INVESTIGATIONS IN FISH CONTROL

41. Identification of MS-222 Residues in Selected Fish Tissues by Thin Layer Chromatography

42. Dynamics of MS-222 in the Blood and Brain of Freshwater Fishes During Anesthesia

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United States Department of the Interior
Fish and Wildlife Service
Bureau of Sport Fisheries and Wildlife

INVESTIGATIONS IN FISH CONTROL

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Continued on inside back cover--

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CONTENTS

IDENTIFICATION OF MS-222 RESIDUES IN SELECTED FISH TISSUES BY THIN LAYER CHROMATOGRAPHY

By John L. Allen, Chemist,
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ABSTRACT.--MS-222, a commonly used fish anesthetic, reacts with the Bratton-Marshall reagents to form a wine-red dye. Residues of MS-222 determined by this reaction are not distinguished from other primary aromatic amines. Thin layer chromatography was used to identify MS-222 in the presence of background primary aromatic amines in fish muscle, brain, and blood. This method, in which the Bratton-Marshall reaction is used to visualize the spots, gave both the specificity of the Bratton-Marshall reaction for primary aromatic amines and the R_f of MS-222 as tools for identification of the residues. Recoveries of 25 to 60 percent were obtained in muscle samples spiked with 2 to 10 ppm of MS-222. Quantitative estimation was difficult in samples spiked with 2 ppm or less, but presence of MS-222 residues could be confirmed in samples spiked with as little as 0.2 ppm. Since the meta-aminobenzoate ester can be identified at these concentrations, this should be a useful ancillary or confirmatory method for determining the rate of disappearance of drug residues in fish flesh and obtaining data for clearance and registration of the anesthetic with the Food and Drug Administration.

MS-222 (methanesulfonate of meta-aminobenzoic acid ethyl ester) is used extensively as an anesthetic for fish. Walker and Schoettger (1967) described a method for quantitative determination of MS-222 residues in fish tissues using a modification of the Bratton-Marshall (1939) method for sulfonamides. MS-222, a primary aromatic amine, gives a wine-red color when reacted with the Bratton-Marshall reagents. Since other primary aromatic amines give the same color, a method for specific identification of MS-222 residues is needed.

Thin layer chromatography has been useful in identifying primary aromatic amines. This technique was used by Bican-Fister and Kajganovic (1963) to visualize sulfonamides on thin layer plates by spraying the plates with modified Bratton-Marshall reagents. We chose to investigate the application of thin layer chromatography, using modified Bratton-Marshall reagents, to visualize MS-222 residues in fish tissue. This system offers both the specificity of the Bratton-Marshall reaction for primary aromatic amines and the R_f of MS-222 as tools for the identification of the compound.

METHODS AND MATERIALS

Experiments on operating parameters

Various solvent systems were investigated for possible use as developers of the chromatograms on silica gel chromatography sheets without fluorescent dye. When a solvent system of 2-percent methanol in benzene was

used, MS-222 gave an R_f of approximately 0.5. However, a substance occurring in certain untreated carp (Cyprinus carpio) and goldfish (Carassius auratus) tissues gave the same red color with about the same R_f as MS-222 when this system was employed. This problem was overcome by using a solvent system of 91 percent benzene, 4 percent acetic acid, and 5 percent ethyl ether. With this system MS-222 gave an R_f of about 0.45, while the interfering substance in carp and goldfish tissue did not migrate above the spotting line.

We used the diazo coupling agent of Bratton and Marshall (1939) to develop a red-colored azo dye in the MS-222 spot on the chromatogram. This diazotization reaction with MS-222 was described in detail by Walker and Schoettger (1967). We found that this coupling reagent in spray solution gave the darkest spot when 0.2-percent sodium nitrite in 1.5-percent hydrochloric acid was used. After the plates were sprayed with the acidic nitrite solution, they were dried in a stream of hot air to destroy the excess nitrous acid. A coupling reagent of 0.1-percent N-1-naphthylethylenediamine dihydrochloride in water was satisfactory.

The minimum time required for the extraction of MS-222 residues from fish muscle in a Soxhlet extractor was determined. Fish muscle samples fortified with MS-222 were analyzed after 4, 8, 12, 16, and 24 cycles. Extraction was essentially complete after 8 cycles.

Reagents and apparatus

Reagents and apparatus were as follows[1]:

1. Methanol, reagent grade.
2. Petroleum ether, reagent grade.
3. Florisil, 100-200 mesh.
4. Alumina, neutral, Brockman activity 1, 80-200 mesh.
5. Developing solution: 91-percent benzene (pesticide grade), 4-percent acetic acid (reagent grade), and 5-percent ethyl ether (U.S.F.).
6. 0.2-percent sodium nitrite in 1.5-percent hydrochloric acid: Dissolve 0.20 g of sodium nitrite in 50 ml of water, add 1.5 ml of concentrated hydrochloric acid and dilute to 100 ml with distilled water. Make fresh daily.
7. 0.1-percent N-1-naphthylethylenediamine dihydrochloride: Dissolve 0.10 g in 100 ml of distilled water. Store in a dark container, refrigerate, and make fresh weekly.
8. Standard solution of MS-222: Dissolve 10.0 mg of MS-222 in 100 ml of methanol.
9. Tissue homogenizer.
10. Silica gel thin layer plates, Eastman Chromogram Sheet without fluorescent dye, 20 x 20 cm.
11. Micropipettes, 1, 5, and 10 μl.
12. Chromatography tank, 4 by 8 by 9 inches, lined with absorbent paper.
13. Chromatographic column, 400 x 24 mm I.D. with sealed in, coarse fritted disk.
14. Soxhlet extraction apparatus, I.D. of extraction tube 30 mm ; 80 x 25 mm thimbles.
15. Teflon coated muffin pan.

Tissue collection

Blood samples are drawn from specimens by cardiac puncture. (Walker and Schoettger, 1967) with a heparinized syringe fitted with an 18- or 20-gage needle.

Other tissue samples are collected from fish after killing them by a blow on the head or by pithing. Brain samples are dissected out of the immobilized fish, and muscle samples are collected by filleting the fish.

Sample preparation and extraction

Blood samples are extracted by adding 0.5 ml blood to 9.5 ml methanol, mixed thoroughly and applied to the chromatographic column for cleanup.

Brain samples are extracted by homogenizing 1 g of brain, or the entire brain if it weighs less than 1 g with 5 ml of methanol. The homogenate is then placed in the chromatographic column for cleanup.

[1]Reference to a company or product does not imply recommendation to the exclusion of others that may be suitable.

Muscle samples (5 g) are taken from a homogenate of the entire fillet, spread thin in the bottom of the muffin pan, and dried at 80°C. for 6 hours. Grind the dried tissue to a powder with a mortar and pestle, transfer to a Soxhlet extraction thimble, and wash with three 15-ml portions of petroleum ether (discard the washings). Air-dry the washed sample and extract with 70 ml of methanol in a Soxhlet for a minimum of 8 reflux cycles. After the final cycle, allow the upper portion of the extractor to fill, but not to siphon over. The extract and the remaining methanol in the boiling flask is ready for column chromatographic cleanup.

Cleanup of extracts

Prepare a 400-mm by 24-mm column by adding 2.5 cm of alumina followed by 7.5 cm of Florisil. Tap the column gently to pack the adsorbent. Prewash the column with 50 ml of methanol. When the methanol wash just sinks into the surface of the column, add the sample extract to the column and begin collecting the eluate in a 100-ml beaker. Discard the methanol prewash. Rinse the flask which contained the extract 3 times with 2-ml portions of methanol, adding each consecutive rinse to the column just as the previous rinse disappears into the surface of the column. As the last of the methanol rinse sinks into the column surface, add 50 ml of methanol and collect the eluate only until the last of the methanol has disappeared into the surface of the column.

Use a hot water bath and a stream of dry air to concentrate the eluate to 3 to 5 ml. Quantitatively transfer the eluate to a 15-ml graduated centrifuge tube with methanol. Place the centrifuge tube in a hot water bath and concentrate the eluate to 0.5 ml under a stream of dry air.

Thin layer chromatography

Mark a spotting line 2.5 cm, and a solvent-front line 12.5 cm, from the bottom of an 8- by 8-inch thin layer plate.

When thin layer chromatography is employed to confirm the presence of MS-222 residues, as determined by the method of Walker and Schoettger (1967), spot a volume of extract equivalent to 0.5 μg of MS-222. To compensate for interferences inherent in the colorimetric procedure, spot 100 μl of extract from samples containing 2.0 ppm or less of MS-222 residue.

When screening unknown samples which may contain MS-222 residues, a maximum of 100 μl of sample extract is spotted on the spotting line. On the same line, spot 50, 10, and 5 μl of extract along with a series of MS-222 standards in the range of 0.1 μg to 1.0 μg.

Thirty minutes before developing the thin layer plate, pour 200 ml of developing solution into a chromatographic tank lined with absorbent paper. Place the thin layer plate in the tank and allow the developing solution to rise to the previously marked solvent-front line. Remove the plate from the tank, mark any deviations of the solvent front, and allow to air-dry in a horizontal position.

Spray the plate with the acidic nitrite solution until the plate is uniformly damp, wait 3 to 5 minutes, and dry in a stream of hot air. When the plate is completely dry, spray with 0.1-percent N-1-naphthylethylenediamine dihydrochloride solution until damp and dry immediately with hot air.

MS-222 is seen as a red spot. The amount of MS-222 can be estimated by comparing the intensity and size of the sample spot to the MS-222 standard spots. MS-222 standards must be run simultaneously with the samples so direct comparison of the R_f of standard and sample can be made on the same plate. After quantitiative estimation is complete, store plate in a dark dry container.

RESULTS AND DISCUSSION

The Bratton-Marshall color reaction is specific for primary aromatic amines. Therefore, any naturally occurring primary aromatic amine or drug containing a primary aromatic amine group develops a color when treated with the Bratton-Marshall reagents.

The presence of low levels of MS-222 residue is difficult to ascertain by the modified Bratton-Marshall method of Walker and Schoettger (1967) because of the background readings obtained from the tissues being analyzed.

Thin layer chromatography separates MS-222 from nine other chemicals containing the primary aromatic amine group. The R_f values for 0.5-μg spots of MS-222 and nine other compounds containing the primary aromatic amine group are shown in table 1. The comparison of R_f values must be made on the same plate since these values may vary between determinations.

The minimum level at which quantitative estimations can be made from MS-222 standard spots was found to be 0.1 μg. The maximum amount of muscle extract that can be spotted on the thin layer plate was found to be approximately 100 μl, which is equivalent to 1 g of tissue.

The efficiency of the method was evaluated by analyzing muscle samples from channel catfish (Ictalurus punctatus) spiked with 0.2 to 10.0 ppm of MS-222 (table 2). Recoveries of MS-222 from samples spiked with 2.0 to 10.0 ppm ranged from 25 to 60 percent. Quantitative estimation becomes difficult at residue levels of 2.0 ppm or less owing to the large amount of sample which must be spotted. When large amounts of samples are spotted, accurate quantitation is prevented by spreading of the spot caused by interfering fats and other extraneous materials. However, presence of MS-222 in muscle tissue was confirmed in samples spiked with as little as 0.2 ppm of MS-222. The samples were spiked by injecting a methanol solution of MS-222 standard into the samples before they were oven-dried.

The method was effective for eliminating background interferences in the analysis of muscle tissue from 8 species of fish (table 3). A red spot was noted only in goldfish and carp, but it did not migrate above the spotting line.

Brain and blood samples from three channel catfish treated with 100 ppm of MS-222 to deep anesthesia were analyzed by thin layer chromatography after 0-hour, 1/2-hour, and 1-hour withdrawals in fresh water. The cleanup on these samples was satisfactory for thin layer chromatography. No recoveries were run on these tissues as the cleanup procedure for blood and brain is identical to that of muscle

Table 1.--R_f values for MS-222 and nine other compounds which produce a red color by the MS-222 thin layer chromatographic method when the 91 percent benzene, 4 percent acetic acid, and 5 percent ethyl ether developing solution was used and 5 μl of a 100-ppm solution of each amine was spotted

Compound	R_f value	R_{MS-222}[1]
Benzocaine	0.48	1.17
MS-222 (tricaine methanesulfonate)	0.41	1.00
Aniline	0.35	0.85
p-Aminobenzoic acid	0.27	0.66
m-Aminobenzoic acid	0.16	0.39
Sulfamerazine	0.07	0.17
Sulfamethazine	0.07	0.17
p-Aminohippuric acid	0.00	0.00
Penicillin G, procaine[2]	0.00	0.00
Sulfanilic acid	0.00	0.00

[1] $R_{MS-222} = \frac{R_f \text{ of sample}}{R_f \text{ of MS-222 standard}}$

[2] Penicillin G, procaine, 300,000 u/cc.

Table 2.--Estimated recovery of MS-222 spiked into 5-g samples of channel catfish muscle as determined by the thin layer chromatographic method

Tr trace

Concentration of spike	Number of fish	Equivalent amount of muscle spotted[1] (g)	Estimated amount of MS-222 detected (μg)	Estimated concentration of MS-222 (ppm)	Percent recovery
Control	4	1.00	0.0	0.0	--
0.2 ppm	4	1.00	Tr	Tr	Tr
0.5 ppm	4	1.00	Tr-0.3	Tr-0.3	Tr-60
1.0 ppm	3	0.50-0.75	Tr-0.6	Tr-0.8	Tr-80
2.0 ppm	4	0.50-1.00	0.5-0.6	0.5-0.6	25-30
5.0 ppm	4	0.10	0.2-0.3	2.0-3.0	40-60
10.0 ppm	4	0.05	0.2-0.3	4.0-6.0	40-60

[1] High and low amounts of tissue spotted do not necessarily coincide with high and low amounts of MS-222 detected.

Table 3.--Analyses of eight species of untreated fish for the presence of naturally occuring interferences by the thin layer chromatographic method for MS-222

Species	Equivalent amount of muscle spotted (g)	Red spot detected	R_f of spot	R_{MS-222}
Northern pike, Esox lucius	1.0	No	--	--
Muskellunge, Esox masquinongy	1.0	No	--	--
Goldfish, Carassius auratus	1.0	Yes	0.00	[1] 0.00
Carp, Cyprinus carpio	1.0	Yes	0.00	[1] 0.00
Channel catfish, Ictalurus punctatus	1.0	No	--	--
Bluegill, Lepomis macrochirus	1.0	No	--	--
Largemouth bass, Micropterus salmoides	1.0	No	--	--
Walleye, Stizostedion vitreum vitreum	1.0	No	--	--

[1] $R_{MS-222} = \frac{R_f \text{ of sample}}{R_f \text{ of MS-222 standard}} = \frac{0.00}{0.41} = 0.00$

tissue. Residues of MS-222 were shown to be present in each sample of blood and brain by a red spot having the same R_f as the MS-222 standards.

Kidney and liver samples could not be analyzed for MS-222 residues by this method, because our cleanup procedure was not effective for these tissues.

Since we were able to effectively isolate, recover, and identify trace concentrations of meta-aminobenzoate ester, this should be a useful ancillary or confirmatory method for determining the rate of disappearance of residues in fish flesh. To obtain clearance and registration of MS-222 as an anesthetic with the Food and Drug Administration, we must generate residue data by analytical methods of sufficient sensitivity and reliability with confirmation by an ancillary method.

REFERENCES

Bican-Fister, T., and V. Kajganovic.
1963. Separation and identification of sulfonamides by thin layer chromatography. Journal of Chromatography, vol. 11, no. 4, p. 492.

Bratton, A. Calvin, and E. K. Marshall, Jr.
1939. A new coupling component for sulfanilamide determination. Journal of Biological Chemistry, vol. 128, p. 537-550.

Walker, Charles R., and Richard A. Schoettger.
1967. Method for determining MS-222 residues in fish. U.S. Bureau of Sport Fisheries and Wildlife, Investigations in Fish Control, No. 14. 10 p.

INVESTIGATIONS IN FISH CONTROL

42. Dynamics of MS-222 in the Blood and Brain of Freshwater Fishes During Anesthesia

By Joseph B. Hunn

UNITED STATES DEPARTMENT OF THE INTERIOR, WALTER J. HICKEL, *SECRETARY*
Leslie L. Glasgow, *Assistant Secretary for Fish and Wildlife and Parks*
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Washington, D.C. · July 1970

CONTENTS

DYNAMICS OF MS-222 IN THE BLOOD AND BRAIN OF FRESHWATER FISHES DURING ANESTHESIA

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ABSTRACT.--Eleven species of freshwater fishes were rapidly anesthetized in solutions of MS-222 containing from 100 to 1,000 milligrams of MS-222 per liter. MS-222 concentrations in blood and brain after 1 minute of exposure indicate that MS-222 rapidly diffuses across the gill and passes the blood-brain barrier. Evidence of metabolism of the drug was seen in the presence of acetylated MS-222 in the blood of all species studied. The concentration of free MS-222 in the brain increased with depth of anesthesia to loss of reflex and then either increased or declined slightly as the fish approached medullary collapse.

MS-222 (methanesulfonate of meta-aminobenzoic acid ethyl ester) is an effective fish anesthetic when administered by immersing fish in a solution or by spraying it on their gills (Schoettger, 1967). In either case, the route of entry is the gills. MS-222 is a lipid-soluble compound which is only 0.01-percent ionized at body pH (Maren, Embry, and Broder, 1968). This lipid solubility most likely accounts for its rapid diffusion across the gills.

Once the drug enters the bloodstream, it is distributed throughout the body. Although the site of action of MS-222 has not been established, it is thought to be in the brain. The blood-brain barrier in fish is known to exclude certain dyes, such as sulfonilic acid, from the cerebrospinal fluid (Rall, 1967). Preliminary investigations by Stenger and Maren (1968) indicate that MS-222 effectively crosses this barrier in the dogfish shark (Squalus acanthias). My studies were designed to extend this observation by measuring the rate of uptake of MS-222 in blood and brain of freshwater fish during the induction of anesthesia.

METHODS AND MATERIALS

Eleven species of fish were obtained from several sources (table 1). All specimens were maintained according to the methods of Hunn, Schoettger, and Whealdon (1968), except carp

Table 1.--Sources and sizes of fish used in the MS-222 uptake studies

Species	Length (inches)	Weight (grams)	Source
Shortnose gar *Lepisosteus platostomus*	21.5-27.5	--	Mississippi River Guttenberg, Iowa
Longnose gar *Lepisosteus osseus*	27.0-33.5	--	Mississippi River Guttenberg, Iowa
Bowfin *Amia calva*	23.0-31.0	--	Mississippi River Guttenberg, Iowa
Rainbow trout *Salmo gairdneri*	11.0-16.0	296-720	National Fish Hatchery Manchester, Iowa
Northern pike *Esox lucius*	10.0-18.0	--	Mead Wildlife Area Marshfield, Wis.
Carp *Cyprinus carpio*	8.5-10.8	140-350	Mississippi River Genoa, Wis.
Spotted sucker *Minytrema melanops*	11.8-14.3	--	Mississippi River Guttenberg, Iowa
Black bullhead *Ictalurus melas*	7.0-9.6	84-220	Mead Wildlife Area Marshfield, Wis.
Channel catfish *Ictalurus punctatus*	9.8-15.8	104-540	Mississippi River Lansing, Iowa
White bass *Roccus chrysops*	12.2-14.0	435-620	Mississippi River Guttenberg, Iowa
Bluegill *Lepomis macrochirus*	7.5-9.3	197-345	National Fish Hatchery Fairport, Iowa

and black bullheads, which were held at 17° C. The anesthetic solution of MS-222 in well water was made up fresh daily. The MS-222 was technical grade (99.4 percent) methanesulfonate of m-aminobenzoic acid ethyl ester obtained from Sandoz Pharmaceuticals. Desired concentrations of the drug were achieved by adding the crystaline material to measured volumes of well water in 5-gallon stainless-steel pails or in 45- or 100-liter polyethlene tanks. Individual fish were immersed in the anesthetic solution for periods of 1, 3, 5, 8, or 11 minutes. All fish were anesthetized to loss of reflex, and most were nearing medullary collapse in 8 to 11 minutes of exposure.

Blood samples were taken by caudal puncture (Steucke and Schoettger, 1967). The spinal cord of the fish was then severed and the brain removed. Concentrations of MS-222 and background primary aromatic amines in whole blood and brain were determined by the Bratton-Marshall method as modified by Walker and Schoettger (1967). The average concentration of background amines was subtracted from total aromatic amines to determine the concentration of MS-222.

RESULTS

MS-222 moves rapidly across the gills and enters the bloodstream of fishes (table 2). Within 1 minute of exposure, the drug concentration greatly exceeds the background level of primary aromatic amines. The ratio of the highest average concentration of MS-222 in whole blood to that of the anesthetic solution ranged from 0.14 in shortnose gar to 0.83 in rainbow trout.

Background primary aromatic amines in whole blood ranged from 0.6 to 5.4 milligrams per liter (mg/l) as free amines, and 0.0 to 4.0 mg/l as acetylated amines.

In seven of the eleven species, the brain concentration of MS-222 exceeded that of the whole blood after the first minute of exposure (table 2). The brains of all species contained amounts of MS-222 in excess of those in the blood after 3 minutes.

Table 2.--Concentration of MS-222 in whole blood and brain of 11 species of fish during the induction of anesthesia

[Condition of anesthesia for each species listed in table 3]

Species and exposure time	Concentration in ppm									Brain-blood ratio
	Whole blood						Brain			
	Free MS-222			Acetylated MS-222			Free MS-222			
	n	Mean	Range	n	Mean	Range	n	Mean	Range	
Shortnose gar:										
0 min.[1]	2	0.6	0.6	2	1.8	1.0-2.6	2	3.6	3.2-4.0	6.0
1 min	2	143.2	93.8-192.6	2	10.3	0.0-20.6	2	135.6	128.8-142.4	0.95
3 min	2	135.0	135.0	2	8.2	7.8-8.6	2	342.8	301.6-384.0	2.54
5 min	2	108.2	105.4-111.0	2	8.8	8.6-9.0	2	269.6	238.4-300.8	2.49
8 min	2	122.8	95.0-150.6	2	0.9	0.0-1.8	2	230.4	190.8-270.0	1.88
Longnose gar:										
0 min.[1]	2	2.1	1.6-2.6	2	1.2	0.6-1.8	2	2.9	2.4-3.4	1.38
1 min	1	74.7	-	1	2.8	-	2	88.3	58.8-117.8	1.18
3 min	2	135.3	126.4-144.2	2	8.5	0.0-17.0	2	265.1	221.6-308.6	1.96
5 min	2	114.3	110.0-118.6	2	1.7	0.0-3.4	2	277.5	262.4-292.6	2.43
8 min	2	127.1	124.8-129.4	2	8.0	5.8-10.2	2	228.7	225.6-231.8	1.80
Bowfin:										
0 min.[1]	2	3.9	3.2-4.6	2	0.2	0.0-0.4	2	8.6	7.2-10.0	2.21
1 min	2	202.2	163.6-240.8	2	105.1	75.0-135.2	2	58.3	45.6-71.0	0.29
3 min	2	178.5	166.6-190.4	2	64.0	6.6-121.4	2	217.3	157.8-276.8	1.22
5 min	2	186.0	184.6-187.4	2	37.0	34.2-39.8	2	188.8	184.8-192.8	1.02
8 min	2	117.3	100.0-134.6	2	0.0	-	2	222.8	192.8-252.8	1.90

See footnotes at end of table.

Table 2.--Concentration of MS-222 in whole blood and brain of 11 species of fish during the induction of anesthesia--Continued

Species and exposure time	Concentration in ppm									Brain-blood ratio
	Whole blood						Brain			
	Free MS-222			Acetylated MS-222			Free MS-222			
	n	Mean	Range	n	Mean	Range	n	Mean	Range	
Rainbow trout:										
0 min[1]	8	1.7	1.3-2.8	8	0.7	0.4-1.1	12	3.2	2.3-5.6	1.83
1 min	4	69.2	43.3-104.2	4	3.8	0.6-7.9	8	116.0	107.7-125.7	1.68
2 min	5	51.9	42.7-66.8	5	3.1	0.6-6.9	8	145.1	136.8-150.7	2.80
4 min	5	68.6	49.9-82.2	5	1.7	0.0-5.3	8	165.4	159.1-169.2	2.41
6 min	4	68.5	60.9-72.6	4	2.0	0.4-3.1	5	156.8	146.1-172.0	2.29
10 min	5	83.1	66.3-94.2	5	2.9	0.0-4.9	5	154.1	144.9-159.6	1.85
Northern pike:										
0 min[1]	6	1.9	1.0-2.4	5	0.7	0.4-4.0	[2]3	1.8	1.6-2.2	0.95
1 min	6	35.9	23.0-57.6	6	1.5	0.0-3.9	[2]3	66.9	60.6-78.4	1.86
3 min	6	81.9	60.4-111.6	5	3.4	1.3-5.3	[2]3	152.9	140.8-158.6	1.87
5 min	6	86.0	73.0-100.4	5	13.1	4.0-16.8	[2]3	204.6	183.6-223.8	2.38
8 min	6	95.7	83.0-105.6	6	7.7	2.4-11.2	[2]3	248.1	230.4-257.8	2.59
Carp:										
0 min[1]	6	1.4	1.2-2.0	6	0.7	0.0-2.8	[2]3	1.1	1.0-1.4	0.79
1 min	6	63.9	34.4-82.0	6	3.8	0.0-17.2	[2]3	54.6	44.2-66.6	0.85
3 min	6	96.0	78.4-114.0	6	2.5	1.4-2.8	[2]3	164.1	155.8-171.4	1.71
5 min	6	90.2	82.8-97.8	6	17.2	3.4-29.8	[2]3	165.8	151.4-192.6	1.84
8 min	6	100.8	88.8-115.6	5	1.7	0.0-2.6	[2]3	190.0	187.0-192.6	1.88
11 min	6	89.5	72.4-105.0	6	3.5	0.0-6.3	[2]3	156.5	134.6-176.2	1.75
Spotted sucker:										
0 min.[1]	6	1.8	1.6-2.2	6	0.0	-	[2]3	4.9	4.0-6.6	2.7
1 min	2	113.4	86.2-140.6	2	0.0	-	[2]P	35.5	-	0.3
3 min	2	100.3	66.0-134.6	2	10.7	7.0-14.4	[2]P	143.7	-	1.4
5 min	2	108.9	58.8-159.0	2	6.6	6.4-6.8	P	190.0	-	1.7
8 min	2	121.0	103.8-138.2	2	0.7	0.0-1.4	P	200.3	-	1.6
11 min	2	102.7	83.2-122.2	2	1.5	1.0-2.0	P	184.1	-	1.8
Black bullhead:										
0 min.[1]	8	2.3	1.6-3.0	8	0.2	0.0-0.6	[2]4	4.8	4.0-5.5	2.09
1 min	6	68.8	42.0-148.0	6	0.4	0.0-2.8	[2]3	111.3	102.8-119.6	1.62
3 min	5	121.5	102.6-174.2	4	23.1	0.0-62.2	[2]3	162.8	140.3-185.7	1.34
5 min	6	145.5	109.0-197.0	5	19.3	12.0-27.4	[2]3	227.1	194.4-259.7	1.56
8 min	6	146.8	130.4-165.8	6	10.9	1.2-15.4	[2]3	266.9	252.0-284.5	1.82
11 min	6	127.9	110.0-156.2	6	11.1	8.0-15.4	[2]3	240.5	220.0-250.5	1.88
Channel catfish:										
0 min.[1]	16	1.2	0.6-1.8	16	1.2	0.6-1.6	8	3.9	2.6-4.6	3.25
1 min	4	114.4	104.4-120.6	4	3.2	0.0-8.2	4	188.6	178.1-198.1	1.65
3 min	4	137.3	123.8-164.0	4	11.7	6.2-14.2	4	260.3	239.7-290.5	1.89
5 min	4	116.4	99.8-120.4	4	11.4	0.8-19.6	4	235.6	227.7-243.7	2.02
8 min	4	106.7	94.0-111.0	4	7.6	2.2-9.8	3	182.3	150.7-240.2	1.71
11 min	4	115.2	104.2-140.8	4	9.2	5.6-13.2	4	217.9	184.5-258.6	1.89
White bass:										
0 min.[1]	3	4.1	3.4-5.4	3	0.4	0.0-1.2	3	2.7	2.0-3.8	0.65
1 min	2	42.8	39.7-45.9	2	3.8	3.6-4.0	2	47.7	45.1-50.3	1.11
3 min	5	86.7	44.3-102.5	5	10.1	2.8-14.4	5	109.7	77.1-132.9	1.27
5 min	2	90.3	84.7-95.9	2	21.2	14.8-27.6	2	115.1	104.9-125.3	1.27
8 min	2	88.9	86.3-91.5	2	13.8	12.8-14.8	2	130.9	125.3-136.5	1.47
Bluegill:										
0 min.[1]	6	1.9	1.4-2.6	6	0.9	0.0-2.2	[2]3	3.1	2.8-3.2	1.61
1 min	6	62.7	27.6-93.4	6	2.9	0.0-8.6	[2]3	89.0	67.6-109.6	1.42
3 min	6	104.3	87.2-123.0	5	5.1	0.0-9.2	[2]3	168.2	152.4-189.2	1.61
5 min	12	121.8	87.7-134.1	11	12.8	0.0-63.9	[2]6	174.2	129.3-206.5	1.43
8 min	6	98.5	91.0-104.6	6	17.5	12.0-20.6	[2]3	196.5	180.0-208.0	1.99
11 min	6	95.5	74.2-117.0	6	8.7	0.0-14.6	[2]3	174.2	163.6-179.6	1.82

[1]Background level of primary aromatic amines.
[2]P = pooled sample, 2 brains per sample.

Table 3.--Concentrations of MS-222 in blood and brain of 11 species of fish at loss of reflex stage of anesthesia

Species	Temperature °C.	Anesthetic concentration (mg/l)	Time in anesthetic at loss of reflex (minutes)	Average concentration of free MS-222[1]	
				In whole blood (mg/l)	In brain (mg/kg)
Shortnose gar	12	1,000	2-3	135.0	342.8
Longnose gar	12	800	2-3	135.0	265.1
Bowfin	12	1,000	2-3	178.0	217.3
Rainbow trout	12	100	3-4	68.6	165.4
Northern pike	12	150	2-3	81.9	152.9
Carp	17	200	3-4	96.0	164.1
Spotted sucker	12	200	2-3	100.3	143.7
Black bullhead	17	200	5-6	145.5	227.1
Channel catfish	12	200	2-3	137.3	260.3
White bass	12	150	2-3	83.6	107.1
Bluegill	12	200	2-3	104.3	168.2

[1] Average concentrations of free MS-222 compiled from table 2.

A minimum concentration of 100 milligrams per kilogram (mg/kg) of free MS-222 appears to be necessary for anesthesia to loss of reflex judging from the average concentrations measured in the brain of 11 species (table 3).

DISCUSSION

Diffusion of MS-222, a highly lipid-soluble nonpolar drug, across the gills of fish is quite rapid. Movement of the drug may be in either direction depending on the concentration gradient. This study has shown concentrations of MS-222 in both blood and brain greatly in excess of background amines after 1 minute of exposure to the anesthetic solution. As shown by Maren, Embry, and Broder (1968) in their study on the dogfish shark, the gill is quite efficient in clearing the blood of MS-222 during recovery from anesthesia. Hunn, Schoettger, and Willford (1968) have indirectly measured the same phenomenon in rainbow trout. Preliminary investigations by Maren, Broder, and Stenger (1968) showed that the nonpolar ethyl m-aminobenzoate and its N-acetyl derivative are both excreted across the gill while the polar m-aminobenzoic acid and its N-acetyl derivative are excreted via the kidney. Most of the MS-222 and its congeners are excreted via the gills during recovery; 95 percent in the dogfish shark (Maren, Embry, and Broder, 1968) and 79 to 85 percent in the rainbow trout (Hunn, Schoettger, and Willford, 1968).

Concentrations of MS-222 in whole blood (table 2) drawn via caudal puncture did not reach the levels in the anesthetic solutions during exposures as long as 11 minutes (fish approaching medullary collapse). This is probably due to the fact that blood drawn by this method is usually venous blood which would contain a lesser concentration of the drug than arterial blood until the drug is in equilibrium between the fish and the anesthetic solution.

The appearance of acetylated MS-222 in most blood samples indicates that all 11 species are able to metabolize it. Highest concentrations of acetylated drug were usually detected after 3 to 5 minutes of exposure. The bowfin had the greatest blood concentration of acetylated MS-222 of any of the 11 species studied, 34 percent after a 1-minute exposure. Concentrations in the 10 species were usually less than 20 percent. Maren, Broder, and Stenger (1968) found the same level of acetylated drug in the plasma of the dogfish shark during recovery from anesthesia.

Stenger and Maren (1968) reported that during MS-222 anesthesia of the dogfish shark, the drug rapidly reaches the cerebrospinal fluid and the brain. My observations confirm this finding. In all 11 species, the concentration of free MS-222 in the brain was significantly above background after 1 minute of exposure. The concentration of drug in the brain

increased with depth of anesthesia to loss of reflex. With deeper anesthesia, the concentration of free MS-222 either increased slightly or declined in comparison with the concentration at loss of reflex. A concentration of at least 100 mg/kg is necessary for anesthesia to loss of reflex in susceptible species like rainbow trout, whereas the more resistant species like black bullhead require approximately 200 mg/kg of the free drug for a similar level of anesthesia.

In a previous paper (Hunn, 1968) I noted that rapid recovery in fresh water is associated with the declining concentration of free MS-222 in the brain of channel catfish. The brain concentration of free drug was 91.6 mg/kg when the catfish righted themselves whereas it was 260.3 mg/kg when they exhibited loss of reflex. Indeed, in all studies published to date anesthesia and recovery in fresh water have been strictly associated with the concentration of free drug in the blood and brain (Schoettger et al., 1967; Walker and Schoettger, 1967b).

SUMMARY

Eleven species of freshwater fish were rapidly anesthetized in solutions of MS-222 containing from 100 to 1,000 mg/l of drug. MS-222 (free and acetylated) concentrations in whole blood and brain after 1 minute of exposure indicate that MS-222 rapidly diffuses across the gill and passes the blood-brain barrier. Blood samples drawn by caudal puncture contained lower concentrations of MS-222 than those of the anesthetic solutions.

The presence of acetylated MS-222 in the blood of all species studied is evidence that fish metabolize the drug. Concentratins of acetylated drug were usually less than 20 percent of the total MS-222 except those in bowfin which had 34-percent acetylation after a 1-minute exposure.

MS-222 rapidly enters the brain from the blood. The concentration in the brain increases with depth of anesthesia to loss of reflex. As fish enter more deeply into anesthesia, the drug concentration either increases slightly or declines in ratio to the levels at loss of reflex. Anesthesia and recovery in fresh water appears to be associated with the concentration of free MS-222 in the blood and brain.

REFERENCES

Hunn, Joseph B.
1968. Anesthetics--physiology. In Progress in Sport Fisheries Research 1967, p. 119-120. U.S. Bureau of Sport Fisheries and Wildlife, Resource Publication 64.

Hunn, Joseph B., Richard A. Schoettger, and Everett W. Whealdon.
1968. Observations on handling and maintenance of bioassay fish. Progressive Fish-Culturist, vol. 30, no. 3, p. 164-167.

Hunn, Joseph B., Richard A. Schoettger, and Wayne A. Willford.
1968. Turnover and urinary excretion of free and acetylated M.S. 222 by rainbow trout, Salmo gairdneri. Journal of the Fisheries Research Board of Canada, vol. 25, no. 1, p. 25-31.

Maren, Thomas H., Lawrence E. Broder, and Vincent G. Stenger.
1968. Metabolism of ethyl m-aminobenzoate (MS 222) in the dogfish, Squalus acanthias. Bulletin Mount Desert Island Biological Laboratory, vol. 8, p. 39.

Maren, Thomas H., Rebecca Embry, and Lawrence E. Broder.
1968. The excretion of drugs across the gill of the dogfish, Squalus acanthias. Comparative Biochemistry and Physiology, vol. 26, no. 3, p. 853-864.

Schoettger, Richard A.
1967. Annotated bibliography on MS-222. U.S. Bureau of Sport Fisheries and Wildlife, Investigations in Fish Control, No. 16. 15 p.

Schoettger, Richard A., Charles R. Walker, Leif L. Marking, and Arnold M. Julin.
1967. MS-222 as an anesthetic for channel catfish: Its toxicity, efficacy, and muscle residues. U.S. Bureau of Sport Fisheries and Wildlife, Investigations in Fish Control, No. 17. 14 p.

Stenger, Vincent G., and Thomas H. Maren.
1968. The pharmacology of ethyl m-aminobenzoate (MS 222) in the dogfish, Squalus acanthias. Bulletin Mount Desert Island Biological Laboratory, vol. 7, p. 51.

Steucke, Erwin W., Jr., and Richard A. Schoettger.
1967. Comparison of three methods of sampling trout blood for measurements of hematocrit. Progressive Fish-Culturist, vol. 29, no. 2, p. 98-101.

Walker, Charles R., and Richard A. Schoettger.
1967a. Method for determining MS-222 residues in fish. U.S. Bureau of Sport Fisheries and Wildlife, Investigations in Fish Control, No. 14. 10 p.

1967b. Residues of MS-222 in four salmonids following anesthesia. U.S. Bureau of Sport Fisheries and Wildlife, Investigations in Fish Control, No. 15. 11 p.

43. Effect of MS-222 on Electrolyte and Water Content in the Brain of Rainbow Trout

By Wayne A. Willford

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CONTENTS

EFFECT OF MS-222 ON ELECTROLYTE AND WATER CONTENT IN THE BRAIN OF RAINBOW TROUT

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ABSTRACT.--Rainbow trout (Salmo gairdneri) were exposed to 100-milligrams-per-liter solutions of MS-222 for 1-, 2-, 4-, and 10-minute intervals and their brains were analyzed for sodium, potassium, calcium, magnesium, zinc, iron, and water content. The mean potassium content decreased 17.7 percent and iron increased 56.2 percent during 2-minute exposures. Sodium and calcium increased slightly (7.4 and 9.4 percent); magnesium, zinc, and water content remained relatively constant. All of the affected electrolytes returned toward control values with 4- and 10-minute exposures. These shifts in electrolytes appear to be related to depth of anesthesia and to the concentrations of free MS-222 in the brain.

MS-222 (methanesulfonate of meta-aminobenzoic acid ethyl ester) has been used extensively as an anesthetic for coldblooded animals including fish and amphibians (Schoettger, 1967). However, little is known about its mode of action.

It is generally believed that anesthetics act by absorption or combination with lipid groups in the cell membrane and somehow alter the cell membrane's function of establishing ionic gradients and regulating respiratory rates in cells (Skou, 1961; Quastel, 1963). The result is disruption of ionic differential, the biopotential and ratios by which nerve impulses are propagated. In addition, upsetting the ionic equilibriums may further affect the rates of reactions which restore standing biopotentials (Hillman, 1966).

Walsh and Schopp (1966) demonstrated that MS-222 and related compounds reduce the frequency of electric organ discharges in the electric fish (Gnathonemus moori). They concluded that these agents apparently inhibit pacemaker cells in the mesencephalic command nucleus. Stenger and Maren (1968) and Hunn (1970) have further shown that MS-222 rapidly crosses the gill of fish and is concentrated in the brain. The depth of anesthesia was associated with the concentration of MS-222 in the brain.

The objective of this investigation was to detect and measure changes in brain electrolytes which are associated with MS-222 anesthesia. The electrolytes chosen for study were those which appear essential to the production and maintenance of nerve potential and metabolic activity.

MATERIALS AND METHODS

Rainbow trout (Salmo gairdneri) were obtained from the National Fish Hatchery, Manchester, Iowa. The fish ranged in weight from 390 to 720 grams and were delivered in two shipments during the 6 months of testing. Each group of fish was held in flowing well water at 12° C. and was fed on a diet of commercial trout pellets supplemented with liver.

The test fish were placed individually into 5 liters of well water containing 100 mg/l (milligrams per liter) of MS-222 for 1, 2, 4 or 10

minutes. This concentration of anesthetic produces deep anesthesia in rainbow trout within 3 minutes and medullary collapse in approximately 10 minutes (Schoettger and Julin, 1967).

After exposure, the fish were removed from the anesthetic and decapitated. Whole brains were removed carefully to avoid contamination and were blotted dry and placed in tared porcelain crucibles. After determination of wet weight, the samples were dried to constant weight at 95° C. Brains of control fish were excised and processed in the same manner.

All samples were dry-ashed according to the approved method for lead using 1 milliliter of 15.5 N nitric acid as the "ash-aid" (Horwitz, 1960). After ashing, 2 ml of 12.1 N hydrochloric acid were added to each crucible, and the resulting solution was concentrated to approximately 1 ml. The concentrate was quantitatively transferred by multiple rinses with distilled, deionized water into a 10-ml volumetric flask containing 1 ml of a 5-percent lanthanum solution in 25-percent (V/V) hydrochloric acid. The lanthanum chloride reduces chemical interference during analysis (Elwell and Gidley, 1966).

The samples were analyzed for sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), zinc (Zn^{2+}), and iron (Fe^{3+}) on an atomic absorption spectrometer. Standard curves were prepared from composite standard solutions of all six elements in the presence of 0.5-percent lanthanum and 10-percent (V/V) hydrochloric acid.

The experiment was performed six times over a 6-month period using three fish for each of the four exposure intervals, and three fish for controls. The data for each brain constituent were analyzed statistically using a two-way analysis of variance to determine the significance ($p \leq 0.05$) of observed changes (Snedecor, 1956).

RESULTS

Anesthesia of rainbow trout in 100 mg/l of MS-222 at 12° C. resulted in significant shifts ($p<0.005$) of K^+ and Fe^{3+} concentrations in the brain (table 1). There was a 17.7-percent reduction in K^+ and a 56.2-percent increase in Fe^{3+} during the initial 2 minutes of exposure as determined by comparison with the controls. With longer exposures, the concentrations of K^+ and Fe^{3+} returned toward control values.

Minor, nonsignificant ($p>0.25$), increases of 7.4 and 9.4 percent were observed in the concentrations of Na^+ and Ca^{2+} respecitvely in the brains of MS-222 anesthetized trout. Though these shifts were not significant, they do suggest an effect of the anesthetic which is similar to that observed in Fe^{3+}. The significance of these measurements may have been masked by indeterminant variation.

The Mg^{2+}, Zn^{2+}, and water contents of the brains remained relatively constant over the entire range of exposures.

In addition to the changes observed during anethesia, significant variation ($p=0.025$ to <0.005) of all the cations and the water content occurred between monthly replicates. The monthly variation in control fish appeared to be random, and resulted in diverse levels of

Table 1.--Brain cation and water content of rainbow trout exposed to 100 mg/l of MS-222 at 12° C. for selected intervals of time

Constituent	Exposed for--				
	Unexposed	1 minute	2 minutes	4 minutes	10 minutes
Na^+ mg/kg[1]	[2] 8,978 ±1,160	9,370 ±1,589	9,646 ±1,119	9,650 ±1,092	9,514 ±1,147
K^+ * mg/kg	14,260 ±1,890	13,140 ±1,850	11,740 ±2,330	13,720 ±2,140	13,920 ±2,310
Ca^{2+} mg/kg	612.6 ±68.2	656.9 ±115.1	670.2 ±120.4	649.7 ±94.4	625.3 ±99.3
Mg^{2+} mg/kg	570.0 ±37.3	593.4 ±55.9	584.2 ±32.4	578.6 ±33.1	576.7 ±47.4
Zn^{2+} mg/kg	47.82 ±5.39	47.00 ±7.63	49.48 ±14.03	48.97 ±9.35	47.48 ±9.10
Fe^{3+} * mg/kg	79.65 ±13.45	104.06 ±29.98	124.38 ±40.46	119.13 ±43.40	103.24 ±33.61
H_2O g/100 g	81.43 ±0.57	81.26 ±0.82	81.14 ±0.73	81.28 ±0.77	81.41 ±0.62

[1] Concentration of all cations based on dry tissue weight.
[2] Mean ± standard deviation (n=18).
*Significant variance attributable to exposure ($p<0.005$).

Table 2.--Monthly variation of brain cation and water content of rainbow trout not exposed to MS-222

Constituent	Sampled on--					
	11/16/67	12/11/67	1/16/68	2/12/68	3/21/68	5/6/68
Na^+ mg/kg[1]	[2] 8,333 ±1,032	7,839 ±128	8,451 ±901	10,353 ±370	9,945 ±1,001	8,952 ±1,081
K^+ mg/kg	12,380 ±3,110	14,990 ±460	14,260 ±680	15,570 ±880	12,950 ±1,800	15,410 ±1,690
Ca^{2+} mg/kg	617.3 ±61.7	549.2 ±31.4	581.9 ±100.1	641.5 ±32.7	596.0 ±51.1	690.3 ±53.8
Mg^{2+} mg/kg	571.0 ±32.2	512.8 ±11.9	566.3 ±29.4	596.3 ±12.1	561.8 ±26.6	612.3 ±12.1
Zn^{2+} mg/kg	51.30 ±3.81	47.43 ±3.88	52.39 ±9.29	43.61 ±1.87	44.27 ±0.44	47.92 ±6.02
Fe^{3+} mg/kg	75.59 ±17.41	80.04 ±3.80	69.06 ±10.94	78.22 ±10.00	82.43 ±6.12	92.57 ±22.66
H_2O g/100 g	80.99 ±0.67	81.35 ±0.54	81.45 ±0.26	81.58 ±0.75	82.08 ±0.42	81.13 ±0.39

[1] Concentration of all cations based on dry tissue weight.
[2] Mean ± standard deviation (N=3).

ions and water in the brain from replicate to replicate (table 2). However, the shift in ions attributable to MS-222 exposure (table 1) was similar within each replicate.

DISCUSSION

The results of this study show that MS-222 disrupts, directly or indirectly, specific cationic equilibrums in the brain of rainbow trout during anesthesia. Disruption of ionic differentials, such as K^+/Ca^{2+} and K^+/Na^+ ratios, has a profound effect on nerve potentials and respiration, this being the basis of the general theory of anesthetic action (Quastel, 1963; Hillman, 1966).

A major decrease in brain K^+, however, is not peculiar to anesthesia. Systemic stress such as anoxia, heat, and cold also produce similar cationic imbalance in fish and mammals in vivo (Benjamin, Anastasi, and Helvey, 1961a; Hickman et al., 1964; Van Harreveld, 1966; Bandurski, Bradstreet, and Scholander, 1968). Benjamin, Anastasi, and Helvey, (1961b) have further shown by in vitro studies with rat brain that temperature does not directly effect K^+ release but both anoxia and lack of glucose do. Since MS-222 reduces the respiratory rate of fish (Campbell and Davis, 1963; Randall, Smith, and Brett, (1965), an associated anoxia or hypoglycemia may have contributed to the K^+ depression which I observed.

Unlike the progressive K^+ loss that reaches a plateau during systemic stress, MS-222-induced anesthesia causes an initial K^+ decrease during 1- and 2-minute exposures followed by a return toward control levels up to the time when the fish is approaching death at 10 minutes. The Fe^{3+} change is the reverse of this pattern.

Hunn (1970) showed that a similar pattern occurs in the concentration of free MS-222 in the brain of rainbow trout during anesthesia. The concentration of MS-222 increases rapidly during the first 2 to 4 minutes of exposure and then slowly declines with longer exposures. The exposure period at which the cation and MS-222 concentrations reverse direction of change coincides with the approximate exposure which produces loss of reflex in the fish. Electrolyte content of the brain appears to be associated with the MS-222 concentration in the brain and the depth of anesthesia.

Hillman (1966) stated that in nearly all conditions examined in mammals, changes in the K^+ content of brain are mirrored in opposite changes in the Na^+ content. In this study, as previously mentioned, the K^+ decrease is mirrored by a marked increase in the Fe^{3+} content and only a slight increase in the Na^+ content. Possibly this phenomenon can be explained in light of the work by Germain and Gagnon (1968). They demonstrated that the blood of hagfish (Myxine glutinosa L.) accumulates in subcutaneous sinuses during MS-222 anesthesia. They postulated that this accumulation was due to reduced respiratory movements and profound changes in hemodynamics during narcosis.

Since the brain is a relatively vascular tissue (Zwehl, 1961; Heisey, 1968), it is possible that a pooling or plasma skimming which concentrates erythrocytes, may occur in the brain during anesthesia. This would tend to mask changes in Na^+ and K^+ concentrations and cause an increase in the Fe^+ content of the brain.

SUMMARY

1. Anesthetizing rainbow trout in 100 mg/l of MS-222 at 12° C. for 2 minutes causes a

significant reduction of K^+ and increase of Fe^{3+} in the brain. A concurrent minor increase in the Na^+ and Ca^{2+} of the brain is observed.

2. Exposure of the fish to MS-222 for periods longer than 2 minutes results in a return of K^+, Fe^{3+}, Na^+, and Ca^{2+} towards control values. This pattern of change appears to be associated with the concentration of free MS-222 in the brain and with the depth of anesthesia.

3. MS-222 anesthesia of rainbow trout does not affect the concentrations of Mg^{2+}, Zn^{2+}, or water in the brain.

4. Whereas shifts in ions appear to be closely associated with anesthesia, it is possible that the large increase in Fe^{3+} may be a secondary effect due to erythrocyte pooling in the brain.

5. Significant monthly variation in the electrolyte and water content of brain occurs in rainbow trout held under the conditions described.

REFERENCES

Benjamin, F. B., Joan N. Anastasi, and W. M. Helvey.
1961a. Effect of stress on potassium content of rat brain. Proceedings of the Society for Experimental Biology and Medicine, vol. 107, no. 4, p. 972-973.
1961b. Effect of stress on potassium release from surviving rat brain. Proceedings of the Society for Experimental Biology and Medicine, vol. 107, no. 4, p. 973/974.

Bandurski, Robert S., Edda Bradstreet, and P. F. Scholander.
1968. Metabolic changes in the mud-skipper during asphyxia or exercise. Comparative Biochemistry and Physiology, vol. 24, p. 271-274.

Campbell, G. D., and D. H. Davies.
1963. Effect of ethyl m-aminobenzoate (MS-222) on the elasmobranch electrocardiograph. Nature (London), vol. 198, p. 302.

Elwell, W. T., and J. A. F. Gidley.
1966. Atomic - absorption spectrophotometry. Second edition, Pergamon Press Ltd., London. 139 p.

Germain, Paul, and André Gagnon.
1968. A simple and reliable method for obtaining large blood samples from the anaesthetized hagfish. Comparative Biochemistry and Physiology, vol. 26, p. 371-375.

Heisey, Richard S.
1968. Brain and choriod plexus blood volumes in vertebrates. Comparative Biochemistry and Physiology, vol. 26, p. 489-498.

Hickman, C. P., Jr., R. A. McNabb, J. S. Nelson, E. D. Van Breeman, and D. Comfort.
1964. Effect of cold acclimation on electrolyte distribution in rainbow trout (Salmo gairdnerii). Canadian Journal of Zoology, vol. 42, p. 577-597.

Hillman, H.
1966. The role of potassium and sodium ions as studied in mammalian brain. International Review of Cytology, vol. 20, p. 125-137.

Horwitz, William. (Editor)
1960. Official methods of analysis of the Association of Official Agricultural Chemists. Ninth edition, Association of Official Agricultural Chemists. Washington, D.C. 832 p.

Hunn, Joseph B.
1970. Dynamics of MS-222 in the blood and brain of freshwater fishes during anesthesia. Bureau of Sport Fisheries and Wildlife, Investigations in Fish Control, No. 42.

Quastel, J. H.
1963. Effects of anesthetics, depressants, and tranquilizers on cerebral metabolism. p. 517-538. In Metabolic inhibitors; A comprehensive treatise. Volume 11. Academic Press, New York.

Randall, D. J., L. S. Smith, and J. R. Brett.
1965. Dorasl aortic blood pressures recorded from the rainbow trout (Salmo gairdneri). Canadian Journal of Zoology, vol. 43, p. 863-872.

Schoettger, Richard A.
1967. Annotated bibliography on MS-222. Bureau of Sport Fisheries and Wildlife, Investigations in Fish Control, No. 16. 15 p.

Schoettger, Richard A., and Arnold M. Julin.
1967. Efficacy of MS-222 as an anesthetic on four salmonids. Bureau of Sport Fisheries and Wildlife, Investigations in Fish Control, No. 13. 15 p.

Skou, Jens Chr.
1961. The effect of drugs on cell membranes with special reference to local anaesthetics. Journal of Pharmacy and Pharmacology, vol. 13, p. 204-217.

Snedecor, George W.
1956. Statistical methods. Fifth edition, Iowa State University Press, Ames, Iowa. 534 p.

Stenger, Vincent G., and Thomas H. Maren.
1968. The pharmacology of ethyl m-aminobenzoate (MS-222) in the dogfish, Squalus acanthias. Bulletin Mount Desert Island Biological Laboratory, vol. 7, p. 51.

Van Harreveld, A.
1966. Brain tissue electrolytes. Butterworth Inc., Washington, D.C. 171 p.

Walsh, Raymond R., and Robert T. Schopp.
1966. Frequency-depressing action of ethylaminobenzoate isomers on momyrid electric fish. American Journal of Physiology, vol. 211, no. 1, p. 1-5.

Zwehl, Vera von.
1961. Über die Blutgefässversorgung des Gehirns bei einigen Teleostiern. Zoologische Jahrbücher, Abteilung Für Anatomie und Ontogenie der Tiere, vol. 79, p. 371-438.

☆ U. S. GOVERNMENT PRINTING OFFICE : 1970 O - 404-780

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