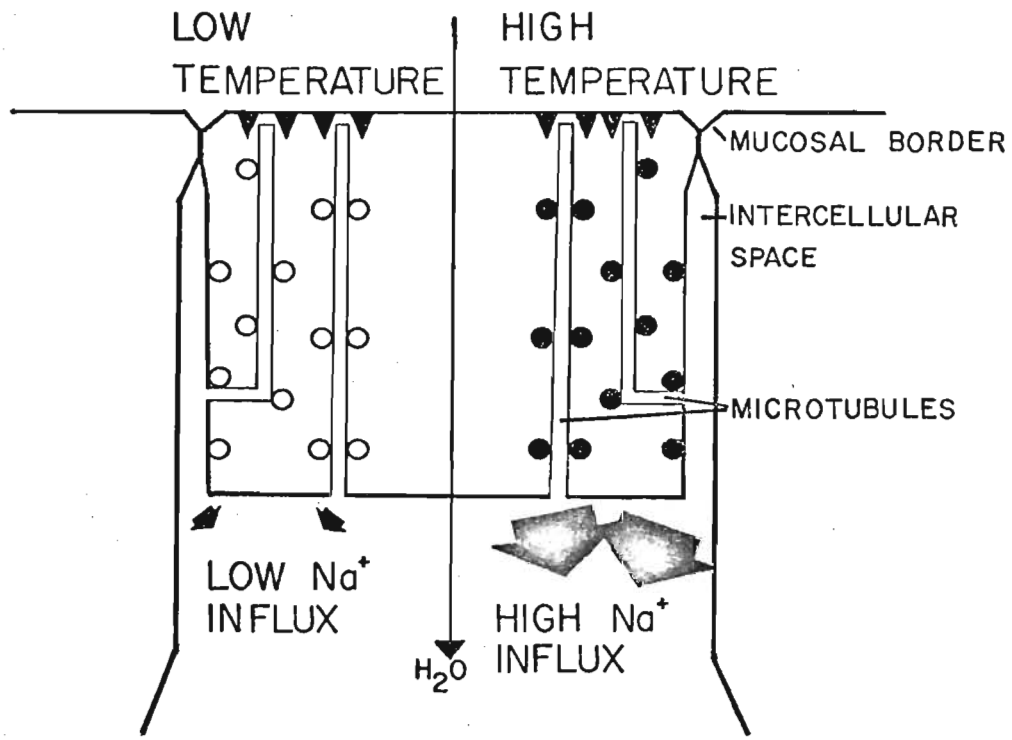
study and by Pfeiler and Kirschner (1972) and the very large thermal coefficient obtained between 2°C and 10°C (27.3), indicates that ($Na^+$-$K^+$)-ATPase may be of little functional importance in the cold-adapted trout. Sodium depletion, however, still takes place, at reduced rates, in the cold. This, plus the limited inhibition of sodium uptake at the gill by ouabain (Payan *et al.*, 1975; Kerstetter and Keeler, 1976), suggests that an alternate mechanism for sodium uptake may exist. Two possible explanations, which are not mutually exclusive, could account for the low activity of ($Na^+$-$K^+$)-ATPase in the cold and its impact on sodium transport. Low activity may reflect low sodium demand, as net ionic losses are significantly reduced in the cold. The limited activity present may, in fact, provide all the transport needed. The sensitivity of the *in vitro* assay may also be a complicating factor here, as the measured activity of the isolated enzyme may represent only a portion of the true *in vivo* capacity. However, it may also be that heteroionic exchange mechanisms provide for minimal or baseline recruitment, while the progressive involvement of ($Na^+$-$K^+$)-ATPase, as suggested by its substantial thermal activation, compensates for the increases in net sodium loss induced by increased acclimation temperature. The role of ($Na^+$-$K^+$)-ATPase, in this case, would be that of a high temperature amplifier of the sodium uptake system. Changes in the gill ($Na^+$-$K^+$)-ATPase/carbonic anhydrase ratio, with temperature, provides support for the latter hypothesis. A further examination of this, in the light of other data, although largely speculative, is interesting. Solute transport is often considered to be a

function, not only of the enzymes present, but of the geometrical relationship of the subcellular components of the cells involved (Diamond and Bossert, 1968; Diamond, 1971). The chloride cell of freshwater trout contains an amplified baso-lateral microtubular system and lateral intercellular spaces (Maetz, 1971; Morgan and Tovell, 1973; Olson and Fromm, 1973). $(Na^+-K^+)$-ATPase appears primarily along the membrane surface of these spaces (Karnaky et al., 1976). A possible explanation of known transport rate changes and $(Na^+-K^+)$ activity changes, with temperature, could be provided by the forward-directed standing-gradient flow hypothesis of Diamond (1971). Heterionic exchange fueled by carbonic anhydrase, occurs at the mucosal border of the cell and may provide , at low temperatures, sodium uptake via the microtubular system and/or the lateral intercellular spaces. Simulation studies by Diamond (1971) indicate that the transport capacity of a mechanism of this type would be severely limited by the length of the channels and the distal location of the transport sites (Figure 13). However, as $(Na^+-K^+)$-ATPase, located along the length of the channels is thermally activated, the capacity of the system increases enormously, and could have the capability of satisfying the increased demand for ion recruitment. In addition, it is conceivable that chloride uptake may be facilitated in a similar manner. Obviously, further study of this model is warranted.

In other cases where sodium transport has been demonstrated, notably amphibian skin, the transepithelial electrical potential (T.E.P.) is such that the body fluids are positive with respect to the exterior (Armstrong, 1971). Only in fish

Fig. 13 A conceptual model illustrating how chloride cell structure, ($Na^+$-$K^+$)-ATPase distribution, and changes in the activity of this enzyme could be used to explain the known changes in $Na^+$ uptake (indicated by arrows) at low (left) and high (right) temperatures.

FIGURE 13 A PROPOSED MODEL RELATING CHLORIDE CELL STRUCTURE AND ENZYME ACTIVITIES



is the T.E.P. negative (Motais and Garcia-Romeu, 1972). It appears that the trout may be an exception, although this is not certain, as changes in the measuring technique can generate in a negative T.E.P. value (Kerstetter and Kirschner, 1974). Needless to say, this descrepancy must be eliminated before detailed models of transport mechanisms can be attempted.

While chloride uptake at the gill is known to involve a bicarbonate/chloride exchange, other aspects of the uptake and the regulatory mechanism governing uptake, are poorly understood (Maetz, 1971; Motais and Garcia-Romeu, 1972). The model suggested by Maetz (1971) locates bicarbonate/chloride exchange at the mucosal border with a linkage to a chloride "pump" at the serosal border; in short, a design analogous to that postulated for sodium transport. Unfortunately, the presently available evidence is insufficient to firmly establish the presence of such a chloride "pump". ($HCO_3^-$)-ATPase has been found in the gill (Kerstetter and Kirschner, 1974), where it has shown some relation to chloride transport (Morisawa and Utida, 1976). That it may be the chloride "pump" is suggested by the fact that it is inhibited, like chloride uptake, by thiocyanate. Unfortunately, chloride uptake and ($HCO_3^-$)-ATPase activity show substantial differences in the character of this inhibition (deRenzis, 1975; Solomon *et al.*, 1975). In addition, *in vivo* inhibition of chloride transport is largely confined to the mucosal border and this is inconsistent with the postulates of Maetz (1971). Furthermore, ($HCO_3^-$)-ATPase activity is not dependent on chloride; something that might be expected if this enzyme was actually involved in chloride transport. However, this objection would have less

force if both bicarbonate/chloride exchange and ($HCO_3^-$)-ATPase activity were independently inhibited by thiocyanate. Unfortunately, data on this point is not available.

Carbonic anhydrase has been shown to be involved with chloride uptake, as inhibition of this system causes decreases in chloride influx (Maetz, 1971). Although Kerstetter and Kirschner (1972) did not find chloride uptake inhibition by acetazolamide in the trout, contrary to findings with goldfish, they provide a possible explanation. Their proposal is that chloride uptake is achieved by a bicarbonate/chloride exchange which is driven by transepithelial diffusion of bicarbonate generated in the blood. Support for this hypothesis is provided in the present study by the occurance of substantially increased blood carbonic anhydrase levels at higher acclimation temperatures. In addition, it would not be unreasonable to expect that, if bicarbonate/chloride exchange produces chloride uptake, the substantial demands created by elevated temperatures would be linked with some reduction in plasma bicarbonate levels, and possibly increased hydronium ($H^+$) concentrations and hence lowered pH. This, in fact, has been observed (Randall and Cameron, 1973).

(b) Kidney

Absorption from the urine is apparently a substantially more complex process than that occuring at the gill. Although intracellular organization is certainly prominent, the structure and conformation of the basic functional subunit of the kidney, the nephron, is of prime importance. This, in conjunction with areas of specialized transport and differential permeability in the nephron confer the ability to eliminate water and recover electrolytes (Hickman and Trump, 1969; Selkurt, 1971;

Stephenson et al., 1976). Since a detailed model of the fish kidney is not available, discussion will procede under the assumption that the gross changes in enzyme activities observed reflect the roles and relative importance of the enzymes measured.

Kidney ($Na^+$-$K^+$)-ATPase does not exhibit the cold sensitivity characteristic of the gill enzyme, although a significant decline is apparent. This is probably a reflection of the fact that renal recovery at low temperatures is substantial. Carbonic anhydrase production of the primary kidney counterion, hydronium, is relatively unimpeded by reduced temperature. The decline in kidney ($Na^+$-$K^+$)-ATPase/carbonic anhydrase ratios, which results from carbonic anhydrase activity increasing faster than ($Na^+$-$K^+$)-ATPase activity, is minor, but perhaps indicative of increasing importance of carbonic anhydrase, with elevated temperature, in electrolyte recovery.

If chloride is not independently transported, and therefore co-transported with sodium, as is usually postulated in other vertebrate kidneys (Selkurt, 1971), the changes in ($Na^+$-$K^+$)-ATPase and possibly carbonic anhydrase, with respect to sodium, should reflect chloride transport changes. If, however, chloride is actively transported, as it now appears that it may be (Selkurt, 1971; Morisawa and Utida, 1976), ($HCO_3^-$)-ATPase may be involved. The thermal response of this system observed in the present study would not be inappropriate under the latter circumstances.

It is obvious that, as pointed out by Cameron (1976), a more complete picture of renal activity, with regard to ion regulation and acid-base balance, is required before the

exact involvement of the kidney in these functions can be confirmed.

(c) Relative Importance of the Gill and Kidney

The data obtained in this study, especially that on gill/kidney enzyme activity ratios, support the contention that at low temperatures renal recovery is of major importance, with only minor gill involvement being required. However, despite temperature-related increases in kidney enzyme activity, and presumably increases in ion recovery rates, the large increases in urine flow still produce increased net ion losses. This, and a rise in gill ion loss, requires compensation. The response appears to be largely a gill function as evidenced by substantially increased branchial enzyme activity. Therefore, at high temperatures the gill becomes the prime site for electrolyte recruitment and regulation.

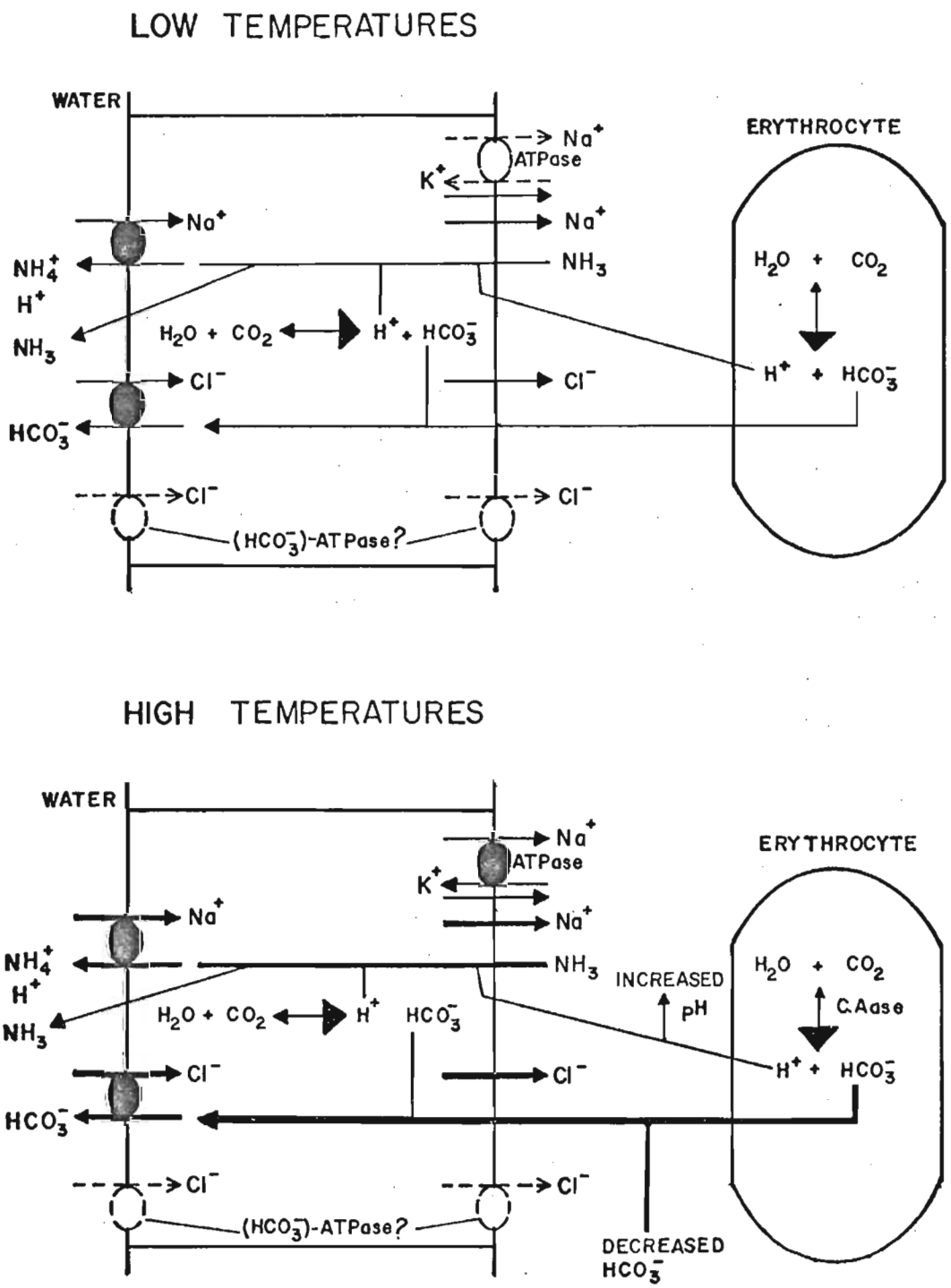
(d) A Model of Ion Exchange

As extensive work has not been performed on the fish kidney, and certain differences in structure and function, compared to higher animals, are present (Hickman and Trump, 1969; Cameron, 1976) a kidney model will not be presented. Although information in this study provides an indication of the relative involvement and function of the enzymes measured, any improvements on the model of Hickman and Trump (1969) - (Figure 2) must await more detailed structural and functional analysis.

However, at the gill some improvements can be made. These are basically changes in emphasis in the original model of Maetz (1971) presented in Figure 4. The model is outlined in Figure 14.

Fig. 14 A conceptual model of chloride cell function at high (lower illustration) and low (upper illustration) temperatures. The location of the various processes is indicated on the figure. Substantial involvement is indicated by solid lines and circles whereas limited involvement or uncertainty of involvement is indicated by broken lines and open circles.

# FIGURE 14 A REVISED MODEL OF CHLORIDE CELL FUNCTION



The model for ion transport at low temperatures takes into account the apparent importance of gill and blood carbonic anhydrase, and of heterionic exchange mechanisms, in ion uptake. Although ($Na^+$-$K^+$)-ATPase activity occurs, its' very low activity and presumably minor involvement in ion transport is alluded to by its' presence in broken lines. A chloride pump, which may be ($HCO_3^-$)-ATPase, is not entirely discounted, although, due to the ambiguity concerning its location and the uncertainty of its involvement, it is presented in broken lines.

The high temperature model is similar to the original Maetz model, but places additional emphasis upon the role of blood carbonic anhydrase. ($Na^+$-$K^+$)-ATPase is now significantly involved, as suggested by its' presence in solid lines. The problems with respect to ($HCO_3^-$)-ATPase remain as above.

In short, in its' simplest form, sodium and chloride uptake at the gill is provided, at low temperatures, by carbonic anhydrase working in association with the heteroionic exchange mechanisms. Gill ($Na^+$-$K^+$)-ATPase and erythrocytic carbonic anhydrase provide the supplemental uptake required at higher temperatures. The intimate interrelationship postulated between plasma sodium and chloride has been observed in several aspects of this study. Although the reason for this is not yet clear, a component common to the two regulatory systems could be involved. Since both sodium and chloride recruitment depend on the products of carbonic anhydrase catalysis, it may be involved in this process.

Finally, the importance of these regulatory systems is not limited to ion regulation, as Cameron (1976) notes that the

sodium/ammonium-hydronium and bicarbonate/chloride exchange systems seem to function to compensate for induced acid-base balance disturbances. Although Kerstetter and Mize (1976) report contrary data, the technique of Cameron appears to be more rigorous. As regulation of the acid-base balance, whether by control of pH, or more probably relative alkalinity - $H^+/OH^-$ ratio, of the body fluids, is of major importance in acclimatory responses, linking ionoregulatory processes with it would further increase the significance of the ion transport systems and regulatory mechanisms (Albers, 1970; Houston, 1971; Rahn and Baumgartner, 1972; Randall and Cameron, 1973).

# VI CONCLUSIONS

1. Over the wide range of acclimation temperatures used in this study, the relatively stenothermal rainbow trout was able to maintain plasma sodium and chloride concentrations essentially constant. Plasma potassium, however, is not so tightly controlled and its concentration increased significantly at higher temperatures, Several aspects of the study, including the constant plasma sodium/chloride ratio and the significant correlation between plasma sodium and chloride concentrations, provided support for the theory that, by some means, sodium and chloride regulatory mechanisms are linked. Carbonic anhydrase, as the products of its catalysis are involved in the uptake of both ions, could be involved in this process.
2. Incubation at <u>in vivo</u> temperatures produced different levels of activity and, in some cases, contrary trends with respect to acclimation temperature when compared to estimates of enzyme activity obtained with incubation at a high common temperature. It is apparent that, especially in studies where thermal influence is suspected, the acclimation temperature of poikilothermic animals must be included in the assay protocol if physiologically-realistic data are to be obtained. The data from this aspect of the study also suggest that limitations in ion transport capacity may be involved in heat death.

3. The presence, in the gill and kidney, of three enzymes implicated in ion transport, ($Na^+$-$K^+$)-ATPase, ($HCO_3^-$)-ATPase, and carbonic anhydrase, was confirmed. In addition, substantial carbonic anhydrase activity in the blood was detected. The changes, with acclimation temperature, in renal ($Na^+$-$K^+$)-ATPase, carbonic anhydrase, and ($HCO_3^-$)-ATPase that were observed are not inconsistant with involvement in kidney function. However, due to the lack of detailed information concerning the piscine kidney, a rigorous analysis of these parameters was precluded.

   Information about the gill was sufficient to enable a model, of ion uptake here to be outlined. It appears that ion transport at low temperatures involves sodium and chloride uptake by heteroionic exchange mechanisms operating in conjunction with carbonic anhydrase. Gill ($Na^+$-$K^+$)-ATPase and erythrocyte carbonic anhydrase seem to provide the additional salt uptake required at higher temperatures. In addition, the standing-gradient flow hypothesis may be used to link chloride cell structure and thermally-induced changes in enzyme activity to observed variations in sodium influx.

4. A determination of the relative importance of the gill and kidney in electrolyte regulation at various temperatures was made possible by the simultaneous measurement, in these organs, of the activities of the enzymes implicated in ion transport. Recovery of ions at the kidney appears to be significant at low temperatures. As temperature increases, gill recruitment becomes increasingly more

important, despite a small rise in kidney uptake.

5. Although weight, plasma ion levels, and enzyme activities demonstrated some relationships in the linear regression analysis, the results of this aspect of the study were not conclusive.

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# APPENDIX

APPENDIX: TABLE I RAW DATA ATPases 2°C Units $\mu$MPi . 15 $min^{-1}$ . mg protein $^{-1}$

| No. | Length (cm) | Weight (g) | Plasma $Na^+$ $mEq.l^{-1}$ | Plasma $K^+$ $mEq.l^{-1}$ | Plasma $Cl^-$ $mEq.l^{-1}$ | GILL ($Na^+$-$K^+$) 2° | GILL ($Na^+$-$K^+$) 25° | KIDNEY ($Na^+$-$K^+$) 2° | KIDNEY ($Na^+$-$K^+$) 25° | GILL ($Mg^{++}$) 2° | GILL ($Mg^{++}$) 25° | KIDNEY ($Mg^{++}$) 2° | KIDNEY ($Mg^{++}$) 25° | GILL ($HCO_3^-$) 2° | GILL ($HCO_3^-$) 25° | KIDNEY ($HCO_3^-$) 2° | KIDNEY ($HCO_3^-$) 25° | GILL ($Mg^{++}$) 2° | GILL ($Mg^{++}$) 25° | KIDNEY ($Mg^{++}$) 2° | KIDNEY ($Mg^{++}$) 25° |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 30.5 | 318.5 | 148.0 | 1.74 | 129.5 | 0.01 | 0.91 | 0.78 | 2.41 | 2.45 | 6.36 | 1.77 | 3.70 | 3.00 | 3.63 | 2.26 | 2.59 | 2.45 | 6.64 | 2.04 | 4.33 |
| 2 | 29.0 | 284.5 | 152.9 | 1.66 | 133.8 | 0.06 | 0.89 | 0.32 | 4.18 | 1.37 | 5.06 | 0.91 | 2.27 | 2.26 | 2.86 | 1.81 | 3.09 | 1.49 | 5.06 | 1.05 | 2.59 |
| 3 | 30.0 | 321.0 | 153.7 | 1.05 | 126.3 | 0.01 | 1.38 | 0.21 | 2.01 | 1.88 | 6.12 | 1.25 | 2.78 | 2.04 | 2.39 | 2.30 | 2.37 | 2.04 | 6.82 | 1.30 | 3.36 |
| 4 | 29.5 | 308.5 | 151.2 | 1.18 | 131.7 | 0.01 | 0.72 | 0.45 | 3.64 | 1.79 | 5.71 | 1.23 | 3.50 | 2.26 | 3.39 | 2.13 | 2.19 | 1.79 | 6.13 | 1.32 | 3.95 |
| 5 | 28.5 | 288.5 | 146.7 | 0.61 | 134.8 | 0.06 | 1.63 | 0.60 | 4.21 | 1.45 | 4.82 | 1.81 | 2.71 | 1.86 | 3.61 | 2.71 | 3.74 | 1.45 | 4.82 | 1.51 | 2.71 |
| 6 | 29.0 | 313.0 | 148.3 | 0.78 | 130.1 | 0.01 | 1.10 | 0.50 | 2.06 | 1.56 | 5.39 | 1.67 | 3.50 | 1.36 | 3.58 | 2.50 | 2.89 | 1.56 | 5.19 | 1.67 | 3.50 |
| 7 | 30.0 | 327.5 | 162.1 | 2.31 | 134.7 | 0.05 | 0.80 | 0.23 | 3.01 | 1.72 | 7.26 | 1.48 | 2.87 | 1.40 | 2.31 | 1.17 | 1.85 | 1.77 | 6.72 | 1.53 | 3.47 |
| 8 | 28.0 | 257.0 | 163.1 | 1.90 | 140.3 | 0.01 | 1.21 | 0.20 | 2.60 | 1.93 | 6.14 | 1.53 | 2.86 | 1.26 | 2.83 | 0.62 | 1.94 | 1.99 | 6.33 | 1.73 | 3.52 |
| 9 | 30.0 | 328.0 | 164.6 | 2.85 | 131.1 | 0.03 | 1.07 | 0.47 | 2.34 | 1.55 | 6.31 | 1.40 | 3.08 | 1.26 | 2.33 | 0.70 | 2.34 | 1.80 | 6.31 | 1.40 | 3.22 |
| 10 | 29.0 | 301.5 | 154.3 | 1.70 | 136.4 | 0.01 | 1.24 | 0.47 | 2.10 | 2.14 | 6.05 | 1.81 | 3.15 | 1.34 | 2.86 | 1.12 | 1.89 | 2.14 | 6.19 | 1.74 | 3.62 |
| 11 | 28.0 | 250.5 | 132.4 | 1.99 | 126.6 | 0.01 | 0.75 | 0.38 | 2.06 | 2.38 | 7.69 | 0.75 | 2.19 | 0.94 | 1.74 | 0.75 | 1.19 | 2.19 | 7.56 | 1.13 | 2.69 |
| 12 | 28.5 | 311.5 | 146.4 | 1.87 | 135.7 | 0.09 | 1.28 | 0.20 | 3.12 | 1.92 | 6.86 | 1.49 | 3.61 | 1.10 | 2.90 | 0.87 | 2.31 | 1.92 | 6.98 | 1.68 | 4.81 |
| 13 | 27.5 | 249.0 | 155.5 | 0.94 | 138.7 | 0.01 | 1.09 | 0.24 | 2.47 | 2.08 | 5.58 | 1.43 | 2.67 | 0.84 | 2.50 | 0.58 | 1.14 | 2.08 | 5.42 | 1.52 | 3.24 |
| 14 | 26.0 | 196.0 | 129.8 | 0.71 | 120.7 | 0.01 | 0.90 | 0.44 | 2.45 | 2.41 | 7.71 | 0.88 | 2.65 | 1.32 | 2.47 | 0.74 | 1.42 | 2.41 | 7.29 | 0.88 | 2.99 |
| *$\bar{X}$ | 28.8 | 289.5 | 150.6 | 1.52 | 132.2 | 0.03 | 1.00 | 0.39 | 2.76 | 1.90 | 6.22 | 1.39 | 2.97 | 1.59 | 2.81 | 1.45 | 2.21 | 1.93 | 6.25 | 1.46 | 3.43 |
| ** High $M_1$ | 29.5 | 312.0 | 156.5 | 1.70 | 133.9 | 0.04 | 1.20 | 0.49 | 3.20 | 2.11 | 6.74 | 1.59 | 3.24 | 1.94 | 3.14 | 1.89 | 2.63 | 2.12 | 6.74 | 1.64 | 3.79 |
| ** Low $M_2$ | 28.1 | 267.0 | 144.8 | 1.34 | 130.4 | 0.01 | 0.78 | 0.29 | 2.32 | 1.70 | 5.70 | 1.19 | 2.69 | 1.24 | 2.48 | 1.01 | 1.79 | 1.75 | 5.76 | 1.29 | 3.07 |

* MEAN ** 95% Confidence Interval

APPENDIX: TABLE II RAW DATA ATPases 10°C  Units $\mu$MPi . 15 $min^{-1}$ . mg $protein^{-1}$

| No. | Length (cm) | Weight (g) | Plasma $Na^+$ mEq.$l^{-1}$ | Plasma $K^+$ mEq.$l^{-1}$ | Plasma $Cl^-$ mEq.$l^{-1}$ | GILL ($Na^+$-$K^+$) 10° | GILL ($Na^+$-$K^+$) 25° | KIDNEY ($Na^+$-$K^+$) 10° | KIDNEY ($Na^+$-$K^+$) 25° | GILL ($Mg^{++}$) 10° | GILL ($Mg^{++}$) 25° | KIDNEY ($Mg^{++}$) 10° | KIDNEY ($Mg^{++}$) 25° | GILL ($HCO_3^-$) 10° | GILL ($HCO_3^-$) 25° | KIDNEY ($HCO_3^-$) 10° | KIDNEY ($HCO_3^-$) 25° | GILL ($Mg^{++}$) 10° | GILL ($Mg^{++}$) 25° | KIDNEY ($Mg^{++}$) 10° | KIDNEY ($Mg^{++}$) 25° |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 29.0 | 318.0 | 161.0 | 1.17 | 131.5 | 0.30 | 0.78 | 1.04 | 3.82 | 2.94 | 6.81 | 2.43 | 3.61 | 2.45 | 3.62 | 1.53 | 2.78 | 2.94 | 6.18 | 3.47 | 3.82 |
| 2 | 31.5 | 420.0 | 152.9 | 0.66 | 129.1 | 0.56 | 1.01 | 0.50 | 2.57 | 4.23 | 8.61 | 2.14 | 2.78 | 2.38 | 2.92 | 1.07 | 1.42 | 4.54 | 8.61 | 2.39 | 3.50 |
| 3 | 30.5 | 262.5 | 146.4 | 2.93 | 138.6 | 0.07 | 0.92 | 0.86 | 1.92 | 4.63 | 6.92 | 2.17 | 2.88 | 2.60 | 3.40 | 2.38 | 2.22 | 4.81 | 6.79 | 2.82 | 3.54 |
| 4 | 31.0 | 388.5 | 150.4 | 0.60 | 137.8 | 0.08 | 0.77 | 0.40 | 1.48 | 4.15 | 9.61 | 1.95 | 3.11 | 3.61 | 3.62 | 3.30 | 3.20 | 3.69 | 8.85 | 2.15 | 3.40 |
| 5 | 29.0 | 356.0 | 156.6 | 0.97 | 133.5 | 0.12 | 0.42 | 0.21 | 0.87 | 1.53 | 2.82 | 1.17 | 1.63 | 1.59 | 1.59 | 1.08 | 1.05 | 1.94 | 2.82 | 1.42 | 1.83 |
| 6 | 30.0 | 335.0 | 153.9 | 1.20 | 132.1 | 0.12 | 0.61 | 0.19 | 0.79 | 1.42 | 2.47 | 1.06 | 1.48 | 1.85 | 1.85 | 1.29 | 1.41 | 1.48 | 2.47 | 1.25 | 1.89 |
| 7 | 29.5 | 303.5 | 159.3 | 0.74 | 132.7 | 0.42 | 0.79 | 0.23 | 1.81 | 3.23 | 6.10 | 1.81 | 2.42 | 2.57 | 3.66 | 1.92 | 2.00 | 3.65 | 5.49 | 2.23 | 2.62 |
| 8 | 31.0 | 392.0 | 147.8 | 1.20 | 131.1 | 0.31 | 0.69 | 0.32 | 2.09 | 2.63 | 5.94 | 1.99 | 3.47 | 2.50 | 2.50 | 2.45 | 2.55 | 2.81 | 5.94 | 2.18 | 3.47 |
| 9 | 30.0 | 362.0 | 153.7 | 0.87 | 130.3 | 0.35 | 1.05 | 0.88 | 1.81 | 3.17 | 8.10 | 2.09 | 3.19 | 3.52 | 3.73 | 1.97 | 2.03 | 3.17 | 7.89 | 2.41 | 3.75 |
| 10 | 28.0 | 238.0 | 163.9 | 1.08 | 136.4 | 0.38 | 1.42 | 0.94 | 2.27 | 4.34 | 8.01 | 1.33 | 2.13 | 3.59 | 4.33 | 3.66 | 2.80 | 4.43 | 7.55 | 3.66 | 2.80 |
| 11 | 31.5 | 355.0 | 151.0 | 2.06 | 135.2 | 0.43 | 1.21 | 1.63 | 3.59 | 4.03 | 7.66 | 1.90 | 2.66 | 5.24 | 4.64 | 4.35 | 4.18 | 4.03 | 6.65 | 1.90 | 2.61 |
| 12 | 27.5 | 253.0 | 153.0 | 1.87 | 138.0 | 0.42 | 1.19 | 0.64 | 2.69 | 3.66 | 5.99 | 2.31 | 2.31 | 3.03 | 3.87 | 3.01 | 3.59 | 3.80 | 5.99 | 2.31 | 3.14 |
| 13 | 27.5 | 232.0 | 154.8 | 1.23 | 136.7 | 0.34 | 1.45 | 0.71 | 2.57 | 4.09 | 7.95 | 2.24 | 2.75 | 4.78 | 4.77 | 3.14 | 4.39 | 3.86 | 7.38 | 2.37 | 2.76 |
| 14 | 27.5 | 237.5 | 147.5 | 1.86 | 126.9 | 0.34 | 0.90 | 0.41 | 1.75 | 3.77 | 7.66 | 2.71 | 3.53 | 3.67 | 3.89 | 2.29 | 2.93 | 3.89 | 7.22 | 3.21 | 3.81 |
| * $\bar{X}$ | 29.5 | 318.0 | 153.7 | 1.32 | 133.6 | 0.30 | 0.93 | 0.64 | 2.15 | 3.42 | 6.75 | 1.95 | 2.71 | 3.10 | 3.43 | 2.39 | 2.59 | 3.50 | 6.42 | 2.41 | 3.07 |
| ** High $M_1$ | 30.4 | 355.0 | 157.3 | 1.69 | 135.7 | 0.38 | 1.12 | 0.87 | 2.65 | 4.00 | 7.92 | 2.22 | 3.10 | 3.70 | 3.98 | 2.97 | 3.19 | 4.05 | 7.50 | 2.81 | 3.46 |
| ** Low $M_1$ | 28.7 | 281.0 | 150.1 | 0.98 | 131.5 | 0.22 | 0.77 | 0.41 | 1.64 | 2.94 | 5.58 | 1.68 | 2.33 | 2.50 | 2.89 | 1.81 | 1.99 | 2.95 | 5.33 | 2.01 | 2.68 |

* - MEAN  ** - 95% Confidence Interval

APPENDIX: TABLE III RAW DATA ATPases 18°C  Units $\mu$MPi . 15 min$^{-1}$ . mg protein$^{-1}$

| No. | Length (cm) | Weight (g) | Plasma $Na^+$ mEq.l$^{-1}$ | Plasma $K^+$ mEq.l$^{-1}$ | Plasma $Cl^-$ mEq.l$^{-1}$ | GILL ($Na^+$-$K^+$) 18° | GILL ($Na^+$-$K^+$) 25° | KIDNEY ($Na^+$-$K^+$) 18° | KIDNEY ($Na^+$-$K^+$) 25° | GILL ($Mg^{++}$) 18° | GILL ($Mg^{++}$) 25° | KIDNEY ($Mg^{++}$) 18° | KIDNEY ($Mg^{++}$) 25° | GILL ($HCO_3^-$) 18° | GILL ($HCO_3^-$) 25° | KIDNEY ($HCO_3^-$) 18° | KIDNEY ($HCO_3^-$) 25° | GILL ($Mg^{++}$) 18° | GILL ($Mg^{++}$) 25° | KIDNEY ($Mg^{++}$) 18° | KIDNEY ($Mg^{++}$) 25° |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 29.5 | 324.5 | 150.5 | 1.11 | 130.0 | 0.37 | 0.74 | 0.66 | 1.78 | 7.09 | 8.96 | 4.06 | 4.46 | 2.83 | 2.10 | 2.72 | 2.08 | 7.34 | 9.47 | 4.11 | 4.90 |
| 2 | 30.0 | 359.0 | 156.9 | 1.00 | 138.5 | 0.37 | 1.24 | 0.70 | 1.43 | 6.62 | 8.32 | 2.91 | 3.48 | 3.08 | 3.02 | 1.74 | 2.39 | 6.99 | 8.97 | 3.26 | 3.91 |
| 3 | 28.0 | 235.5 | 145.8 | 4.52 | 133.5 | 0.19 | 0.67 | 0.25 | 0.66 | 2.79 | 3.65 | 1.28 | 1.48 | 1.64 | 1.07 | 1.68 | 1.07 | 2.98 | 4.32 | 1.38 | 1.79 |
| 4 | 30.0 | 363.0 | 154.4 | 2.67 | 132.4 | 0.33 | 0.50 | 0.52 | 1.04 | 3.00 | 4.08 | 1.19 | 1.29 | 1.84 | 1.50 | 1.66 | 1.24 | 3.58 | 4.58 | 1.29 | 1.86 |
| 5 | 29.0 | 312.0 | 153.9 | 2.97 | 134.8 | 0.33 | 0.50 | 1.09 | 2.13 | 3.33 | 4.58 | 1.53 | 1.73 | 2.33 | 3.34 | 2.23 | 2.23 | 3.58 | 4.58 | 1.73 | 2.13 |
| 6 | 30.0 | 342.5 | 153.9 | 2.35 | 136.9 | 0.37 | 0.57 | 0.94 | 1.46 | 8.21 | 8.49 | 2.34 | 2.55 | 2.93 | 2.83 | 2.13 | 1.94 | 9.62 | 9.43 | 2.66 | 2.96 |
| 7 | 29.0 | 208.3 | 155.5 | 3.62 | 128.7 | 0.40 | 0.78 | 0.88 | 2.27 | 4.44 | 5.89 | 2.03 | 2.03 | 3.14 | 3.47 | 2.15 | 2.15 | 4.52 | 6.29 | 2.38 | 2.44 |
| 8 | 27.0 | 295.5 | 153.7 | 1.63 | 141.5 | 0.33 | 0.56 | 0.37 | 1.72 | 5.50 | 7.19 | 3.01 | 3.56 | 1.88 | 2.50 | 2.78 | 2.41 | 7.06 | 7.81 | 3.10 | 3.15 |
| 9 | 26.5 | 280.5 | 152.0 | 2.19 | 123.8 | 0.45 | 1.14 | 1.48 | 2.29 | 3.94 | 6.14 | 2.43 | 3.34 | 3.50 | 3.51 | 1.82 | 1.81 | 4.39 | 6.14 | 2.56 | 3.34 |
| 10 | 29.5 | 344.0 | 147.3 | 2.97 | 127.2 | 0.44 | 0.96 | 1.18 | 2.50 | 3.95 | 5.79 | 2.36 | 2.36 | 3.51 | 3.15 | 2.60 | 2.83 | 3.77 | 5.53 | 2.12 | 2.97 |
| 11 | 27.0 | 257.0 | 148.4 | 6.25 | 125.9 | 1.00 | 1.05 | 0.39 | 1.41 | 4.44 | 6.11 | 1.73 | 1.73 | 3.78 | 4.00 | 2.44 | 2.37 | 4.44 | 5.44 | 1.73 | 1.73 |
| 12 | 24.5 | 160.0 | 127.6 | 4.40 | 111.4 | 0.66 | 0.76 | 0.70 | 1.48 | 5.94 | 7.92 | 1.41 | 1.55 | 2.83 | 3.96 | 2.61 | 4.57 | 5.66 | 7.36 | 1.76 | 1.76 |
| 13 | 29.5 | 309.5 | 142.7 | 2.06 | 137.1 | 0.59 | 0.78 | 0.87 | 1.63 | 3.33 | 4.51 | 1.74 | 1.92 | 2.55 | 2.74 | 0.93 | 1.40 | 3.33 | 4.12 | 1.74 | 2.61 |
| 14 | 29.5 | 304.0 | 159.0 | 3.40 | 134.7 | 0.64 | 1.03 | 0.77 | 1.70 | 5.87 | 8.49 | 1.46 | 2.15 | 3.80 | 3.97 | 2.38 | 2.62 | 5.71 | 7.54 | 1.85 | 1.92 |
| * $\bar{X}$ | 28.5 | 292.5 | 150.1 | 2.94 | 131.2 | 0.46 | 0.81 | 0.77 | 1.68 | 4.89 | 6.44 | 2.11 | 2.40 | 2.83 | 2.94 | 2.13 | 2.22 | 5.22 | 6.54 | 2.26 | 2.68 |
| ** High $M_1$ | 29.5 | 327.0 | 154.7 | 3.77 | 135.6 | 0.52 | 0.90 | 0.97 | 1.97 | 5.86 | 7.48 | 2.58 | 2.95 | 3.24 | 3.46 | 2.43 | 2.71 | 6.34 | 7.64 | 2.73 | 3.22 |
| ** Low $M_1$ | 27.6 | 260.0 | 145.6 | 2.11 | 126.8 | 0.41 | 0.72 | 0.58 | 1.39 | 3.92 | 5.39 | 1.64 | 1.85 | 2.42 | 2.42 | 1.83 | 1.73 | 4.10 | 5.44 | 1.80 | 2.14 |

* -MEAN  ** -95% Confidence Interval

APPENDIX: TABLE IV RAW DATA CARBONIC ANHYDRASE 2°C

Units - $\mu$M p-nitrophenol + p nitrophenolate . $min^{-1}$ . g $protein^{-1}$

| No. | Length (cm) | Weight (g) | Plasma $Na^+$ $mEq.l^{-1}$ | Plasma $K^+$ $mEq.l^{-1}$ | Plasma $Cl^-$ $mEq.l^{-1}$ | BLOOD 2° | BLOOD 25° | GILL 2° | GILL 25° | KIDNEY 2° | KIDNEY 25° |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 29.5 | 259.0 | 147.6 | 1.9 | 121.5 | 7.4 | 9.0 | 9.9 | 12.1 | 1.2 | 3.0 |
| 2 | 31.0 | 380.0 | 155.0 | 2.2 | 137.8 | 8.5 | 10.6 | 7.0 | 12.6 | -.1 | .4 |
| 3 | 29.0 | 298.0 | 148.9 | 2.2 | 133.2 | 7.8 | 13.9 | 12.6 | 22.7 | 1.7 | 1.0 |
| 4 | 29.0 | 302.0 | 150.4 | 1.6 | 128.1 | 8.7 | 12.1 | 8.8 | 14.5 | -.5 | -.5 |
| 5 | 28.0 | 368.0 | 149.2 | 2.6 | 131.3 | 7.6 | 15.2 | 10.6 | 19.9 | .1 | -1.5 |
| 6 | 30.0 | 258.0 | 155.4 | 4.8 | 136.9 | 8.8 | 11.7 | 9.8 | 19.3 | .5 | .3 |
| 7 | 32.0 | 411.0 | 154.6 | 1.6 | 130.6 | 7.0 | 12.2 | 13.2 | 21.8 | 1.2 | .7 |
| 8 | 29.5 | 344.0 | 148.4 | 1.0 | 125.0 | 11.7 | 15.6 | 12.8 | 23.2 | .7 | 1.2 |
| 9 | 29.5 | 283.0 | 153.6 | 1.1 | 130.6 | 8.7 | 12.5 | 9.5 | 18.8 | -.3 | -.6 |
| 10 | 28.5 | 275.0 | 152.7 | .7 | 133.6 | 8.1 | 14.8 | 10.1 | 15.4 | .1 | .7 |
| 11 | 28.0 | 240.0 | 150.8 | 2.0 | 130.6 | 11.4 | 17.9 | 9.4 | 16.5 | .4 | 2.1 |
| 12 | 32.0 | 400.0 | 156.5 | .9 | 127.2 | 9.1 | 15.1 | 11.8 | 20.2 | .7 | 2.0 |
| 13 | 28.0 | 26.5 | 149.8 | 1.2 | 127.4 | 5.5 | 8.8 | 9.0 | 14.7 | .3 | .2 |
| 14 | 28.5 | 228.0 | 147.9 | 2.1 | 131.8 | 6.6 | 12.1 | 9.6 | 15.9 | -.2 | -1.3 |
| * $\bar{X}$ | 29.5 | 308.0 | 151.5 | 1.85 | 130.4 | 8.4 | 13.0 | 10.3 | 17.7 | .4 | .6 |
| ** High $M_1$ | 30.3 | 343.0 | 153.3 | 2.4 | 132.9 | 9.3 | 14.5 | 11.3 | 19.7 | .8 | 1.3 |
| ** Low $M_2$ | 28.7 | 273.0 | 149.7 | 1.3 | 127.9 | 7.4 | 11.5 | 9.3 | 15.6 | .05 | - .2 |

* - MEAN

** - 95% Confidence Interval

APPENDIX: TABLE V RAW DATA CARBONIC ANHYDRASE 10°C

Units - μMp-nitrophenol + p nitrophenolate . $min^{-1}$ . g $protein^{-1}$

| No. | Length (cm) | Weight (g) | Plasma $Na^+$ $mEq.l^{-1}$ | Plasma $K^+$ $mEq.l^{-1}$ | Plasma $Cl^-$ $mEq.l^{-1}$ | BLOOD 10° | BLOOD 25° | GILL 10° | GILL 25° | KIDNEY 10° | KIDNEY 25° |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 32.0 | 392 | 152.7 | 1.8 | 125.6 | 9.4 | 14.1 | 11.0 | 15.0 | .2 | .3 |
| 2 | 29.0 | 284 | 148.0 | 2.3 | 128.9 | 12.6 | 23.7 | 9.4 | 17.0 | 3.4 | 5.9 |
| 3 | 30.5 | 333 | 153.1 | 2.7 | 128.4 | 8.3 | 11.0 | 10.5 | 13.1 | 1.3 | 2.7 |
| 4 | 31.0 | 340 | 153.6 | 1.6 | 129.4 | 16.4 | 19.6 | 11.6 | 17.9 | 1.1 | 3.0 |
| 5 | 30.0 | 311 | 151.7 | 2.3 | 127.7 | 4.6 | 12.1 | 9.2 | 10.4 | .5 | -1.4 |
| 6 | 31.0 | 334 | 155.0 | 1.5 | 127.5 | 7.4 | 8.3 | 7.6 | 13.7 | .1 | .5 |
| 7 | 32.0 | 342 | 153.6 | 1.3 | 132.2 | 10.9 | 16.4 | 9.0 | 14.2 | 1.0 | 3.7 |
| 8 | 28.5 | 270 | 159.9 | 1.0 | 120.2 | 13.8 | 18.5 | 12.4 | 15.7 | 0.0 | 1.4 |
| 9 | 29.0 | 298 | 156.0 | 2.1 | 127.4 | 8.8 | 13.8 | 12.5 | 15.8 | .4 | 1.5 |
| 10 | 30.0 | 312 | 157.9 | 1.2 | 131.3 | 10.0 | 15.0 | 14.3 | 20.3 | 1.8 | .3 |
| 11 | 26.5 | 248 | 155.4 | 1.1 | 133.9 | 9.1 | 12.6 | 12.0 | 15.2 | -1.1 | -1.7 |
| 12 | 31.0 | 322 | 152.6 | 1.3 | 135.6 | 10.3 | 16.0 | 15.8 | 21.0 | .7 | .5 |
| 13 | 31.5 | 314 | 144.2 | 1.4 | 130.8 | 13.0 | 22.3 | 16.8 | 23.8 | .4 | - .5 |
| 14 | 29.5 | 304 | 145.2 | 1.9 | 129.6 | 10.7 | 16.1 | 14.1 | 23.1 | 1.0 | .9 |
| * $\bar{X}$ | 30.1 | 307 | 152.8 | 1.7 | 129.2 | 10.4 | 15.7 | 11.9 | 16.9 | .8 | 1.2 |
| ** High $M_1$ | 31.0 | 323 | 155.3 | 2.0 | 131.3 | 12.1 | 18.2 | 13.5 | 19.1 | 1.4 | 2.4 |
| ** Low $M_1$ | 29.2 | 292 | 150.2 | 1.4 | 127.0 | 8.7 | 13.2 | 10.3 | 14.6 | .2 | .04 |

* - MEAN

** - 95% Confidence Interval

APPENDIX: TABLE VI RAW DATA CARBONIC ANHYDRASE 18°C

Units - μM p-nitrophenol + p-nitrophenolate . $min^{-1}$ . g-protein$^{-1}$

| No. | Length (cm) | Weight (g) | Plasma $Na^+$ mEq.$l^{-1}$ | Plasma $K^+$ mEq.$l^{-1}$ | Plasma $Cl^-$ mEq.$l^{-1}$ | BLOOD 18° | BLOOD 25° | GILL 18° | GILL 25° | KIDNEY 18° | KIDNEY 25° |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 32.0 | 390 | 141.1 | 2.2 | 120.9 | 15.1 | 21.3 | 4.9 | 9.2 | 2.2 | 2.4 |
| 2 | 27.5 | 267 | 147.6 | 5.7 | 122.6 | 12.3 | 11.3 | 7.1 | 6.0 | 3.5 | 3.8 |
| 3 | 31.0 | 301 | 141.6 | 2.2 | 121.9 | 11.7 | 15.2 | 8.8 | 8.5 | 3.1 | 2.6 |
| 4 | 28.5 | 275 | 148.0 | 2.4 | 122.4 | 16.2 | 14.4 | 5.9 | 8.5 | 4.3 | 4.6 |
| 5 | 28.5 | 270 | 143.4 | 5.0 | 122.2 | 10.3 | 14.1 | 7.8 | 7.0 | 4.7 | 3.5 |
| 6 | 32.0 | 350 | 154.6 | 2.0 | 133.9 | 5.9 | 8.2 | 6.6 | 6.2 | 2.8 | 2.1 |
| 7 | 32.0 | 338 | 150.3 | 1.7 | 133.2 | 24.0 | 28.4 | 5.3 | 6.2 | .7 | 1.3 |
| 8 | 31.0 | 352 | 153.6 | 1.0 | 127.5 | 14.2 | 17.3 | 7.9 | 10.0 | .7 | 1.0 |
| 9 | 32.0 | 412 | 148.4 | 2.0 | 121.4 | 17.5 | 23.8 | 9.9 | 13.2 | .7 | - .2 |
| 10 | 29.5 | 294 | 154.1 | 1.9 | 138.7 | 14.2 | 13.1 | 10.5 | 13.1 | 1.0 | 1.3 |
| 11 | 27.5 | 237 | 155.0 | 2.5 | 133.2 | 11.0 | 16.2 | 9.1 | 9.8 | 1.4 | 1.1 |
| 12 | 29.5 | 261 | 159.6 | 3.0 | 130.5 | 9.1 | 11.4 | 7.6 | 7.2 | - .5 | - .3 |
| 13 | 28.0 | 236 | 149.9 | 4.2 | 129.8 | 12.2 | 12.2 | 13.0 | 15.0 | -1.1 | .7 |
| 14 | 27.0 | 214 | 138.8 | 2.7 | 113.6 | 13.7 | 12.4 | 13.2 | 13.4 | 2.4 | 2.8 |
| * | $\bar{X}$ | 29.7 | 300 | 148.8 | 2.8 | 126.6 | 13.4 | 15.7 | 8.4 | 9.5 | 1.85 | 1.9 |
| ** | High $M_1$ | 30.8 | 334 | 152.5 | 3.5 | 130.5 | 15.9 | 18.8 | 9.9 | 11.3 | 2.8 | 2.8 |
| ** | Low $M_1$ | 28.6 | 265 | 145.5 | 2.0 | 122.6 | 10.9 | 12.5 | 6.9 | 7.8 | .9 | 1.1 |

* - MEAN

** - 95% Confidence Interval