

AN ABSTRACT OF THE THESIS OF

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Title: Thermally Modulated Biliary Excretion of Taurocholate in  
Rainbow Trout

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Biliary excretion of taurocholate in thermally acclimated rainbow trout was stimulated at higher environmental temperature (18 vs. 14 or 10° C). Acute 4° C shifts in body temperature produced more pronounced changes in biliary excretion. Thermally modulated biliary excretion was negatively correlated with plasma half live of taurocholate. Absorption of intraperitoneally injected taurocholate into plasma or tissue distribution were both independent of temperature. Hepatic blood flow as measured by laser doppler velocimetry was also stimulated at 18° C. The correlation coefficient between hepatic blood flow and biliary excretion rate was 0.73 for 7 different thermal treatments. The activities of liver plasma membrane ATPases as analysed by Michaelis-Menton kinetics and temperature responses did not appear to be affected by acclimation to 10 or 18° C. Thermal modulation of hepatic blood flow provides a sound explanation for the observed temperature dependence of the plasma half life and biliary excretion rate of taurocholate in rainbow trout.

Thermally Modulated Biliary Excretion of  
Taurocholate in Rainbow Trout

by

Christopher J. Kemp

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THERMALLY MODULATED BILIARY EXCRETION OF  
TAUROCHOLATE IN RAINBOW TROUT

INTRODUCTION

The process of biliary excretion is affected by a variety of chemicals and drugs in humans and other mammals (Plaa and Priestly, 1977). Animal models used to mimic human hepatobiliary dysfunction have not satisfactorily represented all aspects of clinical syndromes, nor have they produced a thorough understanding of the mechanisms involved in biliary excretion of organic solutes. The study of fundamental processes involved in the operation of the hepatobiliary system can contribute to understanding the basis of dysfunction.

Thermal acclimation of poikilothermic animals provides a unique approach for investigating interrelations of processes involved in hepatobiliary transport for several reasons. First, as discussed by Klaassen and Watkins (1984) hepatic blood flow can be rate limiting in the hepatic clearance of compounds with a high intrinsic clearance. As demonstrated herein hepatic blood flow in rainbow trout was affected by environmental temperature and may play a role in thermally modulated biliary excretion. Second, thermal acclimation also altered membrane lipid composition in rainbow trout liver (Hazel, 1979), eel gill (Thompson et al., 1977), goldfish intestine (Smith, 1976) and goldfish synaptosomes (Cossins, 1977). This process termed homeoviscous adaptation involves a shift in membrane lipid composition and functions to maintain a relatively constant membrane fluidity ( $1/\text{viscosity}$ ) in the face of long-term (2-3 wks) changes in

environmental temperature. The fluidity of the lipid bilayer membrane is directly affected by temperature. At low temperatures the lipid bilayer becomes ordered to the point of undergoing a phase transition from a liquid crystalline to a gel state and at high temperatures becomes disordered and leaky. These changes can profoundly disturb the normal function of the membrane. Further, fatty acid composition and fluidity of the lipid bilayer membrane have been shown to affect the activity of membrane-bound enzymes such as  $\text{Na}^+/\text{K}^+$ ,  $\text{Ca}^{2+}$ , and mitochondrial  $\text{Mg}^{2+}$ -ATPase (reviewed by Kimelberg, 1976; Korenbrot, 1977; and Sandermann, 1978). It is tempting to speculate that the homeoviscous membrane adaptation of poikilotherms functions to maintain the proper environment for membrane-bound enzymes.

Furthermore, it is worth predicting that the many processes which affect membranes may also have some effect on these enzymes and that the many agents which are known to effect membrane enzymes may exert their influence in conjunction with the lipid bilayer. Indeed in a recent review on bile formation by Klaassen and Watkins (1984) it was pointed out that a wide range of chemicals which cause cholestasis also effect  $\text{Na}^+/\text{K}^+$ -ATPase activity,  $\text{Mg}^{2+}$ -ATPase activity and/or membrane fluidity, and more study was recommended.

The advantage of using thermally acclimated rainbow trout is that one can modulate biliary excretion, hepatic blood flow, and membrane fluidity in vivo without the use of chemicals. Therefore we measured several parameters of hepatobiliary transport of taurocholate in 10, 14, and 18°C acclimated rainbow trout. We also looked at the effect of acute shifts in body temperature on the same parameters by



transferring acclimated fish to different environmental temperatures one hour prior to and during an experiment. Next we determined the kinetic properties and temperature activity curves for  $\text{Na}^+/\text{K}^+$ -ATPase and  $\text{Mg}^{2+}$ -ATPase in hepatocyte plasma membrane fractions obtained from 10 and 18°C acclimated trout. Finally, we measured hepatic blood flow in 10, 14 and 18°C acclimated and temperature shifted trout.

## MATERIALS AND METHODS

### Animals and Materials

Shasta strain rainbow trout (Salmo gairdneri) were obtained from Smith Farm, Oregon State University, and held at Oak Creek Laboratory of Biology prior to testing. Fish of either sex weighing between 200-500g were acclimated for 3-4 weeks to 10, 14 or 18°C and fed a maintenance ration (no growth) of Oregon Test Diet (Sinhubber et al., 1977). Construction of food consumption/growth rate curves (Warren, 1971) for cohorts of fish at each temperature allowed adjustment of ration for differences in standard metabolic rate. Fish were housed individually in 300 l aquaria with flow through well water. Water temperatures were held within 0.3°C of that desired with thermostatically controlled immersion coils. A 12 hr light - 12 hr dark photoperiod was used throughout acclimation. Analytical grade reagents were used whenever possible. Oligomycin (65%A, 20%B, 15%C) tris-ATP, ouabain, M.S. 222 (tricaine methanesulfonate), and the sodium salt of taurocholate (98% pure) were purchased from Sigma Chemical Co. Tissue solubilizer (Fisher Scintigest) and scintillation cocktail (Scintiverse I) were from Fisher Scientific Co. and <sup>14</sup>C-taurocholate (s.a. 1.2 mCi/ml) was obtained from California Bionuclear.

### Biliary Excretion of Taurocholate

Taurocholate was used as a model compound because it is well studied in regards to its hepatobiliary transport. <sup>14</sup>C-taurocholate was diluted with the sodium salt of taurocholate to 10 mM (s.a. 0.2 µCi/µm). Purity of this stock and excreted biliary taurocholate was

checked by thin layer chromatography (Eneroth, 1963). Three  $\mu$ l stock taurocholate or 5  $\mu$ l fish bile were applied to activated Analtech silica gel plates using a solvent system of butanol:acetic acid: water at a ratio of 85:10:5. One  $\text{cm}^2$  sections were cut out and analyzed for radioactivity as described below. Only one product was detected in both stock taurocholate and fish bile with an Rf of 0.25 which agreed with the Rf value of 0.22 given by Eneroth and Sjorvall (1969).

Thermally acclimated fish were starved for 48 hr then transferred to static, aerated aquaria at the same temperature of acclimation. Some fish were subjected to an acute  $4^\circ\text{C}$  shift in environmental temperature one hr prior to and during the experiment by transferring them to the appropriate aquaria. Ten and  $18^\circ\text{C}$  acclimated fish were shifted to  $14^\circ\text{C}$  and  $14^\circ\text{C}$  acclimated fish were shifted to 10 and  $18^\circ\text{C}$ . The remaining fish were tested at the same temperature of acclimation i.e., 10, 14 and  $18^\circ\text{C}$ . Fish were removed, injected with  $^{14}\text{C}$ -taurocholate ( $5-10 \times 10^3$  nmol/kg fish, i.p.) and returned to their aquaria. After 60 minutes the fish were sacrificed by a blow to the head and weighed. One ml of blood was drawn from the caudal vein and centrifuged at  $1,200 \times g$  for 5 minutes to obtain plasma. Duplicate 0.2 ml plasma samples were placed in tared vials. The body cavity was opened with a ventral slit and the liver and attached gall bladder removed. The intact gall bladder was dissected free and both liver and gallbladder weighed. Duplicate 0.1 g samples of minced liver were placed in tared vials as were 0.1 ml bile samples. Other tissues sampled included small intestines at the pyloric sphincter and 5 cm caudal, muscle, gill, fat and kidney. Duplicate 0.1 g samples of each

tissue were placed in tared vials. Tissues were minced in the vials then capped and weighed to determine tissue weight. One ml of tissue solubilizer (Fisher Scintijest) and 0.2 ml water were added to each vial. After 24 hours of room temperature incubation tissues not fully digested were incubated at 50°C until solubilized. Nine ml of scintillation cocktail (Scintiverse I) were added and the vials were vortexed and counted on Packard tricarb scintillation counter, Model 3385.

To analyze plasma kinetics of taurocholate, thermally-acclimated trout were anesthetized with M.S. 222 (75 mg/l), spinally transected (Schmidt and Weber, 1973), placed in a plexiglass frame to maintain upright body position and allowed to stabilize for 24 hr in their respective aquaria. The fish were then weighed and injected with  $^{14}\text{C}$ -taurocholate (10  $\mu\text{moles/kg}$ , i.p.). Blood was drawn from the caudal sinus of each fish into heparinized syringes at 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 180, 210 and 240 min post-injection and plasma analyzed for  $^{14}\text{C}$ -taurocholate.

#### Hepatic Blood Flow

Rainbow trout were thermally acclimated to 10, 14 and 18°C for 4 wks. The day before an experiment the trout were spinally transected, placed in a plexiglass frame to maintain upright body position and allowed to stabilize for 24 hr in their respective aquaria. The trout were then positioned ventral side up in the frame in a temperature controlled water bath. A medial incision was made to expose the liver and blood flow measurements were first made at the same temperature of

acclimation (10, 14, and 18°C) followed by another set of measurements after a 4°C shift in body temperature (14°C acclimated shifted to 10 and 18°C and 10 and 18°C acclimated shifted to 14°C. Body temperature was controlled by changing the water temperature and monitored with a thermometer placed under the liver. Blood flow at the liver surface was measured with a laser doppler capillary perfusion monitor (Med Pacific LD5000, Seattle). Like ultrasound this device uses the doppler principle with a laser light to monitor the quantity and speed of moving red blood cells up to 1mm from the surface of the tissue. It is extremely sensitive to changes in blood flow and yields a relative measure of volts (Holloway and Watkins, 1977). Great care was taken to maintain the proper optical coupling between the probe and liver surface. A minimum of 10 measurements were taken at different lateral and medial sites on each liver. The mean blood flow at these two surfaces of the liver were not statistically different and so were combined for analysis. Measurements made on visible blood vessels were significantly higher than the surrounding tissue and were omitted from analysis.

#### Membrane Preparation

A modification of the method of Lutz (1973) as developed by Selivonchick (pers comm.) was used for the isolation of trout liver plasma membranes. Thermally acclimated fish (10 and 18°C) were stunned by a blow to the head and the liver excised. Sufficient fish to yield 8 g liver were used for each preparation. The gallbladder and adherent connective tissue were dissected off and the livers

finely minced with scissors and a razor blade in ice cold 10% sucrose, 50 mM tris (pH 7.4). This preparation was brought to 30 ml with buffer, homogenized with two strokes of a loose fitting pestle in a dounce homogenizer, diluted to 150 ml with buffer and centrifuged at 120 x g for 6 minutes. The supernatant and erythrocyte layer were removed by aspiration, the pellet resuspended in 30 ml of buffer by two strokes in the dounce homogenizer and the suspension filtered through 1 mm nylon mesh. The filtrate was diluted to 150 ml, centrifuged as before and the pellet vigorously homogenized with 15 strokes of the pestle in 45 ml of buffer. For the majority of enzyme characterization this homogenate was centrifuged at 10,000 x g for 30 min, the pellet resuspended in 5-10 mls of ATPase buffer (0.3M sucrose, 0.02M EDTA and 0.1M imidazole, pH 7.2,) (Johnson et al., 1977) and frozen until used. For further purification a discontinuous sucrose gradient was used to separate the plasma membranes from the above homogenate. Twenty ml of homogenate were layered onto each of two discontinuous sucrose gradients consisting of 5 ml 38% sucrose (w/w) in 50 mM tris-HCl, pH 7.4 and 8 ml 33% sucrose in the same buffer. The gradients were centrifuged for one hr in a Sorvall SS 90 vertical head rotor at 20,000 x g with a Sorvall RC-5B refrigerated superspeed centrifuge. Plasma membranes were collected from the 10-33% sucrose interface with a pipette, pelleted at 10,000 x g for 30 minutes, resuspended in ATPase buffer, snap frozen in liquid nitrogen and stored at -20°C until used.

To determine the purity of the plasma membrane preparation a series of marker enzyme assays were conducted. The ratio of

activities of 5'nucleotidase, glucose-6-phosphatase and succinate dehydrogenase in the plasma membrane preparation to the crude homogenate gives an indication of the degree of enrichment or contamination of plasma membranes, microsomes, and mitochondria respectively. This was verified by Statham et al. (1977) using rainbow trout liver. The methods used were those of Dixon and Purdon (1954) for 5'-nucleotidase, Schwartz and Bodansky (1961) for glucose-6-phosphatase, and Pennington (1961) for succinate dehydrogenase. Protein was quantified by the method of Bradford (1976).

Initial difficulties were encountered in detecting consistent  $\text{Na}^+/\text{K}^+$ -ATPase activity, therefore deoxycholate pretreatment was used to activate this enzyme (Jorgenson and Skou, 1970). This treatment was only done on the 3x washed pellet. Five ml were added to 60 ml of 0.6 mg/ml deoxycholate, 25 mM imidazole, pH 7.1, 22°C and gently stirred for 30 minutes. This material was centrifuged at 10,000 x g for 30 minutes and the pellet brought up to 5 ml in 0.3 M sucrose, 0.1 M imidazole pH 7.4. This treatment resulted in a greater than 50% loss of protein but a five-fold increase in  $\text{Na}^+/\text{K}^+$ -ATPase activity. ATPase activity was determined by adding 0.1 ml (20-50  $\mu\text{g}$ ) protein to 0.2 ml salts or inhibitors (see below), incubating for 5 minutes at the reaction temperature then adding 0.2 ml of 3.0 mM vanadium-free tris-ATP, 3 mM  $\text{MgCl}_2$ , 50 mM imidazole, pH 7.4 to start the reaction. The reaction was terminated after 15 minutes with 0.25 ml ice cold 30% TCA and the inorganic phosphate released determined by the method of Fiske and Subbarow (1925). To inhibit potentially contaminating

mitochondrial ATPase, 0.5  $\mu$ l oligomycin in ethanol was initially added to each tube to give a final reaction concentration of 15  $\mu$ M. Total ATPase activity was determined with 100 mM NaCl and 10 mM KCl added as salts.  $Mg^{2+}$ -ATPase activity was the activity remaining with the substitution of  $Na^+$  and  $K^+$  ions with 110 mM choline chloride and 0.5 mM ouabain and  $Na^+/K^+$ -ATPase was calculated as the difference between these two activities. Because it had been reported that the inhibition of  $Na^+/K^+$ -ATPase by ouabain is temperature dependent (Ahmed and Judah, 1965), experiments were first run in the absence of  $Na^+$  and  $K^+$  ions to verify that ouabain inhibited the  $Na^+/K^+$  stimulated ATPase. This, however, caused an increase in basal  $Mg^{2+}$ -ATPase activity. Therefore it was necessary to substitute choline chloride for NaCl and KCl (to maintain constant ionic strength) and to use tris-ATP rather than the sodium salt of ATP.



## RESULTS

Biliary Excretion of Taurocholate

Initial experiments were designed to determine if acclimation temperature affected the absorption or distribution of taurocholate in rainbow trout. Absorption rate constants of 7.0, 7.5 and 6.0 hr<sup>-1</sup> and plasma half-lives of 2.0, 1.8 and 0.9 hr were calculated from plasma kinetic curves of 10, 14 and 18°C acclimated fish respectively (Figure 1).

Distribution of taurocholate to tissues other than liver and bile was negligible and not affected by acclimation temperature. Taurocholate concentrations in kidney, intestine, gill, fat, muscle, and urine were all less than 20 nmoles/gm tissue at 60 min (data not shown). No radioactivity was recovered from the aquarium water.

Biliary excretion rate of taurocholate was affected by temperature (Figure 2). Fish acclimated and tested at 18°C had a significantly higher rate (463 nmoles/gm liver/hr) than those acclimated and tested at 10°C (284 nmoles/gm liver/hr). Fish acclimated and tested at 14°C had an intermediate rate of 302 nmoles/gm liver/hr. Acute shifts in body temperature also affected biliary excretion rate. Fish acclimated at 14°C and shifted to 18°C had a significantly higher rate (666 nmoles/gm liver/hr) than those tested at 14°C (302 nmoles/gm liver/hr) and those shifted down to 10°C (199 nmoles/gm liver/hr). Conversely, fish acclimated at 10°C and shifted to 14°C had a significantly higher rate (466 nmoles/gm liver/hr) than those acclimated at 18° and shifted to 14°C (207 nmoles/gm liver/hr).

Figure 1. Plasma taurocholate kinetics in spinally transected rainbow trout acclimated to 10, 14 and 18°C. The points of each curve represents the mean of 3 fish. Vertical bars represent + 1 SE.

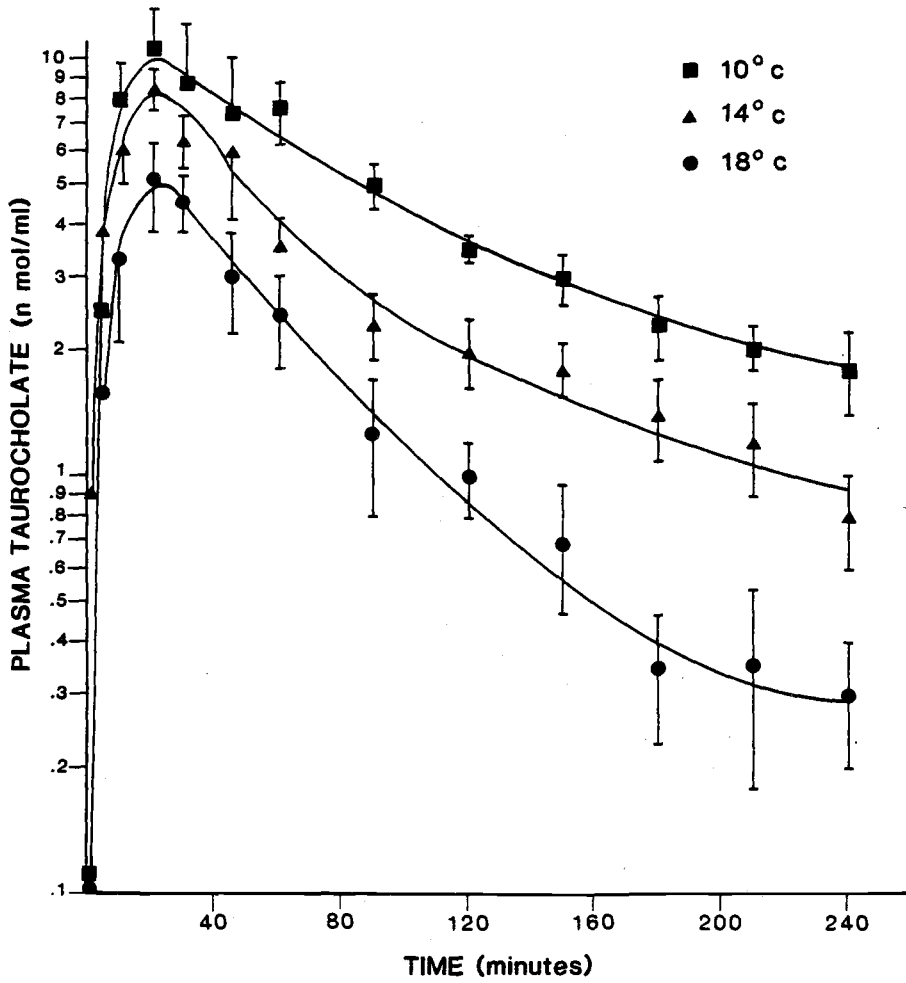


Figure 1.

Figure 2. Biliary excretion rate of taurocholate in free swimming rainbow trout for 7 different thermal treatments. Rate is expressed as total nmoles of taurocholate in gallbladder bile per g liver at 1 hr. Vertical bars represent  $\pm 1$  SE. The Student-Newman-Keuls multiple comparison test (Zar, 1974) was used for the following groups:

\*Acclimated and tested at 18°C significantly higher than 14 at 14°C ( $P < 0.1$ ) and 10 at 10°C ( $P < 0.05$ ) (solid bars) N=5-12 fish at each temperature.

\*\*Acclimated at 14° and shifted to 18° (open bar) significantly higher than 14° at 14° (solid bar,  $P < 0.05$ ) and 14° shifted to 10° (hatched bar  $P < 0.05$ ). N=2-5 fish.

+Acclimated at 10° and shifted to 14° (open bar) significantly higher than 18° shifted to 14° (hatched bar,  $P < 0.05$ ). N=3-5 fish.

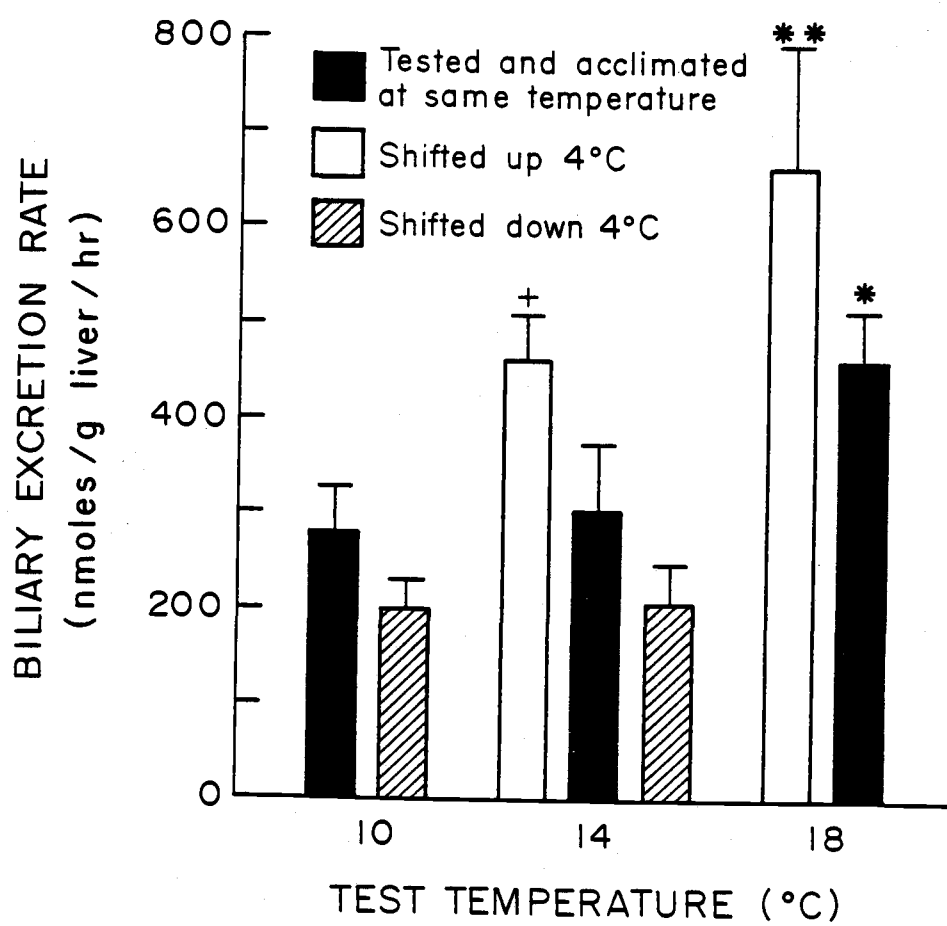


Figure 2.

Hepatic uptake of taurocholate may also have been affected by temperature although these results were more variable and not statistically significant. Taurocholate concentration in the liver at 60 min for fish acclimated and tested at 10, 14, and 18°C were 118, 91, and 154 nmoles/gm liver/hr respectively.

These differences in apparent excretion rates were not artifacts of liver size or bile volume as these quantities were not different from 10, 14, or 18°C acclimated fish.

#### Hepatic Blood Flow

Liver surface blood flow as measured by laser doppler velocimetry was linearly correlated to total liver hepatic blood flow in the isolated perfused rat liver (Shepherd et al., 1983). To verify that in our hands the laser doppler could detect changes in overall hepatic perfusion we occluded the portal vein with mechanical pressure while liver surface blood flow was monitored. As shown in Figure 3 the laser doppler almost instantly detected the decrease in hepatic perfusion. Four replicates of this procedure with the laser probe at four different sites on the liver yielded an average voltage decrease of  $51 \pm 8\%$ . Therefore, although the laser doppler only measures surface blood flow it is also useful as a tool to measure relative total hepatic perfusion in vivo.

Fish acclimated and tested at 18°C had a higher hepatic blood flow (1.75 mv) than fish acclimated and tested at 14 or 10°C (1.03 and 1.14 mv respectively). The mean hepatic blood flow in three of four temperature shift experiments showed a direct relationship to

Figure 3. A representative tracing of laser-doppler velocimetry on the surface of trout liver before and after (†) portal vein occlusion.

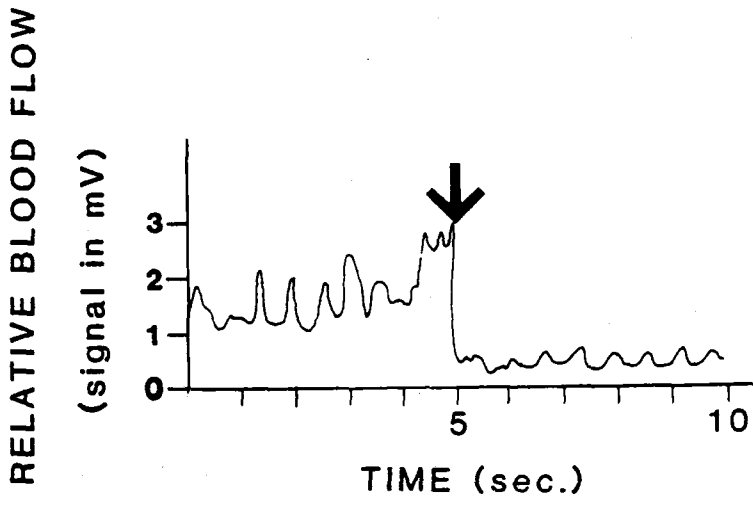


Figure 3.



Figure 4. Hepatic blood flow as a function of temperature in spinally transected trout. Vertical lines represent + 1 SE for 2-4 fish. Bars with no lines represent a single fish.

\*Acclimated and tested at 18° significantly higher than 14° ( $P < 0.1$ ) and 10° at 10° ( $P < 0.05$ ) using Student Newman Keul multiple comparison test.

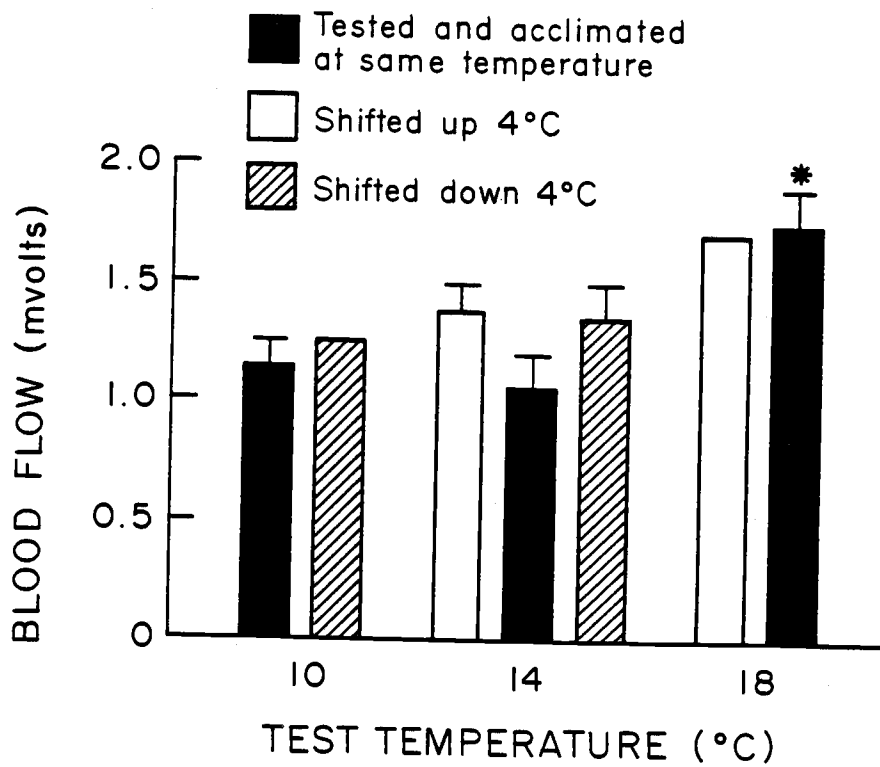


Figure 4.

temperature. Fish acclimated to 10°C shifted to 14°C and 14°C shifted to 18°C showed an increase in blood flow while fish acclimated to 18°C and shifted to 14°C showed a decrease in hepatic blood flow (Figure 4).

The temperature dependence of hepatic blood flow was similar to that for biliary excretion rate of taurocholate (compare Figures 2 and 4). The correlation coefficient was 0.73 between hepatic blood flow and biliary excretion rate for the seven different thermal treatments.

#### Mg<sup>2+</sup> and Na<sup>+</sup>/K<sup>+</sup>-ATPase

There was no apparent difference in purity of plasma membrane preparation from 10 and 18°C acclimated trout. 5'nucleotidase activity expressed relative to crude homogenate activity was 1.0, 4.6, and 30 for crude homogenate, 3x washed pellet and gradient purified membranes respectively. Glucose-6-phosphatase activity for the same fractions was 1.0, 0.5, and 1.9 and succinate dehydrogenase activity was 1.0, 1.5, and 0.6. This indicates selective enrichment of plasma membranes without significant enrichment of mitochondria or microsomes.

The Na<sup>+</sup>/K<sup>+</sup>-ATPase from mammalian plasma membranes has been extensively characterized. Much less work has been done on this enzyme in fish, particularly in hepatocyte plasma membranes. The Mg<sup>2+</sup>-ATPase activity associated with plasma membranes from a variety of cell types has received much less attention to date.

Therefore in the initial series of experiments we attempted to characterize some basic properties of these enzymes from rainbow trout hepatocytes. Some of these properties include the Mg<sup>2+</sup> dependence of the Mg<sup>2+</sup>-ATPase, the more specific Na<sup>+</sup> and K<sup>+</sup> requirement of the

$\text{Na}^+/\text{K}^+$ -ATPase, pH and temperature dependence and Michaelis-Menton constants. Unless otherwise stated the preparation used for ATPase assays was the 3x washed pellet and the assays were run at 37°C with 3mM ATP, 3mM  $\text{MgCl}_2$ , 50 mM imidazole, pH 7.4. To verify the so-called  $\text{Mg}^{2+}$ -ATPase activity was  $\text{Mg}^{2+}$ -dependent, the ATPase assay was run with varying concentrations of  $\text{MgCl}_2$  with no NaCl or KCl present. Activity was negligible (<10%) at 0 mM  $\text{MgCl}_2$  and optimal at 3-4 mM  $\text{MgCl}_2$  verifying that this enzyme was indeed  $\text{Mg}^{2+}$ -dependent. Furthermore, the addition of the divalent cation chelator, EDTA (8mM), to the reaction mixture (3mM  $\text{MgCl}_2$ , 100 mM NaCl and 10 mM KCl) abolished >90% of ATPase activity indicating that the  $\text{Na}^+/\text{K}^+$ -ATPase is also  $\text{Mg}^{2+}$ -dependent. Oligomycin (15  $\mu\text{M}$ ) reduced  $\text{Mg}^{2+}$ -ATPase activity to <20% indicating a small amount of mitochondrial ATPase contamination. All subsequent assays were run in the presence of oligomycin. To verify that ouabain inhibited  $\text{Na}^+/\text{K}^+$ -ATPase, parallel assays were run using choline chloride as a substitute for NaCl and KCl. The percent inhibition of total ATPase in the presence of 100mM NaCl, 10 mM KCl and 0.5 mM ouabain was the same as that with 110 mM choline chloride with no NaCl, KCl or ouabain. These results indicate that these enzymes are similar to those studied in rat hepatocytes and many other tissues.

The temperature response curve for  $\text{Na}^+/\text{K}^+$ -ATPase was nearly identical from 10 and 18°C acclimated fish (Figure 5). The dramatic increase in activity from 10 to 35°C has been reported for plasma membrane  $\text{Na}^+/\text{K}^+$ -ATPase from both homeotherms and poikilotherms including rat brain (Gruener and Avi-Dor, 1966; Bowler and Duncan,

Figure 5. Effect of temperature on ATPase activity of liver plasma membrane from 10 and 18°C acclimated trout. Activity was measured in the 3x washed pellet of liver homogenate as described. A: Mg<sup>2+</sup>-ATPase and B: Na<sup>+</sup>/K<sup>+</sup>-ATPase. Each line represents the mean of 2 separate experiments and each experiment represents material pooled from 3-4 fish. Vertical lines represent + 1 SE.

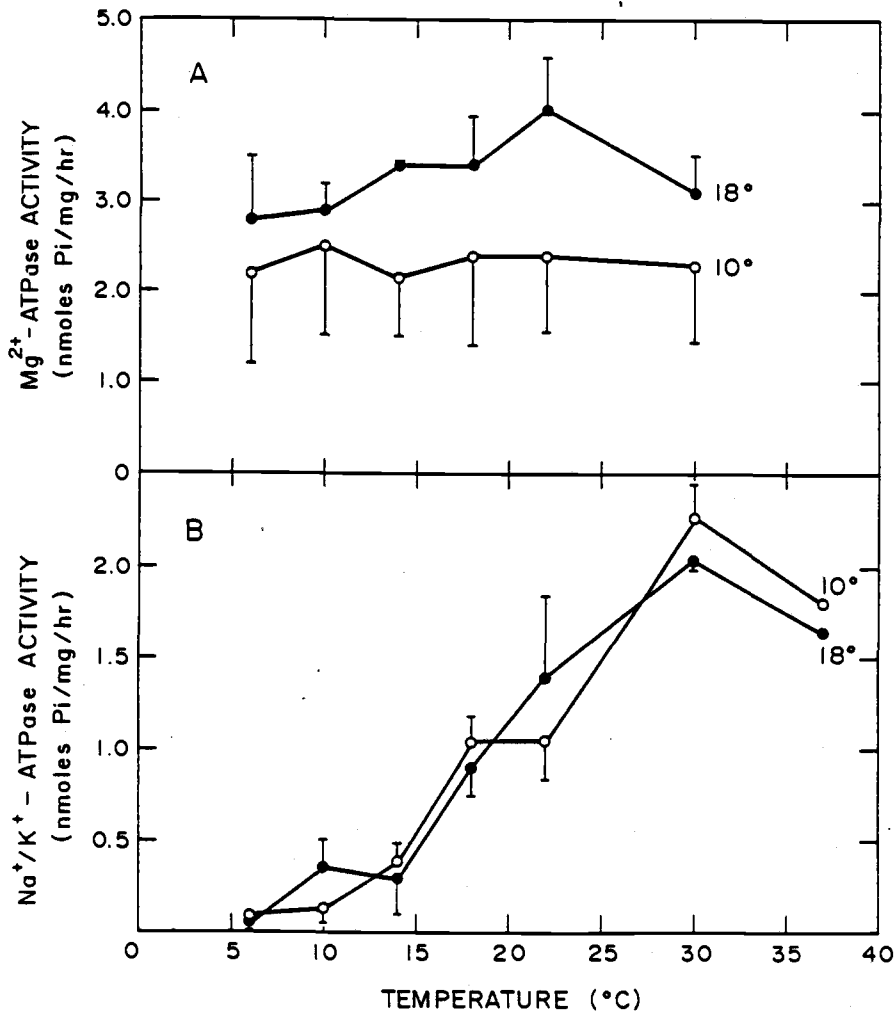


Figure 5.

1968), rat liver (Bakkeren and Bonting, 1968; Boyer and Reno, 1975; Emmelot and Bos, 1968), rabbit kidney (Walker and Wheeler, 1975), toad skin (Park and Hong 1976), turtle bladder (Bourgoignie et al., 1969), goldfish intestine (Smith 1967) and rainbow trout gills (Giles and Vanstone, 1976). However, the study by Smith demonstrated that the intestinal plasma membrane  $\text{Na}^+/\text{K}^+$ -ATPase from goldfish acclimated to 8° and 30°C had quite different temperature response curves and they suggested that the goldfish intestine is using different forms of an ATPase system when acclimated at 30° than when acclimated at 8 °C.

It remains intriguing that the  $\text{Na}^+/\text{K}^+$ -ATPase from poikilotherms has very little activity in vitro below 10°C. It is possible that the decrease in  $V_{\text{max}}$  below 10°C is offset by an increase in E-S affinity (or decrease in  $K_m$ ) a phenomenon which is frequently observed in poikilotherm enzymes (Hochachka and Somero, 1968).

Park and Hong (1976) have reported that the pH optimum of toad skin  $\text{Na}^+/\text{K}^+$ -ATPase increases with decreasing temperature. Therefore we attempted to stimulate the  $\text{Na}^+/\text{K}^+$ -ATPase at low temperatures (10, 14 and 18°C) by varying the pH from 7.2 to 8.0. At 18°C there was a rather distinct pH optimum of 7.4 but at 14 or 10°C the  $\text{Na}^+/\text{K}^+$ -ATPase pH optimum was broad, between 7.4 and 7.8. Therefore, by raising the pH we were unable to significantly stimulate the  $\text{Na}^+/\text{K}^+$ -ATPase at low temperatures.

$\text{Mg}^{2+}$ -ATPase proved to be remarkably temperature insensitive. Figure 5 shows that the  $\text{Mg}^{2+}$ -ATPase activity from the 3x washed pellet from 10° acclimated fish did not change from 6 to 30°C although there

was considerable variability. The  $Mg^{2+}$ -ATPase from 18°C acclimated trout appeared to have a slight temperature optimum at 22°C. The high variability observed among different preparations prompted us to purify this enzyme further using a sucrose gradient to isolate plasma membrane fractions. The  $Mg^{2+}$ -ATPase activity from plasma membrane fractions also had high variability between preparations, but there was not a significant difference in activity or temperature response for this enzyme from 10 or 18°C acclimated fish although above 24° C, the  $Mg^{2+}$ -ATPase activity from 10° acclimated fish had slightly higher activity than from 18° C acclimated fish (Figure 6). A more distinct temperature optimum occurred at around 24°C. Previous work using both homeothermic and poikilothermic membrane preparations have shown the  $Mg^{2+}$ -ATPase to be as temperature sensitive (Walker and Wheeler, 1975, Boyer and Reno, 1975) or more commonly, less temperature sensitive (Bowler and Duncan, 1968; Bakkeren and Bonting, 1968; Bourgoignie et al., 1969; Gruener and Avi-Dor, 1966; Smith, 1967) than  $Na^+/K^+$ -ATPase from the same preparation.

Many comparative enzyme analysis use specific activity as a measure of enzyme performance. Specific activity is usually measured at optimal substrate concentrations ( $V_{max}$ ) a condition which may be rare in vivo. A more realistic measure of enzyme performance may be  $K_m$  (Somero, 1969). As Atkinson (1966) points out at low substrate levels modulator induced changes in E-S affinity may be far more important than changes in  $V_{max}$  in governing enzyme activities. Therefore, we measured both  $V_{max}$  and  $K_m$  of  $Mg^{2+}$  and  $Na^+/K^+$ -ATPase from the 3x washed pellet of 10 and 18°C acclimated trout. These data were



Figure 6. Effect of temperature on  $Mg^{2+}$ -ATPase of sucrose gradient-purified plasma membrane from 10 and 18°C acclimated rainbow trout. Enzyme activity is expressed as a percentage of activity at 24°C. For comparison to figure 5,  $Mg^{2+}$ -ATPase activity at 24°C was  $19.4 \pm 2.0$   $\mu$ moles  $P_i$ /mg protein/hr. Numbers in parenthesis above bars represent the number of independent experiments. Each experiment represents material pooled from 4-5 fish. Vertical lines represent  $+1$  SE.

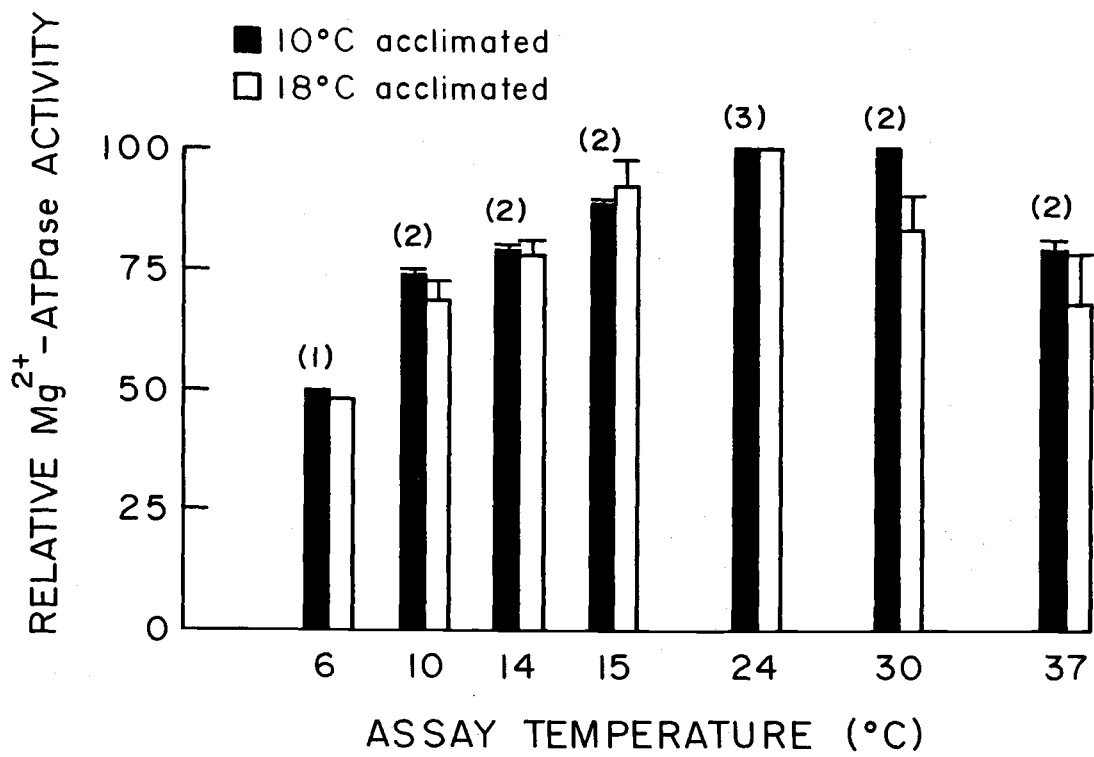


Figure 6.

plotted as a Lineweaver-Burk plot and Michaelis-Menton constants calculated from the line of least squares (Figure 7). Acclimation did not alter the apparent  $K_m$  or  $V_{max}$  for  $Na^+/K^+$ -ATPase. The  $K_m$  was 0.39 and 0.32 and the  $V_{max}$  was 4.3 and 4.0 nmoles/mg/hr for 10 and 18°C acclimated trout respectively. This  $K_m$  is similar to that reported for  $Na^+/K^+$ -ATPase in coho salmon gill (Giles and Vanstone, 1976), toad skin (Park and Hong, 1976) and rat liver (Brivio-Haugland et al., 1976).

Kinetic analysis of  $Mg^{2+}$ -ATPase was again hindered by interpreparation variability. Nevertheless the Lineweaver-Burk plots were linear and permitted estimations of kinetic constants. The  $K_m$  was 0.13 and 0.18 and the  $V_{max}$  was 3.17 and 2.54 nmoles/mg/hr for 10 and 18° C acclimated fish respectively. These values are not significantly different but suggest that the  $Mg^{2+}$ -ATPase from 10° C acclimated fish may perform slightly better at 37° C than the  $Mg^{2+}$ -ATPase from 18° C acclimated fish.

Figure 7. Lineweaver-Burk plot of ATPase activity of liver plasma membranes from 10 and 18°C acclimated trout measured at 37°C. Activity was measured in the 3x washed pellet of liver homogenate. A:  $Mg^{2+}$ -ATPase. B:  $Na^+/K^+$ -ATPase. Each line was calculated by least squares estimation from the mean of 3 separate experiments. Each experiment represents material pooled from 3-4 fish.

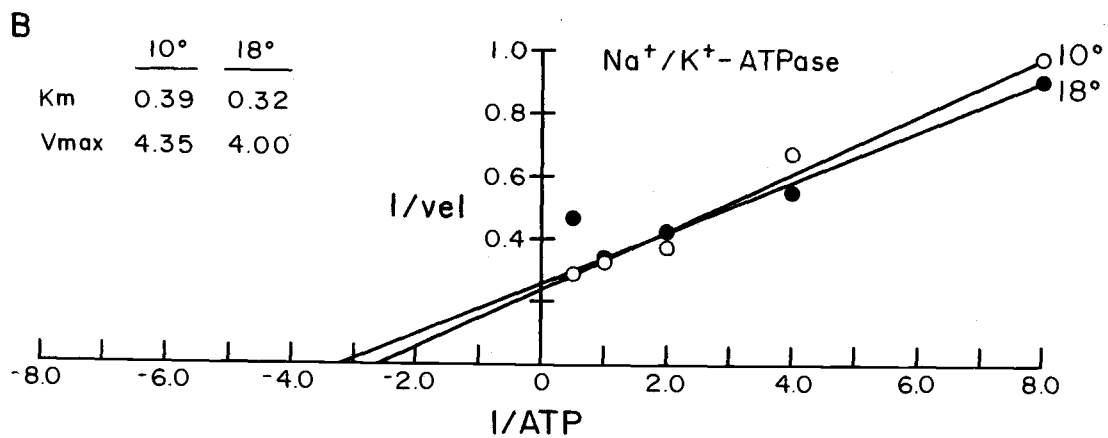
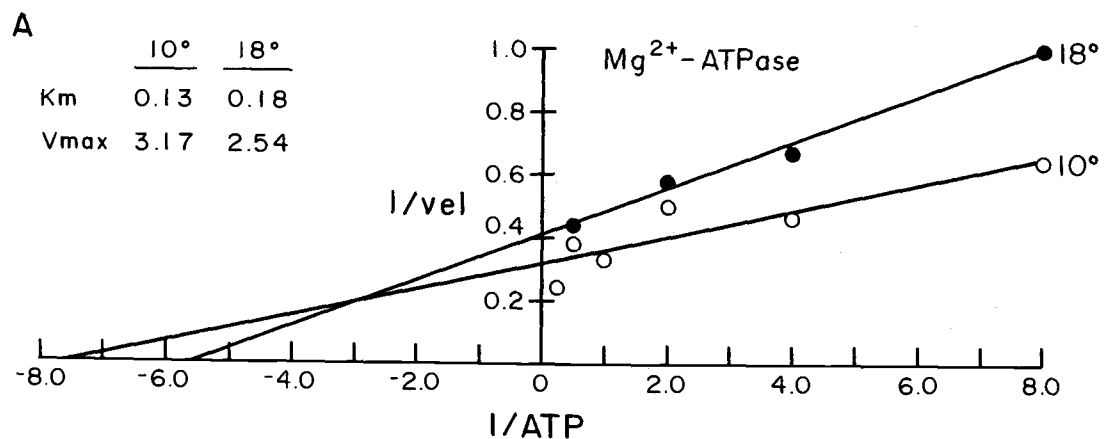


Figure 7.

## DISCUSSION

Biliary excretion of taurocholate was affected by both acclimation temperature and acute shifts in body temperature. This effect was apparently not due to differences in absorption or tissue distribution. Therefore, it must be due to a change in the rate limiting step of taurocholate transport from plasma to bile. The plasma half-lives of 2, 1.8, and 0.9 hr for 10, 14 and 18°C imply an increased rate of hepatic clearance at 18°C. Hepatic clearance for compounds dissolved in plasma is influenced by two independent biological variables: hepatic blood flow and intrinsic clearance (Klaassen and Watkins, 1984). The following equation represents this relationship:  $Cl_H = Q_H \times [(Cl_{int}) / (Q_H + Cl_{int})]$  where  $Cl_H$  is hepatic clearance,  $Q_H$  is hepatic bloodflow and  $Cl_{int}$  is the intrinsic clearance. For compounds with a high intrinsic clearance, hepatic blood flow becomes rate limiting and total hepatic clearance varies in direct proportion to blood flow. Taurocholate has a very high intrinsic clearance in dogs with 90% removed from plasma during a single pass through the liver (O'Maille et al., 1967). This study also clearly demonstrated a linear relationship between taurocholate clearance and hepatic blood flow. The intrinsic clearance of taurocholate in rainbow trout has not been studied to our knowledge but because it is an endogenous bile acid it is quite likely to be high. Therefore, it may well be that hepatic blood flow is rate limiting for taurocholate clearance in rainbow trout. Hepatic blood flow can change dramatically according to the physiological demands

made on the organism (Wilkinson, 1975). In man the normal physiological range is between 0.5 and 2.5 l/min/1.73 m<sup>2</sup> (Wilkinson and Shand, 1975). It is not possible to determine how much hepatic blood flow increased in trout at 18°C vs. 14 or 10°C without calibrating the laser doppler. However, occluding the portal vein immediately reduced the laser doppler voltage by 50%. The voltage reduction for fish measured at 18°C compared to 14 or 10°C was 35-41%. This most likely represents a significant reduction in hepatic blood flow at lower temperatures. This makes sense from a cardiovascular point of view as it has been shown that cardiac rate and cardiac output both decrease as temperature is lowered in rainbow trout (Hughes and Roberts, 1970 and Houston, 1980).

Acute shifts in body temperature appeared to produce an overcompensation in biliary excretion. Fish shifted up 4°C (to 14 and 18°C) had higher rates of biliary excretion than those acclimated and tested at 14 and 18°C. Fish shifted down 4°C (to 14 and 10°C) had lower rates than those acclimated and tested at 14 and 10°C. This phenomenon was not observed for hepatic blood flow. Whether this implies more fundamental structural changes due to acclimation is not known. In attempting to answer this question we measured hepatocyte plasma membrane ATPase activity. It is well known that membrane lipids undergo compositional changes with acclimation. Hazel (1979) showed an increase in unsaturated fatty acids in hepatocyte plasma membranes from 5 vs 30°C acclimated rainbow trout. It is also well known that the temperature responses and kinetic properties of membrane bound enzymes are influenced by their lipid bilayer

microenvironment.  $\text{Na}^+/\text{K}^+$ -ATPase in particular has quite specific lipid requirements for maximal activity (Kimelberg, 1976).  $\text{Na}^+/\text{K}^+$ -ATPase is also a key enzyme involved in sodium-coupled taurocholate uptake into hepatocytes (Blitzer et al., 1982, Scharschmidt and Stephens, 1981 and Schwarz et al., 1975). Our analysis indicated there was no difference in temperature response,  $K_m$  or  $V_{max}$  for this enzyme from 10 and 18°C acclimated trout. However, the ATPase reaction is only a partial reaction of the  $\text{Na}^+/\text{K}^+$ -ATPase and it remains possible that other steps in the transport of sodium or potassium or sodium-coupled taurocholate transport would be affected by temperature-induced changes in plasma membrane lipids.

The function of the  $\text{Mg}^{2+}$ -ATPase associated with many plasma membranes is not known. It is found in high activities at the bile canalicular membrane of rat hepatocytes (Curtis and Mehendale, 1979). Purified plasma membranes from rainbow trout similarly had high  $\text{Mg}^{2+}$ -ATPase activity (Figure 6). Acclimatory effects on the performance of this enzyme if any were slight and do not help explain thermally modulated biliary excretion.

Hepatic blood flow was strongly correlated to biliary excretion rate of taurocholate and provides a sound physiological explanation for the temperature dependent plasma half life and biliary excretion of this endogenous bile acid in rainbow trout.



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