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Project Summary

Level 1 Bioassay Sensitivity

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Environmental assessment bioassays are conducted to detect toxic constituents in complex emissions from industrial sites. Unlike instrumentation used in analytical chemistry, the power (limit of detectability) of the biological techniques to detect specific chemicals or even classes of chemicals is seldom known.

In this report, the published literature is surveyed and used to establish a set of sensitivity estimates for these tests. These estimates will permit a comparison of the bioassays and will also give an estimate of the concentrations of toxic materials that could be in a mixture which registers negative in a particular test (i.e., What does a negative test response indicate?).

Three tests, the Ames *Salmonella*/microsome mutagenesis assay, the *in vitro* rodent cell (CHO) clonal toxicity assay, and the *in vivo* rodent toxicity assay all have substantial published data bases using study designs similar to those employed in Level 1 environmental assessment. The sensitivity limits for these three tests are summarized in this review. Methods developed to assess these data will be applicable to other Level 1 tests such as the rabbit alveolar macrophage (RAM) assay, aquatic tests, and other ecological assays. However, these evaluations await the development of a sufficient data base on a wide variety of pure compounds. Most of the other Level 1 bioassays (e.g., fish toxicity, RAM) have extensive data bases for responses with complex environmental samples, but not with pure chemicals. The insect toxicity test (*Drosophila* LD_{50}) has a large base of published data but the test conditions are extremely variable, making an interpretation of the sensitivity difficult.

This Project Summary was developed by EPA's Industrial Environmental Research Laboratory, Research Triangle Park, NC, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

The primary objective of EPA/IERL-RTP Level 1 Environmental Assessment is to reliably evaluate stationary-source emissions for their toxic potential. With this information, emission sources and process streams can be ranked according to their estimated toxic potency and further testing can be applied efficiently.

One feature of Level 1 Environmental Assessment which needs to be addressed is the level of sensitivity for the chemical and biological methods. There is little problem in defining the limits of sensitivity in chemical analysis; however, the same type of definition for bioassays is extremely difficult. This is especially true for some of the bioassays because the data are both quantitative and qualitative. Qualitative responses are defined as either positive or negative, toxic signs or descriptions of growth. Quantitative effects are those which are specifically measured by counting colonies, animal deaths, or growth-medium turbidity.

This report discusses the problem of determining the levels of sensitivity in biological assays recommended for

Level 1 Environmental Assessment as defined in the IERL-RTP procedures manual (1) and summarizes the sensitivity data where sufficient data exist. Data and sensitivity estimates have been developed for the Ames *Salmonella*/microsome mutagenesis assay, the rodent cell (CHO) clonal toxicity assay, and the *in vivo* rodent acute toxicity assay.

Approach

The proposed data formatting and evaluation scheme in "Level 1 Biological Testing Assessment and Data Formatting" defines the concept of "no detectable toxicity" for each of the bioassays as no significant response at a maximum applied dose (2). This definition implies that the test sample may contain toxicants but that they are below the degree of resolution inherent in the particular assay. The following list contains several factors that are important in influencing the resolution of a test:

1. *The maximum dose/concentration that can be applied to the assay system.*
2. *The inherent response of the assay system to specific classes of chemicals.*
3. ***The number and site of critical targets in the test organism that must be affected to produce a lethal or toxicological response.***
4. ***The validity of extrapolating dose response data for lethality and mutagenesis linearly down to low dose levels.***
5. ***The chemical interactions (synergistic and antagonistic) in complex mixtures.***
6. ***The ability of the assay system to detoxify or eliminate the toxicant will impact on the limit of bioassay resolution and detectability.***
7. ***The ability of the assay system to alter the toxicant metabolically to a more toxic or mutagenic form will affect the limit of bioassay resolution and detectability.***
8. *Nature of the test response.*

Review of Data in General

Defining the lower limits of assay sensitivity is difficult. The critical factor in developing a data base of comparable information is protocol standardization. Data from bioassays using standardized procedures, such as the Ames *Salmonella*/microsome mutagenesis assay and the *in vivo* rodent acute toxicity assay, can be easily collected and compared. Problems arise when data from different protocols are compared. The rather simplistic approach of comparing Level 1 data collected under similar conditions does not tell the entire story about the true level of assay sensitivity or relevance to environmental assessment. Considerations such as the slope of the response curve, the potential for bioaccumulation or biotransformation, and the effect of chronic versus acute exposure ideally should not be ignored.

Additional factors (e.g., the nature of the test response) must be considered when evaluating Level 1 data for levels of sensitivity. Most Level 1 bioassays produce continuous data sets over the range of measured responses. A basic difference exists, however, between Ames mutagenicity data and toxicity data produced by the other assays. In the toxicity assays, any chemical may be expected to exhibit toxicity if tested at a high enough dose. Assay sensitivity is limited by the physical capacity of the system to accept test material and the length of time during which the material is applied. In the Ames assay, on the other hand, a nonmutagenic compound will not produce a positive response regardless of applied dose. Ames data is therefore discontinuous (e.g., a chemical is either mutagenic or nonmutagenic) and only positive (mutagenic) chemicals exhibit a continuous data set. If a sample is positive, it can be further categorized as having high, moderate, or low mutagenicity based on the observed minimum effective concentration. A modicum of uncertainty exists in differentiating between nonmutagenic chemicals and weakly positive chemicals with activity below the threshold of Ames assay sensitivity. Chemicals in both situations are designated as having nondetectable mutagenicity.

Tests with clearly dichotomous responses (e.g., Ames test with data evaluated as mutagenic or nonmutagenic) are more amenable to sensitivity evaluation than tests with continuous responses (e.g., EC_{50} in the CHO clonal toxicity test). For example, it appears that mutation induction is a "single hit" phenomenon and that the level of response is a function of total dose (concentration x exposure time). This means that one should obtain an equal response by elevating the concentration over a short exposure period or by extending the exposure period at a low concentration. Lethality, however, is generally not a "single hit" phenomenon, and distinctly different patterns of lethality will be obtained from experiments when the dose is held constant but the concentration and time parameters are varied. Thus, to define the lower limit effect as a function of dose is meaningful for the Ames test but not for bioassays measuring lethality unless the other parameters are specified.

A Review of Ames Test Data

The Ames *Salmonella*/microsome mutagenicity assay has a well-accepted standardized protocol which has been adapted to Level 1 Environmental Assessment testing. A large amount of Ames mutagenicity data is available that may be used to evaluate the levels of sensitivity and detectability of the Ames assay. For example, McCann *et al.* (3) have compiled a list of data from which this evaluation is drawn.

The minimum effective concentration (MEC expressed as amount-per-plate) can be calculated for these various compounds and compared. Table 1 summarizes the range of reported MEC values for different chemical classes. The chemicals reported in this section are organized by chemical family using the Multimedia Environmental Goals (MEG) (4) classification scheme.

A Review of Rodent Toxicity Data

Level 1 acute rodent toxicity testing is a valuable test method for toxicological assessment of complex effluents. The advantages of the *in vivo* toxicity assays lie mainly in the fact that the testing is performed in whole animals. Also there is a significant background of rodent test data on a wide range of toxicants using standard test protocols, thus supplying needed information for analysis of levels of assay sensitivity and for reliable interpretation of results with complex effluents. The primary disadvantage of the assay is its inability to predict the toxicity induced by long-term/low-level exposures.

Table 2 summarizes the range of LD_{50} values encountered for each chemical (MEG) category in the review of rodent toxicity data. The dose required to kill 50 percent of test animals (LD_{50}) is reported as milligrams of chemical per kilogram of animal body weight. Using the approach discussed above, the sensitivity of the assay to each chemical is determined. This assumes that only the chemical in question exerts toxicity and that no antagonistic or synergistic effects occur.

Table 1. *Level 1 Ames Assay: Range of Minimum Effective Concentration (MEC) Summarized by Chemical Class*

MEG Group[a]	Chemical Class	No. of Entries	MEC[b] Range, Low and High Values, μg/plate	Chemical
6	Glycols, Epoxides	4	7×10^{-2}	Benzo(a)pyrene-4, 5-oxide
			400	1,2,7,8-diepoxyoctane
8	Carboxylic Acids and Derivatives	6	3×10^{-3}	2-(2-furyl)-3-(nitro-2-furyl)-acrylamide (AF-2)
			64	Melphalan
10	Amines	30	6×10^{-3}	2-aminofluorene
			210	N,N-dimethyl-4-(phenylazo) benzeneamine
14	Sulfonic Acid, Sulfoxides	2	49	Methyl methanesulfonate
			217	Ethyl methanesulfonate
16	Halogenated Aromatic Compounds	7	7.8×10^{-2}	9-10-dichloro-methyl anthracene
			2	10-bromoanthracene
17	Aromatic Nitroso Compounds	6	1.4×10^{-2}	2-nitrosofluorene
			73.5	2-nitrosonaphthalene
21, 22	Fused Polycyclic Hydrocarbons	17	1.3×10^{-1}	D aunorubicin · HCl
			120	Benzo(e)pyrene
23	Heterocyclic Nitrogen	10	1×10^{-2}	4-nitroquinoline-1-oxide
			38	U racil mustard
24	Heterocyclic Oxygen Compounds	6	1.2×10^{-2}	Aflatoxin B_1 and aflatoxicol
			42	Aflatoxin B_2
25	Heterocyclic Sulfur Compounds	1	11	Hycanthone methanesulfonate
26	Organophosphorus Compounds	2	1.1	Cyclophosphamide
			60	isophosphamide

[a]MEG = Multimedia Environmental Goals (4); chemical classification scheme developed as part of EPA Level 1 Environmental Assessment testing program. Some chemicals may be placed in more than one group.
[b]MEC = Minimum Effective Concentration. The minimum amount of test material required to give a positive response in the most sensitive tester strain.

A Review of In Vitro Mammalian Clonal Toxicity Assays

Mammalian *in vitro* clonal toxicity assays provide a sensitive and reliable method to measure and compare the cytotoxicity of test agents. The Chinese hamster ovary (CHO) cell clonal toxicity assay is routinely used to measure the toxicity of environmental samples submitted under EPA Level 1 testing of point source emissions. The measured end point is the inhibition of colony formation as a function of dose. The standard parameter for comparison is the dose necessary to reduce the colony-forming ability of quantitatively plated mammalian cells by 50 percent (the EC_{50} value). These survival data are continuous over the doses tested, and the EC_{50} may be determined statistically or graphically from the data.

Quantitative comparisons of toxicity data can only be made from assays conducted under comparable conditions. Since a wide variation in CHO toxicity assay protocols was encountered in the literature review, criteria were developed for selecting cytotoxicity data for comparison. Parameters for which standard ranges were developed included attachment time, exposure period, cell type, serum concentration, and cell density. The amount of comparable CHO clonal toxicity data was greatly reduced when the requirements of a standardized assay design were imposed.

Table 3 summarizes the range of EC_{50} values by chemical (MEG) category from experiments meeting the CHO clonal toxicity assay study design criteria.

Level 1 Bioassays Not Reviewed

The limits of resolution and detectability of the remaining Level 1 bioassays are not addressed in this report. These assays, for the most part, have not been applied on a sufficiently large scale or performed under standardized conditions for any valid comparison of the data to be made. A complete review of the sensitivity of these assays can be made in the future once a larger data base and standard study designs have been developed.

Table 2. *Level 1 Rodent Toxicity Assay: Range of LD_{50}[a] Values Summarized by Chemical Class*

MEG Group[b]	Chemical Class	No. of Entries	LD^{50} Range, Low and High Values, g/kg	Chemical
1	Aliphatic Hydrocarbons	2	800 1440	1-nitropropane nitromethane
2	Alkyl Halides	7	81 3160	2-chloroethanol trichlorovinyl-silicone
3	Ethers	1	170	Bis(2,3-epoxy-propyl) ether
6	Glycols, Epoxides	2	238 4200	9-epichlorohydrin 2-ethyl-1,3-hexanediol
7	Aldehydes, Ketones	5	21 1300	Acrolein Cyclohexanone
8	Carboxylic Acids and Derivatives	9	52 5040	2-fluoroacetamide Citric acid
9	Nitriles	2	65 78	Phthalonitrile Phenylacetonitrile
10	Amines	9	20 1625	2,2-dichloro-N-methyl-diethylamine HCL 2,4-diaminotoluene
11	Azo Compounds; Hydrazine Derivatives	5	33 265	1-methylhydrazine 1,1-dimethylhydrazine
12	Nitrosamines	1	3850	N-nitrosodiphenylamine
13	Thiols, Sulfides, Disulfides	2	38 3700	Methylsulfide Tris (1-aziridinyl) phosphine sulfide
14	Sulfonic Acids, Sulfoxides	2	2000 2100	Sodium lauryl sulfonate Alkyl aryl sulfonate
15	Benzene, Substituted Benzene Hydrocarbons	8	16 3900	n-cumenol methyl carbamate Anthranilic acid methylester
16	Halogenated Aromatics	7	50 4000	Aldrin Hexachlorobenzene
17	Aromatic Nitroso Compounds	3	135 812	1-chloro-2-nitrobenzene p-nitroaniline
18	Phenols	4	300 1600	Phenol Propylgallate
20	Nitrophenols	1	45	2,4-dinitrophenol
21, 22	Fused Polycyclic Hydrocarbons	2	438 700	Carbonyl Phenanthrene
23	Heterocyclic Nitrogen Compounds	4	125 3340	Diquat Nicotine
26	Organophosphorus Compounds	2	135 292	Dichlorvos (DDVP) Metepa
26	Metals and Organometallic Compounds[c]	18	4	Sodium dimethylarsinate
28			4000	Sodium chloride

[a] LD_{50} = Dose lethal to 50 percent of animals.
[b] MEG = Multimedia Environmental Goals classification scheme (4).
[c] Metals and organometallic compounds are summarized as a group and not by individual elements.

Table 3. *Level 1 CHO Clonal Toxicity Assay: Range of EC_{50}[a] Values Summarized by Chemical Class*

MEG Group[b]	Chemical Class	No. of Entries	EC_{50} Range, Low and High Values, (μg/ml)	Chemical
8	Carboxylic Acids and Derivatives	1	5,700	Caprolactam
12	Nitrosamines	7	0.5 10,000	N-nitrosomethylurethane (NMUT) Dimethylnitros-amine
14	Sulfonic Acids, Sulfoxides	5	4.5 317.9	Dimethylsulfate (DMS) Propylmethane sulfonate (i-PMS)
23	Heterocyclic Nitrogen Compounds	1	1	ICR-191
82 68	Metals and Organometallic Compounds[c]	17	0.062 852.7	Cadmium chloride Chromic chloride-hexahydrate

[a] EC_{50} = Effective Concentration of chemical that reduces colony development by 50 percent relative to control levels.
[b] MEG = Multimedia Environmental Goal chemical classification scheme (4).
[c] Metals and organometallic compounds are summarized as a group and not as individual elements.

Health Effects Assays

The rabbit alevolar macrophage (RAM) assay is the only health effects assay without a suitable data base for evaluation. The prospects are good that, as this assay is incorporated into routine testing programs, additional validation work with pure chemicals will be undertaken and reported.

Aquatic Ecological Assays

Aquatic toxicology has had a long record of investigation into the physiologic response of aquatic organisms to specific pure and complex samples. Unfortunately, there is not a large data base for work conducted exclusively with Level 1 protocols using the recommended indicator organisms. It is beyond the scope of this project to compare and interpolate data collected from similar, but basically different, study designs.

Terrestrial Ecological Assays

The three Level 1 terrestrial ecological assays have not been validated as well as have the other Level 1 assays. This lack of published work, both on procedures and assay validation, has prompted EPA's Industrial Environmental Research Laboratory—RTP to direct the production of a laboratory workbook for terrestrial assays (5). No attempt was made to collect and evaluate data from these assays.

Conclusions

It is possible to estimate the level of sensitivity of bioassays used in Level 1 Environmental Assessment. However, with the exception of only a limited number of tests currently proposed, a data base collected under conditions similar to those recommended for Level 1 testing is not available. For those tests it is advised that the data base be developed during ongoing Level 1 analyses. Where the data bases were available, the sensitivity levels were estimated. The mammalian *in vitro* clonal toxicity test and the Ames *Salmonella* test appeared to be quite sensitive compared to the *in vivo* rodent toxicity test. The two *in vitro* tests also approach the sensitivity required of the chemical analyses performed in Level 1 assessments.

Existing data resulting from EPA/IERL-RTP's ongoing Level 1 Environmental Assessment programs may also provide sufficient chemical and biological data to evaluate the sensitivities of the remaining bioassays.

The information in this report might be useful, not only in developing an appreciation of the intrinsic sensitivity of the bioassays, but also in modifying assays to increase their sensitivities.

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The complete report, entitled "Level 1 Bioassay Sensitivity," (Order No. PB 82-221 201; Cost: $9.00, subject to change) will be available only from:

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