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Mammalian Mitochondria as *In Vitro* Monitors of Water Quality

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Public concern about the degradation of surface and groundwater quality has stimulated rigorous efforts to develop reliable biological methods for quantifying aquatic toxicity. Most methods have focused on the use of fish or aquatic invertebrates to assess risk to humans and other species posed by chemical pollutants in water. However, most of these tests are too expensive and time-consuming to form the basis for affordable water quality surveillance programs on an extensive scale. For example, acute fish tests, which are the most commonly used, cost approximately $700 per assay and require 48 to 96 h to determine median lethal concentrations (LC_{50}) of test substances or water samples. Requirements for specialized laboratories, cultivation of test organisms, variations in response related to species difference, and cumbersome statistical procedures are among other important problems that diminish the utility of acute toxicity tests using fish. Only minor improvements in cost, time, and response factors are afforded by acute toxicity tests with aquatic invertebrates such as those based on mortality of the water flea (*Daphnia magna*). Consequently, there is a pressing need to develop rapid and inexpensive, yet reliable and sensitive tests to monitor microcontaminant trends in water quality and to determine toxicity of complex effluents discharged into aquatic systems.

Use of rapid *in vitro* bioassays for monitoring water quality and determining toxicity of effluents or chemical substances is a recent development. Tests based on bacterial responses are at the forefront of this new category of bioassay. Dutka and Kwan (1981) compared four different microbial toxicity testing systems and found the Beckman Microtox™ bioassay, based on reducing light emission from *Photobacterium phosphoreum* due to the presence of toxins (Bulich 1979) to be fastest and most sensitive. However, this appraisal and subsequent studies have suggested that the utility of the 5-min Microtox™ test may be compromised by poor sensitivity and erratic reproducibility (Blondin et al. 1985) and by the occurrence of falsely negative results from environmental samples (Qureshi et al. 1982). Nevertheless, studies with bacterial test systems have established the feasibility of designing rapid and inexpensive *in vitro* bioassays that could be

used as reliable screening devices to differentiate clean and polluted samples prior to more comprehensive toxicity evaluation.

To measure acute toxicity effectively, a system must provide a simple, sensitive, and rapid measurement of physiological parameters which are indicative of overall organism (bacteria) or organelle (mitochondria) viability. To detect a broad spectrum of toxicants, the parameters should be associated with a major metabolic process controlled by interdependent enzyme systems. In the bioassay reported here, phosphorylating submitochondrial particles (SMP), prepared by sonic disruption of the heavy fraction of intact bovine heart mitochondria (Hansen and Smith 1964) serve as *in vitro* monitors of aquatic toxicity. The bioassay is based on the phenomenon of energy-coupled reverse electron transfer (RET), discovered by Chance and Hollunger (1957) in plant mitochondria, and later explored by Low and Vallin (1963) using SMP preparations from mammalian sources. RET responses permit rapid, simple, and sensitive measurement of acute toxicity by spectrophotometric recording of the rate of NAD^+ reduction. The RET reaction measures the rate at which a complex set of energy-producing reactions is operating to produce NADH, which absorbs strongly at 340 nm. The criterion of toxicity in this test is inhibition of NAD^+ reduction in the presence of toxic substances. The RET reaction was chosen over other methods because responses are easily quantifiable and the energy-coupled RET reaction is competent in reconstituted freeze-dried submitochondrial particles (Lenaz et al. 1968). Thus, SMP--a stable biological preparation--can be distributed to other laboratories. This feature is important in selecting a routine bioassay.

MATERIAL AND METHODS

Phosphorylating SMP were prepared from frozen heavy beef-heart mitochondria according to the method of Hansen and Smith (1964). Energy-linked reduction of NAD^+ by succinate and ATP during RET was measured according to the method of Ernster and Lee (1967). The basic assay medium consisted of sucrose (0.18 M), Tris-HCl pH 7.5 (50 mM), magnesium sulfate (6.0 mM), NAD^+ (1.0 mM), potassium succinate (5.0 mM) and antimycin (0.7 µg/mL). Frozen SMP suspensions at 30 mg/mL were thawed before use and diluted to a Biuret protein concentration of 6.0 mg/mL with cold sucrose (0.25 M) in Tris-HCl pH 7.5 (10 mM). Individual assays were performed by adding 2.9 mL of assay medium at 25° to a 1.0-cm pathlength cuvette followed by 20 µL of a serial dilution of toxicants in distilled water, ethanol or dimethyl sulfoxide (DMSO). The preincubation period (10 min) was initiated by adding 0.1 mL of the diluted SMP suspension. Two baseline readings were recorded at the beginning and end of the preincubation period by measuring the absorbance at 340 nm. The RET reaction was initiated by adding 50 µL of a 0.2 M potassium ATP solution. A single endpoint absorption was recorded 2 or 5 min after ATP addition.

All toxicants except two were tested using nominal concentrations of reagent grade or EPA analytical-standard grades of chemicals.

The ethylenebisdithiocarbamate pesticides, Zineb (zinc ethylenebisdithiocarbamate) and Mancozeb (zinc salt of manganese ethylenebisdithiocarbamate), were commercial preparations containing 75% active ingredient. All except two toxicants were analysed using the basic RET assay medium described above. Dose-response curves for cyanide and arsenic were determined in an alternate medium where the reduction is coupled to the aerobic oxidation of reduced (ascorbate) tetramethyl-*p*-phenylenediamine. Microtox 5-min EC_{50} values were compiled from published reports (e.g., Curtis et al. 1982; Bulich et al. 1981); the values quoted represent the means of several independent determinations. Fish LC_{50} values were compiled from handbooks and published reviews (e.g., Johnson and Finley 1980; Brungs et al. 1978); the values quoted are the means of data determined using several species of fish.

RESULTS AND DISCUSSION

Aquatic toxicity is usually expressed as an EC_{50} value, i.e., the concentration of toxicant that reduces the reference or control response by 50% in a specified exposure time. Approximate EC_{50} values can be determined using plots of the logarithm of the concentration of toxicant versus percent inhibition, as illustrated (Fig. 1) by the dose-response curves for pentachlorophenol (PCP), Zineb and *o*-phenylphenol (OPP). Measurable inhibition of RET (10%) occurs with PCP concentrations as low as 5 ppb, and 650 ppb is necessary for the maximum inhibition of nearly 99%. The concentration of PCP producing 50% inhibition (60 ppb) is estimated by interpolation from the dose-effect curve. This experiment was repeated several times by three investigators using different solutions, four batches of SMP and 3 spectrophotometers. Average EC_{50} for PCP was 81 ppb and the standard deviation of 7 replicates from the mean was ± 15% (range ± 2 to ± 21%). Zineb and OPP were tested multiple times by a single investigator using identical solutions, SMP preparation, and spectrophotometer. Mean data values and standard deviations are plotted for these two experiments and average EC_{50}'s were 3.2 ± 0.6 ppm (Zineb, n = 3) and 7.2 ± 0.2 ppm (OPP, n = 5).

Twenty-seven chemicals were tested for their effect on SMP response (Table 1). Overall, values for the replicates deviated from the mean EC_{50} by an average of ± 13%, with a range from about ± 2.8 to ± 23%. Although additional tests are being conducted to evaluate the precision of SMP testing, these data already indicate that EC_{50} reproducibility of the SMP test is similar to that of other toxicity testing procedures.

The questions arose whether the SMP test is a good surrogate for fish bioassays and whether the SMP test shows greater potential than the Microtox™ test along these lines. To examine those possibilities 96-h LC_{50} values for fish and 5-min EC_{50} values for Microtox™ were compiled from published sources. These data (cf. Table 1) are graphically presented in Fig. 2 (A and B). If the *in vitro* and fish data displayed equal sensitivities to the chemicals tested, all 23 data points would have fallen on the line (slope =

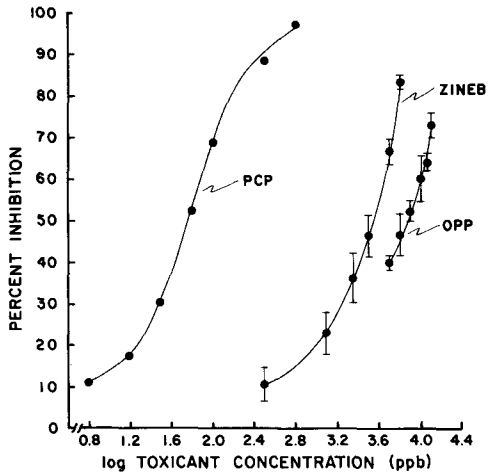

Figure 1. Dose-response curves for the inhibition of RET in SMP by pentachlorophenol, Zineb, and *o*-phenylphenol.

unity) in the figures. This is clearly not the case; data points which fall below the line are more sensitively detected by the *in vitro* test, those above the line by the fish test.

Toxicity data for 17 of the 23 substances were available from both fish and Microtox™ bioassays and were used in regression analyses to compare Microtox™ and SMP toxicity with that of fish. The corresponding log-log plots of the data and fitted models are shown in Fig. 2 (C and D). Also presented are the 95% prediction limits which provide direct estimates of the range of values in EC_{50} that can be expected for given LC_{50}. This approach enables independent correlation of the two *in vitro* tests with fish toxicity, which is regarded as the standard test for aquatic toxicity. The slope of the regression line for the SMP versus fish data (Fig. 2C) is 1.03 indicating that overall the SMP and fish tests are equally sensitive to the toxicants tested. The square of the correlation coefficient (r^2) for the regression is 82%; r^2 is a measure of the similarity in the degree and ordering of responses to the toxicants tested in both systems. The high r^2 value obtained indicates that the RET test is a good predictor of toxicity response in the fish test. The Microtox™ and fish data sets (Fig. 2D) show a poorer correlation. The slope of the regression line is 0.68, i.e. fish were significantly more sensitive than the luminescent bacteria substrate used in the Microtox™ test. For this regression r^2 was only 29% and the 95% prediction limits are considerably broadened.

The stronger correlation between fish and SMP test results is likely due to differences in the structure, biochemistry, and physiology of mitochondria versus bacteria. Both bioassays are based on responses reflecting an array of energy-coupled reactions

Table 1. Relative response of three bioassays to twenty-seven chemcial species.

Toxicant		5 min RET EC_{50}			5 min Microtox EC_{50}	96 h Fish LC_{50}
No.	Name	(ppb)	S.D.	n	(ppb)	(ppb)
1	Rotenone	32	--	1	NA	110
2	Dinoseb[a]	60	4	3	NA	56
3	Cyanide (as NaCN)	70	--	1	8,400	180
4	Pentachlorphenol	81	12	7	510	85
5	Mercury (as $HgCl_2$)	130	14	2	59	280
6	Silver (as $AgNO_3$)	140	--	1	NA	30
7	Copper (as $CuSO_4$)	380	--	1	9,300	360
8	4,6-Dinitro-*o*-cresol	440[d]	--	1	6,400	210
9	Cadmium (as $CdCl_2$)	520[d]	47	2	220,000	1,700
10	Zinc (as $ZnSO_4$)	1,700	110	2	33,000	3,000
11	Sodium Dodecyl Sulfate	1,900	--	1	2,200	6,500
12	Lead (as $Pb(NO_3)_2$)	2,200[d]	440	2	11,000	1,200
13	2,4-Dinitrophenol	2,300	200	2	11,000	520
14	Zineb[b]	3,200[d]	620	3	6,200[e]	7,200
15	Nickel (as $NiSO_4$)	2,200	500	5	900,000	17,000
16	Mancozeb[c]	5,400[d]	1,000	4	80[e]	2,600
17	*o*-Phenylphenol	7,200[d]	200	5	2,100	NA
18	Triton-X100	7,700	--	1	210	NA
19	Aflatoxin B_1	10,000[d]	--	1	23,000	NA
20	*o*-Dichlorobenzene	21,000[d]	--	1	2,600	16,000
21	Arsenic (as Arsenate)	21,000	--	1	38,000	43,000
22	1,1,2-Trichloroethylene	190,000[d]	--	1	130,000	43,000
23	Chloroform	450,000	--	1	44,000	100,000
24	1-Propanol	19×10^6	--	1	18×10^6	NA
25	Acetone	26×10^6	--	1	22×10^6	9×10^6
26	Ethanol	27×10^6	--	1	45×10^6	11×10^6
27	Dimethyl Sulfoxide	$>37 \times 10^6$	--	1	NA	NA

[a] 2-sec-butyl-4,6-dinitrophenol
[b] Zinc ethylenebisdithiocarbamate
[c] Zinc salt of manganese ethylenebisdithiocarbamate
[d] 2 min. EC_{50} values.
[e] 15-min. EC_{50} value.

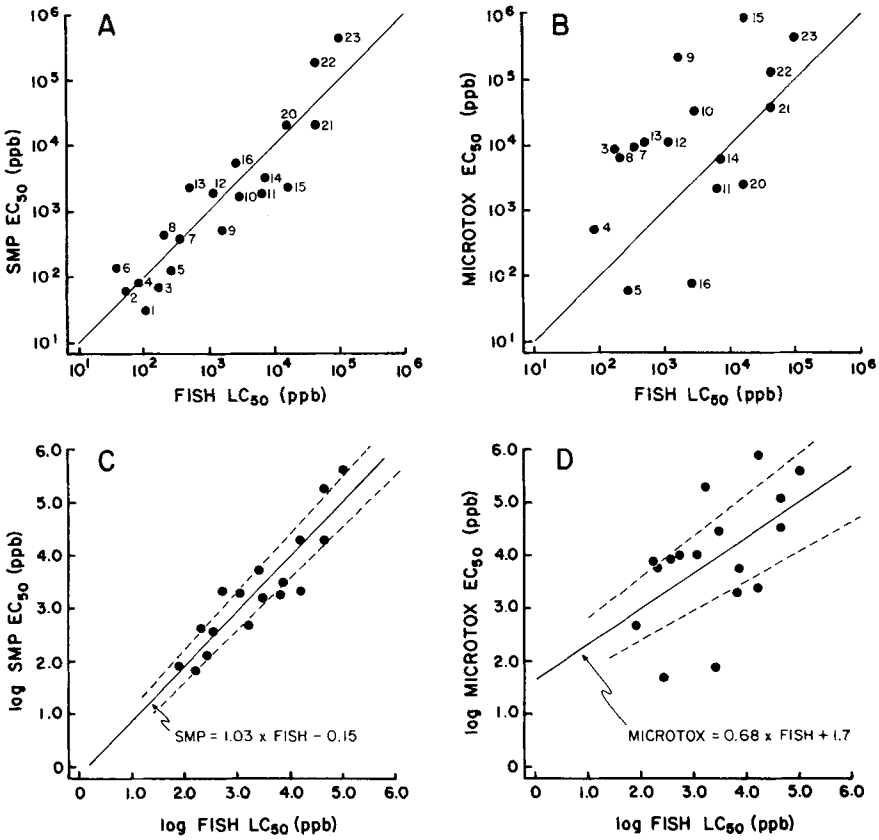

Figure 2. Comparison of *in vitro* EC_{50} data to fish LC_{50} data. Numbers adjacent to data points are keyed to chemical substances identified in Table 1. The broken lines represent 95% confidence bands for the population regression.

that are membrane-dependent and interconnected with total organelle metabolic processes. However, bacteria possess complex, largely impermeable cell walls with selective chemical transport mechanisms. These features are partly responsible for the survival of bacteria in hostile environments and tend to prevent free access of many toxic substances to their internal biochemical machinery, thereby affording a high probability for producing falsely negative results when challenged with diverse toxicants.

Mitochondria share many features with bacteria, from which they are considered to have evolved according to the endosymbiont hypothesis. The average mitochondrian has similar dimensions to *Escherichia coli*. The mitochondrial machinery for protein synthesis is similar to that of bacterial systems. Both contain similar enzyme systems and rely on oxidative phosphorylation rather than glycolysis as a means of energy production. However, mitochondria do not possess a complex and difficultly permeable cell wall. The mitochondrial outer membrane is a porous structure

allowing rapid penetration of small molecules--at least as large as sucrose--without carrier mediation. Moreover, submitochondrial particles, which are remnants of ruptured inner mitochondrial (cristae) membrane which refuse spontaneously to form inverted closed micelles, are completely devoid of the protection of an outer membrane. Consequently, the biochemistry of the SMP-based test system is far more vulnerable to toxins than the bacterial system.

While increasing sensitivity in tests, this increased vulnerability might severely limit mitochondrial usefulness in bioassays because responses to innocuous substances may give rise to falsely positive responses. The most notable concern would be response to calcium ion. Toxicity of Ca^{2+} to energized processes in whole mitochondria is well documented (Hunter and Haworth 1979) and possesses a k_m of only 0.7 ppm. Thus it is important to establish the toxicity of Ca^{2+} in the RET reaction in SMP and the levels of Ca^{2+} encountered in water samples. SMP were challenged by Ca^{2+} in the RET test and no inhibition was found up to 650 ppm. The EC_{50} for Ca^{2+} determined by interpolation from the dose-response curve was 870 ppm. In a study of the Ca^{2+} "trigger site" for this process in whole mitochondria, Hunter and Haworth (1979) identified Mg^{2+} ion as a major inhibitor of the Ca^{2+}-induced transition. Since ATP-driven RET reactions are routinely carried out in the presence of 6 mM Mg^{2+} (~ 140 ppm), the insensitivity of SMP-based RET reactions to Ca^{2+} may be partially explained by a Mg^{2+}-dependent protective mechanism.

Calcium concentrations in the surface waters of the United States rarely exceed 50 ppm. As the major component of hardness in groundwater, Ca^{2+} is usually present in the range 2 to 100 ppm and may reach 200 ppm. Consequently, Ca^{2+} levels in most water samples likely to be tested for toxicity would fall below the limit of detection by RET responses in SMP. As a test of the effect of Ca^{2+} on the SMP bioassay the dose-response curve was determined for PCP in the presence of 100 ppm Ca^{2+} as $CaCl_2$. The EC_{50} for PCP under simulated "hard water" conditions was 91 ppb, a value within the range of earlier experimental results.

The SMP-based RET test has several advantages over whole organism testing as exemplified by the Microtox test: greatly reduced capital and operational costs, rapidity and simplicity of testing procedures, and ease of interpretation. We estimate a cost of less than $2.00 per test to supply all necessary freeze dried biologicals and incubation media components as test-ready materials. SMP can be prepared at a central location, freeze-dried prior to distribution and reconstituted prior to assay with full retention of RET activity. The SMP test offers reproducibility and toxicity responses that correlate closely with fish tests and are superior to results obtained with the Microtox reagent. These findings allow systematic development of a new test to provide a reliable, rapid, and cost-effective method for *in vitro* surveillance of toxicity in water samples.

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REFERENCES

Blondin GA, Knobeloch L, Harkin J (1985) Bioassay of toxic substances in water. Wisconsin Academy Review 32:31-33

Brungs WA, Carlson RW, Horning WB, McCormick JH, Spehar RL, Yount JD (1978) Effects of pollution on freshwater fish. J Water Poll Cont Fed 50:1582-1637

Bulich AA (1979) Use of luminescent bacteria for determining toxicity in aquatic environments. Aquatic toxicol hazard assess ASTM STP 667:98-106

Bulich AA, Greene MW, Isenberg DL (1981) Reliability of the bacterial luminescence assay for determination of the toxicity of pure compounds and complex effluents. Aquatic toxicol hazard assess ASTM STP 737:338-347

Chance B, Hollunger G (1957) Succinate-linked pyridine nucleotide reduction in plant mitochondria. Fed Proc 16:163

Curtis C, Lima A, Lozano SJ, Veith GD (1982) Evaluation of a bacterial bioluminescence bioassay as a method for predicting acute toxicity of organic chemicals to fish. Aquatic toxicol hazard assess STM 755:170-178

Dutka BJ, Kwan KK (1981) Comparison of three microbial toxicity screening tests with the microtox test. Bull Environ Contam Toxicol 27:753-757

Ernster L, Lee CP (1967) Energy-linked reduction of NAD+ by succinate. Methods in enzymology 10:729-739

Hansen M, Smith AL (1964) Studies on the mechanism of oxidative phosphorylation. VII. Preparation of a submitochondrial particle (ETP_H) which is capable of fully coupled oxidative phosphorylation. Biochim Biophys Acta 81:214-222

Hunter, DR, Haworth RA (1979) The Ca^{2+}-induced membrane transition in mitochondria. III. Transitional Ca^{2+} release. Arch Biochem Biophys 195:468-477

Johnson WW, Finley MT (1980) Handbook of acute toxicity of chemicals to fish and aquatic invertebrates. US Fish and Wild Serv Resour Publ No 137, USDI, Washington, DC

Lenaz G, Jolly W, Green DE (1968) Studies on ultrastructural dislocations in mitochondria. I. Reconstitution of oxidative phosphorylation in lyophilized mitochondria by sonic irradiation. Arch Biochem Biophys 103:67-74

Low H, Vallin I (1963) Succinate-linked diphosphopyridine nucleotide reduction in submitochondrial particles. Biochim Biophys Acta 69:361-374

Qureshi AA, Flood KW, Thompson SR, Janhurst SM, Inniss CS, Rokosh DA (1982) Comparison of a luminescent bacterial test with other bioassays for determining toxicity of pure compounds and complex effluents. Aquatic toxicol hazard assess ASTM STP 766:179-195

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