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Assay for Toxic Chemicals Using Bacteria

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A method to measure the toxicity of chemicals is described. Any test for toxic chemicals requires an indicator organism. Toxicity is defined by the damage done to living cells. This assay uses the bacterium *Rhizobium meliloti* as the indicator organism. This assay presents a simple, rapid and inexpensive method to measure toxic chemicals without harming animals. Results are comparable to other methods.

MATERIALS AND METHODS

R. meliloti, Bacillus megaterium, Bradyrhizobium japonicum, Rhizobium leguminosarum, Escherichia coli, Arthrobacter crystallopoites, Salmonella typhimurium, and *Streptococcus lactis* were obtained from the active culture collection at New Mexico State University. *Pseudomonas aeruginosa* was obtained from Dr. Pat Unkefer, Los Alamos National Laboratories. *Rhodopseudomonas sphaeroides* was obtained from Dr. Samuel Kaplan, University of Texas Medical Center, Houston.

The dyes were received from Sigma Biochemicals (St. Louis, MO 63278) and were used as received. Six tetrazolium dyes were tested with the 10 bacteria. These dyes included: TTC, 2,3,5-triphenyl tetrazolium chloride (tetrazolium red); TV, tetrazolium violet ; INT, indolenitrotetrazolium violet ; TBC, tetrazolium blue chloride; MTT, diphenylthiazole tetrazolium; NBT, Nitroblue tetrazolium.

R. meliloti, B. japonicum and R. leguminosarum were grown in CDM medium with 1% mannitol supplemented with 0.1% casamino acids (Gonzalez-Gonzalez et al., 1990). A. crystallopoites and P. aeruginosa was grown in R_2A (Difco). E. coli was grown in LD medium (Difco) or in M63 medium with glucose or succinate as the carbon source (Miller, 1972). S. typhimurium and B. megaterium were grown in LB medium (Miller. 1972). S. Iactis was grown in LB medium supplemented with 1% glucose. R. sphaeroides was grown in LB and in R_2A. Cells were grown at 30° C in an incubator shaker. Cells were collected by centrifugation

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(10,000 x g, at least 10 min) in a refrigerated preparative centrifuge and were washed once with potassium phosphate buffer (0.01 M, pH 7.5). After washing, cells were resuspended in the phosphate buffer to an absorbance = 0.3 (550 nm). Once washed, cells were kept in an ice bath. Cultures were inoculated with a 1% inoculum of *R. meliloti.* The inoculum was prepared by growing cells overnight in CDM with the supplements. Typically cells were harvested with an absorbance at 550 of 0.65 to 1.00, comparable to 230 to 300 µg protein ml^{-1}. Mannitol, the carbon source available during growth of the cells, was added to the washed cells at a final concentration of 0.1%.

To assay a chemical, 1 ml cells was combined with 1 ml Tris buffer (0.1M, pH 7.5). The volume of toxic chemical was varied and water was added to make the final volume of the chemical 1.2 ml. Tubes were mixed and the absorbance was read. 100 µl of the dye were added to a final concentration of 0.019 mM (3mM stock). The absorbance of reduced TTC was read at 485 nm. The absorbance of INT was read at 490 nm. NBT was read at 512 nm. The other dyes were read at 550 nm. The tubes were mixed vigorously with a vortex mixer after each addition. The addition of the dye did not change the initial absorption. After the dye was added, the reaction mixture was incubated at 30°C. After incubation, the absorbance was read again. The time = 0 absorbance was subtracted from the final absorbance. This difference between the absorbance at time = 0 and the final absorbance is considered to be the "absorbance" used in calculations. Reading the absorbance at time = 0 permitted the assays to be run in disposable test tubes rather than in cuvettes. It also permitted a correction for cloudiness in the sample to be introduced. With MTT cells were incubated with the toxin for 20 minutes. Incubation times, with the other dyes' incubations were much longer.

Values were plotted and regression lines were calculated, the slope of the line, the y intercept, and the regression coefficient were determined. Y/2 was the absorbance of controls (samples with 1.2 ml water, no chemical) divided by 2 With this, the equation was solved for x, the concentration of toxic chemical resulting in 50% inhibition of reduction of the dye.

$$x = \frac{Y/2 - b}{m} \qquad \text{(Eqn. 1)}$$

This value is equal to the volume of inhibitory chemical resulting in 50% inhibition of reduction of the dye as estimated from the change in the absorbance of the culture. This is referred to as the IC50 (inhibitory concentration, 50%). From the concentration of the toxic chemical and the volume inhibiting the reduction 50%, the concentration of the toxic chemical corresponding to the volume is determined.

With some chemicals, a linear plot did not provide usable data. A

satisfactory regression line could not be fit to the data. However, when the absorbance was plotted vrs. th log of the concentration of inhibitory chemicals, often a satisfactory regression could be obtained. The equation was modified:

$$\log x = \frac{Y/2 - b}{m} \qquad \text{(Eqn.2)}$$

The antilog of log x was the volume of chemical resulting in a 50% decrease in the absorbance. It was not certain why some chemicals give better data with a linear plot and some with a log plot. It was found that 3(4,5-dichlorophenoxy)-benzoic acid gave good results with a log plot while 3(4-methylphenoxy)benzoic acid gave good results with a linear plot.

If the R^2 for the regression was less than 0.80, the data from that determination were not used. With $R^2 = 0.8$, $R = 0.894$. No values were included unless there were at least 7 samples (5 concentrations of the inhibitory chemical and two controls). The $p = 0.01$ value for a regression, when $v = 5$, was determined to he 0.874. Thus any data from these plots would be significant at the $P = 0.01$ level (Rolf and Sokal, 1981). All the values from determinations resulting in $R^2 > 0.8$ were averaged and the standard deviation calculated. The n reported refers to the number of times the experiment was repeated using a different batch of cells. The variation reported is the standard deviation expressed as a percentage of the mean. The results are presented as the mean for the values in units of ppm, parts per million, mg l^{-1} as is customary in the toxicological literatue

RESULTS AND DISCUSSION.

This assay is based on the observation tha *Rt meliloti* can reduce the tetrazolium dye MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide very readily. The reduction is inhibited by chemicals thought to be toxic. The inhibition is proportional to the concentration of the toxic chemical. The concentration of the dye that inhibits reduction by 50% is calculated. Presumably the dye is reduced by a cytochrome. However, many reductases can use tetrazolium dyes as a terminal electron acceptor (Snowberry and Ottawa, 1965; Berridge and Tan, 1993).

R. leguminosarum, B. japonicum, E. coli, S. typhimurium, A. crystallo-poites, B. megaterium, P. aeruginosa, R. sphaeroides, and S. lactis were tested for their ability to reduce TTC, TV, INT, TBC, MTT, and NBT. Cells were grown, washed, and resuspended as described fo *R.r meliloti. S. lactis* was unable to reduce any of the dyes even after incubating as long as 10 hr. This bacterium lacks all cytochrome activity. Most of the

dyes were reduced by the other bacteria but detectable reduction took several hours . The dyes MTT and INT were reduced more rapidly than the other dyes by all the bacteria . Tetranitroblue tetrazolium (TNB) was tested in *R. meliloti* and *E. coli* and was reduced very little. Nitroblue tetrazolium (NBT) and neotetrazolium chloride (NTC) were tested with *R. meliloti, R. leguminosarum* and *E. coli.* Neither was reduced readily. Several bacteria reduced MTT readily but the inhibition was not affected by toxic chemicals, pentachlorophenol or copper *R. meliloti* reduced DCIP (2,6-dichloro-phenol indophenol) and rezasurin very slowly. It took several hours to see detectable reduction Phenyl hydrazine was the only chemical found to reduce the dye without cells. Of all the bacteria and all the dyes *R. meliloti* reduced MTT most readily and the reduction was inhibited by chemicals considered to be toxic.

Representative data for one experiment are shown in Figure 1

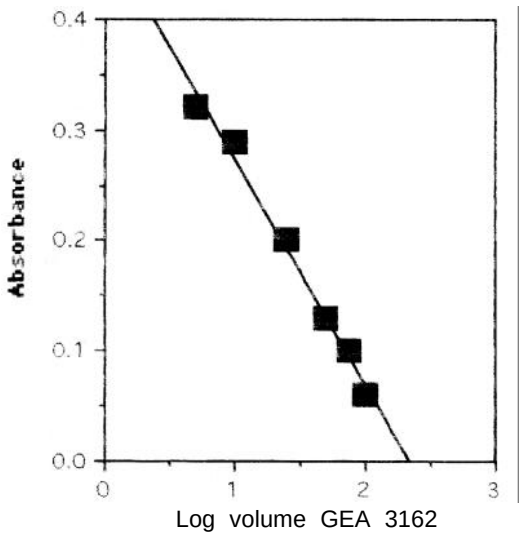

Figure 1 Toxicity of GEA 3162

GEA 3162 is a compound used pharmacologically to generate nitrite ions (Kankaananta et al., 1993) . This graph shows the results of one of six determinations. The regressiol fine for this plot was y = 0.47807 - 0.20395x, R^2 = 0.988. The log of the IC50 was 1.63 or 42.9 µl . The variation in the values was 37% .To correct for the inhibition caused by the DMSO (IC50 for DMSO is 262 µl): 42.9 µl /262 µl = 0.164, 16.4% of the inhibition was due to DMSO. So 42.9 - 0.164(42.9) = 35.9 µl due to GEA 3162. Typically the correction for the solvent changed the value less than 20%.

$$35.9 \times 10^{-6}\ l \times 3300\ mg\ l^{-1} \times \frac{1}{3.3\ ml} = 35.9\ \mu g\ ml^{-1} = 35.9\ mg\ l^{-1}$$

The reaction was characterized with respect to optimal pH, incubation time and the concentration of cells like a typical enzyme catalyzed reaction. With MTT, a 20 minute incubation time was chosen because the reaction was essentially complete at this time. Prolonged incubation did not affect the inhibition of reduction by toxic chemicals. Results remained the same. After washing, placed on ice, cells retained their activity for at least 12 hours.

Values for 60 chemicals are presented in Table 1

Table 1 Toxicity of organic chemicals

Compound	n[a]	mean[b]	variation[c]
1,1,1-trichloroethane	18	46	13 %
1,10-phenoanthroline	8	43	28 %
acetate	6	43	14 %
amino oxyacetic acid	5	507	12 %
carbon tetrachloride	10	1790	12 %
chloroform	12	1786	17 %
cyclohexane	11	431	32 %
dibromomethane	8	58	7 %
dichloroethane	12	240	32 %
ethanol amine	8	257	16 %
GEA-3162	6	39	37 %
hydroxyl amine	10	586	19 %
isopropyl alcohol	3	247	33 %
n-pentanol	4	1623	22 %
octane	5	1	18 %
sodium lauryl sulfate	5	28	15 %
sulfosalcylic acid	5	284	24 %
tetrachloroethylene	10	67	22 %
tricholorethylene	10	411	10 %
CTAB[d]	6	1	32 %
xylene	5	129	26 %
α-naphol	5	87	8 %
benzene	6	840	42 %
benzyl chloride	8	61	8 %

chlorobenzene	25	213	30 %
1,2-dichlorobenzene	11	34	10 %
1,3-dichlorobenzene	7	36	5 %
1,3,5-trichlorobenzene	34	14	39 %
1,3,4-trichlorobenzene	28	3	17 %
ethyl benzene	24	50	22 %
toluene (methyl benzene)	7	213	16 %
dimethyl amino benzaldehyde	10	364	33 %
psuedocumine	13	32	28 %
phenol	7	1433	31 %
catechol	8	4	4 %
2,6-dinitro cresol	9	28	38 %
4,6-dinitrocresol	9	11	32 %
0-nitrophenol	13	38	33 %
trichlorophenol	23	4	72 %
pentachlorophenol	8	0.4	50 %
dimethylamino phenol	10	401	28 %
Toluene	8	213	16 %
2,4,6-trihydroxy toluene	9	65	19 %
dinitrotoluene	4	497	6 %
trinitrotoluene	8	42	34 %
p-cresol	8	74	15 %
Inhibitors of electron transport			
dinitrophenol	11	39	37 %
FCCPe	16	2	50 %
TFFA f	6	6	27 %
sodium azide	10	803	34 %
sodium cyanide	16	21	50 %
Solvents			
acetone	15	68,000	24 %
ethanol	8	73,362	29 %
methanol	9	66,027	41 %
dimethyl sulfoxide (DMSO)	13	88,000	31 %
Dioxanes9			
3(3,5-dicholoro-phenoxy)-benzoic acid	5	37	19 %
3(3,4-dichloro-phenoxy)-benzoic acid	4	45	24 %

3(4-chloro-phenoxy)-benzoic acid	7	34	40%
3(4-methyl-phenoxy)-benzoic acid	6	103	12%
3-phenoxy benzoic acid	4	58	25%
dibenzene furan	5	9	11%

[a]Number samples assayed

[b]Average of values for the samples assayed. Reported as ppm, mg l-1

[c]Variation = standard deviation/mean x 100, expressed as a percentage

[d]hedexyltrimethylammonium bromide

[e]FCCP, triflourocaronlylcyanide phenylhydrazone, inhibits oxidative phosphorylation like dinitrophenol.

[f]TFFA, thenoyltrifluoroacetone, inhibits reduction of cytochrome b (in mitochondria) by succinate.

[g]From Rolf Halden, University of Minnesota, Department of Civil Engineering

Minerals were tested for toxicity (Table 2). Calcium and magnesium were found to be toxic at concentrations comparable to those found in defined laboratory medium. The other minerals found to be inhibitory: cadmium, cobalt, copper, manganese, mercury, nickel, selenium and zinc are usually thought to be toxic and are present in bacterial media in very low concentrations. These minerals were chloride salts Several were assayed as both chloride and sulfate salts with no significant difference . Lead, arsenic, iron and lanthium at concentrations less than 1200 ppm were found to have no effect on the reduction. Sodium and potassium had an inhibitory effect but, at concentrations greater than 15,000 ppm. Inhibition of reduction of dye by calcium was of concern for measuring organic compounds in soil samples It was found that the addition of 0.08 mM EDTA to the reaction relieved the inhibition of the reaction by calcium . The EDTA had a slightly stimulatory effect on the reduction of the dye. EDTA at a final concentration of 0.08 mM relieved the inhibition caused by all the minerals EGTA, considered to be a better chelator of calcium than EDTA, had no effect on inhibition by calcium. Organic chemicals could be assayed in the presence of EDTA and calcium. Presumably the EDTA chelates the mineral to make it inactive.

Table 2. Minerals

Mineral	n	mean	variation
Ba^{+2}	6	22.7	33 %
Cd^{+2}	12	0.791	41 %
Ca^{+2}	13	5.65	33 %
Co^{+2}	11	12.3	46 %
Cu^{+2}	13	0.953	19 %
Mg^{+2}	9	50.8	20 %
Mn^{+2}	11	1.44	35 %
Hg^{+2}	10	0.0159	23 %
Ni^{+2}	11	58.6	11 %
Se^{+2}	9	277	39 %
Zn^{+2}	10	0.847	7 %

[a]As in Table1. Minerals were all chloride salts.

The results of the Rhizobium assay we e compared with values for values using the fathead minnow (Geiger et al., 1990) as well as the Polytox™ and QSAR methods (Sun et al., 1983) and Microtox™ data (Kaiser and Palabrica, 1991) The results of these comparisons are included in Table 3.

Table 3. Comparison of methods for determining toxicity

Method	Comparison	n	Regression equation
Microtox	fat head minnow	20	Y = 0.95 X + 1.20
Polytox	fat head minnow	15	Y = 1.06X + 0.97
Rhizobium	fat head minnow	20	Y = 0.90X + 0.28
Microtox	QSAR	19	Y= 0.93X + 063
Polytox	QSAR	19	Y= 1.00X - 0.12
Rhizobium	QSAR	19	Y = 0.74X + 0.24
Rhizobium	Microto x	30	Y= 0.90X - 0.005
Rhizobium	Polyto x	19	Y = 0.83X + 0.008

This indicates that the three assays using bacteria as the indicator organism give comparable results. All agree well with the fathead minnow assay. QSAR values are generated from a consideration of the structure of an organic compound, the octanol-water partition coefficient, solvatochromic parameters, surface area and molar volume. After very sophisticated calculations, a predicted value for LogS, the log of the toxic concentration of the chemical is derived (Nirmalakhandan and Speece, 1988). The antilog of this value converted to parts per million was used to generate values for the QSAR data. Values were generated by plotting the log of toxicity (in ppm) using one assay against the log of the toxicity using the second assay. If the slope of the regression line = 1, the two methods are comparable.

A variety of different methods for measuring toxic chemicals using bacteria as the indicator organism have been used (Bitton and Dutka, 1986). Only Microtox™ and Polytox™ have been used extensively. In contrast to these assays, this assay is simple. It requires no special equipment. Once marketed, it should be possible for a very minimally equipped laboratory to determine toxicity readily. This should be accepted in third world situations.

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vol. 1-5

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