MOVEMENT OF DRUGS ACROSS THE GILLS OF FISHES

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INTRODUCTION

The physiochemical properties of drugs that influence their movement across biological membranes are fairly well established and include lipid solubility, water solubility, degree of ionization, chemical stability, and molecular weight. In general, any substance that is distinctly charged, or is otherwise fat-insoluble, and has a molecular weight in excess of 100 is almost certain to be virtually excluded from entering cells from the outside unless the particular cell is equipped with a specific transport system for this class of substance (Le Fevre 1).

Historically, much of our knowledge about drug absorption was developed using plant and animal cells. Experimentation on whole animals has mainly been centered on homeotherms with little work on poikilotherms, especially the fishes. The available literature on fishes deals essentially with the influence of lipid solubility on movement of drugs across the gills. Much of the work we reveiw was not performed to study drug dynamics in fishes per se but to evaluate chemicals for use in fisheries. With these limitations in mind, we attempt to use data on toxicity, efficacy, and residues of three chemicals—a weak base (MS-222), a base (quinaldine), and an acid (TFM)—to show that both lipid solubility and degree of ionization play a significant role in the movement of these compounds across the gills of fishes.

FISH: THE EXPERIMENTAL ANIMAL

Fish have been used as experimental animals since the eighteenth century (Nigrelli 2). More recently, fish and goldfish *(Carassius auratus)* in particular have been used in testing the toxicity and pharmacologic properties of drugs (Powers 3, Cutting et al 4, Levy & Gucinski 5, Levy & Miller 6, Gibaldi & Nightingale 7, Nightingale & Gibaldi 8).

One of the major difficulties in using fish as test animals is quality control, especially when they are taken directly from the wild. One normally has to assume a fish is healthy if it shows no overt symptoms of disease and if its behavior is "normal" under the circumstances in which it is held (Hunn et al 9). In working with goldfish, many authors equate size of fish with age. This does not, however, assure that the fish are the same chronological age, because they may be stunted intentionally during culture to keep them of marketable size over various periods of time.

Wild fish are inadvertently exposed to various xenobiotics in their environment that can influence the level of drug-metabolizing enzymes (De Waide 10). On the other hand, species raised under intensive culture may be deliberately exposed to various chemicals such as therapeutic agents, used to control parasites or bacterial infections, and herbicides used to control algae, etc. Many times it is difficult, if not impossible, to get information concerning chemical treatments the fish have been subjected to during their life.

Blood flow through the gills of fishes is variable and can be influenced by the pharmacologic action of the drug being taken up. In vitro studies by Steen & Kruysse (11) and Richards & Fromm (12) have demonstrated a variable shunt system with regulation of filamental and lamellar flow by vasoconstriction and vasodilation. Fromm et al (13) showed that the rate of fluid flow in isolated-perfused gill of rainbow trout *(Salmo gairdneri)* could be reduced as much as 24% by the commonly used anesthetic tricane methanesulfonate (MS-222). Indeed, various studies including those of Houston et al (14) indicate that MS-222 does influence the cardiovascular and respiratory system to the extent that the uptake of the drugs would be affected through reduced blood flow and movement of water across the gills. The movement of the drug across the gills, therefore, should be studied with the possibility in mind that its pharmacological action may well influence the rate of uptake.

A disturbing aspect of the methods used in testing drugs against fish is the use of distilled water as a test medium. Most fish, especially goldfish, are held in tap water or well water that is similar in chemical composition to the water in which they were cultured. Although the fish are hyperosmotic to this medium, it does not impart the osmotic stress that transfer to distilled water does. The use of reconstituted water of known composition that can be buffered to a desired pH is certainly more desirable than distilled water (Marking 15).

LIPID-SOLUBLE DRUGS

Since the turn of the century, investigators have shown that many drugs appeared to penetrate cells by diffusion at rates that could be correlated with their solubility in fat solvents. In addition, evidence has accumulated that indicates that the unionized form of most drugs is the form which penetrates the cell membranes most readily. Despite some well-documented exceptions (Keberle 16), the passage of drugs across cell membranes is governed mainly by physical processes and is predictable from the pK_a and lipid solubility of the drug.

MS-222: A Weak Base

The methanesulfonate of *meta*-aminobenzoic acid ethyl ester has been used to anesthetize poikilothermic vertebrates for over 40 years (Schoettger 17). But only recently have there been attempts to understand the pharmacodynamics of this drug. In the 1960s the toxicity of MS-222 was defined under controlled conditions for four freshwater salmonids and an ictalurid (Marking 18, Schoettger et al 19). Residucs of free and acetylated MS-222 were measured in these species following anesthesia using a modified Bratton-Marshall method (Walker & Schoettger 20). This method of analysis did not distinguish whether or not the residue was an acid or an ester. The residue studies indicated that the drug was eliminated rapidly by these fish, after transfer to fresh water.

In 1968 a study on the marine dogfish, *Squalus acanthias,* established some interesting concepts about the physicial properties of MS-222 as well as the routes of excretion following injection (Maren et al 21). The pK_a of MS-222 was determined to be 3.5, and the partition coefficient using chloroform:water buffered to pH 7.4 was 312, a very high lipid solubility.

A divided box technique was used to separate gill excretion from urinary excretion in dogfish injected intraperitoneally (ip) with MS-222 at the rate of 21 mg/kg. During a 170 min postinjection period, 26,000 μg were excreted across the gills compared to 10 μg in the urine, thus indicating that the gills were the major route of excretion. Using data from the divided-box experiment, the authors calculated a plasma half-life of the drug of 4 hr. This calculation assumed excretion only via the gill, as their data had shown that the renal contribution was negligible. The actual plasma half-life in free-swimming fish was found to be about 1.5 hr, suggesting that excretory mechanisms other than gill or renal function were involved. Biotransformation, biliary excretion, and/or increased blood flow through the gills during swimming could not be ruled out, as they were not investigated. This study did establish, however, that diffusion across the gill, not renal excretion, was the major route of elimination of MS-222 following ip injection. Passage of the lipid-soluble free base through the gill could be expected, as only 0.01% of the MS-222 would be ionized at body pH.

Hunn et al (22) using rainbow trout, *Salmo gairdneri,* found that MS-222 does indeed cross the gills of fishes in either direction. Trout were anesthetized in a 100-mg/liter solution of MS-222, and the excretion of the drug was followed for 24 hr. Whole blood concentrations of the free drug in trout, after 2.0–2.5 min in the anesthetic bath, reached about 74% of the bath concentration. Blood concentrations of MS-222 declined rapidly during the postanesthesia period in fresh water and reached background levels in 8 hr. In trout given ip injections of MS-222 (10 mg/kg), only 15–21% of the dose was excreted via the kidney during 24 hr, postinjection. Injection of 10 to 100 mg/kg ip had no anesthetizing action on the trout. Using data from these studies, the authors indicated that the major route of elimination was extrarenal, presumbaly via the gills. Thus, MS-222 moves rapidly across the gills during the short anesthetization period and given ip in doses as great as 100 mg/kg, it did not induce anesthesia.

The rapid movement of MS-222 across the gills of channel catfish *(Ictalurus punctatus)* was observed during experiments designed to measure brain concentrations of the drug necessary to induce anesthesia (Hunn 23). Catfish were exposed to a 200 mg/liter solution of MS-222 at 17°C for up to 11 min. These fish exhibited loss of equilibrium after 1 min, loss of reflex in 3 min, and were approaching medullary collapse in 11 min. Whole blood concentrations of free drug were 35% of that of the anesthetic bath in 0.5 min and 50% in 1 min. At loss of reflex, the concentration averaged about 70% of that of the anesthetic solution. Brain tissue at loss of reflex contained the highest average concentration of all brain samples taken during the 11 min exposure. Catfish placed into fresh water after 3 min of exposure to a 200 mg/liter solution of MS-222 righted themselves in 2–3 min and were actively swimming in 8 min. The estimated whole blood half-life of free MS-222 in those fish was 6 min. This study again shows the rapid rate of movement of this drug across the gills of fishes. Further studies on nine other species of freshwater fishes confirmed the observations made on the uptake of the drug by channel catfish (Hunn 24).

Studies on anesthesia of brook trout, *Salvelinus fontinalis,* also revealed rapid movement of MS-222 into the blood following immersion in an anesthetic bath at temperatures as low as 3.5°C. Clearance of the anesthetic from the blood was estimated to be 90% in 55 min at 5°C (Houston & Woods 25).

Investigation of the pharmacology of MS-222 in *Squalus acanthias* confirms that the drug can move across the gills of fishes in either direction with great facility (Stenger & Maren 26). This study also shows that the time course of anesthesia is best correlated with the level of free drug in arterial blood. The authors also conclude that MS-222 has a local anesthetic action.

These studies on MS-222 revealed that the drug probably moves across the gills of fishes by diffusion. The rate of diffusion is rapid and apparently associated with high lipid solubility of the free base and the concentration gradient. Other factors influencing the movement of the drug and ultimate equilibration are biotransformation, tissue distribution, plasma protein binding, and renal and biliary excretion. Biotransformation of MS-222 is evident in the appearance in blood and tissues of the hydrolysis product (free acid) as well as in the acetylated amino congener. Preliminary work in this laboratory indicates that the gallbladder may be a site of accumulation of the acid metabolite(s). Species differences in ability to cleave the ester bond of MS-222 also are evident (Luhning 27). Apparently renal excretion of MS-222 or its acetylated derivative is minor; however, the kidney is the major route for elimination of acid metabolites. This deserves further study as most evidence is still circumstantial. In addition, the dymamics of the drug and its metabolites in bile need illumination.

Quinaldine: A Base

The potential of quinaldine (2-methylquinoline) as a fish anesthetic was first reported by Muench (28) in 1958. Greenough (29) patented a fish transport medium containing a buffer, an antibiotic, and quinaldine. Schoettger & Julin (30) evaluated the efficacy of quinaldine as an anesthetic for seven species of fish. In their report they cited a survey of chemicals used at national fish hatcheries that indicated quinaldine had been used as an anesthetic for a wide variety of fish. They reported that quinaldine concentrations of 15 or 16 mg/liter rapidly induced loss of equilibrium in trout and that 15 to 30 mg/liter are effective on channel catfish, bluegill

(Lepomis macrochirus), and largemouth bass *(Micropterus salmoides).* They reported that its major assets include rapid action and prolonged maintenance of anesthesia. However, fish retain a degree of reflex responsiveness.

Baldridge (31) reported in 1969 that quinaldine produced rapid anesthesia in young lemon sharks, *Negaprion brevirostris,* and that recovery was rapid. The following year Trams & Brown (32) found that quinaldine tended to be more concentrated in shark tissues of high lipid content than in muscle or whole blood. Their studies also showed fast uptake of quinaldine from bath solutions and rapid recovery of the sharks from anesthesia following withdrawal from the drug. Brown et al (33) reported rapid uptake of quinaldine by sharks with the highest concentrations found in the brain. They also reported that routes of entrance of quinaldine other than the gill cannot be excluded. They found that an electric eel exposed to 0.5 mmol/liter of quinaldine absorbed the drug at a rate of 4.3 μmol/min. The authors (33) suggest that the gills of this species would not function in the drug absorption process; they therefore concluded that drug uptake proceeded through the integument.

Sills & Harman (34) reported that quinaldine produced rapid anesthesia in striped bass, *Morone saxatilis.* Concentrations of 25 to 55 mg/liter of quinaldine produced loss of equilibrium within 2–5 min. Striped bass exposed to 40 mg/liter of quinaldine for 10 min were analyzed by gas chromatography and were found to contain muscle residues of 2.13 mg/kg. The fish recovered rapidly from anesthesia, and the half-life of the quinaldine residue in muscle was approximately 1 hr. Sills et al (35) found that muscle residues of quinaldine in ten species of coldwater and warmwater fish followed the same general pattern as that found in striped bass.

Unpublished work at our laboratory demonstrates that largemouth bass exposed to 30 mg/liter of quinaldine for 15 min accumulate the highest concentrations (25.4 μg/g) of quinaldine residue in the brain. Brain concentrations of quinaldine after 15 min of withdrawal of fresh water were 4.8 μg/g. Rainbow trout exposed to 1 mg/liter of quinaldine for 12 hr had plasma concentration of 3.1 μg/ml. The plasma concentrations decreased to 0.4 μg/ml after 4 hr of withdrawal from the anesthetic. Two channel catfish were catheterized and exposed to 25 mg/liter of quinaldine sulfate in a metabolism chamber with a flow of 1200 ml/min for 1 hr. Urine collected during 3 hr after termination of the exposure contained 2.7 and 1.8 μg of quinaldine/ml, while those collected 8 hr after termination of exposure contained 1.7 and 0.4 μg of quinaldine/ml. No quinaldine was detected in the urine collected 11 and 24 hr after termination of the exposure. When rainbow trout were exposed to 15 mg/liter of quinaldine sulfate for 30 min, the bile contained 25.7 μg/ml quinaldine residue, which was approximately twice the plasma concentration. The small amount of quinaldine excreted in the urine indicates this is not a major route of excretion of quinaldine. Also, no large concentration of quinaldine has been found in the gallbladder bile. These samples were analyzed by the gas chromatographic and ultraviolet spectrophotometric methods of Allen & Sills (36, 37). Some metabolites would not be detected by these methods. These studies indicate that quinaldine probably moves outward across the gills of fishes by diffusion. Other possible routes of elimination of the drug include renal excretion and biotransformation, although renal excretion of quinaldine appears to be a minor route of elimination of the drug and no metabolites of quinaldine have been found as yet. The drug appears to be excreted in its original form. However, this should be investigated further with ^{14}C-labeled quinaldine.

Schoettger & Julin (30) reported an effect of pH on the efficacy of the anesthetic in 1969. The drug was completely ineffective as an anesthetic on seven species of fish tested at pH 5.0. Also, when smallmouth bass, *Micropterus dolomieui,* were anesthetized with 20 mg/liter of quinaldine at pH 7.0, they recovered from anesthesia within 20 to 25 min after the pH was lowered to 5.0. Channel catfish were not affected by exposure to 50 or 60 mg/liter of quinaldine for 1 hr at pH 5.0. However, when the pH of the former solution was raised to 7.0 and that of the latter to 10.3, the fish were narcotized in 3 to 10 min. Marking (38) noted that quinaldine is more toxic to fish in soft water than in hard water and that the soft water used had a lower pH than hard water. Sills & Allen (39) in 1971 correlated the effect of pH on the efficacy and residues of quinaldine with the ionization constant of quinaldine ($pK_a = 5.42$). Quinaldine was partitioned between hexane and aqueous solutions at pH 4, 5, 6, 7, and 8. The percent of quinaldine found in the hexane at each pH compared very closely with the percent quinaldine existing in the free base form as calculated from the pK_a.

Sills & Allen (39) also demonstrated that the muscle residue of quinaldine in largemouth bass exposed to 35 mg/liter of quinaldine for 15 min was lower at pH 5 (2.15 μg/g) than at pH 7 (5.37 μg/g) or pH 8 (4.89 μg/g). Calculating the amount of quinaldine-free base available in aqueous solutions at pH 5.0, they were able to anesthetize largemouth bass using 125 mg/liter quinaldine (34.4 mg/liter of quinaldine-free base). The residue of quinaldine found in the muscle of these fish (3.91 μg/g) was comparable with the quinaldine residue found in the muscle of those fish exposed to the anesthetic at pH 7 and 8. At the lower pH, 72.5% of the quinaldine is protonated and exists as the lipid-insoluble quinaldinium ion that does not readily cross the gill membrane. At pH 7, 97.4% of the quinaldine is in the lipid-soluble free base form. The biological activity of this compound in fish is altered drastically by the pH of the media in which they are exposed. The effect of the ionic equilibrium of the molecule does not, however, affect efficacy of the compound in most situations where it is used. The efficacy, toxicity, and residues of the chemical all indicate that the lipid-soluble free base of quinaldine is the form that passes across the gills of fishes.

TFM: An Acid

The selective larval lampricide 3-trifluormethyl-4-nitrophenol (TFM) is used to control the sea lamprey *(Petromyzon marinus)* in the Great Lakes (Schnick 40). Work at this laboratory to retain the registered use of this compound has involved studies on the toxicity to and residues in nontarget fishes. The toxicity of TFM to chinook salmon *(Oncorhynchus tshawytscha)* is greater in soft water than in hard water, and is pH dependent (Table 1). Similar results were obtained with six other freshwater species of fish (Marking & Olson, unpublished). Safety of the compound to fish can be predicted knowing the pK_a (TFM—6.07) and the LC50 at a particular

Table 1 Toxicity of TFM (35.7%) to fingerling chinook salmon at selected temperatures, hardnesses, and pH's in reconstituted water

Temp. (°C)	Water hardness	pH	LC50 and 95% confidence interval (μl/l) at 24 hr	96 hr
7	soft	7.5	6.22 5.51–7.03	4.27 3.89–4.69
12	soft	7.5	5.98 5.09–7.03	4.20 3.52–5.02
17	soft	7.5	5.38 4.83–5.99	3.54 3.19–3.92
12	very soft	6.6	3.68 3.20–4.23	1.94 1.62–2.33
12	hard	7.8	16.0 15.2–18.7	10.0 8.98–11.1
12	very hard	8.2	41.5 36.0–47.8	19.0 16.2–22.2
12	soft[a]	6.5	3.12 2.77–3.52	2.45 2.16–2.77
12	soft[a]	8.5	33.4 28.9–38.6	18.0 15.4–21.0
12	soft[a]	9.5	254 215–300	171 154–190

[a] Buffered reconstituted water after Marking (15).

pH. The effect of pH on the toxicity of TFM, therefore, appears correlated with the concentration of lipid-soluble free phenol. This observation is supported further by the partitioning of TFM between an organic solvent, chloroform or hexane, and aqueous buffers between pH's 4 and 9. The amount of TFM in the organic phase followed closely the amounts of free phenol at various pH's.

Residues of TFM in the muscle tissue of fish exposed to the lampricide at various pH's also reflect the unavailability of free phenol at high pH's (Figure 1). For example, when channel catfish were exposed to a 1 mg/liter solution of TFM (18.5°C) for 12 hr at pH's 6, 7, 8, and 9, the free TFM residues in muscle were 3.21, 1.5, 0.33, and 0.03 μg/g, respectively.

Biotransformation probably also plays a role in the movement of TFM across the gills of trout. Lech & Costrini (41) and Lech (42) demonstrated that the major metabolite of TFM in rainbow trout is the glucuronide conjugate which is excreted largely in the bile. Hunn & Allen (unpublished) exposed rainbow trout to a 5 mg/liter solution of TFM in well water at 12°C for 15, 30, 45, 60, and 120 min.

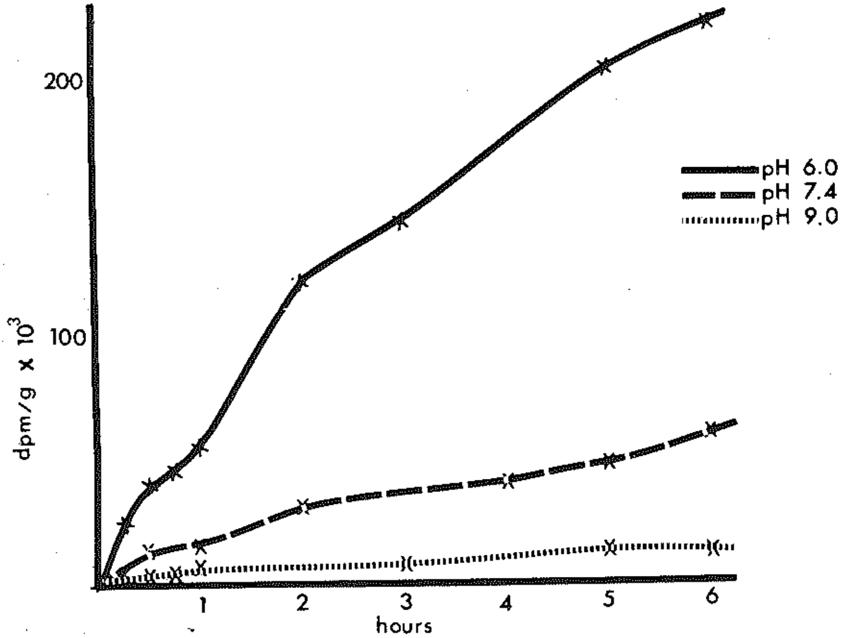

Figure 1 The uptake of ^{3}H-labeled TFM by rainbow trout at three pH's on a whole fish basis.

The concentration of free TFM in the gallbladder bile was 4.12 μg/ml after 120 min while the concentration of TFM glucuronide rose to 196 μg/ml during the same period. The plasma concentration of free TFM was 2.73 μg/ml after 120 min while the glucuronide concentration was 0.87 μg/ml. The rapid rise in accumulation of glucuronide in the bile started between 45 and 60 min of exposure (14 μg/ml to 55 μg/ml). Biotransformation of TFM to the water-soluble glucuronide would reduce its passage across the gills.

No attempts have been made to measure the movement of TFM back across the gills following exposure to the lampricide, although one might predict that outward diffusion may be slower than with the basic compounds discussed previously, because TFM exists mostly as the phenolate ion at the pH of blood. In spite of this, TFM is promptly cleared from fish muscle during 24 hr of recovery in fresh water, and the half-time of free TFM in blood of rainbow trout injected with 2.5 mg/kg TFM ip was shown to be 0.78 hr (Lech et al 43). It is interesting to note that during the rapid decline in free TFM blood levels of ip-injected trout, a large percentage of the injected dose is recovered in bile as the glucuronide conjugate (Lech et al 43). This phenomenon may be related to the lack of rapid gill diffusion due to the presence of high levels of the phenolate ion in blood with subsequent presentation of relatively large amounts of TFM to the kidney and liver for conjugation and excretion via the biliary and urinary routes.

TFM, then, is an example of a phenolic compound whose movement across the gills is influenced both by the pH of the medium the fish is exposed in as well as by the biotransformation and excretion of a metabolite of the parent compound.

ACKNOWLEDGMENTS

The authors wish to thank Dr. John J. Lech of the Medical College of Wisconsin for his encouragement and use of unpublished data.

Literature Cited

1. Le Fevre, P. G. 1972. *Absorption, Distribution, Transformation and Excretion of Drugs*, ed. P. K. Knoefel, 5–38. Springfield: Thomas. 210 pp.
2. Nigrelli, R. F. 1953. *Trans. NY Acad. Sci.* 15:183–86
3. Powers, E. B. 1917. *Ill. Biol. Monogr.* 2:7–73
4. Cutting, W., Baslow, M., Read, D., Furst, A. 1959. *J. Clin. Exp. Psychopathol.* 20:26–32
5. Levy, G., Gucinski, S. P. 1964. *J. Pharmacol. Exp. Ther.* 146:80–86
6. Levy, G., Miller, K. E. 1965. *J. Pharm. Sci.* 54:1319–24
7. Gibaldi, M., Nightingale, C. H. 1968. *J. Pharm. Sci.* 57:226–30
8. Nightingale, C. H., Gibaldi, M. 1971. *J. Pharm. Sci.* 60:1360–63
9. Hunn, J. B., Schoettger, R. A., Whealdon, E. W. 1968. *Prog. Fish Cult.* 30: 164–67
10. De Waide, J. H. 1971. *Metabolism of Xenobiotics.* Drukkerij Leijn: Nijmegen. 164 pp.
11. Steen, J. B., Kruysse, A. 1974. *Comp. Biochem. Physiol.* 12:127–42
12. Richards, B. D., Fromm, P. O. 1969. *Comp. Biochem. Physiol.* 29:1063–70
13. Fromm, P. O., Richards, B. D., Hunter, R. C. 1971. *Prog. Fish Cult.* 33:138–40
14. Houston, A. H., Madden, J. A., Woods, R. J., Miles, H. M. 1971. *J. Fish. Res. Bd. Can.* 28:625–33
15. Marking, L. L. 1969. *Bull. Wildlife Disease Assoc.* 5:291–94
16. Keberle, H. 1971. *Acta Pharmacol. Toxicol.* 29:30–47
17. Schoettger, R. A. 1967. *US Bur. Sport Fish. Wildl. Invest. Fish Contr.* 16:1–15
18. Marking, L. L. 1967. *US Bur. Sport Fish. Wildl. Invest. Fish Contr.* 12:1–10
19. Schoettger, R. A., Walker, C. R., Marking, L. L. 1967. *US Bur. Sport Fish. Wildl. Invest. Fish Contr.* 17:1–14
20. Walker, C. R., Schoettger, R. A. 1967. *US Bur. Sport Fish. Wildl. Invest. Fish Contr.* 14:1–10
21. Maren, T. H., Embry, R., Broder, L. E. 1968. *Comp. Biochem. Physiol.* 26: 853–64
22. Hunn, J. B., Schoettger, R. A., Willford, W. A. 1968. *J. Fish. Res. Bd. Can.* 25: 25–31
23. Hunn, J. B. 1968. *US Bur. Sport Fish. Wildl. Resour. Publ.* 64:119–20
24. Hunn, J. B. 1970. *US Bur. Sport Fish. Wildl. Invest. Fish Contr.* 42:1–8
25. Houston, A. H., Woods, R. J. 1972. *J. Fish. Res. Bd. Can.* 29:1344–46
26. Stenger, V. G., Maren, T. H. 1973. In press
27. Luhning, C. W. In press
28. Muench, B. 1958. *Prog. Fish Cult.* 20: 42–44
29. Greenough, E. E. 1963. Patent No. 3,110,285
30. Schoettger, R. A., Julin, A. M. 1969. *US Bur. Sport Fish. Wildl. Invest. Fish Contr.* 22:1–10
31. Baldridge, D. H. Jr. 1969. *Bull. Mar. Sci.* 19:880–96
32. Trams, E. G., Brown, E. A. B. 1970. *Life Sci.* 9:27–35
33. Brown, E. A. B., Franklin, J. E., Pratt, E., Trams, E. G. 1972. *Comp. Biochem. Physiol.* 42A:223–31
34. Sills, J. B., Harman, P. D. 1970. *Proc. Southeast. Assoc. Game Fish Comm.* 24:546–49
35. Sills, J. B., Allen, J. L., Harman, P. D., Luhning, C. W. In press
36. Allen, J. L., Sills, J. B. 1970. *JAOAC* 53:20–23
37. Allen, J. L., Sills, J. B. 1970. *JAOAC* 53:1170–71
38. Marking, L. L. 1969. *US Bur. Sport Fish. Wildl. Invest. Fish Contr.* 23:1–10
39. Sills, J. B., Allen, J. L. 1971. *Trans. Am. Fish. Soc.* 100:544–45
40. Schnick, R. A. 1972. *U.S. Bur. Sport Fish. Wildl. Invest. Fish Contr.* 44:1–31
41. Lech, J. J., Costrini, N. V. 1972. *Comp. Gen. Pharmacol.* 3:160–66
42. Lech, J. J. 1973. *Toxicol. Appl. Pharmacol.* 24:114–24
43. Lech, J. J., Pepple, S., Anderson, J. 1973. *Toxicol. Appl. Pharmacol.* 25:542–52