Rapid Toxicity Testing Based on Mitochondrial Respiratory Activity

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The need exists for rapid and inexpensive methods to determine the health effects of environmental contaminants on biological systems. Whole organism toxicity assays have been based on measuring induced toxic effects in numerous systems such as tissue cultures (Ekwall 1983), sperm cells (Dinnel et al. 1982), and bacteria (Dorward and Barisas 1984, Karube et al. 1982). However, these tests have inherent disadvantages of requiring special facilities, and assessments may take days to complete. Bacterial assays are inexpensive and rapid (Maugh 1979), but are not always sensitive to toxicants which are lethal to higher species such as fish (Dutka and Kwan 1982). One of the current research approaches for assessing cytotoxicity is to monitor the respiratory activity of the mitochondrion, a sensitive, nonspecific subcellular target site (Blondin et al. 1987, Khan and Culkomp 1982, Masini et al. 1983, Ogata et al. 1983, Pisani et al. 1986). This organelle is common to most eukaryotic cells, and is involved in a number of vital cellular processes. Detected changes in mitochondrial function after the addition of a test chemical could be correlated to toxic effects.

Mitochondrial respiration can be characterized by three indices: state 3 and state 4 respiratory rates, and the respiratory control ratio (RCR). State 4, the idle or resting state, results when coupled mitochondria respire in a medium containing inorganic phosphate and a Kreb's cycle substrate (i.e. succinate) in the absence of a phosphate acceptor such as adenosine diphosphate (ADP). In the presence of ADP the respiration rate increases to a maximum (state 3), accompanied by phosphorylation of ADP to adenosine triphosphate (ATP). The state 4 rate resumes with limiting ADP levels (Lehninger 1976). The ratio of state 3 to state 4, or RCR, indicates how tightly the oxidative phosphorylation process is coupled (Chance and Williams 1956). The RCR may range from 3 to 7, but approaches 1 when the mitochondria are damaged or aged (i.e. complete uncoupling).

The synthesis of ATP by mitochondria is influenced by a number of compounds, most of which are either uncouplers or inhibitors (Figure 1). Uncoupling agents prevent ATP synthesis by promoting the dissipation of energy gained through electron transfer; the state 4 rate approaches that of state 3. Inhibitors of oxidative phosphorylation prevent the ATP-forming mechanism from utilizing the high energy compounds generated from electron transfer. Thus, ATP synthesis is inhibited without increasing the rate of respiration; the state 3 rate approaches a rate similar to state 4. Inhibitors of electron transfer block the utilization of oxygen and

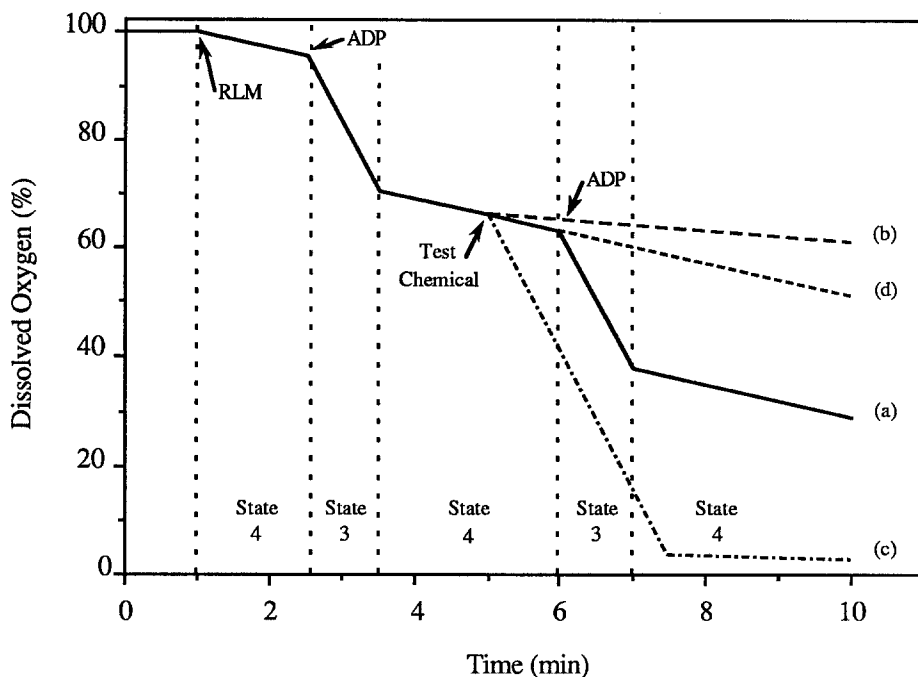Send reprint requests to M.E. Haubenstricker at the above address.

Figure 1. Idealized effects of chemical agents on mitochondrial respiration: (a) no effect detected, (b) inhibition of electron transfer, (c) uncoupling of oxidative phosphorylation, and (d) inhibition of oxidative phosphorylation.

therefore cause cessation of all states of respiration (Tzagoloff 1982).

MATERIALS AND METHODS

The biological oxygen monitoring system (Yellow Springs Instruments, model #53) was calibrated with deionized water having dissolved oxygen (DO) concentrations ranging from 0 - 100% air saturation, as determined by Winkler titrations (American Public Health Association 1975).

Mitochondria from the livers of two month old male Charles Rivers white rats (strain SD) were isolated and prepared according to Kronenberg and Brabec (1982). To minimize variance, the mitochondria from the livers of four rats were isolated and mixed (approx. 15 g·rat^{-1}). The concentration of rat liver mitochondria (RLM) protein was determined by the Biuret colorimetric protein assay (Jacobs et al. 1956), using bovine serum albumin as the standard. The assay medium consisted of 0.15 M sucrose, 5 mM $MgCl_2$, 10 mM KH_2PO_4, 20 mM KCl, 20 mM tris(hydroxymethyl)aminomethane, and 4 mM succinic acid, final pH 7.4.

For each toxicity assay, 25 μL RLM suspension (78.5 ± 1.1 mg protein·mL^{-1}) was injected into the assay chamber containing 2.5 mL air saturated assay media at 30.0°C. After a steady state respiration rate was achieved, 10.0 μL ADP (41 mM; Sigma, grade VI) was injected to induce state 3. After state 4 resumed, an aliquot of test solution was injected. Once state 4 was again established, 10.0 μL ADP was

injected to again produce state 3. Based on 72 assays, average initial state 3 and state 4 values were 68.7 ± 0.1 and 15.3 ± 0.1 ng O_2·mg protein^{-1}·min, respectively, and the average RCR value was 4.54 ± 0.54. Percent change from control values for state 3, state 4, and RCR were calculated from data obtained before and after the addition of the test solution.

Chemicals for assessment of the toxicity sensor were chosen based on water quality criteria as set forth in the United States Environmental Protection Agency (USEPA) list of priority pollutants (1979). Tested compounds included: isophorone, naphthalene, chlordane, n-nitrosodimethylamine, potassium cyanide, chloroform, diethyl phthalate, potassium dichromate, mercuric chloride, cupric chloride, and 2,4-dinitrophenol (2,4-DNP). Malathion was also included in this study as a representative of organophosphate pesticides. Solutions of the test chemicals were prepared in deionized water, stored at 0 - 4°C, and brought to room temperature before use.

RESULTS AND DISCUSSION

The concentration of DO in air saturated RLM assay media was found to be 7.05 mg O_2·L^{-1}. The electrode response to DO in deionized water was linear through the range 0 - 7.45 mg O_2·L^{-1}, with a sensitivity of 36.9 nAmpere·L·mg^{-1}. For all assays: T = 30.0 ± 0.2°C, P = 750 ± 5 torr.

Malathion, isophorone, naphthalene, chlordane, and n-nitrosodimethylamine induced no observable effects on RLM respiratory activity over the tested concentration ranges (195 nM - 81.5 µM, 1.98 nM - 825 µM, 97.8 nM - 40.9 µM, 53.4 pM - 22.6 nM, and 3.96 µM - 1.66 mM, respectively).

Malathion is a phosphorodithioate and acts as an anticholinesterase upon activation. Activation is effected by conversion of the thionates to their corresponding oxygen analogs (Casarett and Doull 1980). The enzyme systems required to catalyze the conversion are found in the cellular microsomes which were not included in the assay media. The time required for each toxicity assay would not favor significant derivative formation even with microsomal activation.

Isophorone is known to affect kidneys and cornea, naphthalene is biotransformed into retinotoxic products, chlordane affects the central nervous system, and n-nitrosodimethylamine ultimately causes fatty acid build-ups and necrosis of the liver (Casarett and Doull 1980). Therefore, although these compounds are also known to be toxic to whole biological systems, there was little indication that they would affect mitochondrial activity.

Cyanide ion and diethyl phthalate produced a progressive inhibition in all respiration indices over the tested concentration ranges (10.6 - 210 µM and 14.7 - 615 µM, respectively). This effect is indicative of electron transfer inhibition. Due to system design and solubility limitations, concentrations of diethyl phthalalte necessary to affect a 50% change from initial rates (EC$_{50}$) were not achieved.

Chloroform and dichromate ion (2.05 - 856 µM and 2.00 - 836 µM, respectively) produced progressive state 3 inhibition but did not affect state 4 rates. This effect isunderstood as an inhibition of oxidative phosphorylation. Again, due to system design and solubility limitations, EC$_{50}$ values could not be determined.
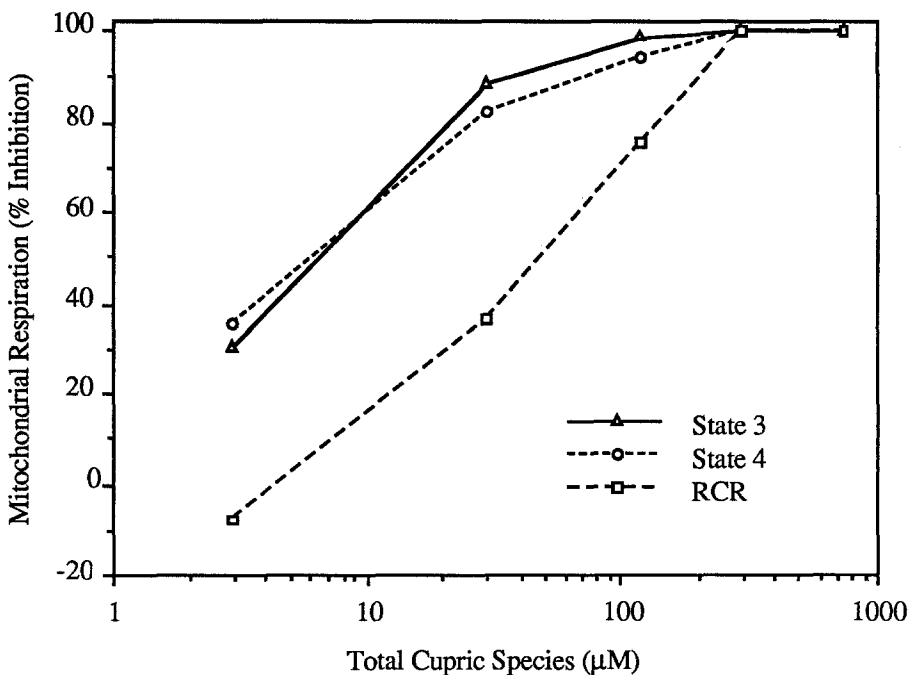
Figure 2. Effect of cupric chloride on rat liver mitochondria respiration.

Mercuric ion, 2,4-DNP, and cupric ion (1.92 - 75.7 μM, 954 nM - 399 μM, and 2.97 - 737 μM, respectively) induced large initial stimulations of state 4 rates at low concentrations (≤ 5 μM), which subsided in about 0.5 min. At higher concentrations (< 150 μM) the three respiration indices for the mercuric and cupric ion tests approached complete inhibition. A typical dose-response curve for cupric ion is presented in Figure 2. These effects are similar to those reported for Zn^{2+} (Kleiner 1974). An initial uncoupling of oxidative phosphorylation in conjunction with electron transfer inhibition could account for the variance in the generated dose-response relationships for these test compounds. From the results of the most concentrated sample of 2,4-DNP assayed, it was unclear whether or not the three respiration parameters were approaching complete inhibition.

This test was designed to be a rapid, non-specific means to assess acute toxicity. However, numerous procedures can be added to provide further information. For instance, the toxic effect from dichromate ion was assumed to be an inhibition of oxidative phosphorylation. This conclusion was clarified when no stimulation in respiration was observed after adding the uncoupler 2,4-DNP. The initial uncoupling effect observed with mercuric and cupric ions could be due to the ions short circuiting the respiratory chain, causing a redox cycle with oxygen. This effect could be determined by addition of an electron transfer inhibitor. When cyanide ion was added to a cupric ion toxicity test, the respiration was inhibited, which indicated that the original effect was due to uncoupling and not redox cycling.

EC_{50} values for the three RLM respiration indicies are presented in Table 1. LD_{50} values for other systems are also included in the table for comparison. Compounds tested that indicated no effect on mitochondrial respiration are not included in the

Table 1. EC_{50} values of isolated rat liver mitochondria (RLM) for selected test chemicals and LD_{50} values for other systems.

| Chemical | -------------- EC_{50} (μM), Mitochondria[α] ------------- | | | Oral Route LD_{50} (mg/kg), Specie[β] |
	State 4 (stimulation)	State 3 (inhibition)	RCR (decrease)	
Potassium cyanide	*	25	80	8.5, mouse; 10, rat; 2.857, human
Mercuric chloride	3	4	3.5	10, mouse; 1.37, rat; 29, human
Cupric chloride	3	6	7	190, mouse; 140, rat
2,4-Dinitrophenol	4	30	15	45, mouse; 30, rat; 4.3, human

[α]Determined concentrations to effect 50% changes from control values
[β]National Institute for Occupational Safety and Health, 1984
*EC_{50} not observed over the concentration range tested

table. There are numerous reasons for poor correlations between the EC_{50} values from this test and reported LD_{50} values. Some compounds known to affect whole systems have no specific effect on the mitochondrial system. Some compounds must first be enzymatically transformed (i.e., the P450 system) into a toxic form before an effect is produced. Structures of an intact organism could also be capable of shielding the mitochondria from compounds that are toxic to these organelles, but are less toxic to components of the whole organism (i.e. the cell membrane).

The mitochondrion is the center of respiration in almost all eukaryotic cells. Numerous studies have used the mitochondrion to assess enzymatic respiratory pathways, which has led to the development of the current bioassay system. This system can serve as a rapid, inexpensive technique to assess the toxicity of certain environmental contaminants on mitochondrial respiration.

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