REGULATION OF BLOOD FLOW THROUGH THE ISOLATED-PERFUSED GILLS OF RAINBOW TROUT: EFFECTS OF VASOACTIVE AGENTS ON FUNCTIONAL SURFACE AREA

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ABSTRACT

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By

Harold L. Bergman

Published studies on gill anatomy and effects of vasoactive agents on vascular resistance in teleost gills have prompted speculation about the physiological significance of different blood flow paths through this organ. In this study the influx of ^{14}C-urea, a passively diffusing molecule, was used to indicate the relative functional respiratory surface area of isolated-perfused rainbow trout gills. Perfusion of vasoactive agents in these preparations significantly altered both ^{14}C-urea influx and branchial vascular resistance. An increase in norepinephrine perfusion from 10^{-9} to 10^{-5}M increased ^{14}C-urea influx 4.6-fold, while an epinephrine increase from 10^{-7} to 10^{-5}M caused a 5.6-fold increase in marker influx. Both catecholamines produced an overall decrease in branchial vascular resistance, but sometimes only after a transient increase. Either α or β adrenergic blockade halved the catecholamine effect on ^{14}C-urea influx while blocking the appropriate increase (α response) or

decrease (β response) in branchial vascular resistance. Perfusion of pharmacological α or β adrenergic stimulants produced effects which mimicked the β or α blocked catecholamine results, respectively. Acetylcholine, when increased from 10^{-8} to 10^{-6}M, decreased ^{14}C-urea influx to 1/5 of its control value, while causing a marked increase in branchial vascular resistance.

Data presented in this thesis supports the contention that gill functional respiratory surface area is controlled by both neural and hormonal mechanisms.

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To Annette, Jill and Peter

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INTRODUCTION

It is widely speculated that teleosts can regulate functional respiratory surface area by adjusting blood flow pattern through the gills. But the existence of some described blood pathways has been disputed, and the physiological mechanisms which regulate blood flow through different pathways remain unclear.

Evidence suggesting functional regulation of blood pathways includes the 5 to 10-fold increase in oxygen uptake reported for several species of fish during exercise (Saunders, 1962; Brett, 1964; Stevens and Randall, 1967a), and the negative Na^+ balance which accompanies increased oxygen uptake in freshwater rainbow trout (Wood and Randall, 1973). The significance of such a regulatory mechanism is that fish could 1) maximize gas exchange during periods of increased activity by perfusing most or all of the respiratory blood pathways, and 2) restrict the undesirable effects of salt and water movements by limiting blood flow to fewer respiratory paths during periods of rest.

The general anatomical features of gills from several teleost species have been described by Hughes and Grimstone (1965), Newstead (1967), and Morgan and Tovell (1973).

The anatomical surface area of each gill arch is greatly increased by its subdivision into two successively smaller units, the filaments and secondary lamellae. The somewhat flattened filaments extend out from the gill arch in two rows. These rows, or hemibranchs, can be extended out to intercept the water which flows through the gill slits on each side of the arch. Numerous platelike secondary lamellae are attached by their edges and extend out from the top and bottom faces of each filament. The secondary (respiratory) lamellae are the functional respiratory units. Basically, each lamella consists of two epithelial sheets which are held together by pillar cells. The lacunar spaces between the pillar cells are large enough to allow passage of red blood cells.

Histological studies have revealed similar circulatory anatomy in the common eel (Steen and Kruysse, 1964), coho salmon (Newstead, 1967) and rainbow trout (Richards and Fromm, 1969). In these species blood flow from the afferent filamental artery to the efferent filamental artery was reported to be through at least two pathways: 1) the flat lacunar secondary lamellae (respiratory pathway), and 2) a central sinus in the filament body (non-respiratory shunt pathway). Steen and Kruysse also observed an additional non-respiratory pathway in the eel, consisting of a direct connection between the afferent and efferent filamental arteries

at each filament tip. Respiratory blood flow through the secondary lamellae is thought to be further subdivided between a preferential path around the free margin of each lamella and the lacunar paths amongst the pillar cells (Hughes and Grimstone, 1964; Newstead, 1967; Skidmore and Tovell, 1972).

In a recent report (Morgan and Tovell, 1973) the non-respiratory filamental sinus pathway in the rainbow trout was not confirmed. Filamental sinuses were observed but were assigned a lymphatic function only, since no red blood cells and no openings from sinuses to filamental arteries were seen. As an alternative to the filamental sinus shunt pathway, the authors supported a recruitment mechanism proposed earlier by Hughes (1972). This proposal suggests that during periods of low oxygen demand respiratory blood flow is directed to the secondary lamellae on the proximal end of the filaments only. During periods of heightened activity when gas exchange demands are increased, additional lamellae toward the distal filament tip are successively recruited to increase respiratory blood flow.

Whether respiratory blood flow is modulated by use of filamental shunts or lamellar recruitment, some mechanism must function to adjust vascular resistances in the appropriate pathways. Hence, a number of workers have sought histological evidence for the presence of vascular smooth muscle in gills.

Morgan and Tovell (1973) reported the presence of a continuous layer of muscle surrounding the endothelial layer of afferent and efferent lamellar arterioles, corroborating observations by Richards and Fromm (1969). Neither study revealed muscular elements associated with the filamental sinus. In an earlier study, Newstead (1967) found no muscle tissue in the region of afferent or efferent lamellar arterioles, but did report what appeared to be muscle filaments in the pillar cells. He, therefore, attributed a contractile function to the pillar cells, supporting a view held by Hughes and Grimstone (1965). This hypothesis was further strengthened by the demonstration that these pillar cell fibers were actomyosin-like proteins (Bettex-Galland and Hughes, 1972), and that the pillar cells were innervated (Gilloteaux, 1969).

Although physiological regulation of gill blood pathways is unclear, it is generally thought to involve hormonal and/or neural inhibition and stimulation of vascular smooth muscle. The approach taken by fish physiologists to elucidate these regulatory mechanisms has, of course, been influenced by knowledge about similar systems in higher vertebrates. Vascular resistance in mammals is controlled by three main chemical transmitters: epinephrine (adrenaline), norepinephrine (noradrenaline), and acetylcholine. Epinephrine and norepinephrine, catecholamines released from the adrenal medulla and peripheral chromaffin cells, increase resistance

in some vascular beds and decrease resistance in others. Ahlquist (1948) postulated that the opposite responses resulted from presence of two different receptors for catecholamines. He suggested that α-adrenergic receptors mediated excitory responses (vasoconstriction), while β-adrenergic receptors mediated inhibitory responses (vasodilation). The neurohumoral transmitters found in higher vertebrates include acetylcholine and, again, norepinephrine. Norepinephrine is the transmitter released at postganglionic sympathetic nerve endings, while acetylcholine is found at all preganglionic autonomic nerve endings and all postganglionic parasympathetic nerve endings. Acetylcholine is also the transmitter at motor nerve synapses with skeletal muscle. Two distinct actions have been attributed to acetylcholine (Dale, 1914) and have also been explained by two different receptor types. The receptors at postganglionic parasympathetic nerve ends could be blocked by muscarine or atropine and were termed muscarinic receptors, while receptors at autonomic ganglia and skeletal neuromuscular junctions could be blocked by nicotine and were called nicotinic receptors.

Physiologists have assumed that regulation of vascular resistance is similar in fish and higher vertebrates, and they have tested the effects of cholinergic and adrenergic drugs and hormones (as well as many other chemicals) on branchial resistance. Some studies have demonstrated changes in

branchial resistance by monitoring dorsal and ventral aortic blood pressures of intact fish during injection of various vasoactive agents (Chester Jones, et al., 1967; Reite, 1969). In studies where only dorsal or ventral aortic blood pressure changes were reported (Mott, 1951; Randall and Stevens, 1967), interpretations about branchial vascular resistance are difficult. Undoubtedly, the injected vasoactive agents affected cardiac output and systemic vascular resistance as well as vascular resistance in the gill, and the pressure responses would reflect a combination of all these effects. Results from experiments with isolated-perfused gills are much more easily interpreted. Vasoactive agent effects on branchial resistance have been detected by observing changes in perfusion pressure (Reite, 1969) or flow rate (Keys and Bateman, 1932, Östlund and Fänge, 1962; Rankin and Maetz, 1971; Randall et al., 1972) in these isolated preparations. The above studies revealed that branchial vascular resistance was reduced by catecholamines and increased by acetylcholine. Using two different methods, Steen and Kruysse (1964) and Richards and Fromm (1969) observed that acetylcholine increased filamental sinus blood flow and epinephrine increased secondary lamellar blood flow. Steen and Kruysse also reported increased oxygen uptake after injection of epinephrine in vivo.

The effects of epinephrine and norepinephrine, coupled with a report that circulating concentrations of these catecholamines are elevated during exercise (Nakano and Tomlinson, 1967), support the hypothesis that blood flow in the gills is at least partly under hormonal control. The reports that norepinephrine and acetylcholine affect branchial vascular resistance also suggest the possibility of autonomic nervous control of blood flow pattern through the gill. The existence of sympathetic and parasympathetic nerve trunks to the gill (Nicol, 1952), and histological demonstration of nerve endings on the pillar cells and afferent and efferent arteries (Gilloteaux, 1969) add impetus to the neural control argument. There have been no reports, however, showing a direct effect of autonomic nerve stimulation on the resistance of any vascular bed in fish (Campbell, 1970).

Existence of a mechanism for adjusting blood flow pattern to regulate functional surface area of the gill has been supported by the anatomical and physiological evidence given above. The anatomical evidence is somewhat tenuous, however, because the proposed filamental sinus pathway has not been confirmed in recent observations, and the recruitment mechanism is still quite speculative with little evidence to support it. The physiological evidence taken as a whole offers the strongest support for the functional surface area hypothesis.

However, alternate explanations can be offered for each physiological observation when taken alone. Increased oxygen uptake during exercise or following epinephrine injection might be accounted for by increased cardiac output and ventilation volume; negative Na^+ balance during exercise could result from altered kidney function; vasoactive agent-induced changes in branchial vascular resistance reflect only the sum of changes in flow resistances through the gill and provide no information about resistance changes in the pathways that have been proposed. Therefore, no single piece of physiological evidence has been strong enough by itself to confirm the hypothesis. A critical series of experiments is needed to provide strong evidence that either confirms or denys the functional surface area hypothesis. Such evidence could be obtained by using isolated-perfused gills to simultaneously measure the effects of vasoactive agents on branchial vascular resistance and functional surface area.

Aside from the basic research interests in this problem, there is a growing need to understand gill structure and function for a more practical reason. The vital functions of fish gills are known to be adversely affected by various aquatic pollutants. We cannot hope to understand or evaluate these abnormal physiological conditions, if we incompletely comprehend gill function in healthy fish.

The objectives of the present study were to 1) perfect an isolated-perfused gill technique which could be used for 3 to 4 hour-long experiments under conditions resembling, as closely as possible, those found *in vivo*, 2) confirm or deny regulation of functional surface area of the teleost gill, and 3) determine the nature of physiological control mechanisms which could be responsible for regulating functional surface area.

In this study the influx of ^{14}C-urea was used as a relative measure of gill functional surface area. Urea is not metabolized in rainbow trout gill tissue (K. R. Olson, personal communication) and is not known to be actively transported by teleost gills. The method is based on the assumption that diffusional influx of ^{14}C-urea is limited principally by the extent of secondary lamellae perfusion--i.e., the functional surface area of the gill available for diffusional influx of the marker.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout (Salmo gairdneri), 200 to 300 grams, were obtained from the Michigan Department of Natural Resources hatchery in Grayling, Michigan. At Michigan State University, the fish were held in flowing dechlorinated tap water at 10-12°C under a controlled photoperiod of 16 hours light per day. Animals were fed a maintenance diet of EWOS 159 salmon pellets (Astra-Ewos, Södertälje, Sweden), but were starved one week prior to use. All experiments were conducted between January and December, 1973.

Experimental Apparatus

Two gill arches could be perfused simultaneously in the apparatus shown in Figures 1 and 2. Perfusion solutions were delivered from polyethylene bottles fitted with glass tubes glued into the bottle bases. Silicone rubber tubing (1.5 mm i.d., 3.5 mm o.d.) coupled the bottles to 4-way stopcocks which allowed perfusing solutions to be conveniently switched during an experiment without introducing air bubbles into the system. A multichannel peristaltic pump (Brinkman Instruments, Inc., Westbury, N.Y.) pumped solutions through the gills.

Figure 1.--Diagram of the gill perfusion apparatus.

Solid lines represent perfusion channels and connections; dashed lines represent electronic connections.
(A) Gill arch in "A" perfusion channel;
(B) Gill arch in "B" perfusion channel;
(D) Photoelectric drop counters;
(FC) Fraction collector;
(FCD) Fraction collector drop count accumulator;
(G) Grass polygraph;
(PC) Pressure calibration manometer;
(PP) Peristaltic pump;
(PS) Perfusing solutions;
(R) Grass polygraph record reading from top to bottom: perfusion pressure in "B" channel, fraction collector turns, perfusion pressure in "A" channel, drops in "A" channel;
(S) 4-way stopcocks;
(T) Pressure transducers.

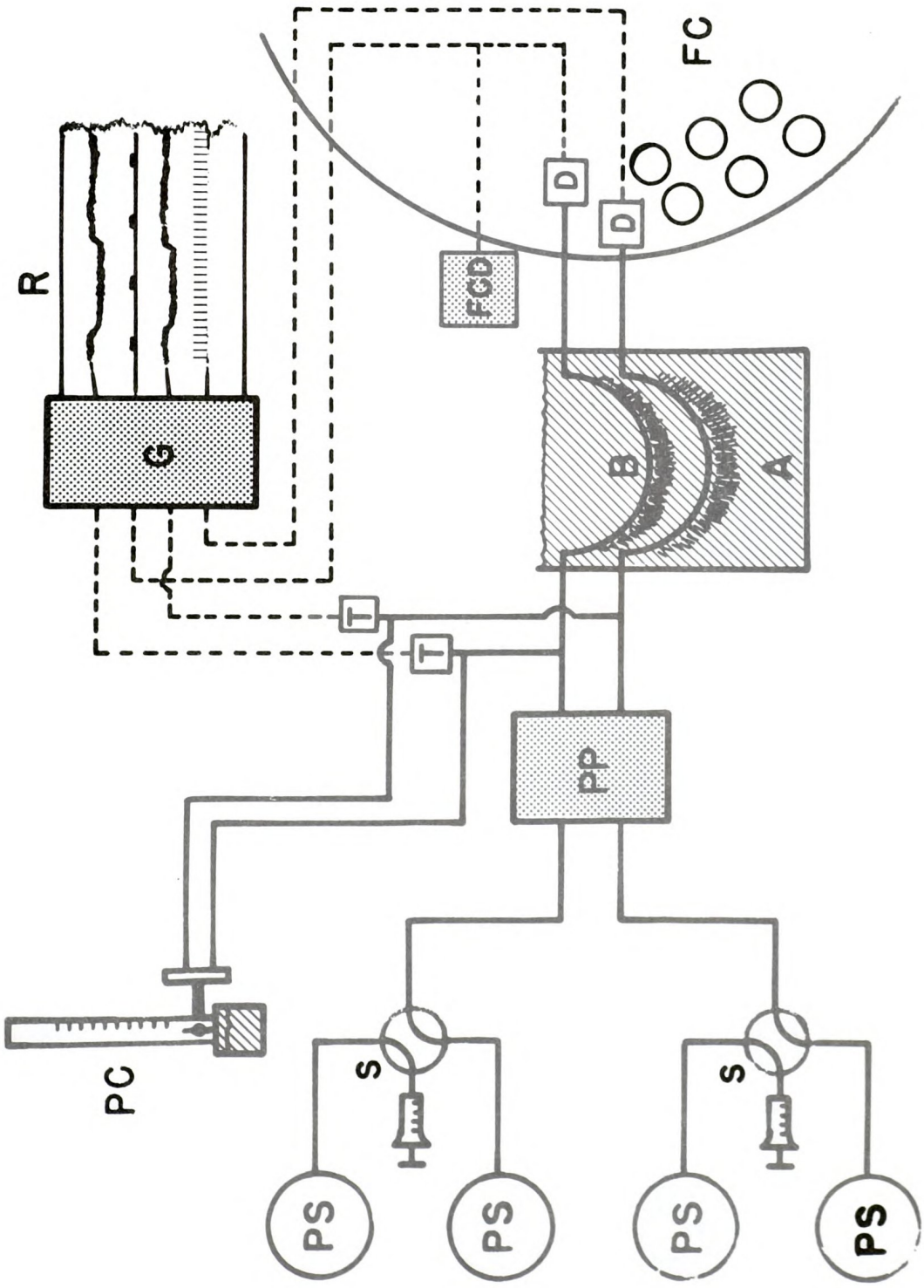

Figure 1

Figure 2.--Photograph of the gill perfusion apparatus.

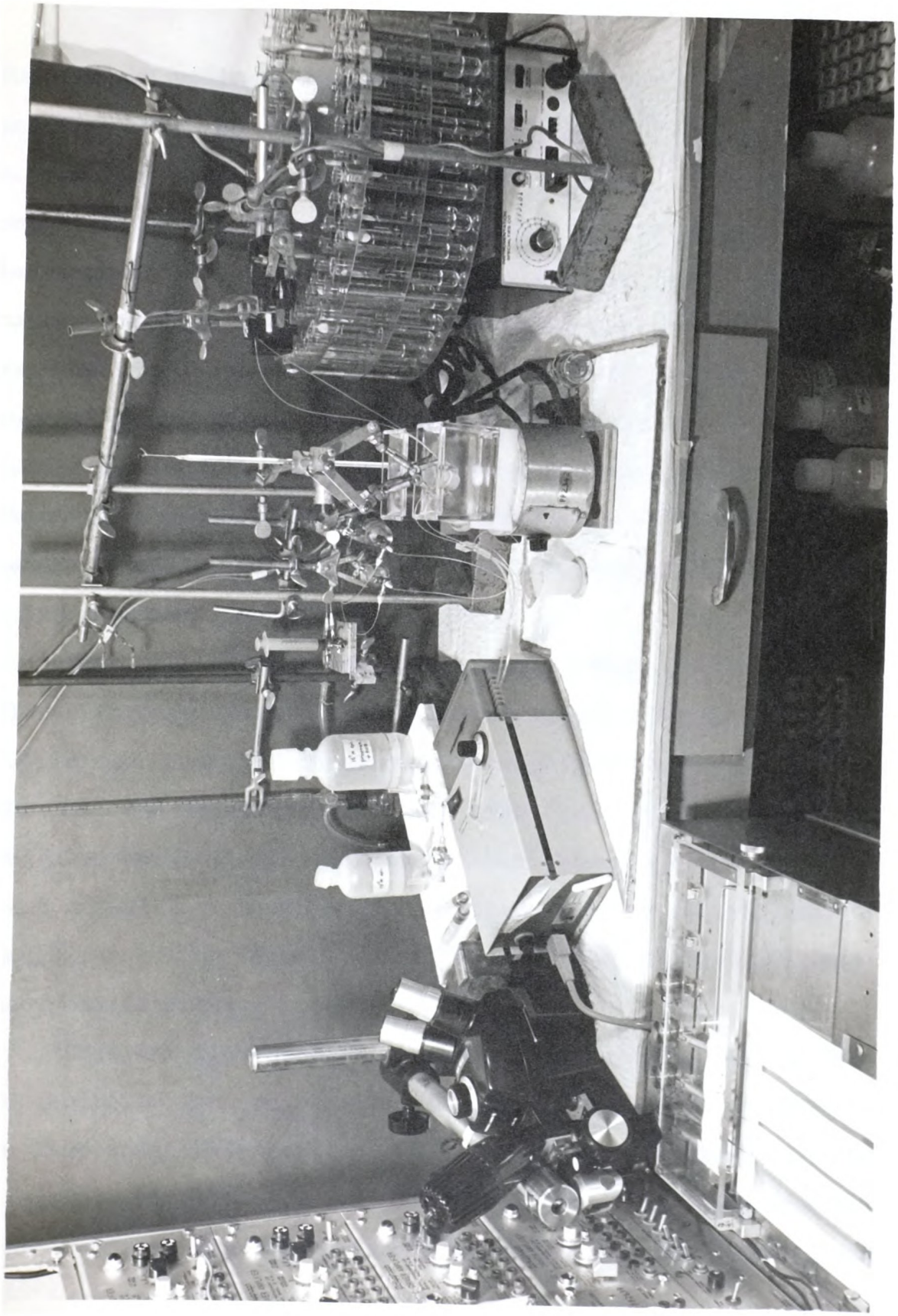

Figure 2

Pump output could be regulated over a wide range, but in practice only two pump settings were used: either 0.25 or 0.50 ml per minute. The lower flow rate was used during cannulation, but before an experiment began the flow was increased to the faster rate. Since the same size pump tubing was used for both perfusion channels, the flow rate was identical, or very nearly so, to each gill arch. Of the several types of peristaltic pump tubing which were tried, Tygon (0.8 mm i.d., 2.4 mm o.d.) gave the most consistent flow and wear characteristics. To maintain calibrated flow rates, however, the pump tubing was replaced after every third experiment.

From the pump to each gill arch, perfusion solutions flowed through 10 cm silicone rubber tubing (1.0 mm i.d., 3.0 mm o.d.), a "T" connector, 10 cm polyethylene tubing (PE 60), and an afferent cannula made from a dulled, cut-off 20 gauge needle. The silicone rubber tubing between the pump and gill arch dampened the pulsatile flow in a manner analogous to the "Windkessel effect" of the conus arteriosus and ventral aorta.

Efferent flow was through a 20 gauge cannula, 32 cm PE 60 tubing, a drop counter assembly, and into sample vials in an Isco model 563 fraction collector (Instrumentation Specialties Co., Lincoln, Neb.). Different drop counter assemblies were used for the two perfusion channels. In the "A" channel,

a Grass model DCA-1 drop counter adaptor (Grass Instrument Co., Quincy, Mass.) was modified by gluing a 2 cm square acrylic plastic block to the top of the adaptor. Holes had been drilled to the center of the block from two adjacent faces so that flow entered a side face of the block and left through the bottom hole which communicated with the hole in the Grass drop counter adaptor. Tubes made from syringe needles were glued into and protruded from the holes in the side (16 gauge tube) and bottom (20 gauge tube) faces of the plastic block. The efferent polyethylene tubing could be firmly inserted into the side face tube and the bottom face tube facilitated delivery of uniformly sized drops. The drops then fell through the light beam of a Grass PTT1 photoelectric transducer. After stepwise amplification by a Grass 5P1K preamp and a Grass 5E driver amp, the transducer signal drove the recorder pen on one channel of a Grass model 5D polygraph. Each pen deflection represented one drop.

In the "B" perfusion channel, the drop counter assembly consisted of another drilled plastic block which was clamped in place above an Isco model 600 photoelectric drop counter. The drop counter signal then drove the fraction collector, which was set to turn the tube reel to the next sample after 64 drops had been collected. A signal from the fraction collector control unit was interfaced through a relay switch (120 volt AC, series 200 relay) to the signal marker input of

the Grass polygraph. Thus, each turn of the fraction collector, representing collection of 64 drops from the "B" perfusion channel, deflected a pen in the signal marker channel of the Grass polygraph.

During all experiments, the gill arches were immersed in baths which consisted of 350 ml of 1% non-nutrient Ringer solution (Appendix) in rectangular glass staining dishes. For ^{14}C-urea influx experiments, enough ^{14}C-urea (International Chemical and Nuclear Corp., Irving, Calif., specific activity equalled 56.3 mc/mM) was added to each bath to obtain about 10^4 dpm/ml. This represented a urea concentration of less than 1.0 μM/l. The baths were continuously stirred with Teflon coated magnetic stirring bars. A sheet of polyurethane foam, 2 cm thick, insulated the baths from heat produced by the magnetic stirring motors. The gill arches were suspended in the baths with gill holders which consisted of a ring stand and movable metal arms terminated with alligator clips. These clips were clamped onto the metal cannulae which protroded from each side of the gill arches. When the gill arches were properly placed in the baths, the vertical distance from the efferent cannula to the drop counter assembly was 20 cm. The pressure on the efferent side of the gill, therefore, was equivalent to 15 mm Hg,

and mimicked the systemic resistance normally present in the intact animal.

To monitor perfusion pressures, the "T" connectors between the pump and gill arches were connected with PE 60 tubing to Statham P23AC pressure transducers (Statham Transducers, Inc., Hato Rey, Puerto Rico). Since the perfusion pressures were measured between pump and gill, they were analogous to measurement of ventral aortic pressures in an intact fish. A pressure calibration manometer was also connected to the transducers with PE 60 tubing. The gill arches, manometer base, and transducers were all in the same horizontal plane to facilitate accurate calibration and measurement of pressures. After stepwise amplification by a Grass 5P1K preamp and a Grass 5E driver amp, each transducer signal drove a recorder pen in each of two channels of the Grass 5D polygraph.

The entire experimental apparatus was assembled in a cold room which was maintained at 11±2°C. Although the air temperature fluctuated through this 9 to 13°C cycle every 20 to 30 minutes, the perfusion and bath solution temperatures did not vary appreciably.

Gill Dissection and Cannulation

Fish were netted, stunned with a sharp blow to the cranium, and immediately decapitated just posterior to the

opercula. The head was placed in a beaker of non-nutrient Ringer solution (Appendix), which contained 2 USP units sodium heparin/ml (Organon, Inc., W. Orange, N.J.). The heart, which continued to beat, pumped this solution through the gills clearing them of blood. After about 15 minutes the head was removed from the beaker, and the ventricle of the heart was quickly cut by entering the pericardial sac from its exposed caudal end. This prevented air emboli from being pumped into the gills during the dissection procedure. The pectoral fins were cut off at this time, so that they would not interfere with later steps in the dissection. With the head held by Kelly forceps clamped to the upper jaw, an operculum was pulled out, one blade of heavy scissors was inserted anteriorly until the tip protruded from the mouth, and the lower jaw was cut (Figure 3A). The operculum was removed by cutting along a line from its dorsal connection to a point just dorsal to the eye and on through to the tip of the upper jaw (Figure 3B). Removal of the remaining operculum was similarly started by cutting the lower jaw, but before the dorsal opercular cut was made the anterodorsal part of the head was cut away just anterior to the origin of the first pair of gill arches in the roof of the buccal cavity (Figure 3C). The remaining operculum was then trimmed off along its dorsal connection.

Figure 3.--Gill arch dissection steps.
See text for discussion.

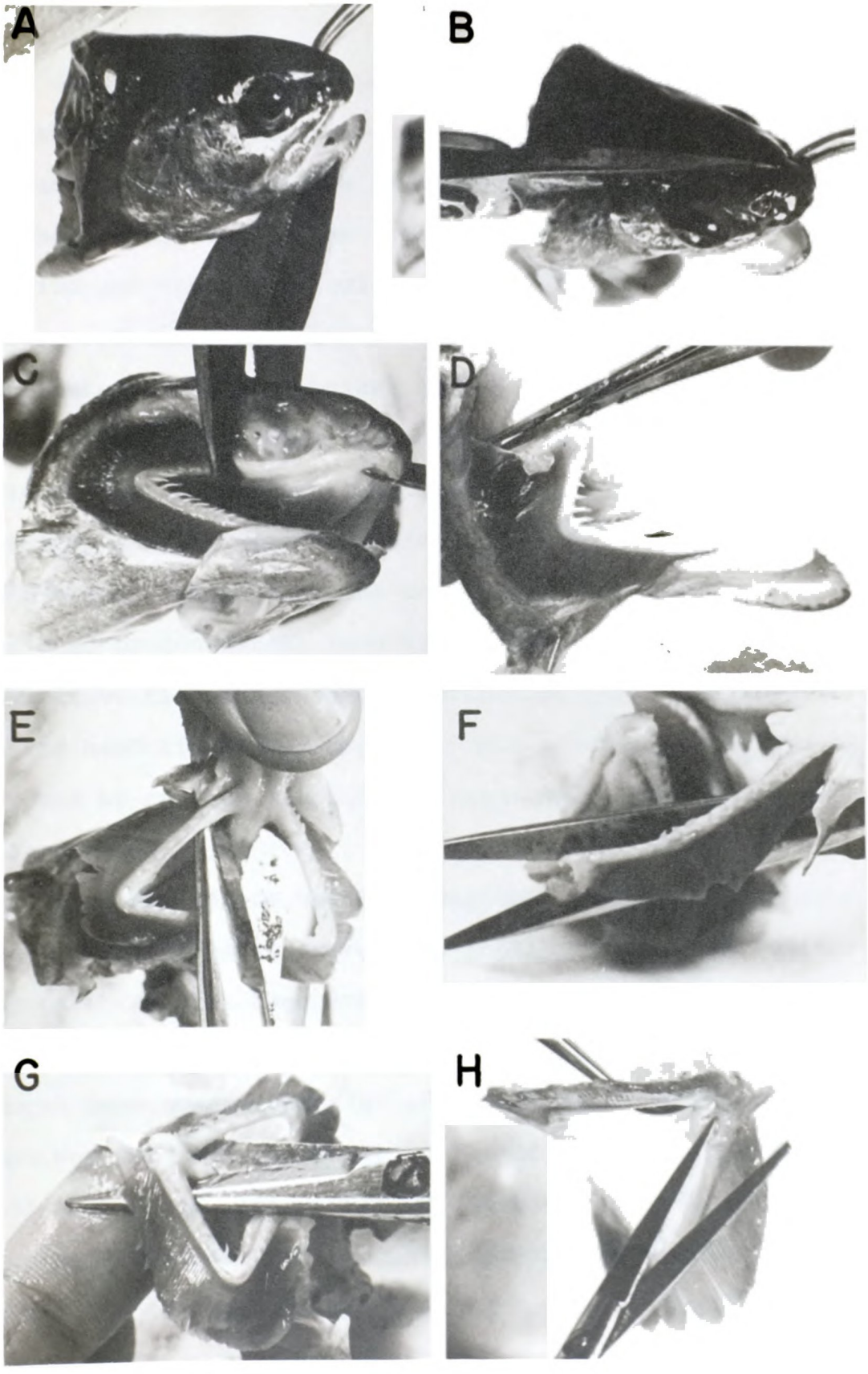

Figure 3

Grasping the exposed anterior and posterior ends of the spinal column between the thumb and forefinger, the cranium was trimmed away from the gill basket with fine tipped scissors. One tip of the scissors was inserted between the cranium and the dorsal origin of the first pair of gill arches and the connecting tissue trimmed back for about one centimeter. The gill basket then tended to hang away from the cranium, stretching the posterior buccal cavity epithelium (Figure 3D). The epithelial tissue was then cut as far as possible on both sides exposing more of the tissue between the base of the cranium and the esophagus. The incision was deepened by continuing to alternately cut esophageal-cranial connective tissue and buccal epithelium until the anterior end of the head kidney was exposed. The cranium was completely removed by cutting through the head kidney and the body wall on each side.

With the tongue held between the thumb and forefinger, the tips of 15 cm scissors were inserted on either side of the basibranchial bone between the ventral origins of the first and second gill arches (Figure 3E). By pushing the tongue down toward the tips of the scissors the basibranchial bone was cut with no damage to the filaments of the second gill arch. The gill basket was then set down so that the cut esophagus and pectoral fin bases rested on the table and the ventral aspect of the animal faced forward. The first pair

of gill arches were cut away one arch at a time by inserting the scissors between the dorsal origins of the first and second arches on one side and cutting diagonal to the midline of the animal (Figure 3F). This cut was more easily made if the tongue was pulled forward so that the first arch was straightened out and pulled against the scissors.

After turning the gill basket 180°, the basibranchial bone between the second and third pair of arches was cut in the same way as the earlier cut between the first and second pair (Figure 3G). To facilitate insertion of the scissor tips through the gill slits for this cut, a forefinger was placed behind the point where the cut was to be made and used to help push the gills onto the scissors. By pushing forward on the ventral side of the animal and pushing down on the scissor handles the second pair of arches bent upward lifting the filaments of the second arch away from the cutting edge of the scissors. After making the cut, the gill basket was again rotated 180°, and the cut ventral end of the second arches was held away from the third arches with teethed forceps. Damage to the arch could be avoided by biting the forceps into the two exposed ends of the cut basibranchial bone rather than the soft tissue around it. With the ventral end of the arches held up and away from the third arches, the scissors were inserted to cut away the dorsal ends. These two cuts were

made in the same way as the earlier cuts to remove the first pair of arches.

With the second pair of gill arches now dissected completely free, fine tipped 10 cm scissors were used to separate the two arches (Figure 3H). The arches were held up with forceps so that the sharp blade of the scissors could be inserted into the caudal end of the cut ventral aorta. The ventral side of the vessel was then laid open exposing the openings to the left and right afferent branchial arteries. Taking care that one afferent branchial artery could be seen on each side of the scissors blade, the dorsal side of the ventral aorta and the basibranchial bone were cut longitudinally. This cut separated the two gill arches and they were ready for cannulation.

For cannulation, a gill arch was placed in a watch glass and covered with non-nutrient Ringer solution to prevent drying. With the pump delivering about 0.25 ml perfusion solution per minute, the afferent cannula was inserted down the afferent branchial artery. A single knot was tied around the arch with number 3 cotton suture to hold the cannula in place. Throughout these procedures the pressure recording for the arch was closely watched as an indicator of flow blockage. If cannulation was successful, initial pressure rarely exceeded 70 mm Hg and usually fell to below 50 mm Hg within several minutes. In a good preparation the pressure

did not increase when the cannula was knotted in place.

To insert the efferent cannula it was usually necessary to use 7 to 10X magnification with a stereoscopic dissecting scope. Black pigmentation spots on the efferent branchial artery facilitated its location, once the surrounding muscle had been carefully teased apart. Holding one edge of the cut end of the artery with number 3 Dumont tweezers, the cannula was inserted. After confirming adequate flow in the efferent tube, the cannula was tied in place in the same way as the afferent cannula. Both cannulae were withdrawn to within several mm of the knots, the arch was suspended in the bath and the cannulae clamped in place with the gill holders. After connecting the efferent tube to its drop counter assembly, the arch was ready for use.

Perfusion Solutions

Perfusion solutions consisted of vasoactive drugs and/or hormones (Table 1), added to a glucose-Ringer solution (Appendix). The Ringer solution was prepared fresh daily from crystalline glucose and stock solutions of the inorganic constituents. Before vasoactive agents were added, the Ringer solution was vacuum filtered through a 0.22 μm Millipore filter. The solution was then vigorously shaken to assure atmospheric equilibration.

Table 1.--Vasoactive drugs and hormones used in ^{14}C-urea influx and perfusion pressure experiments.

Drug generic and trade name	Text abbreviation	Manufacturer or source
Atropine SO_4[1]	----	Sigma Chemical Co. St. Louis, Mo.
Acetylcholine Cl	ACH	Sigma Chemical Co. St. Louis, Mo.
Epinephrine HCl	EPI	Wolins Farmingdale, N.Y.
Hexamethonium Cl[1]	----	Dr. Frank Kutyna MSU, E. Lansing, Mich.
Isoproterenol HCl Isuprel HCl	IPT	Winthrop Laboratories New York, N.Y.
Norepinephrine bitartrate Levophed bitartrate	NEPI	Winthrop Laboratories New York, N.Y.
Phenoxybenzamine HCl Dibenzyline HCl	POB	Smith, Kline & French Labs. Philadelphia, Pa.
Phentolamine[1] Regitine	----	CIBA Pharmaceutical Co. Summit, N.J.
Phenylephrine HCl Neo-synephrine	PEP	Winthrop Laboratories New York, N.Y.
Propranolol HCl Inderal HCl	PROP	Ayerst Laboratories, Inc. New York, N.Y.
Reserpine[1] Serpasil	----	CIBA Pharmaceutical Co. Summit, N.J.

[1]Used only in perfusion pressure experiments.

Experiment Protocols

Usually, two gill arches were perfused simultaneously with the drug or hormone concentration being varied in the experimental arch and either not added or maintained at a constant level in the control arch.

^{14}C-Urea Influx Experiments: These experiments were designed to measure the effect of vasoactive drugs and hormones on perfusion pressure and influx of ^{14}C-urea. Changes in these parameters are taken to reflect 1) changes in the degree of respiratory lamellae perfusion, and 2) concomitant changes in functional surface area of the gill available for diffusional influx of ^{14}C-urea. The experiment protocols are shown in Table 2.

After cannulation, gill arches were perfused for an equilibration period of about one hour before experiments were begun. During this time, arches were perfused with the same solutions that were to be used in the initial period of the experiment. Except when adrenergic blocking drugs were used, the same treatments were applied to both control and experimental arches through the initial period. Starting with the initial period, twenty fractions were collected during each experimental period. In the early studies, all even numbered fractions were collected and counted for ^{14}C-urea activity. Later it was evident that fewer samples would be adequate, and thereafter every fifth fraction was collected

Table 2.--Protocols for ^{14}C-urea influx experiments.

Experiment	Arch[1]	Drug concentrations[2] during different experiment periods[3]				
		Equilibration & Initial	Second	Third	Fourth	Fifth
α Agonist	C	None	None	None	None	---
	E	None	10^{-5}M PEP	10^{-4}M PEP	10^{-3}M PEP	---
β Agonist	C	None	None	None	None	---
	E	None	10^{-7}M IPT	10^{-6}M IPT	10^{-5}M IPT	---
Epinephrine	C	10^{-7}M Epi	10^{-7}M Epi	10^{-7}M Epi	10^{-7}M Epi	---
	E	10^{-7}M Epi	10^{-6}M Epi	10^{-5}M Epi	10^{-7}M Epi	---
Epinephrine	C	10^{-7}M Epi	10^{-6}M Epi	10^{-5}M Epi	---	---
α Block[4]	E	10^{-7}M Epi + POB	10^{-6}M Epi + POB	10^{-5}M Epi + POB	---	---
Epinephrine	C	10^{-7}M Epi	10^{-6}M Epi	10^{-5}M Epi	---	---
β Block[4]	E	10^{-7}M Epi + Prop	10^{-6}M Epi + Prop	10^{-5}M Epi + Prop	---	---
Epinephrine	C	10^{-7}M Epi	10^{-6}M Epi	10^{-5}M Epi	---	---
Double Block[4]	E	10^{-7}M Epi + POB + Prop	10^{-6}M Epi + POB + Prop	10^{-5}M Epi + POB + Prop	---	---
Norepinephrine	C	10^{-9}M Nepi	10^{-9}M Nepi	10^{-9}M Nepi	10^{-9}M Nepi	10^{-9}M Nepi
	E	10^{-9}M Nepi	10^{-8}M Nepi	10^{-7}M Nepi	10^{-6}M Nepi	10^{-5}M Nepi
Norepinephrine	C	10^{-9}M Nepi	10^{-8}M Nepi	10^{-7}M Nepi	10^{-6}M Nepi	10^{-5}M Nepi
α Block[4]	E	10^{-9}M Nepi + POB	10^{-8}M Nepi + POB	10^{-7}M Nepi + POB	10^{-6}M Nepi + POB	10^{-5}M Nepi + POB
Norepinephrine	C	10^{-9}M Nepi	10^{-8}M Nepi	10^{-7}M Nepi	10^{-6}M Nepi	10^{-5}M Nepi
β Block[4]	E	10^{-9}M Nepi + Prop	10^{-8}M Nepi + Prop	10^{-7}M Nepi + Prop	10^{-6}M Nepi + Prop	10^{-5}M Nepi + Prop
Norepinephrine	C	10^{-9}M Nepi	10^{-8}M Nepi	10^{-7}M Nepi	10^{-6}M Nepi	10^{-5}M Nepi
Double Block[4]	E	10^{-9}M Nepi + POB + Prop	10^{-8}M Nepi + POB + Prop	10^{-7}M Nepi + POB + Prop	10^{-6}M Nepi + POB + Prop	10^{-5}M Nepi + POB + Prop
Acetylcholine 1	C	None	None	None	None	---
	E	None	10^{-8}M Ach	10^{-7}M Ach	10^{-6}M Ach	---
Acetylcholine 2	C	10^{-5}M Epi	10^{-5}M Epi	10^{-5}M Epi	10^{-5}M Epi	---
	E	10^{-5}M Epi + No Ach	10^{-5}M Epi + 10^{-8}M Ach	10^{-5}M Epi + 10^{-7}M Ach	10^{-5}M Epi + 10^{-6}M Ach	---

[1] C = control arch, E = experimental arch

[2] Full names and sources of drugs shown in Table 1.

[3] Time of experimental periods: Equilibration = 1 hour, other periods = about 40 min.

[4] Blocking drug concentrations maintained at constant level for entire experiment.
α Blocker = Phenoxybenzamine (POB) at 10^{-5}M.
β Blocker = Propranolol (Prop) at 10^{-5}M.
Double Block = α + β block.

for counting. At the end of a few experiments under each protocol, the vasoactive substance perfused in the experimental arch was restored to the control concentration. This was done to verify return of perfusion pressure and ^{14}C-urea uptake to control values.

Perfusate was collected directly into vials containing liquid scintillation cocktail (Appendix). During each experiment, 3 to 5 successive 100 µl samples of the ^{14}C-urea baths were also taken for counting. All perfusate and bath samples were counted within 48 hours on a Mark 1 liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Using variably quenched ^{14}C standards prepared by Nuclear-Chicago Corp., the channels-ratio method (Bush, 1963) was used to convert counts per minute (cpm) for each sample to disintegrations per minute (dpm). Average background was determined by counting periodic vials containing only scintillation cocktail and was subtracted from all sample counts.

Perfusion Pressure Experiments: Although most of the perfusion pressure data were collected in the ^{14}C-urea influx experiments described above, other experiments were conducted in which only pressure responses were measured, thus, no ^{14}C-urea was added to the baths and no perfusate fractions were collected. These experiments were conducted to determine the effect of several blocking drugs on vasoactive hormone-induced perfusion pressure changes seen in the studies

described above. Usually, the blocking drug was perfused in the experimental arch and the effect of a vasoactive hormone on perfusion pressure was tested in both control and experimental arches. In some cases the blocking agent was removed from the perfusion fluid to determine if control responses were restored.

Data Treatment and Statistics

^{14}C-Urea Influx Data: The ^{14}C-urea influx in any experiment was determined not only by treatment effects but also by such variable factors as the size of the gill arch, the percentage of gill arch perfused due to length of cannulae insertion, the extent of blood clotting in the respiratory lamellae, and bath ^{14}C-urea activity. To separately test the effect of vasoactive substances on ^{14}C-urea influx it was necessary to eliminate sources of variability not due to treatment effects. The ^{14}C-urea count data from individual gill arches were, therefore, converted to percentages of the control ^{14}C-urea counts from the initial experimental period for that arch. The ^{14}C-urea sample activities from the initial experimental period were averaged, and the average was defined as 100% of initial activity. The activities of individual samples collected in later experimental periods were then converted to a percentage of this mean initial activity:

$$\% I = \frac{A_S}{\bar{A}_I} \times 100$$

where:

% I = percent of initial activity

A_S = sample activity in dpm

$\bar{A}_I$ = mean initial activity in dpm

Because high ^{14}C-urea activity in the baths constituted an essentially infinite source of ^{14}C-urea for diffusion into the gill, it was not necessary to correct for the ^{14}C-urea removed from the bath with the perfusion fluid. Occasionally, however, a gill arch leaked enough perfusion fluid into its bath during an experiment to significantly dilute the bath ^{14}C-urea. In severe cases the experiment was abandoned, but where possible a correction factor was calculated from the reduction in bath sample counts. This was done by plotting the fraction of bath activity remaining versus time. The correction factor for any sample was then the fraction of bath activity remaining at the time the perfusate sample was collected. These factors were divided into the perfusate sample % I values to account for progressive reduction of bath ^{14}C-urea available for diffusion into the gill arch.

For each experiment the percent of initial activity values were plotted for both the experimental and control arches. The plots from a typical experiment are shown in Figure 4. Typically, the percent of initial activity increased

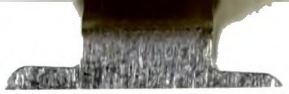

Figure 4.--Results from a typical epinephrine experiment.

Vertical dashed lines delineate experimental periods with epinephrine concentrations in experimental arch (arch 2) shown above. Epinephrine concentration in the control arch was 10^{-7}M throughout. The solid horizontal line represents the control initial activity for both gill arches. Open circles, percent of initial activity in control arch fractions; X's, percent of initial activity for experimental arch fractions.

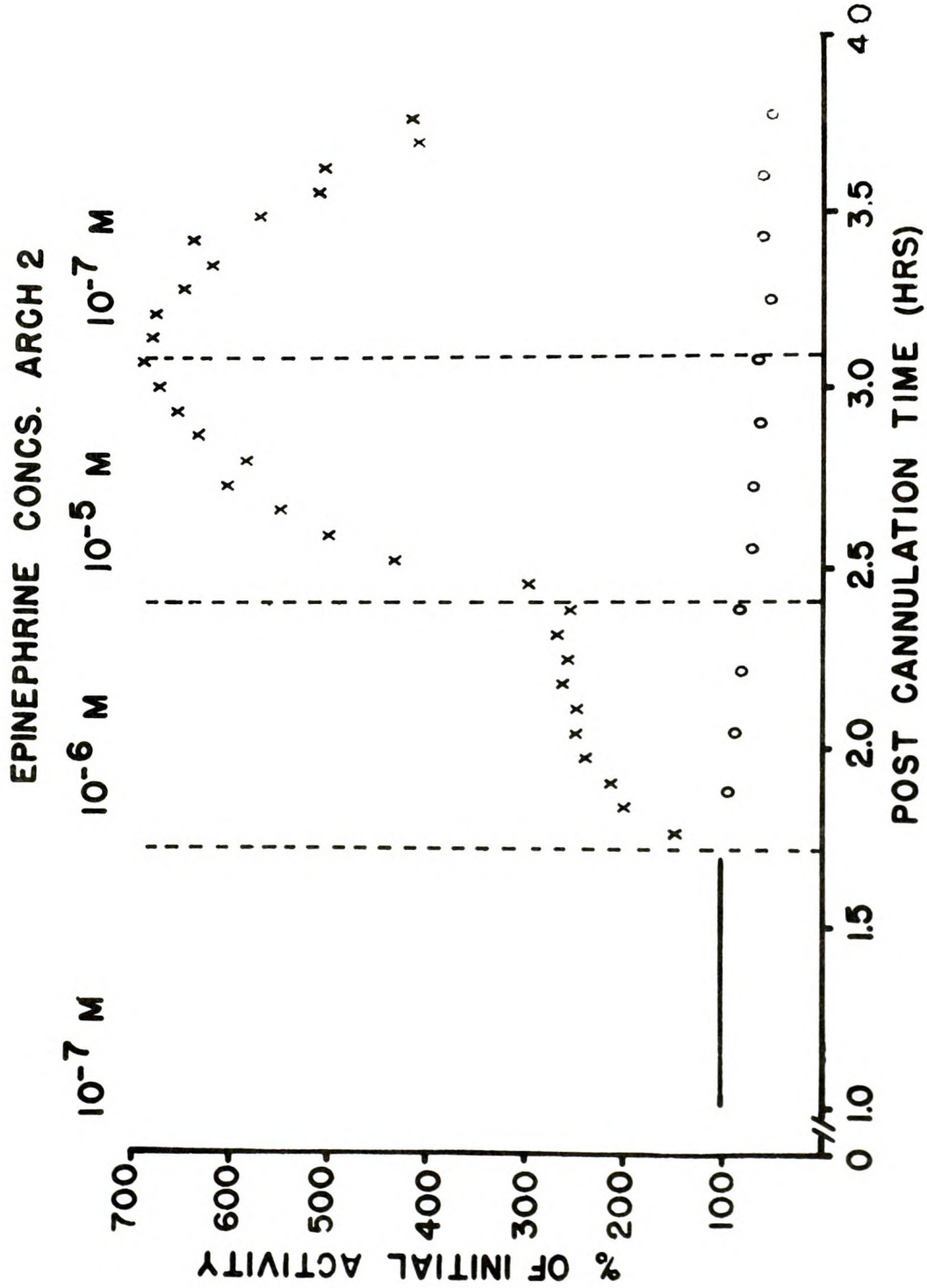
EPINEPHRINE CONCS. ARCH 2
10-7 M
10-6 M
10-5 M
10-7 M
% OF INITIAL ACTIVITY
700
600
500
400
300
200
100
0
1.0
1.5
2.0
2.5
3.0
3.5
4.0
POST CANNULATION TIME (HRS)

Figure 4

or decreased toward new steady-state values in response to step-wise changes in the concentration of vasoactive drug. For all experiments following one protocol, tabulations were made of the steady-state percent of initial activity values which corresponded to the different drug concentrations used. If at the end of an experimental period (corresponding to one vasoactive drug or hormone concentration) a new steady-state had not yet been reached, the percent of initial activity value for the last sample in that period was used as a conservative estimate of the steady-state value. Where the drug or hormone concentration was held constant in the control arch, the percent of initial activity values usually slowly declined or remained constant (Figure 4). For the control arches, then, the percent of initial activity value tabulated for an experimental period was the last sample collected from that period.

Since the variances of the tabulated values were not independent of the means (i.e., as the mean values increased variability increased), all statistics were calculated with log transformed data. The means and 95% confidence intervals were calculated for each treatment and retransformed to linear scale. One way analyses of variance were used for all comparisons and Tukey's ω-procedure (Sokal and Rohlf, 1969) was used for multiple comparisons among means.

Perfusion Pressure Data: To demonstrate qualitative pressure responses to the drug and hormone treatments, typical perfusion pressure records were selected to represent each type of experiment. Most of the perfusion pressure data were presented in this qualitative manner only. Where quantitative expressions of pressure responses were appropriate, the data were treated in the same way as the ^{14}C-urea influx data as described above. This was necessary to eliminate sources of variability not due to treatment effects. Uncontrolled sources of variability that affected pressures included gill arch size and the degree of blood clotting in the gill. For each experiment, the systolic perfusion pressure during the initial experimental period was defined as 100% of the initial pressure. Systolic pressure during later experimental periods was then calculated as a percentage of the initial pressure. This procedure allowed statistical combination of pressure data from all experiments following one protocol, and comparison of results from different sets of experiments. Statistical tests employed to evaluate the pressure data were the same as those used for the ^{14}C-urea influx results.

RESULTS

General Techniques

The isolated gill perfusion technique developed for use in this study appears well suited for the evaluation of certain physiological phenomena in teleost gills. With this preparation it is possible to conduct an experiment over an extended period of time. Only data obtained during a maximum four hour perfusion period were used even though preliminary experiments demonstrated that the preparations were usable for as long as twelve hours. However, if solutions were not filtered before use, perfusion pressure rose steadily, and after two or three hours the pressure was usually too high to continue the experiment.

With the peristaltic pump used, once a gill arch was cannulated flow rate could be changed only by adjusting pump rate. More flexibility could be obtained by using one of several commercially available cardiac pumps. Then stroke volume and pump rate could be varied independently, and flow characteristics could be adjusted to more closely resemble conditions in the intact animal.

^{14}C-Urea Influx

The influx of ^{14}C-urea was altered markedly by the vasoactive drugs and hormones used in this study. Catecholamines and pharmacological adrenergic agonists increased influx, while acetylcholine reduced it.

The effects of the drugs phenylephrine (PEP), an α adrenergic agonist, and isoproterenol (IPT), a β adrenergic agonist, are shown in Figures 5 and 6, respectively. At all concentrations used in this study, PEP significantly increased ^{14}C-urea influx; however, relatively high concentrations were used (10^{-5} to 10^{-3}M PEP). In preliminary experiments, concentrations below 10^{-5}M, PEP appeared to have no effect on influx of the marker. Although the PEP concentration was increased from 10^{-5} to 10^{-3}M, there was no increase in influx beyond that caused by the lowest concentration. A lower concentration range of IPT was used, but only the highest concentration (10^{-5}M) significantly increased ^{14}C-urea influx. It is not known whether the IPT effect was maximal at 10^{-5}M, since higher IPT concentrations were not perfused.

Norepinephrine (NEPI), a catecholamine which stimulates both α and β adrenergic receptors, was perfused at concentrations from 10^{-9} to 10^{-5}M (Figure 7). At the two highest NEPI concentrations (10^{-6} and 10^{-5}M) the ^{14}C-urea influx increased significantly above that of controls perfused with 10^{-9}M NEPI. When either the α adrenergic blocker phenoxybenzamine

Figure 5.--The effect of the α adrenergic agonist, phenylephrine, on ^{14}C-urea influx. During the initial period (not shown) both control and experimental gills were perfused with Ringer solution only. The mean, 95% confidence interval, and N value are shown for each control and treatment from successive experimental periods. Significance for paired comparisons: $0.01<P<0.05$ = *; $0.001<P<0.01$ = **.

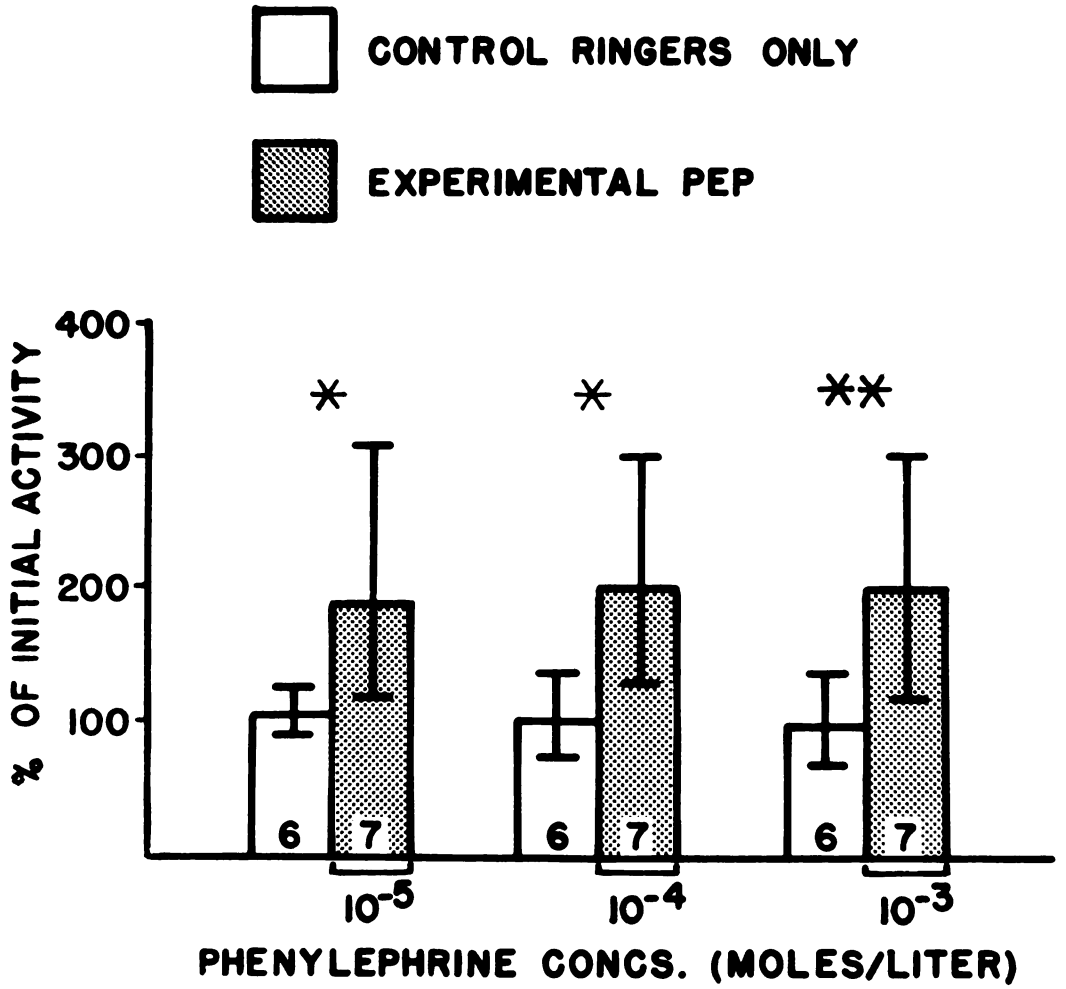
CONTROL RINGERS ONLY
EXPERIMENTAL PEP
% OF INITIAL ACTIVITY
400
300
200
100
*
*
**
6
7
6
7
6
7
10-5
10-4
10-3
PHENYLEPHRINE CONCS. (MOLES/LITER)

Figure 5

Figure 6.--The effect of the β adrenergic agonist, isoproterenol, on ^{14}C-urea influx. During the initial period (not shown) both control and experimental gills were perfused with Ringer solution only. The mean, 95% confidence interval, and N value are shown for each control and treatment from successive experimental period. Significance for paired comparisons: $P<0.001$ = ***.

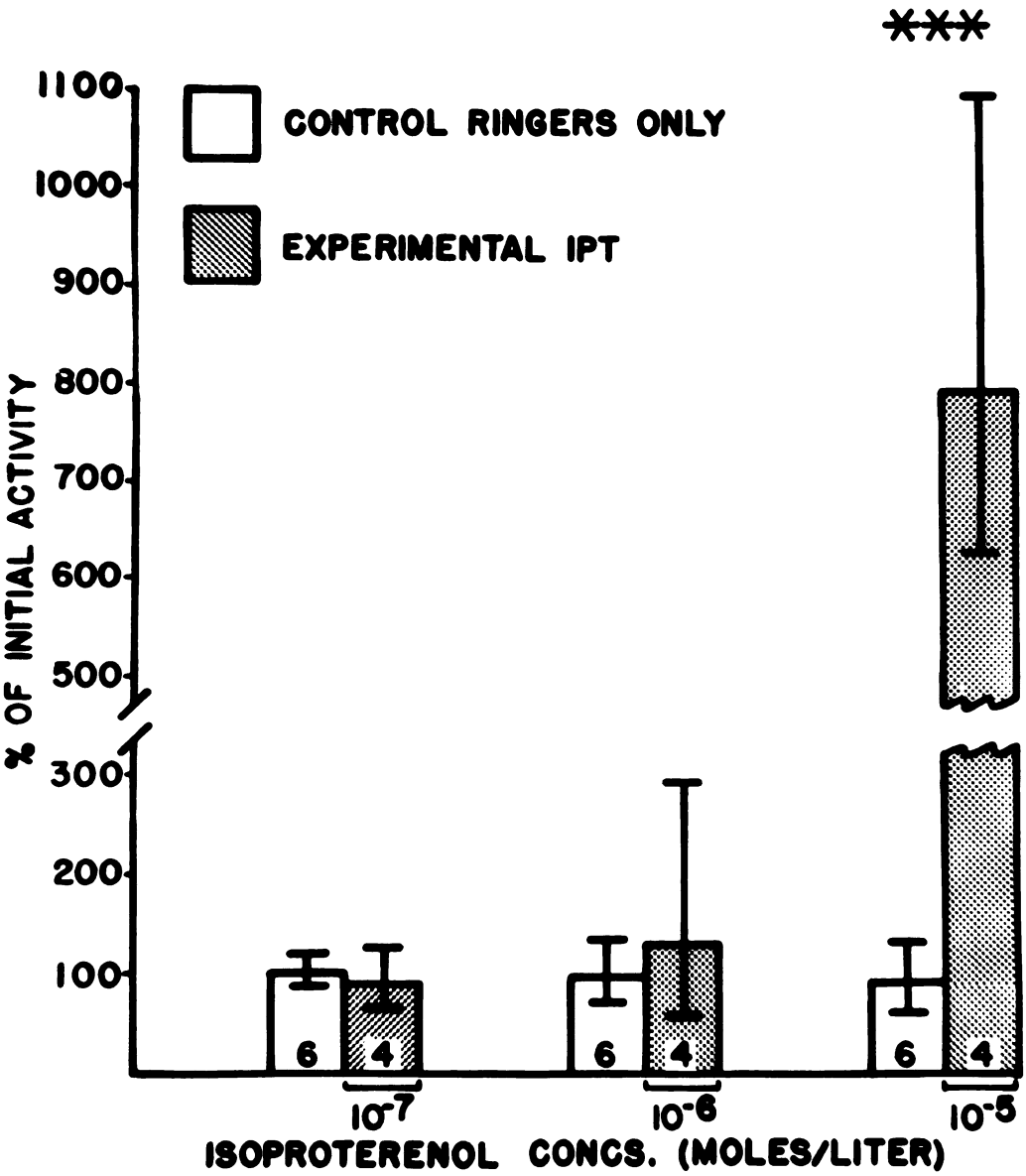
***
CONTROL RINGERS ONLY
EXPERIMENTAL IPT
% OF INITIAL ACTIVITY
1100
1000
900
800
700
600
500
300
200
100
6
4
6
4
6
4
10-7
10-6
10-5
ISOPROTERENOL CONCS. (MOLES/LITER)

Figure 6

Figure 7.--The effect of norepinephrine on ^{14}C-urea influx. During the initial period (not shown) both control and experimental gills were perfused with 10^{-9}M norepinephrine. The mean, 95% confidence interval, and N value are shown for each control and treatment from successive experimental periods. Significance for paired comparisons: $0.001<P<0.01$ = **; $P<0.001$ = ***.

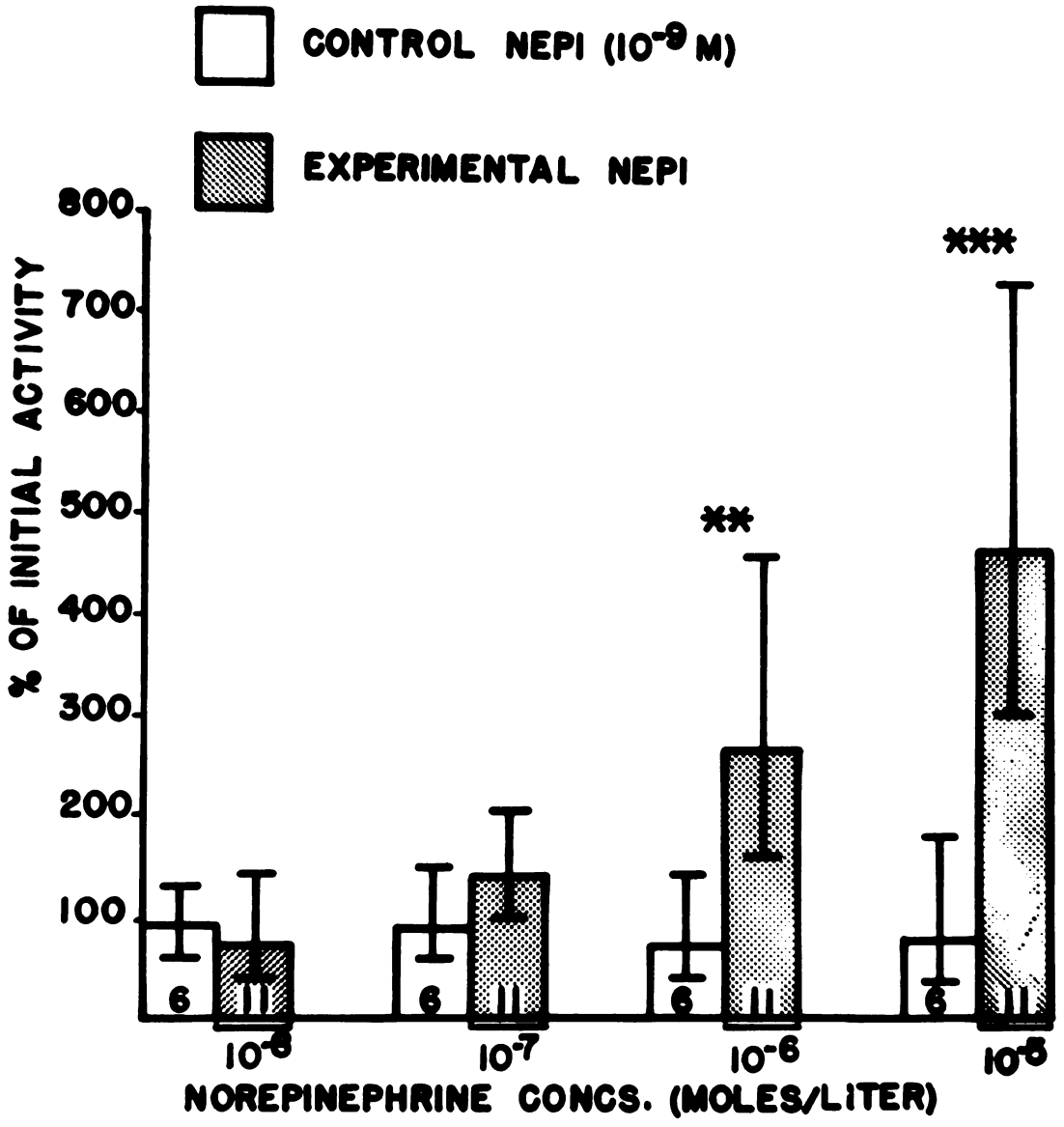
CONTROL NEPI (10^{-9} M)
EXPERIMENTAL NEPI
% OF INITIAL ACTIVITY
800
700
600
500
400
300
200
100
**
***
6
11
10^{-8}
10^{-7}
10^{-6}
10^{-5}
NOREPINEPHRINE CONCS. (MOLES/LITER)

Figure 7

(POB) or the β adrenergic blocker propranolol (PROP) were perfused along with NEPI, the ^{14}C-urea influx caused by NEPI was at least partially reduced (Figure 8). The NEPI effect was completely eliminated when both POB and PROP were perfused with NEPI.

The results from epinephrine (EPI) experiments (Figures 9 and 10) were qualitatively the same as those reported for NEPI, although a narrower concentration range of EPI was used. EPI, like NEPI, is a catecholamine which stimulates both α and β adrenergic receptors. Because ^{14}C-urea influx data were less variable in the EPI experiments than in NEPI experiments, more clear-cut differences were evident when the EPI effect was blocked with POB and PROP (Figure 10). The unblocked 10^{-5}M EPI induced ^{14}C-urea influx is significantly greater than that seen in the control or blocked gills. Influx in the single block experiments (α or β) was still significantly higher than in the controls, and blockade of both the α and β responses to EPI was necessary to reduce influx so that it was indistinguishable from the control. Because the initial period concentrations were different in the NEPI and EPI experiments (10^{-9}M and 10^{-7}M, respectively), statistical tests could not be made between the results for NEPI and EPI shown in Figures 7 and 9. The mean EPI effect at 10^{-5}M is 564% of initial ^{14}C-urea activity, however, while the mean NEPI effect at 10^{-5}M is 466% of the initial activity.

Figure 8.--The effect of adrenergic blockade on norepinephrine induced ^{14}C-urea influx. During the initial period (not shown) all arches were perfused with $10^{-9}M$ norepinephrine. In the blocked arches, the blocking drug(s) was perfused throughout the entire experiment. Phenoxybenzamine at $10^{-5}M$ was the α blocker, and β blockade was with $10^{-5}M$ propranolol. The mean, 95% confidence interval and N value are shown for the control and each treatment. All values are from the same experimental period. Means not underlined by the same line are significantly different ($P<0.05$):

α & β Block	Control NEPI	α Block	β Block	Exp. NEPI
31	83	173	202	466

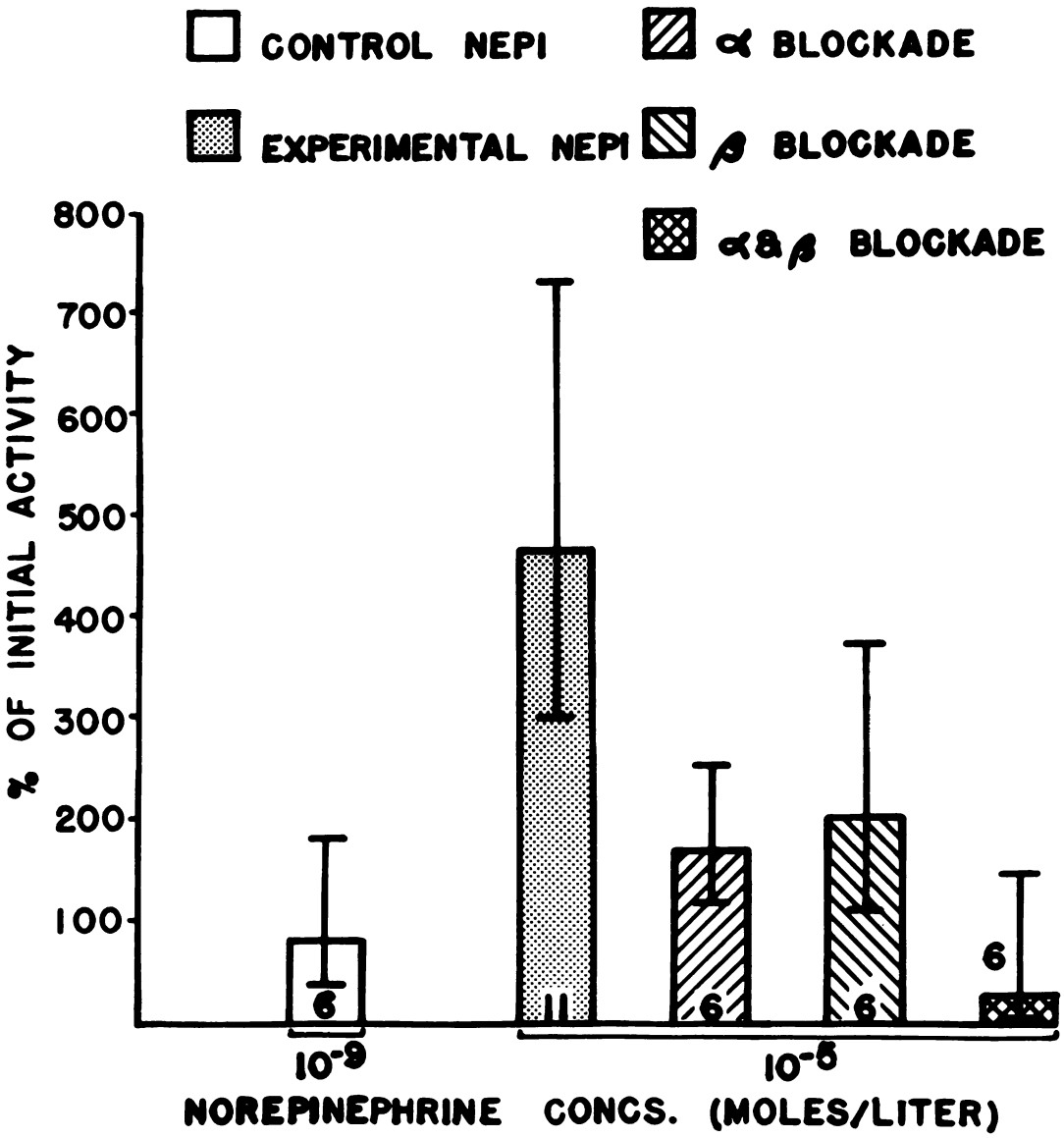
CONTROL NEPI
EXPERIMENTAL NEPI
α BLOCKADE
β BLOCKADE
α&β BLOCKADE
% OF INITIAL ACTIVITY
800
700
600
500
400
300
200
100
6
11
6
6
6
10⁻⁹
10⁻⁵
NOREPINEPHRINE CONCS. (MOLES/LITER)

Figure 8

Figure 9.--The effect of epinephrine on ^{14}C-urea influx. During the initial period (not shown) both control and experimental gills were perfused with $10^{-7}M$ epinephrine. The mean, 95% confidence interval, and N value are shown for each control and treatment from successive experimental periods. Significance for paired comparisons: $P<0.001$ = ***.

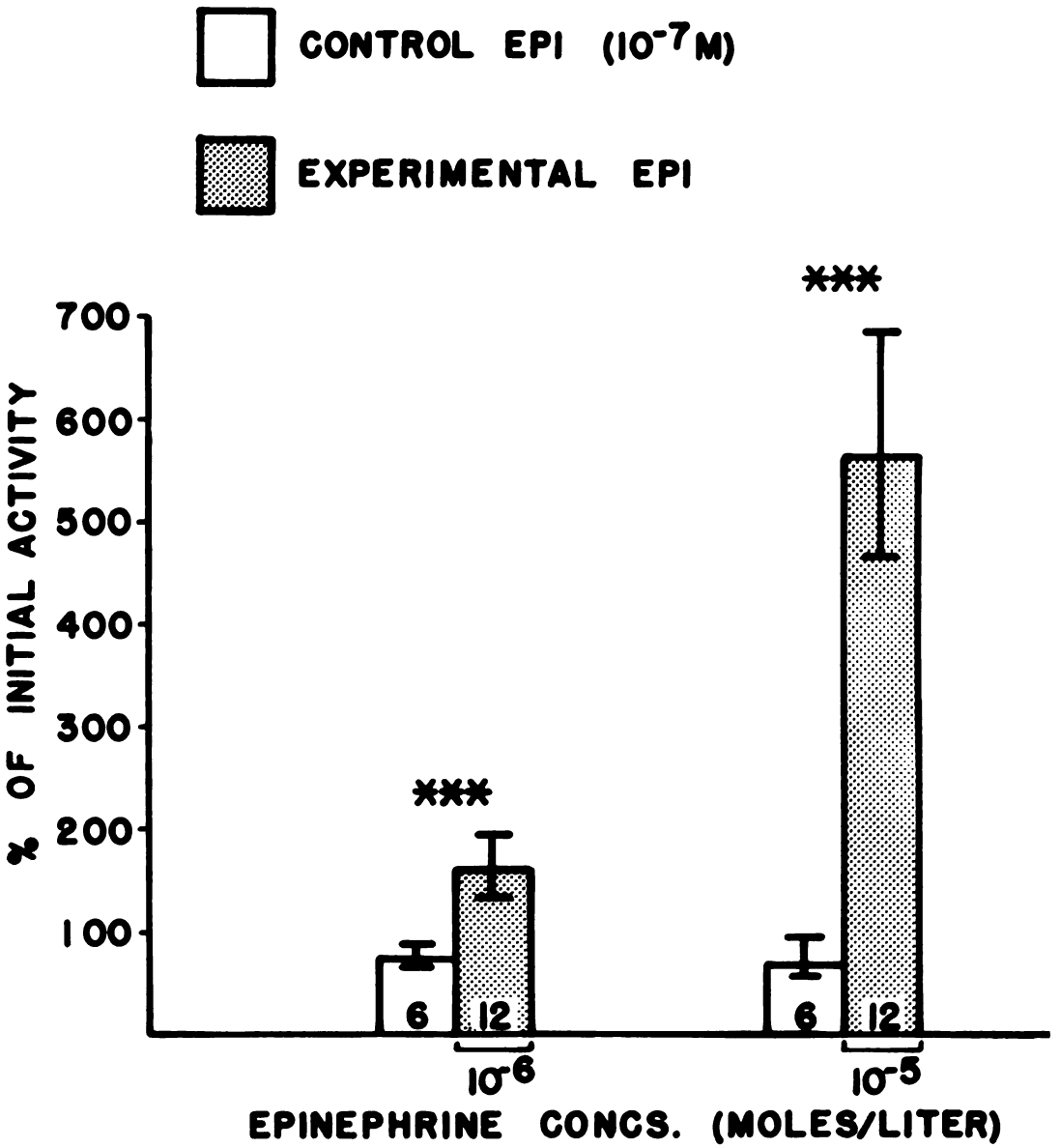
CONTROL EPI (10^{-7}M)
EXPERIMENTAL EPI
% OF INITIAL ACTIVITY
700
600
500
400
300
200
100
***
***
6
12
6
12
10^{-6}
10^{-5}
EPINEPHRINE CONCS. (MOLES/LITER)

Figure 9

Figure 10.--The effect of adrenergic blockade on epinephrine induced ^{14}C-urea influx. During the initial period (not shown) all arches were perfused with $10^{-7}M$ epinephrine. In the blocked arches, the blocking drugs were perfused throughout the entire experiment. Phenoxybenzamine at $10^{-5}M$ was the α blocker, and β blockade was with $10^{-5}M$ propranolol. The mean, 95% confidence interval and N value are shown for the control and each treatment. All values are from the same experimental period. Means not underlined by the same line are significantly different ($P<0.05$):

Control EPI	α & β Block	α Block	β Block	Exp. EPI
72	100	187	189	564

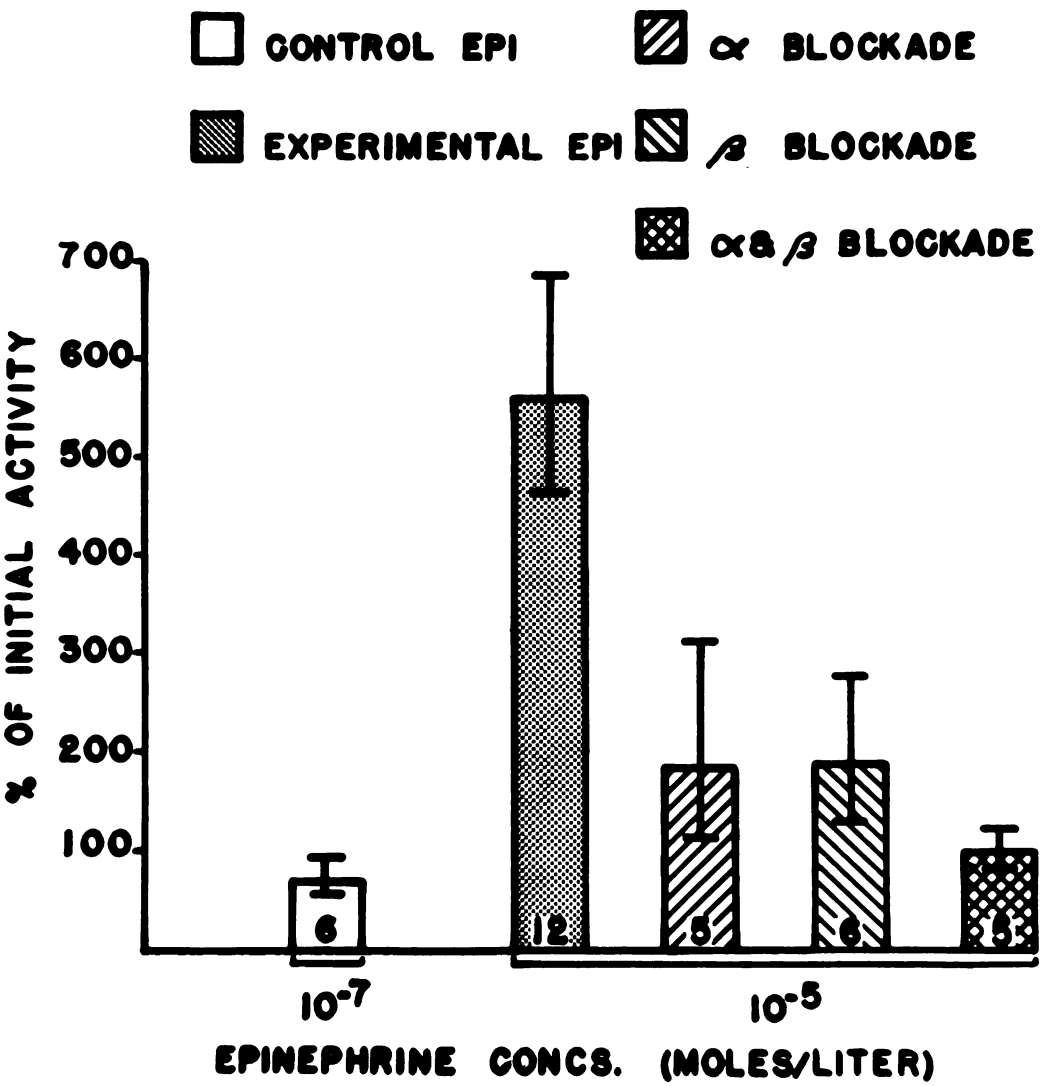
CONTROL EPI
EXPERIMENTAL EPI
α BLOCKADE
β BLOCKADE
α & β BLOCKADE
% OF INITIAL ACTIVITY
700
600
500
400
300
200
100
6
12
5
6
5
10-7
10-5
EPINEPHRINE CONCS. (MOLES/LITER)

Figure 10

This difference may have been greater if EPI had been perfused at the lower concentration (10^{-9}M) during the initial experimental period.

Acetylcholine (ACH), whether perfused alone (Figure 11) or along with 10^{-5}M EPI (Figure 12), significantly reduced ^{14}C-urea influx when compared to controls. The reduction was greater, however, in the experiments where ACH was perfused without the EPI.

Usually, the ^{14}C-urea influx in the control arches steadily declined over the 3 or 4 hour long experiments (Figures 5, 6, 7, 9 ,11 and 12). In no case, however, were these reductions significant.

Perfusion Pressure

The perfusion pressure record segments shown in Figures 13 through 16 are from the ^{14}C-urea experiments previously described. Except when high ACH concentrations were perfused (Figure 16), the perfusion pressures in all experiments were generally within the range reported for ventral aortic blood pressure in resting or swimming rainbow trout (Stevens and Randall, 1967b). For any series of experiments with a vasoactive agent, the pressure effect was qualitatively consistent, but the sensitivity of different preparations to a drug or hormone varied. This may have resulted from differences in prevailing vascular tone. In all cases, however, stimulation

Figure 11.--The effect of acetylcholine on ^{14}C-urea influx. During the initial period (not shown) both control and experimental gills were perfused with Ringer solution only. The mean, 95% confidence interval, and N value are shown for each control and treatment from successive experimental periods. Significance for paired comparisons: $0.01<P<0.05$ = *; $0.001<P<0.01$ = **.

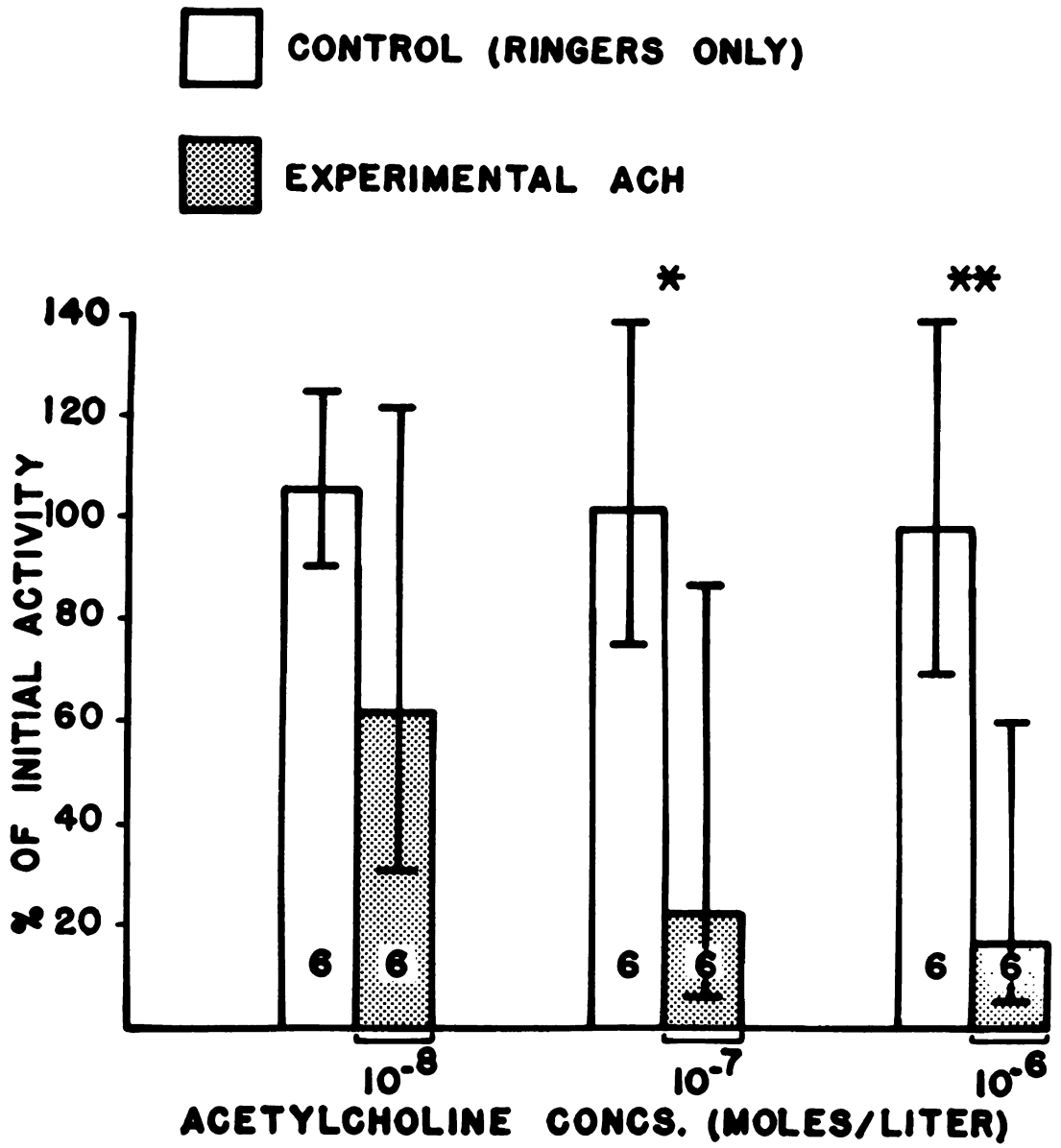
CONTROL (RINGERS ONLY)
EXPERIMENTAL ACH
*
**
140
120
100
80
60
40
20
% OF INITIAL ACTIVITY
6
6
6
6
6
6
10^{-8}
10^{-7}
10^{-6}
ACETYLCHOLINE CONCS. (MOLES/LITER)

Figure 11

Figure 12.--The effect of acetylcholine on ^{14}C-urea influx in the presence of epinephrine. During the initial period (not shown) both control and experimental gills were perfused with 10^{-5}M epinephrine. The mean, 95% confidence interval, and N value are shown for each control and treatment from successive experimental periods. Significance for paired comparisons: $0.01<P<0.05$ = *.

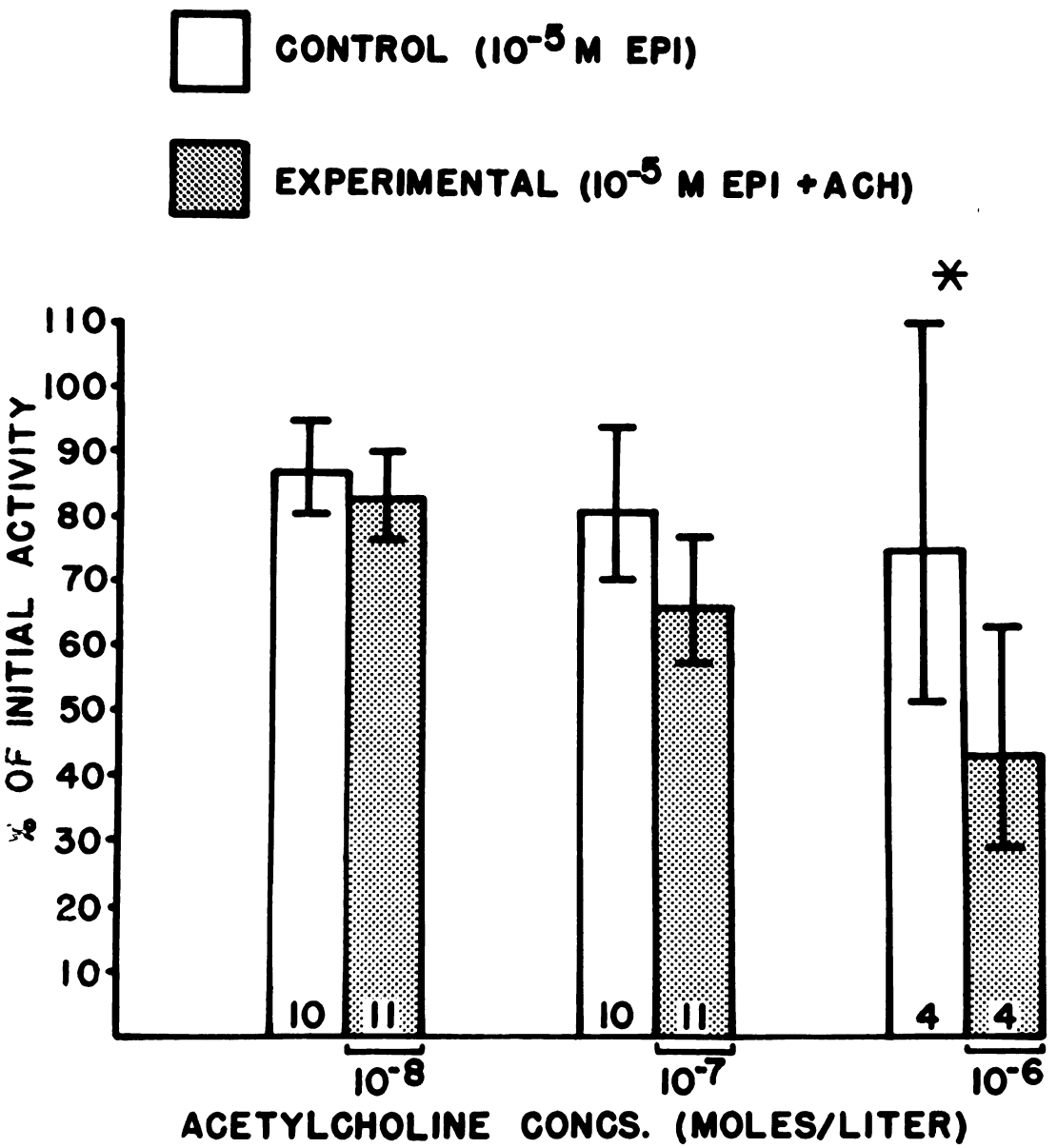
CONTROL (10^{-5} M EPI)
EXPERIMENTAL (10^{-5} M EPI + ACH)
% OF INITIAL ACTIVITY
110
100
90
80
70
60
50
40
30
20
10
*
10
11
10
11
4
4
10^{-8}
10^{-7}
10^{-6}
ACETYLCHOLINE CONCS. (MOLES/LITER)

Figure 12

of β adrenergic receptors caused a pressure decrease, and the effect of cholinergic or α adrenergic receptor stimulation was a pressure increase.

High NEPI concentrations always reduced perfusion pressure (Figure 13A), although in a few experiments a transient pressure increase was seen first (Figure 13B). At 10^{-5}M NEPI the average percent of initial pressure (88%) was significantly lower ($P<.05$) than in the 10^{-9}M NEPI controls. Very little effect on perfusion pressure was seen with NEPI concentrations below 10^{-5}M. In a few experiments when an extremely high (10^{-3}M) NEPI concentration was perfused, a dramatic 3-fold pressure increase followed the usual pressure decrease. Figure 13C, D and E show the typical pressure responses when adrenergic blockers were perfused along with the NEPI increase. With α blockade, pressure decreased in response to increased NEPI just as in the unblocked gills. Blocking the β response to NEPI usually unmasked a pressure increase, and when both α and β blockers were used no pressure change resulted from the increased NEPI concentration.

Perfusion pressure responses in the EPI experiments (Figure 14) were similar to those just described for NEPI. Typically, a pressure decrease was seen, but sometimes only after a transient pressure rise. The mean perfusion pressure at 10^{-5}M EPI was 84% of the initial control pressure, and was significantly lower ($P<.05$) than the pressure in the 10^{-7}M EPI

Figure 13.--Typical effects of norepinephrine on perfusion pressure in the absence and presence of adrenergic blockers. Arrows indicate where norepinephrine concentration was increased from 10^{-6} to 10^{-5}M.

(A) Norepinephrine alone--typically seen pressure decrease;
(B) Norepinephrine alone--infrequently seen transient pressure increase followed by an overall pressure decrease;
(C) α blockade of norepinephrine effect with 10^{-5}M phenoxybenzamine;
(D) β blockade of norepinephrine effect with 10^{-5}M propranolol;
(E) α and β blockade of norepinephrine effect with 10^{-5}M phenoxybenzamine and 10^{-5}M propranolol.

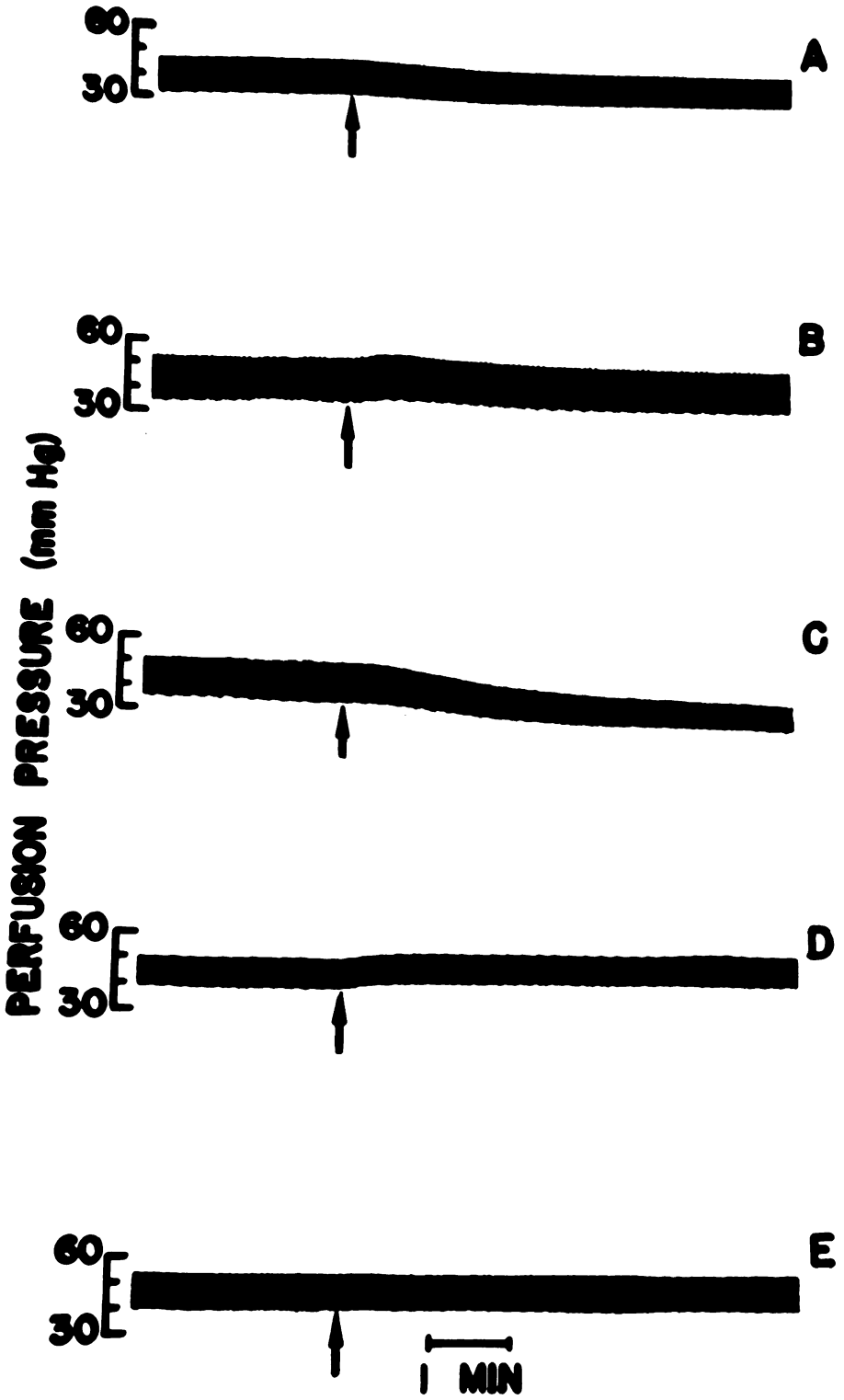

Figure 13

Figure 14.--Typical effects of epinephrine on perfusion pressure in the absence and presence of adrenergic blockers. Arrows indicate where epinephrine concentration was increased from 10^{-6} to 10^{-5}M.

(A) Epinephrine alone--typically seen pressure decrease;
(B) Epinephrine alone--infrequently seen transient pressure increase followed by an overall pressure decrease (beyond the record segment shown, pressure continued to decrease until it was below the initial value);
(C) α blockade of epinephrine effect with 10^{-5}M phenoxybenzamine;
(D) β blockade of epinephrine effect with 10^{-5}M propranolol;
(E) α and β blockade of epinephrine effect with 10^{-5}M phenoxybenzamine and 10^{-5}M propranolol.

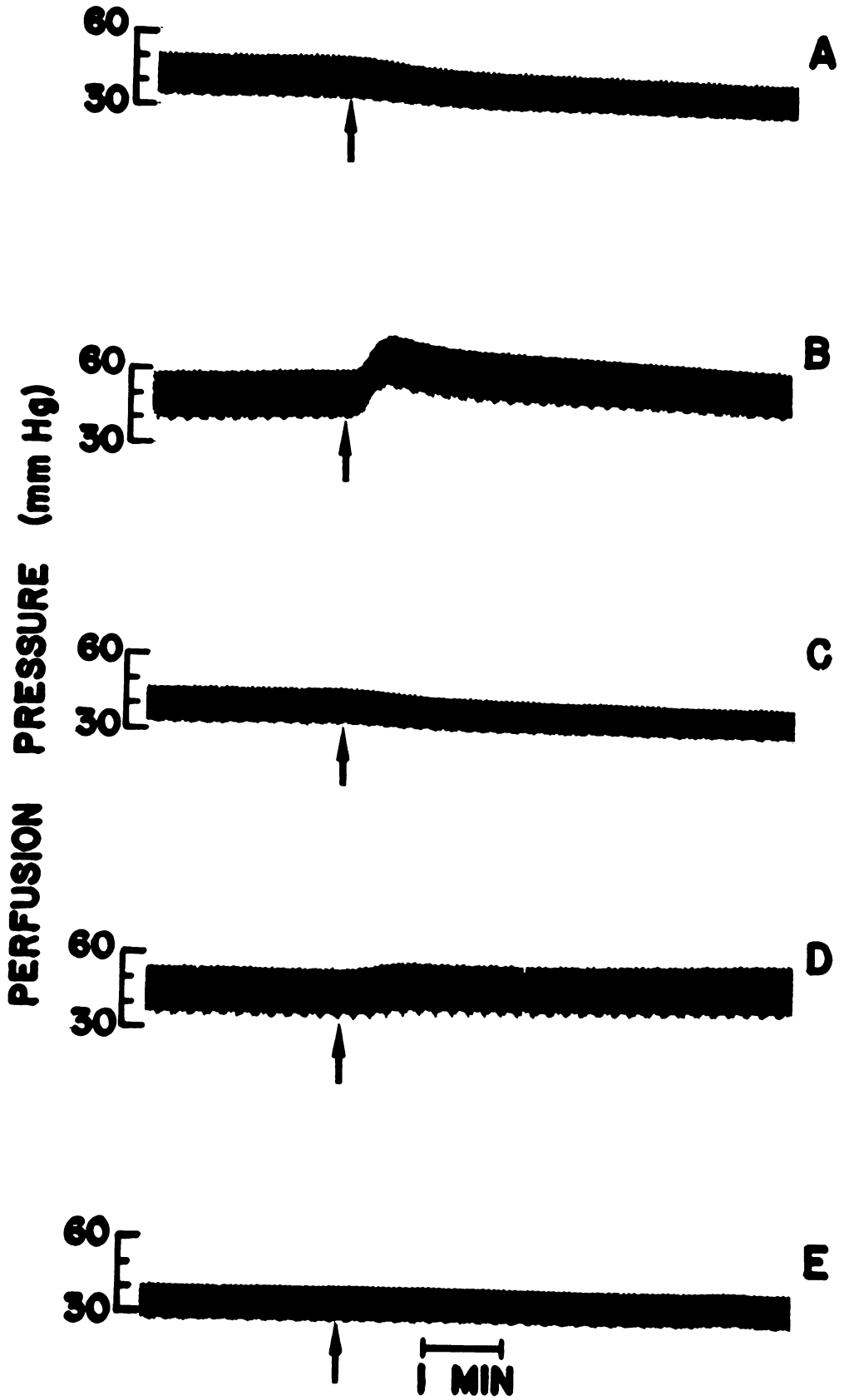

Figure 14

control arches. The qualitative effects of α and/or β adrenergic blockade of the EPI pressure response were exactly the same as those seen in the NEPI experiments.

The α adrenergic agonist, PEP, increased pressure when perfused at 10^{-3}M (Figure 15A); whereas 10^{-5}M IPT, the β adrenergic agonist, decreased the perfusion pressure (Figure 15B). At the concentrations used neither drug significantly changed perfusion pressure. The response to PEP was somewhat delayed when compared to the usual quick response to other drugs and hormones used in this study.

The typical effects of ACH are shown in Figure 16. Raising ACH concentrations from 10^{-8} to 10^{-7}M increased pressure slightly, but another ten-fold increase in ACH to 10^{-6}M resulted in a marked pressure increase. In experiments where 10^{-5}M EPI was perfused with the ACH (see Table 2), mean pressure during the 10^{-6}M ACH perfusion was 229% of the initial pressure (determined when EPI was perfused alone). When ACH was perfused alone, the 10^{-6}M ACH induced pressure increase was 200% of the initial condition (Ringer solution only) pressure. In both the EPI-ACH and ACH protocols, the perfusion pressure was significantly higher at 10^{-7}M ($P<.05$) and 10^{-6}M ($P<.001$) ACH than in the control arches.

Figure 17 shows the effects of five different drugs on the 10^{-6}M ACH induced perfusion pressure increase. Muscarinic and nicotinic (ganglionic) blockers, atropine and hexamethonium

Figure 15.--Typical effects of adrenergic agonists on perfusion pressure. Arrows indicate where agonist concentration was increased.

(A) α agonist phenylephrine concentration was increased from 10^{-4}M to 10^{-3}M;
(B) β agonist isoproterenol concentration was increased from 10^{-6}M to 10^{-5}M.

Figure 16.--Typical effect of acetylcholine on perfusion pressure. Arrows indicate where acetylcholine concentration was increased.

(A) Acetylcholine concentration was increased from 10^{-8} to 10^{-7}M;
(B) Acetylcholine concentration was increased from 10^{-7} to 10^{-6}M.

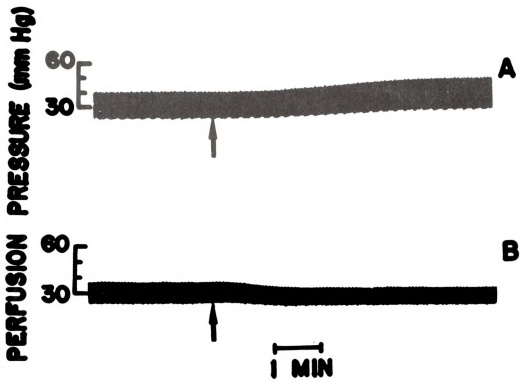

Figure 15

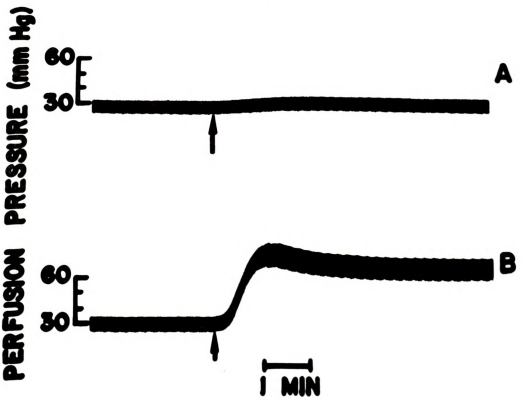

Figure 16

Figure 17.--The effect of various drugs on acetylcholine induced perfusion pressure changes. For each experiment the response to 10^{-6}M acetylcholine is shown before (left) and after (right) perfusion of the drug. The drugs used were:

(A) 10^{-7}M atropine for 5 minutes;
(B) 5×10^{-5}M hexamethonium for 90 minutes;
(C) 10^{-5}M phenoxybenzamine for 80 minutes;
(D) 10^{-4}M phentolamine for 60 minutes;
(E) 10^{-5}M reserpine for 140 minutes.

respectively, both eliminated most of the ACH pressure effect. While the α adrenergic blocker POB completely eliminated the pressure increase, phentolamine (also an α adrenergic blocker) had little effect. POB, however, is known to effectively block ACH in addition to its α adrenergic effect (Goodman and Gilman, 1965). Reserpine depletes tissue norepinephrine stores thereby reducing the effectiveness of sympathetic nerve stimulation. Its effect on the ACH pressure response was not clear-cut. In all experiments where reserpine was perfused, a slow and steady increase was seen in control pressure; but there was little change in the maximum pressure attained when 10^{-6}M ACH was perfused.

DISCUSSION

Although isolated-perfused gills were used in the present study, conditions were maintained as close to those found *in vivo* as possible. Except for the high pressures caused by 10^{-6}M ACH, the perfusion pressures were generally within the range of ventral aortic blood pressures reported for resting or swimming rainbow trout (Stevens and Randall, 1967b). Catecholamine concentrations used in this study were on the high end of the plasma catecholamine concentration ranges reported for resting and disturbed rainbow trout (Nakano and Tomlinson, 1967). While the maximum values Nakano and Tomlinson reported were 5 X 10^{-7}M NEPI and 2 X 10^{-6}M EPI, the concentrations used in this study were 10^{-9} to 10^{-5}M NEPI and 10^{-7} to 10^{-5}M EPI. However, significant effects on ^{14}C-urea influx were seen at NEPI and EPI concentrations at or near their maximum reported values.

Functional Surface Area

The results from this study confirm functional surface area regulation in rainbow trout gills. Stimulation of α and/or β adrenergic receptors with catecholamines or pharmacological drugs produced marked and usually significant increases

in ^{14}C-urea influx. Conversely, ACH significantly reduced influx of the marker. When taken with the attendant drug and hormone effects on vascular resistance, these findings can be accounted for by changes in functional surface area resulting from altered flow pattern through the gills. A different interpretation of the ^{14}C-urea influx results might, at first, seem valid. Namely, vasoactive agents might have affected permeability of the gills to ^{14}C-urea. But it is extremely doubtful that permeability changes could account for the effects of vasoactive agents on branchial vascular resistance. This is especially the case where, with opposite effects on resistance, stimulation of either α or β adrenergic receptors caused an increase in ^{14}C-urea influx. Because of inadequacies in accounting for all the results, major permeability changes which could affect ^{14}C-urea influx can be discounted. Since these experiments used isolated pump-perfused gill arches, the complicating effects of vasoactive agents on cardiac output and ventilation volume were eliminated (pump output and bath mixing rate were held constant for all experiments). The most tenable explanation for a change in marker influx and branchial vascular resistance would be altered perfusion pathway which would result in changed functional surface area of the gill.

At the highest catecholamine concentration used (10^{-5}M), the average increases in ^{14}C-urea influx were 4.6-fold for

NEPI and 5.6-fold for EPI. Using the results shown in Figures 7 and 9, these values can be adjusted downward to estimate the expected ^{14}C-urea influx at the maximum NEPI (5×10^{-7}M) and EPI (2×10^{-6}M) plasma concentrations reported by Nakano and Tomlinson (1967). The estimated increases in influx at the lower catecholamine concentrations are about 2.3-fold for NEPI and 2.8-fold for EPI. Assuming that vascular effects of the two catecholamines are additive at these concentrations, the combined effect of plasma NEPI and EPI in Nakano and Tomlinson's disturbed (exercised) fish could be expected to be about a 5-fold increase in functional surface area (assuming from evidence in the present study that changes in ^{14}C-urea influx are approximately equivalent to changes in functional surface area). Such an extrapolated 5-fold increase in functional surface area cannot account for the large increases in oxygen uptake reported for exercised fish; nor is it meant to. The significance of an increase in functional surface area in exercised fish must also take into account the effects of elevated blood catecholamines (and other control mechanisms) on cardiac output and ventilation volume. In a series of experiments, Stevens and Randall (1967a) observed a 4 to 5-fold increase in oxygen uptake, cardiac output and ventilation volume in rainbow trout subjected to moderate swimming activity. They also found that the percent saturation of arterial blood with oxygen was maintained at

95 to 100 percent whether fish were resting or swimming. These observations, coupled with results from this study, suggest that a 4 to 5-fold increase in blood flow through the gills would probably be accompanied by a comparable increase in functional surface area to maintain the observed 95 to 100 percent oxygen saturation of arterial blood. The large increase in oxygen uptake observed in exercised fish undoubtedly involves a number of hormonally and/or neurally mediated physiological changes that act in concert. Possibly three of the more important of these changes are increases in ventilation volume, cardiac output and functional gill surface area.

Perfusion Pathways

Perfusion pathway alterations are required to explain the vasoactive agent effects on ^{14}C-urea influx and branchial vascular resistance seen in this study. The results, however, do not enable distinction between the filamental sinus shunt and the lamellar recruitment models that have been proposed for blood pathway regulation.

In the filamental sinus shunt model (Steen and Kruysse, 1964; Richards and Fromm, 1969) respiratory blood flow (high ^{14}C-urea influx) is assigned to the secondary lamellae and non-respiratory blood flow (low ^{14}C-urea influx) represents flow through the filamental sinus and/or filamental tip shunts. Respiratory blood flow could then be modulated by adjusting

the degree of secondary lamellae perfusion. To support this model, the α adrenergic response seen in this study (increased resistance and increased ^{14}C-urea influx) must involve reductions in flow through the filamental sinus and filamental tip pathways forcing greater perfusion of the secondary lamellae. The β adrenergic response (decreased resistance and increased influx) could be accounted for by dilation of the afferent and efferent lamellar arterioles allowing greater perfusion of the secondary lamellae. When both α and β adrenergic receptors are acted on with unblocked NEPI or EPI (Figures 8 and 10), the ^{14}C-urea influx is about twice that seen when the α or β receptors are stimulated separately with the same catecholamine concentration. This would be expected with a reduction in the flow through the filamental sinus and tip shunts accompanied by a simultaneous increase in flow through the secondary lamellae. The ACH perfusions (increased resistance and decreased influx) could have caused constriction of vascular smooth muscle around the afferent and efferent lamellar arterioles forcing greater perfusion of the shunt pathways.

The lamellar recruitment model (Hughes, 1972; Morgan and Tovell, 1973) assigns blood flow during periods of low oxygen demand to the secondary lamellae on the proximal ends of the filament. Increased respiratory blood flow is thought to be diverted through an increased number of secondary lamellae toward the filament tip. Adjustment of respiratory blood flow

could then be accomplished by perfusing a variable number of secondary lamellae. To fit the results from the current study to this model, the α adrenergic response could include contraction of the efferent filamental arteries and/or the efferent branchial artery. This would increase resistance across the filaments causing the afferent side pressure to rise enough to exceed the critical closing pressures of the distal afferent filamental arteries and the afferent and efferent lamellar arterioles. The β adrenergic response could be accounted for by dilation of lamellar arterioles and filamental arteries in the distal portion of the filaments.

There is some difficulty fitting the observed ACH effects to the lamellar recruitment model. Again, as in the filamental shunt model, ACH is likely to have constricted the afferent and efferent lamellar arterioles reducing flow to the lamellae. When ACH was perfused alone (Figure 11) there was a marked reduction in ^{14}C-urea influx. In these experiments the initial control perfusion was with Ringer solution only. From the recruitment model it would seem that under these conditions all flow would be through the secondary lamellae at the proximal ends of the filaments. Since no shunt pathways are presumed, there would be no alternate pathway for diverted flow if these basal lamellae were shut off. But since ^{14}C-urea influx was reduced and resistance was increased by ACH, flow probably was diverted to a high-resistance,

non-respiratory pathway. Hughes (1972) has pointed out that red blood cells were found in the filamental sinus only when gills were perfused at excessive pressures. Since perfusion pressures were elevated above physiological levels in some ACH experiments (especially with 10^{-6}M ACH), it is possible that the high pressures ruptured filamental sinuses allowing direct flow from afferent to efferent side. In the ACH-EPI experiments (Figure 12) the initial control perfusion was with 10^{-5}M EPI, and this same level was maintained throughout the remainder of each experiment. Under these conditions most or all of the secondary lamellae would probably be recruited to flow during the initial experiment period. As ACH was added to the perfusion solution, ^{14}C-urea influx was reduced but not as greatly as in the experiments where ACH was perfused alone (Figure 11). The β component of EPI probably antagonized the ACH induced constriction of the lamellar arterioles thereby reducing the ACH effect on ^{14}C-urea uptake.

Hormonal and Neural Control Mechanisms

From observations in this and other studies it is evident that blood pathway and functional surface area regulation in the gill result from changes in the relative vascular resistances of the various pathways. The physiological control mechanisms which are responsible for adjusting these pathway

resistances are probably both hormonal and neural.

When taken with the observation that blood catecholamine concentrations were elevated in exercised fish (Nakano and Tomlinson, 1967), the effects of similar catecholamine concentrations on perfusion pressure and ^{14}C-urea uptake support a regulatory function for these hormones. The catecholamine induced decrease in branchial vascular resistance and increase in ^{14}C-urea influx seen in this study agree with published observations on the resistance (Keys and Bateman, 1932, Östlund and Fänge, 1962; Reite, 1969) and blood pathway effects (Steen and Kruysse, 1964; Richards and Fromm, 1969) of catecholamines. As discussed earlier, the ^{14}C-urea influx data also confirm the catecholamine effects on functional surface area which had been suggested from the blood pathway and resistance observations in the literature.

The characteristics of catecholamine effects in teleost gills remain unclear, however. While all evidence in the literature supports the view that catecholamines act on β adrenergic receptors in the gills, the presence or absence of α adrenergic receptors has become a subject of disagreement in several recent studies. Randall and Stevens (1967) reported the presence of α adrenergic receptors in coho salmon gills based on their observations that phenoxybenzamine (an α blocker) blocked the dorsal aortic pressure rise that accompanied EPI injection or swimming activity. But since the

dorsal aortic pressure rise could also have been blocked by phenoxybenzamine acting on peripheral α receptors, their conclusion is unwarranted. In a later study, Randall and co-workers (1972) observed a decrease in vascular resistance in isolated rainbow trout heads perfused with NEPI. Since norepinephrine is a much more potent stimulator of α receptors than β receptors, they concluded that the NEPI effect demonstrated presence of α receptors in the gills. Two objections can be raised with this conclusion: 1) by definition (Ahlquist, 1948), α adrenergic receptors mediate excitory responses which would, in this case, cause vasoconstriction and an increase in vascular resistance, and 2) the response to NEPI in any vascular bed is also affected by the relative population sizes of α and β adrenergic receptors. As an example of the importance of the relative population sizes of receptors, small coronary vessels in mammals have very few α receptors and are only dilated by NEPI (Zuberbuhler and Bohr, 1965) in spite of NEPI being a more potent α receptor stimulant. Rankin and Maetz (1971) were unable to block the effect of catecholamines on flow rate through isolated-perfused eel gills with phentolamine (an α blocker) and ruled out presence of α adrenergic receptors.

The affects of adrenergic blockade on catecholamine induced changes in ^{14}C-urea influx and vascular resistance show that both α and β adrenergic receptors are involved in blood

pathway regulation in the gill. The ^{14}C-urea uptake caused by NEPI or EPI was reduced by α or β blockade, but the NEPI and EPI effects were not eliminated unless both α and β blockers were used simultaneously (Figures 8 and 10). Double (α + β) blockade was also necessary to eliminate all of the NEPI or EPI effects on resistance (Figures 13 and 14).

Neural control of branchial vascular resistance is suggested by indirect evidence from this and other studies. Published reports on gill innervation support this view (Gilloteaux, 1969; Nicol, 1952) as well. But the probable importance of circulating catecholamines (and possibly other hormones) in controlling resistance is not to be discounted. Rather, it is likely that hormonal and neural controls act in opposition to each other thereby allowing fine adjustment of branchial vascular resistance, blood flow pattern, and functional surface area of the gill.

In this study the rather dramatic effects of ACH on vascular resistance invite speculation that vascular smooth muscle in the gill is under some tonic cholinergic control--a view that has been advanced previously (Richards and Fromm, 1969). The resistances increased significantly when ACH was increased from 10^{-8} to 10^{-7}M or from 10^{-7} to 10^{-6}M. The ACH effect on resistance was blocked by atropine and hexamethonium (Figure 17A and B), which also suggests cholinergic tone. To determine if the ACH effect might be partly due to

stimulation of postganglionic sympathetic fibers causing release of NEPI at neuromuscular junctions, blockade with phenoxybenzamine and phentolamine (both α adrenergic blockers) was attempted (Figure 17C and D). Phentolamine had little effect even at 10^{-4}M, while phenoxybenzamine completely blocked the ACH induced resistance effect. Although this information seemed contradictory, it was later learned that phenoxybenzamine could block ACH effects directly (Goodman and Gilman, 1965). The inability to effect blockade with phentolamine, then, indicates little or no indirect adrenergic (sympathetic) nerve involvement in the ACH effect. The effect of reserpine on the ACH effect (Figure 17E) was inconclusive in that control resistance always increased while the reserpine was perfused, and the maximum resistance obtained with ACH was essentially unchanged.

Since all of the evidence is indirect and/or inconclusive, neural control of branchial vascular resistance remains unproven. It will probably be necessary to show resistance changes in response to autonomic nerve stimulation before neural control can be accepted.

CONCLUSIONS

In this thesis the relative functional surface area of isolated-perfused gills was evaluated by measuring the influx of ^{14}C-urea, a passively diffusing molecule.

1. The functional surface area of rainbow trout gills can be regulated by changing perfusion pathway with adjustments in the relative vascular resistance across the different pathways.

2. The catecholamines, norepinephrine and epinephrine increase functional gill surface area and decrease overall branchial vascular resistance.

3. Acetylcholine decreases both functional gill surface area and overall branchial vascular resistance.

4. Both α and β adrenergic receptors are found in rainbow trout gills.

5. Stimulation of α adrenergic receptors increases both functional surface area and branchial vascular resistance, while β adrenergic receptor stimulation increases functional surface area but decreases branchial vascular resistance.

6. Vascular smooth muscle tone may be under cholinergic (parasympathetic) nervous control, while circulating catecholamines counteract this tonus to effect an overall decrease in branchial vascular resistance and increase in functional surface area.

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APPENDIX

Composition of Ringer Solution

NaCl	7.37 g/liter
KCl	0.31 g/liter
$CaCl_2$	0.10 g/liter
$MgSO_4$	0.14 g/liter
KH_2PO_4	0.46 g/liter
Na_2HPO_4	2.02 g/liter
Glucose	0.90 g/liter

pH 7.3, 290 mOsm/Kg

Composition of Non-Nutrient Ringer Solution

NaCl	8.505 g/liter
KCl	0.105 g/liter
$CaCl_2$	0.205 g/liter

pH 7.8, 290 mOsm/Kg

Composition of Scintillation Cocktail

PPO (2,5-diphenyloxazole)	7.84 g
Bis MSB (p-bis-(o-methylstyryl)benzene)	0.16 g
p-Dioxane	to 1 liter