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Effects of Fishery Chemicals on the *In Vitro* Activity of Glucose-6-phosphate Dehydrogenase

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Many types of chemicals such as piscicides, therapeutants, disinfectants, oxidizing agents, and anesthetics are used by fishery workers. New chemicals must be rigorously tested for toxicity in the laboratory and field to assess their effects on both target and nontarget organisms before they can be registered for fishery uses. Such tests are numerous and expensive. Recently, RUTHERFORD *et al.* (1979) described a simple method of screening petroleum effluents for possible toxic effects in which a simple, inexpensive enzyme assay is used. In their method, toxic substances caused a significant inhibition of the enzyme glucose-6-phosphate dehydrogenase (G6PDH). This study was designed to measure the effects of selected fishery chemicals on the *in vitro* activity of G6PDH and to determine whether the inhibition of enzyme activity and fish toxicity of the chemicals were correlated.

MATERIALS AND METHODS

Thirteen chemicals were used in our experiments (TABLE 1). The G6PDH (EC 1.1.1.49, type XV, isolated from bakers' yeast) and its substrate (Sigma 400-10) were purchased from Sigma Chemical Co., St. Louis, Mo.[1] Heavy mineral oil was purchased locally. Tris buffer (0.3 M, pH 8.3) and phosphate buffer (0.1 M, pH 7.0) were prepared as described by LONG (1961). Stock solutions and all dilutions of the chemicals to be tested were made in phosphate buffer, pH 7.0.

Preliminary experiments were conducted to determine the concentrations of the chemicals needed for maximum inhibition of the enzyme. These concentrations were initially based on their LC_{50} values to fish. If no inhibition, or complete inhibition, of the enzyme was noted, the chemical concentrations were arbitrarily increased or decreased as needed. After the optimum chemical concentrations had been ascertained, we conducted additional experiments to determine the preincubation time required for maximum inhibition of the enzyme. All of the preliminary experiments were carried out by adding 1 mL of the chemical to be tested (four concentrations, made up in pH 7.0 phosphate buffer) and 25 µL of G6PDH (13.75 µg protein or 2.5 enzyme units) to each of four test

[1]Mention of trade names does not imply endorsement by the U.S. Department of the Interior.

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TABLE 1. Identity and source of selected fishery chemicals

Common name or abbreviation	Chemical name	Source
Hyamine 3500	50% n-Alkyl [50% C^{14}, 40% C^{12}, 10% C^{16}] dimethyl benzyl ammonium chloride 50% H_2O and ethanol	Rohm and Haas, Philadelphia, PA
Hyamine 1622	Diisobutylphenoxyethoxy ethyl dimethyl benzyl ammonium chloride 98.8%	Rohm and Haas, Philadelphia, PA
$KMnO_4$	Potassium permanganate	Matheson, Coleman and Bell, Los Angeles, CA
Formalin	Formaldehyde 37%	Ashland Chemical Co., Columbus, OH
Acriflavine	Mixture of 2,8-diamino-10-methylacridinium chloride and 2,8-diamino-acridine	Aceto Chemical Co. Inc., Flushing, NY
$QdSO_4$	Quinaldine sulfate	U.S. Fish and Wildlife Service
MS-222 (Finquel)	3-Aminobenzoic acid ethyl ester methane sulfonate	Ayerst Laboratories, New York, NY
Noxfish	5% Rotenone	S.B. Penick and Co., New York, NY
Pro-Noxfish	2.5% Rotenone	S.B. Penick and Co., New York, NY
Acetone		Burdick and Jackson Laboratories, Inc., Muskegon, MI
Methanol		Burdick and Jackson Laboratories, Inc., Muskegon, MI
Ethanol		Fisher Scientific Co., Fairlawn, NJ
DMSO	Dimethyl sulfoxide	Fisher Scientific Co., Fairlawn, NJ

tubes (7 X 22 mm). A fifth tube contained G6PDH substrate[2] dissolved in 0.5 mL Tris buffer (pH 8.3) and 1 mL of phosphate buffer (pH 7.0), which served as the control. The contents of each tube were mixed, and 0.8 mL of heavy mineral oil was layered over the reaction mixture. Then the tubes and contents were incubated at 27 ± 1°C in a constant temperature water bath. The rate at which the blue dye (2,6-dichlorophenol indophenol) present in the reaction mixture was reduced to its colorless form was followed visually, and enzyme inhibition was then calculated by the following formula:

$$\text{Inhibition (\%)} = \left(1 - \frac{t_{con}}{t_{ch}}\right) \times 100$$

where t_{con} = the time required for color disappearance in tubes incubated with G6PDH only, and t_{ch} = the time required for disappearance of color in tubes incubated with G6PDH and chemical.

In the definitive study, four concentrations of the test chemical and a control were used. Inoculations of 25 µL of G6PDH in each of five tubes were incubated for the optimum time determined by the preincubation study. At the end of the preincubation time, a mixture of G6PDH substrate in 0.5 mL Tris buffer and 1 mL phosphate buffer (pH 7.0) in four replicate tubes was inoculated with 10 µL of the enzyme-chemical mixture from each of the concentrations tested. Mineral oil (0.8 mL) was layered over the reaction mixture and the materials were incubated at 27 ± 1°C. The time required for the substrate to change from blue to colorless, except for a band at the mineral oil-aqueous interface, was recorded and the percent inhibition calculated.

RESULTS AND DISCUSSION

Potassium permanganate was the most inhibitory compound tested. At a concentration of 0.075 mg/L it inhibited the activity of G6PDH by 31% (TABLE 2). Three therapeutants (Acriflavine, Hyamine 1622, and Hyamine 3500) were about five times more inhibitory than the piscicides, anesthetics, and formalin. Five chemicals inhibited the enzyme slightly: two fish toxicants (Noxfish and Pro-Noxfish), two anesthetics ($QdSO_4$ and MS-222), and one therapeutant (formalin).

Since all of the above compounds are water soluble, we attempted to test several other compounds of low water solubility by dissolving them in an organic solvent. However, the concentrations of solvent needed to prevent precipitation of the test chemicals severely inhibited the enzyme (TABLE 2).

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[2]G6PDH substrate consisted of glucose-6-phosphate, sodium, 2.30 µmol; nicotinamide adenine dinucleotide phosphate, 0.065 µmol; 2,6-dichlorophenol indophenol, 0.275 µmol; and phenazine methosulfate as an electron carrier. Sigma Technical Bulletin, No. 400.

TABLE 2. Percent inhibition of the *in vitro* activity of glucose-6-phosphate dehydrogenase and LC_{50} values of selected fishery chemicals.[a]

Chemical	Minutes incubated	Concentration (µg/mL or µL/L) and % inhibition								96-h LC_{50} for[b] rainbow trout
		Conc	%	Conc	%	Conc	%	Conc	%	
$KMnO_4$	30	0.075	31	0.1	43	0.125	51	0.15	53	1.22 mg/L[c]
Acriflavine	60	5	26	15	43	25	57	35	67	19.9 mg/L[d]
Hyamine 1622	30	5	17	15	37	25	40	35	66	3.54 mg/L[e]
Hyamine 3500	30	10	38	15	43	20	54	25	55	2.45 µL/L[f]
Formalin	40	100	12	300	25	500	44	700	63	118.0 µL/L[g]
Noxfish	50	100	0	200	7	300	19	400	32	3.05 µg/L[h]
Pro-Noxfish	30	100	15	200	35	300	46	400	54	1.02 µg/L[h]
$QdSO_4$	60	150	6	225	15	300	26	375	37	31.8 mg/L[i]
MS-222	60	250	20	300	45	350	54	400	65	47.0 mg/L[j]
Acetone	60	5	35	10	100	15	100	20	100	
DMSO	30	5	25	10	27	15	53	20	70	32.3 mL/L[k]
Ethanol	30	10	14	15	20	20	72	--	--	
Methanol	30	5	38	10	36	--	--	--	--	

[a]Percent inhibition calculated as described in text. Dashed line indicates no data. [b]48-hour LC_{50} for Acriflavine. [c]MARKING and DAWSON 1975. [d]WILLFORD 1967a. [e]Unpublished data, National Fishery Research Laboratory, La Crosse, Wisconsin. [f]HOGAN 1969. [g]BILLS et al. 1977. [h]MARKING and BILLS 1976. [i]MARKING and DAWSON 1973. [j]MARKING 1967. [k]WILLFORD 1967b.

There was little correlation between the *in vitro* inhibition of G6PDH and the LC_{50} values for rainbow trout. For example, potassium permanganate was inhibitory to G6PDH at 0.075 µg/g but had an LC_{50} of 1.22 mg/L. On the other hand, the piscicides Noxfish and Pro-Noxfish showed little enzyme inhibition at 100 µg/mL but had LC_{50}'s of 3.05 and 1.02 µg/L, respectively. Correlation coefficients were 0.63 for the 96-h LC_{50} and 50% inhibition values (extrapolated) and 0.46 for the 48-h LC_{50} and 50% inhibition values.

We found that we could use a single enzyme preparation for about 1 week, provided that it was refrigerated. Our results were consistent from day to day if the color in control tubes changed from blue to clear in 40 minutes or less. Several of the compounds that were tested at high concentrations presented a problem because the interface between the aqueous phase and mineral oil never cleared completely.

The inhibitory effects of Acriflavine were perplexing. In the initial experiments, we used concentrations of 100, 200, 300, and 400 mg/L and observed inhibitions of 65, 38, 31, and 32%, respectively. Similar results were obtained in a second experiment. However, concentrations of 20 to 100 mg/L proved much more inhibitory than those of 200 to 400 mg/L (TABLE 2). Since we were interested only in determining whether there was a correlation between enzyme inhibition and toxicity, we did not investigate Acriflavine further.

We concur with RUTHERFORD *et al.* (1979) that the present test is simple and inexpensive. However, it seems clear from our data, that the *in vitro* inhibition of G6PDH cannot be used to predict the toxicity of a chemical to aquatic organisms.

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