*Environmental Health Perspectives*
*Vol. 26, pp. 287–299, 1978*

# Strategy for the Assessment of Neurobehavioral Consequences of Environmental Factors

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**One of the critical issues confronting the evolving discipline of behavioral and neurological toxicology is the general lack of test validation in animal models. This paper seeks to provide a strategy aimed at resolving this important problem. It is proposed that test validation be accomplished by evaluating known neurotoxins in a battery of tests chosen to assess in animal models a wide range of effects on the basis of *reported human toxicosis symptomatology. We propose to measure ongoing home cage motor activity*, food consumption, water consumption, clay consumption (and the diurnal cycling of these), neurological/physiological indices (reflexes, autonomic signs, equilibrium/gait, balance, tremor, reactivity, and muscular strength), and aspects of cognitive and associative behavior involving both endogenous and exogenous (sensory) control of responding. An integrated, time-efficient scheme, covering 90 days of chemical treatment and 30 days of post-dosing recovery will be used.**

**Chemical substances to be evaluated were chosen with the view of representing classes of neurotoxic effects. For initial study, triethyltin was chosen as an agent producing demyelination of nerves, acrylamide as an agent producing "dying-back" neuropathy, and methylmercury as an agent producing mixed central and peripheral neuropathies. Agents which attack specific loci in the nervous system and those producing anoxia will not be assessed in the first stages of this research due to lack of species generality of known effects, present lack of appropriate exposure facilities, or other problems. In addition, two drugs (amphetamine and sodium salicylate) will be investigated to support the generality of the testing procedures.**

**By comparing the observed results of the neurotoxins in the animal models with the predicted effects based on reported human symptomatology, some decision concerning the validity of each procedure will be made. It is expected that the validation of tests to be used in behavioral and neurological toxicology will permit the meaningful assessment of more complex issues, such as the mechanisms by which neurotoxins act.**

## Introduction

### General Aspects of Environmental Toxicology

The patent and potential hazards of exposure to environmental factors such as irradiation, noise, and chemical agents have become of increasing concern to many segments of society. Of particular importance are the possible consequences of contact with the vast number of pharmaceutical, industrial, and agricultural chemicals and their byproducts that have entered the environment in recent years. The benefits yielded by modern technology are clear and, in many parts of the world, the use of products such as pesticides and herbicides contributes directly to human survival. However, even highly useful chemical agents may persist in the environment and incidental exposure of nontarget organisms, including man, to such agents is inevitable. Unfortunately, some of these chemical contaminants affect nontarget biological systems adversely, either directly or through interaction with other environmental factors.

It is clear, therefore, that evaluation of the risk following from exposure to pollutants is required. Environmental toxicology is the emergent discipline devoted to the study of toxic effects of environ-

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mental contaminants, especially as they may apply to human exposure and health. Recognition of the problems entailed in environmental toxicology has evoked at least two major responses from the Federal government. First, regulatory agencies, such as the Environmental Protection Agency (EPA), Food and Drug Administration (FDA), and Occupational Safety and Health Administration (OSHA) have been assigned the responsibility and authority to regulate the levels of chemicals already in, or which might enter, the environment. These governmental agencies also attempt to predict potential ill effects of newly developed chemical entities and to control human exposure to compounds which may be hazardous. For example, in response to the possibility that contact with chemical products may be involved in the etiology of cancer, the Congress of the United States enacted the Toxic Substances Control Act of 1976. This legislation provides the legal basis for the screening or mass testing for possible carcinogenicity and other effects of newly developed chemicals prior to their being marketed.

A second major consequence of the acknowledgment of the hazard of toxic substances in the environment has been an increased awareness that the solution of environmental toxicological problems will require multidisciplinary and interdisciplinary efforts, with contributions necessarily coming from many segments of the biomedical research community. Scientists from many fields of expertise are now studying the biological effects of toxic agents. Intensive efforts are currently under way at centers such as the National Institute of Environmental Health Sciences (NIEHS), the National Center for Toxicological Research (NCTR), and the National Institute of Occupational Safety and Health (NIOSH) to identify the causes, conditions, effects, and mechanisms of toxicosis.

## Evolution of Behavioral Toxicology

In neurotoxicology, the study of toxicities affecting the nervous system, investigators employing methodologies derived from experimental psychology, neurology, neurophysiology, and psycho- and neuropharmacology have begun to form the basis of a new discipline (*1, 2*). The scope and findings of this newly evolved area of research, generally termed behavioral toxicology, have been the subject of several recent reviews and books (*3–7*).

A critical assertion in behavioral toxicology is that the behavior of organisms represents the net result of various sensory, motor and associative functions of the nervous system. Thus, alterations in behavior that follow exposure to low concentrations of toxins may be the result of subtle changes in nervous system function. An important corollary of this argument is that behavioral changes may precede histopathological or morphological changes in nerve tissue and thereby prove to be a highly sensitive component in toxicological evaluations. In fact, many countries, most notably the Soviet Union, have relied heavily upon behavioral tests in their toxicology testing for several years, and it is relevant to this point that the acceptable limits of many toxic substances in the Soviet Union are below those established in this country (*8, 9*). This kind of precedent has stimulated the use of behavioral toxicological testing in the United States, with the subsequent reduction of acceptable limits for chemicals such as trichloroethylene (*5*).

Although behavioral techniques have become relatively commonplace in drug evaluations, recognition of their utility in environmental toxicology is a comparatively recent development (*1*). In fact, some investigators have pointed out the limited agreement as to the sensitivity and utility of many commonly used behavioral tests and procedures (*10*). The Subtask Force on Disease and Injury, Special Problems, of the Second Task Force for Research Planning in Environmental Health Sciences (*11*) lends support to this assertion by recommending the development of strategies for the selection of behavioral procedures for safety evaluation. It is our position that little meaningful progress can be made in behavioral and neurological toxicology until such a strategy has been developed.

In an embryonic area of inquiry, such as behavioral and neurological toxicology, early consensus on aims, strategies, and accepted procedures is unlikely. However, the critical environmental issues that have been and continue to be raised strongly suggest that some effort be made toward providing a relatively coherent, systematic framework for research in this area. It is anticipated that such an attempt would at least provide a basis for discussion among researchers in this field. It is hoped that this interaction will lead to agreement concerning basic research strategy, since this accord should make finding solutions to the critical issues more efficient. A set of common reference points should also facilitate integration of research findings and help establish the credibility of the discipline in the eyes of other scientists, administrators, legislators, and laymen who must cope with the problems of environmental safety.

In this paper, the issue of test validity as the outstanding problem for current research in behavioral and neurological toxicology is discussed. Included here are critical issues related to test validation, among which are criteria for selection of behavioral tests, representative neurotoxins, animal models,

and experimental parameters. Next, we present a specific research program, the goal of which is to provide a valid screening procedure to assess neurobehavioral effects of potentially hazardous environmental factors. The last section presents a matrix of predicted effects of representative chemicals on selected behavioral and physiological measures.

## Overview of Critical Issues Concerning Research in Behavioral and Neurological Toxicology

### Goals and Aims

Neurotoxicologists are confronted with such questions as the mechanisms by which toxins affect the nervous system, the identification of variables that affect the manifestations of toxicosis, and the detection and prediction of neurotoxicity following exposure to low amounts of environmental agents. However, before these interesting and intriguing topics can be systematically investigated, we believe that there is another issue that must be resolved first, and this is the problem of test validation (*12*). We feel that is is imperative first to consider whether or not the tests and methods used in behavioral and neurological toxicology actually measure what they are intended to measure, and whether or not the data obtained from some types of animal models can be logically extrapolated to the human case. To proceed further without first addressing the problem of test validation is, in our opinion, premature and unwarranted.

We believe that the most logical approach to the validation of sensitive and reliable methodologies is to compare compounds known to have specific neurotoxic effects in a battery of tests chosen to detect a wide range of possible effects and to overlap in terms of signs evaluated. By using such a battery, it should be possible to generate a profile of effects characteristic of each compound. This profile could then be used to evaluate the sensitivity and selection of those tests assumed to measure the same neurobehavioral functions. This strategy permits test validation by showing the similarities between procedures assumed or purported to measure the same function and by providing a distinction between procedures assumed to measure different processes. This approach to test validation has been described as the multitrait-multimethod process of validation (*13*).

### Choice of Tests for Assessment of Neurotoxicity

We believe that tests used to assess toxicity in the nervous system should reflect the full range of signs and symptoms reported by humans exposed to neurotoxins. Table 1 summarizes the behavioral and neurological sequelae of human neurotoxicosis; as can be seen, the set of symptoms may be grouped into subcategories of neurobehavioral functions. These include areas of sensory function, motor strength and coordination, associative or cognitive factors, emotionality, and several other symptoms less easily described by a short label (e.g., insomnia, anorexia, hyper- or hypothermia). Thus, selection of neurobehavioral tests should reflect, in a reasonably representative fashion, these areas of neurobehavioral function.

In addition to the relationship between functional tests and potential human symptomatology, there are practical constraints imposed by characteristics of the animal model of choice, the availability of technology for taking the measure, and the cost-effectiveness/time-efficiency factors entailed by the numbers of subjects and numbers of compounds to be tested. Further complications are raised by the generally acknowledged trade-off between sensitivity and complexity of tests and by the frequent desirability of imposing multiple testing procedures on the same subjects. The timing and sequencing of

**Table 1. Symptomatology reported by humans exposed to neurotoxins.**

| Function affected | Symptomatology |
|---|---|
| Sensory | Anosmia<br>Paresthesias in feet, fingers, toes<br>Visual deficits, photophobia, nystagmus<br>Auditory deficits, tinnitus<br>Perceptual dysfunctions, pseudohallucinations |
| Motor | Weakness in hands, arms, legs, paralysis<br>Incoordination, dizziness<br>Fatigue<br>Tremor, convulsions<br>Hyperactivity<br>Slurred speech |
| Affective | Nervousness, irritability, agitation, euphoria, psychosis<br>Apathy, lethargy, depression, compulsive behavior |
| Associative (cognitive) | Impaired short term memory<br>Impaired long term memory<br>Confusion, disorientation |
| Physiological and consummatory responses | Disrupted sleep-awake cycles<br>Hypothermia, hyperthermia, sweating<br>Loss of stimulated appetite<br>Loss or gain in body weight |

**Table 2. Representative neurotoxins classified according to mechanism of action.**

| General mechanism of neurotoxicity | Compounds |
|---|---|
| Agents that produce demyelination | Triethyltin<br>Hexachlorophene |
| Agents that produce "dying back" neuropathies | Acrylamide<br>DDT<br>Leptophos<br>Kepone |
| Agents that produce mixed central and peripheral neuropathies | Methylmercury<br>Inorganic lead<br>Carbon disulfide<br>Tellurium |
| Agents affecting specific CNS nuclear groups | Manganese<br>Monosodium glutamate<br>Salicylates |
| Agents that produce neurotoxicity through hypoxia or anoxia | Carbon monoxide<br>Cyanides and cyanates<br>Azide |
| Agents whose mechanism of neurotoxicity in humans is not yet well defined | Polybrominated biphenyls<br>2,3,5-Trichlorophenoxy-acetic acid<br>Tetraethyllead |

treatments and procedures within the relatively limited lifetime of a typical individual of the species under test are perhaps the final practical boundary conditions on choosing test procedures. Preferred tests are those which are reliable, robust, sensitive, time-efficient, and cost-effective.

## Choice of Representative Agents to Be Used in Validation of Behavioral Tests

It is our position that neurotoxins used for validation of behavioral methods should be selected on the basis of known symptomatology and mechanism or site of action from a standard classification of neurotoxins incorporating those factors described by Norton (*14*). Table 2 summarizes Norton's classification with minor modifications and lists representative compounds in each category.

A second criterion to be used in the selection of candidate neurotoxins for validity testing is that each should be capable of producing neurotoxic effects in the animal model. For example, tri-*o*-cresyl phosphate produces "dying-back" neuropathies in many species, but it is less active in rodent species. Thus, if the rat were chosen as the animal for study, the neurotoxicity of this agent might be detected only after very high doses.

For purposes of comparison between humans and an animal model, it is desirable that adequate information concerning the absorption, distribution, metabolism, and dwell time in the body (half-life) be available.

## Animal Models

The extrapolation of animal toxicological data to man is always tenuous, but, for obvious reasons, animal test models are necessarily used. Unfortunately, there is no single animal model in which effects correlate perfectly with toxicity in humans. In the selection of a suitable animal test model, the following points need to be considered.

If the purpose of a research program is to provide a basis for the development of a test battery to be used in the mass screening of known or suspected environmental toxins, there are obvious economic factors that must be taken into account. In addition to the need for a reasonable cost per experimental subject, factors such as housing and maintenance costs must also be considered, since repeated dosing procedures require adequate space to house large numbers of subjects plus provision for husbandry of them over extended periods of time.

It is also imperative that there be an adequate pharmacological and toxicological data base on the species chosen for study, such that meaningful interpretations of chemically induced effects can be made and appropriate hypotheses about mechanisms and loci of action can be framed.

The use of a single animal species in most, if not all, of the behavioral tests will facilitate comparisons among measures and will help determine the reliability and sensitivity of behavioral methods. Weight should, therefore, be given to those species or strains to which multiple procedures are known to be applicable.

The researcher must also consider various organismal variables. For example, the estrous cycle of female rats may introduce an additional unwanted variance component into the data of some behavioral tests (*15*). Some justification or explanation for the use of female subjects in behavioral toxicological studies would, therefore, be warranted. Another organismal variable is the age of the subject. Developing organisms and older, senescent animals differ from the mature adult in their sensitivities to chemicals (*8*). Unless the research program is specifically aimed at elucidating the effects of chemicals in the developing animal or in older animals, mature adult organisms should be considered as the normative population. We believe that the initial use of the mature, adult organism will provide the opportunity for establishing something of a benchmark against which the possible interactions of age and chemically induced toxicosis can better be evaluated.

## Dosing and Route of Administration

Studies in behavioral and neurological toxicology

involve either single or repeated exposures to the agent being investigated. Assuming that detection of cumulative toxicity following exposure to subthreshold doses is a major research goal, a subacute, multiple-dosing regimen that spans about one-tenth of the expected life span of the tested subject is a typical paradigm (*16*). For example, if the rat were chosen as the animal model, the appropriate dosing period would be three to four months. Neurobehavioral assessments should also be administered for a time following cessation of the dosing regimen since it would be of interest to determine the reversibility of any effects noted during the dosing phase, or possibly to note any delayed post-dosing effects.

In many neurotoxicological studies using a subacute dosing regimen, the rationale for the doses chosen is often missing. One procedure frequently employed to arrive at doses for subacute studies is to use multiples of a previously determined $LD_{50}$ (i.e., dose required to cause lethality in 50% of the animals dosed). One alternative to this approach, at least in the study of anticancer drugs, is to use multiples of the lethal dose (LD), which is defined as the lowest dose producing death in any subject during the period of examination (*17*). However, because the dose response curves of various toxins differ greatly in slope, it is unlikely that either of these two approaches can be used as a standard strategy. Instead, some portion (i.e., one-tenth) of the $LD_{50}$ or LD might be used in a short-term (e.g., 30 days) dose-ranging study designed to detect a cumulative toxic dose (CTD). Once such a dose has been identified, the portions of the CTD might be used in a longer, subacute dosing study.

The acceptance of behavioral and neurological toxicology as a discipline will largely depend upon adherence to basic principles of pharmacological and toxicological research. We believe that it is imperative to establish dose-response relationships between chemicals and behavior, and to study time to the onset, and duration of the observed effects and the cumulative dose required to elicit toxicosis. Under optimal conditions, researchers would be aware of the levels of the agent in the plasma of the subject at the time of testing and would know something about the absorption, distribution and metabolism of the chemical being evaluated.

The choice of route of administration is a critical one for the behavior toxicologist. The chemical's route of entry into the body should be similar to that encountered in the environment. However, adequate facilities for some routes, e.g., inhalation, may not be available and alternative routes should be considered. Since the oral route is frequently encountered, it could be used in most routine toxicological assessments. Of the two usual methods of oral administration, intubation is preferred over ingestion through the diet or drinking water since the amount of chemical delivered to the subject is more precisely controlled.

Finally, the extrapolation of toxicological data from small animals such as the rat to humans is reportedly more precise if dose levels are calculated on the basis of body surface area, rather than body weight (*18*). Researchers using small rodents might consider using the two-thirds power of the body weight factor described to estimate body surface area (*19*).

## Proposed Research Program in Behavioral And Neurological Toxicology

### General Overview of Proposed Research

The purpose of the following research program is to validate behavioral toxicological methods in animals that may be predictive of human behavioral change in toxicosis. As discussed in the previous section, validation of behavioral procedures will be attempted using representative neurotoxins in a battery of tests designed to measure many possible signs of toxicosis.

***Testing Strategy.*** As part of our efforts to validate behavioral methodology, two levels of testing will be employed. At the first level of evaluation, we will use two batteries of tests, the domiciliary and primary screen. In the domiciliary battery we will investigate toxin-related changes in food and water consumption, ingestion of a nonnutritive substance and general motor activity. These measures are known to be diurnally cyclic and periodicities of each will therefore be observed.

A separate group of subjects will be examined periodically in the primary screening tests during exposure to toxins. This battery of tests will consist of body weight measurements and observational ratings of reflexive and physiological functioning. Tests requiring little or no training and having the capacity to test the same subjects repeatedly will be used to assess toxin-related changes in sensorimotor function and CNA excitability. The acquisition and retention of a simple discriminated avoidance task will also be evaluated.

If signs of behavioral or neurological toxicity are observed in the domiciliary or primary screen tests, additional evaluation will occur using procedures thought to be sensitive to subtle changes in sensory, memory, associative capabilities, and/or psychomotor functioning (special tests). Many of these

tests require extended or special training, frequent evaluation and/or manipulation of motivational factors, such as food deprivation or electric footshock. For this reason, the lowest dose producing a significant effect in the domiciliary or primary screen tests will be used at first in validation of the special tests. If it appears that a special test permits the detection of an effect at a lower cumulative dose than that observed in the domiciliary or primary screen tests, lower doses of the chemical will be evaluated.

Depending upon the effect observed in the domiciliary and primary screen tests, one or more of the following special tests or tests to be developed will be considered: measures of pain or reaction threshold, acquisition and retention of an active avoidance response, performance of a temporally based discrimination with and without imposed stimulus-response delays, conditioned suppression of baseline responding by auditory and visual stimuli paired with electric footshock, and responding on an unsignalled continuous avoidance schedule.

*Subjects and Group Assignments.* We propose to use adult male albino rats of the Fisher strain in all studies. Those subjects in the domiciliary test will be housed individually in special cages designed to measure ingestive and motor behaviors. Those animals used for the primary screen and special tests will be housed singly or in groups of two to four, depending upon the procedure employed.

In the domiciliary and primary screen tests, animals will be assigned randomly to one of five experimental groups: nondosed control, vehicle-treated control, and three dosed groups. Unless needed otherwise, only a vehicle treated control group and a treatment group will be used in the special tests. Enough subjects will be used to insure reliable and stable statistical evaluation of results.

*Scheduling of Dosing and Testing.* Prior to the start of the subacute dosing study, a cumulative toxic dose (CTD) will be ascertained by dosing animals for 30 days with 1/10 or 1/100 of the reported or empirically derived oral $LD_{50}$. Dosing will be by gavage, Monday through Friday. Selected behavioral tests from the primary test battery will be used to determine any cumulative toxicity. Once a CTD has been determined in the 30 day dose-ranging study, portions of the CTD (1, ½, or ¼) will be used in the 90-day subacute study. Dosing will be by gavage on Mondays, Wednesdays, and Fridays after all behavioral tests have been completed. Behavioral and neurological assessments will occur in the week prior to dosing (predosing phase) and in the first week and every third week thereafter during the 90 day dosing phase. Tests will also be given during the second and fourth weeks of a 30 day post-dosing phase. Special tests will be administered on a session-by-session basis. The schedule of behavioral testing is summarized in Table 3.

Blood samples will be drawn as needed just prior to dosing on the Friday of each week in which primary screen tests occur. At the end of the entire series, some animals will be euthanized and the brain will be analyzed for histopathology. Other tissue analyses may also be run on a discretionary basis at that time.

**Table 3. Schedule of behavioral testing.**

| Test battery | Time of testing | Variables |
|---|---|---|
| Domiciliary | Daily for 120 days | Frequency and patterning of motor activity, drinking, eating, and kaolin ingestion |
| Primary screen | During predosing phase, every third week of dosing phase and during second and fourth week of post-dosing phase | Gross behavioral ratings<br>Body weight<br>Spontaneous motor activity<br>Visual placement<br>Forelimb grasping<br>Hindlimb extensor response<br>Performance on inclined screen<br>Startle and habituation<br>Tremor<br>Rectal temperature |
| | At 45 days<br>At 90 days | Acquisition of active avoidance<br>Retention of avoidance |
| Special tests | Session-by-session | Temporal discrimination<br>Unsignalled continuous avoidance<br>Conditioned suppression<br>Shock titration-pain thresholds |
| | At 45, 90, or 120 days | Shuttle box or passive avoidance learning |

## Rationale and Description of Behavioral Tests

*Domiciliary Tests.* A general principle in psychology and animal behavior is that the behavior of an organism is likely to be stable and predictable given stable environmental conditions. Another way of saying this is that the probability of occurrence of a given response is relatively fixed under unchanging environmental conditions. It follows more or less directly that differences in response probabilities as a function of chemical administration could be used as indicators of toxicity. In a sense, this theme is characteristic of all behavioral toxicological testing, the major difference being in the initial probability of the response in question (i.e., its baseline rate).

Some responses in any organism's behavioral repertoire are highly probable, some others less so. The measures we propose to take will sample from several levels of response probability. In some, the special tests, response probabilities are actively manipulated through control of stimulus, motivational, and response contingency conditions. In the primary screen tests, the observation of at least some responses is made more probable by imposing appropriate stimulus conditions, e.g., a toe pinch or a touch on the cornea. In the domiciliary test, measures of high probability responses tend to be controlled by internal conditions or states of the organism over relatively extended observation periods (24 hr) (*20*). Such measures include eating, drinking, general locomotor activity, and kaolin clay consumption (geophagia) and are related to the ecological and evolutionary history of the organism.

On the principle that treatment with toxic substances should be discernible in changes in probability of occurrence of a response, it is reasonable to assume that changes in feeding, drinking, and activity patterns will be reliable indicators of toxicosis. Since general cage activity in the domiciliary cage is but a fraction of the animals' total repertoire of spontaneous activity, other tests and apparatus that examine wheel running, exploratory, rearing, and grooming behavior may be necessary. Individual and multivariate assessments of these behaviors will be made to establish baselines for detecting and quantifying the onset and presence of low level toxicosis.

Geophagia, as a further measure, is a low-probability response in normal rats under normal conditions. An increase in the probability of the occurrence of geophagia, however, has been observed under conditions known to produce illness in other species (*21*).

While it is clear that toxicologists and psychopharmacologists have looked at measures of ingestion and locomotor activity as indicators of chemical effects, the scheme proposed here departs significantly from past practice in two respects: (a) its emphasis on cyclicity in those measures and (b) in the introduction of a measure of ingestion of a nonnutritive substance known to be related to illness.

*Primary Screening Tests.* It is relatively standard practice in the evaluation of chemical agents suspected of having effects on the nervous system to subject animals dosed with the agent to a series of simple tests and observational procedures. An example of this approach is detailed by Irwin (*22*). While some of these are not what might be considered behavioral in some pure sense, the measures, taken together, are indicative of the functional integrity of the nervous system.

The measures to be examined fall roughly into categories of neurological screening and general health, activity/reactivity, and sensorimotor functioning.

NEUROLOGICAL SCREENING AND GENERAL HEALTH. Autonomic signs will be scored as present or absent and include observations of salivation, lacrimation, piloerection, exophthalmus, abnormal skin color (further scored as blanched, flushed, or cyanotic if abnormal), and diarrhea. Rectal temperature and body weight measurements will also be taken. Subjects dying during the study will be necropsied for signs of organ disease and pathology possibly related to chemical exposure.

Reflex responses will be scored as normal or depressed for the following: left and right pinna reflexes, left and right eye-blink, left and right ipsilateral flexor reflex, righting reflex, and reflexive responsiveness to pain (tail pinch). The response to tail pinch will be rated as to its intensity as well.

ACTIVITY/REACTIVITY. In addition to the measure of activity obtained in the domiciliary test battery, motor activity will be measured at regular intervals in commercially available activity chambers. Changes in CNS excitability will be measured in the form of startle response magnitude and rapidity of acclimation to a repeated external stimulus such as an air puff, acoustic, or visual signal (*23*).

Included in the category of reactivity is the ability of the subjects to acquire a one-way shock-motivated avoidance response to a combined light-tone signal as described by Clark (*24*). Acquisition will be measured after 45 days of dosing and retention will be assessed after 90 days of dosing. If effects are evident at 90 days, retesting will occur 30 days after cessation of dosing.

SENSORIMOTOR FUNCTIONING. Possible motor dysfunction will be assessed using several indepen-

dent measures. Forelimb and grasping strength will be tested by using a recording grip meter (*25, 26*), while hindlimb extensor reflexes will be evaluated by a technique recently developed in our Laboratory. Overall grip strength and coordination will be assessed by performance on the inclined screen (*26*). Fine motor fasciculations (tremor) will be evaluated by means of a transduction-amplication-recording system similar to that described by Henderson and Wooley (*27*). Orientation to sensory stimulation will be tested using a battery such as that described by Marshall and Teitelbaum (*28*). Head orientation or biting will be scored to presentations of odors, tactile stimulation, visual motion, and auditory clicks. Body orientation to tilt, lateral hopping, and fore and hind paw placing will also be tested.

*Special Tests.* Of the many symptoms reported in clinical cases of toxicosis, perhaps the most difficult ones for which animal models are needed are effects having to do with what might broadly be termed cognitive processes, those involving the acquisition and processing of information, the utilization of response-consequence contingencies, decision-making, and response-initiating functions.

By selectively using the paradigms described below, we will attempt to relate toxicosis to changes in behaviors suspected of being correlated with higher-level nervous system functions listed above.

PAIN THRESHOLDS. Footshock titration procedures have been used to detect both increases and decreases in nociceptive thresholds (*29, 30*). Recently, Sideroff and Santolucito (*31*) reported that the insecticide carbaryl decreased the sensitivity of rats to electric shock, for example.

Because of the apparent utility of operant procedures in the determination of aversion thresholds, rats will be tested in a paradigm similar to that of Weiss and Laties (*32, 33*). After training in 40 min daily sessions, rats will be tested twice weekly during 90 days of dosing and the 30 day post-dosing period. Median shock intensity during each half of the session will be determined.

SHUTTLE BOX AVOIDANCE ACQUISITION. The capability of rats to acquire an avoidance response in the shuttle box is a standard psychopharmacological test of learning and memory in the rat. The subjects will be dosed for 45 days and then given 120 massed acquisition trials in the shuttle box (*34*). The same subjects will be retested in an identical fashion at 90 days of dosing. If significant effects are observed during the 45- and 90-day tests, subsequent tests will be given at the end of the 30 days of post-dosing recovery.

UNSIGNALLED CONTINUOUS AVOIDANCE. This procedure has been used extensively in the evaluation of psychoactive drugs. Since behavior is not under the control of external stimuli, it should be sensitive to changes in central nervous system (CNS) function (*35*). Rats will be trained to postpone electric footshock on an unsignalled continuous schedule (*34*). Training sessions will occur daily until a stable baseline of responding has been established. The rats will then be matched according to baseline rates, assigned to groups, and run twice weekly for the duration of the experiment. Avoidance and escape responses, escape losses, and distribution of lever-press interresponse times (IRT) will be recorded.

CONDITIONED SUPPRESSION OF RESPONDING BY VISUAL AND AUDITORY STIMULI. Many neurotoxic agents produce deficits in visual and auditory processing. For instance, the ototoxicity of methylmercury and kanamycin are well known. Conditioned suppression techniques have been used by various investigators to determine the effects of chemicals on sensory processing (*36*).

Food-deprived rats will be trained to press a lever on a variable-interval 20 sec schedule. Conditioned suppression of schedule-controlled responding will be established using visual or auditory stimuli paired with electric footshock. After obtaining a stable baseline and appropriate suppression of responding, the rats will be assigned to treatment groups and dosed accordingly. Sessions will be conducted 4 days a week for the duration of the study. Average rates of responding and suppression ratios to visual and auditory stimuli will be measured.

TEMPORAL DISCRIMINATION. The ability of rats to maintain a discrimination between two signals differing only in duration is known to be affected by chemical administration (*37*). Further, toxicosis is frequently reported to affect short-term memory. In this study, rats will be trained to discriminate between two temporal durations (nominally 2 vs. 8 sec) prior to the initiation of the dosing regime. The discrimination procedure will be continued through 90 days of dosing and 30 days post-dosing on a Monday-Wednesday-Friday schedule. During dosing, a variable interval (0-15 sec) delay will be interposed between the end of the signal and the animals' opportunity to respond to it; differences in accuracy of discrimination should be inversely related to the length of the delay interval. Accuracy of the discrimination as a function of dose level, delay length, and dosing period will be monitored. Latency of correct and incorrect responses and proportion of left and right responses (response bias) will also be recorded.

## Chemical Agents to be Evaluated

The neurotoxins that will be studied were selected according to the criteria outlined above. In the following section, information is provided about candidate substances to be tested, as well as the symptomatology reported in humans.

*Agents Producing Demyelination of Nerve Tissue.* Triethyltin (TET) and other organotin compounds are environmentally prevalent chemicals used for a variety of purposes, such as molluscicides, algicides, fungicides, and insecticides (*38, 39*). The neurotoxicity of TET is well documented in humans, in which it produces numerous signs of central toxicity, including psychological and visual disturbances and photophobia. Other signs of poisoning include urine retention, vertigo, abdominal pain, weight loss, and hypothermia. Transient pareses and occasionally paralysis have also been observed (*40–42*).

*Agents Producing a "Dying-Back" Axonal Neuropathy.* Acrylamide (ACR) has many industrial uses, waterproofing, strengthening of paper and chipboard, and some types of flocculating processes among them (*43*). This substance produces a "dying-back" neuropathy similar to that resulting from neurotoxic organophosphates such as tri-*o*-cresyl phosphate. The effects of acrylamide in animals closely resemble those observed in humans, hence its use in recent years as a model compound in the study of "dying-back" polyneuropathies (*44, 45*). The symptomatology and morphological effects of acrylamide in humans and animals have been recently reviewed (*43–45*). Initial signs of poisoning include weight loss, fatigue and paresthesias, with numbness in the hands and feet. Weakness in the hands and legs also have an early onset, and fine movements of the hands are impaired. Ataxia and depression of tendon reflexes in the arms and legs are observed, while responses to nociceptive stimulation seem intact. With continued intoxication, more proximal regions of the body are affected and paralysis and death may result. Tremor and bladder incontinence have been reported less frequently. Termination of exposure to acrylamide usually results in an improvement in function, the extent of which depends upon the dose and duration of exposure.

*Agents Producing Mixed Central and Peripheral Neuropathies.* Mercury is a persistent environmental pollutant that has followed the use of mercurials as antifungal agents, slimicides, mildew preventives, and chemical catalysts. Compounds containing mercury have also been used as pharmaceutical agents and in the manufacture of electronic components (*46*). Organic mercury compounds such as methylmercury are highly toxic and produce mixed peripheral and central neuropathies (*46*). Clinically, methylmercury results in a gradual onset of a cluster of symptoms characterized by neurasthenia, weakness, fatigue, headache, memory deficits, emotional irritability, and mood alterations. With continued exposure, methylmercury produces paresthesias and generalized ataxia followed by spasticity and tremor, hearing and visual deficits and eventually coma and death.

*Agents Attacking Specific CNS Nuclear Groups.* An example of this category is manganese or its salts, which occur frequently in many industrial and mining environments. Overexposure to these agents can produce symptoms which in many respects resemble those of Parkinson's disease (*47, 48*). Since the extrapyramidal symptomatology of this syndrome is associated with the deterioration of dopamine-containing pathways in the CNS (*49*), it has been suggested that manganese may also act on these pathways (*48*). These observations indicate that the study of manganese is relevant to the interests of our program. However, it was decided not to include manganese at this time since it has been asserted that albino rats do not display the typical behavioral and neurological signs associated with manganese toxicity in humans (*50*). Manganese has been reported to produce extrapyramidal dysfunction in monkeys and apes (*51*), and this substance will be considered whenever these species are available for study at the Institute.

Other examples of selective neurotoxins, e.g., gold thioglucose, 6-hydroxydopamine, and 5,6-dihydroxytryptamine, were also considered. These agents are not included in the present test battery due to a lack of species generality, inadequate data base, or absence of environmental relevance.

*Agents That Produce Anoxia.* Norton (*14*) discusses several types of compounds that affect metabolism in neuronal tissue by altering the availability of oxygen. Some toxic substances produce anoxia by acting directly on the cardiovascular system and are not relevant to the immediate goals of the program. On the other hand, carbon monoxide, which decreases the amount of oxygen reaching tissue by binding preferentially to hemoglobin, is an environmental toxin with well documented effects on the CNS. Nevertheless, study of this compound is not presently feasible because inhalation facilities meeting the highly varied needs of the program have yet to be developed. Another substance considered was cyanide, which inhibits cellular respiration and produces a cytotoxic anoxia. Since cyanide produces demyelination of white matter in the corpous callosum and associated brain regions in a manner similar to

triethyltin (*52*) and since it was desirable to compare the behavioral effects of neurotoxins having different mechanisms of actions, the study of cyanide will be deferred to a later time.

### Reference Psychopharmacological Agents

In addition to representative neurotoxins from the schema of Norton (*14*), two drugs that have known acute and subacute effects will be assessed in some of the more critical tests of the proposed battery. The information derived from these psychopharmacological standards will help in the interpretation of data obtained from studies with triethyltin, acrylamide, and methylmercury.

*Amphetamine.* Amphetamine (AMP) is a CNS stimulant that has been used at various times in the treatment of obesity, depression, narcolepsy, and more recently, the so-called hyperkinetic syndrome in children. Amphetamine has marked CNS stimulant and analeptic effects. When given in high enough doses or repeatedly, amphetamine may result in tremor, sterotypic motor movements and agitation. In addition, a well-defined psychological syndrome that resembles paranoid schizophrenia has been described in humans (*53*). The administration of very high doses of amphetamine and methamphetamine has been reported to produce morphological changes in the brain that appear to be related to the resultant cardiovascular effects (*54*). The effects of repeated amphetamine administration on animal behavior have been extensively studied (*55, 56*) and have been found to be highly dependent upon pharmacological variables, such as dose and dosing schedule, and behavioral variables, such as the response measured and the schedule of reinforcement (*57–59*).

*Sodium Salicylate.* Aspirin is a ubiquitous ingredient in many medications. The chronic ingestion of relatively high doses of sodium salicylate (SAL) produces a cluster of symptoms including tinnitus, dizziness, confusion, apathy, and loss of hearing (*60*). These effects are generally reversible. The effects of salicylates on a variety of responses controlled by auditory cues has been described recently by Hanson (*61*). It is because of the relatively selective effect of salicylate on hearing function and the potential for possible interaction with numerous environmental chemicals that we chose it as a reference psychopharmacological agent for our studies.

## Predicted Effects of Representative Compounds in Behavioral Tests

If the conditions under which the behavioral and neurological assessments described above are to be made relevant to the signs and symptoms of human toxicosis, it follows then that predictions should be made as to the outcome of tests with the animal model. Indeed, such predictions are necessary in order to demonstrate the adequacy of any given test system, in two senses.

First, prediction of outcomes validates the animal models, in a somewhat limited way, for the class of symptom and the class of neurotoxin in question. As our ability to make such predictions grows (as it must), the efficiency and concomitant economy of behavioral and neurological toxicology is enhanced.

Secondly, predictions which are not upheld are still useful, perhaps even more so than correct projections. When a prediction for a particular procedure–agent combination is not upheld, we expose for further intensive examination a gap in our knowledge, either about the animal model, the parameters of test, the procedure itself or some combination of these. This information can then aid in the evolution of new more refined procedures and/or in the evaluation of the classification scheme, and particularly of the mechanism of the compound in question. Somewhat similarly, the pattern of correct and incorrect predictions may point out inadequacies of the entire test system, leading again to reconsideration of the factors involved in choice of an animal model, choice of test procedures, and decisions about the efficiency of the total set of assessment conditions. In short, then, a comparison of the observed results with predicted outcomes is a critical aspect of this research.

In order to make the desired comparisons, it was necessary to review the human toxicology of the five compounds chosen for study and to establish a symptomatologic profile characteristic of each agent. We then listed the neurobehavioral tests selected for validation in the animal model and generated a pattern of expected effects. Table 4 summarizes these predictions in terms of the presence or absence of an effect, the direction of any effect, the time when an effect may be expected (i.e., first or last half of the dosing phase), and the reversibility or delayed appearance of an effect once dosing has ceased. After all of the compounds have been studied and a profile of observed effects is available for comparison with the expected effects, it will be possible to make some tentative conclusions concerning the validity of each test. Those tests correctly predicting the occurrence, onset, duration, and reversibility of an effect meet the criterion for predictive validity discussed above. Tests supposed to measure the same function or process will also be compared for sensitivity, or the dosage level at which an effect is noted, and for selectivity, or the capability to indicate an effect where one is ex-

**Table 4. Predicted behavioral effects for the highest dose of each representative chemical agent at various times of testing.[a]**

| | 0-45 days | | | | | 45-90 days | | | | | Post-dosing (120 days) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Behavioral test | ACR | TET | MM | AMP | SAL | ACR | TET | MM | AMP | SAL | ACR | TET | MM | AMP | SAL |
| Domiciliary | | | | | | | | | | | | | | | |
| Motor activity | ↓ | ↓ | 0 | ↑ | 0 | ↓ | ↓ | ↓ | ↑ | 0 | 0 | 0 | ↓ | ↓ | 0 |
| Food intake | 0 | 0 | 0 | ↓ | 0 | ↓ | ↓ | ↓ | 0 | 0 | 0 | 0 | ↓ | ↑ | 0 |
| Water intake | 0 | 0 | 0 | ↑ | 0 | ↓ | ↓ | ↓ | ↑ | 0 | 0 | 0 | ↓ | ↓ | 0 |
| Geophagia | ↑ | ↑ | ↑ | 0 | 0 | ↑ | ↑ | ↑ | 0 | ↑ | 0 | 0 | 0 | 0 | 0 |
| Primary screen | | | | | | | | | | | | | | | |
| Neurological screen and general health | | | | | | | | | | | | | | | |
| Autonomics | 0 | 0 | 0 | ↑ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Reflexes | ↓ | ↓ | 0 | 0 | 0 | ↓ | ↓ | ↓ | ↑ | 0 | 0 | 0 | ↓ | 0 | 0 |
| Rectal temperature | 0 | ↓ | 0 | ↑ | 0 | 0 | ↓ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Body weight | 0 | 0 | 0 | ↓ | 0 | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | 0 | ↓ | 0 | 0 |
| Motor activity | ↓ | ↓ | 0 | ↑ | 0 | ↓ | ↓ | ↓ | ↑ | 0 | 0 | 0 | ↓ | ↓ | 0 |
| Forelimb grip | 0 | 0 | 0 | ↑ | 0 | ↓ | ↓ | ↓ | ↑ | 0 | 0 | 0 | ↓ | 0 | 0 |
| Hindlimb extension | ↓ | 0 | 0 | ↑ | 0 | ↓ | ↓ | ↓ | ↑ | 0 | 0 | 0 | ↓ | 0 | 0 |
| Inclined screen | ↓ | 0 | 0 | 0 | 0 | ↓ | ↓ | ↓ | 0 | ↓ | 0 | 0 | ↓ | 0 | 0 |
| Startle | 0 | 0 | 0 | ↑ | 0 | 0 | ↑ | ↑ | ↑ | 0 | 0 | 0 | ↑ | 0 | 0 |
| One-way avoidance | 0 | 0 | 0 | 0 | 0 | ↓ | ↓ | ↓ | 0 | 0 | 0 | 0 | ↓ | 0 | 0 |
| Tremor | 0 | 0 | 0 | ↑ | 0 | 0 | 0 | ↑ | ↑ | 0 | 0 | 0 | ↑ | 0 | 0 |
| Special | | | | | | | | | | | | | | | |
| Pain threshold | ↓ | 0 | ↓ | ↑ | 0 | ↓ | 0 | ↓ | 0 | 0 | 0 | 0 | ↓ | 0 | 0 |
| Temporal discrimination | | | | | | | | | | | | | | | |
| Accuracy | | | | | | | | | | | | | | | |
| Zero delay | 0 | 0 | 0 | ↓ | 0 | 0 | 0 | ↓ | 0 | 0 | 0 | 0 | ↓ | 0 | 0 |
| Max delay | 0 | 0 | ↓ | ↓ | 0 | 0 | 0 | ↓ | 0 | 0 | 0 | 0 | ↓ | 0 | 0 |
| Latency | | | | | | | | | | | | | | | |
| Zero delay | ↑ | ↑ | 0 | 0 | 0 | ↑ | ↑ | ↑ | 0 | 0 | 0 | 0 | ↑ | 0 | 0 |
| Max delay | ↑ | ↑ | 0 | 0 | 0 | ↑ | ↑ | ↑ | 0 | 0 | 0 | 0 | ↑ | 0 | 0 |
| Unsignalled continuous avoidance | | | | | | | | | | | | | | | |
| Avoidances | ↓ | ↓ | 0 | ↑ | 0 | ↓ | ↓ | ↓ | ↑ | 0 | 0 | 0 | ↓ | 0 | 0 |
| Escapes | ↑ | ↑ | 0 | 0 | 0 | ↑ | ↑ | ↑ | 0 | 0 | 0 | 0 | ↑ | 0 | 0 |
| Escape losses | 0 | 0 | 0 | 0 | 0 | ↑ | ↑ | ↑ | 0 | 0 | 0 | 0 | ↑ | 0 | 0 |
| Conditioned suppression | | | | | | | | | | | | | | | |
| Visual, accuracy | 0 | 0 | 0 | ↓ | 0 | 0 | ↓ | ↓ | 0 | 0 | 0 | 0 | ↓ | 0 | 0 |
| Visual, latency | ↑ | ↑ | 0 | 0 | 0 | ↑ | ↑ | ↑ | 0 | 0 | 0 | 0 | ↑ | 0 | 0 |
| Auditory, accuracy | 0 | 0 | 0 | ↓ | 0 | 0 | 0 | ↓ | 0 | ↓ | 0 | 0 | ↓ | 0 | 0 |
| Auditory, latency | ↑ | ↑ | 0 | ↑ | 0 | ↓ | ↓ | ↓ | ↑ | 0 | 0 | 0 | ↑ | 0 | 0 |
| Shuttle box | | | | | | | | | | | | | | | |
| Avoidances | ↓ | ↓ | 0 | ↑ | 0 | ↓ | ↓ | ↓ | ↑ | 0 | 0 | 0 | ↓ | 0 | 0 |
| Escapes | ↑ | ↑ | 0 | 0 | 0 | ↑ | ↑ | ↑ | 0 | 0 | 0 | 0 | ↑ | 0 | 0 |
| Escapes losses | 0 | 0 | 0 | 0 | 0 | ↑ | ↑ | ↑ | 0 | 0 | 0 | 0 | ↑ | 0 | 0 |

[a] Response: ↑ = measure is predicted to be elevated or rate increased; ↓ = predicted to be decreased in magnitude or rate decreased; 0 = no significant alteration in the measure is expected.

pected and no effect where none is expected.

We believe that the test validation scheme discussed in this paper represents a critical step for the growth of behavioral and neurological toxicology as a scientific discipline. Science rests squarely upon the foundation of appropriate methodology. There must be credibility and confidence in the methodological tools of the trade before steps can be made to assess our environmental problems in a meaningful way.

We gratefully acknowledge the direction and insightful suggestions of Dr. Clifford L. Mitchell, Chief, Laboratory of Behavioral and Neurological Toxicology, NIEHS, in the preparation of this paper. The helpful recommendations of our colleague, Dr. R. E. Squibb, Jr., are also acknowledged.

The proposal for the use of naturally occurring behaviors in a home cage environment and for the use of geophagia as an indicator of toxemia in the rat was first recommended to our group by Dr. Denis Mitchell, Department of Psychology, University of Southern California, Los Angeles, California.

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