AN ABSTRACT OF THE THESIS OF

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TITLE: AN INVESTIGATION OF THE BLOOD-BRAIN BARRIER IN THE

RAINBOW TROUT (Salmo gairdneri)

ABSTRACT APPROVED:

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The blood-brain barrier of the rainbow trout has not
been previously investigated. Injection of aniline dyes
and their exclusion from brain tissue have served to
demonstrate the blood-brain barrier in other species. A
method of infusing dyes into a gill arch was devised.
Dyes used in the study included trypan blue, Evans blue,
fluorescein and their respective albumin complexes.

Various challenges were presented to the fish to
determine their effect on the barrier's exclusion mechanism
for aniline dyes. The challenges included brain puncture
wounds (physical challenge), acute temperature change
(thermal challenge) and treatment with toxic chemicals
including alcohols, phenol, mercury, zinc, and the
surfactant Tween 80 (chemical challenge). Absence or
presence of dye extravasation into brain tissue served

as a means of determining the effect of the injury, temperature change, or chemicals on the blood-brain barrier. Certain experiments were repeated on mice and rats to provide a comparison in the mammal.

A blood-brain barrier to aniline dyes and to fluorescein was demonstrated. Most other tissues were intensely stained by the dyes but not the brain. When given a portal of entry (puncture wound) into the brain, the dyes did stain brain tissue. Acute temperature stress had no apparent effect on the dye exclusion mechanism, however the results do not lend themselves to definite conclusions.

An apparent brain-barrier resistance to breakdown by chemical agents such as alcohols, phenol, mercury, zinc, and Tween-80 was demonstrated. Pretreatment of the fish with these chemicals had no observable effect on the dye exclusion mechanism. Phenol caused intravascular coagulation, but no dye appeared to penetrate brain tissue. Mercury may have caused changes in the brain vascular wall since a dye-darkening phenomenon occurred at high doses which appeared attributable to dyes accumulating in the vessel walls. White mice and rats have a similar dye exclusion mechanism to aniline dyes however pretreatment of the rat with mercury caused barrier breakdown and dye extravasation into brain tissue.

Dye exclusion is only a qualitative assessment of barrier integrity. Before conclusions can be drawn as

to barrier resistance to chemical breakdown quantitative

studies must be accomplished.

An Investigation of the Blood-brain Barrier
in the Rainbow Trout (<u>Salmo</u> <u>gairdneri</u>)

by

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I. INTRODUCTION

By the turn of the twentieth century scientists had
theorized the existence of a blood-brain barrier. The
theory was based upon observations with certain injected
aniline dyes, such as trypan blue, which stained all vital
tissues except the brain. On the other hand, scientists
knew that certain drugs, such as the anesthetics, entered
the blood stream and affected the central nervous system.
These observations led investigators to theorize that some
sort of protective or selective barrier, a "blood-brain
barrier," existed between the vascular compartment and the
brain which excluded the dyes but allowed the passage of
anesthetics. During the last 60-70 years much experimenta-
tion has been devoted to testing metabolites, drugs, and
toxicants for their entrance or exclusion from brain tissue.

It has been shown that complex physiological and
biochemical mechanisms are involved in the function of the
blood-brain barrier. Explanations for the exclusion of
chemicals from brain tissue include plasma protein binding,
active transport, molecular and electric charges, molecular
polarity, and lipophobic character of the chemical.

It is assumed that all vertebrates have a blood-brain barrier (2,34), and yet most of the vertebrate work has concentrated on mammals. Investigators (3,4,5,6,33) using electron microscopic techniques on vertebrate brain tissue have come to the conclusion that the blood-brain barrier in most species is formed by tight junctioned endothelial cells in the cerebral vasculature. Capillaries in tissues other than brain have open clefts between cells and a non-specific permeability which is mostly a function of molecular size of the particle (27). High molecular weight and polar substances are known to cross most capillary membranes, however, in the brain, tight junctioned endothelial cells limit the uptake of many materials. To directly affect the central nervous system a material in the blood must pass through this endothelial barrier and enter the extracellular fluid of brain tissue.

The blood-brain barrier of fish has been investigated in the roach, tench and bream (22), perch (1), brook trout (14), carp (36), goldfish (4,9), eel (29), sharks (6, 10-12,16,17,20,21,23) and the hagfish and lamprey (7, 8,25). Sharks differ from other fish and vertebrates in having predominantly open gaps between endothelial cells in the brain vasculature. The site of the blood-brain barrier in the shark has been shown to be the glial cell layer external to the capillary endothelium (6). Other striking differences between fish and mammalian brain

anatomy exist. Fish have a singular primitive meninx, whereas in higher vertebrates a double and triple layered meningeal membrane exists. This anatomical difference limits cerebrospinal fluid, which is in intimate contact with the extracellular fluid of brain tissue, to the ventricles and central canal of the spinal cord in fish. In addition, differences in the distribution of cerebral blood exist in lower vertebrates as compared to higher vertebrates. Heisey (18) has shown that the choroid plexus of cyclostome brain contains sixty percent of total blood volume in the brain, whereas the rat choroid plexus contains four percent. Choroid plexus blood flow in the dogfish shark and rabbit are nearly the same, 2.3 ml/min/gm and 2.9 ml/min/gm respectively (16,30).

Similarities between fish and mammalian tissue uptake or exclusion of certain compounds, notably injected aniline dyes have been reported (22,24). However, uptake and/or exclusion of some compounds is different. Investigators have noted differences in the uptake of injected catecholamines which are not excluded from the midbrain of goldfish or eels as they are in mammals (9,29). Since epinephrine crosses the blood-brain barrier in fish, circulating catecholamines may exert a direct action on brain centers to regulate physiological functions (29).

Differences between uptake of compounds into shark

brain and that of mammals has also been reported. Inulin
and sucrose, which are poorly taken up by rat brain tissue
(32), cross the blood brain barrier more readily in
sharks (16).

Water is a highly diffusable substance into brain
tissue despite its low lipid solubility (26). The tight
junctions in the brain vasculature may serve as a limiting
factor for water permeability since it has been suggested
that highly lipid soluble molecules have, at their
disposal, the entire capillary endothelial surface for
diffusion (28).

A highly lipid soluble molecule may not only traverse
the blood-brain barrier, but may also destroy or damage
cells in the process due to its solvent properties. Cell
membrane lipid structure may be damaged, thus causing
a breakdown in the integrity of the barrier. This break-
down may be assessed by measuring the amount of exogenously
administered test substance taken up into brain tissue
from the blood. Microscopic examination of brain cell
vascular membranes and brain tissue after introduction of
an aniline dye would also determine whether chemical,
thermal or physical challenges had broken down the blood-
brain barrier. Presence of dye in the tissues would
indicate damage to the blood-brain barrier.

Agents which damage the blood-brain barrier have been

classified as either "reversible" or "irreversible."
Reversible agents cause a temporary osmotic imbalance
which shrink cells and/or open the clefts between the
tight junctions in the brain vasculature. Irreversible
agents can destroy cells in the brain vasculature by
denaturing protein. Rapoport (31) demonstrated, in the
rabbit, that barrier opening, caused by certain osmotic
agents such as urea and acetamide, could be reversed by
removing the agent and flushing with an isotonic Ringer's
solution. Extravasation of subsequently administered
aniline dyes into brain tissue was evidence of barrier
damage. Propylene glycol and cyanamide caused permanent
or irreversible damage to the brain barrier, as evidenced
by profuse dye extravasation before and after the saline
flush. However, metabolic inhibitors such as NaCN and
NaF were shown not to impair the integrity of the blood-
brain barrier to HCO_3^-, and the barrier to aniline dyes
was undamaged over a pH range of 1-9.

Using dye exclusion as a criterion for membrane
integrity, Klatzo (20,21) has shown that the blood-brain
barrier of sharks is more resistant to chemical breakdown
and to thermal and physical injury than a mammalian brain.
Heat or cold applied directly to exposed shark brain tissue
did not increase the permeability of the blood-brain
barrier to injected aniline or fluorescing dyes. Diffuse
staining was noted in the cat brain treated in the same

manner demonstrating a major difference in the stability of the barrier when thermally challenged. The shark brain barrier was also shown to be resistant to chemical injury by mercuric ion. Intravenous treatment with mercuric ion followed by injection of dye resulted in no dye extravasation into brain tissue. In the mammal this agent caused irreversible barrier damage and diffuse dye leakage into brain tissue (21).

Since striking differences exist in the characteristics of fish and shark blood-brain barrier compared to mammals it would be beneficial to study a "model physiological system" such as the rainbow trout to gain a more complete understanding of the properties of the exclusion mechanism.

The protection of our waters from pollutants is a topic of environmental concern. The rainbow trout can also serve as a model to assess the environmental impact of thermal and chemical pollution since this species has demonstrated a very low tolerance for habitat change. When temperature and water quality vary outside of certain narrow limits, the rainbow trout have difficulty adapting. An important physiological membrane, the blood-brain barrier, has not been investigated in the rainbow trout. The effects of heavy metals, detergents and lipid soluble molecules on the barrier have also not been studied. Since thermal effluents and chemicals are major contributors

to water pollution it would be important to know their affects on the blood-brain barrier of the trout.

The purpose of this study will be to demonstrate the existence of the blood-brain barrier in the rainbow trout. Various challenges to barrier integrity will be presented to the fish and will include physical, thermal, and chemical injury. Methods, although modified, will closely resemble those used by previous investigators on sharks and mammals. Aniline dye exclusion or extravasation into brain tissue will serve as a means of assessing barrier patency. Certain experiments will be repeated on laboratory rats to provide a mammalian comparison.

II. METHODS

Cannulation

Hatchery rainbow trout weighing 350-750 grams were held at 11-12°C in holding tanks prior to experimentation. Fish were anesthetized with MS222 (tricaine methane sulfonate) at a concentration of 100 mg/l, weighed, placed on a surgery table and strapped down ventral side up. A slow drip of MS222 into a flow of fresh water (150 mg/l) was flushed over gills. This kept the fish oxygenated but anesthetized. A rubber tubing and hook retractor were used to pull the operculum back exposing the gills. A length of "O" suture silk was looped around the first gill arch and the arch was tied off near the ventral aorta. The arch was clamped on the dorsal side and severed near the suture. A suitable length of PE 20 tubing filled with saline-heparin (300 mosM) was inserted 1½ cm. into the arch. The cannula was secured to the arch with several suture wraps and anchored by an attachment to the pectoral fin. The clamp was removed and the fish was placed into a well aerated recovery tank for 24 hours. A cork was placed on the distal end of the cannula which floated to the top of the tank and was readily accessible for subsequent dye

infusion or toxicant administration.

Infusion of Dyes

After a 24 hour recovery period dye was infused into
the fish via the cannula. At least three fish were used
in each of the experiments. One of the following filtered
solutions was used:

1-2% solution of trypan blue (TB) in isotonic saline
(22)

2% solution of Evans blue (EB) in isotonic saline (24)

2% solution of TB or EB in 10% bovine serum albumin
(BSA, Sigma Chemical Co.) (TBA, or EBA) (21)

0.2% fluorescein in 10% BSA solution (F1A) (20)

Using care not to unduly excite the fish by noise
or unnecessary handling, 6 ml/kg of dye solution was
injected over 30 minutes by hand held syringe into the
cannula, or was infused over one hour with a Harvard Model
421 syringe pump. In some cases a second infusion of dye
was given 24 hours after the first to increase the staining
of the tissues. Cannulated controls received isotonic
saline. After the second infusion procedure and a period
of 24 hours to insure dye distribution to tissues, the
fish were decapitated. The brain and other tissues were
removed to determine the extent of staining. Small sections
of tissue were frozen in liquid nitrogen for subsequent

cryostat sectioning and microscopic examination. Tissue from fluorescein treated fish was examined with a fluorescence microscope.

Physical and Chemical Challenges

The integrity of the trout brain dye exclusion mechanism was tested by exposing the animal to various physical challenges and toxic insult. Dye extravasation into brain tissue after the challenge would be evidence of blood-brain barrier breakdown.

a.) Physical Challenge

The trout brain was intentionally damaged to determine the effect of physical injury on barrier integrity, and to observe dye extravasation into brain tissue (after Klatzo, 21). The fish were cannulated as described and while under anesthesia a flap of cartilage over the brain was removed and the optic lobe was exposed. In two fish a sharp 30 gauge needle was inserted into the area and a small amount of dye (100 μl) was injected into the wound. In two other fish the brain was wounded with the needle as above and 1 ml of dye was quickly injected via the cannula. Normal procedure for this test and all subsequent tests was to decapitate the fish within three to ten minutes, remove the brain and examine it for

evidence of the extravasation of dye into the brain tissue.

b.) Thermal Challenge

Direct hot and cold plate applications to exposed brain tissue of mammals and sharks to test barrier integrity have been described (21). The response of fish brain barrier in that study (21) was not tested when the temperature of the environment was acutely altered. Temperature of the environment has been proposed to explain the differences in uptake of certain materials in different species (16).

In the present study four fish were cannulated and held in recovery tanks at $21^{\circ}C$ for 24 hours. Dye was infused as described above. In a second type of heat challenge eight fish were cannulated and allowed to recover at $11^{\circ}C$. After 24 hours the fish were quickly transferred to an aerated tank at $21-23^{\circ}C$, and a dye solution was infused as above. Within ten minutes after dye infusion the fish were decapitated and brain tissue samples were examined.

c.) Chemical Challenge

Toxic chemicals are frequently injected into the blood stream of mammals and fish to test the chemicals' effect on the blood-brain barrier (21,30,31). Heavy metals, (21), phenol (17), alcohols (30) and detergents (17,21) all have known effects in certain species. One

method of assessing the effect of these chemicals on the
blood-brain barrier is to follow the toxicant injection
by an infusion of aniline dye. Dye extravasation into
brain tissue is evidence of barrier damage (17).

Using methods similar to those of previous investigators, chemical challenges were presented to the trout
according to the outline shown in Table 1, Set A. These
injections were followed by 6 ml/kg/hr of one of the
aniline dyes. Animals were decapitated at time intervals
of five to ten minutes and 24 hours, after dye infusion
and brain tissue was examined.

Rapoport (31) has suggested that certain chemical
agents act reversibly on barrier opening. That is, the
barrier opening occurs only when the agent is in direct
contact with the barrier, and as soon as the agent is
flushed away the barrier returns to its original state.
In a second set of experiments, Table 2, Set B, solutions
of the test chemicals were mixed with 2% TBA and injected
via the cannula. The time lag between toxicant introduction
and dye administration was zero. If the agents caused
barrier opening or damage the dye would extravasate into
the brain tissue immediately. Decapitation was performed
within ten minutes and brains were examined.

Table 1: Toxicants used in experimental Set A. Chemical challenge of the blood-brain barrier of the rainbow trout.

Agent	Number of fish	Dose range	Total volume injected	Injection type
$ZnCl_2$	3	3.5-7.0 mg/kg (as Zn^{++})	0.4-2 ml	Bolus, or 0.2 ml injected every ½ hr. for 1-4 hours.
$HgCl_2$	6	0.8-15.0 mg/kg (as Hg^{++})	1-2 ml	Same
Phenol 5-10% in saline	4	20-200 mg/kg	0.1-1 ml	Same
70% ethanol	2	1.4-2.8 gm/kg	1-2 ml	Bolus
70% isopropanol	2	1.4-2.8 gm/kg	1-2 ml	Bolus
10-20% Tween 80	3	400-600 mg/kg	1 ml	Bolus

Table 2: Toxicants used in experimental Set B: Chemical
          challenge of the blood-brain barrier of rainbow
          trout.

Agent	Number of fish	Dose of toxicant	Total volume of dye plus toxicant
$ZnCl_2$	2	1.7-3.5 mg/kg (as Zn^{++})	6 ml/kg over one hour
Tween 80 (20% sol.)	2	200-300 mg/kg	Same
Ethanol (70%)	2	0.7-1.4 gm/kg	5 ml/kg over 30 minutes

In one experiment, phenol was mixed with TBA:

0.3 ml Phenol (10%)	1	120 mg/kg	6 ml/kg/hr

Comparative Mouse and Rat Study

Blood-brain barrier characteristics of ten (CF #1)
mice (25 gm) were tested by injection of aniline dyes.
EBA and TBA at doses of 6 ml/kg/hr, up to 0.3 ml total
volume were administered via the tail vein.

Experimental mice (CF #1) were given bolus injections
of mercury (1.8 mg/kg up to 15 mg/kg) via the tail vein.
These injections were followed by the administration of
EBA or TBA (6 ml/kg/hr). Animals were decapitated at 12
and 24 hours and brains were removed and examined.

Two Sprague-Dawley (Simonsen) rats (250 gm) were

anesthetized with 15 mg intraperitoneal pentobarbital.
The left carotid artery was isolated and cannulated with
PE 10 tubing. A 0.4 ml injection of $HgCl_2$ solution;
(1.8 mg/kg as Hg^{++}, Klatzo, 21), was given over a one
minute period. This was followed by 0.4 ml of 2% EBA.
Controls were injected with saline followed by the same
dye. The rats were decapitated within five minutes.
The brains were removed and examined for dye extravasation.

III. RESULTS

Gill tissue, gonad, kidney, muscle and liver were
all stained by the aniline dyes (Plates 1,2). Dye accumula-
tion was most pronounced in the liver. In the brain the
medulla, cerebellum, optic lobes, and most of the olfactory
lobes were consistently devoid of dye as was the spinal
cord (Plates 3,4). A slight amount of dye could usually
be seen in the anterior portion of the olfactory lobe in
the area of the choroid plexus. This area is highly
vascularized and the dye observed was probably that in
the vessels, not in the brain tissues. No dye was ever
observed to penetrate the saccus vasculosus (Plates 5,6.
See also Appendix A). The hypophysis was always darkly
stained (Plate 5).

Frozen tissue sections (10-40μ) were examined
microscopically. Except for the anterior olfactory lobe
and the hypophysis, no apparent evidence of dye was detected
in the brain or spinal cord sections. A few particles
of dye could sometimes be seen in the lumen of a brain
vessel but never in the wall of the vessel or brain
parenchyma. Diffuse staining by the dye was observed in
sections of liver, gill and intestine.

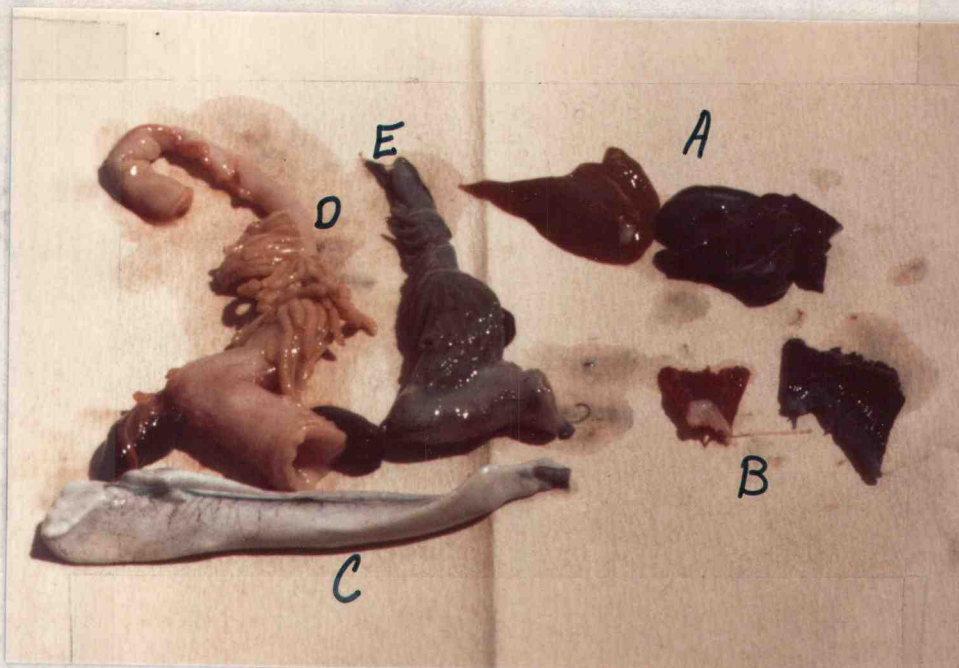The fish were able to tolerate the infusion of dyes

Excised tissue from trout controls vs tissue from trout
treated with two infusions of EBA. Dye treated tissue is
on the right of control tissues. Note intense staining
of liver (A), gill (B), pyloric caeca (D), intestine (E).
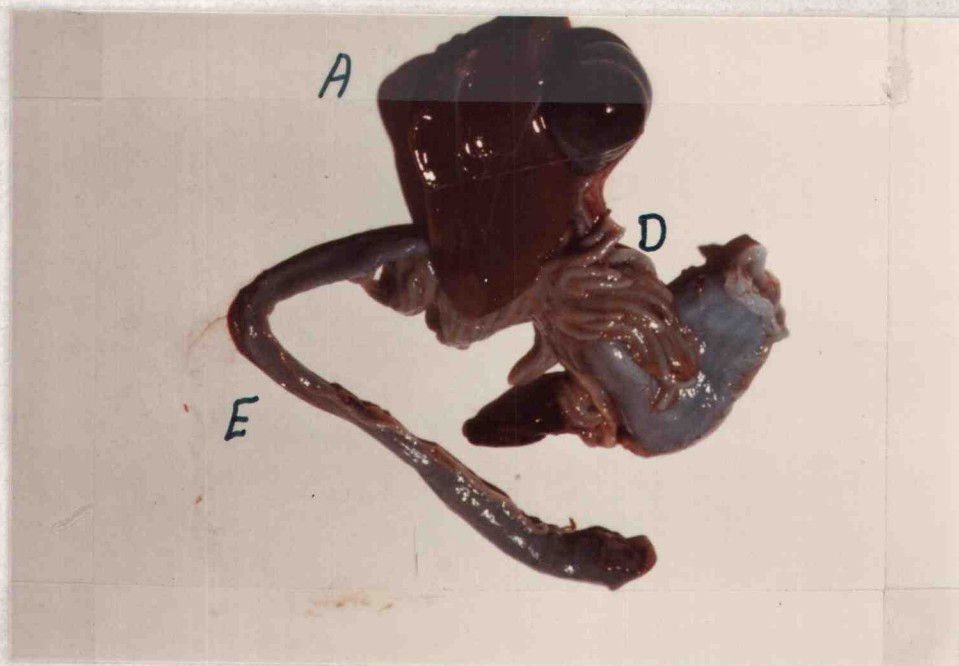Moderate staining of gonad is noted (no control available).

Plate 1

Stained viscera of TBA treated trout. Note more intense
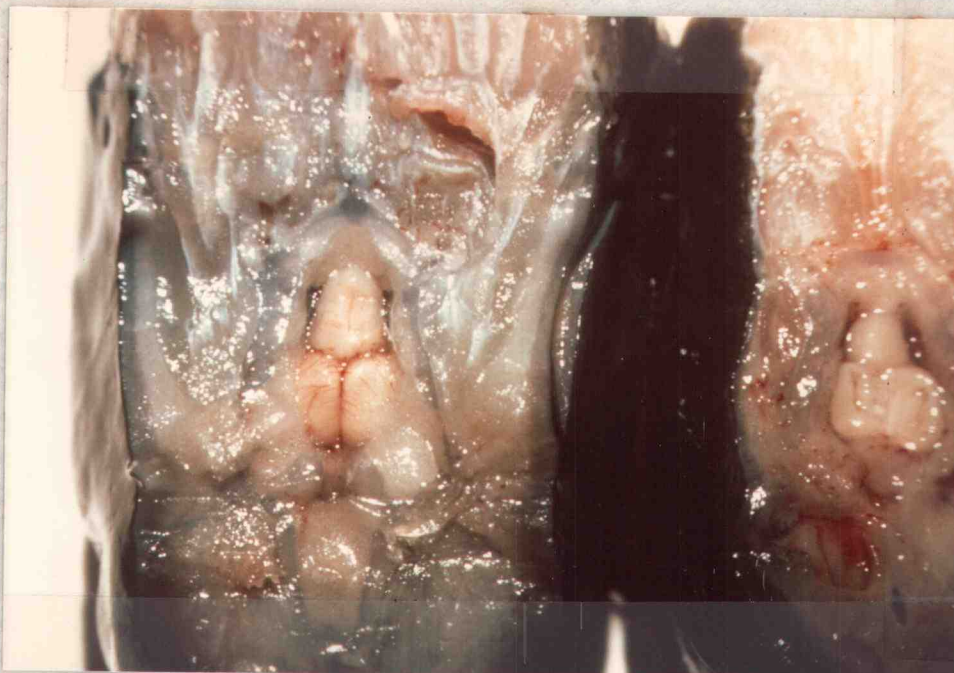staining of tissues using EBA. (A), (D), (E) as above.

Plate 2

Plate 3

Dorsal view of exposed trout brain. Experimental fish on
the left received TBA infusion. Control trout on right.
Note in trout treated with TBA that the dye penetrates
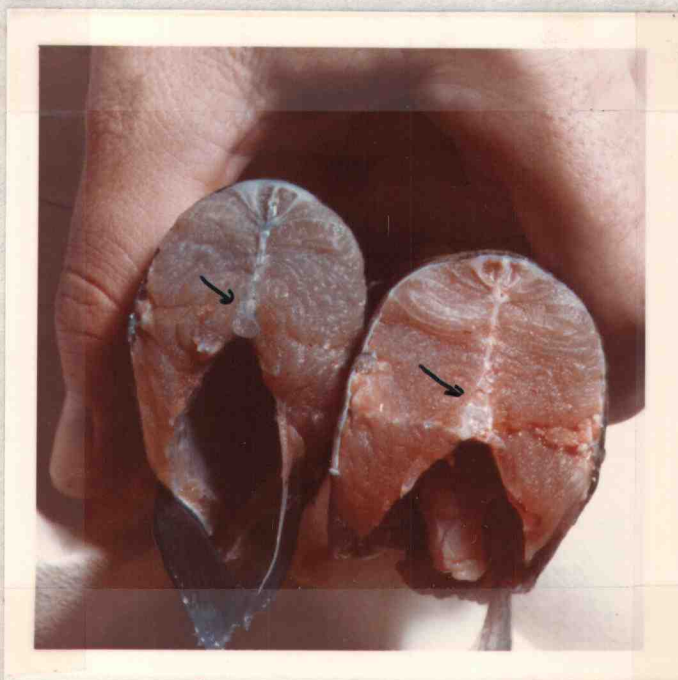surrounding tissues but does not stain brain tissue or
vessels.

Plate 4

Cross sections of trout taken immediately posterior to the
head. Trout on left treated with 2% TBA. Note dye pene-
trates muscle tissue but does not stain the spinal cord
(arrow).
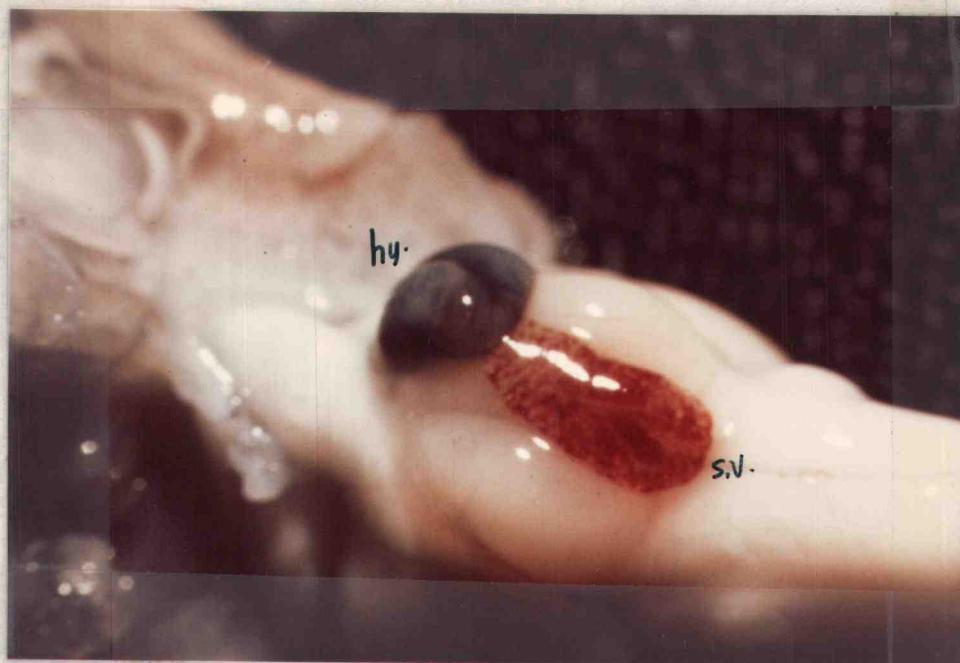
Ventral view of excised brain from trout treated with EBA.
Note intense staining of hypophysis (hy.) and unstained
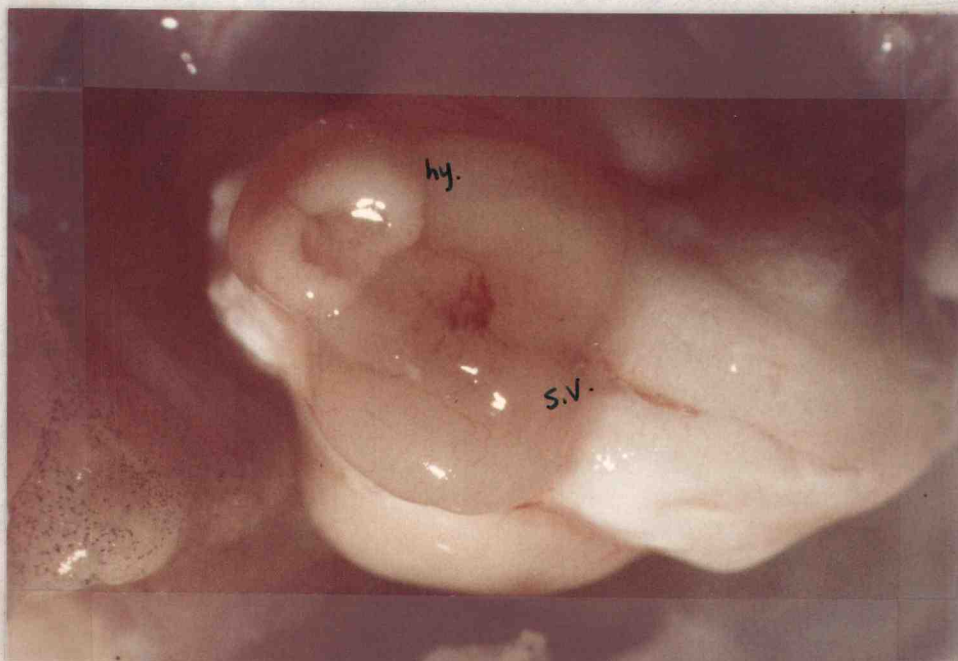saccus vasculosus (s.v.).

Plate 5

Excised control trout brain. Note hypophysis (hy.) and
saccus vasculosus (s.v.). The saccus in this fish was not
filled with blood. In general, the saccus was filled to a
greater extent in those fish treated with dyes or toxicants.

Plate 6

as long as it was accomplished over at least a 30 minute period. If the infusions were done too rapidly the fish became excited or irritated in the tanks and they would tear out the cannulae. Dye-protein complexes provided better staining of tissues than simple 2% solutions in saline. EBA was the more satisfactory of the two aniline dyes, as it generally stained the tissues better than did TBA.

In fluorescein treated fish no fluorescence was detected in the brain tissue sections examined other than a few particles in the lumen of blood vessels. However, thin sections of liver were fluorescent.

Under heavy anesthesia the fish reacted to the needle puncture procedure with violent jerking. The two fish expired within ten minutes. Upon examination the dye had diffusely stained the brain tissue in and around the stab wound. Physical damage to the brain-barrier had allowed dye penetration and diffuse staining of brain tissue. Attempts to infuse the dye through the cannula after the stab wound were unsuccessful.

At 21°C the fish recovered slowly from the anesthetic, and only one out of four fish could tolerate the high temperature for 24 hours. The dye stained the body tissues, however, no dye penetrated the brain parenchyma or the spinal cord.

The fish reacted violently to the quick immersion technique at 21-23°C. They thrashed around in the tanks and within 15 minutes were on their sides in obvious distress. As soon as the fish were immersed, a dye infusion was started. In four out of eight fish about one ml of EBA was infused before the violent thrashing tore the cannulae out. Decapitation was performed after ten minutes. The dye had stained the liver slightly, but it was impossible to determine the staining patterns of other tissues.

When chemicals were injected (maximum rate: 1 ml/min) the fish generally became agitated in the tanks. They became spastic and convulsed within minutes after injections of zinc, mercury, and phenol. However, in contrast to the temperature experiments, less difficulty was encountered when subsequently infusing the dye (EBA 6 ml/kg/hr). Animals were decapitated after ten minutes, and in some cases 24 hours after the dye infusion process and brain tissue was examined. Only about one out of four experimental fish survived any of the acute chemical challenges for more than 24 hours.

The EBA did not appear to penetrate the brain tissue in either the short interval or in the 24 hour interval even after challenge with the highest doses of toxicants. The brain vasculature of the choroid plexus was visibly

reddened, especially in the phenol treated fish (Plate 7).

The saccus vasculosus became engorged with blood and swollen. Administration of isopropanol and ethanol caused the brain to appear "puffy" or swollen, however, subsequently administered dyes still did not appear to penetrate the brain or spinal cord. The puffy appearance may have been due to the protein precipitant action, or osmotic imbalance leading to severe edema of the brain tissue. Conclusions cannot be drawn since only one fish per dose was studied.

When zinc or mercury was injected the dyes took longer to distribute to the tissues than in control fish. The dye could readily be detected in the liver after 30 minutes in control fish, whereas only slight staining was evident in the liver after 30 minutes in those fish pretreated with zinc or mercury. At the higher doses of mercury (5-15 mg/kg) the fish became hyperactive and expired within one hour. Infusion of EBA before death resulted in a slight gray appearance of the entire brain after the highest dose (15 mg/kg) of mercury. There was no apparent dye extravasation into the brain tissue, however, either at the gross or at the microscopic level. None of the other chemicals caused a change in the dye exclusion mechanism in the trout brain.
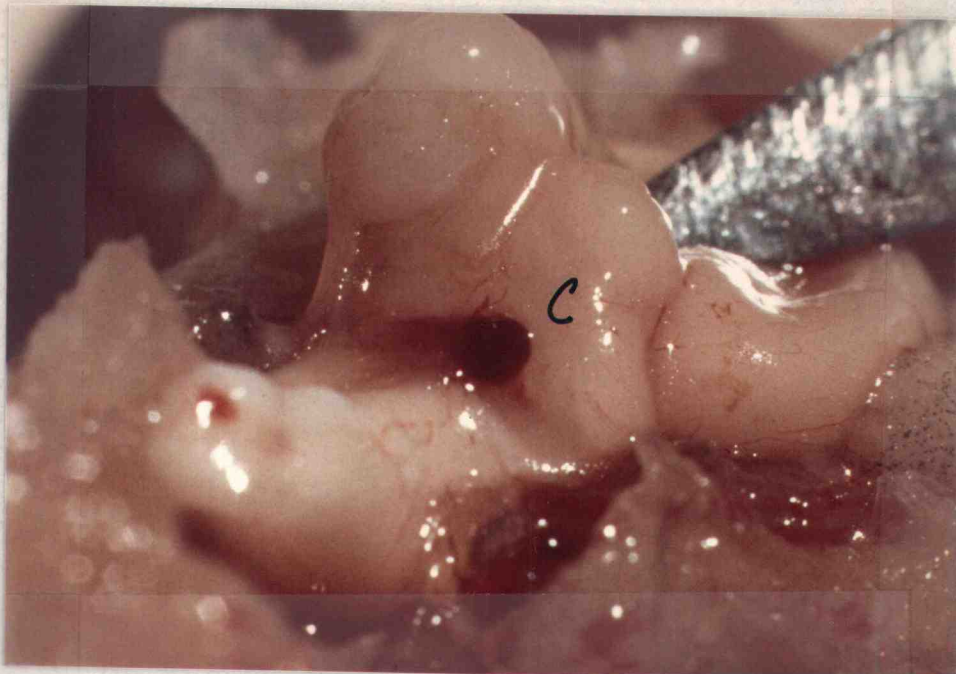
Premixing the chemicals with the dyes in Set B had

Plate 7

Excised, phenol (200 mg/kg) treated trout brain followed
by EBA. Note that phenol causes intravascular coagulation
under the cerebellum (C), however, there is no apparent
extravasation of dyes into the brain tissue.

no effect on the dye exclusion mechanism in the trout brain.
The fish became agitated as before. No dye was apparent
in the brain tissue ten minutes after the dye infusion
process.

TBA and EBA stained muscle, kidney, liver and gonad
of the ten mice. The spinal cord and brain were devoid of
dye. Mouse tissue stained more intensely than that of
fish. Low doses of mercury (1.8 mg/kg) seemed to have
little effect on the mice in the 24 hours they were observ-
ed, however, mercury at a dose of 15 mg/kg caused paralysis
of the hind limbs of the animals within hours after in-
jection and death within 12 hours. There was no difference
in the staining of the tissues in the controls or mercury
treated animals. Mercury did not cause a change in the
dye exclusion mechanism in the mouse brain.

Cannulation and infusion of mercury into the rat
followed by an infusion of EBA resulted in intense dye
extravasation into the brain tissue on the same side as
the cannulation (Plate 8,9). This indicated that mercury
caused a breakdown of the blood-brain barrier in the rat,
and allowed the passage of dye into the brain tissue.

Plate 8

Mercury/EBA dye treated rat brain (right) versus
saline/EBA treated rat brain (left). Ventral view.

Plate 9

Dorsal view of above.

IV. DISCUSSION

The rainbow trout can serve as a model system to assess the impact of water pollutants. The viability of this species can be affected by even small amounts of heavy metals, detergents, or lipid soluble compounds in their environment. Slight variations in temperature can also affect viability. Most toxic compounds (including heavy metals, detergents, and lipid soluble compounds) are known to affect respiration and the gill apparatus of the trout, however, very little is known in the area of the physiology of the brain of the rainbow trout. The effect of temperature and toxic chemicals on the blood-brain barrier of the trout is not known. The blood-brain barrier serves as an important, selective barrier to the entrance of many substances into the brain. It is important to know if toxicants or thermal stress damage the barrier and contribute to the decrease in viability of the trout.

These studies have demonstrated a blood-brain barrier in the rainbow trout to EBA, TBA, their respective protein complexes, and to fluorescein. The methods were patterned after studies on mammals and on other fish. The site of cannulation (the first gill arch) was chosen because of its proximity to the brain. Shutting off the blood supply

to the arch evidently does not incapacitate the fish.

The staining of tissues closely parallels that reported for other species (21,22,24), however, previous investigators indicated that the choroid plexus was stained by the dyes in their respective studies. This was not seen in the present study with the dyes used.

Dye protein complexes provided better staining than the simple dye solutions. Perhaps the albumin is more loosely bound to the dye in the complexes in vitro than the dyes are in vivo to plasma proteins. The complexed dyes would then dissociate more readily and be available to diffuse into the tissues.

Dye extravasation in and around the stab wound showed that, given a portal of entry across the barrier, aniline dyes will stain brain tissue.

Acute heat stress had no apparent effect on the dye exclusion mechanism in the trout brain. The difficulties encountered with the methods employed may have been overcome if the trout were acclimated to the temperature extremes in a slower, stepwise fashion. However, any changes in the exclusion mechanism as a result of temperature extremes might still have been secondary to respiratory difficulty, ionic permeability changes or some other mechanisms. The results of the heat experiments are therefore not conclusive.

Toxic agents, known to alter lipid or protein structure in mammals, apparently did not cause a change in the dye exclusion mechanism in the trout brain. Long term affects (more than 24 hours) of these agents were not studied. However, it is possible that the results might have been quite different if these agents were given in smaller quantities over a longer period. The complex interactions of pollutants with other pollutants and/or temperature were not studied. Mercury (15 mg/kg) caused the entire brain to become darkened. Perhaps this darkening was due to small amounts of dye "leaking" through the barrier due to mercury's protein precipitating effects on membranes. The darkening may have been precipitated mercury itself giving the brain the grayish coloration. This graying effect has been described in the mercury treated shark (21). If this was the case and mercury was in the tissues, the addition or interaction of another toxicant may have marked effects on the brain barrier. Transport of toxic chemicals out of brain tissue back across the barrier must also be a consideration in the study of complex interactions.

Alcohols, although quickly absorbed through the lipophilic barrier (30), did not cause a breakdown, or a temporary opening (31) of the barrier to the dyes. A surfactant-detergent (Tween 80), known to alter lipid

structure (17,21), likewise had no effect on the integrity of the barrier. Phenol is known to cause neuromuscular excitability and to cause intravascular coagulation (35). Coagulation was observed in the choroid plexus of the trout brain, however, the barrier to infused dyes remained intact. The apparent resistance of the trout brain to breakdown by phenol, detergents, and mercury may suggest that an ultrastructural configuration similar to that seen in the shark and in mammals exists, i.e., a protective glial arrangement in the cell layer external to the vascular endothelium. The ultrastructure of the rainbow trout blood-brain barrier has not been described morphologically.

Mercury was expected to cause barrier breakdown in the mice, but could not be demonstrated. No explanation for this can be offered at this time. Mercury did cause a breakdown of barrier integrity in the rat as expected.

The apparent resistance of the rainbow trout brain barrier to breakdown by phenol, surfactants, and heavy metals lead to the conclusion that the trout barrier may have some characteristics closely resembling those of the shark barrier. It is possible that dye exclusion or extravasation is not the best means of assessing barrier patency. Slight amounts of dye may pass through the barrier and may not be seen at the macroscopic or

microscopic levels used in this study. Further study might include electron microscopic examination or radio-isotope studies.

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APPENDIX

APPENDIX A

A part of the trout brain is an obscure organ called
the saccus vasculosus. The saccus is easily visualized
on the exposed ventral side of the intact trout brain due
to its bright red coloration resulting from rich vascu-
larity. The organ is located both posteriorly and dorsally
to the hypophysis. The saccus is lacking in some freshwater
teleosts, present in all marine teleosts and sharks, and
in the freshwater and marine rainbow trout.

Early investigators (13) suggested a sensory function
for this organ but current thought is that it is secretory
and osmoregulatory. The organ has been shown to secrete
mucopolysaccharides (15,19), and to have an active role
in osmoregulation in the sea water adapted rainbow trout
(19). The osmoregulatory activity is paralleled by blood
pooling and swelling of the organ in the sea water adapted
trout (19).

Injected protein bound dyes such as TBA do not
stain the saccus of the freshwater trout (see Results)
or the sea water adapted trout (19), therefore, a blood-
brain barrier to aniline dyes probably exists in the saccus.
In contrast the saccus was stained with aniline dyes in the
shark (21). Possibly sea water adaptation changes the

saccus' barrier exclusion mechamism for aniline dyes in the shark.

In the present study no dye was ever seen to penetrate the saccus vasculosus, even after exposure of the fish to a toxicant. However, after chemical challenge the organ, in many cases, was visibly more swollen and filled with blood. This may suggest some type of a response to the toxicants, but further study is needed before any conclusive statements may be made.