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(6) Formation and Management of an Expert Toxicological Review Team for Literature Search, Evaluation and Organization of Currently Available Rapid Toxicological Tests.

Volume I.

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Battery of Tests	Oxides of Nitrogen	
Benzene	Phosgene	

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The objective of this study was to determine the feasibility of identifying short-term in vivo or in vitro toxicity tests which could be included in a battery of such tests as part of a toxicological screening program.

The study was performed by a panel of ten toxicology experts and a contractor management team, which included scientific information specialists.

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Six compounds (benzene, cadmium, formaldehyde, phosphorus, phosgene, oxides of nitrogen) were chosen as models for analysis by a matrix format. Each matrix was supported by a review article. Panel members selected predictive endpoints from acute and subchronic data taken from the literature. The panel also recommended short-term tests relevant to the endpoints for each compound. Position papers were developed for: pharmacokinetics; behavioral toxicity; in vitro testing; reproductive assessment testing and a concept for toxicological testing. Bibliographies were prepared for the matrix reviews, each position paper, and one for the overall study.

Based on the analyses of the matrices, the position papers and their collective experience, the panel developed recommendations for short-term tests for a minimal toxicology screening program and pointed out gaps wherein additional research was required.

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FINAL REPORT

Formation and Management of an Expert Toxicological Review Team for Literature Search, Evaluation and Organization of Currently Available Rapid Toxicological Tests

Volume I

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April 1978

Supported By:

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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EXECUTIVE SUMMARY

The objective of this study was to identify a battery of such tests that will determine the potential toxic nature of a chemical in the most efficient and cost effective manner using currently available techniques. A panel of ten toxicology experts was assembled, and a contractor team provided management support and selected scientific literature review and analysis.

In order to focus on specific endpoints or effects encountered in acute or subchronic animal studies and to select those endpoints which might be predictive of chronic lesions, the panel recommended a literature review be carried out on specific chemical compounds. Six compounds were selected: benzene, cadmium, phosphorus, formaldehyde, phosgene and oxides of nitrogen. Subsequently, the literature was searched for thirteen categories of effects as reported for each compound.

In order to reduce the data obtained on individual compounds to a manageable form, a matrix was devised. Each matrix contained representative endpoints in the thirteen categories as reported in acute, subchronic and chronic studies. Each matrix was accompanied by a literature review, limited to data on endpoints, animal species and doses administered.

The panel members not only selected predictive endpoints where possible but indicated their choices of short-term *in vivo* or *in vitro* tests which might be employed in screening tests. A summary of endpoints and recommended tests was compiled which then served as one basis for the panel's final recommendations.

The second major basis for decisions of the panel was a series of position papers which are incorporated in the final report of this study. Position papers were prepared on the following subjects: Pharmacokinetics, *In Vitro* Testing, Behavioral Toxicity Testing, Reproduction Assessment Testing, and a Concept for Toxicological Testing.

As a result of evaluation of six compounds with respect to predictive endpoints and short-term tests, review and evaluation of five position papers, and by their collective judgements, the panel of experts made a series of recommendations for a minimal toxicology screening program encompassing short-term *in vivo* and *in vitro* tests. The recommendations made are summarized as follows:

1. Perform complete hematological work-up

2. Carry out bone marrow smears (differential)

3. Conduct a modified one-generation mouse reproduction study

4. Carry out selected short-term *in vitro* tests (cytotoxicity, mutagenicity, carcinogenicity)

5. Perform infectivity test (hypersusceptibility)

6. Conduct standard central nervous system observational evaluations

7. Perform simple motor and sensory function tests and behavioral assessment tests

8. Perform heart and vascular system organ function tests

9. Carry out general and specific biochemical analyses

10. Determine organ/body (or brain) weight ratios and conduct standard histological examinations

11. Perform simple skin and eye irritation tests

12. Conduct, at early stages, pharmacokinetic studies and induction of cytochrome P450

13. Determine physical and chemical properties of each compound including oil/water partition coefficients and stability in aqueous media at pH 4.0, 7.0, and 10.0.

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1.0 INTRODUCTION

1.1 Statement of the Problem

Governmental and private organizations face a formidable task in assessing the potential toxicity and evaluating the safety of a number of chemicals. The number of new chemicals being developed, in addition to numerous existing chemicals, far exceeds present capabilities and budgets available for toxicity testing.

This study was predicated on the basis of comparison and evaluation, by a team of experts, of conventional toxicity tests with a substituted battery of short-term tests. The requirement is to develop a battery of screening tests that would be predictive of long-term toxicological effects of a chemical compound. The battery would consist of a series of simple, rapid, reliable and sensitive tests. Maximum benefit from a battery of tests would be achieved if the screening tests yield parallel information provided by conventional tests. Ideally a battery of short-term tests would yield results providing insight into all currently recognized elements of toxic responses or endpoints. An initial model of a battery of tests may of necessity include simple *in vivo* tests, simple behavioral toxicology tests, and modified or simple measurement of the physical-chemical properties of a compound. The ultimate battery of tests might be ideally reduced to accepted *in vitro* tests.

1.2 Approach to the Problem

An overall approach to the problem of attempting to develop a battery of toxicity screening tests was predetermined in the RFQ (DAMD 17-77-Q-7452). More specifically, the Department of the Army's Medical Research and Development Command wished to utilize the services of a review team or panel consisting of experts in toxicology and related fields. Duties of the expert panel would include evaluation of toxicological test methods, both conventional and those known as short-term tests (both *in vitro* and *in vivo*). The outcome of panel deliberations would be recommendations for the composition of a comprehensive battery of short-term tests to serve as a screening system in lieu of conventional chronic studies with test animals. The role of the contractor would be management of the contract, assistance to the Panel after its selection on the basis of contractor recommendations and Army concurrence, and literature search and retrieval. An initial meeting between sponsor and contractor representatives resulted in selection of the panel as shown in Figure 1. The contractor management team structure and its principal staff members are shown in Figure 2.

Discussions during the first panel meeting were wide-ranging, essentially expressing individual viewpoints on the merits, or lack thereof, of short-term *in vitro* and *in vivo* toxicological tests. A consensus finally emerged, namely, that the literature searching should be predicated on toxic endpoints normally recorded in the course of conventional toxic substances testing in animals. A list of such endpoints was developed by the panel to be used as a preliminary guide for literature searching. It is noted for the record that restrictions imposed on the approach to the problem included exclusion of mutagenesis tests *per se* and tests relating to aquatic systems.

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Figure 1. Toxicology Review Panel Members

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Figure 2. Contractor Management Team Structure

During the second panel meeting (30 November, 1 and 2 December 1977), various members expressed dissatisfaction with the results of the initial approach outlined above. It was recognized by all concerned that the toxicology literature was too voluminous, in view of dollar and time constraints, to exploit it on the endpoint basis. The Panel, after considerable discussion, finally recommended that a matrix analysis should be considered. The general concept of a matrix analysis was based on identifying specific endpoints or lesions resulting from toxic testing (acute, subchronic, chronic) and correlation of less-than-90-day results with long-term (two-year) data. A critical aspect of such analyses is that retrospective data could be obtained only on studies of a specific compound. Species of animal and dose regimens employed would then be the principal variables.

Having achieved a consensus that a matrix analysis approach should be pursued, discussion by the panel members turned to selection of compounds. The Panel was then asked to develop a candidate list of chemical agents from which this selection could be made. The following list is the result of the panel's deliberation on compound selection (principal organ system affected is indicated in parentheses).

1. Chlorinated hydrocarbons (liver)
2. Halogenated hydrocarbons (liver)
   DDT, Dieldrin
3. Aromatic amines (urinary bladder)
   Beta-naphthylamine
4. Benzene (bone marrow)
5. Azo dyes
6. Thiourea - ethyl thiourea (thyroid)
7. Mercury-methyl mercury (kidney, reproduction, behavior)
8. Cadmium (testes, kidney, musculo-skeletal)
9. Paraquat (lung)
10. Irritant gases (lung)
    NO_2, O_3, Phosgene
11. 2,4,5-T (reproduction)
12. Formaldehyde (lung, skin)
13. Nitrilotriacetate (teratogenesis)
14. Carbon tetrachloride
15. Arsenic
16. Triorthocresylphosphate

Selection of compounds was based on availability of Criteria Documents and the extent of data base available. The six compounds selected for preparation of detailed matrices were:

1. Benzene
2. Cadmium
3. Phosphorus
4. Formaldehyde
5. Phosgene
6. Oxides of Nitrogen

It was also decided that each matrix would be supported by a review article containing a description of the experimental data with reference to animal species, dosage, duration of treatment, etc., as they related to the endpoints shown in the matrix. Literature citations employed in the review article for a specific compound were keyed by number in the corresponding matrix. A complete list of references used in compilation of each matrix was attached to the corresponding review article. Each Toxicology Review Panel member was provided with the matrices of all six compounds along with the review articles and literature references. The members were requested to identify predictive endpoints in the matrices and recommend short-term in vitro and in vivo tests that would yield information parallel to that of the long-term animal tests.

The approach to final development of a matrix for a specific compound was to combine panelists' suggestions and recommendations in a "master" matrix. The master matrix for each compound would contain all identified predictive endpoints and suggested short-term tests. The master matrices would then be evaluated during the remaining two panel meetings. A position would finally be established, by the panel of experts, for each compound with respect to recommendations for development of a battery of short-term tests.

Another aspect to the approach strategy of this study was compilation and documentation of U.S. regulatory agency published guidelines for testing of toxic substances. In addition, information relative to industrial organizations' guidelines was to be considered. A list of regulatory guidelines, coupled with a list of endpoints normally observed during conventional animal toxic testing was believed to suffice as background information in panel deliberation. Accordingly, each matrix package described above was accompanied by the compilation of guidelines. References cited in each published guideline were annotated and keyed by number to the guidelines. A hard copy of each literature citation was obtained for reference to experimental details, when desired by panel members. The list of regulatory agency guidelines and samples of industrial protocols and related literature references appear in Section 7.0 of this report.

1.3 Background

In a letter to Science (1), interest in and concern for alternatives to performing expensive chronic studies in determining toxicity of chemical compounds were expressed. In that letter the authors proposed what is now the essence of this contract effort, namely, development of a battery of predictive toxicity screening tests. The economics of substituting a battery of simple tests for conventional protocols was predicted to yield a ten-fold reduction in cost and a five-fold reduction in testing time (1). Other individuals and organizations have been equally concerned with the same monumental problems involved with testing toxicity of chemicals. Pertinent items in this regard are summarized in the following paragraphs.

Stich and co-authors reviewed the status of short-term bioassays for chemical carcinogens in 1975 (2). They recommended a pre-screening program for carcinogens and mutagens which consists of the following tests: 1) Ames Salmonella - strains susceptible to frameshift mutations and base-pair substitutions, plus S-9 or other activation mixtures for precarcinogens and premutagens; 2) Drosophila melanogaster - Recessive mutant test; 3) an in vitro cell transformation assay - morphological and neoplastic transformation of rodent or human cells as endpoints; and 4) DNA damage and DNA-repair synthesis - cultured human cells (normal cells plus cells from high cancer risk persons), plus oxidative/reductive activation mixtures.

In a review of methods of toxicological evaluation in 1976, De Serres (3) commented on the value of newly developing short-term tests for correlation between carcinogenic and mutagenic activity of environmental chemicals. He was specifically referring to studies conducted jointly by U.S. and Japanese scientists, which indicated the good correlations obtained using microbial assay systems, and to other validation tests on-going in 1976 (4). De Serres noted a number of pitfalls associated with employment of short-term assays but believed they should be used to establish priorities for testing in higher animals.

One view on the subject of short-term screening tests for carcinogens was provided by Bridges in 1976 (5). In essence, Bridges opts for the employment of short-term tests with a high predictive value for carcinogenic screening of environmental pollutants. He warns that a battery of tests must be employed for first-tier screening to avoid false negatives which occur with any one test. Bridges also pointed out that the lack of sensitivity of mammalian tests for carcinogens or mutagens impedes validation of microbial screening systems. This is due to "false" positives of microbial tests based on inadequate animal experiments.

The current experimentation with microbial and other short-term screening tests is not limited to governmental agencies and research institutes. In a staff report by Kolata in Science in 1976, she discussed industry's adoption of "quick" tests (6). Motivated by costs, time, and the Toxic Substances Control Act, major chemical companies are apparently turning to microbial (Ames) and other test systems, according to Kolata. She also noted that results from industrial sources will inevitably enhance the data base required for validation and correlation of "quick" test results with conventional mammalian tests.

In a study performed for the Environmental Protection Agency in 1974, Woodard surveyed toxicological test methods employed by nine chemical companies (7). At that time, none of the nine companies were using any but conventional test protocols. Even though short-term microbial and other in vitro tests are still in a transitional stage that stage may be shorter lived than heretofore anticipated.

An example of the intensity of effort expended in the past two to three years to evaluate and validate short-term tests is reflected in a publication from the Imperial Chemical Industries Ltd., Cheshire, UK. Purchase and co-workers (1976) tested 58 carcinogens and 62

non-carcinogens, all organics, using a battery of six tests (8). They evaluated ten short-term tests, empirically, and eliminated four tests but did not depreciate those four for other uses.

Overall results of these tests demonstrated the value of the Ames and cell transformation assay tests. Both tests detected a wide range of carcinogens with a low level of "false positives", according to the authors (8). Deficiencies of short-term tests were discussed and Purchase et al. also recommended that pre-screening include definitive study of molecular structure and physical-chemical properties of new compounds. (A detailed review of the current status of short-term tests for carcinogens and mutagens is given in a feature article in Chemical and Engineering News (9).)

The U.S. regulatory agencies responsible for implementation of laws relating to toxic substances, plus the National Cancer Institute (NCI) and the National Institute of Environmental Health Science among others, are all pursuing validation studies on short-term tests. Studies such as those of NCI were encouraged in a report of the Subcommittee on Environmental Carcinogenesis of the National Cancer Advisory Board (10). The subcommittee expressly stated:

"This subcommittee is enthusiastic about the possible future use of in vitro tests as part of a screening system for potental carcinogens and believes that their further development and validation deserve high priority."

The report also expressed the opinion of the board that short-term in vitro tests do not provide an adequate basis for characterizing an agent as carcinogenic for humans or animals.

Steuer and Ting (1977) reviewed methods being developed for monitoring in vitro carcinogenesis. They pointed out that cell transformation in vitro is meaningless if it cannot be equated with neoplastic transformation (11). The authors concluded that rapid, sensitive, quantifiable in vitro assays predictive of tumorigenicity would provide valuable means of carcinogenic screening of new chemical compounds.

For a somewhat different view of short-term toxicity tests for carcinogenicity, a 1977 article by Grasso and Grant (12) should be consulted. These authors classified short-term tests for carcinogenicity (STTC) as follows: (1) those which lead to the development of tumors as an endpoint, and (2) those in which assessment is based on a biological effect that does not involve tumor production. After a review of both types of STTCs and microbial testing systems, the authors concluded: "none of the tests mentioned provide clear evidence of carcinogenic activity." They also predicted that use of STTCs will lie only in indicating priorities for performing conventional animal testing. McNamara's 1977 article on long-term versus short-term toxicity tests calls for a combination of animal studies of only 90 days duration or less, except for certain suspect carcinogens, and selected in vitro tests (13).

Finally, one should refer to a published comparison of the value of short-term tests in a practical situation. In late 1977, the Office of Technology Assessment (OTA) at the request of Congress (Senate Committee on Human Resources) completed and published a study on the assessement of saccharin as a carcinogen (14). The report indicates the following purposes for conducting the battery of short-term tests: 1) to demonstrate to Congress the nature of the tests, 2) to demonstrate the speed with which they can be conducted (3 months), and 3) to illustrate their usefulness in making regulatory decisions. OTA also hoped that use of the short-term tests would help to clarify uncertainties regarding the carcinogenicity of saccharin. The OTA study also involved critical analysis of earlier animal testing of saccharin ingestion (in high doses) by rats which led to bladder cancer. Three of the short-term tests clearly showed saccharin to be mutagenic. On the other hand, if only *Drosophila*, yeast and the Ames test had been used, the mutagenicity of saccharin would have gone unnoticed.

The above review of current interest in and problems with short-term *in vitro* and *in vivo* tests as substitutes for chronic animal studies is not intended to be exhaustive. The Panel of Toxicological Experts had to be aware of problems existing in the overall milieu of toxicity testing. Efforts of the Panel of Experts, as described in succeeding portions of this report, should provide additional assistance in the selection of short-term tests predictive of chronic toxicological effects.

2.0 RESULTS - DISCUSSION

2.1 The Matrix Approach

Matrices developed for each of the six selected chemical compounds (benzene, cadmium, red and white phosphorus, formaldehyde, phosgene and oxides of nitrogen) are contained in Section 8.0 of this report. Each matrix is supplemented with a review of the literature on that compound as it pertains to endpoints or lesions observed, species of test animal used and the regimen of dosages administered. The literature review provided for each compound was not exhaustive but it contained a significant percentage of the pertinent data desired for review and evaluation by the toxicology panel.

Each matrix and its respective review paper was evaluated in detail by the panel of experts prior to selection of predictive endpoints from acute and subchronic study data. One limitation confronting the panel in the choice of predictive endpoints was the frequent absence of chronic (2-year) data. Based on the predictive endpoints selected, the panel recommended a number of short-term tests that could be used in a battery of tests by the Army to screen compounds. Tests recommended are indicated in the individual matrix for each compound.

A comparison of data recorded in the matrices for each of the six compounds suggests that benzene, cadmium and formaldehyde have been more intensely studied than phosgene, phosphorus, and oxides of nitrogen. In the case of phosphorus, there was a lack of animal data especially for chronic studies. In the latter studies, human results were more frequently reported than experimental results from animal studies. The above factors as well as the lack of information on dose response relationships hindered the panel, to some extent, from selecting predictive endpoints based on acute and/or subchronic effects.

Upon completion of all six matrices panel members reviewed each one again. A number of points were raised questioning the initial inclusion of certain predictive endpoints or short-term tests. A summary of endpoints and short-term tests taken from the six matrices had been prepared by the Tracor Jitco staff. Discussion of the summarized matrix data led the panel into development of a list of chronic effects (Table 1). The panel discussed at length the need to know what effects are commonly produced as a result of chronic exposure of a test animal to a chemical. As shown in Table 1, the panel determined that there were at least 10 major chronic effects experienced generally, plus specific subcategories for fibrosis and neuropathy and impaired performance.

Establishment of the list of chronic effects served a dual purpose. The panel first addressed the question of predictive endpoints based on the six matrices and secondly of determining which test systems might be employed in prediction of chronic effects.

Table 1

Toxicological Effects

Chronic Effect	Predictive Early Effect Test System
1. Neoplasia	1. Cell transformation 2. Covalent binding 3. Hyperplasia 4. Metaplasia 5. Increased unscheduled DNA synthesis (see *in vitro* position paper)
2. Fibrosis	1. Histological examination (Necrosis) 2. Biochemical tests (Collagen synthesis-precursor incorporation)
A. Atrophy (Organ weights reduced)	1. Organ weights
B. Hypertrophy (Increased cell size or size of organs)	1. Metabolic activity increase
C. Hyperplasia (Increased size and number of cells)	1. DNA/RNA Ratio (increased) 2. Thymidine incorporation (increase)

Table 1 (Cont'd)

Toxicological Effects

Chronic Effect	Predictive Early Effect Test System
3. Neuropathy and Impaired Performance	
A. CNS	1. Behavioral tests (selected key aspects from position paper) 2. Histological examination 3. Brain/body/weight ratio
B. Peripheral	1. Standard organophosphate neurotoxicity test in chickens 2. Neurotoxic esterase test 3. Neuromuscular function tests (reflex tests during routine pharmacologic testing-warm water in the ear, pressure on the eye, pressure on the carotid artery, evoked potentials, checklist of pharmacological signs) 4. Neuromuscular function test of medial rectus muscle of the eye (by electron microscopy)
4. Reproduction Impairment	1. Select key aspects from reproduction assessment position paper 2. One-generation mouse test (plus specific tests if required)
5. Mineralization	1. Necrosis 2. Excess intracellular calcium 3. Histochemical Tests 4. Electron microscopy
6. Amyloidosis	No adequate predictive test known

Table 1 (Cont'd)

Toxicological Effects

Chronic Effect	Predictive Early Effect Test System
7. Pigmentation (Excess in tissues or deposition in tissues where it does not belong)	1. Porphyrin (CNS, skin, kidney-urine, sweat, Hardevian glands) 2. ALA synthetase (liver mitochondria) 3. Melanosis (tyrosine metabolism)
8. Reduced Life Span	1. Impaired immunological competence 2. Suggested research items: A. Monitor ethane-pentane production (in vivo lipid peroxidation) B. In vitro cell culture C. Accelerated aging-specific rodents (late adult) D. Use Drosophila, flies or other animals of less than 90-day life span
9. Allergenic Hypersensitivity to Chemicals	1. Guinea pig sensitization test 2. Covalent binding plus a haptene (Research area)
10. Elastosis (Disease of elastic fibers of skin)	1. Physical examination of skin

2.2 Summary Matrix

Mentioned above was the development of a summary matrix designed to assist the panel members in evaluation of predictive endpoints and short-term tests. The summary matrix included all the endpoints and short-term tests recorded in the six individual matrices. The panel reviewed the comprehensive summary matrix and following detailed discussion, it was reduced to include only significant items as shown in Table 2.

Prior to presenting some general comments on the outcome of the matrix approach, it should be recalled that the matrix was initially conceived as a tool. The initial approach to identifying endpoints in the toxicology literature proved to be diffuse which suggested a focus was needed. The matrix concept was therefore one way of focusing on the problem of predictive endpoints and ultimately determining the feasibility of recommending a battery of short-term toxicological tests. Further, it should be noted, as stated in the review papers associated with each matrix, that all endpoints identified in the respective literature articles were not included in the individual matrices. In other words, representative endpoints only were chosen, first for clarity of the matrix and secondly due to the short period of time available to complete this study.

In general, the matrix approach served its purpose well - to focus, on a compound basis, on significant endpoints determined retrospectively from published information. The natures of the six compounds reviewed were diverse enough to yield considerable differences. For example, scanning the vertical columns for formaldehyde, phosgene and oxides of nitrogen in Table 2 reveals far fewer predictive endpoints were selected as compared to benzene and cadmium. In instances where no data were reported or the dose used did not create any adverse effects, no tests were recommended.

Scanning the horizontal columns of Table 2 shows three systems or effects that yielded little or no information - the central nervous system, behavioral, and cardiovascular effects. It would appear premature to judge the merits of these three effects in the overall area of toxic substance testing since only six compounds were considered in this study. Another group of compounds could perhaps yield substantially different results. Reference to the position paper on behavioral toxicology, which is discussed below, clearly shows the importance of that discipline within the overall toxicity testing milieu.

2.3 Short-Term Tests and Position Papers

A prime charge to the panel of toxicologists selected for this feasibility study was to develop a list of short-term in vitro or in vivo tests. Short-term was understood to be a test of 90 days or less. The panel was to recommend a battery of short-term tests which could be used in screening compounds of interest. In the course of selecting or suggesting predictive endpoints in development of the matrices discussed above, panel members also cited a number of short-term tests.

Summary of Endpoints and Recommended Short-Term T

System or Effect	Benzene	Cadmium	Phosphorus	Formaldehyde
1. Hematological Effects	A. Leucocyte Decrease	1. Hemolysis 2. Anemia	1. Leucopenia	None
	B. 1. General hematological work-up 2. Clotting/bleeding times	1. Hematological General work-up	1. General hematological work-up	None
2. Bone Marrow Changes	A. 1. Significant reduction in precursor cells-hemic renewal system	1. Inhibition of hemoglobin synthesis	1. Jaw-bone necrosis	None
	B. 1. Bone marrow differential 2. Turn over and cycle rates	1. Bone marrow differential 2. Turn over and cycle rates	1. Histological study	None

A-Predictive endpoints
B-Short-term tests

Table 2. Summary Matrix

of Endpoints and Recommended Short-Term Tests

	Phosphorus	Formaldehyde	Phosgene	Oxides of Nitrogen
sis	1. Leucopenia	None	None	1. Leucocytosis
logical l p	1. General hematological work-up	None	None	1. General hematological work-up
tion of obin sis	1. Jaw-bone necrosis	None	None	None
arrow ential ver and rates	1. Histological study	None	None	None

Table 2. Summary Matrix

2

Summary of Endpoints and Recommended Short-Term Tests

System or Effect	Benzene	Cadmium	Phosphorus	Formaldehyde
3. Immunological Effects	A. Decreased serum complement	1. Decreased viral antibody titer	None	1. Allergen sensitiz (correla with combinat with met groups i protein (see #7 below)
	B. 1. Globulin level 2. Albumen/ globulin ratio 3. Land-steiner sensi-tivity test	1. Same as benzene	None	1. Same as Benzene
4. Central Nervous System	A. None	None	1. Neural damage 2. Glioma damage	1. Evoked potenti visual of brai changes
	B. None	None	1. Neuroblastoma cytotoxicity test (Research required on these tests)	1. Evoked potenti tests

Table 2. Summary Matrix (cont'

of Endpoints and Recommended Short-Term Tests (Cont.)

	Phosphorus	Formaldehyde	Phosgene	Oxides of Nitrogen
reased al antibody er	None	1. Allergenic sensitization (correlates with combination with methyl groups in proteins) (see #7 below)	None	1. Hypersensitivity 2. Increased susceptibility to infection
e as zene	None	1. Same as Benzene	None	1. Same as Benzene 2. Mouse infectivity test
e	1. Neural damage 2. Glioma damage	1. Evoked potentials in visual center of brain-changes	None	None
e	1. Neuroblastoma cytotoxicity test (Research required on these tests)	1. Evoked potential tests	None	None

Table 2. Summary Matrix (cont'd)

2

Summary of Endpoints and Recommended Short-Term Tes

System or Effect	Benzene	Cadmium	Phosphorus	Formaldehy
5. Behavior	A. None	1. Significantly increased spontaneous locomotor activity		None
	B. Activity-wheel running test (related to blood picture)	1. Observation-routine		None
6. Cardiovascular Effects	A. None	1. Hypertension		None
	B. None	1. Organ-system function tests		None

Table 2. Summary Matrix

nts and Recommended Short-Term Tests (Cont.)

Phosphorus	Formaldehyde	Phosgene	Oxides of Nitrogen
y	None	None	None
	None	None	None
	None	None	None
	None	None	None

Table 2. Summary Matrix (cont'd)

Summary of Endpoints and Recommended Short-Term Test

System or Effect		Benzene	Cadmium	Phosphorus	Formaldehyd
7. Biochemical and Histochemical Effects	A	1. Enzyme changes 2. Reduced protein synthesis 3. Altered liver and kidney functions 4. Cytochrome P450 changes (See No. 12)	1. Decreased calcium serum level (blood) 2. Serum calcium-phosphorus ratio	1. Liver cirrhosis	1. Combina with me groups protein
	B	1. RNAase tests 2. Histology (liver, kidney, P450 changes, spinal cord) 3. Liver and kidney function tests	1. Biochemical tests 2. Histochemical tests	1. Histological tests 2. Biochemical tests	1. Protein synthes inhibit tests

Table 2. Summary Matrix (cont'd)

Endpoints and Recommended Short-Term Tests (Cont.)

	Phosphorus	Formaldehyde	Phosgene	Oxides of Nitrogen
ased um serum (blood) calcium- horus	1. Liver cirrhosis	1. Combination with methyl groups in proteins	1. Depressed A/G ratio	1. Lung damage 2. Elevated IG (possible correlation with hematological effects)
emical chemical	1. Histological tests 2. Biochemical tests	1. Protein synthesis inhibition tests	1. A/G Ratio	1. Lipid peroxidase test

Table 2. Summary Matrix (cont'd)

Summary of Endpoints and Recommended Short-Term Tes

System or Effect	Benzene	Cadmium	Phosphorus	Formaldehy
8. Body Weights, Organs and Tissues	A. 1. Endocrine changes 2. Spleen increased REC (correlation with immunologic effects) 3. Rough endoplasmic reticulum (RER) 4. Liver, fatty, infiltration	1. Decreased mineral content (bone) (correlation with biochemical) 2. Peribronchial fibrous (lung) 3. Decreased insulin (pancreas) 4. Fatty infiltration (liver) 5. Kidney necrosis	1. Kidney degeneration (Red phosphorus) 2. Bone necrosis and atrophy	1. Skin irrita 2. Eye ir 3. Lung c
	B. 1. Organ-body weight ratios	1. In vitro cell chemistry (liver) 2. Zinc/Cadmium relationship (pancreas) 3. Microscopic examination (lung and kidney) 4. Protein synthesis (RNA-DNA) 5. Kidney/liver function tests	1. Histology (kidney) (Red phosphorus) 2. Bone fracture strength test 3. Calcium balance	1. Draize (skin eye) 2. Lung f test 3. Ciliar motion

Table 2. Summary Matrix (cont'd)

dpoints and Recommended Short-Term Tests (Cont.)

	Phosphorus	Formaldehyde	Phosgene	Oxides of Nitrogen
d con- ne) tion cal) chial (lung) d s) fil-	1. Kidney degeneration (Red phosphorus) 2. Bone necrosis and atrophy	1. Skin irritation 2. Eye irritation 3. Lung changes	1. Resistance to gas diffusion	1. Lung damage
 istry ium ship s) pic ion d s) iver tests	1. Histology (kidney) (Red phosphorus) 2. Bone fracture strength test 3. Calcium balance	1. Draize test (skin and eye) 2. Lung function test 3. Ciliary motion test	1. Gas diffusion test 2. Pulmonary edema test (labelled albumen)	1. Pulmonary edema test (labelled albumen)

e 2. Summary Matrix (cont'd)

Summary of Endpoints and Recommended Short-Term Tes

System or Effect	Benzene		Cadmium	Phosphorus	Formaldehy
9. Cytologic and Cytogenetic Effects	A. 1. Chromosomal aberrations		1. Chromatid breaks	None	None
	B. 1. Sister chromatid exchange 2. Chromosome breaks and exchanges		1. Sister chromatid exchange	None	None
10. Molecular Effects	A. 1. RNA-DNA protein synthesis inhibited		1. Decreased RNA-DNA protein synthesis (correlation with liver changes)	None	None
	B. Thymidine-Uridine uptake tests (in vitro) (EPA screening level tests)		1. RNA-DNA ratios	None	None

Table 2. Summary Matrix (cont'd)

Endpoints and Recommended Short-Term Tests (Cont.)

	Phosphorus	Formaldehyde	Phosgene	Oxides of Nitrogen
tid	None	None	None	None
tid ge	None	None	None	None
ged A n sis lation iver s)	None	None	None	None
A ratios	None	None	None	None

Summary Matrix (cont'd)

Summary of Endpoints and Recommended Short-Term Test

System or Effect	Benzene	Cadmium	Phosphorus	Formaldehyde
11. Reproductive	A. 1. Embryonic death 2. Fetal Abnormalities	1. Testicular damage 2. Behavior-temporary loss of copulatory activity 3. Teratogenic effects	None	None
	B. 1. Embryotoxicity screening test 2. One generation mouse test	1. Histological examination (Testes) 2. Reproduction assessment screening (Nardone-Wilson)	None	None

Table 2. Summary Matrix (cont'd)

Endpoints and Recommended Short-Term Tests (Cont.)

	Phosphorus	Formaldehyde	Phosgene	Oxides of Nitrogen
cular e ior- rary loss pulatory rity ogenic ts	None	None	None	None
logical ination es) duction sment ning one- n)	None	None	None	None

Summary Matrix (cont'd)

Summary of Endpoints and Recommended Short-Term Tests

System or Effect	Benzene	Cadmium	Phosphorus	Formaldehyde
12. Metabolism	A. 1. Arene oxide formation	1. Body burden (liver and kidney 2. Excretion (urine)	None	None
	B. 1. Pharmacokinetic studies (absorption, distribution, excretion, body-burden) 2. Induction of cytochrome P450 by sleeping test 3. Extent of covalent binding (liver-kidney, labelled compound)	1. Pharmacokinetic studies (absorption, distribution, excretion, body burden)	None	None

Table 2. Summary Matrix (cont'd)

Endpoints and Recommended Short-Term Tests (Cont.)

	Phosphorus	Formaldehyde	Phosgene	Oxides of Nitrogen
urden and ion)	None	None	None	None
co- c s ption, bution, ion, body)	None	None	None	None

mmary Matrix (cont'd)

Summary of Endpoints and Recommended Short-Term Test

System or Effect	Benzene	Cadmium	Phosphorus	Formaldehyd
13. Carcinogenesis	A. None	None	None	None
	B. 1. Sister chromatid exchange 2. Leucocyte-chromosome damage test (Brewer and Evans)	1. Sister chromatid exchange 2. In vivo and in vitro tests (Ames, Drosophila)	None	None
14. Physical-Chemical Properties	A. None	None		None
	B. 1. Chemical structure relationship to known carcinogens should be examined 2. Identify volume-use characteristics of compound 3. Oil-water partition coefficients 4. Stability at pH 4,7, and 10	Same as Benzene	Same as Benzene	Same as Benzene

Table 2. Summary Matrix (cont'd)

Endpoints and Recommended Short-Term Tests (Cont.)

	Phosphorus	Formaldehyde	Phosgene	Oxides of Nitrogen
	None	None	None	None
r chro- l exchange ivo and itro tests), phila)	None	None	None	None
		None	None	None
as ne	Same as Benzene	Same as Benzene	Same as Benzene	Same as Benzene

2. Summary Matrix (cont'd)

Subsequently, in the course of panel discussion it became obvious that certain categories of toxic substance testing required elaboration prior to making final decisions on short-term tests. Accordingly, the following position papers were prepared by individual members of the panel: (1) Concept for Toxicological Testing; (2) Disposition of Xenobiotics: Pharmacokinetics and Biotransformation; (3) Reproductive Assessment Testing; (4) Toxicity Testing In Vitro; and (5) Behavioral Toxicity Testing. The full text of each position paper may be found in the Section 6.0 of this report. Highlights of each paper are presented below.

2.3.1 Concept for Toxicological Testing

The Concept for Toxicological Testing paper includes a description of several interrelating factors which contribute to variations in toxic testing regimens. The factors range from the purpose of testing to fiscal and other resources available. Attention is drawn to the Environmental Protection Agency's (EPA) three-phased approach for environment source assessment promulgated in 1977. A module concept for testing is described which would be superimposed on a multilevel concept such as that of EPA. Within level I of testing, the use of short-term mammalian and in vitro tests is recommended. The panel of experts, further recommended that the U.S. Army refer to three documents representative of the multilevel testing and module concept. The specific references noted above are cited in the concept paper (Section 6.1).

2.3.2 Disposition of Xenobiotics: Pharmacokinetics and Biotransformation

Pharmacokinetic studies should be performed early in a toxicologic investigation because they provide information that can be useful in setting up and evaluating subsequent tests. Data can be gained rapidly as to whether the agent is absorbed, how rapidly it is eliminated and how it is distributed in the tissues -- information that can predict the course for further testing. Radiolabeling greatly facilitates pharmacokinetic studies; in fact, it would be well to consider the synthesis of a labeled compound as one of the earliest steps in the evaluation of the toxicity of a compound.

In vitro studies of the biotransformation of toxic agents are useful, but in vivo studies usually provide more meaningful information. Metabolism studies should be conducted in two stages. In the first stage, the degree of biotransformaton is assessed without identifying the metabolites. Again, radiolabeling is greatly facilitative. The second stage is concerned with the identification of the metabolites. This frequently can be accomplished most readily by using the gas liquid chromatography-mass spectroscopy technique. Biotransformation studies may predict the mechanism by which the compound produces overt signs of toxicity. For example, hydroxylation reactions frequently involve the formation of intermediate metabolites which combine covalently to cellular macromolecules, thereby causing cellular damage or carcinogenesis. When radiolabeled compounds are available, covalent binding can be readily assessed.

Drug metabolizing systems are frequently highly inducible by foreign compounds. The induction of these enzyme systems have important toxicologic implication because these systems not only detoxify compounds, but in some cases, increase toxicity by causing the formation of toxic metabolites. Thus, depending upon the compound, induction may decrease or increase toxicity and thereby greatly influence the course of chronic toxicity tests. Induction can be evaluated by examining hepatic liver preparations for their cytochrome P-450 content or by performing "sleeping time" or "paralysis time" tests in intact animals using hexobarbital or zoxazolamine, respectively.

2.3.3 Reproductive Assessment Testing

Reproductive assessment entails the evaluation of those factors which lead up to and make possible pregnancy and embryonic development. While in vitro tests (cell, tissue and organ culture) may be useful for selected experiments their use in a battery of screening tests is not encourgaged at this time.

In lieu of use of the current 90-day mouse tests, a modified one-generation mouse test (65 days) is described and recommended for Army consideration.

The position paper also lists a number of non-mammalian reproductive assessment tests including the advantages and limitations of each system. The panel suggested further that as these tests are validated, they should be considered for inclusion in a battery of screening tests.

Reference to the recommendation section of this report reflects the panel's evaluation of the importance of reproductive assessment testing.

2.3.4 Toxicity Testing In Vitro

The thrust of this paper is on in vitro tests involving the use of mammalian cells and tissue cultures. Advantages and limitations are exemplified, and the use of in vitro tests primarily in screening programs is stressed. Cytotoxicity tests which have the widest acceptance and have been validated to varying degrees include cell viability, cell proliferation, and mutagenesis and carcinogenesis tests of different kinds.

A qualifying statment in the in vitro paper is worthy of quoting here: " In vitro cellular toxicology is at a crossroad in development." Tests and systems which are ready for validation and exploitation are detailed. This paper also contains recommendations for a comprehensive testing program which includes "minimal tests" and "supplementary tests". In vitro tests for cytotoxicity, mutagenesis and carcinogenesis, used in the first level of testing, coupled with animal studies, could serve as a basis for decision-making and prioritization of resources.

The panel recognized the emergence of in vitro testing as a new factor in toxicological testing which is not fully accepted but in concert with the position paper, the panel made specific recommendations on in vitro testing (see Recommendations Section).

2.3.5 Behavioral Toxicity Testing

The complex area of behavioral toxicity testing is dealt with in this paper under four categories: (1) motor performance, (2) sensory processes, (3) complex learned behavior, and (4) emotional behavior. Background information contained in the discussions of these four categories was the basis for presenting an overall strategy for the use of behavioral toxicity studies.

The overall strategy for use of behavioral toxicity screening tests includes use of rats only and three sets of procedures (neurological, motor integrity and sensory function, and complex learned behavior). Options and trade-offs are appropriately listed.

The panel's decision to include behavioral toxicity testing in its recommendations reflects the members' awareness of and concern for this subject.

In addition to the information provided in the behavioral toxicity testing paper discussed above, a behavioral toxicology protocol was made available to the panel. The protocol is included in this report as an addendum to the position paper and is found in Section 6.6. The protocol is currently in use in the Chemical Systems Laboratory, U.S. Army Armament Research and Development Command, Aberdeen Proving Ground, Maryland.

3.0 CONCLUSIONS

The panel of toxicology experts agreed that the matrix approach described in this report provided a focus for determining predictive endpoints. As with any retrospective analysis of research literature, the uniformity of available data was less than desirable. The matrix analysis permits identification of gaps or lack of information on the toxicology of a specific compound. Its application to a class or group of similar chemical compounds should be considered by the Army in a follow-on to this initial effort.

Opinions on the merits of current *in vitro* short-term tests as substitutes for chronic animal studies were not necessarily unanimous among panel members. Nonetheless, the panel fully agreed on the increasing need for development and validation of *in vitro* tests. Indeed, the panel concluded that additional research and development should be encouraged not only in such areas as *in vitro* cell culture but also, for example, in the use of test animals with less than a 90-day life span and in covalent binding.

The panel also endorsed the inclusion of behavioral toxicity testing as a significant factor in screening programs for the Army. The panel further concluded, based on its position paper for behavioral toxicity testing, that the number of simple tests now existing provides suitable opportunity for selection of specific tests as part of a battery of tests. These are specified in the recommendation section of this report.

Considerable attention was given to the areas of pharmacokinetics and biotransformation. The panel concluded that specific recommendations for such testing of compounds at the early stages of an Army program were warranted. The panel also concluded that these were areas requiring additional research.

The panel concluded that a one-generation reproduction study was sufficient for Army screening purposes. Only in a few cases would it be necessary to expend additional time and money to conduct the more elaborate three-generation tests. The panel concluded that use of *in vitro* systems involving cell, tissue and organ cultures for reproduction studies in a battery of short-term tests should be discouraged at this time. Special technical skills, high cost and limited information obtained from such tests are factors limiting their effectiveness.

In considering the role of a battery of short-term tests in an overall Toxicity Testing Program, the panel concluded that a spectrum of tests, viewed as modules, should be considered. Various combinations and sequences of the modules could satisfy a wide variety of needs ranging from screening to assessment of risk to man and prioritization of resources. The minimal tests recommended by the panel are compatible with the module concept.

4.0 SHORT-TERM TESTS RECOMMENDED FOR A TOXICOLOGY SCREENING PROGRAM

1. Perform a complete hematological work-up

2. Prepare bone marrow differential smears

3. Perform a one-generation reproduction study
   a. Reproductive assessment can be achieved by a modified one-generation mouse test. Sexually immature mice of a stable heterogeneous stock are required for the test which covers about 65 days. (See the position paper on reproductive assessment for details)
   b. Use of *in vitro* cell, tissue and organ culture systems for reproductive assessment in a battery of screening tests is not recommended at this time.

4. *In vitro* testing
   It is recommended that *in vitro* tests for cytotoxicity, mutagenesis and carcinogenesis be used in the first level of testing. Results of such tests, coupled with those of animal studies, can be used in decision-making regarding further testing and prioritization of resources. Specific tests recommended are as follows:

   Cytotoxicity

   Minimal Tests
   1. Cell viability with two established cell lines (ECL). Requires 3 to 5 days.
   2. Cell proliferation with two established cell lines (ECL). Requires 3 to 5 days.

   Supplementary Tests
   1. Cell viability using ^{51}Cr and two established cell lines. Requires 3 to 5 days.
   2. Cloning efficiency with two established cell lines (ECL). Requires 2 weeks.
   3. Gross cytology with two established cell lines (ECL). Requires 3 to 5 days.
   4. Macromolecular synthesis (RNA/DNA synthesis) with two established cell lines (ECL). Requires 3 to 5 days.
   5. Liver cell function with primary liver epithelial cell culture. Requires 3 to 5 days.
   6. Alveolar macrophage (phagocytosis) with rabbit alveolar macrophages. Requires 3 days.

   Mutagenicity

   Minimal Tests (In addition to Ames, Drosophila)
   1. Chromosome damage (Sister chromatid exchange) with ECL.
   2. Gene mutation with mouse lymphoma (L5178Y). Requires two weeks.

Supplementary Tests

1. Unscheduled DNA synthesis with WI38 cells. Requires 1 week.
2. Single strand DNA breaks with WI38 cells. Requires 3 to 5 days.

Carcinogenesis Tests

Minimal Tests

1. Cell transformation with mouse embryo cells C3H/10T1/2/CL8. Requires 4 to 6 weeks.
2. Cell transformation with Syrian hamster embryo. Requires 4 to 6 weeks.

Supplementary Tests

Cell transformation with hamster embryo, transplacental. Requires 4 to 6 weeks.

5. Perform microbial infectivity test to demonstrate animal hypersusceptibility to infectious organisms.

6. Carry out standard central nervous system observational evaluations.

7. Perform motor and sensory function and behavior assessment tests using rats and three sets of procedures: neurological, motor integrity and sensory function, and complex learned behavior. (See the position paper on behavioral toxicity testing for details).

8. Carry out organ function tests in the heart and the vascular system.

9. Carry out biochemical tests including as a general procedure covalent binding and *in vivo* lipoperoxidation tests for ethane or pentane production. Specific biochemical tests are recommended on a compound basis.

10. Organ morphology procedures recommended are determination of organ/body (or brain) weight ratios and standard histological examinations.

11. Skin and eye irritation and skin sensitization tests should be performed.

12. A basic pharmacokinetic study is recommended in the early stages of a toxicology test program. Tests for induction of cytochrome P450 are also recommended with emphasis on the indices, for example, hexabarbital ("sleeping time") and zoxazolamine ("paralysis time").

13. The minimum tests recommended for obtaining information on a compound's physical and chemical properties are:
    a. Oil/water partition coefficients
    b. Stability in aqueous media at pH levels of 4.0, 7.0, and 10.0.

5.0 LITERATURE

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6.0 POSITION PAPERS

6.1 Concept for Toxicological Testing

The many and varied circumstances which are associated with any major producer or user of potentially toxic substances preclude the adoption of a single, inflexible, standard regimen for testing all chemicals of interest. Among the often interrelating factors which contribute to the variation from one producer/user to another and for a single producer/user at different times are the following:

1. The purpose(s) of the tests. The approaches and tests that are used should be selected to satisfy specific objectives. Those that are used as primary screens in the identification of potential problem areas will not necessarily satisfy regulatory agencies. Also, methods used for the assessment of risk to man or to other parts of an ecosystem will differ.

2. The number, nature and variety of the potentially toxic substances. The situation may be affected by a need to test a large number of chemically and physically unrelated substances which have varied or unknown toxicities, involve several different exposure routes, and which may have an additive, synergistic, or antagonistic effect when administered in concert.

3. The magnitude of the problem. At times, the magnitude of the problem can be readily discerned because of earlier testing on the same or related chemicals, or earlier studies on their distribution and cycling in nature, and knowledge of the quantities of chemicals involved. In the absence of earlier studies, the range of tests to be used is expanded.

4. The time frame for testing. The lack of a knowledge base on which to base decisions regarding substances already in use as well as an awareness of a potentially serious problem which may not be readily contained, creates a time imperative quite different from that which is attendent to the orderly development and testing of a new product.

5. Degree of certitude necessary for decisions. Toxicity testing regimens often represent the result of a number of "trade-offs" which affect risk assessment. While it is axiomatic that the highest degree of certitude is to be sought, practical considerations often preclude this. A particular producer/user may recognize that because of existing knowledge and/or the amount of material under consideration a high degree of certitude may be absolutely mandatory in some instances and not as critical in other instances.

6. Fiscal and other resources. Available funds, technical personnel, and facilities are among the many circumstances which shape testing regimens.

Testing Objectives

Testing objectives and their associated descriptive statements may be broad or narrow. In either event, it is imperative that they be unambiguous. Every comprehensive testing program entails both types which often are related to a testing sequence and to a need to satisfy different confidence requirements.

The U.S. Food and Drug Administration proposed that mutagenesis testing proceed through a Tier System involving three levels of increasing complexity, with each tier serving to answer different questions. Tier 1 questions whether or not a compound is a potential mutagen; Tier 2 questions whether or not a presumptive mutagen (Tier 1 positive) is mutagenic in mammals; Tier 3 questions what is the potential risk to man from exposure to a mutagen (Tier 2 positive).

Woodard reported in 1974 that the major industries covered in a survey of testing practices ordinarily engage in four levels of environmental testing which "correspond to the same number of levels of exposure of either man or his environment. These levels are derived from consideration of length of exposure, extent (avoidable or not), numbers of people at risk, and the portion of the environment exposed".

In 1977, the Industrial and Environmental Research Laboratory of the EPA developed a three phased approach for environmental source assessment with Level 1 serving to segregate out the "bad actors" from substances which are probably innocuous. The "bad actors" are evaluated more critically in Level 2 while the presumptive innocuous substances are assigned a lower priority for futher testing. The objectives of Level 3 are to monitor the problems identified in Level 2 and to assess the chronic and ecological effects of the components of an industrial process.

The environmental source assessment phased approach, which is designed to monitor industrial processes and their effluents, comes closest to satisfying the needs of the Department of the Army. Nevertheless, the Department of the Army does have some unique problems including a military imperative, a back-log of varied chemicals to be tested and unique use and dispersal situations.

The Module Concept in Toxicity Testing.

The above-mentioned factors have served to emphasize that flexibility is mandatory if the varied and pressing problems confronting the Department of the Army are to be addressed in a logical and realistic fashion.

Ideally, the Department of the Army should have access to a spectrum of tests, which should be viewed as modules to be used in various combinations and sequences to satisfy virtually every conceivable situation and need, ranging from primary screening to definitive assessment of potential risk to man and which could be used in the prioritization of time and resources.

Through the use of the MODULE CONCEPT, superimposed on the multilevel testing concept, it should be possible to readily select appropriate combinations and sequences of tests which best relate to a clearly specified testing objective. In this way, the toxicological testing efficacy can be more readily focused. The system also lends itself to a continuous assessment of opportunities for pruning and "trade-off" in order to be more efficient and to satisfy constraints and imperatives of time.

General Recommendations for Testing.

a. It is recommended that for all chemicals for which there is no adequate data base, a testing objective comparable to EPA Level, Health Effects Assessment be adopted. It is recommended further that Level 1 testing have the following as its objectives:

1. Preliminary acute toxicity, mutagenicity and carcinogenicity assessment (using short-term mammalian and *in vitro* tests).

2. The use of Level 1 assessment for the prioritization of resources and effort vis-a-vis further testing of some of these compounds in Level 2.

b. It is recommended that Level 2 testing have the following as its objectives.

1. Testing of Level 1 positives, and when necessary, other substances and their relatives reported to be potentially harmful, using tests which will permit a more reliable estimation of the nature and degree of risk to man and his environment.

2. The use of Level 2 assessment for the prioritization of resources and effort vis-a-vis further testing and control of exposure to toxic substances.

It is recommended further that the module concept for test construction be used for the selection, temporal arrangement and decisions leading to termination of specific Level 2 tests.

Level 2 modular test construction and utilization also may be appropriate in other circumstances - regardless of Level 1 testing or its outcome. It is recommended that Level 2 testing be performed when the following circumstances exist:

1. When previously published information indicates the existence of a potential problem with the same or related substances.

2. When the substance is produced in large quantities.

3. When the use of the substance is concentrated in a given locale.

4. When the stability and/or cycling of the substance in nature suggests that it may persist for a long time or be concentrated by physical or biological factors.

5. When the risk of accidental breakdown of containment is significant.

6. When the length of exposure and/or number of people at risk is high.

The kinds of tests and the species used in Level 2 testing will be governed by a number of factors among which will be the following: degree of certitude required; the magnitude of the potential problem; the anticipated lesion (mutation, teratogenesis, etc.); the probable route of exposure; time constraints; fiscal constraints.

This testing approach is growing in acceptance, and has been described in a number of documents, including several published under the aegis of Federal regulatory agencies. Representative among them are the following:

Food and Drug Administration Advisory Committee on Protocols for Safety Evaluation. (1971). Panel on Carcinogenesis report on cancer testing in the safety evaluation of food additives and pesticides. Toxicol. Appl. Pharmacol., 20: 419-438.

National Academy of Sciences - National Research Council. (1964). Committee on Toxicology. Principles and procedures for evaluating the toxicity of household substances. NAS Publ. no. 1138. Washington, D.C.

Food and Drug Administration. (1976). Criteria for evaluation of the health aspects of using flavoring substances as food ingredients. Prepared for Bureau of Foods, FDA. Life Sciences Research Office, Federation of American Societies for Experimental Biology, Bethesda, Md.

The following list of references as well as the examples of how multilevel modular testing could be applied have been reproduced from the EPA-sponsored study, "Testing for Health Effects of Fuels and Fuel Additives" published in 1977 (Gause, M. et al., 1977).

References for Possible Testing Approaches
(from Gause, E.M., et al., 1977)

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TOXICITY	MUTAGENICITY	CARCINOGENICITY	TERATOGENICITY
General	Gene Mutation	Chronic in vivo	Teratogenesis
Acute in vivo	3 of these 5	inhalation	test
Dermal/eye	Bacteria		
Chronic in vivo	Eukaryote		
inhalation	Insect		
Pulmonary	Mammalian cells		
Inhalation in vivo	Rodent (specific locus)		
acute	Chromosomal Effects		
sub-chronic	3 of these 4		
Central Nervous	Insect		
System	Rodent cytogenetic test		
	Rodent dominant lethal		
	Rodent heritable tanslocation		
	DNA Damage and Repair		
	2 of these 4		
	Bacteria		
	Yeast mitotic recombination		
	and gene conversion		
	Mammalian cell DNA repair		
	Mammalian cell sister-chromatid		
	exchange		

HEALTH RISK ASSESSMENT

Figure 1. AN EXAMPLE OF A MATRIX APPROACH TO TESTING
(from Gause, E.M., et al., 1977)

	TOXICITY	MUTAGENICITY	CARCINOGENICITY	TERA
	General Acute in vivo Dermal/eye	Gene Mutation Bacteria Yeast	Mutagenesis Tier 1 tests	Tera i
TIER 1 (Level 1)	In vitro Pulmonary Acute inhalation	DNA Repair Bacteria Chromosomal Effects Yeast		
	Low Toxicity High Toxicity	Negative Positive	Negative Positive	
	Pulmonary sub-chronic inhalation	Gene Mutation Plants, insects, Mammalian cells	Neoplastic Transformation in vitro	
TIER 2 (Level 2)	Central Nervous System	DNA Repair Mammalian cells Chromosomal Effects Insects, mammal- ian cells, rodent		
	Chronic In vivo inhalation	Gene Mutation Rodents	Chronic In vivo inhalation	
TIER 3 (Level 3)		Chromosomal Effects Rodents		

HEALTH RISK ASSESSMENT

FIGURE 2. AN EXAMPLE OF AN HIERARCHIAL APPROACH TO TESTING.

(from Gause, E.M., et al., 1977)

CARCINOGENICITY

TERATOGENICITY

Mutagenesis
Tier 1 tests

Teratogenesis
in vivo

SCREENING FOR BIOLOGICAL ACTIVITY (Acute)

ffects

Positive

Negative

Positive

Neoplastic
Transformation
in vitro

sects,
cells

cells

CONFIRMATORY TESTS (Sub-chronic)

ffects
mmal-

Chronic
In vivo
inhalation

RISK ASSESSMENT (Chronic)

ffects

HEALTH RISK ASSESSMENT

MPLE OF AN HIERARCHIAL APPROACH TO TESTING.

m Gause, E.M., et al., 1977)

2

6.2 Disposition of Xenobiotics: Pharmacokinetics and Biotransformation

Pharmacokinetics is concerned with the absorption, distribution, biotransformation and excretion of foreign compounds (xenobiotics). Although biotransformation is a component of pharmacokinetics, it is best discussed under a separate heading.

PHARMACOKINETICS

Pharmacokinetic studies should be performed early in a toxicologic investigation because they provide information that can be useful in setting up and evaluating subsequent tests. For example:

1. A pharmacokinetic study would establish whether or not a substance is absorbed. High molecular weight polymers (e.g., nitrocellulose) and some very insoluble substances are not absorbed. When this is the case, further toxicological testing may not be necessary.

2. The volume of distribution of a compound, which can be calculated from the rate of disappearance of the compound from the blood, can tell something about the distribution of the compound in the tissues; e.g., a very high volume of distribution can mean that the substance is deposited in body fat.

3. Rapid and complete elimination of the substance may mean that ingestion of trace amounts of the substance does not constitute a hazard and that a short-term (90 days) repeated ingestion study should adequately assess its relevant toxicological properties.

The amount of the compound to be administered can be predicted by its LD_{50}. Studies should be performed using a toxic dose (e.g., an LD_{25} dose) and a very low dose which does not produce obvious signs of toxicity. The route of exposure would be determined in part by the kind of exposure that humans might be expected to experience. For example, if humans were to be exposed by breathing air contaminated by the substance, administration by inhalation would be emphasized. In any event, more than one route should be employed. The decision as to what animal species should be used is difficult. While absorption, distribution and renal excretion of most compounds are quite similar among laboratory animals, rates of biotransformation are not, and it is therefore not possible at this time to predict the species that will biotransform a given compound as man does. At selected intervals after the administration of the compound, feces, urine and expired air would be collected and examined for their contents of the compound and its metabolites. Serum samples would be collected at more frequent intervals. In some cases it might be advisable to examine the carcass at the end of the experiment for its content of the compound and its metabolites.

The development of methods for the determination of the compound and its metabolites in body fluids, tissues and excreta would usually present such a formidable task that pharmacokinetic studies could not be undertaken as a screening procedure. However, it is often possible to synthesize a ^{14}C-labeled compound, and when this is the case,

pharmacokinetic studies can be greatly simplified. In fact, it might be well to consider the synthesis of a labeled compound as one of the earliest steps in the evaluation of the toxicity of a compound. The radioactivity count of the various specimens to be analyzed does not distinguish the compound from its metabolites, but for a screening test this is not usually necessary. Pertinent information as to the longevity of the compound in the animal, serum levels of the drug, rates of excretion, and volume of distribution can be obtained with radio-labeled compounds. A very prolonged urinary excretion of ^{14}C often means that metabolites of the compound have entered the carbon pool.

BIOTRANSFORMATION

Xenobiotics are biotransformed by four kinds of reactions: oxidation, reduction, synthesis and hydrolysis. A compound may be involved in one or more of these reactions; the reactions may occur independently or sequentially. For example, the compound may be hydrolized and one or both of its products may then be oxidized; the oxidized product may then be conjugated by one of several mechanisms (glucuronidation, sulfation, acetylation, etc). The kinds of reactions which will biotransform a given xenobiotic can be largely predicted from its structure. Biotransformation usually results in products which are less toxic and more readily excreted than the parent compound. Thus biotransformation usually means detoxification; the more extensively a xenobiotic is metabolized, the less likely it is to accumulate in the tissues and produce toxic effects. There are notable exceptions to this generalizaton; some compounds are metabolized to active intermediate products which damage cells by reacting covalently with cellular macromolecules. In fact, some compounds, notably certain polycyclic hydrocarbons, are thought to manifest their carcinogenicity in this way. Biotransformation studies are important in the overall evaluation of the toxicity of a compound because they may provide some insight as to the mechanism by which the compound produces overt signs of toxicity. This in turn may predict the severity of the toxicity of related compounds. The identification of certain metabolites may send up warning signals; for example, hydroxylation reactions frequently involve the formation of intermediate epoxides; epoxides are known to provide the opportunity or covalent binding to cellular macromolecules.

In vitro studies. Most biotransformations of xenobiotics occur mainly in the liver. In vitro studies are therefore usually performed only with liver preparations unless there is some reason to suspect that other organs may contribute to the metabolism of the compound. Hepatic microsomal preparations are employed for oxidative reactions, but other cell fractions are required if synthetic biotransformations are to be observed. In vitro studies may provide useful information, but it is unlikely that this information can be as useful as that obtained from in vivo studies. To be generally applicable, an in vitro screening test would necessarily employ tissue preparations that would contain the enzymes and cofactors needed for all possible reactions. Obviously, no single preparation would be suitable for a screening test. Moreover, in vitro tests do not always predict what will occur in vivo, largely because it is not possible to duplicate in vivo conditions with respect to available cofactors, available enzyme, oxygen supply, membrane

effects, etc. In view of these considerations, it is not likely that meaningful in vitro tests can be performed as readily as in vivo tests. This does not exclude in vitro tests which might be indicated for certain compounds. For example, if the structure of a compound suggested that it could act as an anticholenergic agent, one might wish to see what effect it might have on cholinesterase.

Many xenobiotics are oxidized by cytochrome P-450-dependent monooxygenase systems located in the endoplasmic reticulum (microsomal fraction) of the liver. These monooxygenase systems are frequently induced by the xenobiotic in question such that not only is its own rate of biotransformation enhanced greatly, but that of many other xenobiotics is also induced. The induction of these enzyme systems have important toxicologic implications. The increase in the rate of biotransformation produced in this way may greatly increase the rate of detoxification of the compound, or in cases where a toxic intermediate metabolite is formed, the toxicity may be enhanced. Induction may be a particularly important factor when exposure to more than one toxic agent occurs. Induction involves an increased biosynthesis of hepatic cytochrome P-450. Cytochrome P-450 content of the liver is usually determined by difference spectroscopy of hepatic microsomes. The isolation of microsomes requires a high speed centrifuge that will attain a speed of 100,000 x g. The procedure consists of placing a suspension of microsomes in two cuvets contained in a spectrophotometer, which are then balanced spectrally to eliminate the spectrum produced by cytochrome b_5, the only other chromaphore found in hepatic microsomes. Dithionite is added to both cuvets and carbon monoxide is then bubbled through the sample cuvet. A tracing is made of the spectrum. The magnitude of the peak at 450nm (OD 450-490 nm) determines the amount of cytochrome P-450. A simplified procedure which uses whole liver homogenates can be used which gives results very similar to those obtained with microsomes. Homogenates are balanced spectrally in two cuvets contained in a spectrophotometer, carbon monoxide is bubbled through both cuvets, dithionite is added to the sample cuvet, a tracing of the spectrum is made, and the content of cytochrome P-450 is calculated from the magnitude at peak absorption (450 nm). This procedure circumvents the interference caused by contaminating hemoglobin and eliminates the need for a high speed centrifuge and the two centrifugations (one at 10,000 x g to remove nuclei and mitochondria, and the other at 100,000 x g to sediment microsomes) required for the isolation of microsomes.

The degree of induction depends on the compound as well as the dose. Maximal induction may require as little as two days (as with 3-methylcholanthrene and many other polycyclic hydrocarbons) or as long as two weeks (as with chlordane). This should be taken into consideration when compounds of unknown inducing capacity are tested.

Some xenobiotics cause a loss of cytochrome P-450 when administered (e.g., carbon tetrachloride, seconal and certain other compounds that possess an allyl function, and all interferon inducing agents that have been tested). It would be important to know when this occurs because the loss of cytochrome P-450 would affect the toxicity of the compound in question as well as that of other xenobiotics to which the human might be exposed simultaneously. Any change in the cytochrome P-450 level of the

liver, whether an increase or a decrease, will affect the pharmacokinetics of the compound that produces the changes if it is biotransformed by cytochrome P-450.

"Sleeping time" or "paralysis time" tests are frequently employed to evaluate induction or depression of cytochrome P-450-linked monooxygenase systems. The length of time an animal will sleep or remain paralyzed after the administration of a barbiturate or zoxazolamine, respectively, may be a measure of the rate of in vivo metabolism of these two drugs. If an agent prolongs sleeping or paralysis time it may mean that the agent has impaired a drug metabolizing system; if these times are shortened, drug metabolizing systems may have been induced by the agent. The other interpretation is that the agent has an affect on the central nervous system not related to drug metabolism. In this case, the agent may produce obvious CNS effect when administered without the barbiturate or zoxazolamine. In any event, if a prolongation or shortening of sleeping or paralysis time is noted, a determination of the blood level of hexobarbital or zoxazolamine at one or two time intervals after administration will reveal whether drug metabolism is involved. If an agent causes a prolongation of sleeping time, the agent should be administered at the moment the animal awakens (rights itself). If the effect of the agent is on the CNS, the animal will go back to sleep; if it does not, the prolongation of sleeping time is most likely due to delayed hexobarbital metabolism. Hexobarbital is used because it has a relatively short half life. Zoxazolamine is used because those cytochrome P-450 systems which are not involved in hexobarbital metabolism are usually involved in zoxazolamine metabolism. These tests not only tell us something about the effects agents may have on cytochrome P-450 linked monooxygenase systems, but if it is shown that alteration of sleeping or paralysis time is not due to altered drug metabolism, they may also tell us something about the effects of a given agent on the CNS.

Metabolites

Metabolite studies should be conducted in two stages. In the first stage the degree of biotransformation would be assessed without identification of specific metabolites. Urine, feces and breath would be processed for thin layer, liquid-liquid chromatography, GLC; etc. If a ^{14}C labeled compound is used, the radioactivity of the spots or fractions would be counted.

The second stage would be concerned with the identification of the metabolites. There is no way to screen the identity of metabolites. However, an investigator who is experienced in the field of metabolism of xenobiotics can usually predict what metabolites can be formed from a given substance, and this narrows the search considerably. Fortunately, modern technology in the form of gas liquid chromatography-mass spectroscopy has provided the means for isolating and identifying extremely small amounts of metabolites. GLC-MS has reduced the time required for the identification of metabolites from weeks or months to days or hours. It has reduced the time required for identification of metabolites to what one would hope to achieve with a screening test.

Most organic xenobiotics and their metabolites bind loosely to proteins and therefore exist in equilibrium between free and bound forms in the tissues. However, it is now known that certain highly reactive intermediate metabolites of many xenobiotics may combine covalently with cellular proteins and other macromolecules. In certain cases, cellular damage or carcinogenesis occur as a consequence of covalent binding. Although it is known that not all covalent binding of intermediate metabolites is damaging to the cell, knowledge of its existence should signal a more than routine search for cellular damage, cancer or mutagenesis. Methods for the determination of covalent binding usually involve a labeled compound. The radioactivity of various tissues is determined after extraction procedures have been employed to remove all but the covalently bound radioactive metabolites.

6.3 Position Paper on Reproductive Assessment Testing

The following summarizes our views regarding reproductive assessment testing as part of a battery of tests for the evaluation of the potential toxicity of chemicals in the environment.

Reproduction in higher mammals involves as many as thirteen different episodes including those attendant to copulation, fertilization, implantation, histogenesis, and organogenesis. Hence, reproductive assessment entails factors which lead up to and make pregnancy, as well as embryonic development, possible. Embryonic development *in utero* is characterized by a number of unique interrelationships involving the mother and the embryo which have a significant impact on how a chemical may affect an embryo. These include metabolic alteration of a chemical, its excretion, and its impact on maternal and embryonic homeostasis. Hence, a reproductive assessment testing program must encompass tests which are indicative of effects on pre-fertilization and post-fertilization factors and which reflect awareness of the unique maternal-embryonic interrelationships.

At the present time, cell, tissue, and organ culture approaches can be used to study only selected aspects of reproduction such as fertilization, blastocyst development, histogenesis and organogenesis. Such *in vitro* systems, indeed, may be useful and/or necessary for selected experiments addressed to mechanistic questions. Their use in a screening battery reproductive assessment is to be discouraged at this time, however. Mitigative against their use is the fact that the information which can be obtained is limited and conditional due to the artificial circumstances. Furthermore, special technical skills and high costs make the systems inappropriate for screening purposes.

Among the important problems confronting those responsible for chemical testing are problems related to time and cost. Limitations in availability of a chemical or a desire to minimize the distribution of a potentially harmful substance, as well as cost and time factors, suggest that alternatives to a 90-day mouse study warrant serious consideration.

It is recommended that reproductive assessment be done using a modified one-generation mouse test over a period of approximately 65 days. A brief outline of the test and the types of data to be obtained follow.

Sexually immature mice of a stable heterogeneous stock (eg., Swiss-Webster) are treated daily with the test substance or a suitable vehicle beginning on day 40 of life. A minimum of 10 males and 30 females are to be treated at each dosage level and as concurrent controls.

After 20 days of continuous treatment, mating is begun by caging one male with three females. Daily observations of females for vaginal plugs are then initiated. Breeding and treatment are continued until all females are observed to have vaginal plugs or until a further 20 days have elapsed. As females are found to have plugs, they are successively assigned to three groups, A, B and C. Females not observed to have

vaginal plugs after 20 days of exposure to a male are killed, weighed, and ovaries and uteri examined histologically for signs of cyclic activity. Data recorded: % females not inseminated and % not running estrous cycles.

Group A females are killed 12 days after observation of vaginal plug, and total number of corpora lutea on both ovaries and total implantations in uterus counted. Data recorded: total corpora lutea, total implants, % resorbed implants, % failed implantation of ovulated ova.

Group B females are maintained to day 19 after observation of plug and then killed. Their uteri are then examined for surviving fetuses which are weighed and evaluated for developmental abnormality. Data recorded: intrauterine death and/or resorption, growth retardation and developmental abnormalities.

Group C females are allowed to deliver at term (20 days after plug) and to nurse their young for 5 days after which all females and young are killed and weighed. Data recorded: number of females failing to complete parturition, number of females failing to nurse or care for young, % of young stillborn, % of young failing to survive to 5 days, growth deficiency in surviving young.

Males surviving after 40 days of treatment are killed and weighed and those not having inseminated at least one female are examined for testicular and accessory organ weights and histologically for spermatogenesis. Data recorded: presence or absence of sterility and whether attributable to deficient reproductive behavior or endocrinology, or inadequate spermatogenesis.

An example of a one-generation reproduction study in rats is shown in Figure 1 (10).

Reproduction

The foregoing mouse reproduction test is not a comprehensive test of all aspects of reproductive function; for example, in case of reproductive failure it does not always permit assignment of the primary cause of failure to either sex or both sexes. To determine whether males or females are at fault, it may be necessary to repeat the test by pairing treated females with control males and treated males with control females. It does provide information on several critical aspects and should be adequate to alert the testing agency that more rigorous tests are needed if human or animal exposures to more than negligible concentrations are anticipated. Although less time consuming and expensive to conduct than currently approved mammalian tests, this abbreviated version does not remove the need for faster and less costly screening procedures.

The following table summarizes an evaluation of several non-mammalian tests that could be useful in a reproductive assessment program after appropriate validation.

NON-MAMMALIAN REPRODUCTIVE ASSESSMENT TESTS (TERATOGE

Reference	Organisms or Species	End-points	Time	Advantages	I
1.	Drosphila	Morphological anomalies; mouth, wing, etc.	15 days	Large numbers; low cost; time; clear endpoints	
2.	Oryzias latipes (a fish)	Extra-embryonic circulation; external and internal anatomy	20 days	Large numbers; low cost; ease of handling, studies can readily be timed; broad spectrum of responses	
3.	Amphibian embryos	Arrested development	Several days	Broad range of responses possible; tetrapod development; cost; time	
4.	Quail	Beak and leg development	15-25 days	Mother administered chemical prior to egg laying; low cost	
5.	Amphibians	Behavioral disturbances; retarded development; morphological changes	25 days	Large numbers; intact embryo; tetrapod development; low cost	

REPRODUCTIVE ASSESSMENT TESTS (*TERATOGENESIS*)

Time	Advantages	Limitations	R & D Required
15 days	Large numbers; low cost; time; clear endpoints	Non-placental; one dose study	Wastage data; sterility data; several doses; validation with variety of mammalian teratogens.
20 days	Large numbers; low cost; ease of handling, studies can readily be timed; broad spectrum of responses	Non-placental; spawning in lab not assured	Validation with few teratogens exists, more is needed. Good potential.
Several days	Broad range of responses possible; tetrapod development; cost; time	Seasonal availability only limited range of responses has been studied; jelly coat may be a barrier	Consequences of removal of jelly coat; extension of range of responses.
15-25 days	Mother administered chemical prior to egg laying; low cost	Maternal to ova transfer implies chemical must have an affinity for yolk constituent; not to be confused with transplacental transfer; seasonal availability; small sample number	Good potential requiring extensive validation.
25 days	Large numbers; intact embryo; tetrapod development; low cost	Seasonal availability; penetration problem may not mirror mammalian situation	Penetration problem can be circumvented by injection.

NON-MAMMALIAN REPRODUCTIVE ASSESSMENT TESTS (TERATOGENESIS) (

Reference	Organisms or Species	End-points	Time	Advantages	Lim
6.	Pigeons	Interference with histogenesis	20 days	Low cost; may be a model system for histogenesis induction capability	
7.	Newt	Inhibition of tissue and organ growth; differentiation	5-52 days	A tetrapod; clear end point large numbers	
8.	Chicken	Embryo lethality; developmental abnormalities	21 days or less	Cost, time, large number; this is the most thoroughly studied non-mammalian system	
9.	Chicken	Abnormal neural fold and segmentation of paraxial mesoderm	2 days	Rapid, inexpensive, well-studied system	

RODUCTIVE ASSESSMENT TESTS (TERATOGENESIS) (CONT'D)

ime	Advantages	Limitations	R & D Required
0 days	Low cost; may be a model system for histogenesis induction capability	Seasonal availability	Needs validation.
-52 days	A tetrapod; clear end point large numbers	Injection of chemical is tedious; analogy that morphogenesis and embryogenesis are somewhat equivalent remains to be proved; seasonal availability	Further validation with mammalian teratogens.
1 days or ss	Cost, time, large number; this is the most thoroughly studied non-mammalian system	Avian system; distribution of chemical in yolk	Some additional validation. Excellent potential.
days	Rapid, inexpensive, well-studied system	Explanted chick embryo culture requires special skills; sample size may be limited by above	Additional validation.

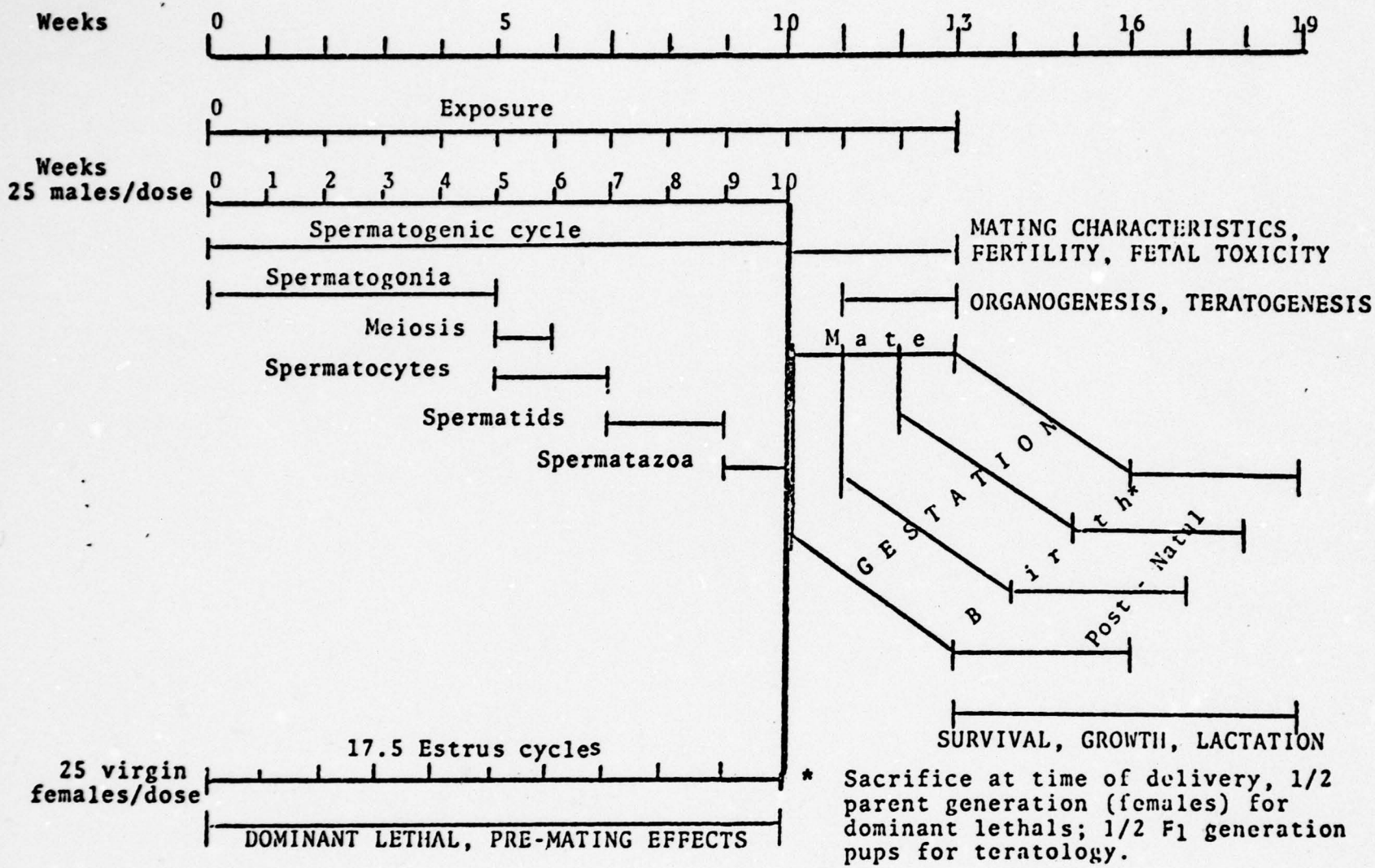

Figure 1. One Generation Reproduction Study in Rats

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6.4 Toxicity Testing In Vitro

Introduction

Among the many in vitro systems that have been used in toxicological studies are those involving pro- and eukaryotic microbial cells, cells of vertebrates of all classes, and embryonated eggs. Nevertheless, for the purposes of this paper, in vitro testing will focus on mammalian cell and tissue culture.

The above-mentioned in vitro systems have been used for studies aimed at an understanding of the mechanism of action of toxic substances (Dawson, 1972) as well as for screening purposes (Nardone, 1977).

In order to evaluate the real and potential usefulness of in vitro systems for toxicity testing it is necessary to have an appreciation of their limitations as well as their attributes. In most instances, advantages of time, cost, accessability, genetic manipulation and control, and control of the the chemical and physical environment accrue to the user of in vitro systems. It is recognized that some of these advantages stem from the creation of a life-style which may not reflect the in situ situation. With the disruption of organismal integrity and the use of an artificial environment, the risk of an ill-founded extrapolation to what happens in a whole animal exists. Nevertheless, awareness of these pitfalls coupled with caution and good judgment regarding the kinds of questions to be asked and how the answers are to be applied has enabled in vitro testing to be an important, integral part of toxicity testing programs.

It is axiomatic from what has been said above about disruption of organismal integrity, as well as from an ever-expanding list of experiments involving several aspects of cytotoxicity, mutagenesis, and carcinogenesis, that in vitro systems are most useful when they are used to assess the effect of a putative toxin on molecular, subcellular, and cellular phenomena that are not dependent upon or influenced by other cells and tissues and when they are used to assess toxicity without attempting to express in a quantitative way the potential risk to man. Hence, we witness the use of in vitro tests primarily as screens in contemporary testing programs.

Those tests which have gained the widest acceptance and have been validated to varying degrees include cell viability, cell proliferation, mutagenesis, and carcinogenesis tests of different kinds. Other cytotoxicity tests which are used less frequently but are useful in particular circumstances include plating efficiency determinations, macromolecular synthesis studies, assessment of gross cytological damage such as nuclear blebbing and cytoplasmic vacuolization, and a variety of differentiated function tests such as phagocytosis, ciliary beating, hormone production, and cardiac cell contraction.

Each of the commonly used cytotoxicity tests - cell viability and cell proliferation - have clear end points which are readily quantified, and which could result from a variety of cellular lesions. For example, a cell cannot proliferate at a normal rate to form adaptive (fit) descendents should there be severe distortions in any one of a long list of interrelated cellular activities such as DNA, RNA, and protein synthesis, bioenergetics, microtubule assembly, ribosome biogenesis, regulation of influx and eflux, and template transcription. Furthermore, there is great commonality among cells of diverse types and species regarding these processes. Hence, the information gained from in vitro studies is readily applicable to cellular damage in general and to the in vivo situation.

The same logic applies to mutagenesis and carcinogenesis. Genes and chromosomes of diverse species are relatively similar in composition, mode of reduplication, and expression. Hence, barring differences in repair capability, metabolic activation, and permeability, similar mutagenic and carcinogenic responses should be experienced by eukaryotic cells of diverse sources, in vitro and in situ.

Validation

Confidence in the role in vitro testing should play in a toxicity testing program must stem from well-controlled comparative studies. While many of these exist, the field, for the most part, has grown in an almost amorphous way with retrospective analysis providing the bulk of the support.

A variety of studies, prospective and retrospective, show correlations between toxicity, mutagenesis and transformation in vitro and in animals or humans. These include studies with environmental samples (Christian, 1978), biodegradable materials (Hegyeli, et al.) phthalate esters (Autian and Dillingham, 1978), drugs (Dawson, 1978) and potential industrial mutagens and carcinogens (Fishbein, 1977).

It should be recognized that differences in sensitivity often exist when in vitro and whole animal studies are compared, with the former usually being more sensitive.

In vitro cellular toxicology is at a crossroad in development. There are many well defined and reproducible systems which could be adopted and incorporated into testing regimens in order to ascertain the effect of toxic substances on differentiated cell types and the expression of cell-specific endpoints. Among the systems which are currently ready for validation and exploitation are the following cell types - cell specific endpoint combinations.

Neuroblastoma - neuronal cell functions such as neurotransmitter chemicals and action potentials.

Glioma - glial cell functions such as specific protein (S100) synthesis

Type II Cell - surfactant producing alveolar Type II cell

Mammary epithelial cells - hormone receptors

Primary liver epithelial cells - glycogen, glucose-6-phosphatase, a-2-globulin

Tissue and organ culture applications are lagging behind; however, the significant progress recently made in the in vitro maintenance of tissue and organ integrity with skin, whole mammary gland, lung, and whole embryos (Nardone, 1977) suggests that at some time in the near future we will be able to study in vitro the effect of toxins on processes which are affected by cell to cell interaction and which are accompanied by temporally related changes, such as keratinization in skin.

Recommendations

It is recommended that in vitro tests for cytotoxicity, mutagenesis and carcinogenesis be used in the first level of testing. The results of such tests, coupled with those of animal studies, are to be used in decision making regarding further testing and prioritization of resources.

A comprehensive testing program will require the "Minimal Tests" listed in the appended program and could be augmented by those tests labeled "Supplementary". It should be recognized that mutagenesis tests could also be predictive of carcinogenicity.

Table

Cytotoxicity Tests

"Minimal"	End-Point	In Vitro System	Time Required
A)Cell viability	Dye exclusion	Two established cell lines (ECL)	3-5 days
B)Cell proliferation	Culture growth	ECL	3-5 days
"Supplementary"			
A)Cell viability	^{51}Cr efflux	ECL	3-5 days
B)Cloning efficiency	Clonal growth	ECL	2 weeks
C)Gross cytology	Nuclear and cytoplasmic anomalies	ECL	3-5 days
D)Macromolecular synthesis	DNA, RNA and protein synthesis	ECL	3-5 days
E)Liver cell function	Glucose-6-phosphatase; a-2-globulin	Primary liver epithelial cell culture	3-5 days
F)Alveolar macrophage	Phagocytosis	Rabbit alveolar macrophage	3 days

Mutagenesis Tests (to be coupled with microbial, Drosophila, and other tests. See appended "Predictive Testing Scheme for Carcinogenicity or Mutagenicity of Industrial Chemicals" (Fishbein, 1977)).

Table

"Minimal"	End-Point	In Vitro System	Time Required
A)Chromosome damage	Sister chromatid exchange	ECL	1 week
B)Gene mutation	Forward mutation at the hypoxanthine-guanine phosphoribosyltransferase locus (HGPRT+/-)	Chines hamster ovary (CHO) or lung (V79)	2 weeks
Gene mutation	Forward mutation at the thymidine kinase locus (TK+/-)	Mouse lymphoma, L5178Y	2 weeks
"Supplementary"			
A)DNA alteration	Unscheduled DNA synthesis	WI38	1 week
B)DNA damage	Single strand breaks	WI38	3-5 days
Carcinogenesis Tests			
Minimal Tests			
A)Cell Transformation	Altered growth patterns, focus assay	C3H/10T1/2/CL8 cells (mouse embryo)	4-6 weeks
Cell Transformation	Altered growth patterns, focus assay	Syrian hamster embryo	4-6 weeks
Supplementary Tests			
A)Cell Transformation	Altered growth, clonal assay	Hamster embryo, transplacental	4-6 weeks

Table 1 (From Fishbein, 1977)

A Predictive Testing Scheme for Carcinogenicity of Mutagenicity of Industrial Chemicals

Phase 1: initial screen

(a) Screening test with sensitive micro-organisms
  (i) Salmonella tyhpimurium TA 1538 (frame shift)
  (ii) Escherichia coli WP2 (base-pair substitution)
  (iii) Saccharomyces cerevisiae (mitotic gene conversion)

(b) Microsomal assay using rat liver homogenate with the above four micro-organisms.

(c) Cytotoxicity study with HeLa cells and cultured rat liver (RL_1) cells

(d) Chromosome study in cultured rat liver cells

(e) Short-term exposure of rats by a relevant route to the highest tolerated dose followed by histological examination and analysis of chromosome damage

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Phase 2:

(a) Microsomal assay using liver homogenates from mice and other species

(b) Dominant lethal assay in male mice

(c) Assay of gene mutation in cultured mammalian cells

(d) Assay of malignant transformation in cultured cells or by a host-mediated approach

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Phase 3:

(a) An in vivo assay of gene mutation

(b) Dominant letal assay in male rats

(c) Dominant lethal assay in female rats

(d) In vivo chromosome study in Chinese hamsters or mice or both

(e) Long-term carcinogenicity studies in one or two species

(f) Pharmacokinetic studies and biochemical studies at the sub-cellular level

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Table 2 (From Fishbein, 1977)

Framework of Carcinogenicity Test Procedures

Valid Data on	Test System	No Data On
	Carcinogenic in man	Threshold dose; individual risk
Target organ in man; high risk groups	Epidemiological studies	Level A
	Positive	Predictive value for estrapolation (at present limited); target organ; threshold dose
Species and organ specificity; dose response in animals	Carcinogenicity test in animals	Level B
	Positive	Species and/or organ specificity; correlation between mutagenic and carcinogenic potency
Mechanism of metabolic activation in animals and man; type of genetic damage	Mutagenicity tests Microbial, mammalian, human cells/activation in vivo and in vitro Chemicals	Level C

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6.5 Behavioral Toxicity Testing

Outline

A. *Introduction*

B. Motor Performance
   1) Spontaneous motor activity
   2) Coordination
   3) Strength and endurance
   4) Tremor

C. Sensory Processes
   1) Vision
   2) Audition
   3) Pain sensitivity

D. Complex Learned Behavior
   1) Rate of responding
   2) Discrimination
   3) Learning new behavior
   4) Memory

E. Emotional Behavior
   1) Sexual behavior
   2) Aggressive behavior

F. Overall Strategy for Behavioral Toxicity Testing

A. Introduction

Those charged with the assessment of behavioral toxicity share with other toxicologists the difficult task of having to affirm the negative; of always wanting to conclude that, given exposure to a particular concentration of a chemical for a particular time, no effect has been produced. For each type of behavior to be examined, this negative conclusion is most effectively established if a functional relationship is first determined between exposure level and effect, with some levels not producing an effect whereas higher levels do so.

But note that this procedure must be followed for each type of behavior in which there is any interest. How can one conclude that a substance has no behavioral effects whatsoever without first testing every conceivable behavior? The answer is one cannot. After all, establishing that a chemical does not affect seeing says nothing about how it affects hearing; examples abound of chemicals that affect one sensory system while sparing others.

Because we lack knowledge of the behavioral interdependencies, we cannot confidently generalize from negative results on one aspect of behavior to conclude that no other behavioral effects will be found. However, we obviously can never test the integrity of all behaviors. At the moment, the solution to this dilemma is to be found only in sampling widely, hoping not to miss any important aspect of behavior.

In the following sections, I will specify some of the aspects of behavior that appear important enough to warrant attention in any program aimed at affirming that no behavioral effect has been seen with a particular chemical. For each aspect I will offer my judgment on how this can be done today most quickly and with the least expense. Usually the best, spare-no-expense method will also be specified. As we will see, there are inevitable trade-offs between speed and quality.

B. Motor performance.

Measures of motor performance abound and vary greatly in complexity. We shall consider these in four categories: spontaneous motor activity; coordination; strength and endurance; and tremor.

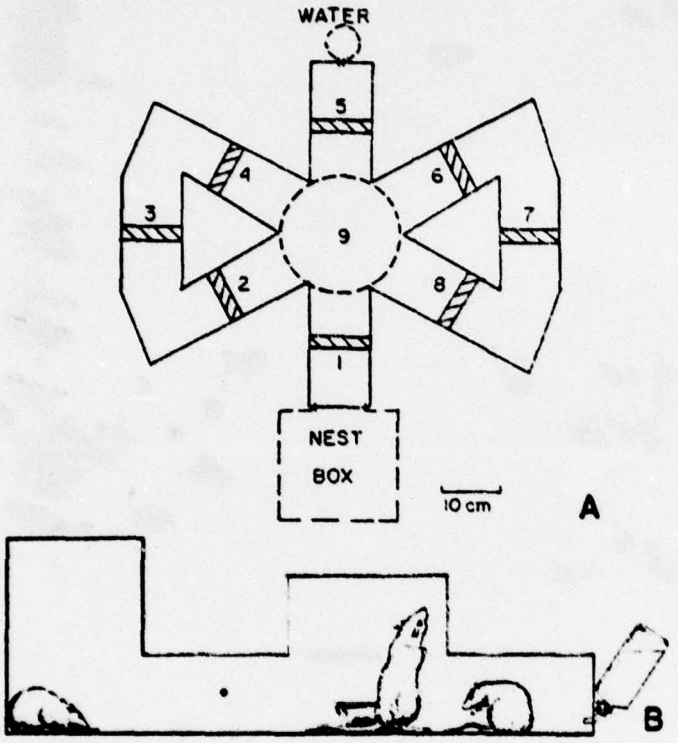
1) Spontaneous motor activity

Most animals display a low level of general activity that seems to have no obvious cause; for this reason, it is sometimes labelled "spontaneous motor activity". This is usually taken to include such acts as walking, running, sniffing, rearing, scratching, etc., in rats or mice, and really is defined by exclusion of all activity that has an obvious cause. For instance, sexual activity would be studied separately as would aggressive behavior, if one knew that such behavior was likely. Otherwise, these would simply increase the total "spontaneous" activity counted.

The actual composition of the activity count is heavily dependent upon type of apparatus. A large number of methods have been used: photocell cages, running wheels, jiggle cages, direct observation, and a variety of electronic sensors that reflect movement in a field. Some combination of direct observation and either photocells or electronic sensors makes the most sensible choice at present. Direct observation is essential to pick up behavioral changes that are not sensed by automatic devices; it would seem important to note, for instance, whether or not a substance was producing an increase in stereotyped sniffing or rearing, activities which may not produce lawful changes in a photocell activity cage. Some hint of this type of change should come from observations made during chronic toxicity testing. It is best to make such observations systematically, using an appropriate rating scale in order to increase the reliability of the measure. Several are given or referenced in Robbins (1977).

Kinnard and Watzman (1966), Finger (1972), and Robbins (1977) review the advantages and disadvantages of the various methods of recording activity, without, however, evaluating the most recently developed devices. It appears likely that at least two of these may offer advantages over those methods with much longer histories. One, typified by that used by Fechter and Annau (1977), uses tuned oscillator coils to detect horizontal movement in a plastic box located just above the coils. Since the sensitivity of such devices can be varied by the experimenter and since they can detect very slight movements, they offer some advantage over photocell arrangements. Several are commercially available. However, I know of no head-to-head comparisons that demonstrate their sensitivity relative to any other activity measurement devices.

Photocell activity cages continue in active use and appear to be quite sensitive to toxic substances (e.g., Kurtz, 1976). One recent development that has increased interest in them within toxicology is their use with rats housed as a group. Before studying activity, a decision must be made on whether measurements should be on isolated animals in order to avoid the complications of social influences or to



A. Top view of residential maze. Numerals indicate location of 8 photocells and raised area (Area 9).

B. Side view of residential maze showing next box at left and raised area in center (Area 9 in A).

Figure 1

study several animals together in order to avoid the complications associated with isolation. Lately, some investigators have opted for the latter course. The most prominent example, that of Norton and her colleagues, has been named a "residential maze". The one described by Norton, Culver and Mullenix, (1975), and used by Culver and Norton (1976) in work on carbon monoxide, is shown in Fig. 1.

Four rats were allowed to live in the apparatus, usually for four or more consecutive days, and activity was measured by the photocell-operated counters. Separate totals were presented for diurnal (12:00 noon to 6:00 PM) and nocturnal (6:00 PM to 6:00 AM) activity, as well as for what the authors called "exploratory activity", which refers to activity recorded during the first two hours after the apparatus was cleaned, etc. each morning.

The finding that CO-exposed rats showed greater changes in activity when studied during the night (Culver and Norton, 1926) emphasizes the necessity of attending to circadian rhythms when studying toxic substances even though these rhythms may not always be affected differentially (cf. Kotsonis and Klaassen, 1977). Note that it is not yet clear that this particular grouped animal preparation is either more or less sensitive than the more traditional single animal techniques.

The idea of measuring the activity of a group of animals living together has also been applied to mice (Ely et al. 1976).

I think that either the traditional photocell or newer electronic activity meters would be first choices for quick looks at spontaneous motor activity. It probably would be a good idea to measure animals separately, something frequently not done with these techniques, because it would simplify interpretation of the results. (If animals are studied in small groups, the correct unit of analysis is the number of such groups, not the numbe of individuals.) The residential maze has much to recommend it, especially if one wishes to measure simultaneously both exploratory behavior and general activity, or is interested in studying diurnal cycles.

2) Coordination

Two general classes of experiments appear here. In one, an animal is trained to make a response that demands a certain amount of coordinated activity in order to earn a reward or avoid a shock. Examples can be found in the work of Clark et al. (1962), who trained monkeys to hold a lever within narrow limits in order to avoid shock; and in the work of Falk (1969), who taught rats to exert a specified amount of force on a lever in order to get food pellets. These techniques demand an experimenter with a good deal of behavioral sophistication. They involve the forelimbs or paws.

The second general class involves unlearned behavior. If a measure of coordination of running by rats or mice is satisfactory, then one of the many measures of running on a rotating cylinder would be appropriate. A review of such devices appears in Watzman and Barry (1968). The one used by Kaplan and Murphy (1972), which features an

electrode floor that discouraged rats from voluntarily dropping off the rotating rod before they were forced to fall due to its increasing speed, appears quite satisfactory. So does the rotating cone of Christensen (1973), which forces the animal to walk against increasing surface speed until it falls off. (This one was designed for mice but could easily be adapted to rats.) Several versions of the rotating rod are available commercially. A treadmill that has recently been developed seems to offer no advantages over the rotating rod (Gibbins, 1968). Spyker et al. (1972; see also Spyker, 1975) used a simple preparation, merely placing mice into a deep glass tank that was filled with room temperature water. Swimming was observed and quality of coordination of the swimming noted.

Another test that is probably related to coordination was devised by Edwards and Parker (1977) and involves measuring the amount of splaying of the hindlimbs of rats that were dropped 32 cm onto a lab bench, having been held dorsal side up and horizontal. The position of the fourth digit of each hindlimb upon landing is marked and the distance measured. Rats given acrylamide, 50 mg/kg ip 3 times weekly, showed substantial increases in splaying after only three doses.

I recommend trying the rotating rod described by Kaplan and Murphy (1972), unless one of the commercially available devices proves satisfactory. I do not know from personal experience how quickly rats or mice learn that they can jump off without penalty rather than playing the experimenter's game. It may be that this is not a problem except in repeated tests over many months with the same animals. A second relatively simple task is swimming as used by Spyker et al. (1972). The measurement of hindlimb splaying (Edwards and Parker (1977) is also promising if a good way of making more objective the actual measurement could be devised. The authors considered it a measure of peripheral neuropathy. And simplest of all as a measure of motor integrity is the righting reflex, which could be included in the simple neurological battery described below in section F.

3) Strength and endurance

Closely related to coordination tests are those reflecting strength and endurance. For instance, whereas Clark et al. (1962) taught their rhesus monkeys to position a lever that was easy to move, Dews and Herd (1974) trained theirs to exert a force of about 80% of their body weight, sustaining such pulls for from 30 to 300 seconds. Clark et al. were interested in how well the subject could position a lever; Dews and Herd were trying to induce changes in mean arterial blood pressure via sustained static work. Both methods are of interest to behavioral toxicologists but both are too complex to serve as routine tests.

It is probably necessary to turn to unlearned behavior for a more rapid method for judging strength and endurance. Swimming seems a likely candidate for measuring endurance. It has been used by Tusl et al. (1973) among others, and appears sensitive to various environmental insults. Tusl used rats that were required to swim from a starting platform to a second platform some distance away; the second platform then was lowered, forcing the rats to swim again, while the first platform was raised to serve as the goal this time. Control animals took about 20 seconds to make the swim.

Cabe et al. (1978) have described a simple way to measure grip strength in the rat. The animal is allowed to grasp a 45 mm diameter ring attached to a strain gauge; it is then pulled away smoothly from the ring and the force required to break its grip is measured. This measure was sensitive to PBBs, age and sex.

4) Tremor

A simple method for the detection of tremor was used by Ambani and Van Woert (1972) in a study of tremorigenic drugs; it seems suitable for use in screening for tremor changes after the administration of toxic substances. They used a commercially available activity platform (Lafayette Instruments, Inc.: Model 501), setting the sensitivity control so as to minimize counts due to exploratory behavior. They measured the tremor of two rats at a time, a procedure that does not make too much sense; I would recommend that a single rat be studied, with a small box used to keep it from roaming.

It should be recognized that there are much more sophisticated ways of studying this phenomenon. Rapid changes in the availability and price of computers may make better methods competitive with simpler methods very soon. For instance, Wood et al. (1973) studied the tremor induced by elemental mercury poisoning and found that both the amplitude of tremor and its frequency spectrum changed as plasma mercury levels dropped during recovery from the poisoning; the simple activity measurement device mentioned above would not give information on frequency.

C. Sensory Processes

A crude neurological examination can be carried out on a rat that has been given a toxic substance. One such is described by Marshall et al. (1971) (see also the comments on it by Deuel (1977). Visual functioning, for instance, is tested by bringing into view small bits of white paper, coming at the rat from over its head. The rat usually turns toward the paper when it enters its peripheral field of vision. Auditory functioning can also be tested. Clicks are presented just behind the ear; the rat will turn and orient to the source of the noise if it can hear. Pinching the rat's hind leg, for example, is used to test for somatosensory responsiveness. If these examinations are done blind by a bright technician, with the results recorded on rating forms that provide for at least crude quantification of responses, a rough indication of large changes in sensory function can be found. Any hints of loss of function, can be pursued with more sophisticated tests such as those given below for vision, audition and pain sensitivity. Taste and smell can be tested in analogous ways. And note that loss of weight may indicate losses in taste and smell.

1) Vision

A more quantitative way to test an animal's vision is to train it to respond on one lever in the presence of one light and on a second lever in the presence of another. The two lever situation guarantees that cessation of responding is not mistaken for a loss of discrimination;

this type of error is possible where only a single lever is used, and the animal is taught to respond in the presence of a light but not in its absence. It is possible to study any aspect of vision separately. Thus, for example, it is possible to train an animal to respond only in the presence of a particular wavelength and then discover whether or not sensitivity to that wavelength has been changed by a compound. Unfortunately, I know of no way that one might test in a simple fashion whether or not a small change in sensitivity has taken place with regard to some aspect of visual performance. Particularly disconcerting is the fact that changes in, for instance, ability to discriminate between forms may appear at a time when no changes in ability to discriminate brightnesses have yet appeared (Evans et al. 1975). Thus, a simple test of brightness discrimination in this case would have not turned up the deficiency in vision that had occurred.

2) Audition

There appears to be no simple way to ascertain whether or not an animal's hearing has been impaired (see D'Arcy and Harpur (1977) for a recent review of many proposed simple screening tests). It is necessary to train the animal in some way so that an auditory stimulus has control over some observable behavior and then to watch for changes in that behavior in order to detect changes in hearing. Unlearned responses to sound tend to be unreliable or transient. The conditioned suppression technique seems to be the easiest to use with rats. In it, as described by Kelly and Masterson (1977), "... the animals were water deprived... and then trained to lick a spout for water reinforcement in the test apparatus. Once steady licking had been established on a variable ratio schedule (3%-6%), the animals were given further training in which the offset of a 10-sec tone was paired with a shock to the feet. After a few tone-shock pairing, the onset of the tone elicited a freezing response incompatible with licking. Thereafter, the cessation of suppression of licking was used as an indication of an animal's ability to hear the tone" (p. 931). From then on it is simply a matter of varying the tone intensity and recording the reaction of the rat. This is done for a wide variety of frequencies to produce an audiogram for each subject. (Sidman et al, 1963, describes the use of the same technique with mice).

Methods using positive reinforcement are preferred for the monkey (Stebbins, 1970), but the general idea of first training the animal to make a discriminative response lies behind all techniques of this sort.

3) Pain Sensitivity

A simple test of whether any changes have occurred in sensitivity to pain can be made by using the classic tests for analgesic drugs, e.g., the hot plate technique. Mice or rats can be used. They are placed on a commercially available hot plate that is electrically heated and thermostatically controlled. Time to the first lick of a hind paw and time to the first jump can be measured. The method is capable of detecting both increases and decreases in sensitivity. Further work on pain could use the titration technique in which the subject sets its own threshold by working to decrease the level of shock, while the apparatus is programmed to increase the shock level periodically (Weiss and Laties, 1970). However, this is not as yet a simple screening method.

D. Complex learned behavior

The only way to learn anything about the effects of a chemical on complex learned behavior is to study complex learned behavior. I know of no shortcut that can get us past this truism and allow us to substitute simple methods for complex ones. Even the ones that I label "simple" in the next few paragraphs are full of traps for the unwary, and the literature is full of examples of experiments done by perons who had not mastered the techniques of their science well enough to save them (and their readers) from error. But the problems posed by the complexity of this behavior will not go away without study. My point is that we are stuck with this difficult subject matter and might as well get on with the job of studying it intelligently, even if it costs more to do so that we would like to spend. The alternative is to ignore it, a course of action we take at our peril: this type of behavior represents much of what we mean when we speak of the ordinary behavior that man displays.

Here I shall consider only four aspects of behavior: rate of response; discriminative control of responding; learning new behavior; and memory.

1) Rate of response. The rate at which an animal makes responses depends in large part upon how it is rewarded for making the responses. If the reinforcements are unpredictably related to the responses, the animal will adjust its response rate at a particular level for a particular level of reinforcement rate. That level will be changed by many drugs and also by many toxic substances. A straightforward way to examine changes in response rate is to use the variable interval schedule of reinforcement to generate a steady rate (Ferster and Skinner, 1957). If, for some reason, one wished to avoid positive reinforcers, one could use a free operant avoidance baseline to produce the steady rate (Sidman, 1966). Neither procedure is very complicated but both demand careful work and close control over other variables. For instance, the animal's weight must be closely monitored and deprivation level kept constant if food is to be used as the reinforcer for performance on the variable interval schedule.

The response rate itself can be specified more closely by making reinforcement dependent upon explicitly designated pauses between responses. Such schedules are called interresponse time schedules. If the animal is rewarded with food for waiting at least 20 seconds, it would come respond appropriately, waiting long enough to ensure reinforcement for its response perhaps half the time. This performance has been shown to be sensitive to many CNS drugs (e.g, Sidman, 1955) and to such physical variables as non-ioning radiation (Thomas et al. 1975). The influence of a toxicant on this baseline is of interest because it says something about how well an animal can inhibit its responding, since that is what it has to do to succeed in gaining reinforcements on the schedule: refrain from responding until the appropriate time has passed. Of course, no external signal is given to the animal as to when the required time has elapsed.

2) Discriminative control of responding. Behavior that is reinforced in the presence of a particular stimulus comes under the control of that stimulus. That is, it then becomes possible to get the animal subject to emit the response simply by turning on the stimulus. Experimental preparations suitable for the exploration of questions of discriminative control abound. A handy example in the toxicology literature occurs in the work of Hanson (1975). He trained pigeons to respond in the presence of light of certain wavelengths but not in the presence of others and then studied the effects upon this performance of the anti-depressant drug, pheniprazine. Hanson showed that prolonged treatment with the drug abolished the discrimination, a finding that confirmed similar findings with human color vision.

The same general principle can be used to examine the integrity of performance under the control of less obvious stimuli. Thus an animal can be trained to discriminate a certain amount of its own behavior, making a response only after it has first emitted that much behavior. For example, a rat (Mechner, 1958) or a pigeon (Laties, 1972) can be trained to make eight or more responses on one switch before making a response on another; the response on the second switch is reinforced if the requirement has been met. If, on the other hand, the minimum number of responses has not been made, nothing is given for the response on the second switch and the animal has to begin its count over again.

3) Learning new behavior. There are as many ways of studying learning as there are behaviors that can be learned. One frequently used method involves discrimination reversals, with the animal taught to respond on the basis of one set of external stimuli and then, after performance has reached a high standard, the cues are reversed and the animal required to relearn the task with these reversed again, and this procedure continues from session to session, with the rate of learning the reversals serving as the measure of interest. This method was used by Smith et al. (1976) in a recent study of dieldrin.

The most important recently developed method for studying learning involves the repeated acquisition of sequences of responses. It is a highly sophisticated method but is not yet a cheap, easy-to-use tool (Boren and Devine, 1968; Thompson and Moerschbaecher, in press). The discrimination reversal method is likewise not cheap or simple. I am afraid that there is no simple way to study learning.

4) Memory. Perhaps the most commonly used method for the study of memory is the "passive avoidance" procedure, which involves punishing a response by a mouse or rat and then seeing whether the animal "forgets" that it has been punished when next confronted with the same situation. A recent version was used by Flood et al. (1978). A mouse is put in the black compartment of a two compartment box. A mouse hole leads to a white compartment. The white compartment has a grid floor through which shock can be administered to the feet of the mouse. The subject will almost invariably go from the black to the white compartment as soon as it sees the hole. There it receives its shock and is immediately taken from the box. When retested a week later, a normal mouse, presumably

remembering the shock, will not move from the black to the white compartment. A substance that interferes with its memory will lead it to re-enter the white compartment as if it had never been shocked. The animal is used only once.

More complicated methods are also available for studying memory. For instance, much work has been done with what is called "delayed matching to sample", a task in which the animal is taught to press a panel just like the one to which it has previously been exposed. If it has just seen a picture of a ball, it must now chose out of, say, three pictures, the one of the ball. It is rewarded for successful choices and the interval between the presentation of the sample picture and the group from which it must make a choice is varied in an effort to determine how the duration of this interval affects its performance. This type of procedure is required if repeated measurements over many weeks or months on the same subject are necessary to the project. I believe that the simple passive avoidance procedure is preferable for toxicity screening purposes, with different groups of mice exposed to different durations of exposure providing a possible design to examine effects of exposure duration.

E. Emotional behavior

Under this heading I shall discuss only sexual and aggressive behavior. I had originally intended including a section on exploratory behavior, covering the work done with the open-field test, but decided to omit this. My reading has convinced me that interpretation of the results from such experiments is impossible without a full-scale series of experiments covering the multitude of possible variables influencing performance on this test. The experimental situation is simplicity itself: a rat is placed in an open field that is completely bare. It is much larger than the animal's home cage; in one recent example (Seliger, 1977), the field was 4 ft x 4 ft. The field is ruled off into squares so that the animal's activity can be scored in terms of squares entered. A rat placed in such an environment usually "freezes" for a short while before starting to explore the area. It is also likely to urinate and defecate freely in the unfamiliar environment; boli are usually counted and taken as a measure of "emotionality". Unfortunately, these measures--and there are many more (Walsh and Cummins, 1976 list about 30) -- seem very susceptible to influence by many procedural variables and usually correlate only slightly with one another even though they are supposed to be measuring the same underlying process. Factor analytic techniques have recently been used to make some sense out of a confusing literature (Royce, 1977) but I think that the technique produces only confusion when used as a screening technique in toxicology.

1) Sexual behavior. If a test of reproductive competence is done, further tests of sexual behavior may be given a very low priority; one can argue that adequate reproductive performance presupposes adequate sexual behavior. The paper by Wilson and Nardone (unpublished) should be consulted for details of suggested methodology for the assessment of reproductive performance. If a complete reproductive assessment is not contemplated or if a rapid indication of interference with sexual behavior itself is desired, testing such as that carried out by

Madlafousek et al. (1971) would be appropriate. They examined the way in which cadmium affected the sexual behavior of male rats by presenting sexually receptive females to sexually inexperienced males and measuring such aspects of performance as time to first intromission, time from the first intromission to the appearance of the ejaculatory behavior pattern, time between consecutive mounts, number of incomplete mounts, etc. A brief description of the types of recording done in studies of sexual behavior may be found in Miczek and Barry (1976); the measurements that Madlafousek et al. (1971) used are described in detail in Larsson (1956).

2) Aggressive behavior. Miczec and Barry (1976) list eight different ways to induce aggressive behavior that have commonly been used in studies of drug action on rodents. These are:

Putting together previously isolated male mice (a certain proportion of such isolated mice will fight when first put together);

Introducting a strange rat (or mouse) into the home environment of another (fighting may ensue, but the incidence of such fights is low and variability high, and repeated measurements are impossible);

Painful stimulation, usually shock to the feet (a good procedure in that reliable behavior can easily be generated; bad in that its relation to naturally-occurring aggressive behavior is remote);

Changing a positive reinforcement schedule to extinction; i.e., no longer giving food for responding (this type of procedure has been studied with elegant automatic recording of the attack behavior; repeated measurements designs would be difficult to use);

Electrical stimulation of points in limbic, diencephalic and mesencephalic structures or destruction of such structures as the olfactory bulbs or the septum (the effects are sometimes only transitory, procedures are quite tricky with precise placement of the electrodes difficult, and interpretation of resulting aggression problematic in terms of naturally-occurring aggression;

Administering particular doses of drugs such as amphetamine and apomorphine (the need for high doses, the bizarre behavior produced, difficulties of interpretation, all combine to make this an unsuitable method for our purposes);

Putting animals in competition for food, water or a sexual partner (one drawback is that any substance may have effects upon hunger, thirst or the sex drive, independent of the aggressive behavior presumably being studied as the way the animals are resolving their competition);

Mouse killing (some rats will kill a mouse put in its cage; interpretation of this behavior is clouded by the fact that little is known of its causes; the incidence of killing tends to drift with repeated trials, with some rats showing a new, higher level of mouse killing after a treatment that has induced it to kill more than usual, a finding that complicates interpretation of experiments involving a series of repeated measurements).

The above comments within parentheses come largely from the Miczek and Barry review. It appears that there is no single perfect way to study aggressive behavior but the first and last mentioned methods offer the most promise for short tests. Neither the use of previously isolated mice nor the use of mouse killing demands much in the way of equipment. It appears that an isolation period of about four weeks is needed to ensure that most of the mice will actually show aggressive behavior when put together; that such factors as strain and the precise measure used to indicate aggression are very important in producing reliable results; and that blind recording of the behavior is essential. Strain of rat used is quite important in determining the level of mouse killing. Sprague-Dawley rats show kill rates of 10% to 30% whereas Long-Evans rats have kill rates of 50% and higher.

A simple way to detect the presence of pain-induced aggression is to pinch the forepaw of a rat; this was the test used by Marshall et al. (1971) in their examination of the effects of lateral hypothalamic damage. It is hard to quantify such a measure but it may still be useful as a crude first look at the existence of a change in level of aggressive behavior, with any hint of such a change to be followed up with other tests.

We should recognize here that we have been treating aggressive behavior as if it it were a unitary phenomenon when it most likely is not (Moyer, 1971). The various types of agggressive behavior have different physiological and biochemical bases and therefore would react differently to toxic chemicals. It may thus be necessary, or at least desirable, to study them all separately in order to arrive at a definitive picture of a substance's effect on "aggression".

F. Overall strategy for behavioral toxicity testing

Here is one of the many possible approaches to the task of examining an unknown chemical for behavioral toxicity. For other approaches, see Laties et al., 1977; Weiss et al., 1975; EPA Workshop, 1977. I will assume that the work is to be done with rats and that exposures will be made at levels appropriate to the questions to be answered about the particular chemical at issue.

1. A crude "neurological" examination, such as that described by Marshall et al. (1971) and further elaborated by Dueul (1977), could serve for a first look at any obvious effects on behavior (see above, page 8, for a brief description of part of the sensory examination). Turner (1965) also describes many simple ways of looking at reflex functions.

2. Motor integrity could be examined by using a combination of simple procedures, such as an activity measuring device, a rotating rod, a swimming task, and grip strength The last-named could be done as part of the original neurological examination.

3. Sensory function and complex learned behavior could be examined together by training rats to work on a multiple schedule of reinforcement, consisting of two simple schedules with each under the control of a different sensory stimulus. For instance, a light and a tone could be used as controlling stimuli with them alternating every 15 minutes. The trained animal would switch quickly to the pattern of responding appropriate to the schedule in force at the time. Changes in its behavior after exposure to a chemical could reveal much about the substance under review although the complexity of the situation has deliberately been chosen to require further work to pin down precisely which aspect of the behavior is responsible for any change. For instance, if the rat starts to respond at the same rate during each period, regardless of which stimulus is present, it may not be capable of seeing the light or hearing the tone. Such suspicions could be investigated with the methods described in Section C above. However, further work to determine which sensory defect has occurred may be of only academic interest in view of the profound damage done by the chemical. If the rat works in a very desultory fashion on the task, pausing for long periods, it may be that the substance has interfered with its appetite; again, more work would have to be done to tease this out as an unique effect. We may also learn something about the chemical's effects upon the discriminative control exerted by the two stimuli, apart from any frankly sensory defects produced. If the rat remained under good control of some other sensory stimulus, such as a light that was associated with delivery of the food pellet, we may be able to conclude that it can indeed still see. The chemical's effect on response rate itself would of course be measured here. And if the performance remained intact from day to day, we probably would conclude that no great changes in memory were being produced. If one component of the multiple schedule involved shock to the feet, as in the free operant avoidance schedule, an absence of changes in rate would assure us that no changes in sensitivity to painful stimulation had occurred.

The exact schedules of reinforcement to be chosen are less important than the fact that two of them are to be examined. One choice would be fixed interval and fixed ratio schedule combination recommended in Laties et al. (1977), which has the virtue of having been the subject of a great deal of prior work in behavioral pharmacology (McMillan and Leander, 1977). Work with a great many combinations can easily be defended. The best thing that could happen to behavioral toxicology at present would be to have many different experimenters try out different schedules in order to find out their relative sensitivity to toxic substances. Premature freezing of procedures is unwarranted.

4. The three suggested groups of tests just outlined do not constitute a hierarchy of tests; it does not seem possible at this time to order tests in such a way that negative results on some tests imply that negative results will be obtained on all those below. It may be possible to make defensible inferences about the prospects for performance on the more expensive and complex tests from the results on the simpler ones by "trading", as it were, between exposure level and expense. For example, one could assume that a chemical that does not disturb a rat's coordinated motor activity at one exposure level would not distrub complex performance on a reinforcement schedule at some small fraction of the level.

The determination of toxicity requires more information about the relative sensitivity of various types of behavior to chemical insult. Researchers should be encouraged to make systematic comparisons among behaviors part of any future behavioral toxicology work.

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6.6 Addendum: Behavioral Toxicology Protocol

General

Three different behavioral tests are in routine use. Each employs the rat as the test subject and requires twenty-five minutes to complete (a test session). The Sequential Response Test (SRT) described below requires pretrained rats and is used to assess the effects of drugs and chemicals on learned behavior. The SRT has also been used, though not routinely, to examine response behavior during extinction. The Spontaneous Activity Test (SAT) described below, requires experimentally naive rats and is used to assess the effects of drugs and chemicals on rats' spontaneous unconditioned movements or behavior. The Passive Avoidance Test (PAT) described below, is a one-trial test which also employs naive rats and is used to assess the effects of drugs and chemicals on learning behavior.

Routine Tests

Sequential Response Test (SRT): A rat, conditioned to lever press, is placed in a specially designed test environment containing four levers and a liquid dipper mechanism mounted on one of the walls. The rat is required to press the levers in the sequence 1, 2, 3 and 4, in order to gain access for five seconds, to a cup containing 0.2 ml of water (a reward or reinforcement). The levers are a hard, clear plastic material, each back-lighted with a 5 watt bulb. As each lever is pressed in accordance with the demands of the schedule, it lights up. However, any response (R) occurring out of sequence or more than one R per lever, resets the sequence (turning off whatever lever lights were lit) and requiring the rat to start over again at lever one.

A rat is conditioned to the four lever chained schedule, in five stages: (1) Lever pressing behavior is hand shaped using the principle of rewarding successively closer approximations to the desired response, until the animal finally presses the lever. (2) The rat is then rewarded for responses on each of the four levers, randomly, until it learns to move rapidly from lever to lever without favoring any particular lever. (3) Gradually the rat is introduced to a multi-lever chained schedule; rewarded on lever 2 after pressing lever 1, then on lever 3 after pressing lever 1 and 2, and finally on lever 4 after pressing 1, 2 and 3. Early in this stage the rat is randomly rewarded on lever 1 as well as on the terminal lever of the other three chained schedules. Gradually, rewards on lever number 1 are eliminated, then on the 1-2 chain etc, until the rat reaches stage (4) where it is being rewarded only on the four lever chain. The lever lights are not used until the beginning of stage (3) and the sequence reset is added (stage 5) to the schedule only after the rat achieves 60% correct responding (or more) and is receiving 60 to 70 rewards per test session, in stage (4). Conditioning in stage (5) is considered complete when the daily plot of each rat's percent correct responding begins to asymptote. When this

point is reached, the percent correct responding for individual rats ranges from about 70 to over 90%. Although all the parameters vary considerably from rat to rat, they are remarkably stable for each rat, from one test session to the next.

The basic data collected during a test session are the total number of rewards, the number of incorrect Rs on each lever, the total Rs and the total trials (a trial is terminated by either a reward or an incorrect R). In addition to the percent correct responding, the percent correct trials are calculated and recorded also.

It requires from nine to twelve weeks to fully condition a rat to this schedule of reinforcement.

Spontaneous Activity Test (SAT): A rat is placed in a plexiglass cage mounted on a special sensor plate that detects movement electronically (Stoelting Electronic Activity Monitor - EAM). Four sensor units are housed in a sound-retardant cabinet. A low gain white noise is piped into the cabinet through a 2 inch PM speaker, located adjacent to each activity cage.

This system takes advantage of the fact that when a capacitance (rat's body) is moved in a radio frequency field (generated by an oscillator and broadcast in a very restricted area around the sensor plate) it generates a small voltage in the plate, proportional to the magnitude of the movement. This voltage is sensed by a detector and chopped above whatever peak voltage level the experimenter has it calibrated for. This digitized signal is reshaped, reamplified and counted as an activity count. Two adjustable activity detectors are connected to each sensor plate. One detector is calibrated to pick up all animal movements down to the level of muscle tremors (L-1). The other detector is calibrated to pick up motion of the magnitude of locomotion or greater (L-2). The counts on counter (L-2) are recorded as Gross activity and the (L-1)-(L-2) counts are recorded as Fine activity. A ratio of F/G movements is also calculated and recorded for each rat.

Passive Avoidance Test (PAT): A rat is placed in a test box containing a house light and a grid floor through which an electric shock can be delivered. Located in each of the four corners of the box are a pair of photodetecting units (each unit consists of a photosensor and a light source), mounted so that the adjacent beams are parallel to the floor but perpendicular to each other, intersecting at a point 5.5 cm from both walls and 3 cm above the floor. Each pair of corner units are connected to a 28 vdc controlling circuit. That circuit is opened (off) only when both photobeams in a corner are interrupted simultaneously. Although the light sources for all the photosensors are on during a test, only the two sensors associated with one corner (the correct corner) control the operation of the shock generator.

A test session begins when the house light is turned on. Starting five seconds later and repeating every five seconds thereafter, a 0.5 second scrambled shock (0.6 ma, 300 volts) is delivered to the grid floor until the rat terminates the shock by entering the correct corner. As

long as the rat remains in that corner it will not get shocked, hence the term, passive avoidance. Whenever the rat moves far enough from the corner so that at least one photocell beam is made again, the rat will get shocked until it once more fully re-enters the corner.

The normal behavior of the rat in this test situation is to run around the inside perimeter of the box during each shock, but stopping and remaining apprehensively still between shocks. The rat usually discovers the correct corner by the coincidence of 'freezing' in that corner between shocks. However, after being there for a short while without being shocked, most rats will finally wander to the corner and consequently get shocked. This cycle repeats with the rat rapidly learning that entering and remaining in the correct corner prevents or avoids shock.

The basic data are the total number of times that the rat enters and remains in the correct corner, for a minimum duration of five seconds. This is termed a Passive Avoidance Response (PAR). A learning curve can be constructed by cumulatively plotting the PARs recorded each minute of the test session. In addition, the total number of shocks delivered and the total time the rat is out of the correct corner are also recorded.

Procedures

When dose response studies are done, a minimum of 48 rats are used. They are apportioned equally among six experimental groups; one receives saline (or other vehicle) and the remaining five groups are each administered different doses of the compound being studied. The material being studied is usually administered intraperitoneally.

The continuous data generated in the behavioral tests are converted to probit data by comparing each treated animal's particular behavioral parameter to the 95% confidence limits of the control mean, for the same parameter ($\bar{M} \pm t0.05 \times SD$; df=n-1). Values that fall outside these limits are scored as an effect and those that fall within these limits are considered as no-effect. The dose response regression line and limits are then computed using the Bliss method.

Rats being utilized in other types of toxicity testing are occasionally submitted for routine behavioral toxicity evaluation in the SAT and PAT. These rats, kept in whatever cages they arrive in, are stored overnight in the behavioral laboratory animal room. They are allowed water ab libitum, but are deprived of food. The following day each rat is tested first in the SAT and then in the PAT (four rats are evaluated simultaneously in each system). Immediately after testing, the rats are returned to the laboratory from which they came.

Where it is appropriate to do so, the mean, standard deviation and standard error are calculated for each test parameter. A standard Students 't' test is routinely employed to determine significant differences between experimental and control mean values. The level of significance differences accepted is p 0.05.

Animal Care & Use

Rats obtained for long term use in the behavioral toxicology laboratory are housed in stainless steel cages containing chopped corn cob bedding. Up to four rats are housed in a single cage and these cages are changed weekly. The rats are fed and given fresh water daily. They are also ear coded on arrival and then handled individually and weighed daily for at least two weeks (10 working days) before being used.

Rats to be conditioned in the SRT are deprived of water for 72 hours prior to the first attempt to shape lever pressing behavior. During conditioning, the contingencies or reinforcement are arranged to insure that each rat receives at least 15 to 20 ml of water daily during the work week. On Fridays the rats are allowed water ad libitum for a minimum of one hour and then deprived for the week-end.

The shock duration, frequency and dose employed in the avoidance procedure is discomforting enough to motivate a rat to learn how to avoid it, but is otherwise harmless.

When a rat is no longer needed it is euthanized either by cervical dislocation or some other painless method.

7.0 REGULATORY AGENCY GUIDELINES AND INDUSTRIAL PROTOCOLS

Table 8

Summary of Regulatory Guidelines for Toxicity Testing

A. Environmental Protection Agency

Conventional Tests - adapted from FR 40 #123 6/25/75 (Pesticides) #162.81 Hazards to Humans and Domestic Animals

			References
1.	Acute Tests		
	Acute oral LD_{50} (single dose)	Rat preferred	1,2,3,4,6
	Acute dermal LD_{50} (single dose)	Rabbit preferred (Guinea pig and rat acceptable)	5,22
	Acute primary dermal irritation	Rabbit preferred (Guinea pig and rat acceptable)	13
	Acute primary eye irritation	Rabbit acceptable	12
	Acute inhalation LC_{50}	Rat preferred	10,11
	Acute by other routes (intravenous, intraperitoneal)	Same species as for acute	None
2.	Subacute Tests		
	Subacute (1/2 lifetime of organism)		
	Subacute dermal (multiple exposure)	Rabbit - subacute dermal Guinea pig - skin sensitization	13
	Subacute inhalation	Rat preferred	None
	Subacute oral	At least 2 mammalian species, one a non-rodent, but excluding the rabbit	None
3.	Teratology	One mammalian species that has a hemochorial placenta (rat, mouse, non-human primate), dog may also be used	15,16,18,20
4.	Neurotoxicity	Adult hen (acceptable for determining effects on myelin sheath); rat or dog (for demonstrating acetylcholinesterase inhibition)	26

Table 8 (Cont'd)

5.	Metabolism	Rat or dog for extrapolation to man	27,28
6.	Chronic tests (1/2 lifetime of organism)		
	Oncogenicity	Lifetime feeding studies on at least 2 mammalian species (rat and mouse or hamster)	None
	Feeding	At least 2 mammalian species, one of which must be the rat	9
	Reproduction	Must be performed on at least one mammalian species using one of the same rodent species used in the feeding studies.	17,18
	Other chronic tests (usually the oral route) Effects on pesticides on: Hematopoiesis Endocrine systems Histopathology of various tissues and organs, particularly liver and kidney		5,6,7,8
7.	Special studies (Required under special conditions)		
	Mutagenicity	Conducted on in vivo mammalian test systems	15,19,20,21
	Potentiation studies		23,24,25
8.	#162.82 Hazards to Fish and Wildlife		
	Avian acute oral LD_{50}	Single Dose - Mallard preferred or quail	
	Avin subacute dietary LD_{50}	8-day protocol - one water fowl and one game bird	
	Fish acute toxicity 96-hr, LC_{50}	One cold and one warm water fish (Rainbow Trout and Bluegill)	

Table 8 (Cont'd)

Invertebrate acute toxicity 96-hr, LC_{50}	Daphnia Sp.

Mammalian toxicity data (acute and sub-acute) usually adequate for for wild mammals
Acute toxicity 96-hr, LC_{50} with shrimp and crabs for estuarine or marine environments
Acute toxicity 96-hr, LC_{50} with oyster larvae or shell deposition data with representative marine mollusc for marine or estuarine environments
Effects on flora and fauna in aquatic environments (case by case basis)
Chronic tests
Avian reproduction studies (Bobwhite and Mallard)
Subacute or chronic fish and/or invertebrate reproduction studies

CRF 40 1/1/72	Section 162.8 - Economic Poisons Highly Toxic to Man	
Toxicity		
Oral - Single Dose	14-day LD_{50}	Male and Female Rats
Inhalation	14-day LC_{50}	Male and Female Rats
Skin absorption (No References)	14-day LD_{50}	Rabbits

Consumer Product Safety Commission

FR 38 #187 9/27/73 #1500.50	Methods of Testing Toxic Substances
Acute dermal - single exposure	Rabbit (24 hours; two weeks observation)
Primary skin irritation (Patch-test technique)	Albino rabbit (24 hours, 72 hours)
Eye irritant test (0.1 ml Liquids; 100 mg Solids)	Six albino rabbits per substance (readings at 24,48,72 hours)

(No references)

Note - The Consumer Product Safety Commission commissioned The National Academy of Sciences to update "Principals and Procedures for Evaluating the Toxicity of Household Substances" (NAS-NRC Publication 1138, 1964). The 1964 edition has been referenced by EPA as a suggested source for guidelines and protocols. The 1977 edition will likely also be referenced by EPA. This document gives the most detailed guidance for toxicological testing of any published to date.

Table 8 (Cont'd)

Department of Transportation

CFR 49 Parts 100-199 12/76 Section 173.343 Poison B.

Oral toxicity - single dose - rats
Toxicity on inhalation - single dose - rats
Toxicity by skin absorption - rabbits

A substance is labeled a class B poison when it produces death within 48 hours in half or more than half of a group of 10 or more animals (Rats, rabbits).

(No References)

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(Tables 8 and 9)

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Distribution: RHC(71)EWG(1)GHS(1) DTH(1)WCK(1)HT(1)MTC(1)FW(1) AW(1)WHM(1)REH(1)CAI(1)RWB(1) CB(1)LHF(1)KWH(1)JB(1)DG(1) JAB(3)TWM(1)JDR(1)KDM(1)RF(1)	Division: HUMAN SAFETY Section: IX. STANDARD TEST METHODS	Page: 5 Issue No.: 1 Date: 12/20/76 Supersedes: Page: Issue:

Standard Procedure #1A for Toxicological Evaluation

Chronic Oral Toxicity

Purpose: To assess the chronic toxicity of a test substance.

Animals: Assign 20 male and 20 female weanling (30-35 days of age) Charles River CD (Sprague-Dawley) rats to each experimental or control group. Distribute the animals among the groups evenly according to sex and weight. Mark each animal for permanent identification.

Animal Care: House the animals in individual cages at 22-25°C and approximately 45% relative humidity. Expose them to alternating 12-hour light and dark periods, unless otherwise specified.

Feeding Levels: Establish one control group, receiving ground Purina Laboratory Chow. Establish 3 test groups, each receiving a different level of the test substance in Purina Chow. The lowest level is intended to be a no-effect level. The highest level should be the highest dose not expected, from subacute studies, to produce an adverse effect other than a slight weight reduction when compared to the control. An intermediate level is chosen in the expectation of seeing a dose-related response.

Feeding Conditions: Offer food and tap water ad libitum. Prepare fresh chow diets weekly, and store them in the dark at 3 ± 2°C until they are put into feed cups. Submit each batch of diet for analysis to insure that the test material has been incorporated at the prescribed level. Submit samples of the pure test material at intervals to insure that it has not changed during storage.

Data: Maintain careful records of each individual animal. Record values of body weight, feed consumption, and feed efficiency weekly for 13 weeks, and monthly thereafter. Calculate the quantities of test material ingested per unit of body weight at each of these intervals. Make daily gross observations of the animals, and record any perceived abnormalities. Continue the experiment for 12 months.

Distribution: RHC(71)EWG(1)GHS(1)DTH(1)WCK(1)HF(1)MPC(1)FW(1)AW(1)WHM(1)REH(1)CAI(1)RWB(1)CB(1)LHF(1)KWH(1)JB(1)DG(1)JAB(3)TWM(1)JDR(1)KDM(1)RF(1)	Division: HUMAN SAFETY Section: IX. STANDARD TEST METHODS	Page: 6 Issue No.: 1 Date: 12/20/76 Supersedes: Page: Issue:

Standard Procedure #1A for Toxicological Evaluation

Chronic Oral Toxicity (cont'd)

Kidney & Liver Functions:

If organ damage is detected in early autopsies, perform urinalyses, kidney function, and liver function tests before later sacrifices (Appendix I).

When urinalyses, kidney function tests, and liver function tests are to be performed, use the ten animals from each group that are to be sacrificed. Place these animals in metabolic cages which have been thoroughly washed and rinsed in distilled or dionized water, for 3 days to adapt to their new environment before collecting samples. Record food consumption while the animals are in the metabolism cages. Allow one week between sample collections and sacrifice so that the collections can be repeated if necessary.

Collect urine samples under toluene for a 24-hour period. Freeze individual urine samples and store them until all the data have been collected and analyzed at each necropsy period.

Necropsies:

Sacrifice and necropsy 5 males and 5 females from each group at 3 months and at 6 months. Select these animals before the start of the study, from a table of random numbers. Take samples for histology and hematology and as listed in the Appendix II.

Animals that die or are judged moribund during the experiment are to be grossly examined by a pathologist, searching for tumors and evident cause of death. Make these examinations even in animals that autolyze extensively between death and discovery. Tissues are taken and placed in the appropriate fixative using sufficient volume to insure complete fixation.

Distribution: RHC(71)EWG(1)GHS(1) H(1)WCK(1)HT(1)MTC(1)FB(1) AW(1)WHM(1)REH(1)CAI(1)RWB(1) CB(1)LHF(1)KWH(1)JB(1)DG(1) JAB(3)TWM(1)JDR(1)KDM(1)RF(1)	Division: HUMAN SAFETY Section: IX. STANDARD TEST METHODS	Page: 7 Issue No.: 1 Date: 12/20/76 Supersedes: Page: Issue:

Standard Procedure #1A for Toxicological Evaluation

Chronic Oral Toxicity (cont'd)

At termination of the study, all animals are necropsied. Tissues are taken and preserved as described above. Tissues from all controls and high treatment groups will be examined. In addition, all tissue masses, suspected tumors and lesions are to be examined microscopically by a pathologist. Additional tissues from low dose group(s) animals should be examined if indicated by findings in higher treatment groups.

Report: Prepare a comprehensive report giving all experimental details, body weight gains, feed efficiencies, organ/body weight ratios, hematological values, longevity values, incidence, location and description of all tumors or lesions, and all pertinent tests of statistical significance. File the final report within 3 months of completing the experimental work.

Protocol: These studies are always to be carried out under individually prepared protocols. The previous description is a guideline for protocol preparation.

Distribution: RHC(25) REW(47) ENG(5) CHS(1) DTH(1) WCK(1) HT(1) MTC(1) TWB(1) AJW(1) WHM(1) REH(1) GWH(1) CAI(1) RWB(2) JTR(1) LHF(1) KWH(1) JB(1) DG(1) GCC(1) APW(1) DGC(1) TWM(1) KDM(1) RF(1) JTO(1) NA(1) LB(1) JRD(1) DAN(5) DS(1) RET(1)	Division: HUMAN SAFETY Section: IX. STANDARD TEST METHODS	Page: 25 Issue No.: 2 Date: 6-24-77 Supersedes: Page: 25 Issue: 1

Standard Procedure #7 for Toxicological Evaluation

Subchronic Oral Toxicity - 28 and 91 Day Feeding Studies

Purpose: To assess the toxicity of a substance over an extended time period and/or to determine the doses of the substance that will be appropriate for use in a chronic feeding study.

Animals: Weanling (~28 days) rats, of a strain and source to be specified for each experiment (usually Cox or Charles River [SD] Caesarean derived).

Dosage Level: Choose dose levels on the basis of data from pilot studies or previous experience.

Procedure: House incoming animals in quarantine (3 animals of like sex per cage) for 4-7 days after arrival. Feed Purina Laboratory Chow-Meal and water ad libitum.

Exclude all animals of questionable health or outlying body weight (60-90 gm). Divide the animals (20 males and 20 females/group) (1 per cage) between the number of experimental groups specified, including one control group (Laboratory Chow-Meal).

Administer the test material for 28 or 91 days at the designated levels in Purina Laboratory Chow-Meal. Prepare diets at the appropriate level one day prior to the start of the test in sufficient quantities to last for 7 days. Mix subsequent diets at weekly intervals. Discard all diet not consumed within 7 days. Store test materials and diets not placed in animal feed jars in a dark, cool area (38°F-42°F). Take a random sample of each batch of diet (~30-50 gms) for analysis and analyze as appropriate. Record individual animal body weights and feed consumption weekly and process the data through the computer for determination of group statistical significant differences for body weight gain, feed consumption and feed efficiency.

Observations: Check animals in their cages daily, and observe more closely when weighing for physical appearance, local systemic toxicity, abnormal tissue masses and mortality.

Distribution: RHC(25) REW(47) EMG(5) CHS(1) DTH(1) WCK(1) HT(1) MTC(1) TWB(1) AJW(1) WHM(1) REH(1) GWH(1) CAI(1) RWB(2) JTR(1) LHF(1) KWH(1) IB(1) DG(1) GCC(1) APW(1) DGC(1) TWM(1) KDM(1) RF(1) JTO(1) NA(1) LB(1) JRD(1) DAN(5) DS(1) RET(1)	Division: HUMAN SAFETY Section: IX. STANDARD TEST METHODS	Page: 26 Issue No.: 2 Date: 6-24-77 Supersedes: Page: 26 Issue: 1

Standard Procedure #7 for Toxicological Evaluation

Subchronic Oral Toxicity - 28 and 91 Day Feeding Studies (cont'd)

Necropsy: Upon initiation of the study, schedule the animals for necropsy with, as nearly as possible, an equal distribution as to sex and group number for each necropsy day. An ascending order of animal numbers should be followed.

Perform a gross necropsy and take tissues from all animals that die or become moribund. At the conclusion of the study, necropsy all surviving animals. Anesthetize the animals with sodium pentabarbital (I.P. 5 mg/100 gms body weight). Exsanguinate ~5 ml of blood from the posterior vena cava using a needle no smaller than 23 guage. Do not withdraw the syringe plunger faster than the blood can flow into the syringe to prevent hemolysis. Transfer the blood to a vacutainer tube containing EDTA by inserting the needle into the rubber stopper. Allow the vacuum in the tube to empty the syringe. Process the blood to determine values for hemoglobin, hematocrit, white blood count, red blood count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, segmented neutrophil, non-segmented neutrophil, lymphocyte, monocyte, eosinophil and basophil.

Dissect all animals, including those which die or become moribund, take tissue specimens and trim as listed on the attached sheet. Preserve all tissues in 10% neutral buffered formalin*. Process tissues from all animals and evaluate histologically.

Report: Report mortality, body weights, body weight gains, organ weights, organ to body weight ratios, feed consumption, feed efficiency, and hematology values. Analyze the data statistically using the analysis of variance method (LSD and $F0_5$ tables). Report any clinical or behavioral abnormalities that are observed during the study and any abnormalities that are observed at necropsy. File the final report within 6 weeks of completion of the experiment.

*15 parts fixative: 1 part tissue

Distribution: PHC(25) REW(47) EMG(5) GHS(1) DTH(1) WCK(1) HT(1) MFC(1) JVB(1) AJW(1) WHM(1) REH(1) GWH(1) CAI(1) RWB(2) JTR(1) LHF(1) KWH(1) JB(1) DG(1) GCC(1) APW(1) DGC(1) HWM(1) RDM(1) RF(1) JTO(1) NA(1) JB(1) JRD(1) DAN(5) DS(1) RET(1)	Division: HUMAN SAFETY Section: IX. STANDARD TEST METHODS	Page: 27 Issue No.: 2 Date: 6-24-77 Supersedes: Page: Issue:

Standard Procedure #7 for Toxicological Evaluation

Necropsy - Tissues and Trimming Methods

Tissue	Trimming Method
Lung (2)	Left lung - longitudinal section Right lung - longitudinal section from diaphragmatic lobe only
Heart (2)	Cross section through the upper portion of both ventricles including the interventricular wall Paramedian section of left auricle and aorta
Aorta	Cross section of the straight portion of the thoracic aorta
Tongue	Cross section just anterior to dorsal prominence which lies midway between tip and base of tongue
Trachea, Esophagus, Thyroid (Parathyroid)	Obtained together by transversely sectioning at the level of the thyroid cartilages and at the level of the first tracheal rings.
Submandibular Lymph Node	Section through greatest dimension (left for section; right for save).
Ileocecal Lymph Node	One for section; one for save
Stomach (Fundic, Cardiac, Pyloric Regions)	Open the stomach along its greatest curvature and observe and remove ingesta. Cut a strip of stomach by making an incision parallel to the first. The section will include the cardiac, fundic and pyloric portions of the stomach.
Liver	Section two large lobes of the liver
Duodenum	Cross section
Jejunum	Cross section
Ileum	Cross section
Cecum, Colon	Cross sections of each
Urinary Bladder	Separate from reproductive organs and cut in half anterior to posterior.
Kidneys	Cross section the mid portions of both kidneys.

Distribution: RHC(25) REW(47) EWG(5) GHS(1) DTH(1) WCF(1) HT(1) MTC(1) FWB(1) AJW(1) WHM(1) REH(1) GWH(1) CAI(1) RWB(2) JTR(1) LHF(1) KWH(1) JB(1) DG(1) GCC(1) APW(1) DGC(1) TWM(1) KDM(1) RF(1) JTO(1) NA(1) LB(1) JRD(1) DAN(5) DS(1) RET(1)	Division: HUMAN SAFETY Section: IX. STANDARD TEST METHODS	Page: 28 Issue No.: 2 Date: 6-24-77 Supersedes: Page: Issue:

Standard Procedure #7 for Toxicological Evaluation

Necropsy - Tissues and Trimming Methods (cont'd)

Tissue	Trimming Method
Reproductive Tract	Male - Half of the prostate and seminal vesicle. Open tunic of the testes and place 1 testis in vial for sectioning. Female - Both ovaries are taken intact. Cross section both uterine horns. Cross section vagina.
Adrenals	Left intact adrenal is taken for sectioning; the right for save.
Thymus	Section through the greatest dimension
Psoas muscle	Cross section
Spleen	Longitudinal section
Pancreas	Longitudinal section
Bone Marrow	Total left femur
Skin	Dorsal cervical section
Brain	Medial longitudinal section (includes cerebrum, mid brain, cerebellum, stem)
Submandibular Salivary Gland	Section through greatest dimension. Left for section, right for save.
Eyes	Both eyes are taken intact.
Lesions	

Determine relative and absolute weights for the liver and kidneys.

Distribution: RHC(25) REW(47) EWG(5) CHS(1) DTH(1) WCK(1) HF(1) MTC(1) FWB(1) AJW(1) WHM(1) REH(1) CWH(1) CAI(1) RWB(2) JTR(1) LHF(1) KWH(1) JB(1) DG(1) GCC(1) APW(1) DGC(1) FWM(1) KDM(1) RF(1) JTO(1) NA(1) LB(1) JRD(1) DAN(5) DS(1) RET(1)	Division: HUMAN SAFETY Section: IX. STANDARD TEST METHODS	Page: 29 Issue No.: 2 Date: 6-24-77 Supersedes: Page: Issue:

Standard Procedure #7A for Toxicological Evaluation

Fourteen-Day Oral Toxicity Study

Purpose: To provide the information that is needed for designing a 91-day oral toxicity, especially information about the dose levels to be used and the gross toxic effects that may be expected.

Animals: Five male and 5 female rats per group. The rats are to be conditioned for a minimum of 7 days and be 4-5 weeks old when the experiment begins. Their strain and source are to be specified for each study. House the animals one per cage. Assign the rats randomly to 3 test groups, which will receive different levels of test material, and to one control group. Exclude animals of questionable health or outlying body weight.

Procedure: Supply feed and water ad libitum. Administer the test material either by oral intubation in a specified vehicle or by incorporation into the diet, as specified for each study. Choose 3 levels for administration on the basis of the known or anticipated toxicological properties of the material, and give one level to each of the 3 test groups. Ideally the levels should be so chosen that the highest level produces a response, the low level produces no response and the intermediate level then indicates the nature of the dose-response relationship. Dose the rats daily (by oral intubation) or continuously (by dietary inclusion) for 14 days.

Observations: Observe the rats daily for physical appearance, signs of local or systemic toxicity, and mortality. Weigh the animals before the experiment and after 1 and 2 weeks. Determine feed consumption and feed efficiency weekly. Perform gross necropsies on any animals that die or become moribund during the study.

Termination: On the 15th day, sacrifice all surviving animals by administration of excess anesthetic (sodium pentobarbitol - I.P.). Perform gross necropsies on all animals. Preserve any tissues showing gross lesions in 10% neutral buffered formalin* for possible future pathological examination.

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* 15 parts fixative: 1 part tissue.

Distribution: RHC(25) REW(47) EWG(5) HS(1) DTH(1) WCK(1) HT(1) MTC(1) TB(1) AJW(1) WHM(1) REH(1) GWH(1) AI(1) RWB(2) JTR(1) LHF(1) KWH(1) JB(1) BG(1) GCC(1) APW(1) DGC(1) WM(1) KDM(1) RF(1) JTO(1) NA(1) B(1) JRD(1) DAN(5) DS(1) RET(1)	Division: HUMAN SAFETY Section: IX. STANDARD TEST METHODS	Page: 29-a Issue No.: 2 Date: 6-24-77 Supersedes: Page: Issue:

Standard Procedure #7A for Toxicological Evaluation

Fourteen-Day Oral Toxicity Study (cont'd)

Report: Report body weights, feed consumption, and feed efficiencies with appropriate statistical analyses. (Analysis of Variance [LSD and Fo_5 tables]) Report mortality data, cause of death where it can be determined, and any abnormalities that are observed in the living animals or at necropsy. File the report within 4 weeks of completing the study.

Distribution: RBC(25)REW(47)EWG(5) HS(1)DTH(1)WCK(1)HT(1)MFC(1) WB(1)AJW(1)WHN(1)KEH(1)GWH(1) CAI(1)RWB(2)JTR(1)LHF(1)KWH(1) JB(1)DG(1)GCC(1)APW(1)DCC(1) JWN(1)KDM(1)RF(1)JTO(1)NA(1) JE(1)JRD(1)DAN(5)DS(1)REF(1)	Division: HUMAN SAFETY Section: IX. STANDARD TEST METHODS	Page: 30 Issue No.: 2 Date: 6-24-77 Supersedes: Page: Issue:

Standard Procedure #8 for Toxicological Evaluation

Guinea Pig Immersion

Purpose: To determine relative levels of primary irritation.

Animals: Hartley albino guinea pigs, three per group, ~300-350 gm each.

Dosage Level: Concentration of material is based on previous experience or preliminary results.

Procedure: Prepare 2,000 milliliters of the appropriate concentration of test material. Put ~220 milliliters of the solution in a 600-milliliter beaker, one for each animal. Place the beakers in a water bath set at 39° Centigrade until the test solution reaches bath temperature. Ear punch the animals for group identification and place the animals in perforated restrainers. Place the animal and restrainer in the beaker so as to cover the entire trunk of the animal for 4-1/2 hours daily for 3 consecutive days. Wash the animals after each exposure with tepid tap water and dry with terry cloth towels. Use fresh test sample daily.

Shave the abdomens of the animals on the sixth test day with a small animal clipper. Score reactions following a pictorial guide (on file at MCL Biological Testing Facility) on a scale of 1-10. Rate the reactions as follows:

10 = Normal Skin 7 = Scaling 5 = Fissuring 3 = Fissuring and Bleeding

Average the individual scores. Necropsy and report animals that die from aspiration of the test solution or from loss of fluid due to excessive primary irritation. Exclude these animals from scoring.

Report: Individual animal skin reactions, deaths, and systemic toxicity systems are reported. The report should be filed within 3 weeks of the time the experiment is finished.

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Standard Procedure #13 for Toxicological Evaluation

Teratology in the Rat

(Segment II of 1966 FDA Guidelines)

Purpose: To determine the effects of a material on teratogenesis in the rat.

Animals: Rats, Charles River CD, Sprague-Dawley originated, sexually mature, weighing approximately 200 g at the time of receipt, 30 females and 15 males per group. All animals will be housed in individual stainless steel cages with raised wire floors and have free access to feed and water. The males will be used only for mating and will receive no treatment; no records will be kept on them. All animals will be acclimated to the laboratory for 1-2 weeks, while being fed Purina Chow pellets or their equivalent. If the animals are to be treated by gavage, they will remain on Purina Chow pellets. If the test material will be administered in the feed, the animals should be placed on ground Purina Chow at the end of the acclimation period. The females will be assigned unique numbers and be identified with ear tags.

Procedure: Take daily vaginal smears to determine length and normality of extrous cycles. At the beginning of the third cycle expose females to the males (2 females to 1 male). The presence of sperm in the vaginal smear will indicate day "0" of pregnancy. On days 6 through 15 inclusive of gestation, treat the females with test compound, either by gavage or in the feed. Test two, preferably three, or more levels of the test material, chosen on the basis of intended use (human exposure), general properties and acute toxicity. Naive and vehicle controls will be included where appropriate. During each pregnancy, measure and record the feed consumed for the periods 0-5, 6-15 and 16-20. Weigh the females on days 0, 3, 6, 9, 12, 15 and 20, in order to monitor maternal toxicity and to adjust dosages during the treatment period. The records for the animals sacrificed on day 13, will end on that day.

On day 13, sacrifice one-half of each group of females, which had been randomly assigned on day "0", by excessive ether. Remove the uteri and ovaries. Record the numbers of corpora lutea of pregnancy, implantations and resorptions, to determine early embryotoxicity. On day 20 of gestation sacrifice the remaining one-half of each group of pregnant females with excessive ether and open the abdominal cavity.

Distribution: HC(25)REW(47)EEC(5) JRS(1)DTH(1)RCK(1)HT(1)MFC(1) [illegible]ER(1)AES(1)WHM(1)REH(1)GWH(1) [illegible]AI(1)RWB(2)JTR(1)LHF(1)RWH(1) [illegible]G(1)DG(1)GCC(1)APW(1)DGC(1) [illegible]AN(1)KDM(1)RF(1)JTO(1)NA(1) [illegible]R(1)JRD(1)DAN(5)DS(1)RET(1)	Division: HUMAN SAFETY Section: IX. STANDARD TEST METHODS	Page: 39 Issue No.: 2 Date: 6-24-77 Supersedes: Page: 39 Issue: 1

Standard Procedure #13 for Toxicological Evaluation

Teratology in the Rat (cont'd)

(Segment II of 1966 FDA Guidelines)

Record the numbers of corpora lutea, specifying the number on each side. Open the cornua and remove the fetuses, but also note any resorptions and dead fetuses. Remove fetuses, cutting umbilicus approximately mid-distance between fetal abdomen and placenta. Blot fetuses dry with soft paper toweling, inspect for gross abnormalities, determine the sex and weigh. The fetuses should be numbered consecutively from the distal end of the cornua on the animal's right side to the distal end of the left side in a counter-clockwise fashion. Resorption sites should be indicated in their appropriate number place.

Each fetus will be identified with an appropriate tag fastened to a limb or around the neck, showing a code number which identifies the group, dam and fetal position. Randomly select one-third of the fetuses in each litter for skeletal examination with the remaining two-thirds receiving soft-tissue examination. However, the random selection should be altered if a fetus has an external condition that warrants examination by a particular method e.g. a fetus with spina bifida or micropthalmia would be examined by soft-tissue methods.

The fetuses to be examined for skeletal defects will be eviscerated, cleared with KOH and stained with alizarin. The method used should be Staples and Schnell (Stain Tech. 39, 1964) or an equivalent method. The fetuses from a single litter can be put into a single jar for processing. The jar should be identified as to study number, group or treatment number and dam or litter number. The remaining pups will be fixed in Bouins fixative for two weeks, again using a single jar for each litter and labelled as above. These fetuses will be razor-blade sectioned and examined for soft-tissue abnormalities (Wilson, Teratology, Principles and Techniques, 1965). Dead, near-term fetuses should be included in the soft-tissue examination. Edematous fetuses or hemorrhagic blebs will be considered as late resorptions.

During the sketetal examination record numbers of ribs and sternebrae, indicating degree of calcification. Examine vertebrae for number and the degree of calcification, as well as for obvious defects. During the soft-tissue examination record variations such as hydronephosis and folded retina.

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Standard Procedure #13 for Toxicological Evaluation

Teratology in the Rat (con't)

(Segment II of 1966 FDA Guidelines)

Tissues will not normally be taken from the dams for histopathology, but all gross lesions should be recorded. If it is deemed appropriate by the investigator and resident pathologist that tissues should be taken, the number, kinds and methods will be determined at that time.

At the time of making the test solutions or diets, a 100 g sample will be taken of each such solution or diet to the presence of the test material and the appropriate level in the vehicle. These should be labelled with study number, test material number, group or treatment number, date made and investigator's name. These samples should be stored in a manner to prevent deteriation.

Report: Data to be reported are:
Body Weight changes for the three periods, 0-5, 6-15 and 16-20 days.
Feed consumed for the three periods.
Total amount of test material ingested per animal.
Daily amount of test material ingested per animal expressed as mg/kg of body weight.
Number of pregnancies per group at 13 and 20 days.
Number of corpora lutea of pregnancy per litter at 13 and 20 days.
Numbers of implants per litter at 13 days.
Number of resorptions per litter at 13 days.
Number of live fetuses per litter at 20 days.
Number of dead fetuses per litter at 20 days.
Number of resorptions per litter at 20 days.
Numbers and weights of males and females per litter at 20 days.
Number of fetuses examined for soft-tissue defects by litters and groups.
Number with soft-tissue defects.
Number of fetuses examined for skeletal defects by litters and groups.
Number with skeletal defects.
Types of soft-tissue and skeletal defects.

The final report should be completed within 6 weeks after completing the experimental work.

Distribution: RHC(71)EWG(1)GHS(1) TH(1)WCK(1)HT(1)MTC(1)FW(1) AW(1)WHM(1)REH(1)CAI(1)RWB(1) CE(1)LHP(1)KWH(1)JB(1)DG(1) JAB(3)TWM(1)JDR(1)KDM(1)RF(1)	Division: HUMAN SAFETY Section: IX. STANDARD TEST METHODS	Page: 36 Issue No.: 1 Date: 12/20/76 Supersedes: Page: Issue:

Standard Procedure #12 for Toxicological Evaluation

Acute Inhalation Toxicity

Purpose: To determine the concentration of a material that will produce pharmacological changes or death in rats when they are exposed to it in the form of a particulate solid aerosol, a liquid aerosol, a vapor, or a gas.

Animals: Albino rats, of strain and source to be specified for each experiment, weighing 200-300 g, randomly assigned to groups of 10 males and 10 females.

Procedure: Arrange to generate an air stream containing the test material at specified concentrations, to introduce this air stream into an inhalation chamber, and to monitor the concentration of the material in the chamber. Expose the animals to this atmosphere, under dynamic conditions, for 1 hour. Remove the animals to standard individual cages and normal air; retain the survivors for a 14-day observation period.

Concentration of Test Substance: Unless otherwise indicated by knowledge of the test material, expose the first group of animals to a concentration of 200 mg of test substance per liter of air. If more than 90% of the animals die during exposure, repeat the experiment at lower levels until a level is found that produces 10-90% mortality. From the numbers of deaths, calculate the LC_{50} for the material (1).

Observations: During the 1-hour exposure period and daily for the next 14 days, observe the animals for mortality, behavioral abnormalities, and other evidences of morbidity. Necropsy animals that die or appear moribund, and, at the end of the experiment, necropsy all survivors. The lungs, trachea, liver, and kidneys, as well as any other organs that look grossly abnormal are preserved in the appropriate fixative using a volume to ensure preservation of the specimens. If required by special protocol, histological examination of these tissues will be made. Weigh the lungs, liver, and kidneys and calculate organ/body weight ratios.

(1) W. R. Thompson, Bacterial Rev., Part I, 11, 115-145 (1947); C. S. Weil, Biometrics, 8, 249 (1952).

Distribution: RHC(71)EWG(1)GHS(1)OTH(1)WCK(1)HT(1)MTC(1)FB(1)AW(1)WHM(1)REH(1)CAI(1)RWB(1)CB(1)LHF(1)KWH(1)JB(1)DG(1)JAB(3)TWM(1)JDR(1)KDM(1)RF(1)	Division: HUMAN SAFETY Section: IX. STANDARD TEST METHODS	Page: 37 Issue No.: 1 Date: 12/20/76 Supersedes: Page: Issue:

Standard Procedure #12 for Toxicological Evaluation

Acute Inhalation Toxicity (cont'd)

Report: Report the means used to generate the test atmosphere and results of monitoring its concentration. Report mortalities and LC_{50}. Report organ/body weight ratios, with appropriate analyses. Report the results of histological examination, and report any other abnormalities observed.

File the report within 4 weeks of completing the experiment.

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