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Use of Submitochondrial Particles for Prediction of Chemical Toxicity in Man

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Three bioassays which use submitochondrial electron transport particles (ETP) to evaluate chemical toxicity have been developed. These tests were initially designed for use in water quality monitoring and are currently being applied for that purpose. However, they are also valuable for assessing the toxicity of new and existing chemicals. To date 92 substances have been tested using this technology. Median effective or lowest observable effect concentrations (EC50s/LOECs) for most of these chemicals are highly correlated to median lethal concentrations (LC50s) determined following aquatic exposure to fish; r^2 = 0.92 (Blondin et al. 1987; 1989a,b). The mitochondrial assay results are also closely related to EC50 values from cell culture analyses; r^2 = 0.86 (Knobeloch et al. 1990). These strong correlations indicate that the mitochondrial tests are good indicators of the relative toxicity of many xenobiotics to cells and fish. The current investigation studies the ability of these procedures to predict *in vivo* tissue concentrations associated with clinical illness in man.

To examine this potential, data obtained using the mitochondrial tests were compared to chemical concentrations measured in human blood samples obtained during the acute stage of chemical-induced illness. Twenty-nine chemicals were used in the comparison including 6 metals, 8 pesticides, 5 drugs, 4 solvents and 3 alcohols. Only non-fatal, symptomatic blood levels were included since fatal blood levels reported in the medical literature may significantly exceed minimum fatal levels. The results of this study support the hypothesis that the mitochondrial bioassays can successfully predict the *in vivo* toxicity of many diverse chemicals. Properly performed and evaluated these short-term tests may be useful in identifying potential environmental pollutants, selecting compounds for market development and prioritizing substances for more extensive testing in animals.

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MATERIALS AND METHODS

Three complementary mitochondrial test systems were used to analyze chemical toxicity. Most of the data were obtained using the RET test, so named because it is based on the ability of ETP to perform reverse electron transport (Chance and Hollunger 1957). In this system, diagrammed in Figure 1, energy supplied by ATP drives electrons from succinate in a thermodynamically unfavorable direction, through mitochondrial respiratory enzyme Complex I to NAD^+, reducing it to NADH. The accumulation of NADH is monitored spectrophotometrically by measuring the increase in absorbance at 340 nm (Blondin et al. 1987; 1989 a,b). This test is used to determine EC50 values for chemicals which inhibit functional protein subunits within mitochondrial respiratory-chain enzyme Complexes I, II, or V (ATPase); or which have a chaotropic or uncoupling effect on the membrane. However, this system lacks sensitivity to a small number of environmentally important chemicals including polychlorinated biphenyls (PCBs), phthalates and organochlorine pesticides. A second assay based on conventional forward electron transport (ETR test) was used to establish EC50s for these toxicants which inhibit the flow of electrons through Complexes III and IV (Blondin et al. 1987; 1989 a,b).

In this assay NADH is added as an electron donor, and is oxidized by the electron transport system to form NAD^+ (Figure 2). This spontaneous, non-energized process is also monitored spectrophotometrically by measuring the decrease in absorbance at 340 nm. This system is not sensitive to inhibitors of Complexes II and V, or to uncouplers of oxidative phosphorylation such as pentachlorophenol. However, the RET and ETR tests are both sensitive to inhibitors of enzymes in Complex I, such as metals and rotenone, and to membrane-disruptive agents.

A third assay was used to analyze toxicants such as paraquat and 4-nitroquinoline-*N*-oxide which exert their toxicity by inducing a prooxidant state *in vivo* (Cerutti 1985). This test, illustrated in Figure 3, is based on a mechanism in which electrons are withdrawn from the electron transport chain by the toxic chemical to form an unstable free radical intermediate (X·) which quickly reduces oxygen to produce the superoxide anion radical ($\cdot O_2^-$) (Sata *et al.* 1983). The electron withdrawal mediated by these chemicals is facilitated by the addition of antimycin A which blocks the flow of electrons through Complex III. Therefore, this test is called the facilitated electron withdrawal (FEW) assay (Knobeloch et al. 1989). It requires nonphosphorylating particles which have been treated with ethylenediaminetetraacetic acid (EDTA) to inactivate superoxide dismutase (SOD), an enzyme normally present in the matrix of mitochondria which catalyzes the destruction of superoxide radicals. The superoxide anions generated in this test system are detected using the adrenochrome reaction (Misra 1985). Responses are measured in terms of lowest observable effect concentrations (LOECs) defined in the experimental section.

Except for the pesticides, all chemicals were ordered from Sigma Chemicals, St. Louis, MO and are analytical grade; pesticides were obtained from the U.S. EPA or their manufacturers in the purest forms available. Electron transport particles were prepared as described below. These can be obtained through the Wisconsin Alumni Research Foundation, PO Box 7365, Madison, WI 53707 (608-263-2828).

Phosphorylating electron transport particles for the RET and ETR tests were prepared from bovine heart mitochondria according to established methods (Hansen and Smith 1964). Nonphosphorylating-EDTA particles were prepared using the protocol described by Lee and Ernster (1967). Both particle preparations can be stored at -70° C for several months without loss of enzyme activity. ETP were thawed immediately prior to use and reconstituted to the appropriate protein concentration (PC) in 0.25 M sucrose, 10 mM HEPES buffer, pH 7.5.

The Reverse Electron Transfer test protocol: The incubation medium containing 0.25 M sucrose, 50 mM HEPES buffer, 6.0 mM $MgCl_2$, 5.0 mM potassium succinate and 0.7 ug/ml antimycin was adjusted to pH 7.5, warmed to 25° C, and dispensed in 2.9 ml aliquots into 1-cm pathlength glass cuvettes. Toxicants dissolved in distilled water, absolute ethanol or dimethylsulfoxide or, for controls, appropriate diluents were added in 20-50 ul volumes. A 10-minute preincubation period was initiated by adding 0.6 mg of ETP (100 ul, 6 mg protein/ml) and inverting the cuvettes to mix the contents. The baseline absorbance at 340 nm was recorded and the reaction was started by adding 50 ul of a 0.2 M potassium ATP solution. An endpoint absorption was read 5 minutes after ATP addition and used to calculate EC50 values.

The Electron Transfer test protocol: The incubation medium containing 0.25 M sucrose, 50 mM HEPES and 6.0 mM $MgCl_2$ was adjusted to pH 7.5 and 25°C, and dispensed in 2.9 ml amounts into 1-cm pathlength glass cuvettes. Toxicants dissolved in distilled water, ethanol or dimethyl sulfoxide, or diluents were added in 20-50 ul volumes and a 5-minute preincubation was begun by adding 0.1 mg of ETP (50 ul, 2 mg protein/ml) and inverting the cuvettes to mix the contents. The ETR reaction was initiated by adding 50 ul of freshly prepared 4 mM NADH solution. The endpoint absorption was read 20 minutes after NADH addition and used to calculate EC50 values.

The Facilitated Electron Withdrawal test protocol: 1.8 ml of 50 mM HEPES buffer, pH 7.2 was placed into 1-cm pathlength glass cuvettes. Toxicants dissolved in distilled water or ethanol or, for controls, appropriate diluents were added in a volume of 20-50 ul such that the final concentration ranged between 0 and 100 mg/L. Then 1 mM epinephrine, 0.4 uM antimycin A and 0.5 mg of SOD-depleted ETP were added. The cuvettes were inverted three times to mix the contents. Baseline absorbance readings were taken at 480 nm and the reaction was started by adding 50 ul of 5 mM NADH and inverting the cuvettes to mix the contents. Final

Table 1. Comparison of mitochondrial test data and toxic human blood levels.

Compound	ETP EC50/LOEC (mg/L)	Human Blood Level (mg/L)	Symptoms
Mercury	0.13[R]	0.18	nervousness
Dieldrin	0.15[E*]	0.20	seizures
Cyanide	0.23[E]	0.18	mild cyanosis
Copper	0.38[R]	2.87	mild toxicity
Cadmium	0.47[R]	1.20	acute exposure
Aroclor	0.59[E]	0.40	nausea
Endrin	0.67[E]	0.05	convulsion
Chlordane	1.26[R]	2.70	seizures
Zinc	1.80[R]	1.50	lethargy
Nickel	2.20[R]	3.00	nausea
Lead	2.40[R]	1.10	abdominal pain
Paraquat	3.50[F]	0.60	vomiting
Chloroquine(SO_4)	17.00[E]	6.10	moribund
Phenytoin	20.00[R]	20.00	teratogenicity
Lindane	26.00[R]	0.84	seizures
Hexachlorobenzene	33.00[E]	0.38	porphyria
Arsenic	35.00[R]	1.60	intoxication
Diazinon	39.00[R]	1.70	intoxication
Carbon tetrachloride	115.00[R]	20.00	intoxication
Amobarbitol	117.00[E]	43.00	unconciousness
Halothane	134.00[R]	80.00	anesthesia
Trichloroethylene	225.00[R]	64.00	anesthesia
Chloroform	450.00[R]	92.00	anesthesia
2,4-D	561.00[R]	400.00	hyperthermia
Aspirin	937.00[R]	500.00	toxicity
Isopropanol	18620.00[R]	1280.00	coma
Acetone	26200.00[R]	2500.00	lethargy
Ethanol	27000.00[R]	7800.00	intoxication
Methanol	41700.00[E]	1300.00	poisoning

ETP = Electron transport particle, EC50/LOEC = Median effective or lowest observable effect concentration.

ETP test used: R = Reverse electron transfer E = Forward electron transfer F = Facilitated electron withdrawal.

All blood level data and patient symptoms are quoted from Baselt (1982).

*EC50 cannot be determined due to low water solubility of dieldrin. Value listed is an EC20 concentration.

2,4-D = (2,4-dichlorophenoxy)acetic acid.

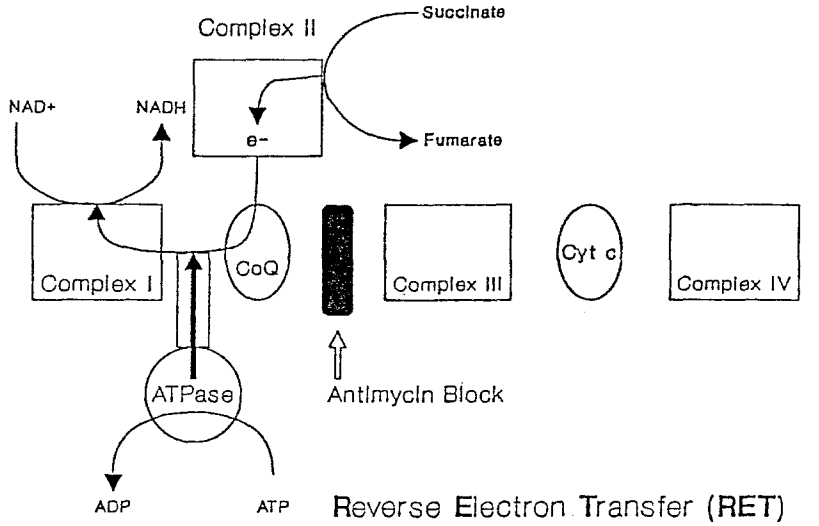

Figure 1 Diagram of the reverse electron transfer test showing the flow of electrons from succinate to NAD+. Energy is supplied by the ATPase (Complex V), and antimycin A is added to block the normal forward flow of electrons through Complex III. Complex I contains the NADH:ubiquinone oxidoreductase, Complex II the succinate:ubiquinone oxidoreductase, Complex III the ubiquinol:cytochrome c oxidoreductase and Complex IV the ferrocytochrome c:oxygen oxidoreductase.

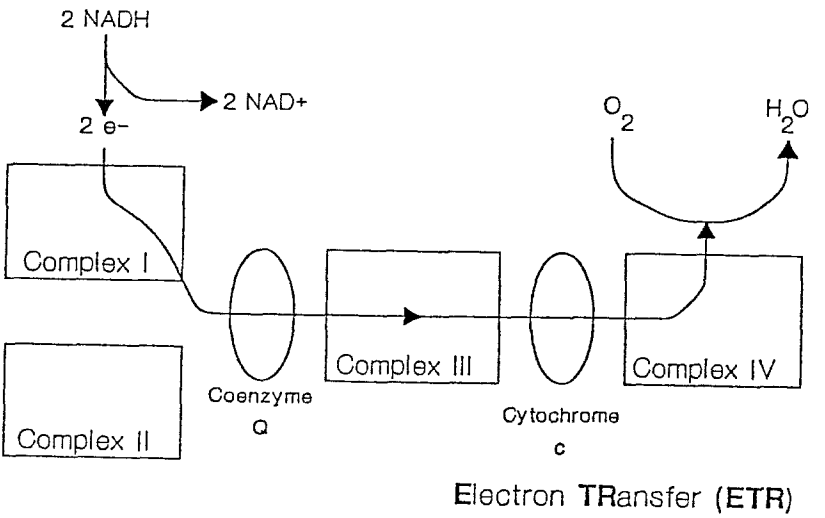

Figure 2 Diagram of electron flow in the electron transfer test. Electrons flow spontaneously from NADH through Complexes I, III and IV to molecular oxygen.

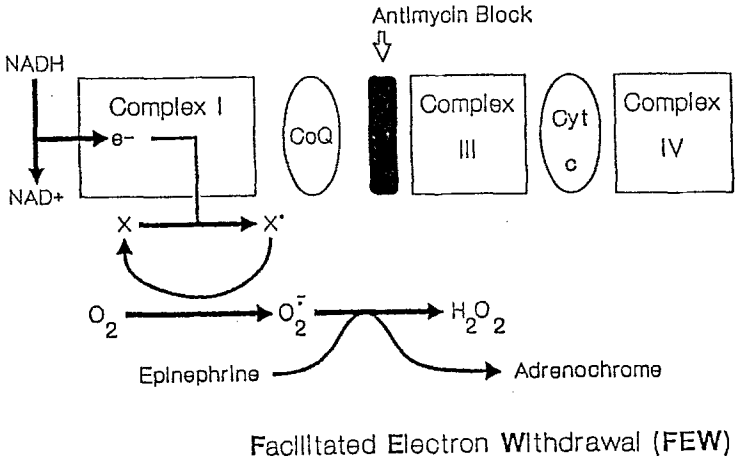

Figure 3 Diagram of the facilitated electron withdrawal assay. Chemicals withdraw electrons from Complex I to form an unstable radical (X·) which quickly reacts with oxygen to produce the superoxide anion radical. This highly reactive molecule is quantitated using the adrenochrome reaction.

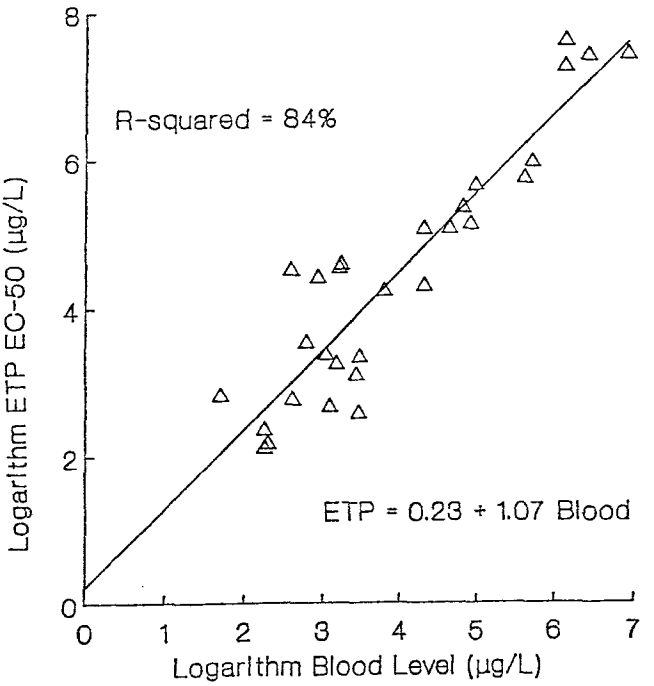

Figure 4 Linear regression analysis of data from the submitochondrial electron transport particle tests versus toxic human blood levels. All data are listed in Table 1. Since the data span six orders of magnitude a logarithmic transformation was used.

absorbance readings were taken after a 10-minute incubation at room temperature and used to calculate a lowest observable effect concentration (LOEC) for the chemical tested. The LOEC, used in this test instead of an EC50, is the concentration which gives a response three standard deviations above the mean control blank value. It is analogous to the limit of detection (LOD) utilized for environmental samples subjected to conventional analytical determinations (Keith et al. 1983).

RESULTS AND DISCUSSION

Results from the three mitochondrial tests and toxic human blood levels are displayed in Table 1. All data are listed in order of decreasing toxicity as determined by the mitochondrial tests. A statistical regression of these data is presented in Figure 4. Since the data span six orders of magnitude, a logarithmic transformation was used. The coefficient of determination (r^2) for the regression is 0.84 indicating a strong linear relationship between these values.

The excellent correlation between chemical concentrations that altered enzyme function or membrane stability in the electron transport particles and human blood levels which produced illness suggests that these mitochondrial tests could be useful in the fields of human risk assessment and chemical safety evaluation. These tests offer several advantages over current testing methods. They are much easier, faster and less expensive to perform than whole animal tests or cell culture analyses. Each of the protocols can be completed in less than 30 minutes at a projected cost well below $100 per analysis. Submitochondrial particles are commercially available and can be stored frozen until needed. The biologicals and reagents needed for these protocols are inexpensive and readily available. The only items of equipment necessary to perform the analyses are a visible-range spectrophotometer, pH meter, metric balance, and household refrigerator.

Many toxicologists believe that *in vitro* tests should be used to reduce the need for whole-animal testing. However, it is difficult to validate such methods using existing data collections which are primarily based on oral LD50 analyses because *in vitro* assays, designed to detect cellular or molecular toxicity, cannot account for the gastrointestinal uptake, hepatic metabolism and excretion processes that occur in live animals. We have avoided this dilemma by comparing mitochondrial data to toxic human blood levels. Our findings partially validate the role of short-term tests in assessing the human health risks associated with exposure to chemical toxins.

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