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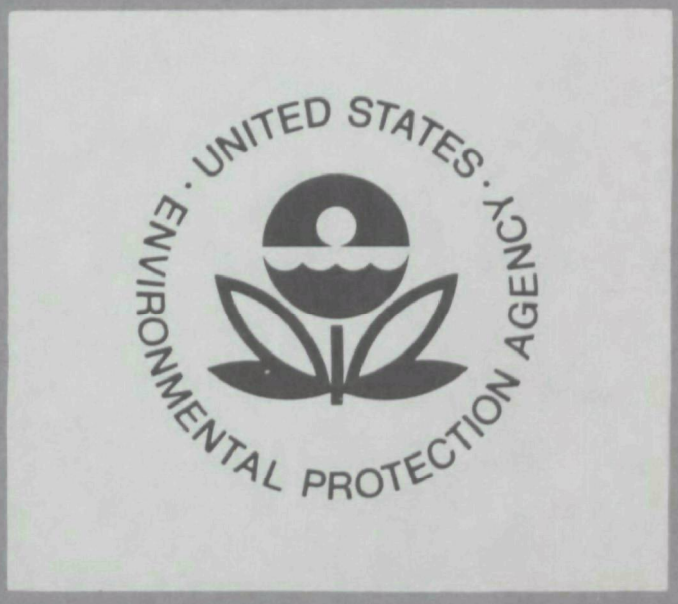
Ecological Research Series

TOXIC EFFECT OF WATER SOLUBLE POLLUTANTS ON FRESHWATER FISH



Environmental Research Laboratory
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TOXIC EFFECT OF WATER SOLUBLE POLLUTANTS ON FRESHWATER FISH

by

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FOREWORD

Our nation's freshwaters are vital for all animals and plants, yet our diverse uses of water -- for recreation, food, energy, transportation, and industry -- physically and chemically alter lakes, rivers, and streams. Such alterations threaten terrestrial organisms, as well as those living in water. The Environmental Research Laboratory in Duluth, Minnesota develops methods, conducts laboratory and field studies, and extrapolates research findings

--to determine how physical and chemical pollution affects aquatic life,

--to assess the effects of ecosystems on pollutants,

--to predict effects of pollutants on large lakes through use of models, and

--to measure bioaccumulation of pollutants in aquatic organisms that are consumed by other animals, including man.

While the results of this project do not provide "numbers" for direct agency use, the study is no less important to EPA. Only by understanding modes of action and physiological pathways can aquatic toxicologists predict effects of pollutants without long and expensive testing. Otherwise our data produced are applicable only to the conditions studied.

This report moves us another step closer to better predictions of toxicity.

Donald I. Mount, Ph.D.
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ABSTRACT

Studies of the effect of inorganic and organic mercury on trout indicated that uptake was primarily via the gills in non-feeding fish. Organic mercury entered fish at a faster rate than inorganic mercury. Exposure of trout to 10 µg Hg/l (methyl form) had no effect on the gill oxygen consumption measured in vitro or on the plasma electrolytes. The hematocrit index increased significantly. Studies of the metabolism of iron by normal and iron deficient trout (made deficient by bleeding) indicated that the liver, spleen, and head kidney are the major iron storage organs. Liver iron was reduced by bleeding whereas splenic iron was unaffected. In iron deficient fish more radioiron appeared in erythrocytes than in normal controls. Studies of isolated-perfused gills revealed the presence of both α and β adrenergic receptors and the data obtained indicate the functional surface area of trout gills can be regulated by changes in perfusion pathway through the gills. Use of perfused gills appears to be a very sensitive model to detect deleterious action of pollutants on fish. Evaluation of heat exchange in perfused gills indicates that the presence of epinephrine increased the transfer maximum of the gill but they were unaffected by the administration of acetylcholine. Analysis of a simple model indicated that the gills may account for as much as 60% of the total heat exchange by trout.

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FIGURES (continued)

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ACKNOWLEDGMENTS

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Principal Investigator: Paul O. Fromm, B.S. Zoology, M.S. Physiology, Ph.D. Physiology all from Univ. of Illinois, Urbana, Ill. Professor, Department of Physiology, Michigan State University, East Lansing, Mi.

Support personnel: All persons other than R.L. Bergman who worked on or were employed on this grant project have been graduate students in the Department of Physiology at Michigan State University. Appointments were usually on a one-half time basis and were entitled 'Special Graduate Research Assistant'. A brief biographical sketch is given with each person listed.

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SECTION 1

INTRODUCTION

The research work supported in part by Environmental Protection Agency Grant No. R-801034 was conducted over a period of approximately three calendar years. There were essentially three general research areas, all of which involved laboratory experiments with rainbow trout. All projects were carried out in the Comparative Physiology Laboratory, Department of Physiology, Michigan State University, East Lansing, Michigan. The results of most of the research work have previously been published and the reader should consult the respective publications for a more detailed discussion than that which appears below. The information on the effects of exposure to mercurials on the ultrastructure of gills is being prepared for publication and the report of heat flux across the gills has been accepted for publication in the Journal of Comparative Physiology.

The experimental animals used in all experiments discussed herein were hatchery-reared rainbow trout (Salmo gairdneri). Fish were obtained from the Michigan Department of Natural Resources hatchery at Grayling, Michigan and a few were supplied by the Great Lakes Fishery Laboratory, Bureau of Sport Fisheries and Wildlife at Ann Arbor, Michigan. The most recent experiments have been conducted with fish purchased from Midwest Fish Farming Enterprises, Inc., Harrison, Michigan. During the period of acclimation to laboratory conditions fish were kept in large (300 liter) fiberglass tanks in flowing dechlorinated tap water at about 12° C under a controlled photoperiod of 16 hours light per day. In our holding facilities the fish were fed commercial trout food, either EWOS or Purina Chow pellets. Subsequent treatment and feeding of fish varied with each experiment and are so indicated below.

SECTION 2

CONCLUSIONS

Twenty-four hour uptake rate of either $^{203}HgCl_2$ or $CH_3{}^{203}HgCl$ by rainbow trout was not affected by esophageal ligation. Uptake of these two mercurials in non-feeding trout appears to be by way of the gills. Methyl mercury enters the fish at a faster rate than the inorganic form and anomalous tissue distribution of these two mercurials suggests that inorganic mercury does not require methylation prior to entry into the fish. In vitro experiments using radioactive mercurials demonstrated high affinity of methyl mercury for red cells, up to 90% was bound to red cells in 40 minutes. Only 9% of inorganic mercury was taken up by red cells, but, this percentage was increased up to 65% if the cells were washed and suspended in Ringer solution prior to incubation with mercury.

Mercury was found in gills of rainbow trout which had been exposed to inorganic mercury but not in those exposed to methyl mercury. No specific site for mercury uptake was identified and it is suggested that inorganic mercury enters the gill across the general lamellar surface. High concentrations were found associated with the gill cartilage. Since little ion diffusion occurs during tissue preparation, localization and/or identification of tissues can be accomplished by scans for various elements: sodium, potassium chlorine and sulfur. The technique is not suitable for identification of highly volatile compounds such as methyl mercury due to the necessity of subjecting tissues to high vacuum conditions, however, electron probe analyses may prove to be useful in studies of active ion transport systems in gill tissue and in investigations of the effects of heavy metal pollutants on fishes.

Exposure of fish to methyl mercury for 12 weeks had no effect on the oxygen consumption of gill tissue or on plasma electrolyte concentration but the hematocrit index increased significantly.

In studies of iron metabolism by control and iron deficient (bled) trout we found that within 24 hours after injection most radioiron was absorbed from the peritoneal cavity and that clearance of iron from the plasma is a 3 component exponential process. The erythropoietic response to bleeding is slow. The incorporation of radioiron into red cells probably represents (a) incorporation of iron for hemoglobin formation and (b) exchange of iron between cytoplasmic non-heme stores and the plasma. The liver, spleen and head kidney are the main iron storage organs in trout and bleeding significantly reduced the liver iron content but had little effect on splenic iron. Iron metabolism in rainbow trout appeared to be a closed, recycling system as essentially no radioiron

was excreted in feces and urine.

The relative functional surface area of isolated-perfused gills was evaluated by measuring the influx of radiocarbon labelled urea, a passively diffusing molecule. The catecholamines, norepinephrine and epinephrine, increase functional gill surface area and decrease overall branchial vascular resistance. Surface area and resistance effects of adrenergic agonists and blocking agents demonstrated the presence of both α and β adrenergic receptors in rainbow trout gills. Stimulation of α adrenergic receptors increased both functional surface area and branchial vascular resistance, while β adrenergic receptor stimulation increased functional surface area but decreased branchial vascular resistance. Acetylcholine decreased functional gill surface area and increased overall branchial vascular resistance. The data presented strongly indicate that the functional surface area of rainbow trout gills can be regulated by changing perfusion pathway with adjustments in the relative vascular resistance across the different pathways.

Casts of the gill vasculature of rainbow trout perfused with Microfil were examined visually. The most conspicuous feature seen was enlargements, called blebs, of the afferent filamental vessels that were located in the outermost region of the interfilamental septa. Several lamellae were supplied by profusely branched afferent lamellar vessels, whereas most lamellae were individually connected to the efferent filamental vessel. Vessels contained in the septal area between filaments were also visualized as well as vessels that ran parallel to the long axis of the filaments.

Heat exchange was evaluated in isolated-perfused second gill arches and the intact branchial basket of rainbow trout. The presence of 10^{-5}M epinephrine in the perfusion solution increased the transfer maximum of the gill suggesting a change in perfusion pathway and/or vascular dimensions. Changes in perfusion flow altered heat exchange by the gill which is again due in part to changes in vascular dimensions. A model for the evaluation of the gills in relation to whole body heat exchange indicated that in the range of perfusion flows from 4 to 20 ml min^{-1} the gill may account for as much as 30 to 60% of the total heat exchange in the animal.

SECTION 3

RECOMMENDATIONS

1. The electron microprobe should be satisfactory for use in investigations of active ion transport systems in gill tissues and in research on the effects of heavy metal ions (pollutants) on fishes. The technique is not suitable for identification of highly volatile compounds such as methyl mercury due to the necessity of subjecting tissues to high vacuum conditions.

2. Incorporation of radioiron into erythrocytes is recommended as a very sensitive parameter for study of the effects of environmental pollutants on fish. The study of any one parameter, however, would probably provide insufficient information on which a meaningful diagnosis could be based, thus it is suggested that determinations of blood hemoglobin and erythrocyte volume (hematocrit) as well as reticulocyte counts be made concurrently with experiments using radioiron.

3. It is highly recommended that the isolated-perfused gill preparation be further developed and standardized for use as an assay procedure to determine the effects of water soluble pollutants on fish. Using this preparation one could quantitatively describe the effects of pollutants on gills in terms of changes in gill permeability to water, gill ion transport and possible biotransformation of pollutants by pulmonary (gill) metabolism.

SECTION 4

EFFECTS OF METHYL MERCURIC CHLORIDE AND MERCURIC CHLORIDE ON RAINBOW TROUT

A STUDY OF UPTAKE PATHWAYS INTO WHOLE FISH

Uptake of mercury by fish can occur via three possible routes, the gastrointestinal (GI) tract, the skin, or the gills. The importance of the GI tract in mercury uptake has been demonstrated by Hannerz (1968), Backström (1969), Miettinen et al. (1969, 1970), Miettinen (1970), and others. Although orally administered mercury was often regurgitated in their experiments, the general consensus was that mercury, especially methyl mercury, is absorbed through the GI pathway. In nonfeeding, freshwater teleosts, which also accumulate high tissue levels of mercury, the amount of mercury absorbed via the GI tract may be limited by a low drinking rate (around 13 µliters/hr per 100 g for a freshwater adapted eel (Maetz and Skadhauge, 1968). However, if exposure to mercury stimulates drinking rates, the GI tract pathway could play a significant role in mercury uptake. To our knowledge there have been no efforts to delineate the importance of each of these pathways in nonfeeding teleosts.

Methods

In order to determine the significance of the GI tract in mercury uptake by nonfeeding trout, the GI pathway was eliminated in one group of fish by esophageal ligation. The fish were divided into three groups: control, sham ligated, and ligated. The purpose of the sham ligation procedure was to determine if any mercury was taken up through the body wall incision. Eight fish from each group were placed into 100 liters of aged tap water than contained 275 ng/liter Hg as either $^{203}HgCl_2$ or $CH_3{}^{203}HgCl$. After 24-hr exposure fish were randomly removed and blood samples taken from the caudal vein. They were then killed and tissue samples from the gills, liver, kidney, heart, skeletal muscle, stomach and intestine were removed and placed into preweighed vials. Tissue activity was determined with a 2-inch thallium activated sodium iodide crystal well detector and Nuclear Chicago analyzer/scaler. Tissue dry weights were also determined. Analysis of the data was done on a Control Data Corp. 6500 computer. One way analysis of variance was used for all statistical comparisons.

Results and Discussion

Uptake of divalent mercuric ions, as well as methyl mercuric ions, occurs primarily by way of the gills. Ligation of the esophagus has no apparent effect on 24-hr accumulation of either form of mercury by any tissue with the

exception of gills from fish exposed to inorganic mercury. In this instance gills from the sham-operated fish had the highest mercury concentration. There was a noticeable trend, although not statistically significant, toward increased tissue mercury concentrations for sham-operated fish in both experiments. The explanation for this is unknown but it possibly could be related to variations in surgical trauma. In both methyl and inorganic mercury-exposed fish, dry weight to wet weight ratios remained constant for all tissues except liver and kidney. Even though there was an increase in hydration of liver and kidney tissue the change was no greater than 1.5% and probably does not reflect gross inward water flux which would be needed to significantly affect tissue mercury concentrations.

Methyl mercury was taken up by all tissues at a much faster rate than inorganic mercury over the 24-hr period and the large concentration differences in gill tissue observed between the two compounds (28 vs. 405 ng Hg/g dry weight) can be explained only by faster penetration of the methylated form. Mucus which continually coats the gill epithelium can act as an ion binding resin to trap mercury and prevent its access to the tissue. Since ambient concentrations of both mercurials were identical for the experiments and assuming both ion species were trapped or precipitated by the mucus, the concentration of inorganic mercury at the gill should be no less than one half that of the organic form. This is based on the assumption that one sulfhydryl (SH) binding site is occupied by each monovalent methyl mercuric ion and a maximum of two SH sites by each divalent inorganic mercury cation.

TABLE 1

THE 24-HR UPTAKE OF MERCURY BY TROUT EXPOSED TO 275 NG/LITER MERCURY AS EITHER CH_3HgCl or $HgCl_2$ (n = 8)

Tissue	Tissue activity (ng Hg/g dry wt.) CH_3HgCl	$HgCl_2$	Ratio Methyl Hg: inorganic Hg
Gill	404.6	28.1	14.4
Liver	29.3	2.6	11.3
Stomach	9.6	2.3	3.5
Intestine	15.9	12.3	1.3
Heart	30.7	2.6	11.8
Kidney	92.1	4.6	19.8
Muscle	2.4	0.2	15.4
Blood	81.8	15.4	5.3

A ratio of gill methyl mercury concentration to gill inorganic mercury concentration (Table 1) of 14.4 indicates that the increased uptake of the methylated form must reflect either entry of mercury into the gill tissue where additional binding sites are available or increased mucus secretion which binds the methylated form on the exterior of the gill. Observations of fish from this and numerous other experiments have shown that methyl mercury does not stimulate greater mucus secretion than inorganic mercury and, in fact, the opposite is often the case. The increased penetration of methyl

mercury into gill tissue may simply reflect the greater lipid solubility of this compound over that of inorganic mercury. This explanation would hold if mercury gains entry into gill epithelial cells across a lipid-containing cell membrane and not via water-filled pores. On the other hand the bivalency of inorganic mercury may promote tighter binding to mucoproteins thereby restricting its diffusion into gill tissue.

Comparison of the distribution of the two mercury compounds within a tissue demonstrates preferential accumulation of mercury based on its chemical form. Ratios of methyl mercury to inorganic mercury in various tissues after a 24-hr exposure are given in Table 1. The magnitude of these ratios is partially attributable to increased uptake of methyl mercury by the gill, however, all the tissues should exhibit approximately the same ratios if there is no selectivity by the tissues for specific forms of mercury. The differences in these ratios show that tissues such as the kidney, muscle, gill, heart and liver preferentially accumulate the methylated form and blood, stomach and intestine do not. Low ratios for the latter three tissues could be due to selective accumulation of the inorganic form which is masked by higher uptake rates of the organic compound. Using radioautography Halbhuber et al. (1970) have demonstrated selective accumulation of radiomercury by the Paneth cells in the intestine of the rat and guinea pig. It was postulated that one function of these cells is to transfer divalent heavy metal ions from the body fluids into the lumen of the gut. Similarly Paneth cells in the trout gut might sequester divalent mercury but not methylater forms.

Backström (1969) administered methyl and inorganic mercury intravenously to speckled trout. After 24 hr the distribution patterns of methyl mercury were similar to our data for rainbow trout with the exception of the low values for the gills. He reported highest inorganic mercury concentrations in the kidney; values for blood and gill were about 50% lower. These small discrepancies in distribution when compared to data presented here are probably due to the mode of administration of the mercurial. The tissue mercury distributions reported by Hannerz (1968) for cod exposed for 24 hr to waterborne mercurials are similar to those found in the present study. He postulated gill uptake as the principal mode of entry of mercury into cod and pike.

The wide variation in organic/inorganic tissue concentration ratios that we noted supports the idea that inorganic mercury can cross the gill epithelium as divalent mercury ions or as some complex without prior methylation. The present experiments have eliminated the GI tract as a major pathway for mercury uptake in nonfeeding trout and it is widely accepted that the skin is relatively impermeable (Prosser, 1973, pp. 48).

UPTAKE OF MERCURY BY TROUT ERYTHROCYTES IN VITRO

Methods

Heparinized whole blood or washed cells were placed in 25-ml stoppered Erlenmeyer flasks. The mercury solution was added to the blood at time zero and the flasks stirred at 16 rpm with a multipurpose rotator. At timed intervals 0.2 ml of blood was removed and placed in a 0.4 ml polyethylene

microcentrifuge tube. Packed red cells were then resuspended in Ringer solution, centrifuged and the subsequent supernatant was placed in a third microcentrifuge tube. Hematocrits were determined in duplicate for all blood samples incubated. The concentration of mercury from the packed red cells, plasma and wash was determined by scintillation counting. Percent uptake of mercury into the cellular fraction was standardized by correction for equal volumes of red cells and plasma. All glassware used was coated with silicone to minimize absorption of the mercurial onto the glass.

Results and Discussion

The percent uptake of both mercurials into erythrocytes as a function of time is plotted in Figure 1. With the exception of methyl mercury incubated with whole blood, equilibrium was reached in less than 10 minutes. Uptake of methyl mercury did not reach equilibrium when incubated with whole blood until after 30 to 40 minutes. Analysis of this reaction revealed a two-component uptake system, the first having a half time of under 1 min, the second about 11 min. (Uptake values of less than 1 min are only estimates due to the inherent time lag incurred during blood centrifugation and separation.) This can be explained by initial binding of a small percentage of mercury to plasma proteins or thiols, the remainder being taken up by the red cells as a function of the free or nonplasma bound mercury. After the red cells have removed most of the unbound mercury from the plasma a new equilibrium (slow component) is established that is dependent on the dissociation of bound mercury from 'plasma sites' prior to red cell uptake. This postulate is further supported by the mode of uptake of methyl mercury into red blood cells suspended in Ringer solution. In this instance the uptake rate is so fast that only one component can be determined. The increased CH_3Hg^+ binding at equilibrium by washed cells in Ringer solution compared to cells in plasma (95% vs. 90%) is probably indicative of some irreversible mercury binding to plasma protein. Failure of the washed cells to bind 100% of the mercury could be due to slight hemolysis coupled with some mercury binding by the incubating media or absorption on glassware.

Inorganic mercury incubated with whole blood binds almost exclusively to the plasma fraction; less than 9% was taken up by red cells after 1440 min (not shown in Figure 1). Washed cells suspended in Ringer solution take up 9-10 times more inorganic mercury than cells do in plasma. Removal of plasma proteins undoubtedly is the critical factor. There is also a significant difference in percent uptake by red cells that is dependent on the suspending media; cells suspended in Abbott Ringer take up less Hg^{++} than cells suspended in phosphate buffered Ringer solution. Since the only difference is the presence of magnesium and the buffering capacity of the PBR either the magnesium or pH or perhaps both factors must enhance equilibrium binding by red cells.

In other experiments it was shown that uptake of either methyl or inorganic mercury was, in all instances, independent of the length of the incubation period which preceded addition of mercury to the cell suspension. Thus, secretion of a chelator by trout red cells is not a factor in the failure of these cells (especially washed cells) to bind all mercury present. As

indicated in Figure 1 less than 100% of the available methyl or inorganic mercury was taken up by red cells in these experiments.

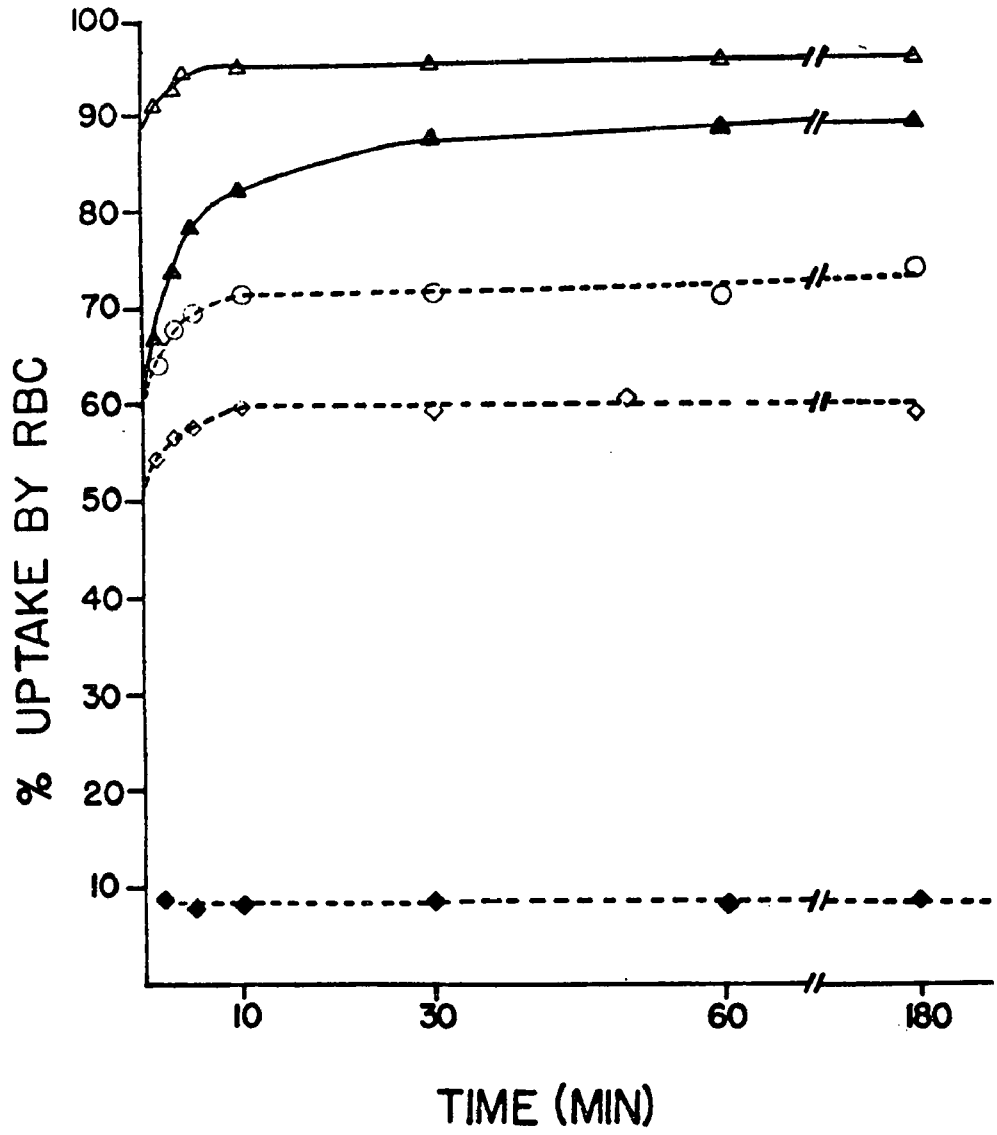

Figure 1. Percent uptake of methyl mercury or divalent mercury by red blood cells as a function of time. Δ = CH_3HgCl incubated in whole blood (15); = $HgCl_2$ in whole blood (6); Δ = CH_3HgCl incubated with washed cells suspended in Abbott Ringer (4); = $HgCl_2$ incubated with washed cells suspended in Abbott Ringer (4); ° = $HgCl_2$ incubated with washed cells suspended in Phosphate Buffered Ringer (4). Exposure concentrations approximately 0.0133 total ppm CH_3HgCl and 0.054 ppm total $HgCl_2$. Number of observations (N). Standard errors are not shown on the graph as they were smaller than the diameter of the data points.

It appears that saturation of trout erythrocytes with mercurials must involve either (1) some group(s) such as SH, amono, or other which are responsible for cell membrane integrity and are not saturated at the time of hemolysis or (2) a disruption of ionic balance and subsequent osmotic hemolysis caused by mercury levels below those required for total cell saturation. SH-containing compounds such as albumin, cysteine and reduced glutathione failed to remove either inorganic or organic mercury previously bound to red cells, which suggests strong mercury-red cell binding.

MERCURY UPTAKE AND ION DISTRIBUTION IN GILLS OF RAINBOW TROUT

Efforts to localize and/or characterize the pathway of uptake of mercury in gills of trout using radioautographic techniques were largely unsuccessful. Autographs of gills of fish exposed to either methyl or inorganic mercury exhibited a diffuse grain pattern which precluded identification of the uptake site. Lack of grain density in areas containing many chloride cells probably indicates that these cells are not involved in the uptake of either mercurial. The thinness of lamellae and the small interlamellar distances prevented localization of preferential mercury binding sites. Dense grain patterns due to $^{203}Hg^{++}$ were found associated with the filamental cartilage. Our second attempt to localize the gill pathway for mercury uptake was done with the electron microprobe. The following is a report of these experiments.

Methods

Fish were exposed to the mercurials which were added to water in 100-liter tanks. The concentrations used were 0.25 and 0.05 ppm Hg for inorganic and organic mercury, respectively. After 23-hr exposure a second amount of mercury equal to the first was added to the tanks and fish were removed 1 hr later and killed. The second pair of gill arches were removed from mercury-exposed fish within 15 sec after the fish was killed. The arches were quick frozen in isopentane which has been cooled to near -160° C with liquid nitrogen. Details of mounting, embedding, sectioning and preparation of sections for use in the ARL Microprobe are given by Olson and Fromm, 1973. Tissue scans and image scan micrographs were recorded on Polaroid 200 black and white film and the line scans were recorded on a Hewlett Packard x-y recorder. Low peak to background rations during mercury analysis made the tissue scan procedure difficult to interpret, however, line scan procedures proved adequate for mercury identification. Line and tissue scans were also used for detection of other elements as both an aid in tissue orientation and a check on diffusional ion movements which might have occurred during sample preparation. The 0.5 μ diameter of the primary electron beam permits fairly high resolution in the localization of ions within a given area especially with the line scan procedure. All analytical data obtained were qualitative or only semiquantitative as the refinements and sophistication required for quantitative determinations were beyond the scope of this preliminary study.

Results and Discussion

For the line scans as the beam entered the area known to contain large numbers of chloride cells, concentrations of all ions except mercury

increased. Interlamellar areas contained definite mercury peaks and the location of lamellae was marked by increases in the concentrations of Na, K, Ca and Cl. Mercury content appeared to be inversely related to concentrations of the other elements and it did not increase until the beam entered the space normally filled by environmental water. This confirms results of radioautographic studies and supports the concept that inorganic mercury is not selectively taken up by chloride cells nor concentrated by them in 24 hr. It should be stated that we were unable to detect mercury in gills from tout exposed to methyl mercury even though our previous study showed that fish exposed to methyl mercuric chloride contained 10 times more mercury than those fish exposed to mercuric chloride. The inability to detect mercury after methyl mercury exposure was probably due to the loss of this element during tissue preparation. Crystals of methyl mercury will sublime at room temperature, therefore, mercury could be lost during freeze drying, carbon evaporation or in the vacuum volumn of the microprobe. All of these procedures require a high vacuum.

The mercury peaks observed were due in large part to mercury which was added 1 hr prior to sampling and demonstrated the effectiveness of the interlamellar mucus to trap ions. Presumably mercury is contained in the mucus from which it can either enter the gill or be eliminated with the sloughed off mucus. McKone *et al.* (1971) exposed goldfish to 0.25 ppm $HgCl_2$ for 3 hr and found that 79.3% of the mercury in excised gills was removed by washing with 80% ethanol. They concluded that this fraction represented mercury trapped by the mucus coating the gill, however, they did not examine gill tissues after the ethanol wash to verify that only mucus was removed. Gills are very fragile and it is quite possible that some epithelium was also removed in the wash process.

Tissue scans of other material showed that mercury concentrations were highest in cartilage and were correlated with high calcium, sulfur and phosphorus levels. Microprobe (and radioautographic) studies have clearly indicated that there is no preferential accumulation of mercury in lamellae. It appears that mercury can enter gills through the general lamellar surface, thus active uptake is probably of little consequence in mercury influx. Once in the filament, much inorganic mercury is accumulated by cartilagenous tissue. Other gill tissues exhibit no preferential uptake of mercury with respect to a given cell type, i.e. epithelial cells, pillar cells, or chloride cells.

Preparation of tissues for electron microprobe analysis avoids the use of various aqueous solvents as are commonly employed in routine analytical histochemistry. Thus, localization of many ions (e.g. small molecular weight monovalents) with the probe probably reflects a fairly true tissue distribution of these ions. We suggest that electron probe analyses should prove beneficial in a study of ion distribution in chloride cells of fish exposed to various salinities or to pharmacological agents. Further information on the ion transfer role of mucus, which covers the surface of chloride cells from marine species, could be gained by studies utilizing the electron microprobe.

EFFECT OF METHYL MERCURY ON GILL METABOLISM AND BLOOD PARAMETERS OF TROUT

The gills of freshwater teleosts function as the primary site for the active absorption of ions from the external media and for the exchange of respiratory gases. Large amounts of water must be passed over the gill surfaces to meet the oxygen demands of the fish consequently when fish are in contaminated waters their gills can be exposed to large amounts of water soluble pollutants. The experiments discussed below were performed to determine if methyl mercury has any effect on the metabolism (oxygen consumption) or physiological function (plasma electrolyte regulation) of the gill.

Methods

The experimental design involved exposing a group of starved fish to 10 µg Hg/liter administered as methyl mercuric chloride while another group of starved fish served as controls. The mercury was administered to the experimental fish using a gravity feed system in which the flow rate of the concentrated mercury solution into the tank was correlated to the flow of water through the tank to maintain a concentration of 10 µg Hg/liter. At the end of 4, 8 and 12 weeks the fish were killed and determinations of the following parameters were made: hematocrit, oxygen consumption of gill tissue, protein content of gill sample and plasma electrolyte concentrations (Na^+, K^+, Cl^-, Mg^{++} and Ca^{++}). Plasma sodium and potassium were determined on a Beckman flame photometer, magnesium and calcium were determined on a Perkin Elmer atomic absorption spectrophotometer and chloride was measured with a Buchler chloridometer. All plasma electrolyte determinations were done in duplicate. Samples of gill filaments from the second and third branchial arches and were suspended in 3 ml of 10% and 100% phosphate buffered saline (PBS). The oxygen consumption of the filaments was then measured polarographically at 12° C with a YSI biological oxygen monitor. Calculation of oxygen consumption was based on the solubility of oxygen in 10% and 100% PBS at 12° C and the percent of initial oxygen consumed during a 10 minute period. Following oxygen consumption measurements the 3 ml samples of PBS containing the gill filaments was sonified to remove as much tissue as possible from the cartilaginous skeleton and the protein content of the mixture was determined.

Results and Discussion

Results from this investigation (Table 2) indicate that up to 12 weeks exposure to methyl mercuric chloride (10 µg Hg/liter) does not significantly affect the *in vitro* metabolism of the gill or the concentration of plasma electrolytes in rainbow trout. There was a significant increase in the hematocrit of experimental fish after 12 weeks exposure. Webb (1966) pointed out that many mercurials are known to cause hemolysis of RBC's which would tend to cause a decrease in the hematocrit index, not an increase as we found in our experiments. Possibly methyl mercury directly stimulated erythropoiesis causing an increase in hematocrit of trout or the fish may have overcompensated erythropoietically in response to RBC loss resulting from exposure to mercury.

TABLE 2

VALUES FOR SOME BLOOD PARAMETERS OF CONTROL AND MERCURY EXPOSED (10 NG Hg/LITER) RAINBOW TROUT

Blood Parameter	Length of Exposure (Weeks) 4	8	12
Plasma Na^+ (meq/L)			
Control	140.08±1.35(12)	142.68±3.48(8)	139.37±3.13(8)
Hg-treated	142.58±1.93(12)	149.20±1.83(12)	133.75±7.66(8)
Plasma K^+ (meq/L)			
Control	2.74±0.17(12)	1.36±0.22(8)	1.70±0.27(8)
Hg-treated	2.61±0.13(12)	1.42±0.19(12)	1.77±0.36(8)
Plasma Cl^- (meq/L)			
Control	123.36±0.95(12)	122.02±5.13(8)	121.75±4.00(8)
Hg-treated	123.27±2.07(12)	128.73±1.71(12)	110.85±11.14(8)
Plasma Ca^{++} (meq/L)			
Control	4.36±0.28(12)	4.00±0.15(8)	3.76±0.15(8)
Hg-treated	3.97±0.25(12)	4.15±0.26(12)	3.73±0.25(8)
Plasma Mg^{++} (meq/L)			
Control	1.82±0.05(12)	1.96±0.04(8)	2.15±0.09(8)
Hg-treated	1.66±0.09(12)	2.02±0.06(12)	1.92±0.23(8)
Hematocrit (% RBC)			
Control	19.44±1.28(12)	19.59±2.63(8)	21.31±1.46(8)
Hg-treated	18.00±0.88(12)	21.52±1.38(12)	30.34±2.40(8)

Mean±S.E.(N)

Although there were no significant differences in plasma electrolyte levels for paired comparisons between control and mercury treated fish after 4, 8 and 12 weeks exposure, over the 12 week period several of the electrolytes did vary considerably. Plasma sodium for controls remained fairly constant at about 140 meq/liter while those for experimental fish varied between 133 and 149 meq/liter. This greater variability in the experimental fish may represent some effect of methyl mercury on the active transport of sodium by gills but further work is needed to substantiate this hypothesis. No plausible explanation can be offered for the increase in plasma magnesium levels of control fish after 12 weeks exposure.

Gill samples from control and mercury exposed fish measured in 100% PBS showed a significantly higher rate of oxygen consumption than samples measured in 10% PBS (Table 3). This could be the result of the sudden transition of gill tissue from fresh water (5-10 mOsm) to 100% PBS (285-290 mOsm) which represents both an osmotic shock and a probable increased salt load for the tissue. Chloride cells present in the gill filaments could conceivably have responded with an increased rate of active transport to maintain osmotic equilibrium and thus account for at least some of the difference in oxygen consumption. It is interesting to note that the decrease in oxygen consumption for control and mercury treated fish in 10% PBS occurred during the same period of time that a decrease in plasma potassium concentration was

seen. It would appear that these changes represent some form of accommodation to the effects of starvation since they were observed in both control and experimental fish.

TABLE 3

IN VITRO OXYGEN CONSUMPTION OF GILL TISSUE FROM CONTROL AND MERCURY EXPOSED (10 µG Hg/LITER) RAINBOW TROUT IN 100% AND 10% PHOSPHATE BUFFERED SALINE (PBS)
Data expressed as µl O_2 consumed/hr/mg protein.

Sample	Length of Exposure (Weeks)		
	4	8	12
100% PBS			
Control	17.35±0.74(12)	15.56±0.98(8)	16.47±2.07(8)
Hg-treated	18.53±1.37(12)	14.48±1.15(12)	17.27±1.15(8)
10% PBS			
Control	14.13±0.78(12)	8.74±0.53(8)	9.78±0.77(8)
Hg-treated	13.13±0.64(12)	8.64±0.50(12)	9.57±0.68(8)

Mean±S.E.(N)

The data presented above seems to indicate that longterm exposure to methyl mercuric chloride does not alter the metabolism of the gill or affect its role in plasma electrolyte regulation. Such a generalization may at best be premature. McKim et al. (1970) reported significant changes in seven blood parameters of brook trout after exposure to three different concentrations of copper for periods of 6 and 21 days, however, when the experiment was extended to 337 days five of the seven parameters were similar to control values. It is possible that methyl mercury exerted a similar transient effect on the fish in this study and remained undetected or possibly a longer period of exposure is necessary before significant changes in oxygen consumption or plasma electrolyte concentrations occur. The need for longer term studies with shorter time intervals between samplings is evident and would do much to further our understanding of the physiological effects of mercurials on fish.

EFFECT OF MERCURY EXPOSURE ON RAINBOW TROUT GILL ULTRASTRUCTURE

Several investigators using light microscopy and standard histological techniques have described the gross histopathological or structural changes in the gill epithelium after exposure of fish to mercurials. Schweiger (1957) found that fish exposed to 0.03 mg Hg/liter survived longer than seven days and considered this concentration harmless. At higher concentrations the survival time was progressively shortened and massive damage to the respiratory epithelium was observed. Below is a description of some morphological changes that occurred in the ultrastructure of gills of rainbow trout which were exposed to methyl mercuric chloride and mercuric chloride. Using light microscopy, no significant histopathological changes were noted in these gills when compared to untreated controls.

Methods

Rainbow trout which had been exposed to monomethyl mercury for 4 weeks or longer were obtained from the Great Lakes Fisheries Laboratory, Bureau of Sport Fisheries and Wildlife, Ann Arbor, Michigan. These fish ranged from 35 to 60 g and were exposed to an average concentration of 200 ng Hg/liter (as CH_3HgCl) at 5, 10 and 15° C in a constant flow bioassay apparatus. Gill samples from these fish were collected and fixed at Ann Arbor. For transmission electron microscopy (TEM) individual gill filaments were dissected from supporting tissue of the second gill arch of a freshly killed fish, fixed in 4% glutaraldehyde buffered with Sørensen buffer and post fixed in 1% osmium tetroxide. The tissues were then dehydrated in graded concentrations of ethanol or acetone and embedded in Spurrs embedding media. Thin sections were stained in uranyl acetate and lead citrate and were examined with a Philips EM 300 electron microscope. For scanning electron microscopy (SEM) the second gill arch (contralateral) side was removed, rinsed with tap water and fixed in 50% glutaraldehyde. After six rinses of 30 minutes each in Sørensen buffer the tissues were either frozen in isopentane cooled with liquid nitrogen and subsequently lyophilized or they were dehydrated with ethanol and amylacetate and dried by the critical point method. Dried filaments were separated from the supporting cartilage, attached to coverslips and coated with carbon and gold palladium. The tissues were then examined with an AMR Model 900 scanning electron microscope.

Results and Discussion

For detailed descriptions of normal rainbow trout gill ultrastructure the reader should consult Olson and Fromm (1973) for SEM work and Morgan and Tovell (1973) for TEM observations. Investigations with the SEM indicated that a 24 hr exposure to 0.05 mg/liter methyl mercury produced some slight disarrangement of the microridges present on the outer surface of lamellar epithelial cells although junctions between epithelial cells appeared essentially normal. This same exposure resulted in what is believed to be an increase in the degenerative process in chloride cells which, in SEM micrographs is typified by loss of surface microvilli. There was also an increase in the number of degenerating chloride cells in gills of fish exposed to 200 ng/liter methyl mercury for 4 to 8 weeks. Exposure to $HgCl_2$ produced somewhat similar changes in the surface features of chloride cells and also led to the appearance of many smooth non-ridged lamellar epithelial cells.

Observations with the TEM revealed that exposure to 0.05 mg/liter methyl mercury had relatively little effect on the appearance of the epithelial microridges which appear more like villi in TEM micrographs. Some vacuolation of chloride cells was apparent but many of these cells appeared normal. A 4 week exposure to 200 ng/liter methyl mercury produced red cell vacuolation and slight loss of epithelial cell ridge integrity. The vascular space was often filled with debris, possibly the contents of ruptured red cells. Degenerating or abnormal chloride cells appeared to be present in greater numbers but normal chloride cells were also present. Extensive vacuolation of chloride cells was noted in fish exposed to 200 ng/liter methyl mercury for 8 weeks. This vacuolation extended throughout the cell and appeared to be

associated with the smooth endoplasmic reticulum (SER) of these cells. In addition to their becoming vacuolated these cells were often detached from subepithelial structures. Many red cells in fish exposed to this concentration appeared to be normal but vacuolation was not uncommon. Chloride cells in gills of fish exposed for 5 days to 0.25 mg/liter inorganic mercury and those exposed to lower concentrations for longer periods of time showed extensive vacuolation and loss of surface microvilli. Small vacuoles with a low electron density were found near the apical area of the chloride cells, whereas, large (up to 0.5 μm diameter) vacuoles were located in the basal portion of the cell and were more electron dense. This is in contrast to methyl mercury exposed chloride cells in which the small vacuoles were present throughout all but the extreme apical portion of the cell. This difference may reflect different penetration rates of these two mercurials or different biochemical interactions. Cross sections through the smooth areas on the lamellar epithelial surface revealed that these areas were the outer or apical surface of large cells which appear to push apart and replace regular epithelial cells on the lamellar surface. These smooth cells are somewhat larger and less electron dense than normal epithelial cells; they contain a relatively large nucleus, numerous mitochondria and cytoplasmic vacuoles. Except for the absence of a slightly ridged epithelial surface, these cells are similar to the 'new' type of epithelial cell seen in methyl mercury exposed fish.

SECTION 5

IRON METABOLISM

METABOLISM OF IRON BY NORMAL AND IRON DEFICIENT RAINBOW TROUT

Iron is an essential element involved in the physiological functions of oxygen transport and cellular respiration. An enormous volume of literature has resulted from measurements made on human subjects and rats which deals with iron absorption, the mode of iron transport via the plasma, and the utilization and conservation of iron. There is, however, relatively little information concerning iron metabolism in lower vertebrates. The study reported here was designed to increase our knowledge of the metabolism of iron in salmonidae. The objectives were: (1) to study iron distribution and utilization in normal control and iron deficient trout over a 30 day period following an intraperitoneal (i.p.) injection of ^{59}Fe, (2) to investigate the absorption of iron into red blood cells and (3) to evaluate the relative importance of various iron storage pools in providing iron to be used during time of increased erythropoietic activity by head kidney tissue.

Methods

One group of fish were made iron deficient by removal of 40% of the blood volume in four bleedings of 10% of the blood volume over a seven day period with 48 hours between bleedings. On the day of the last bleeding both experimental and control fish received an i.p. injection of 1 $\mu Ci\ ^{59}FeCl_3$/100 g fish in 0.9% saline. Six experimental and six control fish were sacrificed 1, 2, 4, 8, 11, 16, 23, and 30 days following ^{59}Fe injection. Blood samples were taken via the hemal arch and fish were killed with a sharp blow to the head. Tissue samples were taken and weighed using a Roller-Smith Balance. Intestinal contents were tare weighed on a Mettler Model B5 Balance. Tissues and intestinal contents were stored at 0°C in glass culture tubes until analyzed for total iron and/or ^{59}Fe content.

The percentage of pack red blood cells was determined by the microhematocrit method and hemoglobin by the cyanmethemoglobin method using Hycel (Houston, Texas) standards and reagents. Reticulocyte counts were made on blood smears prepared by supra-vital staining. After staining a total of 1000 cells, both immature and mature red cells, were counted using an ocular grid. The number of reticulocytes was expressed as a percentage of this population. Plasma iron was determined colorimetrically and expressed as µg Fe/100 ml plasma (µg%). Plasma from any hemolyzed samples was discarded. The plasma total iron binding capacity (TIBC) was calculated from the plasma iron content and the unsaturated iron binding capacity (UIBC). Tissue iron was measured colorimetrically after wet-ashing in sulfuric-nitric acid (5:1). Total blood volume and tissue blood volume was determined using ^{51}Cr-labelled RBCs. The tissue blood volumes were used to correct tissue ^{59}Fe activity and total iron concentration for the blood iron content. To evaluate

absorption of radioiron by the intestine the method of De Benedetto and Farmanfarmaian (1975) was used to measure renal excretion of radioiron the urinary bladders of trout were cannulated and urine collected in a manner described by Fromm (1963).

Results and Discussion

With respect to normal control rainbow trout the data presented in Table 4 indicate that the spleen has the highest iron concentration but the liver due to its mass, represents the prime storage area for iron in trout. Tissue iron concentration and/or total iron content corrected for blood iron content remained constant over the 30 day study period. Plasma contained very small amounts of iron but plasma does contain transferrin, the iron transport protein which shuttles iron back and forth between the various iron pools, thus it plays an essential role in the metabolism of iron by trout.

TABLE 4

CALCULATED VALUES FOR THE IRON CONTENT OF TISSUES OF A HATCHERY-REARED 100 G RAINBOW TROUT

Tissue	µg Fe/g (X + S.E.)	µg Fe Total
Liver	168 ± 13	185.0
Spleen	381 ± 46	81.2
Head kidney	122 ± 11	24.3
Caeca	36 ± 3	45.3
Intestine	34 ± 3	14.3
Plasma	0.55 ± 0.03	1.2

The clearance of iron from the plasma of rainbow trout following i.p. injection of ^{59}Fe was determined. When plotted as log concentration against time, radioiron content of the plasma presents the clearance curve seen in Figure 2 upper left. The curve is composed of three exponential components C_1, C_2 and C_3, similar to those characterized for human plasma iron clearance. The equation for the line is given at the top of Figure 2 where: C_t = fraction of the initial dose of ^{59}Fe present in the plasma at time t; Co_1, Co_2, Co_3 = y-intercepts for components 1, 2 and 3 expressed as decimal equivalents of initial dose; k_1, k_2, k_3 = rate constants for components 1, 2, and 3; e = base of natural logarithms and t = time in days.

The first component (C_1) with a half time (T1/2) of 5.98 hours represents the flux of radioiron-labelled transferrin from the plasma into the tissue extravascular spaces. The second component with a half-time of 1.16 days represents the return of ^{59}Fe-transferrin to the plasma pool, a process which is responsible for the change in the slope of the clearance curve between days 2 and 8 following i.p. injection of radioiron. The attainment of equilibrium between radioiron in the plasma pool and the erythropoietic and storage labile iron pools occurs after day 8 and is represented by the third component of the plasma clearance curve (t1/2 = 39/38 days). As indicated above fish were made iron deficient by removal of 40% of their blood volume. No fish died as a result of the bleeding. Recovery from hemorrhagic shock was

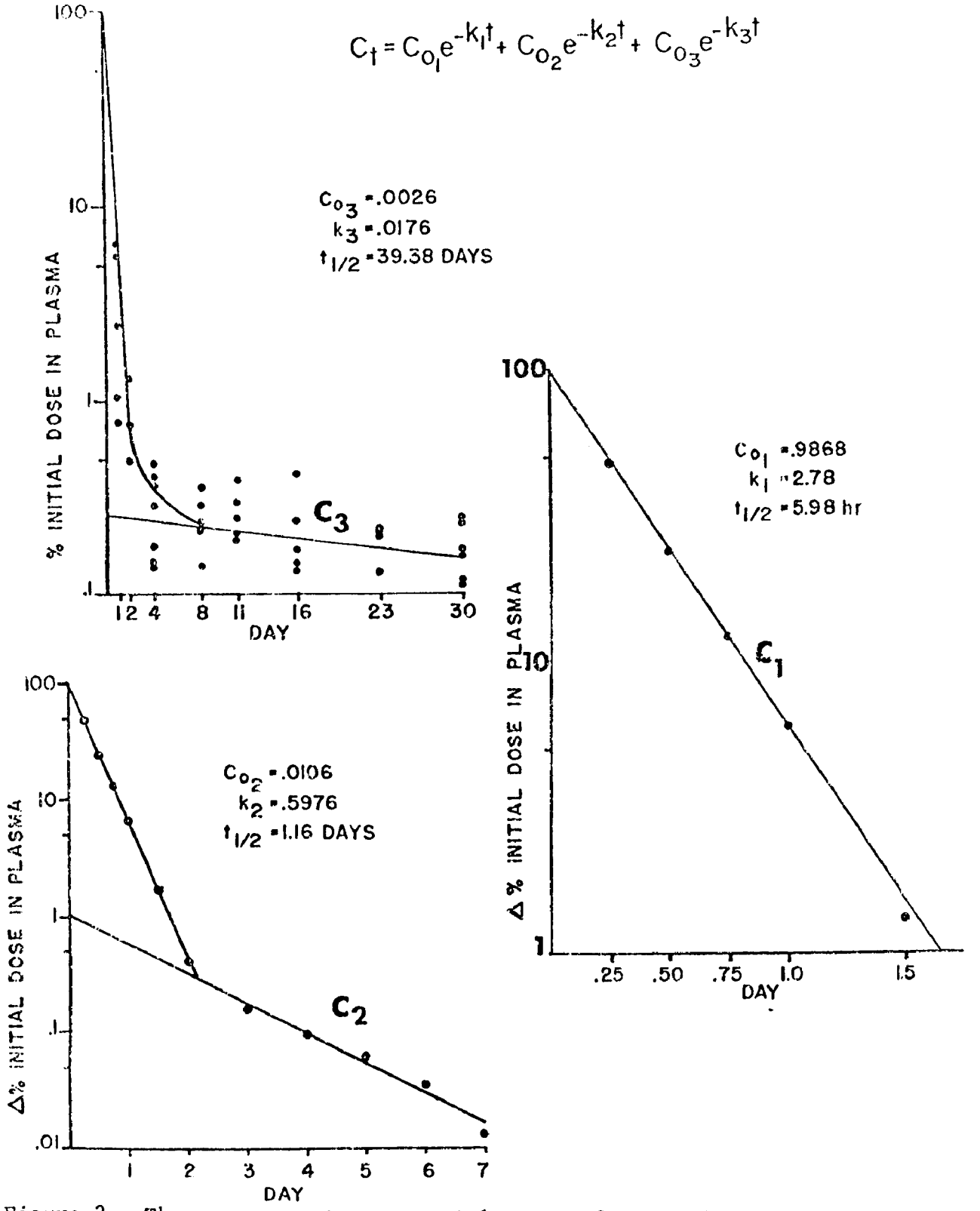

Figure 2. Three component exponential curve of iron clearance from plasma of rainbow trout following i.p. injection of ^{59}Fe. Each component is graphed separately to show its y-intercept (Co), T1/2 and slope (k). Definitions of the symbols used in the equation are given in the text.

rapid and fish were actively swimming within minutes after bleeding. Some fish gupled air at the surface but the majority exhibited normal activity. There was very little change in total iron concentration in the various tissues except the liver during the 30 day period following the final bleeding and averages for the different tissues did not differ from those for controls. The iron conent of the liver dropped significantly after day 11 and was significantly below that of controls. The plasma clearance curve for iron deficient fish was similar to that shown in Figure 2. The T1/2 for the C_1 component appeared to be shorter than the control but statistically there was no difference between the two curves.

The movement of ^{59}Fe to and from the extravascular spaces of tissues is indicated by plots of data in Figures 3 and 4 for controls and iron deficient fish respectively. It is evident that ^{59}Fe activity of tissue is in equilibrium with the iron pool of the plasma some 8 days after administration of radioiron. The clearance of ^{59}Fe from spleen, head kidney and cecal tissue was rapid and muscle (not shown) was similar in that respect. The specific activity of muscle iron was the lowest of any tissue and only 3 to 5% of the initial injected activity remained in the total muscle mass 16 days after injection (controls). In contrast, an average of 15% of the initial injected radioactivity remained in the liver of controls over the 30 day experimental period and the specific activity of iron in the liver was higher than that for any tissue from day 4 through day 30 (Figure 3). This indicates that a significant amount of ^{59}Fe was deposited and/or stored in the liver and remained there throughout the 30 day study period. In iron deficient fish the initial increase in liver radioiron between days 1 and 4 is similar to that seen in controls but thereafter the ^{59}Fe activity decreased steadily in the liver of bled fish whereas it remained unchanged in controls. There is some indication that the head kidney of bled fish is more active in sequestering ^{59}Fe than those from untreated controls. Mean values for specific activity (and % of initial dose given) are higher in the experimental fish head kidney than in the controls but since these values were not statistically different we are unable to state that bleeding had any verifiable effect on head kidney tissue. Thus the experiments with iron deficient trout tend to indicate that the liver contains the most important labile iron storage pool which can be utilized for the accelerated erythropoietic process. We found that the spleen iron stores were not significantly reduced following bleeding thus this storage pool apparently is non-responsive to acute induced iron deficiency.

Between 70 and 80% of the injected dose of radioiron was found in the red blood cells of bled fish by day 16. The difference in red cell iron uptake between the bled and control groups was statistically significantly different. The peak erythrocyte radioiron content coincided with the increase in reticulocyte population (Table 5). Following day 16 there was a decline in red blood cell radioactivity similar to that seen in control fish. In cells from control fish initial uptake of ^{59}Fe was rapid and reached a maximum of 55 to 60% on the initial injected activity by day 11.

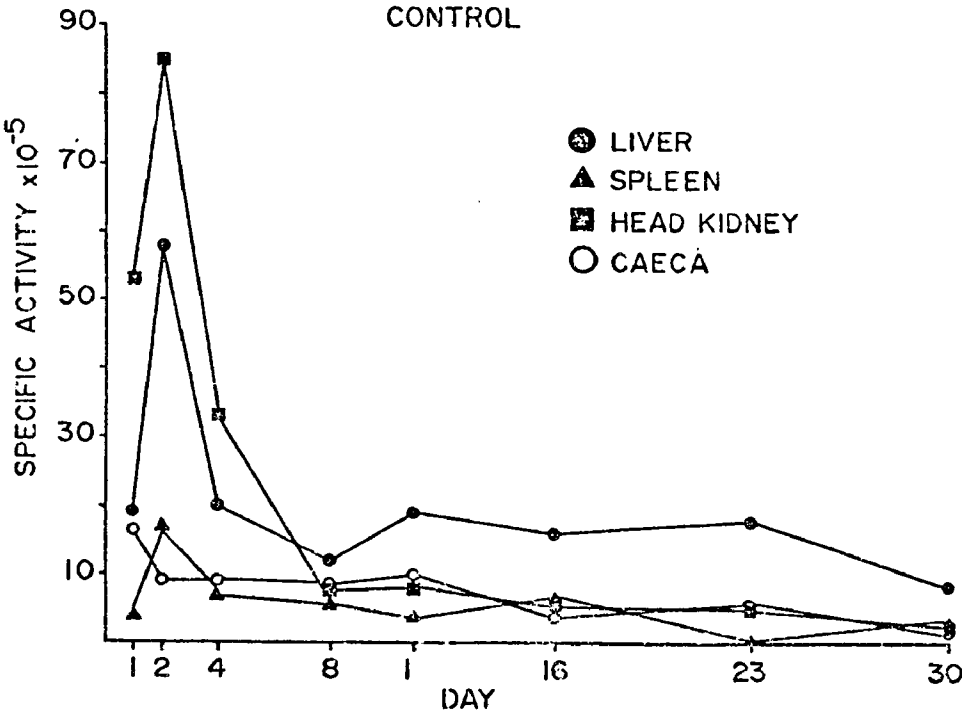

Figure 3. Specific activity of iron in tissues of trout following intraperitoneal injection of ^{59}Fe.

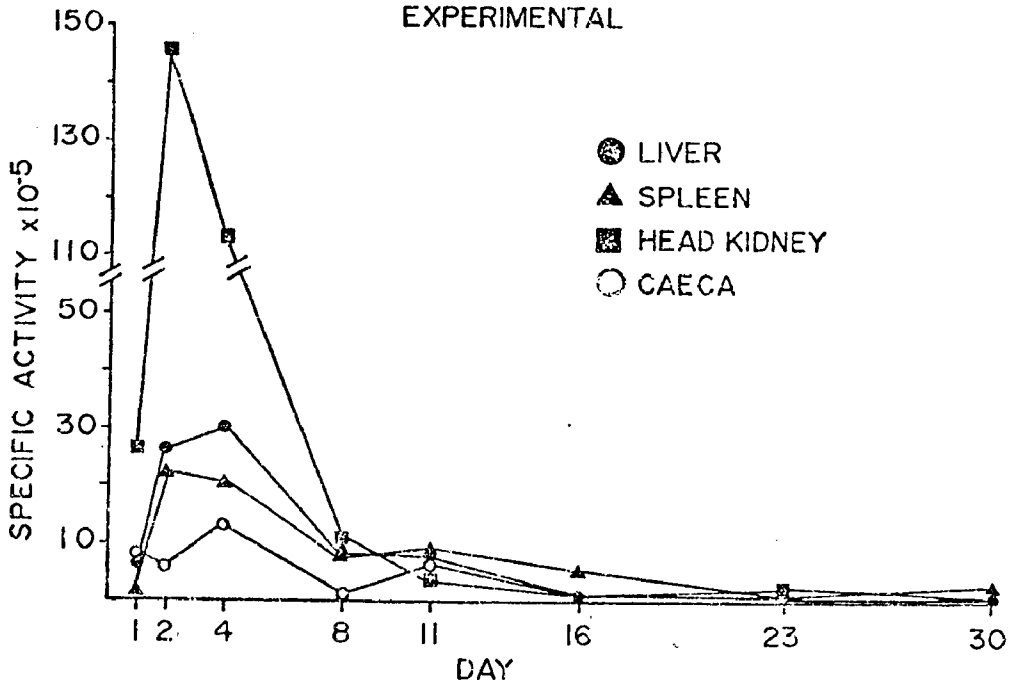

Figure 4. Specific activity of iron in tissues of iron deficient trout following intraperitoneal injection of ^{59}Fe.

TABLE 5

HEMATOCRIT, HEMOGLOBIN, MCHC, AND PERCENT RETICULOCYTES OF BLOOD SAMPLES FROM CONTROL AND EXPERIMENTAL FISH. INCLUDED ARE MEAN ± S.E. (N).

Days after inj.	Group	Hematocrit	Hemoglobin (gram%)	MCHC	Reticuloytes (%)
1-30	Control	37.7 ± 1.1(48)	7.9 ± 0.2(48)	20.8	2.75 ± 0.3(28)
1	Exp.	16.3 ± 2.2 (6)*	3.5 ± 0.4 (6)*	21.3	6.98 ± 0.8 (5)*
2	Exp.	14.2 ± 1.9 (6)*	2.9 ± 0.4 (6)*	20.1	
4	Exp.	19.8 ± 2.0 (6)*	3.2 ± 0.3 (6)*	16.2	5.00 ± 1.5 (2)
8	Exp.	26.3 ± 2.3 (6)*	4.7 ± 0.5 (6)*	17.7	11.07 ± 5.5 (5)
11	Exp.	24.7 ± 1.8 (6)*	3.9 ± 0.3 (6)*	15.9	17.65 ± 6.7 (5)*
16	Exp.	31.7 ± 1.0 (6)*	5.1 ± 0.1 (6)*	16.0	10.62 ± 2.1 (6)*
23	Exp.	33.5 ± 2.7 (6)	5.9 ± 0.6 (6)*	17.7	5.07 ± 2.1 (6)*
30	Exp.	35.2 ± 3.8 (5)	5.8 ± 0.7 (5)*	16.5	5.00 ± 0.9 (2)

* Significantly differnet from controls at p = 0.05

As shown in Table 5, the hematocrit index was lowest 2 days after the last bleeding but thereafter the red cell volume gradually increased and was back to the control level by day 23. Although the red cell volume was normal by day 23 the hemoglobin concentration remained below normal during the 30 day study period. The man corpuscular hemoblobin concentration (MCHC) decreased from about 21% to 15-16% and remained below the normal level throughout the remainder of the 30 day experimental period. Apparently the red cell volume was restored to normal by the release of young cells, which lacked functional hemoglobin, into the circulating blood. Even though the accuracy of reticulocyte counting is poor there was some evidence of an increase in the immature red cell population by day 11 to replace those cells lost via bleeding. It is apparent that the processes involved in hemoglobin formation are significantly slower than those involved in the formation and/or release of red blood cells from the erythropoietic head kidney.

The total iron binding capacity (TIBC) of the plasma, which is an indirect indicator of the transferrin concentration, was measured. There was a noticeable and significant increase in the TIBC following bleeding, however, there was also an increase in the TIBC for controls during the same period. This nullified any attempt to relate the increase to induced iron deficiency. The TIBC values for trout plasma (400-700 µg%) are higher than values reported for humans (250-350 µg%) and for the tench (250 µg) reported by Hevesy et al. (1964). The values for trout blood were reproducible with the method used and human plasma tested in the exact same manner yielded values within the accepted range.

The results of the absorption studies indicated that there was a direct correlation between the amount of iron injected into the gut segment and the

amount recovered in the tissues of the trout. The dose given was insufficient to exceed the absorptive capacity of the gut, thus it was not possible to establish a miniumum daily requirement for iron by trout based upon intestinal absorption. Possibly some iron enters trout by absorption across the gill and uptake by this pathway has yet to be investigated. However, it seem unlikely that absorption of iron by the gill would be of any great importance since we have shown that iron is readily absorbed via the gut. Following i.p. injection, very little radioiron is lost from trout via the feces. The radioiron in fecal material taken from the intestine of fish used in this study was initially as high as 4% of the injected dose, but by day 2 it was barely detectable and less than 0.08% was found infecal material after day 8.

To check iron loss in urine a group of trout which had been on the same feeding regimen as controls were cannulated (urinary bladder cannulation) and urine was collected over a 4 day period following i.p. injection of ^{59}Fe. No detectable quantity of radioiron was found in the urine. Thus the data from experiments with the gut and kidney indicate that the iron cycle in fish is a closed recycling system with very little iron input or excretion and in this respect is similar to the iron cycle in humans.

Summary

Iron metabolism in normal and iron deficient rainbow trout was studied after intraperitoneal injection of ^{59}Fe. In both groups most of the ^{59}Fe was absorbed from the peritoneal cavity within 24 hours and equilibrium between the plasma ^{59}Fe pool and that of the tissues and attained 8 days after i.p. injection. Liver iron, the main storage pool in trout, was reduced from the control level of 185 µg/g to below 100 µg'g 16 days after bleeding, whereas, splenic iron stores were unaffected. In iron deficient fish the RBC ^{59}Fe content increased to 70-80% of the injected dose by day 16 compared to 50% in controls. This was attributed to the difference in reticulocyte count which was 10-20% for the bled fish and 2-3% in controls. Some iron accumulated by erythrocytes is temporarily stores as non-heme iron by these cells. An average of 15% of the i.p. injected ^{59}Fe was taken up by hepatic tissue of control fish and remained there throughout the 30 day study. In iron deficient trout liver radioiron was reduced from a high of 15% on day 2 to less than 1% of the initial dose by day 16 post bleeding. There was essentially no detectable loss of ^{59}Fe in the urine or feces of either normal control or iron deficient fish.

SECTION 6

RESPIRATORY PHYSIOLOGY

CONTROL MECHANISMS FOR REGULATING THE FUNCTIONAL SURFACE AREA OF FISH GILLS

Research on the effects of water borne pollutants is gradually moving from the realm of determination of LD-50 concentrations or TLm values into that of clinical determinations of the cause of death of test animals. Information on the toxicological and/or pharmacological action of pollutants obtained from experiments with fish may prove to be very beneficial in assessing the effect(s) these materials may have on mammals, including man. During the past several years we have been forced to interpret data on the toxic action of materials on fish in light of information from experiments with rats or other 'higher' experimental vertebrates. With the advent of many suitable micro-procedures which can be used to obtain physiological and biochemical data and with more sophisticated chemical and physical analytical systems available we are in a position to extend our collection of information on the toxic action of pollutants on fish.

Ventilation of the gills of fish maintains at the gill surface the concentration of any pollutant that is in the aquatic environment. It follows that the study of gill structure and function can contribute valuable insight into the action of pollutants. Suspended solids and sulfite liquor from paper-mill wastes, ions of heavy metal salts from mining industries, detergents, phenols and a variety of other discharges from agricultural practices and industrial processes as well as specific fish poisons, e.g., rotenone, are known to affect the gills of fishes.

Thickening of the gill epithelium is one of the first changes noticeable in fish that are exposed to heavy metal salts, detergents and phenols. Epithelial thickening is usually followed by fusion of adjacent secondary lamellae and detachment of the epithelium from the basement membrane of the gill filaments and their secondary lamellae. Changes in the gill epithelium during poisoning by detergents are accompanied by vasodilation of the gill arterioles and blood spaces of the secondary lamellae, producing extensive vascular stasis or hematomos. Heavy metal ions are known to coagulate mucus secreted on the gills and the gills of chronically exposed fish have a thinner epithelium covering the lamellae than those acutely exposed. These changes occur to varying degrees in different parts of the gill so that diagnosis must be based on a substantial sample of filaments from different parts of gills.

It is important to note that even though histological changes found after exposure to various toxic materials have been described in detail, they do not necessarily indicate the preceise cause of death. Heavy metals

may react with enzymes in gill epithelial cells causing formation of lysosomes, vesicles and vacuoles in the gill. Damage to the epithelium may affect gas exchange, extrarenal excretion or gill ion exchange although osmotic or ionic imbalance has not been found to be the precise cause of death after exposure to zinc or mercury. It is probable that in many cases where fish are exposed to toxic materials, death is due to hypoxia resulting from the detachment of the gill epithelium from the vascular bed of the lamellae causing a lengthening of the water-blood pathway. Under these conditions a significant decrease in the diffusing capacity of the gills would result and even greatly increased gill ventilation would be unable to meet the oxygen requirements of the fish. Water borne toxic materials might also have serious detrimental effects on blood flow through the gills so that even with adequate external ventilation the internal perfusion might not be adequate for transport of oxygen from the gills to the various body tissue where it can be utilized.

It is apparent from the above discussion that more detailed experiments involving use of the isolated gill preparation are in order. From experiments of this type information should be obtained relating to critical or detrimental effects that water soluble pollutants, or changes in certain water quality criteria, might have on freshwater fish. The objectives of the experiments discussed below were to (1) modify published isolated-perfused gill techniques to permit longer experiments under conditions resembling, as closely as possible, those found in vivo, and (2) to add to our existing knowledge of the physiology of the fish gill so that the preparation could be utilized for studies of the effects of pollutants on gills. With respect to the latter we wished to confrim or deny regulation of functional surface area of the teleost gill and determine the nature of physiological control mechanisms which would be responsible for regulating functional surface area. For these experiments the influx of ^{14}C-urea was used as a relative measure of gill functional surface area. Urea is not metabolized by rainbow trout gill tissue and is not known to be actively transported by teleost gills. The method is based on the assumption that diffusional influx of ^{14}C-urea is limited principally by the extent of secondary lamellae perfusion, i.e., the functional surface area of the gill available for diffusional influx of the marker.

Methods

Previously described methods for isolated gill perfusion studies were modified by using a peristaltic pump to deliver perfusion solutions and by collecting perfusate samples in a fraction collector (Figure 5). Two independent perfusion channels were used to simulataneously perfuse the second pair of gill arches from each fish. All experiments were conducted in a cold room at 11 ± 1°C. The peristaltic pump aspirated perfusion solutions from polyethylene bottles and delivered them through PE 60 polyethylene tubing and the afferent cannulae to the gills. Perfusate leaving the gills passed through efferent cannulae and PE 60 tubing to photoelectric drop counters above the fraction collector. The channel B drop counter signal drove the fraction collector, which was set to turn after a pre-selected number of drops had been collected. Fraction collector turns along with the individual drop signals from the channel A drop counter were recorded

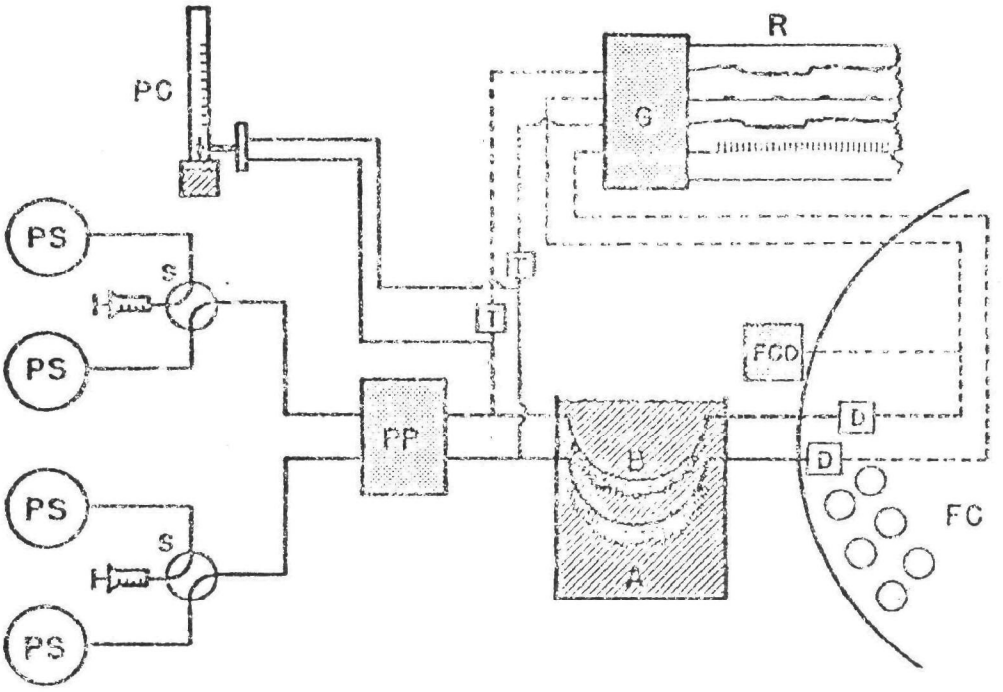

Figure 5. Diagram of gill perfusion apparatus. Solid lines represent perfusion channels and connections; dashed lines represent electronic connections. A and B gill arches in A and B perfusion channels; D photoelectric drop counters; FC fraction collector; FCD fraction collector drop count accumulator; G Grass polygraph; PC pressure calibration manometer; PP peristaltic pump; PS perfusion solutions; R polygraph recording; S 4-way stopcocks; T pressure transducers.

on a Grass 5_D polygraph. To monitor perfusion pressures, 't' connectors between the pump and gill arches (10 cm from the afferent cannula tips) were connected to Statham $P23_{AC}$ pressure transducers. A calibration manometer was also connected through valves and PE 60 tubing to the transducers. The cannula tips, manometer base, and transducers were all positioned in the same horizontal plane to facilitate accurate calibration and measurement of pressures. Output from each transducer was recorded continuously on the Grass 5_D polygraph.

The gill cannulation procedure has been described in detail (Bergman et al. 1974). After cannulation, the pump output to each arch was increased to 0.5 ml/min and held constant throughout the remainder of each experiment. Since only about 70% of the filaments were perfused in these gills the 0.5 ml/min flow rate was consistent with the expected single arch flow rate (0.75 ml/min, assuming equally divided flow to all eight gill arches) calculated from cardiac output estimates for resting 200-400 g rainbow trout. Drop size calibration for the photoelectric drop counters permitted calculation of flow rate through each gill arch. In preparations where severe leakage developed the measured flow rate fell below pump output and data from these experiments were discarded. Pressures usually ranged within the limits reported for ventral aortic blood pressure in rainbow trout. To mimic

systemic resistance, efferent pressure was set at 15 mm Hg by elevating the efferent tube outflow 20 cm above each gill arch.

Perfusion solutions consisted of vasoactive drugs or hormones added to Ringer solution. At 11°C the pH of this solution was 7.5 which compares closely to mixed venous-arterial blood pH for the same species and temperature in this laboratory. However, this value is lower than the arterial blood pH of 7.9 reported at 10.5°C by Randall and Cameron (1973) for rainbow trout. Before vasoactive agents were added, the perfusion solution was vacuum filtered through a 0.22 μm millipore filter. The solution was then vigorously shaken to assure atmospheric equilibration.

After cannulation, gill arches were perfused for an equilibration period of about one hour before experiments were begun. During this time each gill arch was perfused with the same solution that was to be used in that arch during the initial period of the experiment. After the initial period, drug or hormone concentrations were increased in 10-fold steps in the experimental arch while they were either not added or held constant in the control arches. At the end of a few experiments under each protocol the vasoactive perfusion pressure and ^{14}C-urea flux to control values. Starting with the initial period, perfusate fractions of equal volume were collected directly into vials for determination of ^{14}C-urea activity in a liquid scintillation counter. The ^{14}C-urea counts from the initial experimental period for the arch. The sample activities from the initial period were averaged for each gill arch, and the average was defined at 100% of initial activity. The activities of samples collected during the remainder of the experiment were then converted to a percentage of this mean initial activity. To demonstrate qualitative pressure responses to the drug and hormone treatments, typical perfusion pressure records were selected to represent each type of experiment. Where quantitative expressions of pressure responses were appropriate, the data were treated in the same way as the ^{14}C-urea data. All statistics were calculated with log transformed data, and the means of 95% confidence limits were used for all comparisons and Tukey's w-procedure was used for multiple comparisons among means.

Results and Discussion

The influx of ^{14}C-urea into isolated-perfused gills was altered markedly by the vasoactive drugs and hormones used in this study. The results of a typical epinephrine (EPI) experiment are depicted in Figure 6. In the figure the open circles = control arch fractions and x = experimental arch fractions. Vertical dashed lines delineate experimental periods with EPI concentrations in the experimental arch shown above. EPI concentration in the control was 10^{-7}M throughout. The solid horizontal line represents the control initial activity for both gill arches. Typically, the percent of initial activity increased (or decreased depending upon the agent used) toward some new steady-state value in response to step-wise changes in the vasoactive drug concentration. For all experiments following one protocol, steady-state percent of initial activity values are tabulated for the drug concentrations used.

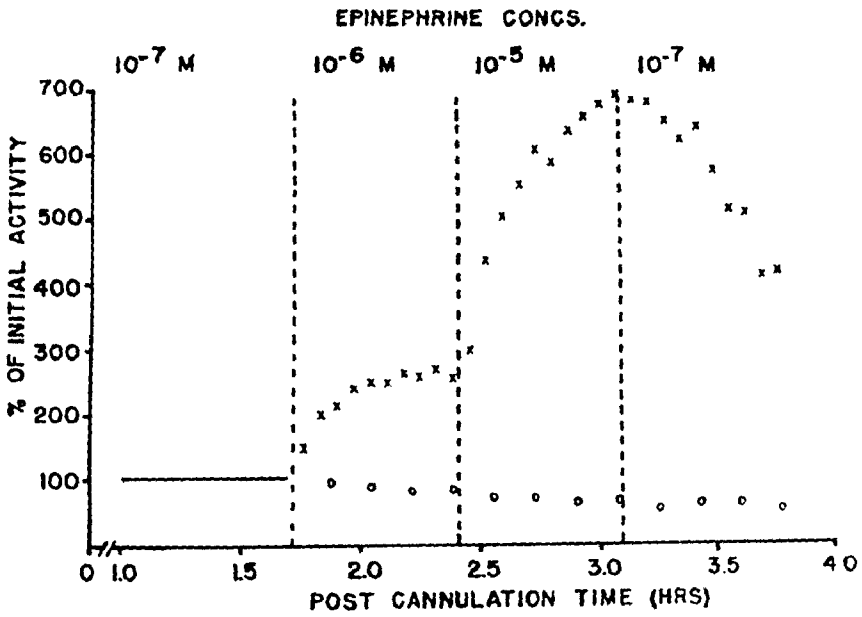

Figure 6. Results from a typical epinephrine experiment. See text for details.

The results from epinephrine (EPI) experiments (Figures 7 and 8) were qualitatively the same as those found for norepinephrine (NEPI), although a narrower concentration of EPI was used. EPI, like NEPI, is a mixed agonist which can stimulate both α and β adrenergic receptors. Because ^{14}C-urea influx data were less variable in the EPI experiments than in NEPI experiments, more clear-cut differences were evident when the EPI effect was blocked with phenoxybenzamine (POB) and propronolol (PROP) as noted in Figure 8 below. The unblocked 10^{-5}M EPI induced ^{14}C-urea influx is significantly greater that seen in the control or blocked gills.* Influx in the single block experiments (α or β) was still significantly higher than in the controls, and double blockade (α and β) of the EPI effect was necessary to reduce influx to the control value.

Acetylcholine (ACH) significantly reduced ^{14}C-urea influx when perfused alone (Figure 9) or when perfused along with 10^{-5}M EPI. The reduction was greater in experiments where ACH was perfused without the EPI. Stimulation of α and/or β adrenergic receptors with catecholamines or pharmacological drugs in this study produced marked and usually significant increases in ^{14}C-urea influx, whereas ACH significantly reduced influx of the marker. The most tenable explanation for the observed changes in ^{14}C-urea influx and branchial vascular resistance is that alterations in the internal perfusion pathway resulted in changes in functional surface area of these gills. It could be aruged that the vasoactive agents may have affected the permeability of the gills to ^{14}C-urea but it is doubtful that the changes in permeability could account for the effects these agents had on branchial vascular resistance (Figures 10 and 11). This is especially true in the case where, with opposite effects on resistance, stimulation of either α or β adrenergic receptors caused an increase in ^{14}C-urea influx. We conclude that these vasoactive

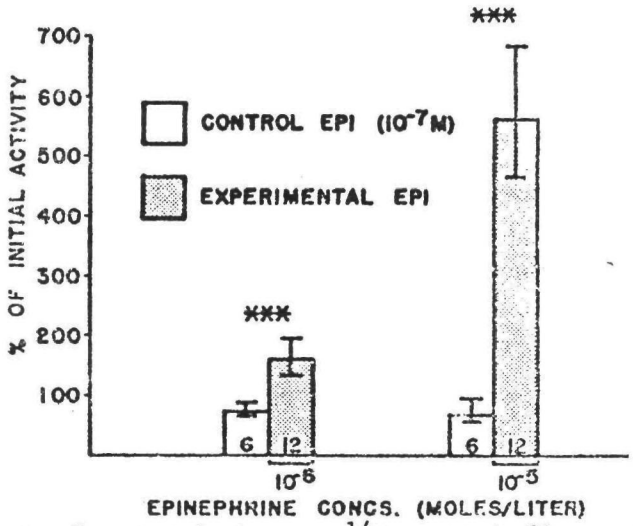

Figure 7. The effect of epinephrine on ^{14}C-urea influx. During the initial period (not shown) both control and experimental gills were perfused with 10^{-7}M epinephrine. The mean, 95% confidence interval, and N value are shown for each control and treatment from successive experimental periods. Significance for paired comparisons: *0.01 P 0.05; **0.001 P 0.01; *** 0.001.

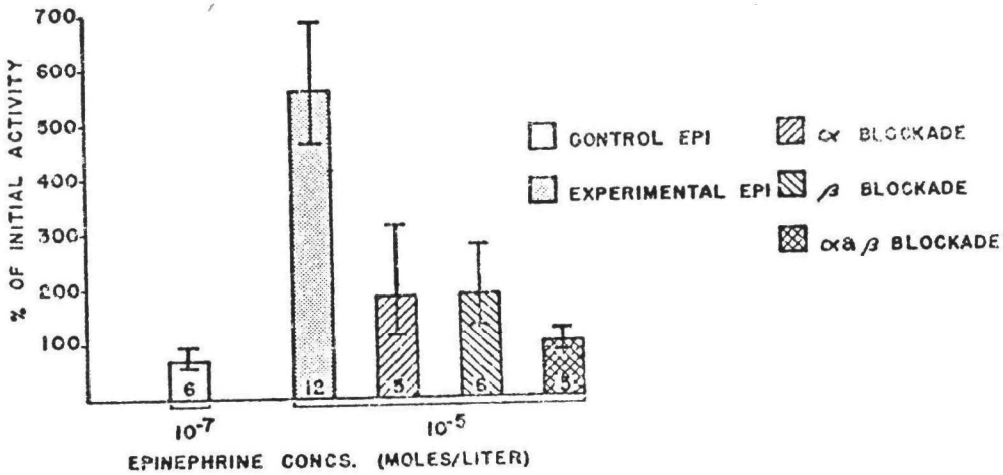

Figure 8. The effect of adrenergic blockade on epinephrine induced ^{14}C-urea influx. During the initial period (not shown) all arches were perfused with 10^{-7}M EPI. In blocked arches, the blocking drug(s) was perfused throughout the entire experiment. All values are from the same experimental period. α block = 10^{-5}M POB; β block = 10^{-5}M PROP. The means, 95% confidence interval and N values are shown. The means listed below which are not underlined by the same line are significantly different (0.05):

Control	$\alpha + \beta$	α	β	Exp.
EPI	block	block	block	EPI
72	100	187	189	564

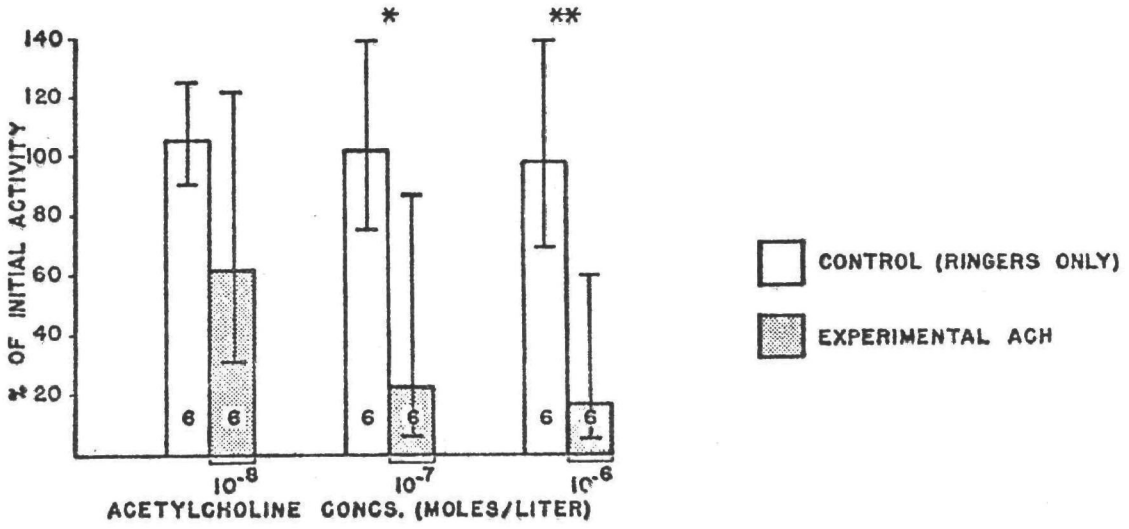

Figure 9. The effect of acetylcholine on ^{14}C-urea influx. During the initial period (not shown) both control and experimental gills were perfused with Ringer solution only.

agents had little effect on permeability and that the observed changes in ^{14}C-urea uptake reflect changes in the relative functional surface area of the gills. For any series of experiments with a vasoactive agents, the pressure effect was qualitatively consistent, but the sensitivity of different preparations to a drug or hormone varied. This may have resulted from differences in prevailing vascular tone.

Perfusion pressure was reduced by NEPI (Figure 10), although in a few experiments a transient pressure increase was seen first (Figure 10B). At 10^{-5}M NEPI the average percent of initial pressure (88%) was significantly lower than in the 10^{-9}M NEPI controls. Although not statistically significant, reductions in perfusion pressure were observed in some preparations with NEPI concentrations as low as 10^{-8}M. Figure 10C, D and E show the typical pressure responses when adrenergic blockers were perfused along with NEPI just as in the unblocked gills. After β blockade, however, a pressure increase was usually unmasked and when both α and β blockers were sued no pressure change resulted from the increased NEPI concentration. Qualitative pressure responses in gills perfused with EPI alone or with adrenergic blocking agents were identical to those seen in the NEPI experiments, hence these data are not presented. When the ACH content of the perfusion solution was increased from 10^{-8} to 10^{-7}M a slight but significant increase in perfusion pressure was noted (Figure 11A). Another ten-fold increase to 10^{-6}M ACH resulted in a very significant pressure increase (Figure 11 B). The effects of four different drugs on the increased perfusion pressure induced with 10^{-6}M ACH were also noted. The muscarinic and nicotinic (ganglionic) blockers, atropine and hemamethonium respectively, both eliminated most of the pressor effect of ACH. The α adrenertic block POP completely eliminated the pressure increase whil phentolamine, also an α adrenergic blocker, had little effect.

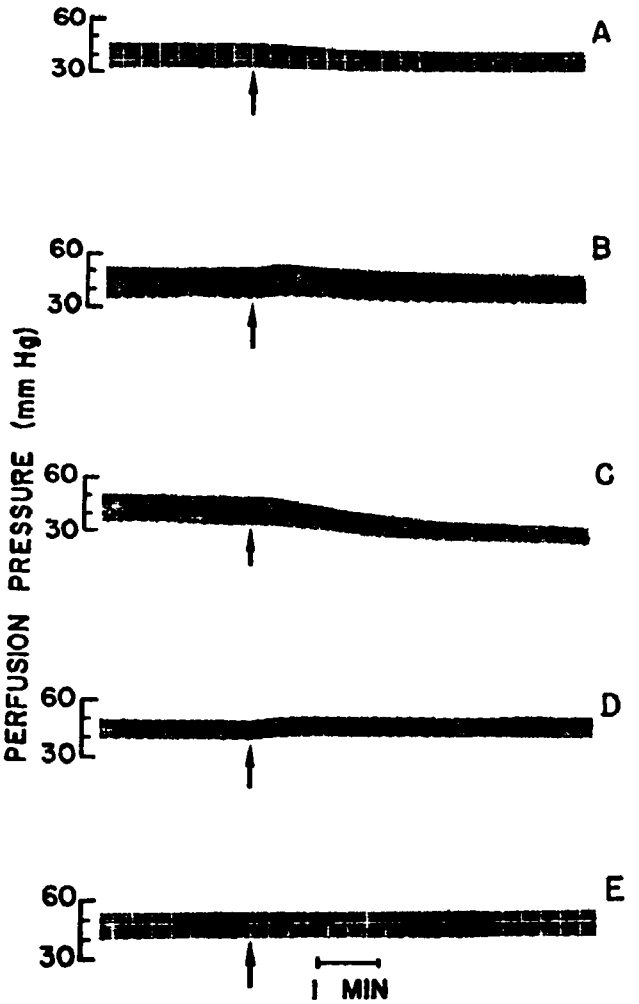

Figure 10. Typical effect of norepinephrine on perfusion pressure in the absence and presence of adrenergic blockers. Arrows indicate where NEPI concentration was increased from 10^{-6} to 10^{-5}M. (A) and (B) NEPI alone; (C) α blockard of NEPI with 10^{-5}M POB; (D) β blockade of NEPI with 10^{-5}M PROP; (E) α and β blockade of NEPI with 10^{-5}M POB and 10^{-5}M PROP.

Summary

The physiological viability of the perfused gills in this study was probably due partly to the use of filtered perfusion fluid (Rankin and Maetz, 1971) as well as pulsatile pump-perfusion, which appeared qualitatively superior to perfusion with a hydrostatic head (Richards and Fromm, 1969; Rankin and Maetz, 1971; Randell et al. 1972; Wood, 1974) or a constant pressure syringe pump (Shuttleworth, 1972). The appearance of the gill arches usually remained good during and after perfusion except that secreted mucus accumulated on the surface of a few gills. Although no edema was evident, colloid osmotic pressure was low in our perfusion fluids and some edema could have developed. However, branchial vascular resistance remained constant and vasoactive agent effects were reproducible for as long as 12 hours. With flow rates which approximated the expected single arch flow rate in intact, resting rainbow trout (Stevens and Randall, 1967a), the perfusion pressures in these experiments were generally within the range of ventral aortic blood

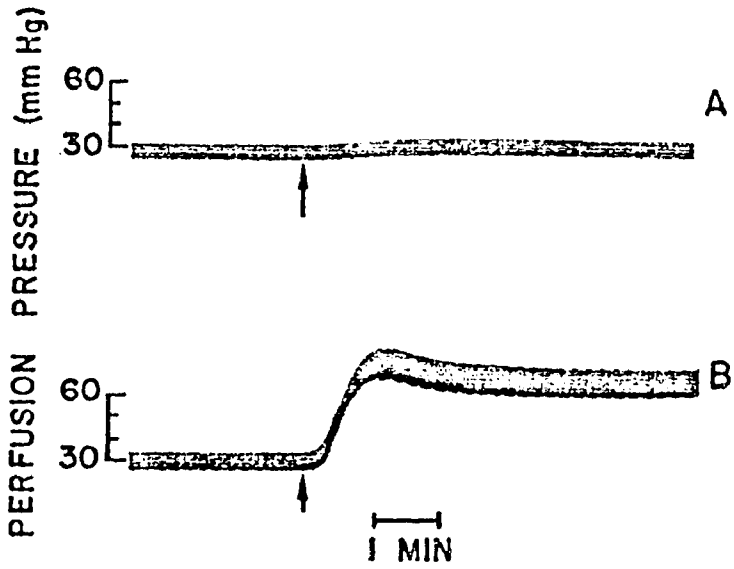

Figure 11A and B. Typical effect of acetylcholine on perfusion pressure. Arrows indicate where ACH concentration was increased. (A) ACH increase from 10^{-8} to 10^{-7}M; (B) ACH increase from 10^{-7} to 10^{-6}M.

pressure reported for this species (Stevens and Randall, 1967b). Pressures in excess of the normal range were observed only in the gills perfused with 10^{-6}M ACH. Significant effects on resistance and ^{14}C-urea uptake were seen at NEPI and EPI concentrations which were comparable to the maximum plasma catecholamine levels reported for distrubed rainbow trout (Nakano and Tomlinson, 1967). However, the adrenergic agonist concentrations which produced threshold effects on resistance were sometimes 100-fold higher than previously reported for the same drugs and hormones in isolated teleost gill preparations (Rankin and Maetz, 1971; Wood, 1974). The reasons for this are probably related to differences in pH, temperature, perfusion pressure and composition of the perfusion fluids.

The physiological control mechanisms responsible for adjusting the resistance to flow in teleost gills are probably both hormonal and neural in nature. The catecholamine-induced decrease in branchial vascular resistance increase in functional surface area seen in these experiments agree with published observations on the resistance (Keys and Bateman, 1932; Östlund and Fänge, 1962; Reite, 1969) and blood pathway effects of catecholamines (Steen and Kruysse, 1964; Richards and Fromm, 1969). These findings, combined with the observation that plasma catecholamine concentrations are elevated during exercise (Nakano and Tomlison, 1967), strongly support hormonal control of gill blood flow and functional gill surface area. Wood (1974) has proven the involvement of β_1 adrenergic receptors in this catecholamine effect. Although the critical catecholamine potency-series experiments have not yet been reported as proof, evidence in this and other studies suggest α adrenergic receptor involvement in the catecholamine effect as well.

THE ROLE OF FISH GILLS IN HEAT EXCHANGE

Another series of experiments dealing with heat exchange of fishes were conducted using isolated-perfused gills as well as isolated-perfused heads of rainbow trout. Investigations dealing with rates of thermal exchange in

fish have been few in number. Stevens and Fry (1970, 1974) have reported values for a coefficient of temperature exchange in a variety of species, however, there appear to be no data directly in terms of heat exchange by a teleost. Fry (1967) speculated that the gills play a major role in overall thermal exchange but pertinent experiments to substantiate this hypothesis are lacking.

In attempting to quantitate heat exchange by the gills of rainbow trout a generalized equation for a tubular heat exchanger was adopted (Kay, 1963):

$$Q = hA\Delta_{ln} \qquad (1)$$

Where Q = total heat flux in cal min^{-1}; h = heat transfer factor in cal $min^{-1}°C^{-1}cm^{-2}$; A = surface area in cm^2 and ΔT_{ln} = logarithmic mean temperature gradient in °C. The value of h will be determined by three factors: (1) convective transfer to the inner wall of the vasculature, (2) conduction through the vessel wall and (3) convective transfer from the gill surface to the environment. The surface area will be determined by the mean length and mean diameter of the vasculature. Because we were unable to accurately determine a value for surface area (A), Equation 1 was solved for the quantity hA following determinations of Q and T_{ln} using Equations 2 and 3 respectively (Kay, 1963):

$$Q = f\ Cp\rho(T_i - T_o) \qquad (2)$$

$$T_{ln} = \frac{T_i - T_o}{\ln \frac{T_i - T_o}{T_o - T_b}} \qquad (3)$$

Where f = perfusion flow rate in ml min^{-1}; C_p = specific heat of perfusion fluid (= 0.962 cal $gm^{-1}°C^{-1}$ for 0.1% NaCl, CRC Handbook); ρ = density of perfusion fluid flowing into the gills, out of the gills, and of the bath respectively. Equation 3 represents the mean temperature gradient for a tube of uniform dimensions and may not be rigorous for the case of the branching vasculature. However, this equation provides the best means of assessing the mean gradient driving heat across the gill. Also, with unidirectional ventilation, the increase in temperature of the ventilatory stream as it passes the gills may be accounted for an equation similar to (3), but makes an insignificant contribution to the results reported here.

The experiments performed were designed to provide information on (1) the magnitude of heat exchange occurring in isolated-perfused gills of rainbow trout, (2) the effect of changes in vasculature gemoetry (i.e., changes in functional surface area) on heat exchange and (3) the effect of ventilation on heat exchange. Finally, a simple model is evaluated in an attempt to relate gill heat exchange to the whole body thermal response.

Methods

The second gill arches from 200-400 gm rainbow trout (Salmo gairdneri) were prepared for cannulation as outlined by Bergman et al. (1974). Inflow, outflow and bath temperatures were measured (± 0.05°C) with thermistors (32A7, Victory Engr.,) using a continuous balancing Wheatstone bridge re-

corder. Inflow and outflow thermistors were inserted in the cannulas to within 1 cm of the supportive branchial bar of each gill arch. Heat exchange was determined in gills perfused at 0.5 ml min^{-1} by a Ringer solution alone or Ringer solution which contained 10^{-5}M epinephrine (EPI). With T_i held constant at 10 $\pm$ 1°C, data were obtained and Equation 1 solved for hA at steady state values for T_b of 2, 5, 8, 13, 16, and 19°C. Statistical treatment of the data was performed using the Student's t test for sample means.

For whole head preparations fish were decapitated just posterior to the opercula and the intact branchial basket was allowed to clear of blood as described by Bergman et al. (1974). A cannula with a thermistor probe threaded through the lumen to the cannula tip was inserted into the ventral aorta and secured with a ligature around the bulbus arteriosus. A similar cannula was placed in the dorsal aorta and secured by a ligature around the dorsal aorta and spinal column. A third ligature was placed around the cut end of the esophagus. The head was transferred to a temperature regulated water bath and a ventilatory flow ($\dot{V}_g$) was diverted into the mouth via a flowmeter and varied between 40 and 130 ml min^{-1} (Figure 12). Perfusion solutions were delivered by a positive displacement type pump with an independently adjustable stroke volume and rate. Perfusion pressure was monitored continuously with mean perfusion pressure of all control experi- thermistor for a reliable measure of T_o precluded the use of an outflow resistance resulting in larger than normal perfusion pulse pressures. Isolated heads were perfused with four different solutions: Ringer solution alone and Ringer solution which contained 10^{-5}M EPI, 10^{-8}M or 10^{-7}M ACH. Heat exchange was determined in the steady state with an inflow temperature (T_i) of 12 $\pm$ 1°C and bath temperature (T_b) of 8 $\pm$ 0.1°C. The thermistor probes were calibrated against a differential thermometer and temperatures recorded $\pm$ 0.01°C. All experiments were preceded by a 30 min equilibration period. Changes in drug concentration were followed by a 30 min equilibration period and changes in perfusion flow were followed by a 15 min equilibration period.

Results and Discussion

In the isolated-perfused heads, the influence of ventilatory flow on heat transfer was evaluated and is shown for a representative experiment in Figure 13. Values for hA at $\dot{V}_g$ below 400 ml min^{-1} were not determined because of marked variations in hA due to (1) extraneous heat picked up by the fluid passing through the flowmeter which was in contact with ambient temperature (20°C) and (2) possible mixing of the low temperature bath with the warmer ventilatory fluid by back flow into the opercular chamber at low $\dot{V}_g$. Despite these experimental limitations, two constraints may be placed on the values of hA with increasing $\dot{V}_g$. Since $\dot{V}_g$ influences only the convective heat loss from the gill surface, as $\dot{V}_g$ increases a point is reached where heat transfer is limited solely by the transfer of heat to the inner vessel wall and the conduction of heat through the vessel wall and hA becomes independent of $\dot{V}_g$. This constraint represents an asymptotic maximum of hA (hA_{max}). In a similar manner, for 0 ml min^{-1} $\dot{V}_g$ there will ideally be no heat lost from the system in steady state and hA must be zero. We chose to use a rectangular hyperbolic function to predict hA for a given $\dot{V}_g$:

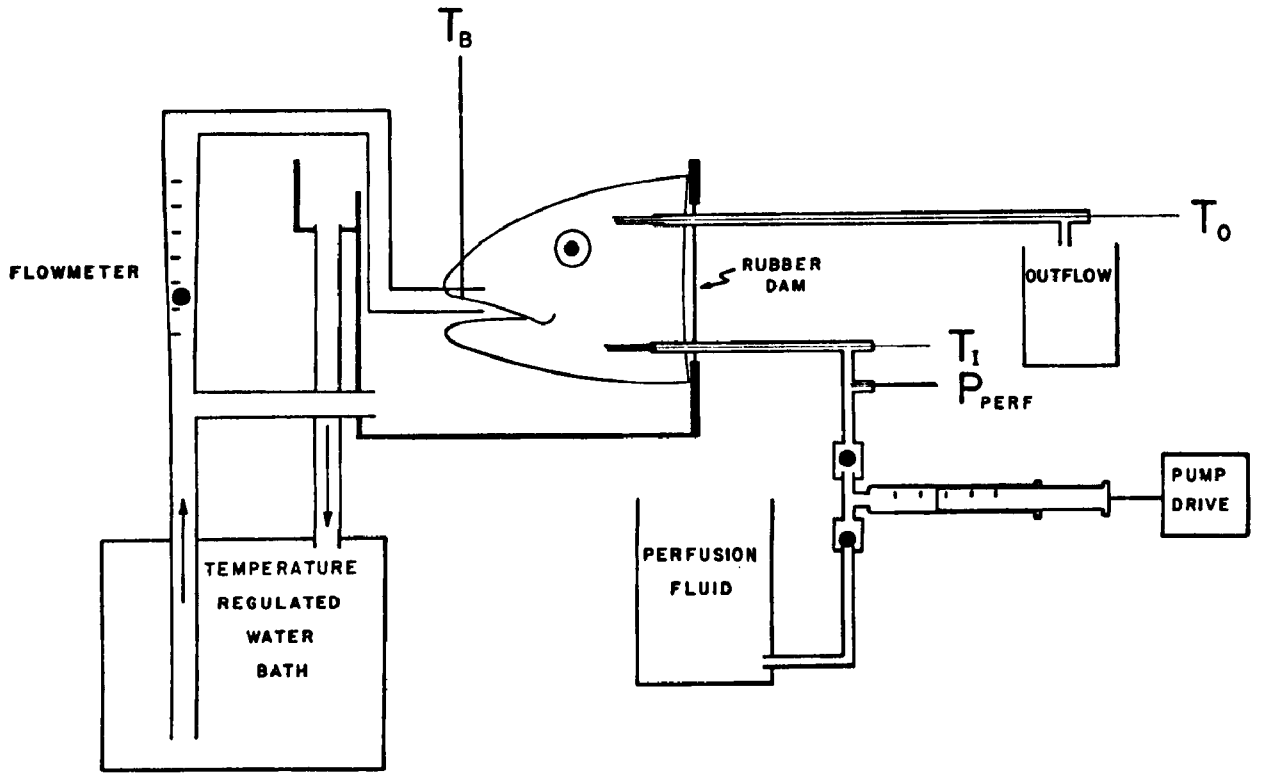

Figure 12. Diagram of apparatus used to perfuse isolated heads of rainbow trout. See text for details.

$$hA = \frac{hA_{max} V_g}{V_g - Vasym} \qquad (4)$$

The term -Vasym also represents the value of V_g necessary to reach one-half of hA_{max} ($V_{0.5\ max}$) and Equation 4 becomes:

$$hA = \frac{hA_{max} V_g}{Va + V_{0.5\ max}} \qquad (5)$$

From equation 5, the transfer maximum (hA_{max}) and ventilatory flow at one-half the transfer maximum ($V_{0.5\ max}$) were obtained from a linear regression of hA_{max} -1 on V_g -1. Changes in hA_{max} and $V_{0.5\ max}$ were evaluated using a 2 way analysis of variance and when appropriate a Student-Newman-Keuls test for significance group means (Sokal and Rohlf, 1969).

Values for heat transfer (hA) in the isolated-perfused gills are shown in Table 6. Perfusion with 10^{-5}M EPI significantly increased hA. There were no uniform or systemic alterations in hA or perfusion pressure associated with changes in bath temperatures.

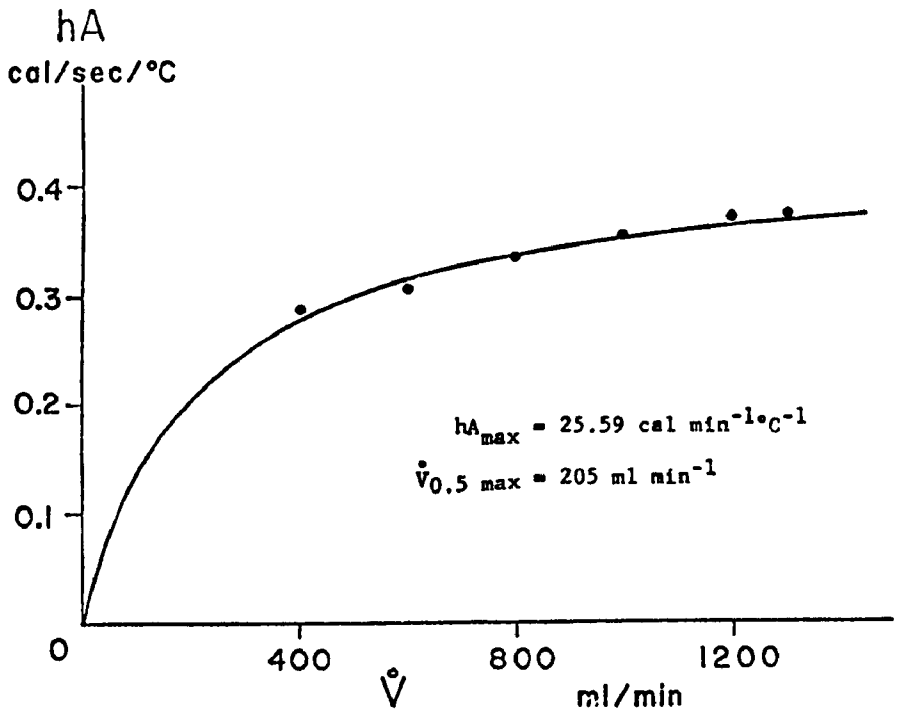

Figure 13. Experimental data for heat transfer plotted as a function of ventilatory flow ($\dot{V}_g$) for one isolated head perfused at 20 ml min^{-1}. hA_{max} and $V_{0.5max}$ were determined by a double reciprocal linear regression and the solid line represents the theoretical curve.

TABLE 6

HEAT TRANSFER (hA) OF ISOLATED-PERFUSED SECOND GILL ARCHES OF RAINBOW TROUT. PERFUSION RATE = 0.5 ML MIN^{-1}.

Perfusion Fluid	Heat transfer (hA) cal $min^{-1}°C^{-1}$
Ringer solution	0.774 ± 0.024*
10^{-5}M EPI in Ringer solution	0.858 ± 0.024*+

*$\bar{X}$ ± SE; + p less than 0.05

Values of hA_{max} and $\dot{V}_{0.5max}$ at perfusion flow rates of 16 and 20 ml min^{-1} for the initial control periods of all experimental groups are summarized in Table 7. The effects of altering perfusion flow are shown in Figure 14.

TABLE 7

HEAT TRANSFER FOR ISOLATED-PERFUSED HEAD OF RAINBOW TROUT DURING CONTROL PERIODS. VALUES (X ± SE, n) ARE GIVEN FOR FLOW RATES OF 16 and 20 ML MIN^{-1}.

	Perfusion flow rate	
	16 ml min^{-1}	20 ml min^{-1}
hA_{max} (cal $min^{-1}°C^{-1}$)	21.27 ± 0.57 (21)	24.79 ± 0.77 (21)
$\dot{V}_{0.5max}$ (ml min^{-1})	144 ± 17 (21)	183 ± 23 (21)

Increasing the perfusion flow increased hA_{max} but had no effect on $\dot{V}_{0.5max}$. Also, at a given perfusion flow rate, hA_{max} and $\dot{V}_{0.5max}$ were unaffected by a 20% change in stroke volume.

The effects of drug infusions are shown in Figure 14 (below) for a perfusion flow of 20 ml min^{-1}. Each experimental group was followed through control, drug infusion and post-control periods. During each period hA was determined for perfusion flows of 16 and 20 ml min^{-1} at a stroke rate of 50 min^{-1}. $\dot{V}_{0.5max}$ was unaltered for any of the experimental treatments. With infusion of 10^{-5}M EPI hA_{max} was significantly increased above the values obtained during the pre- and post-infusion control periods, whereas, perfusion of solutions containing 10^{-8}M and 10^{-7}M acetylcholine had no effect on hA_{max}. These results are qualitatively the same at both perfusion flow rates. When perfusion flow was increased from 16 to 20 ml min^{-1} during constant infusion of either EPI or ACH the change in hA_{max} was identical to that seen in the control experiments (Figure 15).

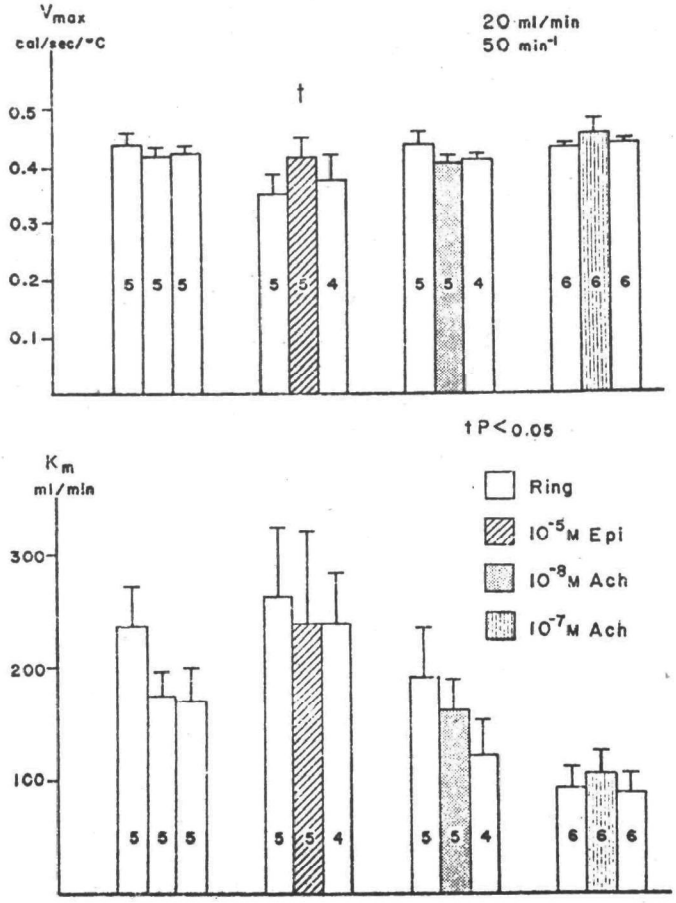

Figure 14. The effects of vasoactive agents on hA_{max} and $\dot{V}_{0.5max}$ ($X \pm SE$) of the perfused head at a perfusion flow of 20 ml min^{-1}. + significant change in hA_{max} during 10^{-5}M EPI from pre- and post-infusion control periods.

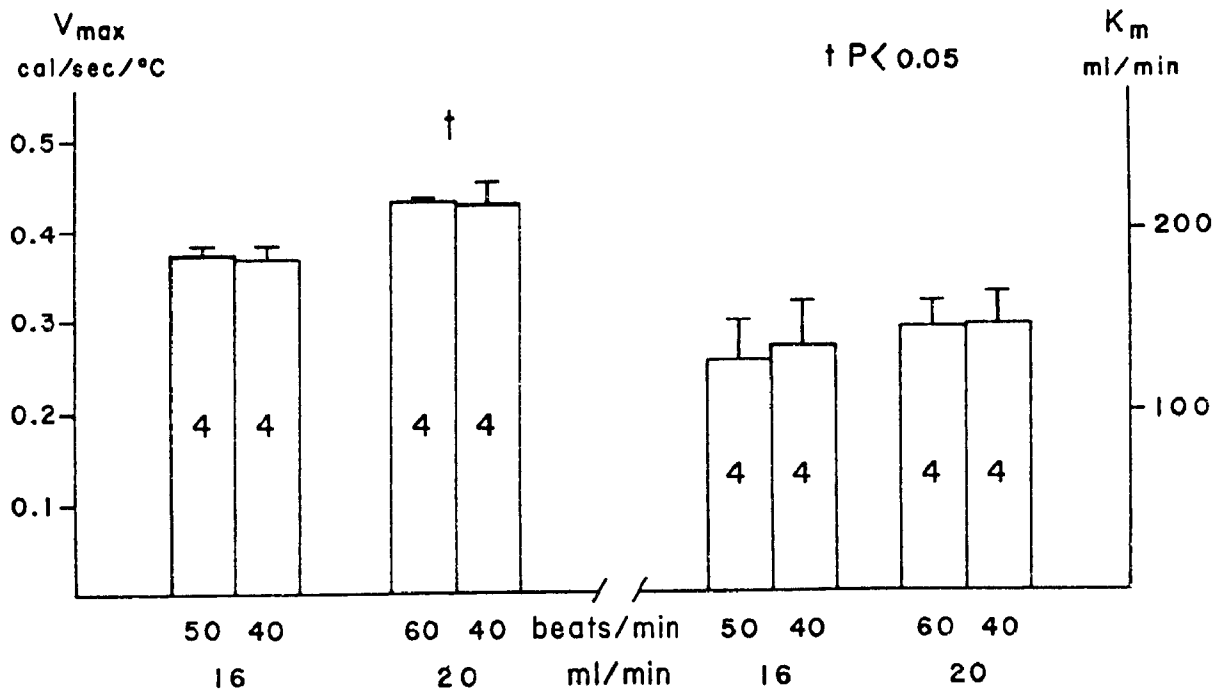

Figure 15. The effects of alterations in perfusion flow and heart rate on hA_{max} on the left and $\dot{V}_{0.5max}$ on the right (X $\pm$ SE) for the isolated-perfused head (n = 4). + significant change in hA_{max} at perfusion flow of 20 ml min^{-1} over 16 ml min^{-1}.

In interpreting changes in heat transfer (hA) following experimental manipulations, the basic determinants of h and A must be considered. These determinants are not independent. A change in vascular dimensions will alter not only the length and diameter of the vessels, but wall thickness and perfusate velocity such that the value of both h and A are affected. The effects of ventilatory flow on external convection will be determined by the velocity of the stream as well as its relation to the path of internal perfusion.

The use of values for maximal heat transfer (hA_{max}) and ventilatory flow at one-half hA_{max} ($\dot{V}_{0.5max}$) allows some of these factors to be evaluated independently. An increase in $\dot{V}_{0.5max}$ without a change in hA_{max} would result in a decrease in hA at any given $\dot{V}_g$. Assuming the internal dimensions and hence internal convection and conduction are stable during the determination of hA_{max} and $\dot{V}_{0.5max}$, the only alteration which would account for the increase in $\dot{V}_{0.5max}$ would be a decrease in convective loss from the gill surface. A decrease in external convective loss can be brought about by two means: (1) a conformational change of the gill in the ventilatory stream or (2) a change in the mean ventilatory velocity over the perfused lamellae. If a gross conformational change of the gill has not occurred, the patterns of ventilatory velocity over the gill should be unaltered. The increased $\dot{V}_{0.5max}$ would then indicate a change in perfusion pathway away

from the better ventilated secondary lamellae with a resultant increase in ventilation-perfusion inequality. In contrast to $\dot{V}_{0.5max}$, hA_{max} provides a $\dot{V}_g$ independent measurement of heat transfer and should be altered only by changes in internal thermal convection from the perfusate to the vessel wall and thermal conduction through the vessel wall. By evaluating the two parameters of the hyperbolic function relating hA to $\dot{V}_g$, independent changes in either $\dot{V}_{0.5max}$ or hA_{max} will provide information regarding alterations in (1) the ventilation-perfusion relationship of the gill (hA_{max}). Simultaneous changes in hA_{max} and $\dot{V}_{0.5max}$ would indicate some indeterminate combination of these factors.

The theoretical determinants of hA_{max} could be correlated to experimental observations by noting the effects of increasing perfusion flow. The increased flow and resultant increase in perfusion pressure may cause an increase in mean perfusate velocity and/or distension of the vasculature. These changes alter the internal convection and conduction processes and result in a significant increase in hA_{max} in response to the increased perfusion flow (Figure 14). We were unable to detect any significant alteration in $\dot{V}_{0.5max}$ following our experimental manipulations. This may be due in part to a basic insensitivity of $\dot{V}_{0.5max}$ to detect ventilation-perfusion changes. However, the importance of ventilation-perfusion matching cannot be overemphasized when evaluating exchange processes in any respiratory organ.

Holeton and Randall (1967) reported that trout exposed to a hypoxic enviroment exhibit a marked bradycardia with no alteration in cardiac output. In this case, the animal should attempt to maximize oxygen transport by minimizing ventilation-perfusion inequalities. The evidence for a capactivie 'bleb' in the afferent filamental vessels (Fromm, 1975 and see below) gave rise to speculation concerning the frequency dependence of the distensible gill vasculature. If the gill vessels could exhibit a frequency dependent nature to allow for passive changes in perfusion pathways in response to changes in heart rate, the hypoxic bradycardia could give rise to a passive redistribution of perfusion to aid in oxygen transport. Thi is apparently not the case since, as shown in Figure 14, a 30% change in heart rate (40 to 60 min^{-1}) did not alter the $V_{0.5max}$ or hA_{max} of the gills. However, the diffusion distance and membrane permeability characteristics than heat, thus there are many changes that may be occurring in vascular flow and the lamellar membrane that will alter oxygen uptake, but have an insignificant effect on thermal exchange. If a passive vascular response to changes in heart rate occurs, this could be coupled with an active mechanism(s) to efficiently regulate ventilation-perfusion ratios.

Along with the possibility changes in the gill vasculature, there have been several investigations dealing with active regulation of the gill vasculature (Bergman et al., 1974; Steen and Kruysse, 1964). Bergman et al., (1974) concluded that EPI increased functional surface area for

^{14}C-urea uptake and that it was decreased by acetylcholine. The primary changes in these experiments were attributed to perfusion pathway changes which should be reflected by alterations in the hA_{max} value for heat exchange. As shown in Table 6 and Figure 15, 10^{-5}M EPI significantly increased hA in the isolated perfused gill and hA_{max} in the isolated perfused head. This increase is not as dramatic as that seen by Bergman *et al.* (1974), and is indicative of the relative sensitivities of the two markers used. In the brachial basket the major change in vascular dimensions is presumed to take place at the secondary lamellae which would greatly alter the ^{14}C-urea uptake and also change heat transfer. However, the remainder of the large vessels in the gill are readily exchanging heat but not ^{14}C-urea indicating there is a greater effective surface area for thermal exchange than for molecular exchange. This could explain why there was a much smaller increase in total heat flux (13%) than total ^{14}C-urea flux (400%, Bergman *et al.*, 1974) for the same perfusion pathway change. Acetylcholine at concentrations of 10^{-8}M and 10^{-7}M did not significantly alter hA_{max}. There are several possible reasons for this lack of supportive evidence of ACH modulation of the exchange process: (1) the relative sensitivities of a molecular versus thermal diffusion phenomena to small changes in vascular geometry, (2) the initial control state of the gill vasculature and (3) the possibility of a differential response by the gill arches to ACH that would mask overall changes in the brachial basket but not in the isolated gill arch. This final point has received little attention and the redistribution of perfusion pathways at the gill arch level as well as the lamellar level should be considered for all vasoactive agents.

Aside from the basic quantitation of heat exchange by the gill, a simple model (see Figure 16) was constructed to determine the relative contribution of the gill to whole body heat exchange. The body of the fish consists of a cylinder with thermal conductivity (k) of 1.4×10^{-3} cal°cm^{-2} (k for water, Crc Handbook) and an internal core of 0.5 cm radius maintained at some temperature (T_i) above ambient (T_b). Blood is pumped through an exchanger and returned to the body at T_o. To simplify the model it was assumed that: (1) there is no convective mixing in the body, (2) there is no interference with thermal conduction from the body due to the flow of blood through the gills, and (3) there is no heat conducted from the ends of the cylinder. Thus, the total heat flux (Q_{tot}) will be the sum of the convective loss from the gill (Q_{cv}) and the conductive loss from the body (Q_{cd}):

$$Q_{tot} = Q_{cv} + Q_{cd} \qquad (5)$$

Q_{cd} can be determined using Equation 6 (Bennett and Meyers, 1962).

$$Q_{cd} = k\ 2\ \ l\Delta T_i/(\ln r_2/r_1) \qquad (6)$$

Where l = length of cylinder; r_1 = internal radius of cylinder; r_2 = external radius of cylinder and $T_i = T_i - T_b$. Q_{cv} can be determined by using Equation 2. The solid lines in Figure 17 represent a plot of the percent of total heat flux (% Q_{tot}) exchanged via the model gill as a function of perfusion flow when the % equilibration $(1 - T_o/T_i)100$ is held at 100% and 50%. The maintenance of a constant % equilibration in the face of increasing perfusion flow will probably not occur and the actual model response should tend to

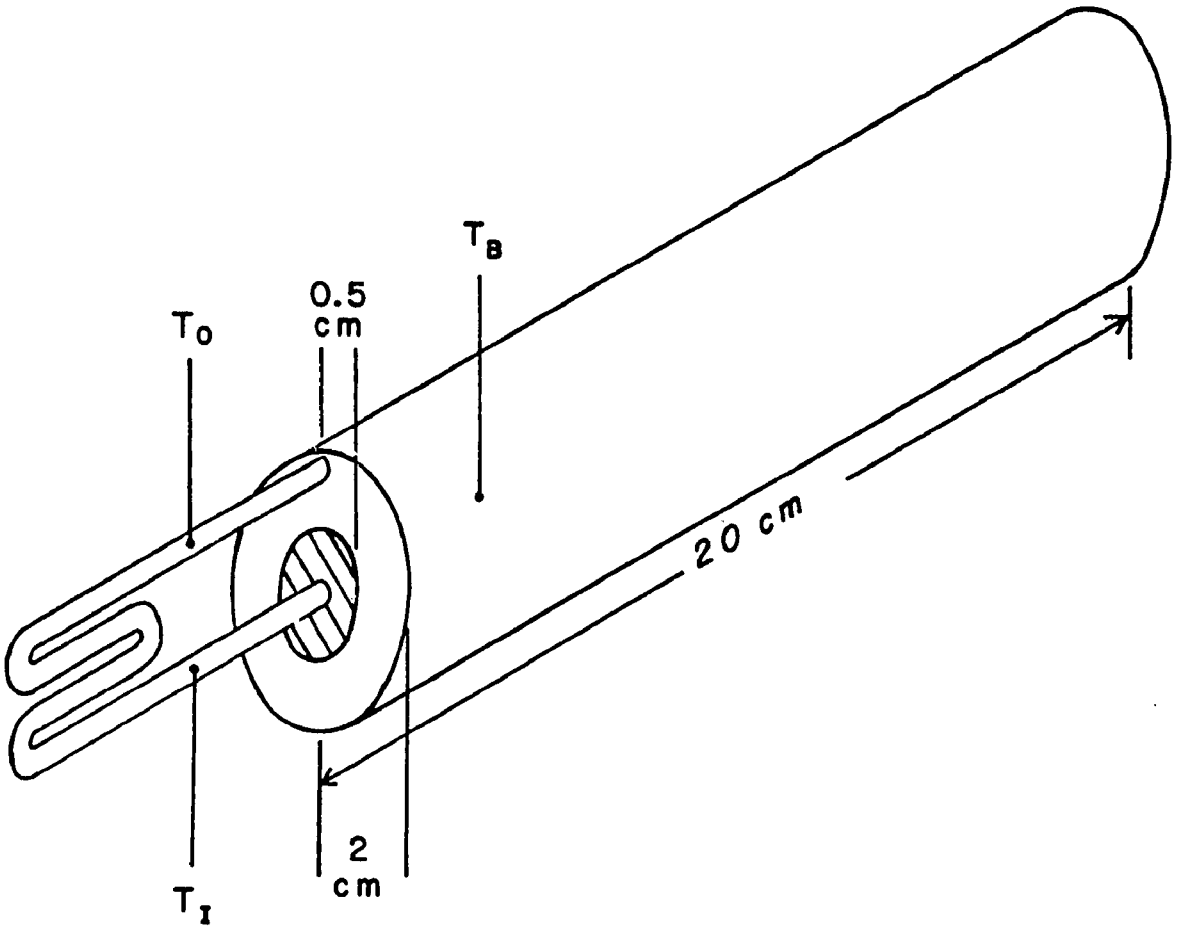

Figure 16. Diagram of model used to evaluate gill heat exchange in terms of whole body heat exchange. r_1 = 0.5 cm, r_2 = 2.0 cm. Other terms as given in the text.

flatten as the heat delivered by the perfusion fluid exceeds the exchange capability of the gills.

Experimental data were evaluated in a similar manner. Since $Q_{cv} = f\ C_{p\rho}\ (T_i - T_o)$(Equation 2) and $Q_{cv} = hA\Delta T_{ln}$ (Equation 1) if we express T_o as a fraction, X, of T_i then:

$$f\ C_{p\rho}\ (T_i - T_o) = hA\ \ln \frac{T_i - XT_i}{\frac{T_i - T_b}{XT_i - T_b}}$$

and:

$$\ln \frac{-T_i - T_b}{XT_i - T_b} = \frac{hA\ (T_i - XT_i)}{fC_{p\rho}\ (T_i - XT_i)}$$

Subtracting T_b from all temperatures, i.e., measuring all temperatures relative to T_b:

$$\ln \frac{(T_i - T_b)}{X(T_i - T_b)} = \frac{hA}{f\ C_{p\rho}}$$

Solving for X:

$$X = e^{\frac{hA}{f\ C_{p\rho}}} \qquad (7)$$

and:

$$\% \text{ equilibration} = \left(1 - e^{\frac{-hA}{f\ C_{p\rho}}}\right) 100$$

Using this value of X:

$$Q_{cv} = f\ C_p\ (T_i - XT_i)$$

and:

$$\% Q_{tot} = \frac{f\ C_p\ (1 - X)}{f\ C_p\ (1 - X) + k\ \frac{2\pi 1}{\ln \frac{r1}{r_2}}}\ 100 \qquad (8)$$

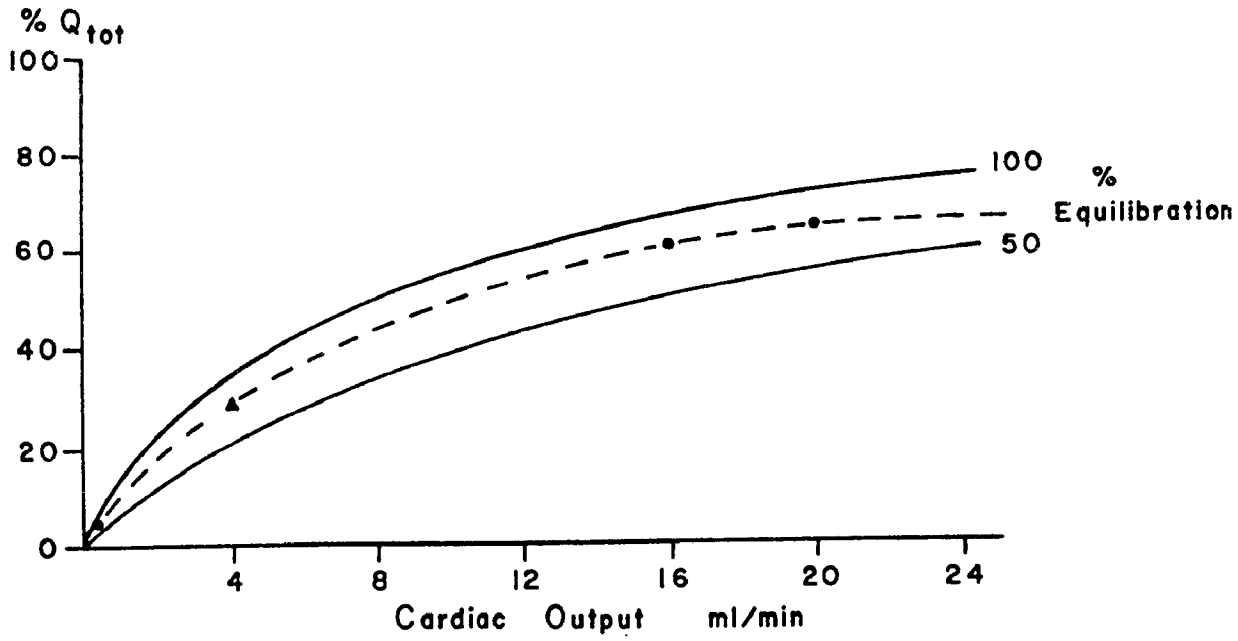

Figure 17. Graph of percent of total heat flux (% Q_{tot}) from the model gill with increasing perfusion flows. Solid lines indicate the model response at a constant % equilibration, 50 and 100%. Dotted line indicates model response using experimental data.

For the specific model of Figure 16, Equation 8 was solved using experimental data. In Figure 17 the data point at 0.5 ml min^{-1} is the value obtained for a single gill arch (Table 6) and the point at 4 ml min^{-1} is extrapolated assuming 8 independent gill arches all exchanging at the same rate as the single arch perfused at 0.5 ml min^{-1}. The points at perfusion flows of 16 and 20 ml min^{-1} were calculated using the hA_{max} values given in Table 7. The dotted line connecting these data points lies between the 50 and 100% equilibration curves for the idealized model and % Q_{tot} is leveling off with higher flow rates.

The model may also be used to determine the influence of ventilation on % Q_{tot} exchanged at the gills. Values of hA as a function of $\dot{V}_g$ were determined using Equation 5 and substituting for hA_{max} and $\dot{V}_{0.5\ max}$ at perfusion flows of 16 and 20 ml min^{-1}. Values for % Q_{tot} were obtained by solving Equation 7 as a function of $\dot{V}_g$ and using Equation 8 to determine the % Q_{tot}. The marked effect on % Q_{tot} as $\dot{V}_g$ is increased is shown in Figure 18. Several cautions should be placed on the interpretation of the model responses.

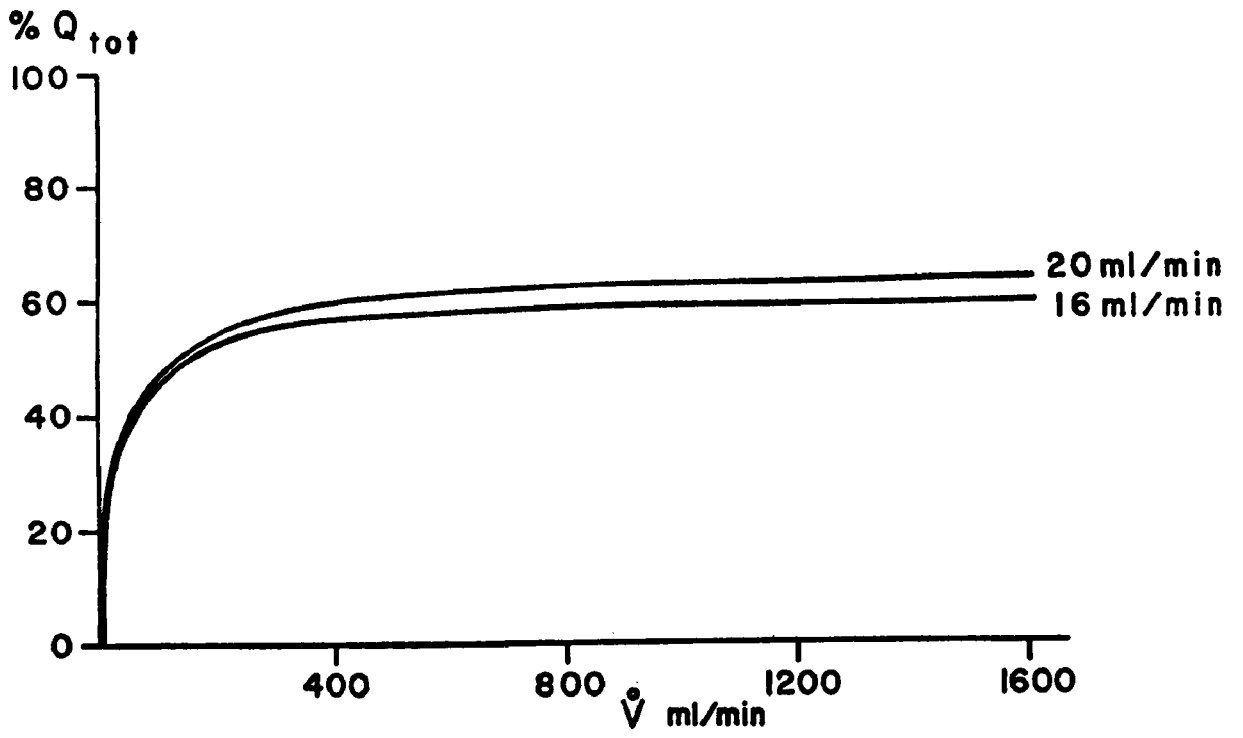

Figure 18. Model prediction of % Q_{tot} as a function of ventilatory flow ($\dot{V}_g$) using experimental data at perfusion flows of 16 and 20 ml min^{-1}.

First, we are only able to estimate the effect of perfusion flow on the maximal rate of heat transfer by the gills, the in vivo state will lie at some point below the dotted curve of Figure 17. Second, we are only able to estimate the effect of a continuous ventilatory stream at perfusion flows of 16 and 20 ml min^{-1}. Data for intact animals may be at some point below the

curves of Figure 18 due to lower cardiac outputs and the complicating factor of a phasic respiratory flow. Finally, the model of the fish body as being a purely conductive cylinder will be complicated by the convective mixing of the blood. This factor will tend to raise Q_{cd} and lower all the values of gill heat transfer when expressed as % Q_{tot}. The model is primarily illustrative of the maximum relative contribution the gills may make to whole body heat exchange. The actual contribution to heat exchange by the gills awaits further investigation.

Summary

The heat exchange has been evaluated in isolated-perfused second gill arches and the intact branchial basket. The presence of 10^{-5}M epinephrine increases the transfer maximum of the gill suggesting a change in perfusion pathway and/or vascular dimensions. Changes in perfusion flow alter heat exchange by the gill which is again due in part to changes in vascular dimensions. A model for the evaluation of the gills in relation to whole body heat exchange indicates that in the range of perfusion flows from 4 to 20 ml min^{-1} the gill may account for as much as 30 to 60% of the total heat exchange in the animal.

THE VASCULARITY OF TROUT GILLS

The final project which received financial support from Grant R-801034 was work which was an outgrowth of studies on circulation in trout gills injected with latex and examined after acid digestion (Richards and Fromm, 1969). More recently Microfil, a vulcanizing liquid silicone compound that flows through vessels of capillary size much more readily than latex, became available. The following is a summary of observations made on gill circulation in specimens infused with this compound.

Methods

Rainbow trout were anesthetized with MS-222 and placed ventral side up in a V-shaped trough, and an incision was made to expose the heart and bulbus arteriosus. Freshly mixed Microfil (2.5 ml MV 112 white, 2.0 ml MV diluent, and 0.135 ml curing agent)(Canton Bio-Medical Products, Inc., Boulder, Colo.) was drawn into a 5 ml syringe and infused via a polyethylene cannula (PE 90) that had been inserted and secured into the ventral aorta. Gentle pressure on the syringe plunger was applied manually and excessive distention of the ventral aorta avoided by extending the infusion over a period of 12-15 min. The infusion pressure was not recorded. About 5 ml Microfil was injected into fish weighing around 200 g, a much larger volume than that required to fill the gill vasculature. Significant amounts passed through the gills and appeared in vessels of the musculature and the splanchnic area. After injection, the specimens were stored in a refrigerator overnight to allow complete polymerization of the Microfil. They were then cleared in methyl salicylate (Merck), and selected casts with adhering cleared tissue were submerged in methyl salicylate and photographed (see Figure 19).

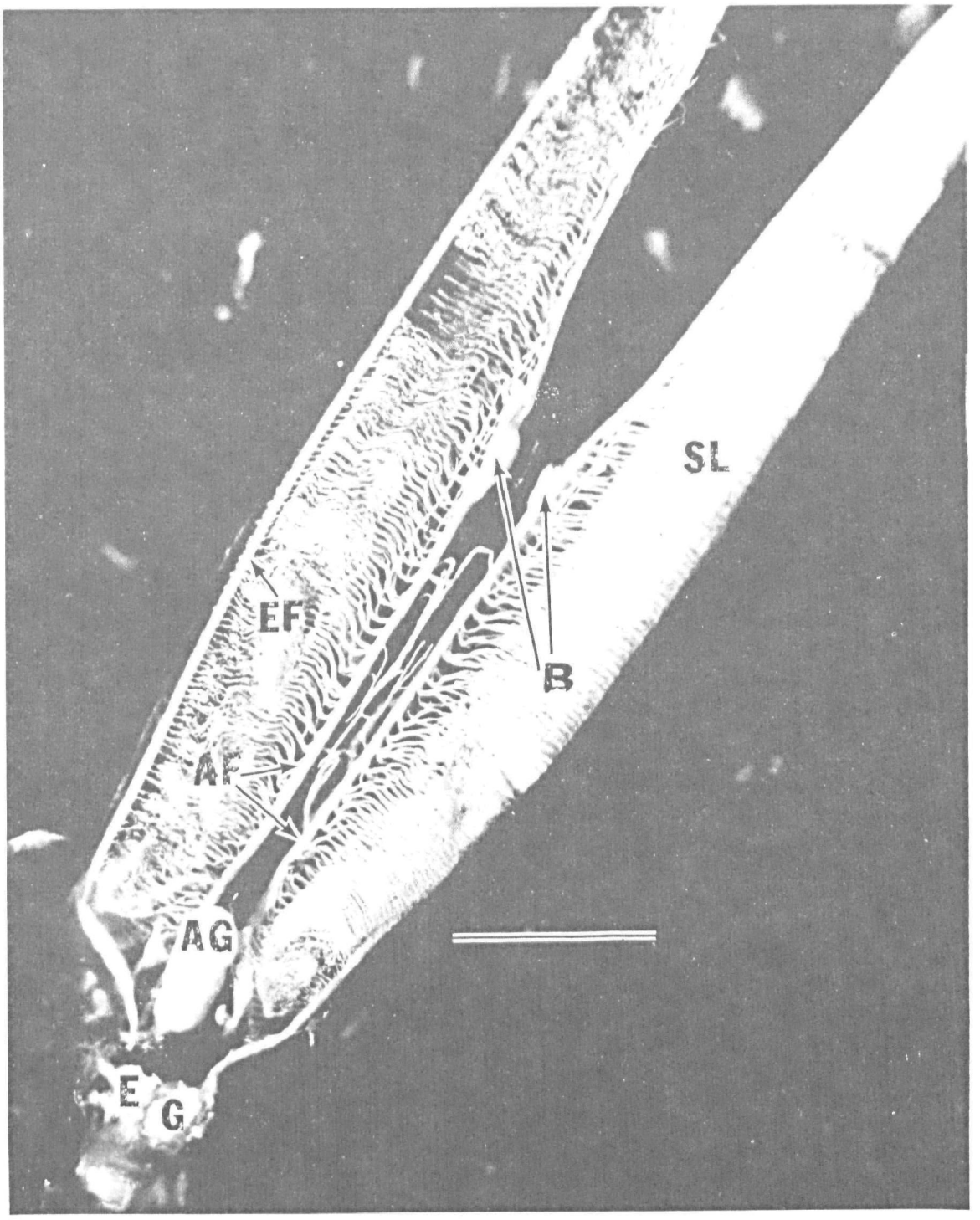

Figure 19. Microfil cast of a single pair of gill filaments from rainbow trout. AG, afferent gill vessel; AF, afferent filamental vessel; EG, efferent gill vessels; EF, efferent filamental vessel; B, afferent filamental blebs; SL, secondary lamellae. Calibration bar = 1 mm.

Results and Discussion

The trout circulatory system from the ventricle to the secondary lamellae fit the classical description except that the efferent gill vessel (EG) was found to leave the arch a short distance below the upper limit of the gill, leaving a cul-de-sac extension to drain blood from vessels of the more dorsally located filaments. Many vessels that arise from the base of the afferent filamental or from the afferent gill vessel were observed in the interfilamental septal area. Vessels on either side of the septum were seen to loop toward the base of the filament and then leave the septal area and run parallel to the afferent filamental vessel. Although it is not as clearly visible, a second somewhat larger vessel, distal to the bleb, also ran parallel to the afferent filamental vessel toward the tip of the filament. It appeared as if this vessel might provide cross connections between afferent lamellar vessels at the point where they empty into the space surrounding the pillar cells. Another septal vessel of smaller diameter than the efferent filamental vessels was found in several but not all gill arches. This vessel was located in the basal portion of the septal area and ran parallel to the afferent gill blood vessel. Several additional short intraseptal vascular connections between successive pairs of filaments on a gill arch were also noted.

Perhaps the most conspicuous feature of the gill casts was the presence of blebs. These aneurysm-like enlargements of the afferent filamental vessels were located in the most distal part of the septal area, roughly one-third of the distance from the base to the tips of filaments. The casts appeared as if those on opposing filaments would make contact with one another when fully distended but no vascular connections between blebs were found.

It appears presumptive that the blood vessels in the interfilamental septal area and those that run parallel to the afferent filamental vessels are not involved in gas exchange. The function of the filamental blebs, on the other hand, is even more speculative.

In these experiments we were aware that perfusion pressure during casting was rather critical, especially with respect to flow into the central filament space between afferent and efferent filamental vessels. It was not practical to record perfusion pressure but care was taken to avoid excessive distension of the ventral aorta during infusion. There was no evidence of the application of excessive pressure, e.g., rupture of gill vessels; thus, we believe that the casts represent fairly accurately the size and shape of vessels and blebs in trout gills.

Using a dissecting microscope and intense direct illumination we have observed a turbulent, pulsatile flow of blood into the bleb area of rainbow trout gills *in situ* and the frequency of this flow into the blebs was found to correlate perfectly with a simultaneous recording (ECG) of heart rate. Also when a large nerve carrying fibers to an isolated-perfused gill arch was stimulated, adduction of the filaments occurred, accompanied by an increase of about 15% in the perfusion pressure. When stimulation was extended for as long as 60 sec the increased pressure rapidly subsided, lasting

only about 15-20 sec. It is tempting to speculate that contraction of the adductor muscles may have increased tissue pressure surrounding the blebs and facilitated their emptying. If rhythmical isotonic or isometric contraction of these muscles occurred synchronously with heart beat, the pumping effect would be analogous to that of gill hearts that aid in forcing blood through the gill vasculature. Unfortunately, this idea is the observation by Bijtel (1949) that adduction movement of the filaments (which might not occur with isometric contraction of adductor muscles) appears sporadically and then only in connection with coughing movements.

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15. SUPPLEMENTARY NOTES

16. ABSTRACT

Studies of the effect of inorganic and organic mercury on trout indicated that uptake was primarily via the gills in non-feeding fish. Organic mercury entered fish at a faster rate than inorganic mercury. Exposure of trout to 10 μg Hg/l (methyl form) had no effect on the gill oxygen consumption measured in vitro or on the plasma electrolytes. The hematocrit index increased significantly. Studies of the metabolism of iron by normal and iron deficient trout (made deficient by bleeding) indicated that the liver, spleen, and head kidney are the major iron storage organs. Liver iron was reduced by bleeding whereas splenic iron was unaffected. In iron deficient fish more radioiron appeared in erythrocytes than in normal controls. Studies of isolated-perfused gills revealed the presence of both α and β adrenergic receptors and the data obtained indicate the functional surface area of trout gills can be regulated by changes in perfusion pathway through the gills. Use of perfused gills appears to be a very sensitive model to detect deleterious action of pollutants on fish. Evaluation of heat exchange in perfused gills indicates that the presence of epinephrine increased the transfer maximum of the gill but they were unaffected by the administration of acetylcholine. Analysis of a simple model indicated that the gills may account for as much as 60% of the total heat exchanged by trout.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
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