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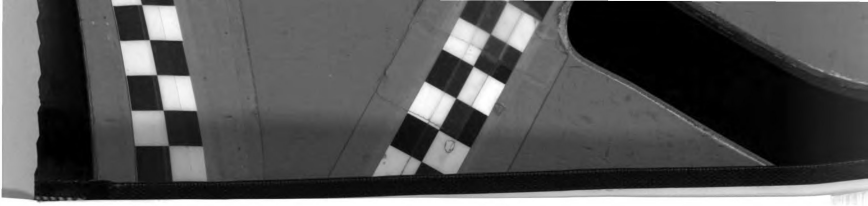

ABSTRACT

ELECTROPHYSIOLOGICAL RESPONSES OF THE LATERAL LINE AND HEART TO STRESSES OF HYPOXIA, CYANIDE, AND DDT IN RAINBOW TROUT

By

Thomas Gordon Bahr

Studies of lateral-line nerve and heart responses in rainbow trout, Salmo gairdnerii, to certain pollutional stresses are presented. Spontaneous and evoked lateral-line neural discharges and electrocardiograms were measured in situ from curarized preparations exposed to hypoxic and asphyxic conditions, and cyanide and DDT poisoning.

Asphyxiation and cyanide poisoning caused a reduction in heart rate, changed wave forms of the electrocardiogram, and depressed both spontaneous and evoked activity from the lateral-line nerve. Evoked responses persisted longer under stress than spontaneous activity. Discontinuation of stress was followed by recovery of both heart and lateral-line activity. Normal function of the lateral line is dependent on blood circulation and it is believed that ischemic conditions in the lateral line were responsible for the depressing effects of the stresses.

Thomas Gordon Bahr

Intravenous injection and water exposure to DDT caused little if any change in lateral line or heart activity. No change of these parameters was observed in fish suffering characteristic DDT poisoning tremors.

Hypoxic conditions resulted in a slight decrease of the heart rate and changes in the electrocardiogram were noted. Electrical activity recorded from the lateral-line nerve remained unchanged.

The methodology developed for this study proved to be a useful tool for analyzing neurotoxic effects of pollution-related stresses.

ELECTROPHYSIOLOGICAL RESPONSES OF THE LATERAL LINE AND HEART TO STRESSES OF HYPOXIA, CYANIDE, AND DDT IN RAINBOW TROUT

By

Thomas Gordon Bahr

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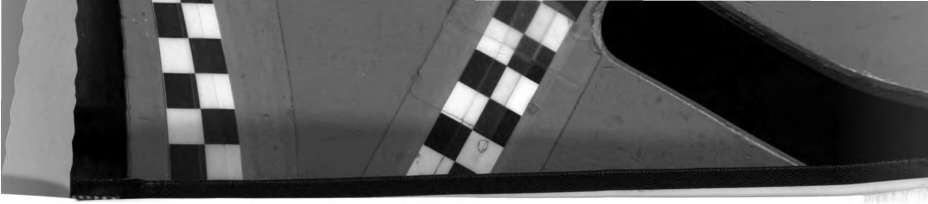

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INTRODUCTION

Studies in the past which have been concerned with effects of pollution stress upon physiological changes in fish species have largely ignored effects of toxic pollutants upon functional changes in the nervous system. This is unfortunate since a very large number of toxic substances are recognized as specific nervous system poisons. In addition to specific neuro-toxins there are a number of chemicals not normally considered to be neurotoxic which can alter physiological processes within the body in such a manner as to have profound secondary effects upon the nervous system. Because the nervous system is extremely sensitive to changes in the internal environment of the body and because it is unparalleled in its role of immediate usefulness to the animal it is not surprising that a large variety of substances can cause severe disability or even death by secondarily affecting the nervous system.

It is believed that a more detailed understanding of neural mechanisms in fish, coupled with knowledge of effects of various toxic substances associated with pollution are necessary preliminaries for understanding the complexity of

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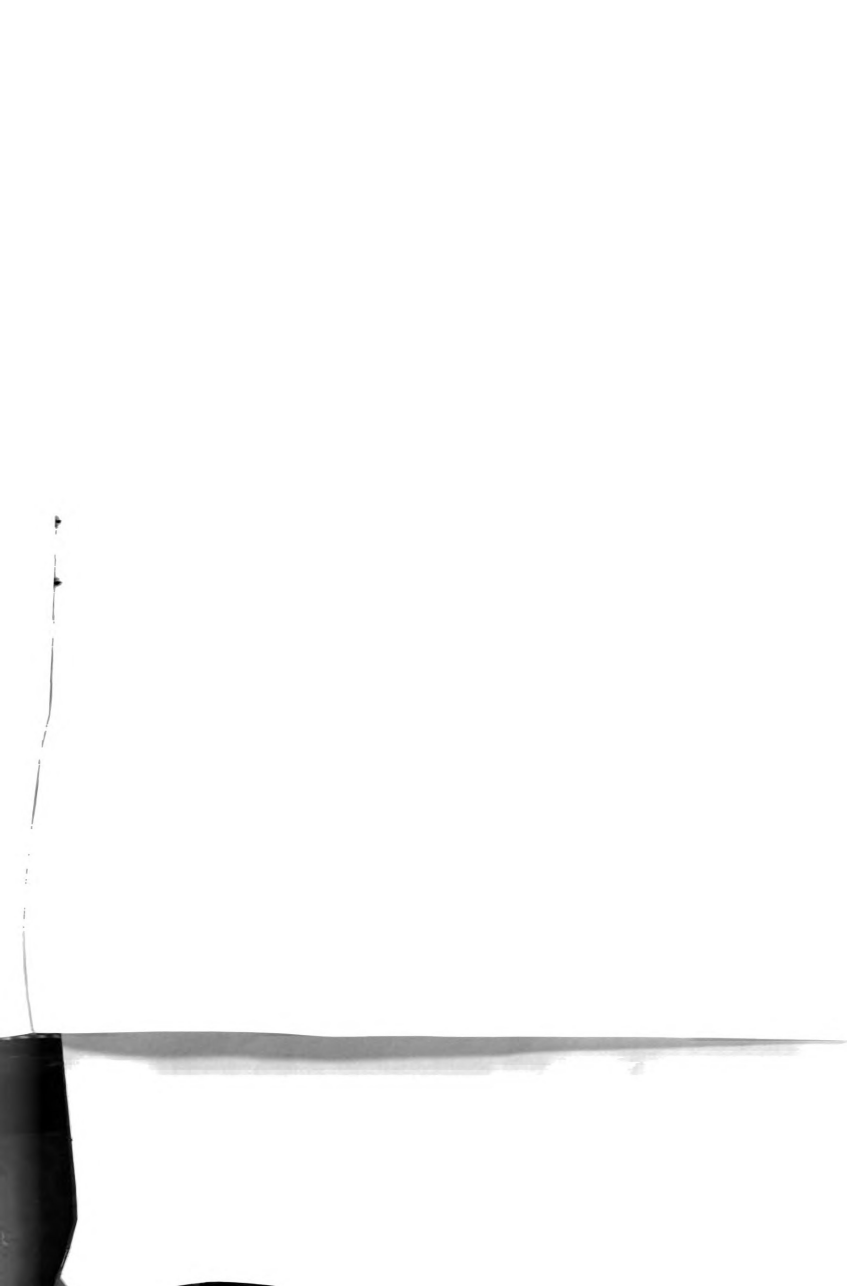
biological changes occurring in inhabitants of water receiving toxic wastes.

Most of the information regarding effects of toxicants upon the nervous system has been obtained from experiments on animals other than fish, in particular, mammals. There is a growing awareness that applying this type of information to fish can be wholly inadequate because of the numerous biochemical and physiological differences existing between the two classes of animals.

The objective of this study was to develop electrophysiological methodology for investigating one aspect of the nervous system in trout, the lateral-line system. In order to describe the basic physiological characteristics of the lateral-line system suitable procedures were tested and adopted. In the second phase of the study I attempted to demonstrate the effects of some stresses encountered in a pollutional situation. In addition to furnishing knowledge of immediate usefulness, this study may serve more importantly by providing much needed background information for more intensive investigations of effects of pollution on fish neurophysiology.

This thesis will present data on the following electrophysiological measurements obtained from rainbow trout:

1. Spontaneous neural discharge from the lateral-line nerve.
2. Evoked neural discharge from the lateral-line nerve following mechanical stimulation.



3. Electrocardiograms (ECG's).

The above measurements were made on fish that had been subjected to a number of stressful situations. Because of the nature of this study the majority of the fish used for experimentation served as their own controls. In other words, a "base line" for each measurement to be made in a particular fish was established from that same fish prior to subjecting it to an experimental stress condition. This thesis will present results from four groups of experiments considering the following stresses:

1. Exposure to the insecticide, DDT, via the water and also via the blood stream.
2. Exposure to reduced partial pressures of dissolved oxygen in the water.
3. Exposure to conditions causing asphyxiation.
4. Exposure to the metabolic inhibitor, potassium cyanide.

The insecticide, DDT, is reported to have a direct action on the nervous system. Exposure to conditions of reduced oxygen tension (hypoxia), and asphyxia are presumed to have a more or less indirect action on the nervous system. The poison, potassium cyanide, produces widespread metabolic depression and may also have direct effects on the nervous system.

Rainbow trout, Salmo gairdnerii, were chosen as the experimental fish for these reasons:

1. They were readily available from local hatcheries.
2. They are easy to maintain under laboratory conditions.
3. They have a well-developed lateral-line system.
4. Background information for physiological application is documented.
5. They are considered an important sport fish and are empirically designated as a pollution intolerant species.

The choice of using the lateral-line system in these experiments was made for a number of reasons. The first concerns the possible role of the lateral line with the symptoms commonly observed in fish suffering DDT poisoning. One of the first symptoms of poisoning is characterized by hyperexcitability. This is evidenced by violent locomotor activity on the part of the fish following slight mechanical disturbances in the water. These characteristic motor responses in poisoned fish might be partially explained by malfunction in sensory inputs to the central nervous system. The lateral-line system is one such input. It is conceivable that excessive motor activity by the fish may be initiated by a prolonged period of repetitive after-discharge from lateral-line receptors following a single stimulus.

Secondly, the lateral-line nerve and associated receptor organs are located just under the skin in very close proximity to the water bathing the fish. This not only allows

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easy access to the nerve for dissection and recording purposes but is also significant since waterborne toxicants may possibly enter the endolymph of the lateral-line canal through the small pores in the skin immediately above the canal. If toxicants entered the canal in this manner they would come in immediate contact with the sensory neuromasts lining the base of the canal.

Thirdly, the lateral-line nerve is in a state of continuous spontaneous neural discharge. The spontaneous activity presumably reflects physical and/or chemical events occurring at the junction of the receptor cells and the afferent lateral-line nerves. If a toxicant were to change physiological conditions at these junctions the spontaneous activity might be expected to change. By recording the frequency of neural discharge prior to a given stress situation one has a convenient base line against which to measure effects of the stress.

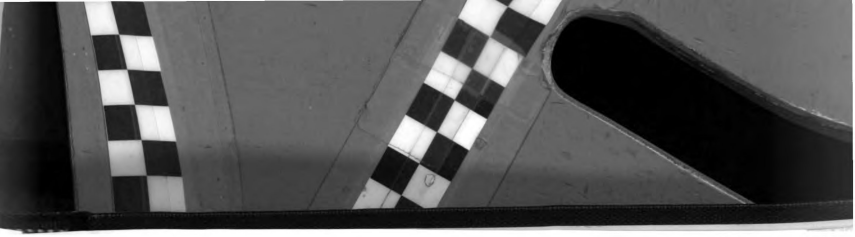
LITERATURE REVIEW

The lateral-line system in aquatic vertebrates has been a subject of widespread research activity in recent years. Interest in this subject was primarily aroused by the development of sonar techniques during World War II. Because of the intimate relationship between underwater acoustics and function of the lateral-line system there has been active support for physical and biological investigation of this sensory system.

Literature concerning the morphology and physiology of the lateral-line system in trout is unfortunately scarce. Only one publication was found that dealt specifically with the lateral-line system of trout (Hoagland, 1933b). Hoagland reports that the trunk lateral-line nerve of brook trout, Salvelinus fontinalis (Mitchill), is in a state of continuous spontaneous activity and this activity can be reduced by cutting the nerve or by cooling it. Hoagland was able to obtain neural responses from a single fiber and record its frequency. However, his work with trout was not extensive; his research effort was concentrated on the catfish, Ameiurus nebulosus Les. (Hoagland, 1933a, 1933b, 1934a, and 1934b).

Although not primarily interested in the function of the lateral-line as a system, Tasaka (1939) and Cragg and Thomas (1957) used the lateral line of trout in studies of neural conduction velocity and strength-duration relationships. They do, however, mention that the lateral-line nerve is spontaneously active and that the spontaneous activity will cease if the nerve is removed from the fish or if the circulation to the nerve is stopped.

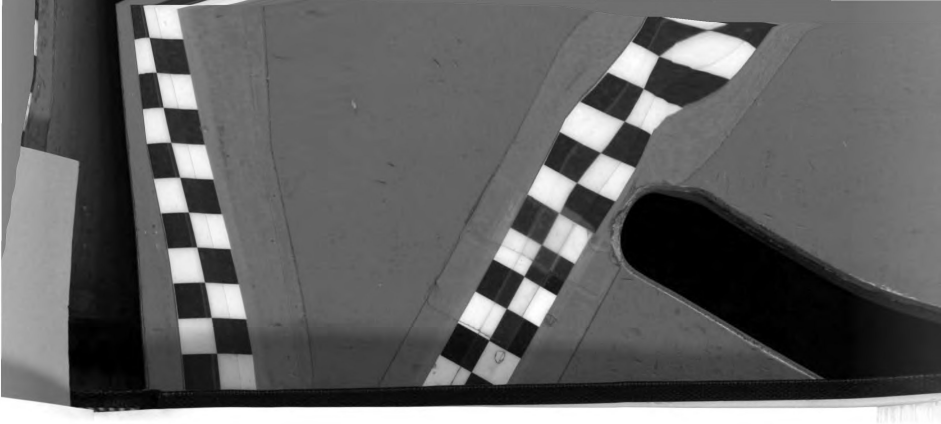
Although morphology of the lateral-line system has been described in many fish by a large number of investigators, little has been done on trout. Some similarity probably exists between the lateral-line organs in trout and those in other species. Dijkgraaf (1962) presents a very comprehensive review of the literature concerning the morphology and physiology of the lateral-line system. Dijkgraaf distinguishes between ampullary lateral-line organs, found in elasmobranchs, and "ordinary" lateral-line organs, found in most fish and amphibians. He subdivides the ordinary lateral-line organs into free sensory neuromasts and canal organs. The lateral-line system in trout is of the canal organ type. Briefly, the canal organ of the type found in trout consists of a longitudinal canal located just under the skin which courses the entire length of the flank on either side of the fish. Protruding into the fluid-filled canal are numerous sensory endings arising from patches of sensory neuromasts lining the length of the canal.



Endings within the canal itself consist of numerous sensory hairs, embedded in a jelly-like substance called the cupula. Shearing movement of the cupula with respect to the top of the sensory cell is responsible for initiating the evoked neural response in the afferent nerve. Sensory neuromasts are innervated by fibers of the lateralis branch of the vagus nerve. Cell bodies of these fibers are found in the acoustic tubercle of the medulla oblongata.

A search of the literature revealed no studies concerning effects of pollution stress on the neurophysiology of the lateral line in any species.

Neurophysiological studies of effects of the insecticide DDT have been conducted almost exclusively on insects and other invertebrates. Classical symptoms in DDT-poisoned insects include hyperexcitability, convulsions, and eventual paralysis (Welsh and Gordon, 1947; Roeder and Weiant, 1948; Yamasaki and Ishii, 1952; Lalonde and Brown, 1954). Similar symptoms are also common in fish, birds, and mammals. The mode of action of this insecticide is still unclear although there have been numerous revealing studies. Yamasaki and Narahashi (1957a, 1957b) were first to indicate that DDT specifically affected the nerve action potential. Their work with axons of the American cockroach demonstrated that DDT treatment caused an increase in the duration of the negative after-potential of the nerve action potential. More recently, Narahashi and Haas (1968) using voltage-clamp



techniques on lobster giant axons were able to clearly demonstrate that DDT inhibits the turning-off process of the peak transient current during an action potential and also that the steady state current is inhibited.

Matsumura and O'Brian (1966a, 1966b) working with the molecular structure of the site of action of DDT upon nerve membranes in cockroaches have demonstrated that the insecticide forms a charge-transfer complex with a component of the membrane. It is possible that this interaction with the membrane is responsible for the conductance changes observed by Narahashi and Haas.

Effects of cyanide upon the nervous system were studied by Schoepfle and Bloom (1959) and Schoepfle (1963). These studies demonstrated that the spike height of an action potential becomes smaller even though there is no change in the resting membrane potential. This finding indicates that cyanide affects sodium conductance before cyanide has a significant effect on the metabolic Na-K exchange mechanism. A later study by Schoepfle and Eikman (1967) demonstrated this more directly by measuring the efflux of radioactive sodium from the sciatic nerve of the frog. Cyanide did not affect the sodium extrusion rate for the first 20 to 40 minutes and there should have been no change in the intracellular sodium concentration or sodium equilibrium potential during this period. Nevertheless, reduction in the height of the action potential was, in fact, observed within the first 20 minutes.

The effects of cyanide on trout have largely been bioassays, testing survival times of test fish after exposure to various concentrations of the poison (Herbert, 1952; Downing, 1954; Burdick et al., 1958). Effects of cyanide on cardiac rhythm in embryos of Fundulus heteroclitus (killifish) were determined by Brinley (1930). By visual observation of the heart in the embryos he found that the heart rate would decrease after exposure to the poison and upon removing the stress the heart rate would return to normal. He also found that the auricles of the heart were more resistant to cyanide poisoning than the ventricles. No literature could be found concerning effects of cyanide on fish electrocardiograms.

Effects of low dissolved oxygen conditions on heart activity have been studied by a number of investigators (Garey, 1962; Holeton and Randall, 1967a, 1967b; Randall and Shelton, 1963; Randall and Smith, 1967). Compensatory decreases in heart rate occurred in trout when exposed to hypoxic environmental conditions. Increased activity in inhibitory neurons of the heart created the reduced rate. This effect was blocked by the drug, atropine. Electrocardiograms were used to monitor the heart rate in these studies, however, no detailed description of changes in the ECG are given. Normal ECG's in several species of fish have been described in the past (Oets, 1950; Kisch, 1948; Robertson et al., 1966).

When cardiac activity becomes severely reduced the heart may no longer be able to perfuse body tissues. Lack of blood

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flow in a tissue is termed ischemia. Effects of ischemia on peripheral nerves have been well documented in mammals and to a lesser extent in amphibians, primarily frogs (Cooper, 1923; Forbes and Ray, 1923; Heinbecker, 1929; Lehmann, 1937; Magladery et al., 1950; Fox and Kenmore, 1967). No similar studies were found relating to fish species. In short, studies have demonstrated that central nervous system neurons could not withstand more than a few minutes of ischemia before becoming permanently damaged. Peripheral nerves, however, were more resistant to ischemia and were able to recover even after blood flow had ceased for more than an hour.

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METHODS

Fish Holding and Feeding

Fish used in this study were two-year old rainbow trout, Salmo gairdnerii, weighing from 170 to 340 grams each. They were furnished by the State of Michigan Conservation Department, Research and Development Laboratory, Grayling, Michigan. About 50 fish were kept in each of two 150 gallon circular Fiberglass holding tanks manufactured by Frigid Units, Inc. of Toledo, Ohio. A continuous flow of dechlorinated tap water, about 3 l/min, was sprayed through a small plastic nozzel at an angle to the surface of the water. Dechlorination was accomplished by passing tap water through a 5 cu ft activated charcoal filter. The fine spray of water into the tanks was sufficient to maintain the dissolved oxygen level at near saturation and also had enough force to set up a current of approximately 2 ft/sec within the tanks. Fish maintained their position in the tanks by swimming against the current and by doing so obtained a small amount of exercise. The cold tap water maintained a temperature of about 13° C which varied less than 1° C per day. The center of each tank was equipped with two standpipes, concentrically placed (outer standpipe open at the bottom, inner standpipe

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open at the top), allowing the feces which accumulated on the bottom to flow through the standpipe system to the drain.

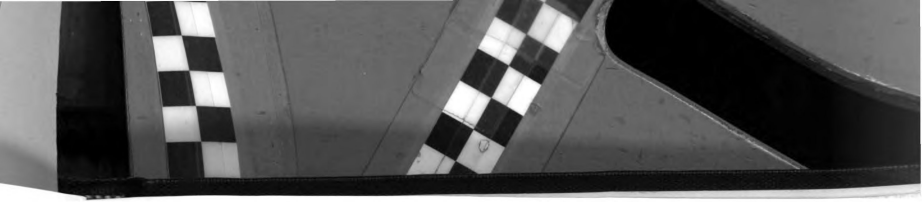
The fish were fed daily on a diet of dry pellet trout food (Formula 65-W, Glencoe Mills, Glencoe, Minn. and Strike Fish Feed, Country Best, Agway, Inc., Syracuse, N. Y.) furnished by the Michigan Conservation Department. From time to time this diet was supplemented with chopped beef liver. Fish were maintained in very good health on this diet and noticeable growth was observed during the study period.

Holding tanks were illuminated with incandescent lamps which were electrically switched on and off with a timer to coincide with the natural photoperiod.

Experimental Fish Chamber

For purposes of obtaining electrophysiological recordings under controlled conditions fish were individually transferred from the holding tanks to a small rectangular Plexiglas chamber. The chamber was slightly larger than the fish itself and was provided with a continuous supply of fresh oxygenated water. The chamber provided a convenient place to conduct surgery, to confine fish after attachment of recording electrodes, and to expose fish to toxic chemicals. The design of the chamber and associated equipment allowed the following conditions to be satisfied:

1. Fish could be completely covered with water.
2. The water temperature could be held constant



throughout an experiment.

3. A constant flow of water could be passed over the gills of the fish.
4. Oxygen tension in the water could be carefully controlled and monitored during an experiment.

Immobilized fish were placed on their side in the chamber and the water inlet tube was inserted into the mouth and secured by a steel pin which passed through the tube and through the lower jaw of the fish. Fish were further secured by fixing the dorsal fin to the side of the chamber with the aid of a spring loaded clamp. Water left the chamber through an adjustable siphon at the rear. By moving the siphon either up or down one could adjust the water level in the chamber.

Water to the chamber was at the same temperature and from the same source as that supplying the holding tanks. A constant flow of water into the chamber inlet was accomplished by first passing it into a 2-liter head tank located about 2 feet above the level of the chamber. The constant head was maintained by passing an excess flow of water into the tank and allowing it to overflow through a drain port located near the top. Pure oxygen was bubbled into the head tank through an air stone. Spherical float flowmeters (RGI, Inc., Great Neck, N. Y.) were placed in line with the water and oxygen inputs to the head tank. The oxygen tension in the water could be quickly changed to any desired level by

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adjusting the oxygen and water flows to predetermined values found by prior calibration. The dissolved oxygen level of the dechlorinated water entering the head tank was less than 1 ppm, thus to achieve low oxygen levels it was not necessary to use oxygen stripping techniques on the water supply to the chamber. To achieve oxygen saturation in the water to the fish chamber the flow of oxygen to the head tank was increased. Flow rates required for water saturation, for example, were 1.5 l/min of water and 0.4 l/min of oxygen. Water flowed to the fish chamber at a rate of 0.67 l/min and the remaining 0.83 l/min left the overflow port in the top of the head tank.

A polarographic oxygen sensor (Beckman Model 777) was placed between the head tank and the fish chamber to monitor the dissolved oxygen during the entire course of an experiment. The sensor was passed through a hole cut in a rubber stopper and the stopper containing the sensor was inserted through a hole cut into the side of a 2 inch diameter piece of plastic pipe. Using large stoppers this pipe was placed in the water-flow line between the head tank and the fish chamber (Figure 1). The water flow into the sensing chamber was directed at the surface of the oxygen sensitive area of the probe. This provided a continuous water flow, the velocity exceeding the minimum required for accurate operation of the sensing unit. The sensing chamber was tilted to prevent entrapment of air bubbles within the chamber and on the sensitive area of the probe.

The oxygen meter was calibrated prior to each experiment by exposing the probe to the air then setting the meter to read a partial pressure of oxygen of 160 mm Hg. Previous experimentation proved that readings from the oxygen meter calibrated in this fashion agreed very closely to dissolved oxygen measurements done on the same water with chemical oxygen determinations using the Winkler Method (Standard Methods, 1960).

In one series of experiments fish were exposed to various water concentrations of potassium cyanide. This was accomplished by metering a known flow of the chemical from a reservoir to the inflowing water supply to the fish chamber. A Beckman Model 746 Solution Metering Pump was used for this purpose. The flow rate from the pump could be adjusted to the nearest 0.01 ml/min from 0.00 to 20.00 ml/min by turning a calibrated dial which in turn would increase or decrease the length of travel of a Teflon piston within the pump. Flow from the pump would pulsate in two second intervals. To prevent pulses of concentrated cyanide solution from passing over the gills of the fish as a result of the pulsating nature of the pump, a mixing chamber was placed between the inlet of the fish chamber and the juncture of the pump inlet to the water line (Figure 1). Mixing occurred in the turbulence created by water entry into the mixing chamber and the volume of the mixing chamber (250 ml) was sufficiently large to damp out the cyanide pulses. By pumping dye into the system the mixing proved to be very good.

Figure 1.--Diagram of the experimental fish chamber, head tank, oxygen metering equipment, and toxicant metering system. Water flow through the system is indicated by arrows.

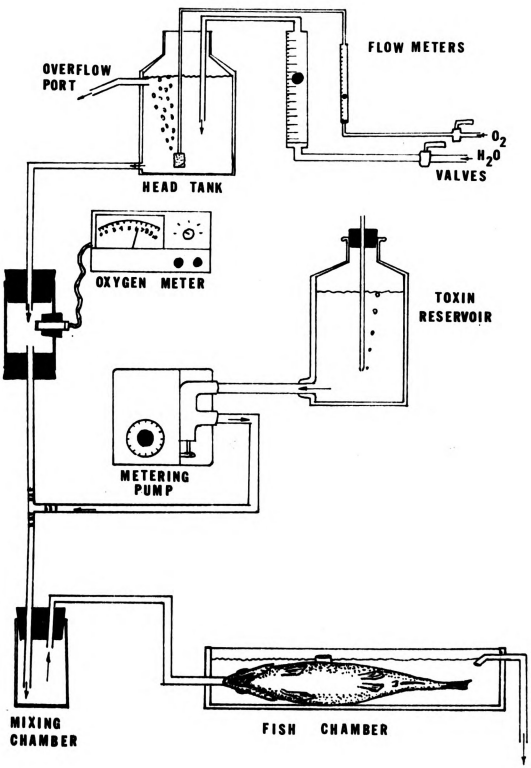
OVERFLOW PORT
FLOW METERS
O_2
H_2O
VALVES
HEAD TANK
OXYGEN METER
TOXIN RESERVOIR
METERING PUMP
MIXING CHAMBER
FISH CHAMBER

Figure 1

Surgical Methods

Immobilization

Fish to be used in the chamber just described were removed from the holding tanks with a net and anesthetized in a solution of 100 ppm MS-222 (tricaine methane sulfonate, Sandoz) for five minutes. The fish were then weighed and given an intramuscular injection of d-tubocurarine chloride (Sigma Chemical Co.) at a dosage of 20 mg/kg live weight. Tubocurarine is a drug which causes muscle paralysis by inhibiting neural transmission at the motor end plate region of the muscle. This drug enjoys widespread use in neurophysiological investigations because it achieves total immobilization of the test animal without severe depression of the central nervous system.

To determine whether or not tubocurarine had an effect on neural discharge from lateral-line organs in the fish used in this study, a number of separate experiments were conducted where the animals were immobilized by pithing and lateral-line discharges measured before and after injections of the drug. Pithing was accomplished by either severing the spinal cord in the anterior cervical region with a steel needle or by completely destroying the brain and spinal cord by running a copper wire through the brain and down the entire length of the spinal column. Electrodes were attached to the lateral-line nerve (as described in the next section) and control recordings were made of both spontaneous and evoked

neural activity before administration of the drug. Tubocurarine was then injected intramuscularly in doses two to three times those normally given and the lateral line recordings were closely observed. Results from these experiments did not demonstrate any depressing effect of this drug on the lateral-line nerve and also the mode of pithing seemed to have little effect on the nerve.

Prior to placing the curarized fish in the experimental chamber the gills were rinsed with fresh water to rid them of excess mucus which accumulated during anesthetization. Very frequently fish would vomit stomach contents shortly after curarization. When this occurred fish were massaged to remove remaining stomach contents then the mouth and gills were rinsed again with fresh water. Since a curarized fish is unable to move its mouth and operculum it is not only unable to ventilate but also unable to conduct gill cleaning reflexes. Failure to rinse the mouth and gill region prior to placing the fish into the chamber did, on several occasions, result in asphyxiation of the test animal.

The fish was then placed into the chamber on its side and the water input tube inserted into its mouth. At this time a unipolar electrocardiogram (ECG) electrode was inserted into the base of the left pectoral fin and pushed down just under the skin to a midventral position about one centimeter posterior to the heart. Construction of the ECG electrode will be discussed later. The water level in the

chamber was then raised above the fish and the ECG trace on the oscilloscope observed for about one hour. If the heart continued to function properly during this period the experiment was continued. If the heart showed signs of failure at this time the fish was discarded and a new fish used.

Exposure of the lateral-line nerve

Following a one hour period of normal heart function the next step was to isolate the lateral-line nerve and attach recording electrodes. In most of the fish tested the lateral-line nerve was found just under the skin lying one or two millimeters dorsally to the lateral-line markings on the surface of the fish. The lateral-line nerve (lateralis branch of the vagus) leaves the cranium behind the gill region and courses the entire length of the trunk to the caudal peduncle. There is one nerve trunk on each side of the fish.

To isolate the nerve a shallow longitudinal incision was made with a sharp blade just through the skin about 5 mm below the lateral line and at a point about one-third of the way down the trunk toward the tail of the fish. The length of the incision was about 1 cm. Using forceps and a blunt probe the skin overlying the musculature surrounding the lateral-line nerve was separated by running the probe between the skin and muscle. An area 2 cm in diameter directly over the lateral-line nerve was freed in this manner and a

circular piece of skin, 1 cm in diameter, was cut free from the fish with scissors. This exposed a circular area of flesh containing a small longitudinal groove. This groove was covered with a thin layer of membranous tissue or was an open canal depending on the depth of the dissection. This canal, running the entire length of the flank from operculum to caudal peduncle, is the lateral-line canal. The sensory neuromasts of the lateral line are found along the base of this canal. Afferent nerves from the neuromasts join the main lateral-line nerve trunk immediately beneath the canal. As the nerve trunk courses toward the head it picks up more and more of these afferent fibers.

With the aid of a dissecting microscope the nerve was separated from the surrounding musculature by spreading the muscle apart with fine forceps. At times it was necessary to cut away strands of connective tissue with small scissors in order to completely free the nerve. It is important not to stretch the nerve which may cause permanent damage. If the dissection was done correctly there was very little bleeding.

A small glass cylinder, 1.5 cm in diameter and about 1 cm long, with a flanged lip at one end was then positioned into the circular incision by forcing the lip under the loosened skin surrounding the incision. This was done by placing a portion of the lip under the skin at a convenient starting point and stretching the skin over the remaining lip

by inserting a blunt probe between the skin and the glass lip, then working the probe around until the entire lip was covered with skin. When completed the skin fit tightly against the glass cylinder forming a water-tight seal. The water level in the chamber could then be raised over the entire fish and still provide a water free area of flesh within the cylinder. A cross section of the glass cylinder after being positioned in the fish is shown in Figure 2.

Two fine silver wire electrodes were attached to the top of the glass cylinder with beeswax and soldered to two-conductor shielded cable. The electrodes were constructed from 26 gauge silver wire that had been electrolytically etched to fine points in nitric acid. The tip of each electrode was bent to form a hook and both were bent so that the tips would extend into the interior of the glass cylinder.

In most experiments the nerve was cut centrally (between the brain and the recording electrodes) and the distal portion of the nerve lifted over the electrodes. The cut end of the distal portion of the nerve was firmly secured to the anterior-most electrode by pinching the wire hook tightly against the nerve. The other electrode was simply looped under the nerve a few millimeters posterior to the first. The nerve and electrodes were then covered with mineral oil to prevent the nerve from drying out.

Many electrode preparations were tried during this study but the one just described proved to be the best. Using this

Figure 2.--Electrode apparatus used for recording neural activity from lateral-line nerve in rainbow trout. Flanged glass cup inserted under skin excludes water from electrode area. Top diagram is an enlarged cross section of area indicated in lower figure.

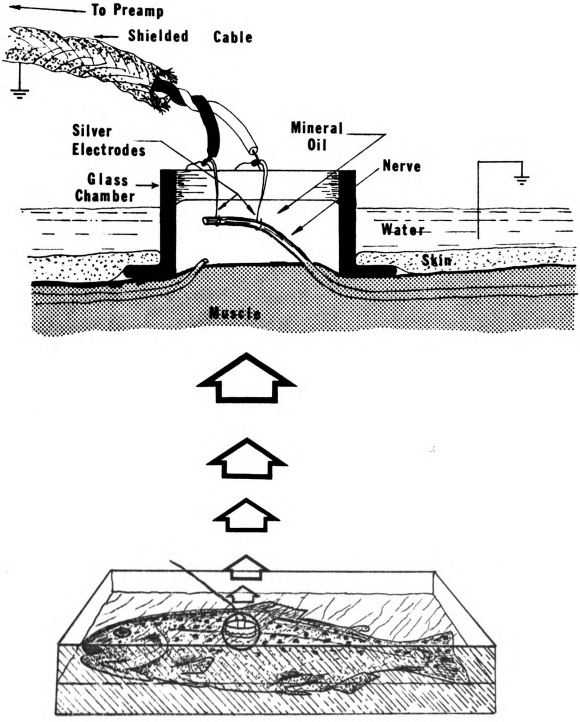
To Preamp
Shielded Cable
Silver Electrodes
Mineral Oil
Glass Chamber
Nerve
Water
Skin
Muscle

Figure 2

technique a fish could be completely covered with water, the nerve was kept from drying out, and recordings could be made from the lateral-line nerve for many hours with no apparent ill effects to the fish nor to the lateral-line responses. No other method tested had all of the above characteristics.

Canulation of the cardinal vein

In experiments requiring blood injection of chemicals a canula was inserted into the posterior cardinal vein near the tail. Because small amounts of heparin (an anticoagulant) were introduced into the blood circulation during this process the clotting ability of the blood was reduced. Consequently, the lateral-line incision was made prior to the canulation to prevent hemorrhage at the lateral-line site. Very little bleeding occurred at the lateral-line incision if done first.

The posterior cardinal vein was exposed by making a 2-3 cm longitudinal incision just anterior to the caudal peduncle, about 5 mm below the lateral line. The underlying muscle was cut and separated down to the spinal column and the incision held open with the aid of retractors. The flesh adhering to the ventral spines of the vertebrae was removed with a blunt probe and forceps exposing the vein coursing caudad between the bony spines.

The canula was fashioned from a 20 cm length of Clay-Adams PE 10 Intramedic Polyethylene tubing, inside diameter

technique a fish could be completely covered with water.
the nerve was kept from drying out, and respiration could
be made from the lateral-line nerve for many hours with
no apparent ill effects to the fish nor to the lateral-line
responses. No other method tested had all of the above
characteristics.

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0.011 inches, to which was fitted a 26 gauge needle. The other end of the canula was attached to a 1 ml tuberculin syringe. The canula and syringe were filled with Courtlands' saline, pH 7.40 (Wolf, 1963), and contained 1 percent heparin to prevent clotting. The needle was inserted into the vein in the direction of the blood flow (caudad) with the aid of a dissecting microscope. After successful insertion the retractors were closed tightly against the canula. This held the tubing firmly against the fish, hence, there was no need to suture it in place. Best results were obtained when the sharp beveled edge of the needle was dulled before being placed into the vein. This prevented excessive cutting of the vessel wall and resulted in very little hemorrhage. On a few occasions, however, faulty placement of the canula resulted in hemorrhage and when this happened bleeding was so severe it completely obstructed the view of the canulation site, making corrective measures very difficult. It was necessary to discard these fish.

Injection of solution was accomplished by drawing blood into the tubing up to a point just below the needle, removing the needle from the tubing, then inserting a needle from a second syringe (containing the solution to be injected) into the blood-filled tube. The plunger of the second syringe was depressed until all of the blood in the canula was displaced by the injection fluid. A measured amount of solution could then be accurately injected into the vein. After the solution

had been injected the original syringe containing the heparinized saline was replaced in the reverse order of the procedure described above.

Electrophysiological Methods

Recording from the lateral-line nerve

Nerve impulses conducted toward the central nervous system along the lateral-line nerve were recorded using the extracellular silver wire electrodes described in the previous section. As was stated, the distal portion of the centrally cut nerve was attached to two electrodes as shown in Figure 2. The electrode nearest the cut end of the nerve was grounded at the preamplifier and the other electrode was connected to the input of the preamplifier. With the electrodes attached in this manner the nerve impulses, as they passed the first electrode, would cause a negative deflection of the oscilloscope beam after being amplified through the preamplifier. Such a recording is termed monophasic, that is, one nerve impulse causes one deflection of the oscilloscope beam. Because the frequency of neural discharge was an important measurement in this study it was necessary to display the nerve impulses in a manner which allowed accurate counting. Monophasic recording techniques proved to be the best suited for this purpose.

Recording electrical activity from the heart

Electrocardiogram (ECG) measurements were routinely taken during most of the experiments as a check on heart function. The main purpose of conducting these measurements was to use the ECG as a convenient cardiotachometer. After examining many ECG records from fish subjected to various stresses certain characteristic changes were observed in the pattern of electrical activity from the heart. For this reason it was decided to standardize the ECG recording method so that comparisons could be made between different fish. Construction and placement of the ECG electrodes are described below.

Because fish used in this study were covered with water it was not possible to make good ECG recordings using common surface electrodes. To avoid this difficulty electrodes had to be placed under the skin and kept from making electrical contact with the water. The electrodes were constructed by soldering steel pins to insulated wire, then insulating the pin and wire with several coats of insulating varnish. The insulation at the sharp point of the pin was then scraped off leaving a few millimeters of electrically conductive metal at the end of the pin. The pin was inserted at the base of the pectoral fin and then pushed just under the skin until the end of the pin reached the ventral midline one centimeter posterior to the heart. Insertion of this electrode was done the same way in every fish. The wire from this electrode

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was directly connected to the vertical amplifier of the lower beam of the oscilloscope. Another electrode was grounded to the water, completing the circuit. The voltages recorded from the heart using this method were about one millivolt.

Instrumentation

Before weak electrical signals from the lateral-line nerve could be displayed on an oscilloscope it was necessary to amplify them. This was done in the majority of the experiments with a Grass P8 A.C. Pre-amplifier (Grass Instrument Co., Quincy, Mass.). The pre-amplifier could be operated either differentially (amplifying the voltage difference between two electrodes) or single-ended (amplifying the voltage difference between one electrode and ground). The open-end mode was used most frequently because it would give a larger signal to noise ratio than the differential mode. The open-ended mode of operation, however, was more prone to pick up external 60 Hz (60 cycles per second) electrical noise than was the differential mode. This was eliminated to a large extent by proper grounding of the preparation. The pre-amplifier was equipped with high and low frequency noise filters which were set to exclude electrical signals above 4 KHz (half amplitude frequency) and signals below 0.5 Hz. The high frequency filter did not appreciably attenuate the wave form of a nerve impulse recording.

Leads from the nerve-electrode preparation to the pre-amplifier and from the pre-amplifier to the oscilloscope

were shielded and the shields grounded. The fish chamber and pre-amplifier were enclosed in a common shielded cage which was also grounded. The peak-to-peak noise level of the system as observed on the oscilloscope was less than 10 μV.

Electrical activity from both the lateral-line nerve and the heart was displayed on the screen of a Tektronix Type 502-A Dual Beam Oscilloscope (Tektronix, Inc., Portland, Ore.), the lateral-line activity on the upper beam and the ECG on the lower beam. Permanent records of oscilloscope traces were made by photographing the screen with a C-27 Oscilloscope Camera (Tektronix Inc.).

Frequency of lateral-line impulses was measured either directly from the photographs or was electrically determined with the use of a counter. Direct frequency measurements from photographs involved counting the number of spikes (nerve impulses) appearing over a known time interval. For example, if 10 spikes were counted over a distance of 10 cm on the oscilloscope screen and the sweep rate of the beam was 10 msec/cm the impulse frequency would be 10 impulses per 100 msec or 100 per sec.

Electrical frequency determinations were done with a decade scaler, the same type of scaler as used in radioactive isotope counting. Initial attempts to count nerve spikes by connecting the pre-amplifier directly to the scaler were unsuccessful. This was because the input gate of the scaler

would exclude electrical pulses with rise times greater than 10 μsec. A nerve impulse from the lateral line has a rise time of about 500 μsec, much too slow to pass the input gate of the scaler. For this reason it was necessary to alter the wave form of a nerve impulse to decrease its rise time. This was accomplished by placing an electrical trigger circuit between the pre-aimplfier and the scaler. The circuit is a bistable multivibrator, commonly termed a Schmitt Trigger, which I constructed from a circuit described by Brophy (1966). An amplified nerve impulse, a relatively slow rounded wave, is changed by the trigger into a rectangular wave with a rise time of about 1 μsec which could then pass the input gate of the scaler and be counted.

Understanding the principle of operation of the trigger is a necessary prerequisite for interpreting data obtained from it. It operates as follows: If an input voltage exceeds a given threshold the resting voltage of the trigger output rapidly switches to a higher value. When the input voltage then falls below this threshold the output voltage returns to its resting value. The threshold voltage required to "fire" the trigger can be adjusted by changing the amount of voltage bias at the input. This was done by turning a calibrated ten-turn potentiometer which was part of a voltage divider circuit placed across the input of the trigger. Supplying positive voltage to the input with this voltage divider lowers the threshold of the trigger enabling nerve

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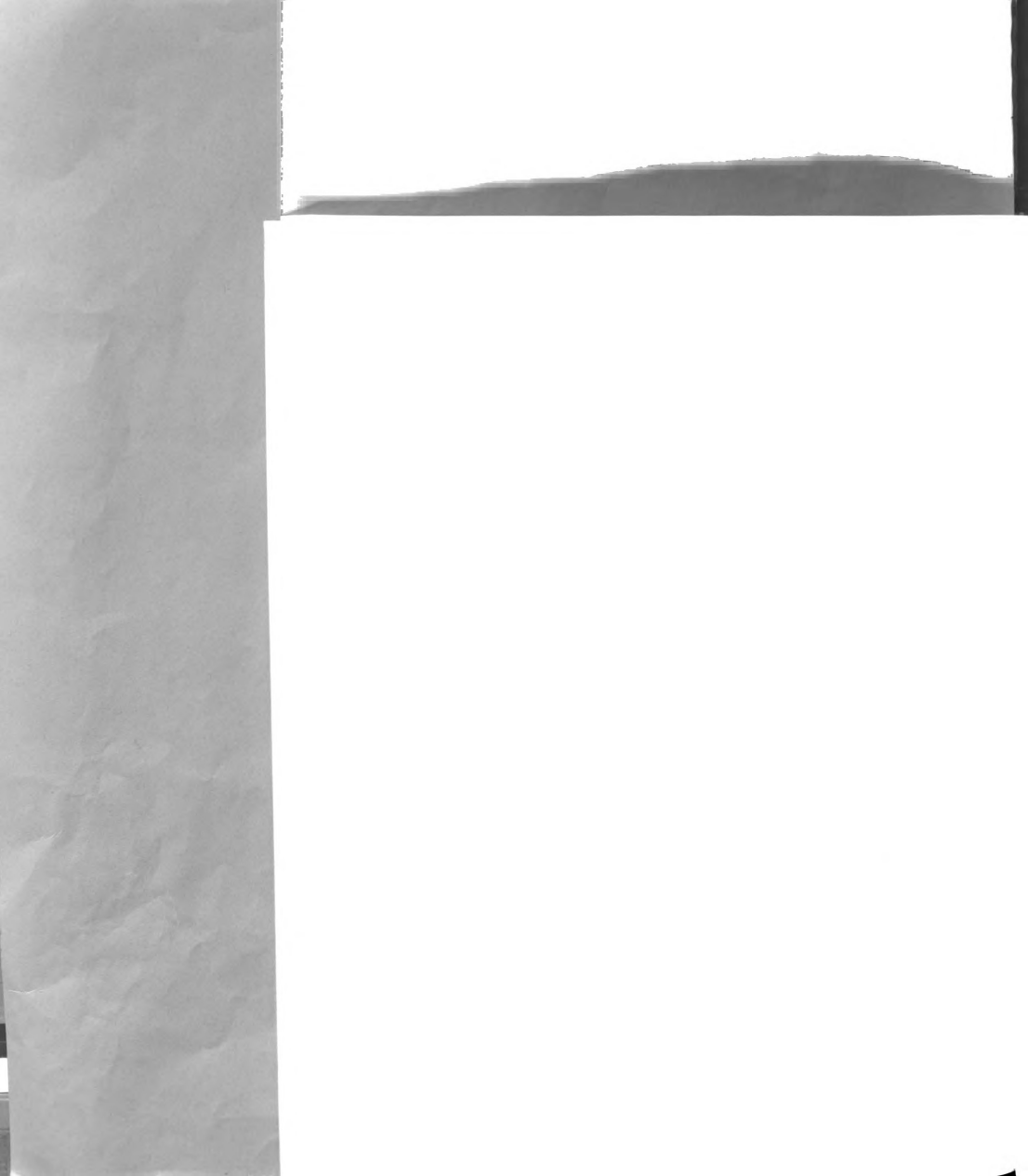
impulses of lower voltage to fire the trigger. The exact amount of bias given to the input of the trigger was determined by counting the number of turns which the potentiometer was moved (the potentiometer was connected to a mechanical revolution counter subdivided into 100 graduations). By displaying the output of the trigger on one beam of the oscilloscope next to the input signal of the trigger (amplified nerve impulses), displayed on the other beam of the oscilloscope, it was easy to see which nerve spikes had the ability to fire the trigger. By careful visual observation of these two simultaneous events it was possible to calibrate the potentiometer settings of the bias circuit to the size of the nerve spike (voltage) one desired to count. It should be emphasized that this system did not have the capability of selecting impulses of a certain voltage range (a window), but rather could only select a threshold, all nerve impulses of sufficient voltage to exceed this threshold being counted and those below the threshold excluded.

There is one difficulty encountered with this counting system that should be mentioned at this time. This occurs when two or more nerve impulses reach the recording electrodes very close together. The second impulse may reach the electrodes before the first has totally passed. This event is seen on the oscilloscope as a multiple peaked wave. The difficulty arises at the input of the trigger circuit. If the first nerve impulse does not return to below threshold



voltage before the second reaches the input of the trigger, the output of the trigger will remain at its higher voltage until the input voltage does fall below the threshold. As a result only one impulse will be recorded at the counter when actually two or more nerve impulses actually occurred. During the experiments, however, this effect was largely eliminated by reducing the firing frequency of the lateral line by cutting the nerve until neural activity from only a few receptor units was being recorded. By reducing the firing frequency the occurrence of overlapping impulses was greatly reduced.

To determine impulse frequency using the counter system described above, one connects the pre-amplifier to the trigger-counter circuit, selects the threshold, and counts the number of nerve impulses occurring over an interval of time, usually one minute. The average impulse frequency was found by dividing the number of impulses by the time interval.

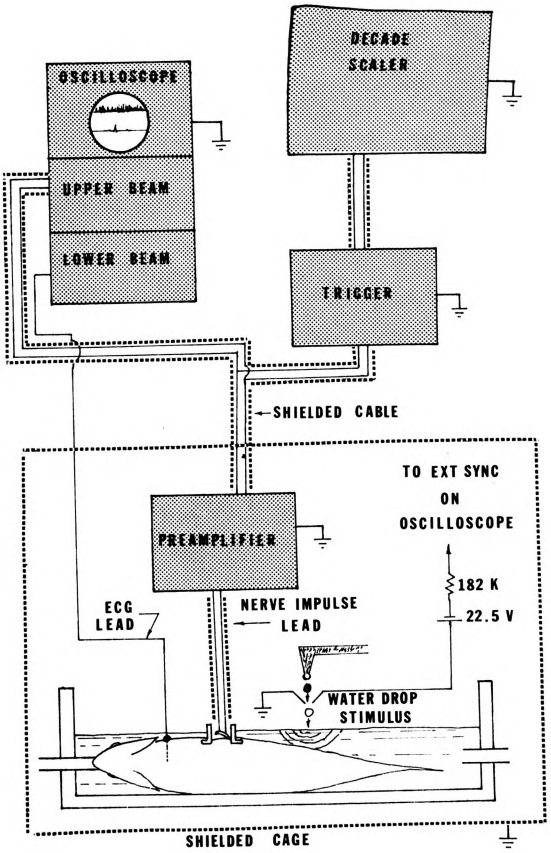
Stimulation of lateral line receptors

Methodology described to this point has been involved primarily with recording spontaneous neural activity from the lateral-line nerve. In many experiments the receptors of the lateral-line system were stimulated and the evoked neural response was recorded. The standard stimulus used for this purpose was to drop water from an opening in a glass tube on to the surface of the water directly over the lateral line of the fish. The water drop apparatus was located in a fixed

position over the fish chamber so that the drop of water would always hit the surface of the water at the same place and fall from a given height. So that the evoked neural response resulting from this mechanical stimulation could be easily seen on the oscilloscope it was necessary to synchronize the sweep of the oscilloscope beam with the falling drop of water. This was done by placing two silver wires immediately below the opening of the glass tube. As a water drop fell from the end of the tube it would simultaneously make contact with the end of both wires, completing a circuit consisting of a 22.5 v battery placed in series with the external D.C. synchronizing input of the oscilloscope. When the circuit was closed by the falling water drop the beam of the oscilloscope would start to sweep across the screen and the nerve impulse burst (resulting from mechanical stimulation of the lateral line receptors) would always appear in the same position on the screen. If the sweep of the beam was not synchronized to the falling of the water drops the bursts of neural activity would appear in different positions along the sweep of the beam making it very difficult to see, much less photograph.

A block diagram of the instrumentation is shown in Figure 3. Figure 4 is a photograph of a fish with ECG and lateral-line nerve electrodes in place. Figure 5 is a photograph of the entire experimental setup.

Figure 3.--Block diagram of instrumentation used for recording electrical activity from the heart and lateral-line nerve. Nerve activity displayed on upper beam of oscilloscope and heart activity (ECG) on lower beam. In experiments of evoked responses, water drop stimuli synchronize sweep of scope by completing the circuit shown just above the fish chamber. In spontaneous activity experiments nerve impulses are electronically counted on a decade scaler for frequency analysis.


DECADE
SCALER
OSCILLOSCOPE
UPPER BEAM
LOWER BEAM
TRIGGER
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TO EXT SYNC
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PREAMPLIFIER
182 K
22.5 V
ECG
LEAD
NERVE IMPULSE
LEAD
WATER DROP
STIMULUS
SHIELDED CAGE

Figure 3

Figure 4.--Secured fish showing position of electrocardiogram electrode (A) and lateral-line nerve electrodes (B).

rocardio-
rve

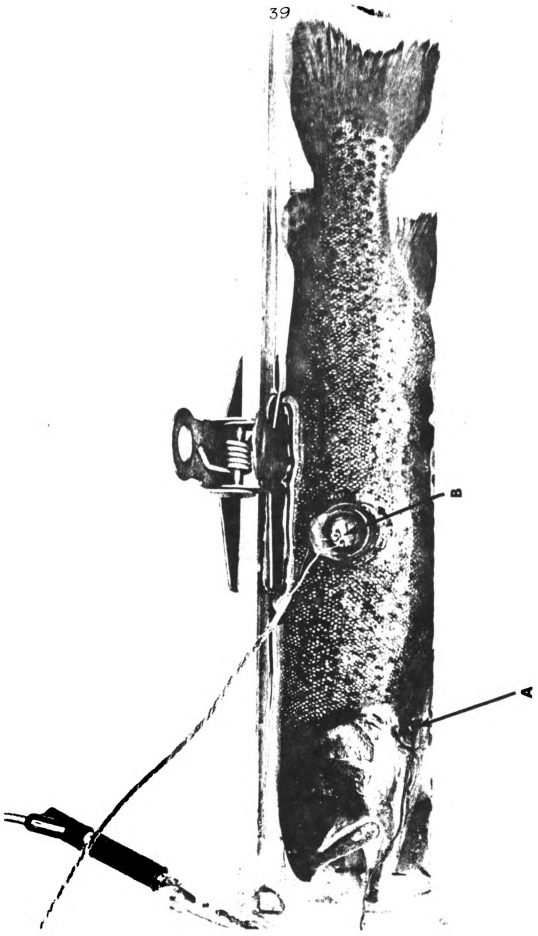


Figure 4

Figure 5.--Entire experimental setup.

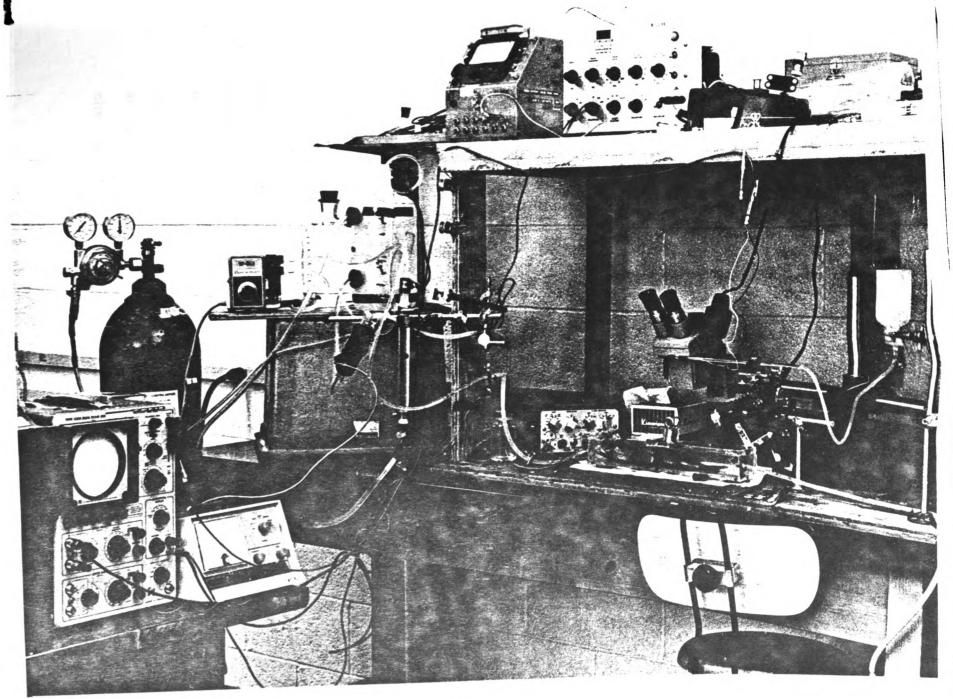


Figure 5

Chemical Methods

DDT

The DDT used in this study was a 99-plus percent pure p,p' isomer of 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane, M.W. 306.48. The chemical was obtained from the City Chemical Corp. of New York, N. Y., and is considered a Pesticide Reference Standard by the Entomological Society of America.

Stock solutions of DDT were made up by dissolving the chemical in absolute ethanol. The best method found to inject the chemical into the blood stream was to first make a saline-DDT suspension (10 percent ethanol) by rapidly mixing the DDT-ethanol solution with the fish saline. This suspension was immediately injected. Waiting for only a few minutes would cause the micro-crystals of DDT to precipitate in the form of large particles. These particles would get caught in the small opening of the 26 gauge needle at the end of the canula preventing successful injection of the insecticide into the vein.

Direct injection of the DDT-ethanol solution into the vein proved to be very unsatisfactory. Upon contact with the blood the solution would rapidly precipitate serum proteins and the DDT would crystallize at the same time. Such injections severely clogged the canula and if large enough amounts of the precipitated material were allowed to enter the vein heart failure soon followed.

Attempts were made to inject DDT into the vein using soybean oil as a carrier. In less than ten minutes following injections of 0.1 to 0.5 ml of pure soybean oil the heart would show signs of failure. It was assumed that the high viscosity of the oil was partially responsible for the drastic effect on the heart. No other oils were tested as possible carriers for the DDT.

Early in the study, before the solution metering apparatus was used in conjunction with the fish chamber, a few experiments were conducted whereby fish were exposed to very high levels of DDT in water. In these experiments the purpose was to expose fish to the chemical for a period of time long enough to induce tremors and convulsions. To accomplish this, fish were individually placed in 5 gallon aquaria which contained a concentration of 15 ppm DDT. The DDT was added to the water in an ethanol solution, then carefully mixed. The aquaria were aerated, covered, and placed in a cold water bath at 13°C. Fish were exposed in this manner for about six hours, time enough for fish to show advanced symptoms of DDT toxicity.

Blood and nerve tissue from a few fish exposed to DDT were analyzed for their DDT content. This was not a major objective of this study and consequently will not be discussed in detail. Briefly the chemical analysis consisted of converting all of the DDT to DDE by treatment with alcoholic KOH, extracting the DDE in petroleum ether, drying the ether in

sodium sulfate, and then analyzing the final solution for DDE in a gas chromatograph employing an electron capture detector. Unknowns were compared with standards and the results were expressed as ppm p,p' DDT.

Cyanide

Stock solutions of potassium cyanide were made by dissolving the chemical in distilled water. Fresh solutions were made every other day and stored in the refrigerator.

Tubocurarine

Stock solutions of d-tubocurarine chloride, 5 mg/ml, were made by dissolving the chemical in distilled water. At this concentration the use of a magnetic mixer is highly recommended to aid in dissolving the chemical. The curare solution was kept refrigerated at all times, and the powder was kept in a dessicator and also refrigerated.

Saline

The trout saline solution used for tissue moistening and as a DDT carrier for intravenous injection was made according to the formula described by Wolf (1963). It consisted of the following:

NaCl.	7.25 g
$CaCl_2 \cdot 2H_2O$.	0.23 g
KCl	0.38 g
$NaH_2PO_4 \cdot H_2O$	0.41 g
$NaHCO_3$.	1.00 g
$MgSO_4 \cdot 7H_2O$.	0.23 g
Glucose	1.00 g
Water	1000 ml

The pH of the saline was adjusted to 7.40 by adding either HCl or NaOH. Very small amounts of acid or base were required for this adjustment, hence, the ionic strength of the saline was changed very little.

To give the saline anticoagulant properties when used to fill canulae, a small amount of heparinized-saline was made by dissolving enough heparin in the saline to give a one percent heparin solution. The heparin was a Grade I sodium salt obtained from the Sigma Chemical Co.

RESULTS

Experiments on Control Fish

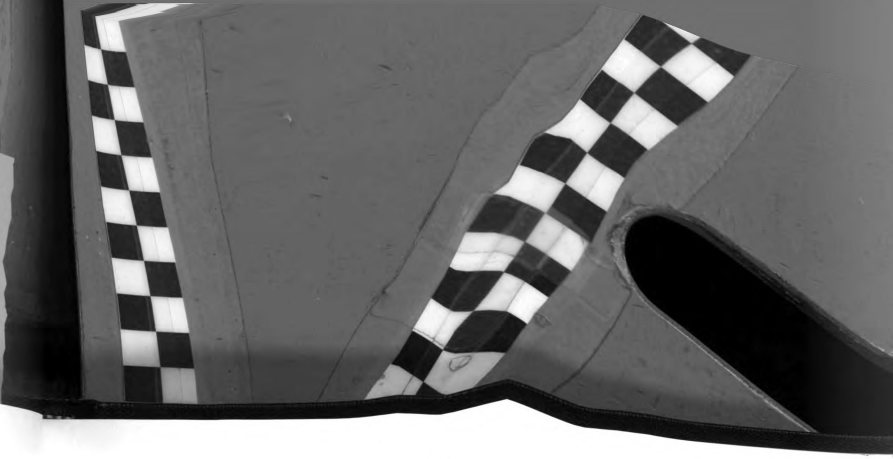
Spontaneous activity from lateral line

General observations. A continuous discharge of high frequency nerve impulse activity was typically observed in unstimulated lateral-line nerves, neural activity seemingly the result of independent spontaneous firing of individual neurons within the main nerve hundle. In recordings from over 180 different fish preparations less than 10 were found lacking spontaneous activity. It is presumed that the lateral-line nerve from the fish having no spontaneous activity was damaged during dissection.

It was found that rough handling of the nerve would often damage it. Gently pulling on the nerve with a forceps would often cause severe reduction in the level of spontaneous activity and many times completely silenced the nerve. If a large fish was forced into the fish holding chamber causing dorso-ventral compression of the lateral-line, the fibers innervating the compressed area would be permanently silenced. It is possible that this type of compression damaged the delicate fibers innervating the neuromasts in this area or damaged the neuromasts themselves.

As was stated in the previous section the lateral-line recordings were made on preparations in which the lateral-line nerve was cut free from its central connection with the brain. Results of preliminary experiments did not demonstrate any obvious change in the spontaneous activity upon cutting the nerve centrally. It should be mentioned that spontaneous activity was observed from the centrally cut end of the nerve on several occasions. This activity, however, did not seem to arise from the brain, but rather from a location within a few millimeters from the recording electrodes. The spontaneous activity may have originated from a neuromast group which was still attached to the main nerve at the recording electrode site or it may represent an injury discharge of the type described by Hoagland (1934b). Similar discharge was also seen from the distal end of the nerve, arising from an area a few millimeters distal to the recording electrodes. This type of discharge occurred only rarely and was not present in preparations which were intended for analysis of spontaneous activity frequency. One could quickly determine its presence by noting the lack of response to mechanical stimuli.

Spontaneous activity did not seem to be influenced by the contralateral nerve (the lateral-line nerve on the other side of the fish). Leaving it intact, cutting it free from central connection with the brain, or completely removing it from the fish had no effect on the level of spontaneous



activity in the opposite nerve. Similarly, transection or complete destruction of the spinal cord also had little influence on lateral-line spontaneous activity.

Nerve cutting experiments. Counting the number of nerve impulse peaks (spikes) over a given time interval from oscilloscope photographs of spontaneous activity revealed impulse frequencies exceeding 2000 spikes per second. This figure represents a conservative estimate because many of the larger spikes which were counted as single impulses may actually have been the summated effect of two or more smaller spikes. Summation will occur if two or more impulses, conducted in separate fibers, reach the recording electrodes simultaneously. One thus observes a single large spike rather than two or more smaller ones. To demonstrate this, the frequency of spontaneous discharge may be purposely reduced by cutting through the nerve at points caudal to the recording electrodes. This will eliminate spontaneous activity arising from neuromasts caudal to the cut. With the spontaneous activity reduced in this manner the probability of any two (or more) impulses reaching the electrodes at the same time is reduced. If summation is responsible for creating the large spikes one should observe a decrease in the relative numbers of large spikes after such an operation.

A series of ten experiments were conducted to test this hypothesis. In each experiment the recording electrodes were attached to the centrally cut nerve (Figure 2) at a point

one-third of the way to the tail (Figure 4). The spontaneous activity from this preparation was then photographed from the display on the screen of the oscilloscope. Successive transverse cuts were then made into the skin and through the nerve starting near the tail and progressing toward the recording electrodes. After each cut the spontaneous activity was photographed and the distance between the point where the nerve enters the body at the recording electrodes to the point where the nerve was cut was measured. The frequency of spontaneous activity was determined after each cut by counting the number of distinct spikes on the photographs over a time interval of 200 msec.

A nerve length after each cut was expressed as a percent of its original length, the distance between entry of the nerve into the body at the recording electrodes to its termination in the caudal peduncle representing 100%. Similarly, spontaneous neural activity after each cut was expressed as a percent of its original frequency. Expressing neural activity and nerve lengths in terms of percentages enabled fish of different lengths to be quantitatively compared and compensated for unavoidable differences in placement of recording electrodes along the side of the fish. Frequency measurements in uncut nerves varied from fish to fish depending on where the recording electrodes were placed. If placed closer to the head the frequency would be greater than if placed closer to the tail. This was a result of greater or

fewer numbers of neuromasts contributing to the overall frequency. By defining the initial frequency as 100% these differences were eliminated.

Results of the ten experiments demonstrated that as more and more of the distal portion of the nerve was removed a corresponding decrease in the number of large spikes (150-200 μV) always occurred. The decrease in numbers of large spikes continued until approximately 60% of the nerve distal to the recording electrodes was removed. With additional cutting the decrease in number of large spikes becomes difficult to distinguish, however, there was a noticeable reduction in the total number of spikes. This decrease in frequency continued until the nerve was cut at the point where it leaves the body for attachment to recording electrodes. When cut here all neural activity stopped.

An example of the spontaneous activity patterns associated with progressive elimination of distal portions of the lateral-line nerve is shown at the top of Figure 6. Each photograph corresponds to the oscilloscope display of spontaneous activity immediately after each cut was made. The percentages to the right of each trace represent the amount of intact nerve distal (caudal) to the recording electrodes. Notice the decreased number of large spikes and eventually the decreased frequency as the nerve is pared down.

At the bottom of Figure 6 is a plot of the frequency of spontaneous discharge (expressed as a percent of its initial

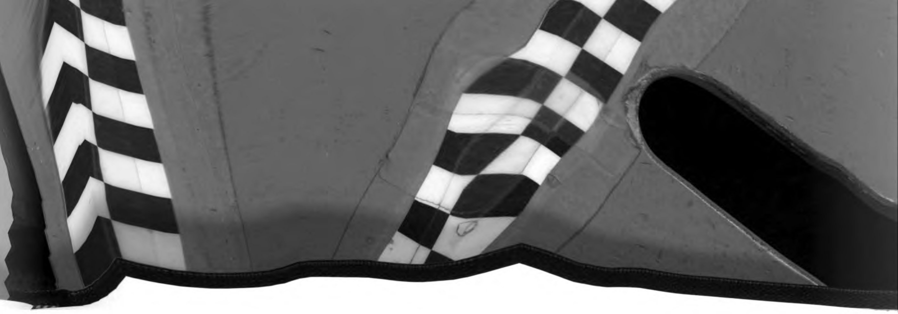
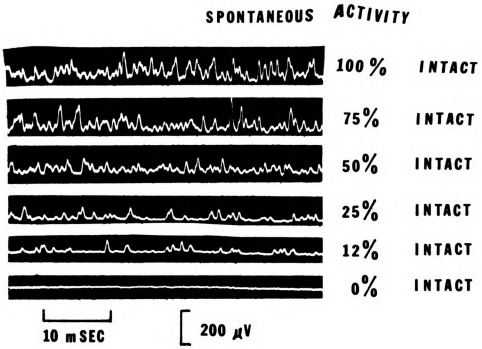


Figure 6.--Spontaneous lateral-line activity in control fish demonstrating reduced frequency upon progressively cutting the nerve. Top--Oscilloscope traces of neural activity corresponding to percent of remaining original nerve length distal to recording electrodes. Recordings from one fish. Bottom--Plot of spontaneous activity (percent of initial frequency) vs. percent of intact nerve length distal to recording electrodes. Results taken from ten different fish.


SPONTANEOUS ACTIVITY
100 % INTACT
75% INTACT
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25% INTACT
12% INTACT
0% INTACT
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200 μV

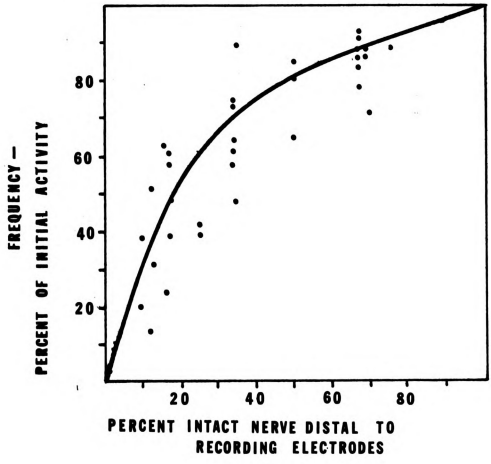
FREQUENCY — PERCENT OF INITIAL ACTIVITY
80
60
40
20
20
40
60
80
PERCENT INTACT NERVE DISTAL TO RECORDING ELECTRODES

Figure 6

frequency) versus the percent of the intact nerve distal to the recording electrodes. This plot was made from results of the ten experiments just described. The frequency of spontaneous discharge does decrease with progressive elimination of distal portions of the nerve. The curve is steep to the left and becomes much flatter to the right. This observation supports the assumption that summation is occurring at levels of high spontaneous activity. With summation one is unable to distinguish all of the impulse spikes thus counting efficiency will be low. This condition corresponds to points on the right hand side of the plot. Decreasing the frequency of spontaneous activity by cutting the nerve will reduce the number of large spikes but at the same time allow the previously hidden smaller spikes to now be seen. As a result, the rate of decrease of activity will remain low until summation has been significantly reduced. Summation was significantly reduced after about 60% of the nerve was eliminated and as can be seen on the plot in Figure 6 the curve tends to become steeper to the left.

In fish preparations to be used for lateral-line frequency measurements later in the study the lateral-line nerve was pared down by cutting through the nerve about 3 cm distal to the recording electrodes. The resulting spontaneous activity frequency was about 500 spikes per second. By paring the nerve down in this manner the problem of summation was largely eliminated. This enabled the observer to

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accurately distinguish individual impulses and detect subtle frequency changes which might otherwise be hidden.

Evoked activity from the lateral line

After recording electrodes were attached to the spontaneously firing lateral-line nerve it was found that a number of mechanical disturbances were able to elicit bursts of neural activity clearly seen above the background of spontaneous activity. This evoked activity was present in intact nerve preparations as well as pared-down preparations. Bursts of evoked neural activity could be induced by tapping gently on the side of the fish holding chamber, by dropping water on to the surface of the water overlying the lateral line, by rippling the water within the chamber, by gently tapping the fish with a probe or by numerous other methods. The lateral line was very sensitive to even the slightest disturbances. The almost unnoticeable vibration set up by a cooling fan in an amplifier on the same table as the fish preparation would evoke very clear responses from the lateral line. All of the evoked responses just mentioned were not artifacts introduced into the electronics of the system but were actually bursts of neural activity. This was clearly demonstrated by the disappearance of the response after the nerve had been cut at the point where it leaves the body for connection to the recording electrodes. Examples of a few types of evoked responses are shown in Figure 7. It is interesting to note that the stimulus need not be

Figure 7.--Examples of various evoked responses produced by stimulating the lateral line. (A) Normal background of spontaneous activity; (B) Lateral line stimulated by scraping probe on rough edge of fish chamber; (C) Stroking finger across skin over lateral line; (D) Gently tapping head of fish with probe; (E) Directing a jet of water directly on the lateral line; (F) Tapping side of fish chamber with fingernail.

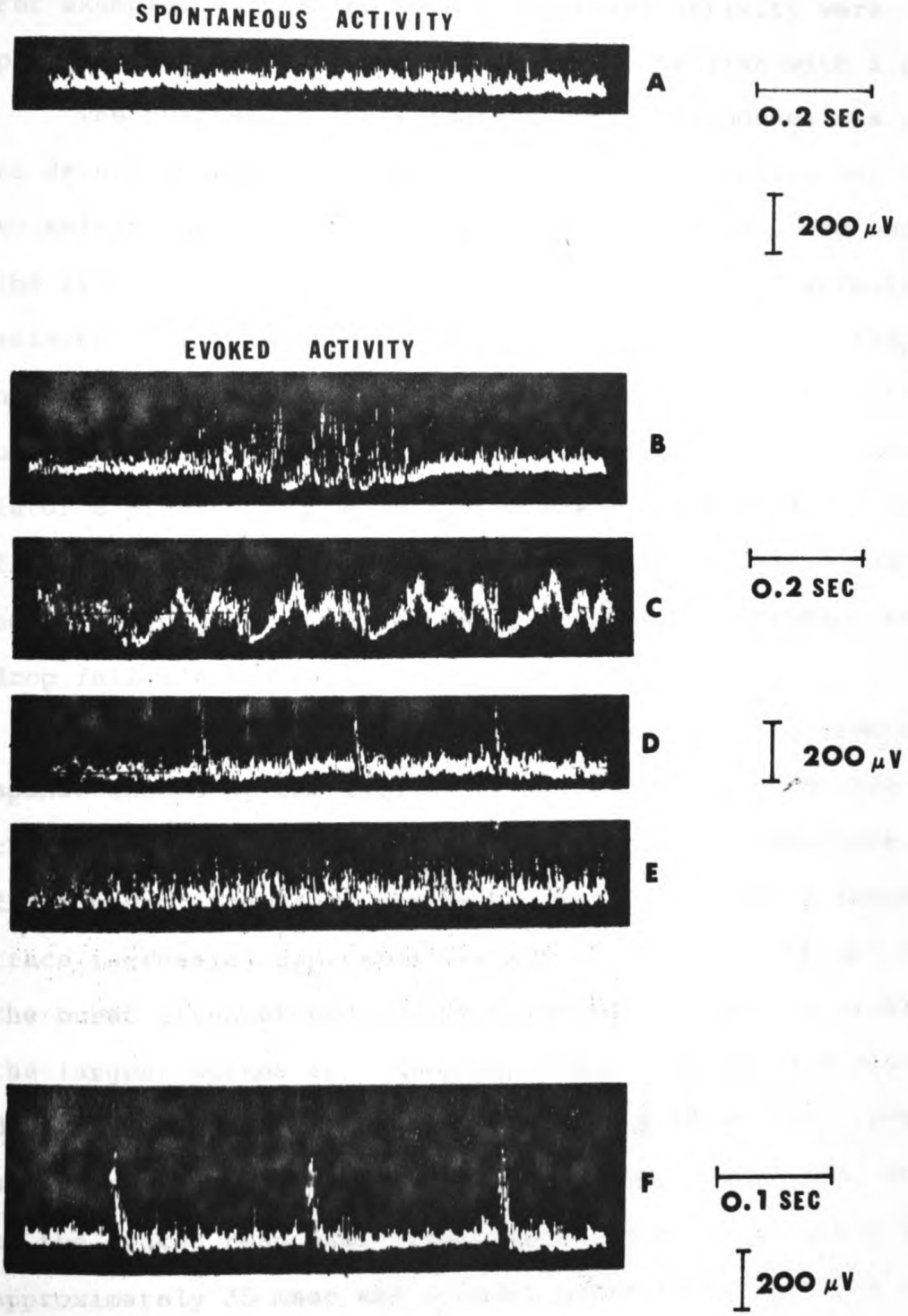

SPONTANEOUS ACTIVITY
A
0.2 SEC
200 μV
EVOKED ACTIVITY
B
C
0.2 SEC
D
200 μV
E
F
0.1 SEC
200 μV

Figure 7

directly applied to the lateral line to evoke a response. For example, very sharp bursts of neural activity were produced by gently tapping the head of the fish with a probe.

The purpose of investigating evoked responses was not to determine what constitutes an adequate stimulus but rather to select one type of stimulus and note its response under the stress conditions described previously. The stimulus selected for this purpose was the impact of a water drop hitting the surface of the water in the fish chamber directly over the lateral line. With the aid of a mechanical manipulator a pipet could be held at a fixed position above the fish, directing water drops to the preparation from the same height throughout an experiment. In every experiment the drop fell a distance of 10 centimeters.

The top trace in Figure 12 shows a typical evoked response caused by the falling water drop. The burst can be characterized by an immediate increase in the amplitude and frequency of impulses, the base line of the oscilloscope trace increasing approximately 200 μV. The first spikes of the burst often exceed 600 μV in amplitude and are usually the largest spikes in the entire burst. There is a gradual decrease in spike height as the burst continues and occasionally a second series of large spikes is seen near the end of the evoked response. The initial burst of activity lasts approximately 35 msec and in most cases is followed by a period of about 20 msec of reduced neural activity.

following the period of decreased activity there is a secondary period of evoked neural activity which corresponds to reflected surface waves created by the falling water drop (surface waves were allowed to stop before another drop of water fell to the preparation).

By synchronizing the falling of the water drop to the sweep of the oscilloscope as described earlier it was possible to display the evoked response in the same position on the screen of the oscilloscope with each drop. Because each evoked burst appeared in the same place it was possible to examine an "averaged" response. This was done by photographing consecutive oscilloscope sweeps, superimposed upon each other by keeping the shutter of the camera open for 20 complete sweeps. Any spike occurring in the same position sweep after sweep could be seen on the photograph as a single, well defined, spike. The shape of this spike could be considered an "average" of 20 spikes. Spikes due to spontaneous activity, having no fixed time relation to the falling of the water, would appear randomly along the movement of the beam and appear as a cloudy area above the base line when observed on a photograph.

For purposes of this study it was desirable to ascribe a quantitative measurement to the evoked response in order to evaluate changes in it induced by stressful conditions. By employing the averaging method discussed above it was possible to do so. For lack of a better method it was decided

to use the amplitude of the first spike in the evoked response for this quantitative measurement. The large initial spike of the evoked response actually consists of many smaller, summated spikes. A common term for a spike of this nature is "compound action potential." The fact that this spike is compound can be demonstrated by noting its gradual decrease in amplitude as the nerve is progressively pared down by partially cutting through the nerve a few centimeters caudal to the recording electrodes. The more the nerve was cut the greater was the reduction in the amplitude of the first spike. Had this spike been the result of an action potential conducted in a single fiber it would have maintained its original size until the single fiber was cut. At this time the spike would have completely disappeared.

A measurement of the amplitude of the first spike of the evoked response is a measure of the number of active fibers contributing to it. The greater the number of fibers contributing, the larger the spike (Katz, 1966).

The amplitude of the first spike in an averaged evoked response was quite well defined and not difficult to measure. An example of such a spike can be seen at the top of Figure 16b.

The amplitude of the first spike of the evoked response under pre-treatment conditions of an experiment was measured, and subsequent changes in its amplitude were expressed as a percent of the pre-treatment value. As with spontaneous

activity, using percents with evoked responses allowed comparison among different fish to be put on the same basis. In experiments to be discussed later, the evoked activity as well as the spontaneous activity were measured in lateral-line nerve preparations which had been pared down by cutting through the nerve about three centimeters caudal to the recording electrodes.

Electrical activity from the heart

The top oscilloscope trace in Figure 8 is an electrocardiogram (ECG) from a curarized rainbow trout showing five consecutive heart beats. An enlarged trace of the electrical activity from one beat is shown in the second trace. On it can be seen the three main waves of the ECG, the P-wave, QRS complex, and the T-wave. The P-wave is the first wave of the ECG and corresponds to electrical activity created by myocardial depolarization of the atrium. The QRS complex is the wave of electrical activity resulting from depolarization of the ventricle and repolarization of the atrium. The beginning of this wave complex started about 0.2 seconds after the start of the P-wave. Repolarization of the ventricle gives rise to the last wave of the ECG, the T-wave. The T-wave began approximately 0.4 seconds after the start of the QRS complex.

The P-wave was typically monophasic, rising in the positive direction, then falling directly to the base line. The QRS complex was also monophasic in the positive direction,

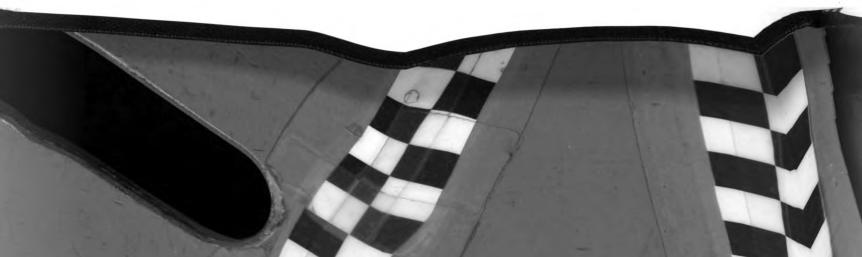
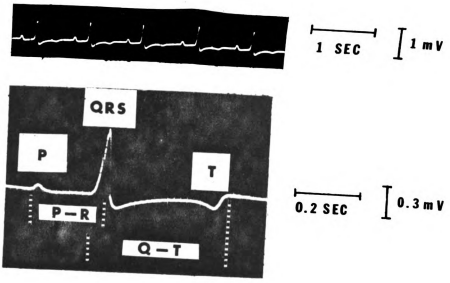


Figure 8.--Typical electrocardiogram (ECG) from rainbow trout using a unipolar electrode. Top--Oscilloscope traces of ECG indicating positions of the P, QRS, and T waves, and the P-R and Q-T intervals. Bottom--Histogram of average rate, P-R interval, and Q-T interval. Data obtained from 27 separate fish. Vertical bar indicates one standard deviation.



from rabbit
e. Top--
pacing positi
the P-R and Q-T
average rate
Data obtained
bar indicates

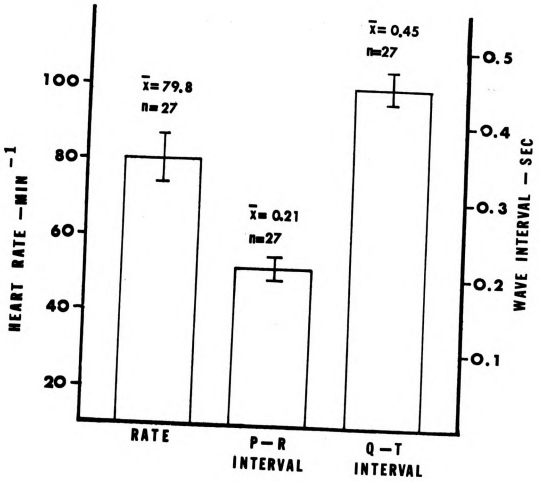
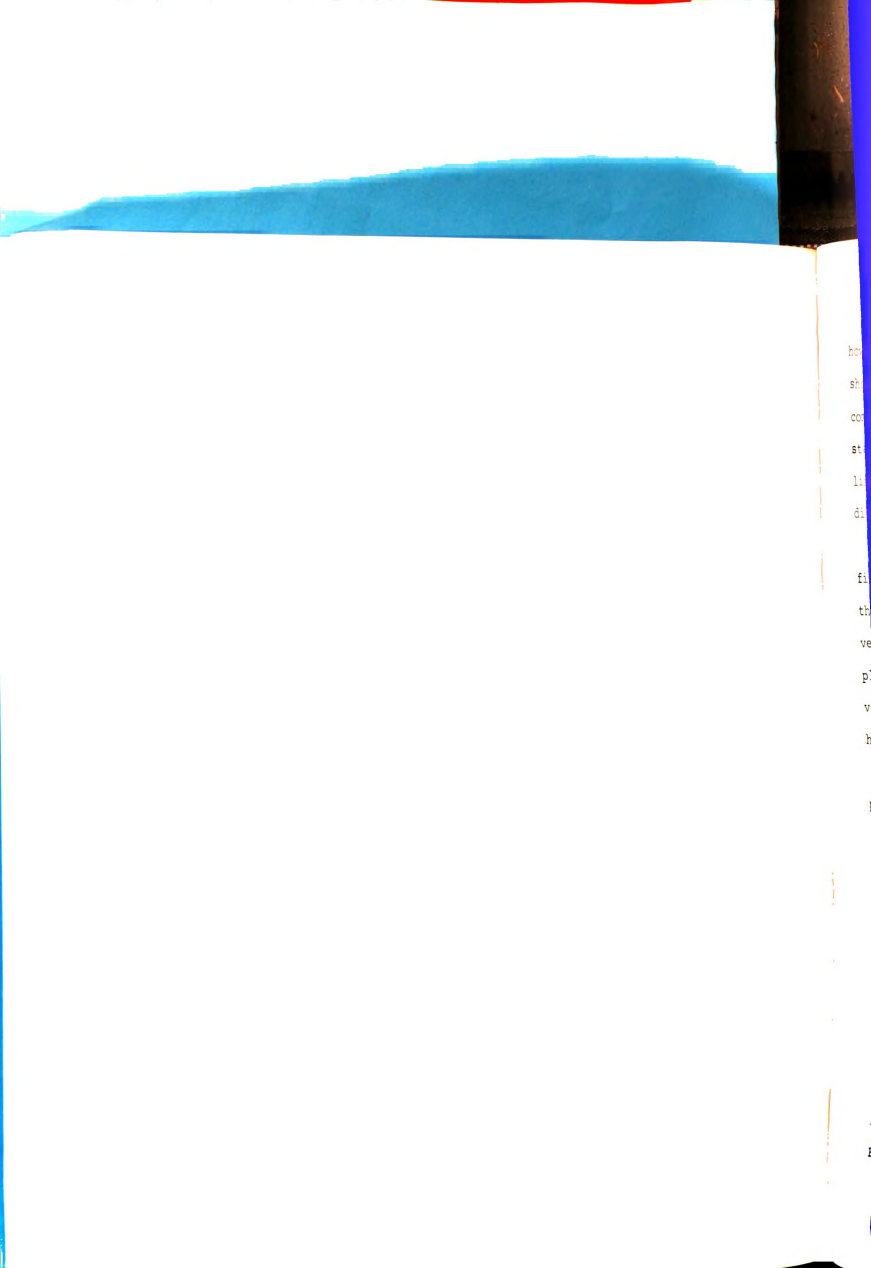


Figure 8



however it demonstrated a slight amount of negative overshoot at the completion of the wave. The T-wave was either completely monophasic in the negative direction or biphasic, starting in the negative direction, overshooting the base line in the positive direction, then finally returning directly to the base line.

The exact placement of the ECG electrode within the fish had a large influence on the amplitude (voltage) of the ECG waves. If placed deeper into the surrounding muscle ventral to the heart, the waves would be much larger than if placed just under the skin. Careful measurement of wave voltages was not an important consideration of this study, however.

The ECG parameters which were not dependent upon precise placement of the electrode included the heart rate, and time intervals between the various waves. The two intervals measured from the ECG were the P-R interval and Q-T interval. The P-R interval, time from the beginning of the P-wave to the point of maximum voltage in the QRS complex, is the time required for the myocardial depolarization to travel from the pacemaker region in the atrium to the ventricle. The Q-T interval, time from the start of the QRS complex to the end of the T-wave, is the time required for complete depolarization and repolarization of the ventricle. The P-R and Q-T intervals are indicated just below the second trace in Figure 8.

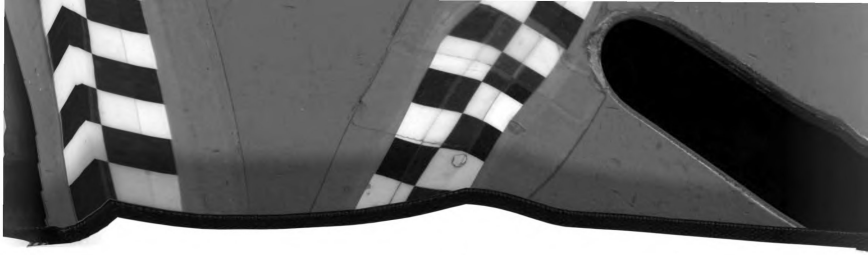
Measurements of heart rate, P-R interval, and Q-T interval were made on 27 curarized fish. Water temperature within the fish holding chamber in these as well as all other experiments was 13°C. The results of the 27 experiments are shown in the histogram at the bottom of Figure 8. The mean heart rate was 79.8 beats per minute, the mean P-R interval was 0.21 seconds, and the mean Q-T interval was 0.45 seconds.

Experiments on Stressed Fish

DDT experiments

Spontaneous activity from the lateral line. Saline suspensions of DDT were perfused into the posterior cardinal vein of curarized trout preparations. The frequency of spontaneous neural discharge from the distal end of the centrally cut lateral-line nerve was monitored using the Schmitt trigger-decade scaler counting system. The Schmitt trigger was adjusted to include nerve impulse spikes of all sizes.

In six experiments at six different injection levels of DDT, each fish received a 0.4 ml injection of the suspension. The spontaneous activity from the lateral-line nerve was recorded for one hour before and for two hours after injection of the insecticide. The dosage level in each injection ranged from 0.01 mg to 4.0 mg. One fish was used at each injection level. The blood concentration of DDT resulting



from these injections was calculated to range from about 2 ppm to slightly over 750 ppm immediately after injection. Calculations were based on an assumed blood volume of 2.25% of the live weight of the fish (Schiffman and Fromm, 1959). In addition to the six experiments listed above, four fish each received a control injection of 0.4 ml saline (10 percent ethanol). Frequency measurements of spontaneous activity were done in a similar manner.

Figure 9 shows the results of these ten experiments. At the top of this figure is a plot of the frequency of spontaneous activity (percent of initial frequency) versus elapsed time following control injections of saline. The bottom of Figure 9 shows a similar plot of experiments where DDT was included in the injections. The six dosages used in these experiments are indicated at the bottom of the plot. None of the spontaneous activity measurements in the six DDT experiments appeared to differ enough from similar measurements in the four control experiments to warrant statistical analysis of the data.

In a series of six similar experiments where fish were immobilized by spinal transection rather than by curarization the same results were obtained. The spontaneous discharge frequency in four fish receiving 0.1 mg, 0.5 mg, 1.0 mg, and 4.0 mg of DDT respectively did not appear to differ from the measurements recorded in two control fish. These experiments were also continued for two hours after injection.

Figure 9.--Effect of intravenous injection of DDT on spontaneous activity recorded from the lateral-line nerve. Top--Control injections (0.4 ml) of saline. Four experiments. Bottom--Injections of DDT-saline suspensions (0.4 ml). Dosage of DDT per injection is indicated at the bottom of the plot. Six experiments, one at each dose.

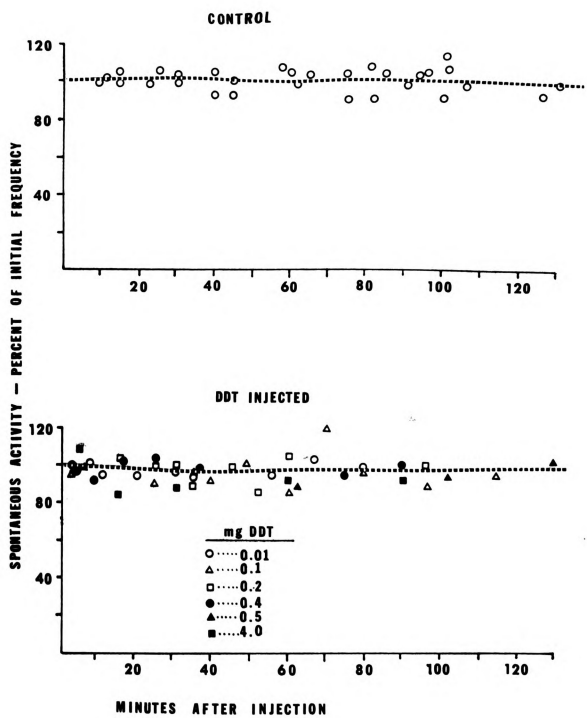

CONTROL
120
80
40
20
40
60
80
100
120
DDT INJECTED
120
80
40
mg DDT
0.01
0.1
0.2
0.4
0.5
4.0
20
40
60
80
100
120
SPONTANEOUS ACTIVITY — PERCENT OF INITIAL FREQUENCY
MINUTES AFTER INJECTION

Figure 9

An interesting observation was made in this series of experiments. At dosage levels of 0.5 mg DDT and above, fish would exhibit eye twitching movements and occasional trembling of opercular and jaw muscles. These are symptoms commonly observed in fish that have been poisoned by DDT. The control fish showed no such symptoms. The muscular activity was restricted to the head region and no movement was observed below the level of the spinal transection. It is believed that the symptoms observed in these fish was induced by the action of DDT. The observations just mentioned demonstrate that spontaneous activity from the lateral-line nerve remains unchanged even in the presence of obvious symptoms of DDT poisoning.

In four experiments individual fish were placed into water containing a concentration of 15 ppm DDT. The purpose of these experiments was to observe the spontaneous activity from the lateral-line nerve and note any abnormal changes in discharge frequency or discharge patterns after the fish had reached the terminal stages of DDT poisoning. Shortly after one hour of exposure to the insecticide fish became hyperexcitable, demonstrated occasional periods of violent swimming movements, and exhibited erratic eye and jaw movements. These symptoms increased in severity for about three hours. After about four hours of exposure fish became calmer and laid almost motionless on the bottom of the aquaria. The only movements that could be observed were erratic eye

m

t

d

t

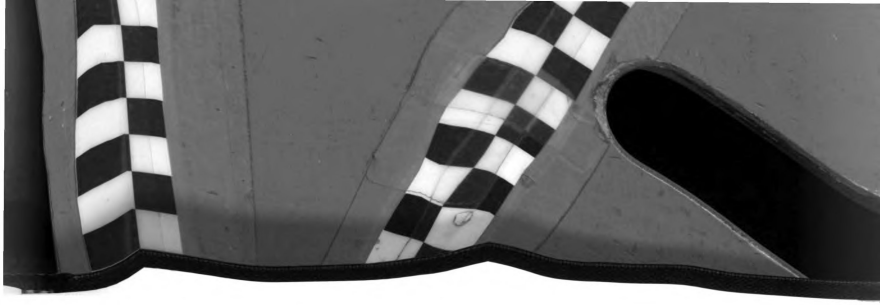
t

movements, opercular trembling, and occasional trembling in the trunk. Fish were kept for two more hours in this condition (six hours total exposure) before being removed for the preparation table. Fish were then pithed and connected to the recording apparatus as before. None of the four fish showed any signs of abnormality in the discharge of spontaneous activity from the lateral-line nerve.

On three occasions successful attempts were made at injecting a saline suspension of 100 ppm DDT directly into the lateral-line canal. Recordings of neural activity were made before and after the injection as in other experiments. Here, as before, no change could be detected in the frequency of lateral-line discharge.

Evoked activity from the lateral line. Measurements of evoked neural responses from the lateral line were also conducted in all of the DDT experiments. No changes could be found in the amplitude of the response nor any other parameter associated with the evoked burst following any of the DDT treatments.

Heart activity. The heart rate was the only ECG parameter measured in the DDT experiments. None of the DDT treatments had a significant effect on the heart rate. This was also true for fish that had been exposed for six hours to 15 ppm DDT in the water. Casual observation of the ECG waves in these fish also showed no apparent abnormalities.



Hypoxia experiments

The oxygen tension in the water perfusing the gills of the fish preparation was lowered by reducing the flow of oxygen to the head tank (Figure 1). A total of nine fish were used in these experiments, three fish at each of three reduced oxygen levels. The three dissolved oxygen levels used, expressed as oxygen partial pressures (pO_2), were 50, 40, and 30 mm Hg. On a part per million basis these values correspond to 3.5, 2.8, and 2.1 ppm, respectively.

Spontaneous activity from the lateral line. Measurements of spontaneous activity were conducted for two hours with the fish exposed to oxygen saturated water, pO_2 of 160 mm Hg, and then for three hours after the oxygen tension had been reduced. Frequency measurements of neural activity were obtained from oscilloscope photographs rather than using electrical counting methods because of an unexpected malfunction in the preamplifier. Although much more time consuming than electrical counting this method proved to be entirely adequate.

None of the reduced dissolved oxygen experiments demonstrated significant changes in the frequency of spontaneous neural activity from the lateral-line nerve.

Evoked activity from the lateral line. Evoked bursts of neural activity also remained unchanged following the stress of hypoxia.

Heart activity. Electrocardiograms were recorded in all of the experiments listed above. Figure 10 summarizes the heart rate data from the above experiments. The three plots in this figure correspond to heart rates obtained at reduced pO_2 levels of: 50 mm Hg, (A); 40 mm Hg, (B); and 30 mm Hg, (C).

At a pO_2 of 50 mm Hg the heart rate decreased slightly over the three hour period of exposure, dropping only about 10% from its pre-treatment rate. At a pO_2 of 40 mm Hg the heart rate decreased to approximately 80% of its initial value over the first hour of exposure. During the second hour of exposure the heart rate gradually increased by about 10% then decreased approximately 20% in the third hour. A pO_2 of 30 mm Hg had the greatest effect on the heart rate. At this level the heart rate decreased over 20% during the first hour. Following this initial decrease the heart rate stabilized at approximately the same level for the second hour and then began to gradually decrease again, approaching a rate of 60% of its pretreatment value after three hours.

Electrocardiograms taken during the course of these experiments demonstrated some interesting changes. Within the first 20 minutes of exposure to a pO_2 of 30 mm Hg the P-R interval of the ECG increased from a normal value of about 0.2 seconds to nearly 0.4 seconds. During the same time period the wave form of the QRS complex changed from a typically large monophasic wave to a much smaller biphasic

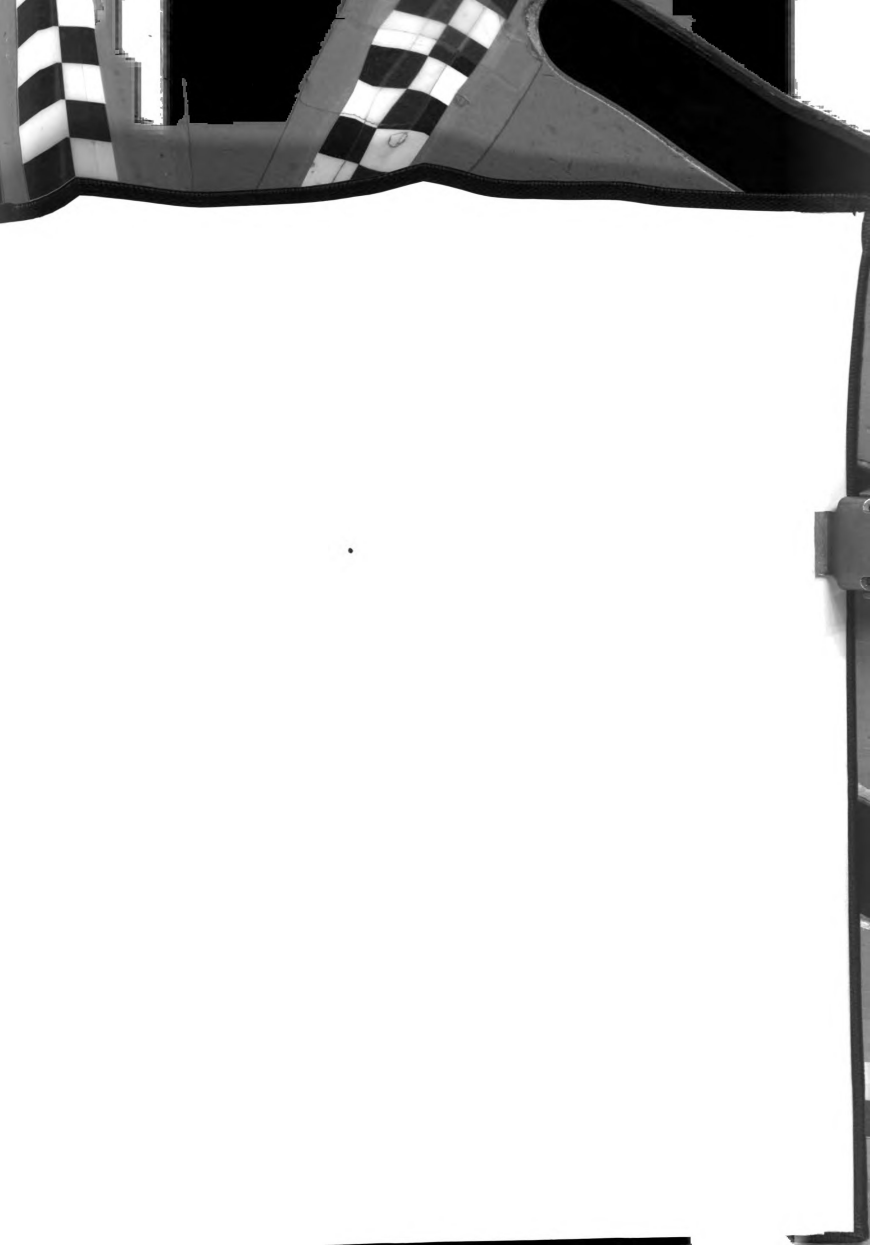


Figure 10.--Effect of lowered oxygen tension in water on heart rate. Top--Oxygen partial pressure of 50 mm Hg; Center--Oxygen partial pressure of 40 mm Hg; Bottom--Oxygen partial pressure of 30 mm Hg. Three fish used in each experiment. Heart rate is expressed as a percent of the initial rate in the unstressed condition.

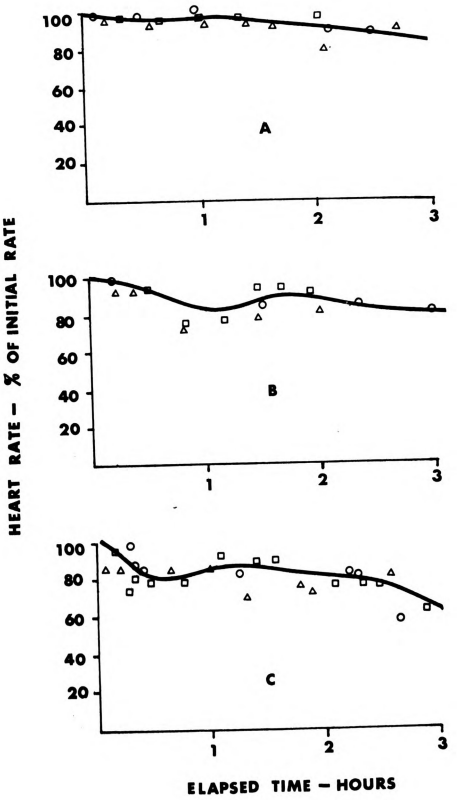

HEART RATE — % OF INITIAL RATE
100
80
60
40
20
A
B
C
1
2
3
ELAPSED TIME — HOURS

Figure 10

wave. The T-wave also changed during this period, becoming progressively larger both in amplitude and duration. The Q-T interval was very difficult to measure because the end of the T-wave was not well defined.

The delay of ventricular depolarization and repolarization created by the prolonged P-R interval caused the T-wave to nearly coincide with the P-wave of the next heart beat. This event, however, did not seem to change the basic heart rate. For the duration of the first hour the changes just described for the first 20 minutes persisted. The P-R interval remained unchanged, the QRS complex continued to demonstrate a lower-than-normal voltage, and the T-wave continued to slightly increase in size. Occasionally the QRS complex would regain its monophasic form, but only for a few minutes at a time and at a subnormal voltage.

The decrease in heart rate during the first hour did not appear to be caused primarily by a decreased pacemaker rate because consecutive P-waves maintained normal intervals on the ECG. However, an entire beat would occasionally be missed, the ECG showing no activity from the end of a T-wave to the beginning of a later P-wave. This silent period would last for the duration of one cardiac cycle and then the P-wave would reappear exactly one beat later. Less frequently the silent period would last for a duration of two beats. The heart rate was calculated by counting the number of complete cardiac cycles over a period of one minute. The apparent

decrease in heart rate was primarily due to missed beats, not to a reduced peacemaker rhythm. This was largely true for the first hour. During the second hour, however, the pacemaker rhythm did decrease. This was evidenced by an increase in the interval between consecutive P-waves. Also during the second hour the heart became more regular, missing very few beats. The regularity of the heart during the second hour accounted for the apparent plateau in Figure 10c. The decrease in rate during the third hour resulted from a combination of reduced pacemaker rhythm and reappearance of missed beats.

Although changes in the ECG patterns given above were responses to a pO_2 of 30 mm Hg, similar changes were observed in experiments conducted at oxygen partial pressures of 40 and 50 mm Hg. In these cases the exposure times required to elicit similar changes were longer, but nonetheless present.

Figure 11 shows a series of ECG records from two fish after various exposure times to a partial pressure of oxygen of 30 mm Hg. The top six traces in the figure (a-f) demonstrate most of the abnormalities discussed above. The first trace (a) is a normal ECG from a fish exposed to oxygen-saturated water (pO_2 of 160 mm Hg). The second trace (b) is the ECG in the same fish after ten minutes of exposure to a partial pressure of 30 mm Hg. Note the increased P-R interval, the biphasic QRS complex, and enlarged T-wave. In this trace, as well as in those following, the T-wave partially overlaps

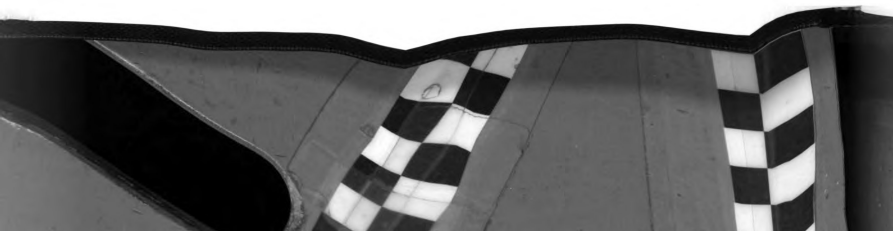


Figure 11.--Examples of ECG changes during hypoxia. Oxygen partial pressure in the water was reduced to 30 mm Hg. Top--(a), ECG at 160 mm Hg pO_2; (b-f), ECG at times after the pO_2 in the water had been reduced. Bottom--Same experimental conditions upon a different fish. (a), ECG at 160 mm Hg pO_2; (h-j), ECG at times after pO_2 was reduced.

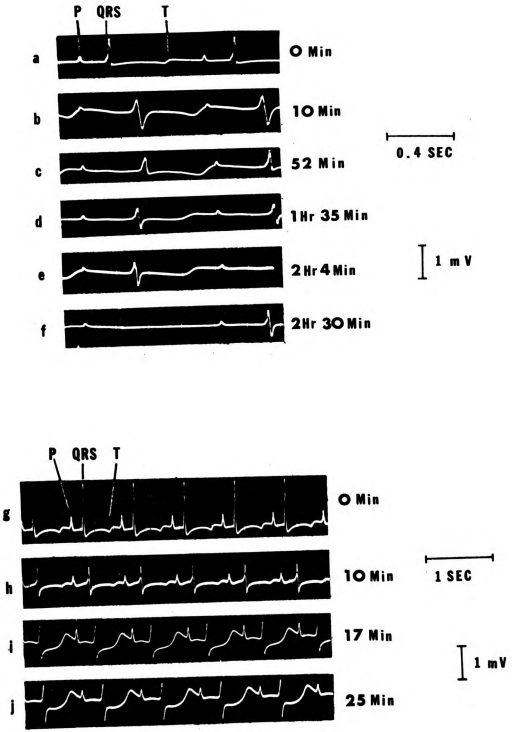
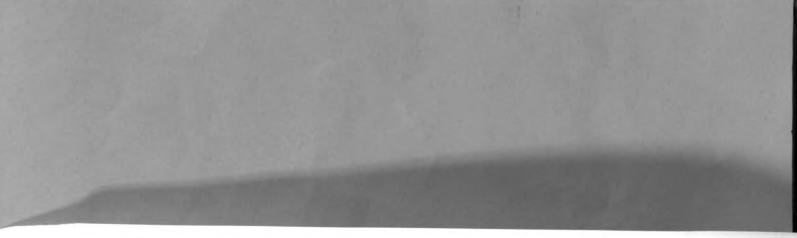

P
QRS
T
a
0 Min
b
10 Min
c
52 Min
0.4 SEC
d
1 Hr 35 Min
e
2 Hr 4 Min
1 mV
f
2 Hr 30 Min
P
QRS
T
g
0 Min
h
10 Min
1 SEC
i
17 Min
1 mV
j
25 Min

Figure 11



with the P-wave of the next heart beat. The third trace (c) shows a QRS complex after it had temporarily regained its monophasic shape. Its amplitude, however, is much smaller than normal. Trace f shows a missed ventricular beat evidenced by two consecutive P-waves in the absence of a QRS complex, a phenomenon occurring quite frequently in later stages of hypoxic stress. The four traces (g-j) were obtained from a different fish under the same experimental conditions and are given for comparison. Note the very large T-wave after the oxygen tension had been lowered for 17 and 25 minutes (i and j).

Two of the fish tested at an oxygen partial pressure of 30 mm Hg were allowed to recover from the hypoxic stress. After three hours of exposure to this reduced dissolved oxygen level, the saturation level was re-established. The ECG from both fish showed signs of recovery within minutes. The biphasic QRS complex returned to its normal monophasic shape within eight minutes. Within 20 minutes the ECG appeared normal except for a slower rate. In both fish it took approximately one hour for the rate to fully recover. The ECG was carefully observed for about three hours after complete recovery and no ill effects were noted in the ECG.

Asphyxia experiments

Spontaneous activity from the lateral line. Nine fish were subjected to conditions of asphyxia by discontinuing the flow of oxygenated water over their gills. This was done

by clamping the input and output tubes of the fish chamber.

The effect of asphyxiation upon spontaneous activity from the lateral line was quite severe. Reduction of discharge frequency occurred soon after the water flow had ceased; six out of nine fish exhibited complete loss of spontaneous activity in less than ten minutes. The other two fish lost spontaneous activity in about 20 minutes.

After the spontaneous neural discharge had ceased the clamps were removed from the input and output tubes and oxygenated water was again allowed to perfuse the gills. In every case the spontaneous activity returned to normal within 40 minutes, the majority attaining normal levels in 20 minutes.

Evoked activity from the lateral line. The discharge of neural activity resulting from water drop stimuli was also measured in these experiments. It was found that the evoked response was much more resistant to this stress than was the spontaneous activity. At the time spontaneous activity had disappeared about 50% of the evoked response still remained. With the spontaneous activity gone the evoked response could be clearly seen rising above the silent base line. Figure 12 shows six oscilloscope traces of the typical response of spontaneous and evoked activity to conditions of asphyxia. Neural activity to the left of the arrow at the bottom of the figure is spontaneous activity and neural activity to the right is evoked. Note the rapid

Figure 12.--Effect of asphyxiation on spontaneous and evoked neural discharge from lateral-line nerve. Numbers to the right represent the elapsed time in minutes after water flow over the gills was discontinued. Arrow at the bottom marks the time when the water drop stimulus hit the water over the lateral line. Spontaneous activity to the left of the arrow and evoked activity to the right.

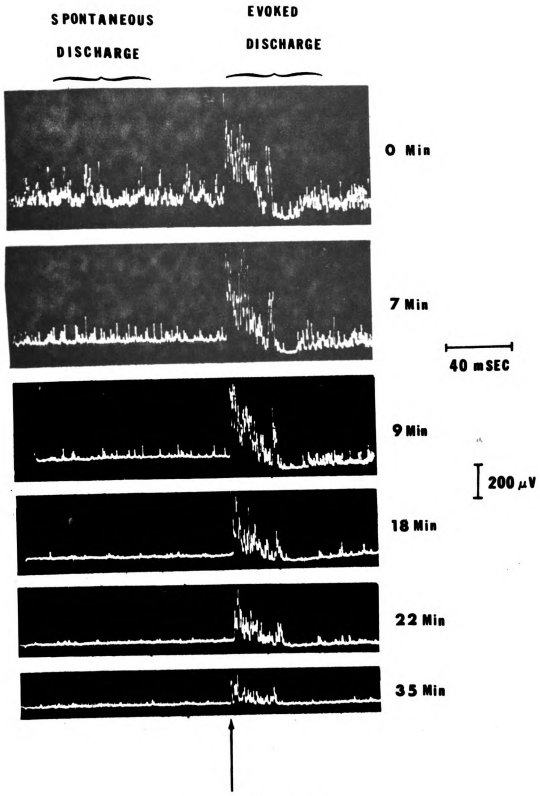

SPONTANEOUS
DISCHARGE
EVOKED
DISCHARGE
0 Min
7 Min
40 mSEC
9 Min
200 μV
18 Min
22 Min
35 Min

Figure 12

decrease in spontaneous activity and the relative persistence of the evoked activity.

The relationship between spontaneous and evoked activity can be best described by the lower plot in Figure 17. In this plot each point represents the evoked activity which corresponded to the spontaneous activity at a given time during asphyxia. It should not be implied by the position of the ordinate and abscissa that the evoked activity is necessarily dependent on the spontaneous activity. The graph was constructed for the purpose of demonstrating a relationship rather than dependence. Evoked activity is expressed as a percent of its initial amplitude (first spike) and spontaneous activity expressed as a percent of its initial frequency. The curve through the points was fitted by eye. When spontaneous activity was reduced by half the evoked activity was still at 80% of its initial level. When spontaneous activity disappeared the evoked activity had decreased 50%.

Recovery of the evoked response was also observed when oxygenated water was again allowed to perfuse the gills. Full recovery was observed in about 20 minutes.

Heart activity. Within four minutes after the onset of asphyxia the amplitude of the QRS complex decreased by more than 70%. The P-R interval increased and the duration and amplitude of the T-wave increased. The heart rate decreased

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from a normal value of about 80 beats per minute to less than 40 per minute. After seven minutes the heart rate fell to about 20 beats per minute and the T-wave of the ECG was almost gone. Electrical activity from the heart at this time was very erratic and difficult to interpret. In the following minutes the waves of electrical activity weakened and the heart would beat only once every five to seven seconds. Within ten minutes after the onset of asphyxiation it was judged that heart function, at best, was very poor.

When water flow across the gills was again resumed after being off for a total of from 20 to 40 minutes the heart recovered rapidly. Regular rhythm was restored in less than two minutes and the normal rate in less than five minutes. The wave form of the ECG appeared normal within two minutes.

Cyanide experiments

A total of nine fish were used in cyanide experiments, three fish at each of three cyanide concentrations (0.3 ppm, 1 ppm, and 4 ppm KCN). In each experiment the spontaneous activity, evoked activity, and electrical activity from the heart were measured.

Spontaneous activity from the lateral line. Frequency analysis of spontaneous neural discharge was conducted using photographic rather than electrical counting techniques. Measurements were made for a two-hour period before and up to ten hours after exposure to the cyanide.

The bottom (solid) lines on the plots in Figures 13, 14, and 15 correspond to the spontaneous activity measurements in the three series of experiments done at 4 ppm, 1 ppm, and 0.3 ppm, respectively. Spontaneous activity is plotted against accumulated dose per gram of fish rather than time because of the size differences encountered between fish. Small fish (less than 200 g) reacted much quicker to the poison than the larger fish (over 300 g) because a constant cyanide flow was being forced over the gills irrespective of the size of the fish. A small fish was thus forced to receive a larger dose of cyanide per unit weight than a larger fish. From a toxicological standpoint it was more meaningful to take fish weight into consideration after studying the conditions of these experiments.

As can be seen from Figures 13, 14, and 15 the spontaneous activity from the lateral-line nerve decreased markedly following exposure of fish preparations to the three levels of cyanide. The most rapid decrease in spontaneous activity occurred at the higher exposure concentrations. At 4 ppm (Figure 13) the spontaneous activity decreased to half its original level after about 150 μg/g of cyanide had passed over the gills and approached a zero level of activity after about 700 μg/g. At exposure levels of 1 ppm (Figure 14) the spontaneous activity approached half of its original value after approximately 300 μg/g of cyanide had passed the gills and after 700 μg/g the spontaneous activity was about 20% of its

Figure 13.--Effect of 4 ppm KCN on spontaneous and evoked neural discharge from the lateral-line nerve. Abscissa represents the amount of KCN passed over the gills per gram of fish. Ordinate represents the lateral-line neural discharge expressed as a percent of the initial (pretreatment) activity. Top curve (dashed) is the evoked discharge and the bottom curve (solid) is the spontaneous discharge. Symbols similarly shaped represent the same fish.

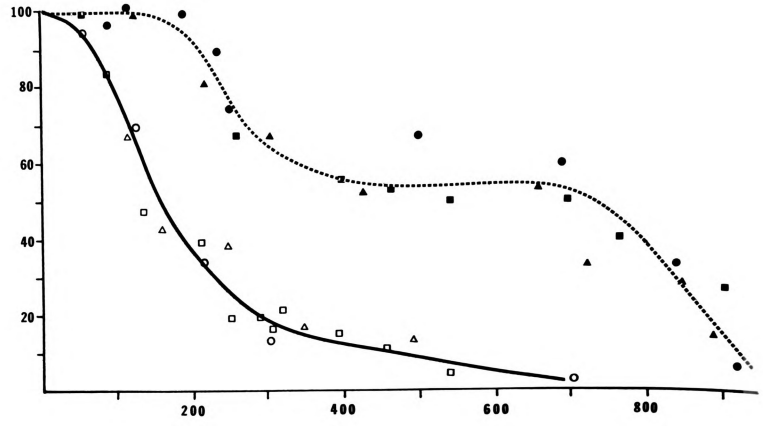

PERCENT OF INITIAL NEURAL DISCHARGE
100
80
60
40
20
200
400
600
800
AMOUNT OF CYANIDE PASSED OVER GILLS -- µg KCN/g OF FISH

Figure 13

Figure 14.--Effect of 1 ppm KCN on spontaneous and evoked neural discharge from the lateral-line nerve. Abscicca represents the amount of KCN passed over the gills per gram of fish. Ordinate represents the lateral-line neural discharge expressed as a percent of the initial (pretreatment) activity. Top curve (dashed) is the evoked discharge and the bottom curve (solid) is the spontaneous discharge. Symbols similarly shaped represent the same fish.

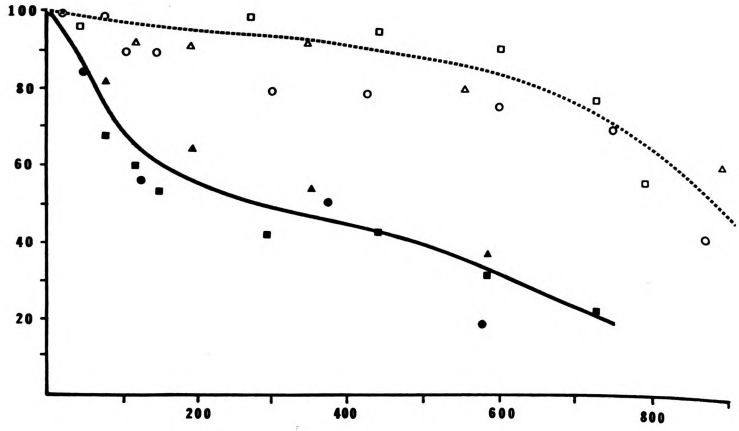

PERCENT OF INITIAL NEURAL DISCHARGE
100
80
60
40
20
200
400
600
800
AMOUNT OF CYANIDE PASSED OVER GILLS -- μg KCN/g OF FISH

Figure 14

500
400
300
20
40
60
80
100

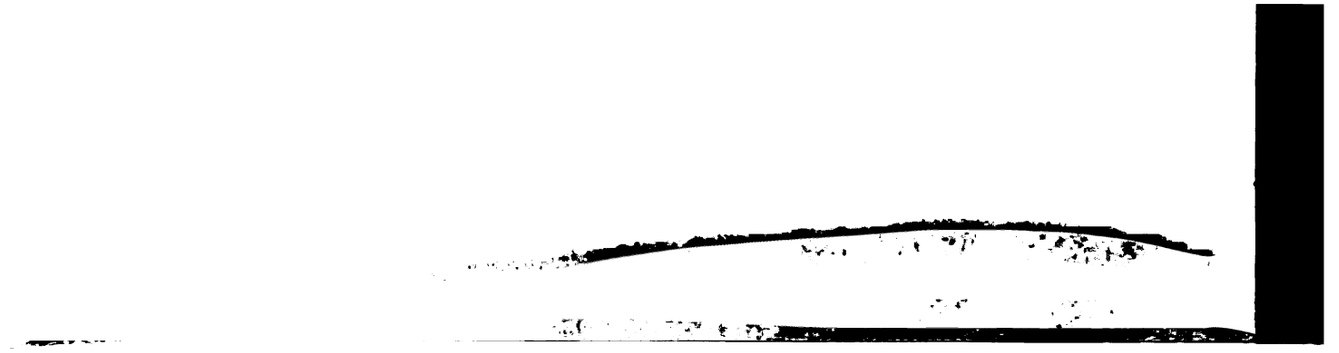


Figure 15.--Effect of 0.3 ppm KCN on spontaneous and evoked neural discharge from the lateral-line nerve. Abscissa represents the amount of KCN passed over the gills per gram of fish. Ordinate represents the lateral-line neural discharge expressed as a percent of the initial (pretreatment) activity. Top curve (dashed) is the evoked discharge and the bottom curve (solid) is the spontaneous discharge. Symbols similarly shaped represent the same fish.

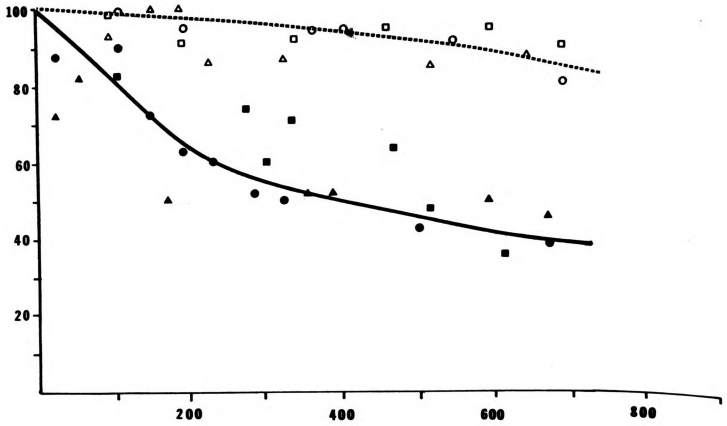

PERCENT OF INITIAL NEURAL DISCHARGE
100
80
60
40
20
200
400
600
800
AMOUNT OF CYANIDE PASSED OVER GILLS -- µg KCN/g OF FISH

Figure 15

initial value. Fish exposed to 0.3 ppm of cyanide (Figure 15) did not react as fast to the chemical as with levels of 1 ppm and 4 ppm. Fish required nearly 400 μg/g of cyanide before the spontaneous activity from the lateral line fell to 50%, and after exposure to 700 μg/g of the poison the activity was still at 40%. Figure 16 (top) shows oscilloscope traces of spontaneous activity in one fish after exposure to 1 ppm KCN. For this particular fish approximately 180 μg KCN per gram of fish passed over the gills per hour.

Evoked activity from the lateral line. The top lines (dashed) in Figures 13, 14, and 15 correspond to the evoked activity measurements after exposure of fish preparations to the same three concentrations of cyanide. In every case the evoked activity decreased more slowly than the spontaneous activity. In experiments with cyanide concentrations of 4 ppm (Figure 13) the evoked activity fell to approximately 60% of its initial value after slightly over 300 μg of cyanide per gram of fish had passed the gills. The evoked activity remained at this level until about 700 μg/g passed the gills; then the evoked response fell off sharply. After approximately 900 μg/g had passed over the gills the evoked response nearly disappeared.

Experiments at 1 ppm cyanide demonstrated a more gradual decrease in evoked activity (Figure 14). The evoked response gradually declined until the accumulated cyanide dose was 700 μg per gram of fish. The evoked activity then fell off

Figure 16.--Effect of 1 ppm KCN exposure on spontaneous and evoked neural discharge from the lateral-line nerve. Top--Spontaneous activity at indicated elapsed times during KCN exposure. Bottom--Evoked discharge from lateral line at indicated times after exposure. Each trace represents 20 superimposed sweeps of the oscilloscope beam.

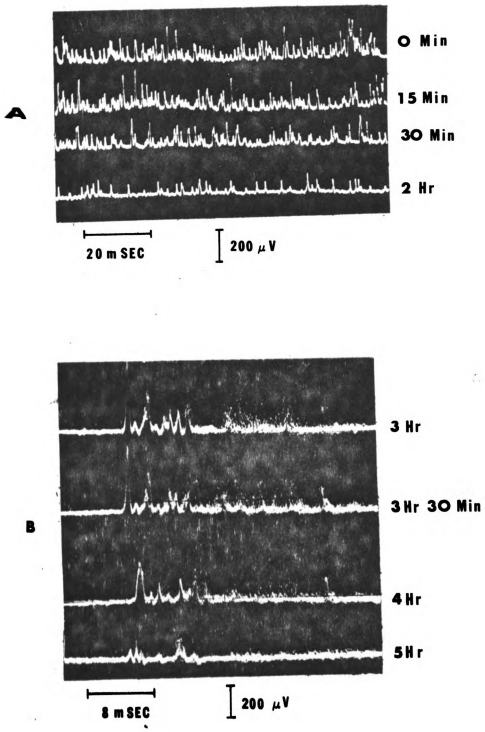

A
0 Min
15 Min
30 Min
2 Hr
20 m SEC
200 μV
B
3 Hr
3 Hr 30 Min
4 Hr
5 Hr
8 m SEC
200 μV

Figure 16

more sharply, reaching 50% of its initial value after exposure to 900 μg/g.

Exposure to 0.3 ppm of cyanide (Figure 15) resulted in a very mild decrease in evoked activity from the lateral line. The decrease was nearly linear, the response falling only 20% after the fish was exposed to 700 μg of cyanide per gram of fish.

The decrease in evoked response during 1 ppm experiments is shown in the bottom series of oscilloscope traces in Figure 16. Traces at the top and bottom of the figure were obtained from the same fish.

The relationship between spontaneous and evoked activity is shown at the top of Figure 17. This is the same type of plot that was described in the section on asphyxia. Each point represents the evoked response observed at a given level of spontaneous activity. Data from all three dosage levels are included in the plot. As can be seen there was very little difference in the spontaneous-evoked relationship between cyanide experiments and asphyxia experiments.

Recovery of the spontaneous activity and evoked response was found to occur after the flow of cyanide to the preparation was discontinued. All three fish exposed to the 4 ppm level recovered, both the spontaneous and evoked neural activity returning to normal levels after about one hour. Recovery experiments were not done at the other two cyanide levels.

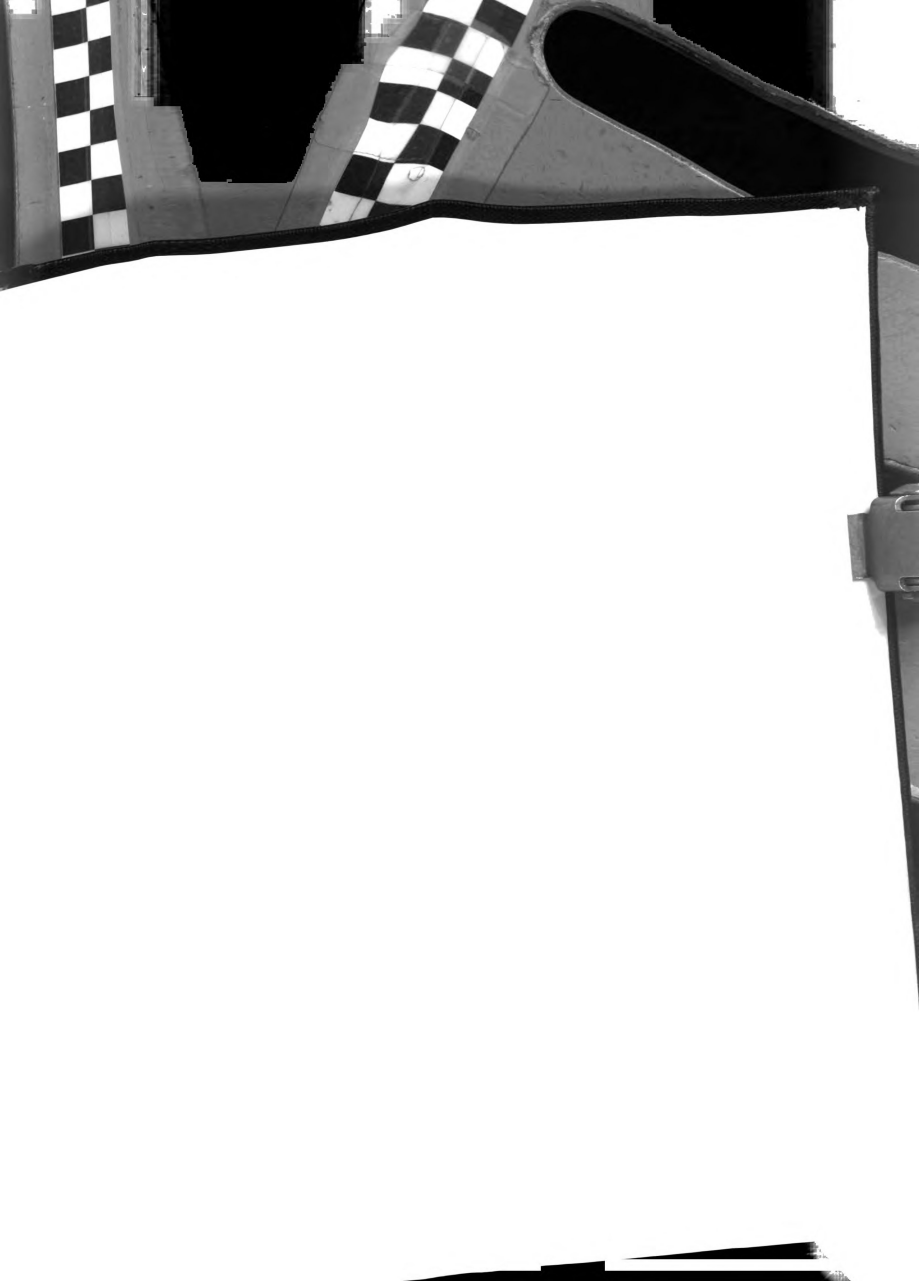


Figure 17.--Relationship between spontaneous and evoked neural activity during asphyxia and cyanide exposure. The abscissa represents the spontaneous activity (percent of the initial frequency) and the ordinate represents the evoked activity (percent of the initial amplitude). Plot at the top was constructed from data obtained from cyanide experiments and the bottom plot from data obtained in asphyxia experiments.

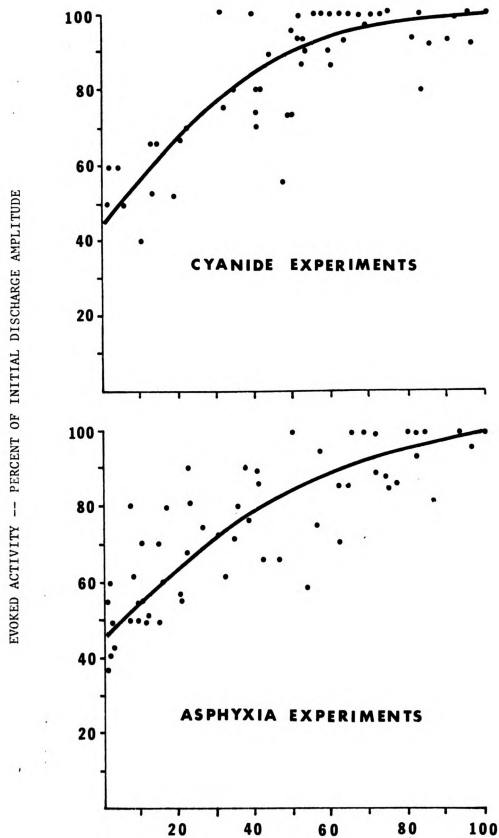
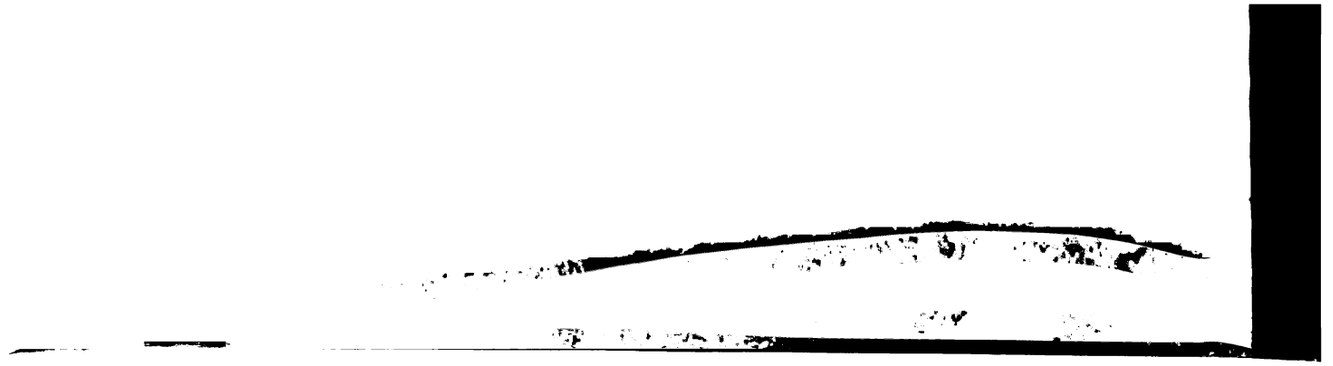

100
80
60
40
20
CYANIDE EXPERIMENTS
100
80
60
40
20
ASPHYXIA EXPERIMENTS
20
40
60
80
100
EVOKED ACTIVITY -- PERCENT OF INITIAL DISCHARGE AMPLITUDE
SPONTANEOUS ACTIVITY -- PERCENT OF INITIAL FREQUENCY

Figure 17

Heart activity. Electrocardiograms were recorded throughout the cyanide experiments. Figures 18, 19, and 20 show the effect of cyanide upon the heart rate. The percent of the initial rate was plotted against the accumulated dose of KCN which passed over the gills per unit weight of the fish. Nearly every fish in the three series of experiments demonstrated an initial rate increase of 5 to 10%. Following this initial increase the heart rate dropped sharply to about 20% of the initial rate in the experiments conducted at 4 ppm KCN, and to approximately 40% in experiments at 1 ppm and 0.3 ppm. The low point of this rapid rate decrease occurred at nearly the same point in each series of experiments: after about 100 μg of cyanide per gram of fish had passed the gills. After this period of reduced heart activity the heart rate increased. The heart rate in the 4 ppm experiments increased from 20% to roughly 50% by the time 300 μg/g had passed the gills. From this point on the rate gradually decreased, falling just under 20% after an accumulated exposure of 1000 μg/g. In experiments conducted at a concentration of 1 ppm KCN the heart rates demonstrated similar increases. The heart rates rose from 40% to approximately 60% after the fish were exposed to 300 μg/g and in two of the three fish the rate remained near this level until exposed to 800 μg/g. The rate in the third fish fell in a manner very similar to heart rates in fish exposed to 4 ppm KCN. The heart rates in the



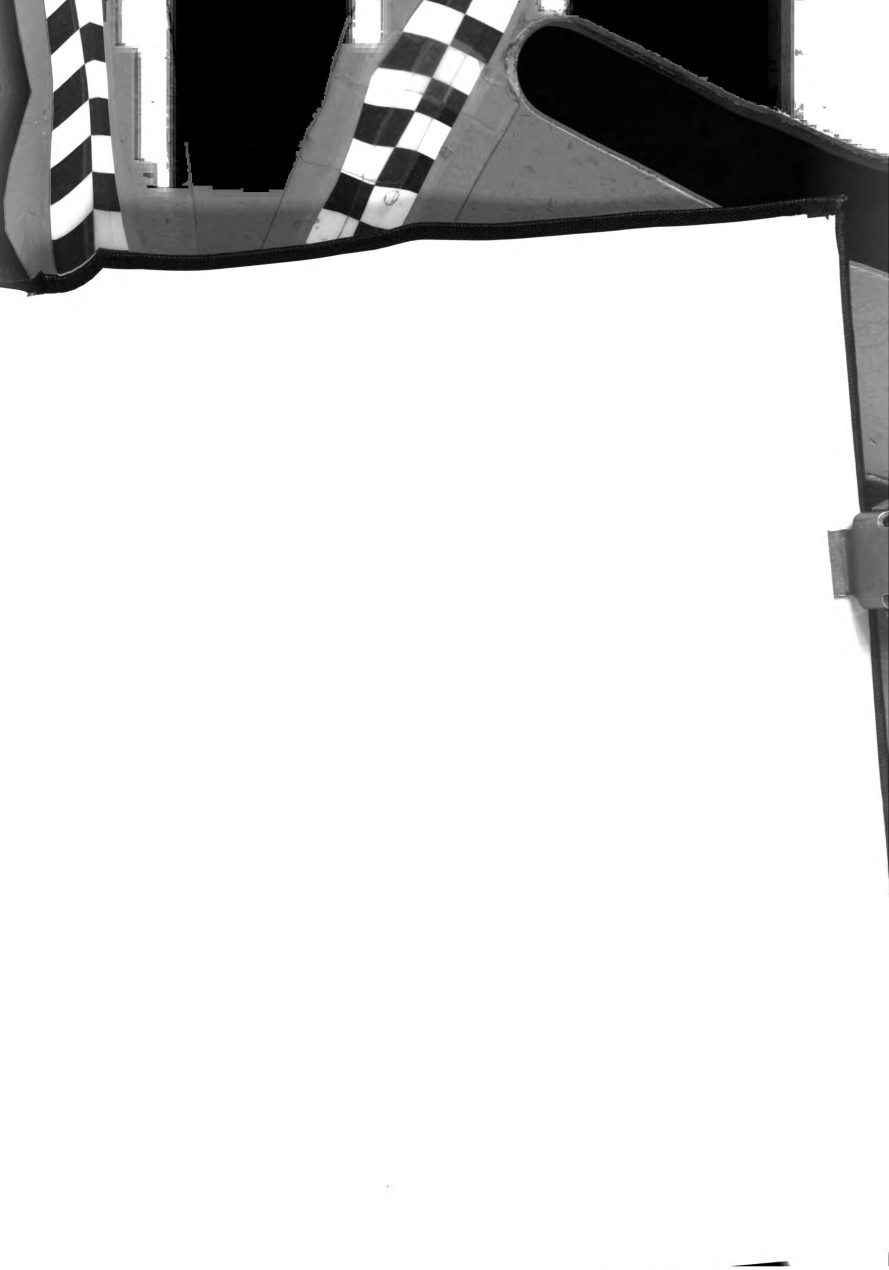


Figure 18.--Heart rate effects during exposure of fish preparations to 4 ppm KCN. Abscissa represents the amount of KCN passed over the gills per gram of fish. The ordinate represents the heart rate expressed as a percent of its initial value. Different symbols represent different fish.

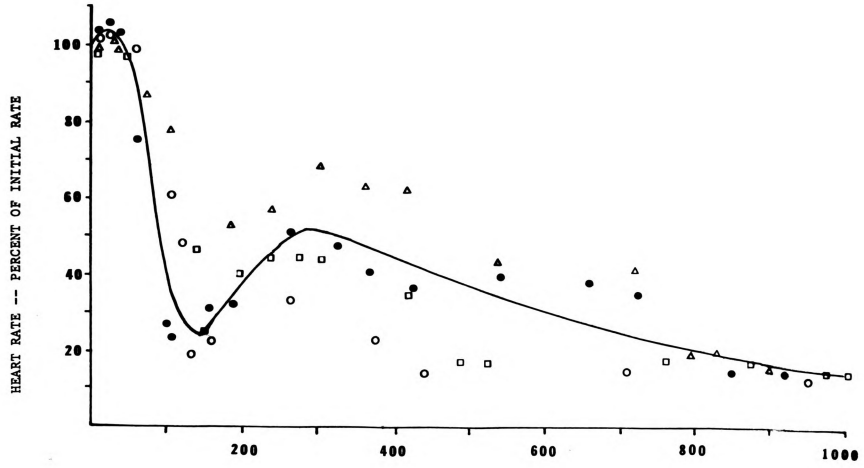

HEART RATE -- PERCENT OF INITIAL RATE
100
80
60
40
20
200
400
600
800
1000
AMOUNT OF CYANIDE PASSED OVER GILLS -- µg KCN/g OF FISH

Figure 18

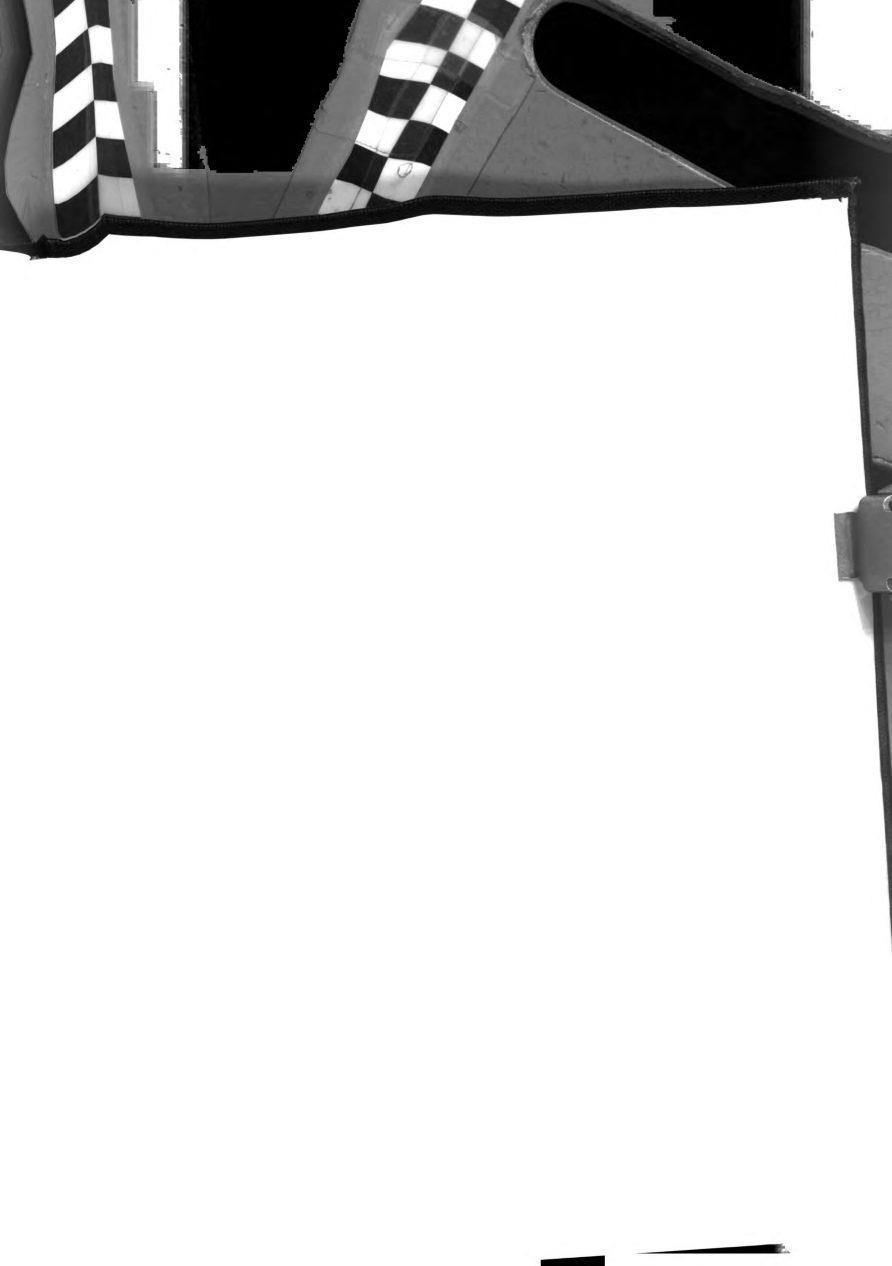


Figure 19.--Heart rate effects during exposure of fish preparations to 1 ppm KCN. Abscissa represents the amount of KCN passed over the gills per gram of fish. The ordinate represents the heart rate expressed as a percent of its initial value. Different symbols represent different fish.

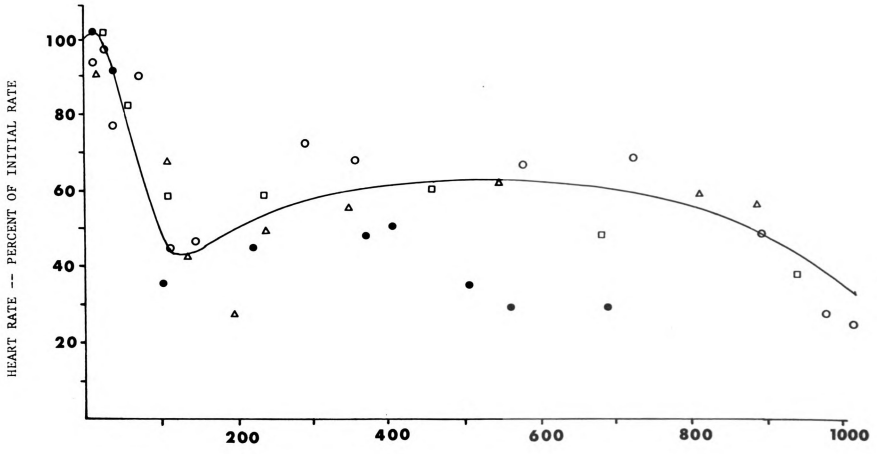
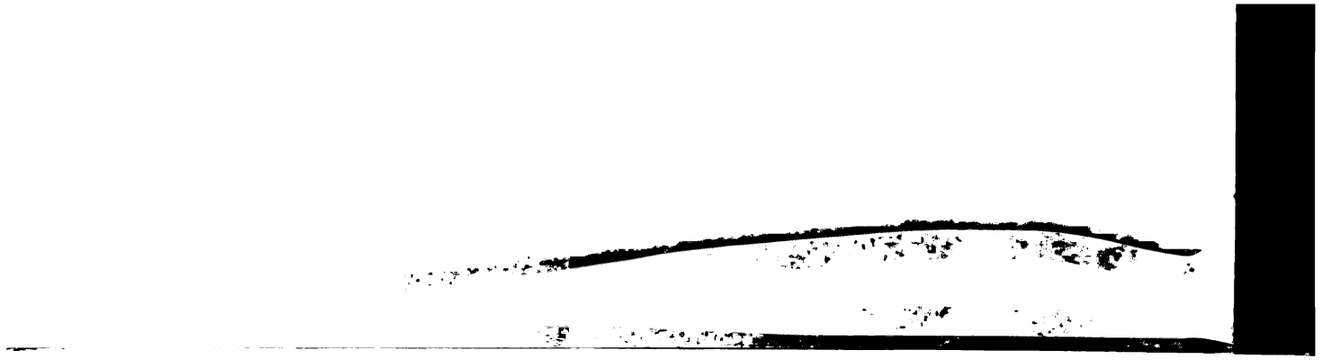

HEART RATE -- PERCENT OF INITIAL RATE
100
80
60
40
20
200
400
600
800
1000
AMOUNT OF CYANIDE PASSED OVER GILLS -- µg KCN/g OF FISH

Figure 19



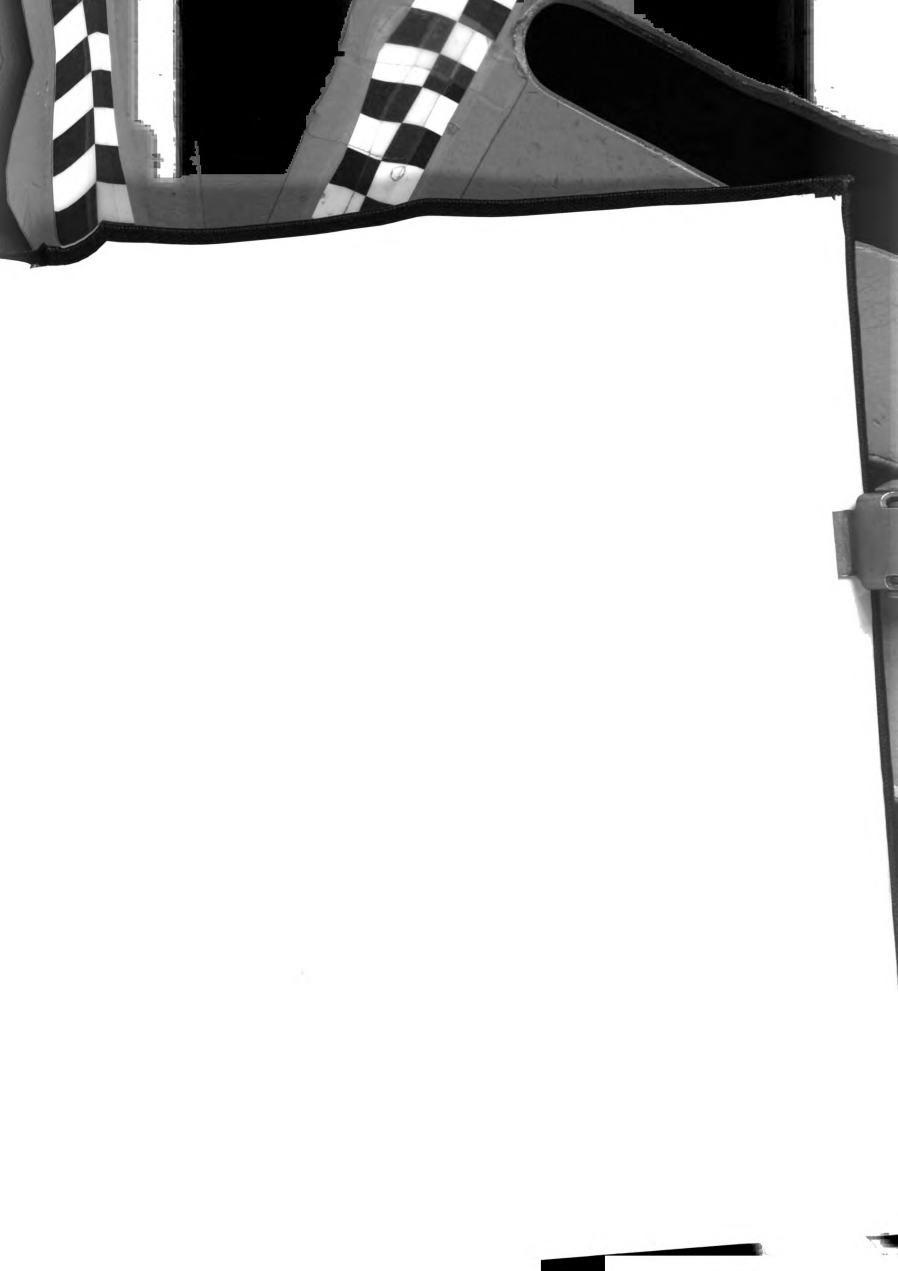


Figure 20.--Heart rate effects during exposure of fish preparations to 0.3 ppm KCN. Abscissa represents the amount of KCN passed over the gills per gram of fish. The ordinate represents the heart rate expressed as a percent of its initial value. Different symbols represent different fish.

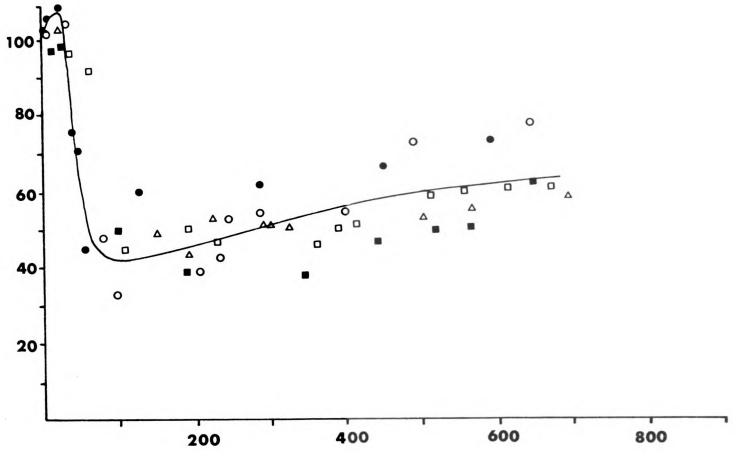
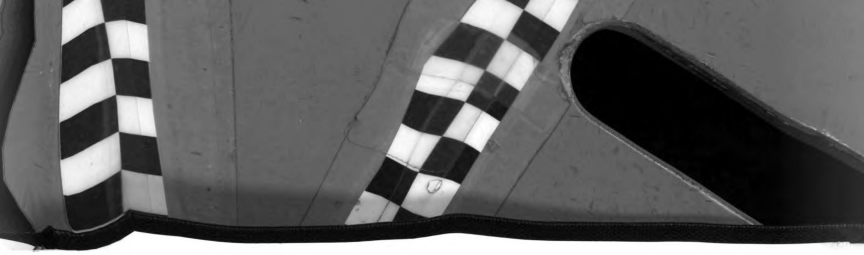

HEART RATE -- PERCENT OF INITIAL RATE
100
80
60
40
20
200
400
600
800
AMOUNT OF CYANIDE PASSED OVER GILLS -- µg KCN/g OF FISH

Figure 20



other two fish decreased to 30% of original levels after 1000 μg/g. An increasing heart rate after an initial drop was also observed in fish that were exposed to 0.3 ppm KCN. This increase was very gradual, reaching the 60% level of heart activity after an accumulated exposure of 700 μg/g.

The wave forms observed from the ECG recordings taken during these experiments demonstrated changes that were nearly identical to those observed in the hypoxia experiments. These included: reduction in the amplitude of the QRS complex; change in the QRS complex from a monophasic wave form to a biphasic wave form; increased length of the P-R interval; and increased size of the T-wave. The slight increase in heart rate at the beginning of the cyanide experiments resulted from a slight increase in the rate of the pacemaker as evidenced by the shorter intervals between successive P-waves. None of the other measurements conducted on the ECG demonstrated any changes during this period. The changes in the ECG described above began during the rapidly falling phase of the heart rate. The decrease in rate resulted mostly from missed beats but the pacemaker discharge rate also decreased slightly. After the initial drop in rate the heart became much more rhythmic, beating at regular intervals. This accounted for the rate increase observed after the large initial drop. The rate never reached its pretreatment value because of the substantially decreased pacemaker rhythm. The eventual decrease in heart rate resulted from a combination of reduced pacemaker rhythm and missed beats.

In the recovery experiments after exposure of fish preparations to 4 ppm KCN the heart rate in each fish approached its pretreatment value within about 40 minutes. Recovery of the ECG waves to their normal shape occurred within 20 minutes.

DISCUSSION

DDT Experiments

The finding that DDT exposure did not change any electrophysiological measurements conducted on the lateral line was quite interesting. These were unexpected results considering the profound effects of this chemical on the nervous systems of other animals. The first explanation which came to mind concerning these "negative results" was that the insecticide was not actually getting to the lateral-line tissue in large enough amounts to elicit changes. This seemed to be a valid consideration until a few revealing observations were made later in the study.

The most direct approach to the question of whether or not DDT was able to get to the lateral-line nerve was to expose fish to the chemical and then measure the amount of DDT in the lateral-line nerve. This was done on three fish. Two fish were given intravenous injections of 2 mg DDT and the third fish was exposed to 15 ppm DDT in the water for six hours. A fourth fish, not exposed to DDT, was used as a control. The lateral-line nerve from the fish exposed to 15 ppm DDT in the water for six hours contained a DDT concentration of about 3 ppm. The lateral-line nerves from the

DISCUSSION

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two fish receiving the intravenous injection contained 2.2 and 2.5 ppm DDT two hours after injection. No DDT was found in lateral-line nerves from the control fish.

The blood concentration of DDT was measured in one fish at various times after injection. Samples of blood were taken from a second canula placed caudal (upstream) to the injection canula. The initial blood concentration of DDT was calculated to be roughly 400 ppm. After 20 minutes the measured blood concentration of DDT was only 15 ppm; after one hour, 8 ppm; and after two hours, 5 ppm. This evidence, although obtained from a single fish, demonstrated that the injection technique was successful in introducing the insecticide into the systemic circulation. Disappearance of the DDT from the blood indicates that it is presumably entering other body tissues, as evidenced by the appearance of DDT in the lateral-line nerve from the same fish.

Whether or not DDT was able to get to the lateral line is probably irrelevant when one considers the effects of DDT on the whole animal. As was shown earlier, injections of DDT did elicit the characteristic poisoning symptoms and at the same time no changes could be identified in the discharge of neural activity from the lateral line. This observation strongly indicates that the lateral line plays a minor role in the symptoms characteristic of acute DDT poisoning. Injections of very large amounts of DDT might well affect the lateral-line system, however, from the evidence given above

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above it is likely that if these effects did occur they would be of minor importance to the fish. By this time the fish would already have been suffering the terminal effects of the insecticide by its action on another part of the body.

Hypoxia Experiments

Perhaps the most significant finding in hypoxia experiments concerns heart function. Briefly restating the results of these experiments I found that exposure of fish to oxygen partial pressures of 40 and 50 mm Hg for three hours reduced the heart rate by 20% and exposure to 30 mm Hg for the same length of time reduced the rate by approximately 40%. After two hours of exposure the heart rate was at the 80% level at all three of the reduced oxygen levels.

Randall and Smith (1967) describe different results. They demonstrate that when rainbow trout were exposed to hypoxic conditions the heart rate rapidly decreased at oxygen partial pressures below 80 mm Hg. For example at a pO_2 of 50 mm Hg the heart rate decreased 50% and at 40 mm Hg it decreased approximately 60%. These values are well below those observed in my experiments.

The reason for this apparent discrepancy is probably due to the fact that Randall and Smith did not immobilize their fish with curare as I did. Curare is a powerful acetylcholine antagonist and can block impulse transmission across synapses utilizing acetylcholine as a chemical mediator.

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Randall and Smith have shown that the heart of the rainbow trout is innervated by cholinergic inhibitory fibers from the vagus nerve. A cholinergic nerve is one which releases acetylcholine at a synapse or neuromuscular-junction. These inhibitory fibers are responsible for causing reflex inhibition of heart activity during hypoxic conditions. Because curare is an antagonist of acetylcholine it is suspected that the heart rate was partially prevented from slowing down during the hypoxic conditions of my experiments because inhibitory impulses to the heart were blocked by the action of curare. Randall and Smith have shown that atropine (another acetylcholine antagonist) has a similar effect.

Lateral-line function did not appear to be affected by the hypoxic conditions of the experiments. It appears as though the lateral-line nerve is able to maintain normal function as long as blood circulation continues.

Asphyxiation Experiments

The rapid decline in normal neural activity of the lateral-line system under conditions of asphyxia appears to be closely related to heart function. During asphyxia the heart rate rapidly decreased and in about ten minutes the heart had nearly stopped beating. It is probably safe to assume that blood circulation was severely reduced and perhaps even lacking in some tissues. Ischemic conditions (lack of blood flow) not only result in oxygen lack at the tissue level

but also cause a build-up of metabolites because there is no means for their removal.

The accumulation of metabolic products in the vicinity of the nerve is probably responsible for decreased neural activity in the lateral-line nerve during asphyxia. Metabolites which can occur include potassium, lactic acid, carbon dioxide, and perhaps others. Hashimura and Wright (1958) state that progressive anoxia in frog nerves results in an accumulation of extracellular potassium. It has been indicated by Adrian (1956) that excessive amounts of extracellular potassium will cause hyperpolarization of neural membranes and eventually render the nerves functionless. It is possible that potassium will also accumulate extracellularly in trout under the conditions of asphyxia.

Holeton and Randall (1967b) found that lactic acid will accumulate extracellularly in trout exposed to hypoxic conditions, presumably due to increased anaerobic metabolism. It is very likely that this occurred in my asphyxiation experiments. Carbon dioxide is also known to accumulate under anoxic conditions. Both lactic acid and carbon dioxide will depress nerve function. The resulting decrease in pH raises the surface tension causing the nerve cell membrane to increase its resistance to water soluble ions (Fox and Kenmore, 1967). Sodium and potassium passage across the membrane is hindered and the net effect is hyperpolarization of the nerve membrane. Hyperpolarization, as was demonstrated

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The accumulation of metabolic products in the vicinity of the nerve is probably responsible for decreased neural activity in the lateral-line nerve during hypoxia. Decreases which can occur include increases in lactic acid, carbon dioxide, and perhaps others. Hopkins and Wrabe (1970) state that prolonged anoxia in lung tissue results in an accumulation of extracellular K+. It has been indicated by Adrian (1969) that excessive amounts of extracellular potassium will cause hyperpolarization of neural membrane and eventually render the nerve inoperative. It is possible that potassium will also accumulate extracellularly in tissue under the conditions of asphyxia.

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in the case of excessive amounts of extracellular potassium, will depress nerve function. Nerve depression is brought about by increasing its threshold of excitability.

Asphyxiation caused by discontinuing the flow of oxygenated water across the gills led to conditions which were favorable to accumulation of metabolites in the vicinity of the lateral-line nerve. It is the action of metabolites on the nerve which is believed to have caused the depressed activity in spontaneous and evoked neural activity from the lateral-line nerve.

During hypoxia experiments the heart continued to function throughout the test period hence, a build-up of metabolic products in the vicinity of the lateral-line nerve was probably prevented. This would explain the inability of the hypoxic stress to depress neural activity in these experiments.

It was interesting to note that the evoked activity from the lateral-line nerve was more resistant to conditions of asphyxiation than was the spontaneous activity. It is tempting to explain this phenomenon based on a theory that different fibers are responsible for conducting evoked and spontaneous nerve impulses. If fibers responsible for carrying the evoked nerve impulses were less susceptible to ischemic conditions than were the fibers carrying spontaneous impulses it follows that the spontaneous activity would be first to disappear. I have no evidence to support this however.

It is more likely that both spontaneous and evoked impulses are carried in the same fibers. This is evidenced by the findings of Sand (1937) who demonstrated that the frequency of spontaneous discharge in single fibers increased during stimulation. His work was conducted on one of the lateral-line canal organs in the head of the skate. When one records activity from a nerve trunk containing hundreds of individual fibers a simultaneous increase in firing frequency of a number of these fibers will appear as a burst of evoked activity. The evoked activity discussed throughout this thesis is probably a summated burst of this type and is not the result of newly recruited fibers.

Ischemic conditions resulting from asphyxiation will cause hyperpolarization of neural membranes. In the lateral-line nerve the hyperpolarization presumably affects all of the fibers equally. It is possible that events responsible for initiating evoked and spontaneous activity differ in their ability to elicit a nerve impulse. If the threshold of the nerve is raised by hyperpolarization the "stronger" of the two events would be more able to initiate an impulse. I maintain that the evoked activity is more resistant to ischemic conditions than the spontaneous activity for the above reason.

The biochemical and physiological basis for the events which cause spontaneous and evoked activity have been discussed in detail by Harris and Flock (1967). Their model is based

on results of experiments conducted on the Asian toad, Xenopus laevis, and probably applies to fish as well. Their model is this: An afferent fiber of the lateral-line nerve makes synaptic contact with the base of a sensory hair cell. The top of the hair cell is equipped with the cupula which moves during mechanical stimulation. Next to the area of synaptic contact with the afferent fiber are numerous synaptic visicles located within the hair cell. Each vesicle contains a discrete quantum of chemical transmitter. When the transmitter contacts the postsynaptic membrane (afferent nerve membrane adjacent to the sensory hair cell) after being released into the synaptic cleft (space between hair cell and nerve terminal) the postsynaptic membrane is depolarized. Many efferent terminals from adjacent hair cells unite to form a single fiber. If enough of the terminals are depolarized by simultaneous release of transmitter from the hair cells the resulting waves of depolarization (excitatory postsynaptic potentials, EPSP's) will summate and exceed a threshold in the region where the fibers join. This is the region where the regenerative action potential (nerve impulse) begins. Depolarization of this region beyond a certain threshold value will initiate events leading to an "all or nothing" action potential which then is conducted to the central nervous system.

Spontaneous neural activity is presumed to result from random leakage of transmitter substance from the hair cell

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Xenopus laevis, and probably applies to frog as well.
Their model is this: An afferent fiber of the Interstitial
nerve makes synaptic contact with the base of a Basilar Hair
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which moves during mechanical stimulation. Next to the site
of synaptic contact with the afferent fiber are numerous
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the transmitter contacts the cell membrane (afferent
nerve membrane adjacent to the hair cell) it is released into the synaptic cleft and nerve terminal the membrane is depolarized. Many efferent terminals from adjacent hair cells unite to form a single fiber. If enough of the terminals are depolarized by simultaneous release of transmitter from the hair cells the resulting waves of depolarization (excitatory postsynaptic potentials, EPSP's) will summate and exceed a threshold in the region where the fibers join. This is the region where the regenerative action potential (nerve impulse) begins. Depolarization of this region beyond a certain threshold value will initiate events leading to an "all or nothing" action potential which then is conducted to the central nervous system.

Spontaneous neural activity is presumed to result from random leakage of transmitter substance from the hair cell.

into the synaptic cleft. When enough quanta are simultaneously released from different hair cells, the EPSP in the regenerative region will exceed threshold and a spontaneous impulse will ensue. An evoked action potential is not the result of random leakage of the transmitter. Upon stimulating the sensory cells in a given region of the lateral line, the cells will simultaneously release quanta of transmitter substance giving rise to a large EPSP and eventually an action potential in the nerve. These events do not depend on the probability of several hair cells releasing transmitter quanta at the same time as was true for spontaneous impulses.

As the nerve terminals encounter conditions of ischemia the nerve membranes begin to hyperpolarize due to the action of the accumulating metabolites discussed earlier. This progressive hyperpolarization increases the threshold of excitability in the regenerative region of the efferent fiber. As a result, more and more hair cells must simultaneously release transmitter substance in order to develop an EPSP which is large enough to exceed the rising threshold. The EPSP's developed from random leakage of transmitter material are generally much smaller than the EPSP's created by the simultaneous evoked release of the transmitter after stimulation. Thus, as the threshold rises, spontaneous nerve impulses will disappear before the evoked impulses. During recovery from ischemic conditions, the evoked impulses will be first to appear followed by the spontaneous impulses.

into the synaptic cleft. When enough quanta are simultaneous-ly released from different hair cells, the EPSP in the regen-erative region will exceed threshold and a propagated impulse will ensue. An evoked action potential is not the result of random leakage of the transmitter. Instead [illegible] the sensory cells in a given [illegible] the cells will simultaneously [illegible] quanta [illegible] stance giving rise to [illegible] potential in the nerve. [illegible] probability of several [illegible] quanta at the same time as was [illegible]

As the nerve terminals [illegible] ischemia the nerve membranes begin [illegible] due to the action of the accumulating metabolites discussed earlier. This pro-gressive hyperpolarization increases the threshold of excit-ability in the regenerative region of the afferent fiber. As a result, more and more hair cells must simultaneously release transmitter substance in order to develop an EPSP which is large enough to exceed the rising threshold. The EPSP's developed from random leakage of transmitter material are generally much smaller than the EPSP's created by the simultaneous evoked release of the transmitter after stimula-tion. Thus, as the threshold rises spontaneous nerve im-pulses will disappear before the evoked impulses. During recovery from ischemic conditions, the evoked impulses will be first to appear followed by the spontaneous impulses.

The observations made concerning the disappearance and reappearance of evoked and spontaneous neural activity in my asphyxiation experiments agree very well with the model described above.

The rapid decrease in heart rate which occurred during the asphyxiation experiments probably resulted from the direct effects of anoxia upon myocardial tissue rather than reflex inhibition mediated by inhibitory neurons to the heart. This was evidenced by the observation that gradual slowing of the basic heart rate did not occur. Rather, the rhytum was progressively interrupted by periods of missed beats and the wave patterns on the ECG became very erratic and weak. Reflex neural inhibition would cause a rate decrease in the absence of such drastic changes in the ECG.

Cyanide Experiments

All three concentrations of cyanide used in these experiments (0.3, 1, and 4 ppm KCN) caused reduction in heart rate, spontaneous activity, and evoked activity from the lateral-line nerve.

Doudoroff et al. (1956), demonstrate that molecular HCN is responsible for the toxic effects of cyanide compounds and not the ionized CN^- form. The cyanide ion is only found in minute quantities after KCN is dissolved in acid, neutral, or slightly alkaline water. At a pH 8.0, 93% of the KCN exists in the undissociated HCN form (Wuhrmann and Woker,

1948) and at lower pH's this percent increases. The pH of the water used to perfuse my fish preparations was less than 8,0, thus it can be assumed that nearly all of the potassium cyanide was in the form of the undissociated HCN molecule.

In considering both the lateral-line nerve and the heart responses to cyanide it was evident that the higher concentrations of cyanide would elicit changes faster than the lower concentrations. From this observation it was surmised that the response time was a function of the accumulated amount of cyanide which had passed over the gills of the fish preparation. It was also observed that smaller fish would react quicker to the cyanide than would the larger fish at a given concentration. This indicated that the weight of the fish should be taken into consideration when comparing cyanide effects between different fish. For these two reasons the magnitude of the physiological responses observed in the cyanide experiments was plotted against the amount of cyanide which passed over the gills per unit live weight of fish. When the data were plotted in this manner (Figures 13 through 18) the position of a major physiological change would usually occur at the same position on the abscissa. This was largely true for heart rate data. For example, the rapidly decreasing phase of the heart rate occurred when approximately 50 μg of KCN per gram of fish has passed over the gills (Figures 16, 17, and 18). Lateral-line responses did not coincide as well, however.

Decreases in spontaneous and evoked activity from the lateral line were found to be greater at the higher cyanide concentrations, even though the same amount of cyanide had passed over the gills per unit weight of fish. This might be explained by an excretion or detoxifying process if the following assumptions are made: (1) The detoxification or excretion process proceeds at a relatively fixed rate below that of the cyanide uptake rate; and (2) The rate of uptake of cyanide across the gills is a relatively linear function of the cyanide concentration in the water.

If these assumptions are true, at lower cyanide concentrations a greater fraction of the cyanide crossing the gills would be detoxified or excreted than higher cyanide concentrations. The net effect of such a process is that higher concentrations of cyanide will "load" the fish with the poison faster than at lower concentrations. This could explain why higher concentrations of the chemical have a greater physiological effect than lower concentrations even though the accumulated dose per gram of fish was the same.

Lateral-line responses in cyanide experiments were similar to those observed in asphyxiation experiments. Evoked responses persisted long after the spontaneous activity had ceased, indicating that a hyperpolarization process in the nerves may have been occurring. Heart function was severely affected and ischemic conditions may well have been developing the lateral line. Anoxic conditions were also very likely.

Cyanide affects the electron transport system so that oxygen cannot be biochemically utilized by body tissues. This causes tissue anoxia even though the blood may be saturated with oxygen. Cyanide may also directly affect the nerve cell membrane (Schoepfle, 1963) but this was difficult to determine from these experiments.

An interesting finding was that fish preparations were able to recover from cyanide poisoning. Recovery was noted in the heart and also spontaneous and evoked activity from the lateral-line nerve. This suggests that a continuous detoxification or excretion process is in effect.

The effect of cyanide on the heart was puzzling. The rapid reduction in heart rate during the early exposure periods was probably due to anoxia of the myocardial tissue. The heart has a very high metabolic demand for oxygen and cyanide could easily block its ability to use it. Because the myocardium cannot tolerate an oxygen debt it is not surprising that it reacted so quickly to cyanide poisoning. What is puzzling is the temporary recovery of the rate following the rapid decrease. This finding was reproducible in nearly every fish tested. Perhaps this temporary rate recovery was not at the expense of an increased energy expenditure. For instance, the rate could be higher but at the same time the force of contraction could be less. This was perhaps evidenced by the observation that the voltages recorded from the ECG were lower during this period of temporary recovery.

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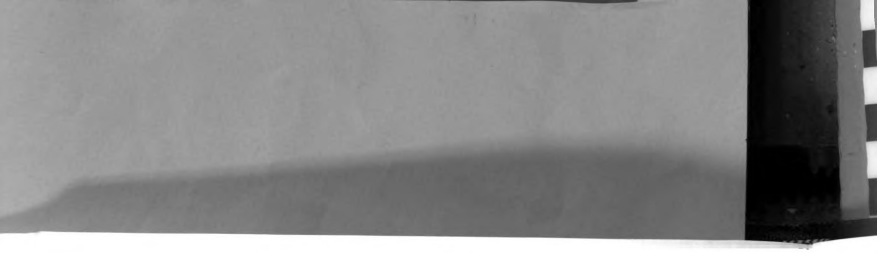

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