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PREFACE

This report has been prepared by ECETOC for use by a Special Expert Group of DG V who are expected shortly to examine the neurotoxic potential of solvent chemicals in the context of their Indicative Limit Value programme. The report is based on ECETOC Monograph No. 18 "Evaluation of the Neurotoxic Potential of Chemicals".

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KEY DOCUMENT TO ASSIST IN THE INTERPRETATION AND EVALUATION OF THE NEUROTOXIC POTENTIAL OF CHEMICALS IN ANIMALS

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INTRODUCTION

The ultimate objective of animal toxicity studies is to provide information that will enable the safe use of the chemical through hazard identification, hazard assessment and risk assessment. This document provides a scientific view on how to effectively evaluate the neurotoxic potential of chemicals in laboratory animals. A more detailed monograph on this subject is available from the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC, 1992).

Toxicity tests in laboratory animals are a major component of hazard identification, hazard assessment and risk assessment. Standard toxicity studies with rats, mice, rabbits and dogs provide many opportunities to identify target organs and treatment-related effects in all organ systems including the nervous system. Thus, a significant amount of neurotoxicity data can be obtained from standard toxicity studies.

Neurotoxicology is a relatively new branch of toxicology and relies on a number of disciplines including psychology, neurology, physiology, pharmacology, biochemistry and pathology. Each discipline has a range of methods, with varying complexity, specificity and applicability for investigating neurotoxic effects of chemicals.

Potential damage to the nervous system is difficult to assess because of wide variations in normal function of the nervous system as well as its plasticity, residual capacity and compensatory mechanisms (WHO, 1986). Furthermore, there may be difficulties with the interpretation and assessment of the relevance of the results from certain tests. Essentially all substances could be classified as "neurotoxic" since all chemicals are toxic at some dose level and will induce some behavioural change at these dose levels. Therefore, detection of those

compounds that may cause direct, persistent, adverse effects on the nervous system should be given the most critical attention in order to protect human health in the workplace and the environment.

Evaluation of the neurotoxic potential of a chemical should include descriptions of functional and morphologic effects as well as the determination of the dose response, time course, reversibility of effects and the NOEL. Differentiation between direct and indirect neurotoxic effects of a substance is critical for scientific and regulatory considerations. Also, investigation of species specificity, toxicokinetics and the mechanism of a neurotoxic effect may provide useful information for the risk assessment process.

UNDERSTANDING AND DEFINING NEUROTOXICITY

A simplistic definition of neurotoxicity (or a neurotoxic effect) is an adverse change in the structure or function of the nervous system that results from exposure to a chemical substance. A recent publication (OTA, 1990) cautions that the definition of neurotoxicity "hinges on interpretation of the word 'adverse,' and there is disagreement among scientists as to what constitutes 'adverse change.'"

Interpretation of the results from neurotoxicity studies, in terms of potential human health effects, requires an understanding of several significant issues:

- What constitutes an adverse effect on the nervous system?

- What distinctions should be made between transient effects and persistent effects?

- What is the significance of direct as opposed to indirect effects on the nervous system?

- When should a compound be considered a neurotoxic substance?

ADVERSE EFFECTS

A judgment as to what constitutes an adverse effect on the nervous system depends primarily on the nature of the change (morphological, neurochemical, neurological, or behavioural), the degree of change and whether the effect is transitory or persistent.

A reasonable definition of **an adverse effect is any treatment-related change which interferes with normal function and compromises adaptation to the environment.**

The term "adverse" should suggest the concept of disease and implies that the change interferes with normal function (e.g., the change is maladaptive to the individual). Adverse effects should be considered only in a toxicological sense rather than as any unwanted effect. Effects without recognised maladaptive consequences may have beneficial, indifferent or unknown consequences, but are not necessarily adverse.

Most morphological changes such as neuronopathy, axonopathy or myelinopathy would be considered adverse even if the changes were mild and/or transitory. On the other hand, transitory hypertrophy of astrocytes could be viewed as an adaptive, physiological response. A more complicated example is the transitory reduction in the number of dendritic spines on pyramidal neurons in rats exposed *in utero* to ethanol (Ferrer et al, 1988). Although a reduction in the number of dendritic spines in 15-day old rats was followed by morphological recovery at 90 days of age, the results would not necessarily prove functional recovery.

Neurochemical changes in the nervous system of experimental animals should not necessarily be interpreted as adverse without additional correlative information. For example, gerbils exposed to near-lethal levels of dichloromethane (DCM) for 2 months and examined 4 months post-exposure, were reported to have changes in brain levels of DNA as well as glial fibrillary acidic protein (GFAP) and S-100 protein (Rosengren et al, 1986). A subsequent study was conducted in order to determine if exposure to DCM was associated with any toxicological adverse effects on the nervous system (Mattsson et al, 1990a). Detailed clinical, neurofunctional and neuropathological examinations of rats exposed to high levels of DCM for 13 weeks did not reveal any persistent post-exposure, treatment-related effects and call into question the relevance of the changes reported in the initial study.

Although neurotypic and gliotypic proteins have been proposed as biochemical markers of neurotoxicity (O'Callaghan, 1988), the EPA Science Advisory Panel concluded that radio-immunoassay measurement of GFAP in the brain is not sufficiently validated as an indicator of neurotoxicity to be incorporated in test guidelines (SAP, 1989). Likewise, there is considerable doubt about the validity of plasma and red cell cholinesterase inhibition as an indicator of neurotoxicity (SAB/SAP, 1990) since these biochemical measures are not correlated with recognised adverse effects. The Panel recommended that adverse effects on the nervous system should be defined on the basis of functional measures (behavioural, electrophysiological) accompanied by morphological indices such as histochemical techniques.

TRANSIENT VERSUS PERSISTENT EFFECTS

Transient, acute neurological effects of compounds should be distinguished from permanent or chronic effects. Many chemicals can have non-specific

pharmacological effects on the nervous system at high exposure levels, but these effects are not relevant for prediction of neurotoxic effects at occupational or environmental exposure levels.

The potential consequences of neuropharmacologic effects vary significantly. Similar to the situation with anaesthetics, some reversible functional or behavioural changes that are not associated with permanent morphological alterations would not be considered adverse in themselves. On the other hand, pharmacologic effects in humans may induce adverse consequences by a reduction in vigilance which possibly could result in accidents. Furthermore, chemicals such as organophosphates clearly have adverse acute neurotoxic effects because of their pharmacologic mechanism of action.

Dichloromethane (DCM) is an example of a substance with transient neuropharmacological effects, but no confirmed neurotoxic effects (Mattsson et al, 1990a). DCM causes central nervous system depression at relatively high concentrations and also alters neurophysiological functions of rats during exposure to 2000-5000 ppm DCM. Post-exposure functional tests (observational battery, grip strength, and evoked potentials) along with detailed pathological examinations of rats exposed to as much as 2000 ppm DCM for 6 h/d, 5 d/wk for 13 weeks did not reveal any treatment-related changes. Thus, even though DCM does have sedative and anaesthetic properties at relatively high concentrations, brain injury appears unlikely.

DIRECT VERSUS INDIRECT EFFECTS

Another important concept in the practical definition of neurotoxicity is the significant difference between direct (primary) as opposed to indirect (secondary) effects on the nervous system. A wide variety of disease conditions such as nutritional and metabolic disorders, diabetes mellitus, liver disease and kidney disease may have secondary effects on the function and structure of the nervous system, such as encephalopathy or polyneuropathy (Robbins and Cotran, 1979). Exposures which do not cause primary toxicity in the liver or kidney, for example, would not result in any secondary effects on the nervous system. Thus, differentiation between direct and indirect effects of a substance on the nervous system is critical for scientific and regulatory considerations.

The indirect effect of substances on the nervous system is a major concern for the interpretation of acute toxicity studies which are conducted at very high dose levels. Substances which are not directly toxic to the nervous system can cause signs such as convulsions, tremors or ataxia at toxic or lethal dose levels. Thus, essentially all substances could be classified as "neurotoxic" since all chemicals are toxic at some dose level and will induce some behavioural change at these very high dose levels. These concerns were summarised by the EPA's Science Advisory Panel (SAP, 1989) when they stated "The Panel has serious reservations about the validity of neurotoxicity studies in which the high-dose level results in gross changes which exceed the MTD [maximum tolerated dose] or in which the normal metabolic processes of the body are severely compromised."

The interpretation of neurofunctional data from routine toxicity studies may be confounded by systemic toxicity. Gerber and O'Shaughnessy (1986) evaluated the specificity of several functional and behavioural tests for nervous system toxicity. The results indicated that impairment of organs other than the nervous system as well as reduced food and water intake can mimic the behavioural effects of standard neurotoxic agents. In fact, Gerber and O'Shaughnessy (1986) suggest that "before it can be concluded that a compound is neurotoxic on the basis of behavioural test results, it must be ascertained that non-neural organs have not been damaged by the test compound, and that food and water consumption have not been severely decreased."

Further studies of dietary restriction in rats revealed significant effects on physical, behavioural and neurophysiological parameters after 4 weeks of dietary restriction (Albee et al, 1987). Thus, the relevance of neurofunctional changes is obscure in the presence of general toxicity and in the absence of neuropathological changes (Mattsson et al, 1989).

Interpretation of results from motor activity tests is difficult especially in terms of direct versus indirect effects on the nervous system. A dose-related change in motor activity reflects an effect on the nervous system only in the absence of general toxicity; additional information is required to determine that such an effect actually is adverse (Maurissen and Mattsson, 1989). Conclusions about nervous system involvement based on motor activity can be made only by exclusion and thus, interpretation should be limited to a dose range not associated with general toxicity.

Neurotoxicity should imply a direct effect on the nervous system while behavioural or neurofunctional effects are terms that indicate a more general functional change, whether or not such an effect originates in the nervous system (Maurissen and Mattsson, 1989). Therefore, indirect effects that are detected by neurofunctional tests such as clinical observations, motor activity and other behavioural tests, clearly should be distinguished from direct neurotoxic effects.

DEFINITION OF A NEUROTOXIC SUBSTANCE

Designation of a substance as neurotoxic should be reserved for those xenobiotic compounds or their metabolites that produce adverse effects as a result of direct interaction with the nervous system. Substances which are not directly toxic to the nervous system, but result in neurobehavioural signs as a result of damage to other organ systems, should not be considered as neurotoxicants.

An assessment of potential neurotoxicity should be based on a number of different parameters that are derived from a variety of toxicological tests at relevant dose levels. A combination of functional and morphological tests enhances the ability to discover neurotoxicity.

Inappropriate categorisation of a substance as "neurotoxic" could be best avoided by a clear understanding of the general toxicity of a chemical prior to specialised neurotoxicity studies. Subsequent specialised tests for neurotoxicity should only be conducted at dose levels where no adverse general effects were detected with routine toxicity studies. Thus, a critical point in the evaluation of potential neurotoxicity is the threshold dose for neurotoxicity; substances with neurotoxic effects at levels which are less than the NOEL for other toxic effects would be of concern.

Criteria for categorisation of a substance as a human neurotoxicant were proposed for solvents by Spencer and Schaumburg (1985). Three questions must be answered affirmatively before a solvent is accepted as a human neurotoxicant:

- Does the substance or mixture produce a consistent pattern of neurological dysfunction in man?

- Can this entity be induced in animals under comparable exposure conditions?

- Are there reproducible lesions in the nervous system or special sense organs of exposed human beings and/or animals, and do these abnormalities satisfactorily account for the neurobehavioural dysfunction?

These criteria also are applicable to chemicals other than solvents and would be useful in the categorisation of substances in regard to neurotoxicity for man.

A substance could be considered a 'possible human neurotoxicant' if animal toxicity studies reveal limited evidence of direct, adverse, persistent effects on the nervous system. Indirect effects on the nervous system that clearly are secondary to systemic toxicity would not be a basis for even this category.

When animal neurotoxicity studies provide sufficient evidence of direct, adverse, persistent effects on the nervous system and are likely to be consistent across species, the substance could then be considered as a 'probable human neurotoxicant'.

Finally, fulfillment of criteria similar to those of Spencer and Schaumburg should be applied in order to categorise a substance as a 'known human neurotoxicant'.

METHODS FOR THE EVALUATION OF POTENTIAL NEUROTOXICITY

The design of neurotoxicity studies should incorporate clear objectives and produce interpretable data. The World Health Organisation (WHO, 1986) defined the objectives of neurotoxicity testing as:

- identify whether the nervous system is altered by the toxicant (detection);

- characterise nervous system alterations associated with exposure;

- ascertain whether the nervous system is the primary target for the chemical;

- determine dose- and time-effect relationships in order to establish a no-observed-adverse-effect level.

These objectives translate into a series of questions about the toxicity of a chemical that may be answered with standard toxicity tests as an initial screen and/or more specialised neurotoxicity studies.

STANDARD TOXICITY STUDIES

The term "standard toxicity studies" refers to the toxicity test guidelines of OECD (1981), EEC (1983), EPA/TSCA (1983-84), EPA/FIFRA (1991) and Japan/MAFF (1985). Relevant information concerning potential target organs including the nervous system can be obtained from acute (single dose), subacute, subchronic and chronic toxicity studies and reproduction studies.

Information on Potential Neurotoxicity from Standard Toxicity Studies

Standard toxicity studies are important in the assessment of potential neurotoxicity of a compound because these studies are conducted at relatively high doses, with different duration and routes of administration as well as with several species of animal. Metabolism and pharmacokinetic data often are developed in support of the standard toxicity studies for many compounds. Standard toxicity studies evaluate functional, behavioural and morphological endpoints for the nervous system which may give preliminary or definite indications of the neurotoxicity of xenobiotics (Steinberg, 1987).

The variety of dosing regimens for standard studies is important because some chemicals induce effects after single exposure (e.g., trimethyltin, Hagen et al,

1988; organophosphates, Abou-Donia and Lapadula, 1990) whereas others require repeated exposure (e.g., acrylamide, Bogo et al, 1981). Not only is the duration of exposure important but, for some chemicals, the exposure pattern is important in determining whether or not the material is neurotoxic. For example, exposure to hexane at 1000 ppm 24 h/d, 5 d/wk for 11 weeks produced clear and long-lasting neurotoxicity but exposure at 24,000 or 48,000 ppm for brief (10 minutes) periods 6 or 12 times per day (i.e., equivalent or higher total exposure) produced only slight effects (Pryor et al, 1982).

Contribution from Clinical Observations. Clinical observations included in standard toxicity protocols usually are obtained by cage-side monitoring of animals, as well as during handling at the time of dosing or body weight determination. Clinical observations may indicate changes in motor function (e.g., disturbances of gait, abnormal posture or muscle tone), arousal state (e.g., hyperactivity, apathy or lethargy), psychological state (stereotypes, aggression, biting, licking, self mutilation) or indications of pharmacological effects (sedation, anaesthesia).

Indirect evidence of neurotoxicity from standard toxicity studies may be suggested by the general physiological state of the test animals. The integrity of the autonomic nervous system can be assessed with observations of specific functions such as salivation, lacrimation, urination or defecation.

Clinical observation of adults or pups in standard reproduction studies may give an indication of altered neuromotor functions or arousal states that may be affected by developmental neurotoxicants. Successful mating, delivery and rearing of pups depend on normal behaviour and appropriate function of multiple organ systems including the nervous system. Also, physical and functional landmarks of pups are sensitive parameters of development. Furthermore, reproduction by F1 animals provides additional information on nervous system development.

Contribution from Morphological Examinations. Standard toxicity studies generally include gross examination of most organs and tissues, measurement of the weight of organs including the brain, and histopathological evaluation of brain, spinal cord, peripheral nerve, muscle, eyes as well as many other tissues. Thus, a broad range of cellular elements and functional entities which comprise the nervous system is evaluated by standard toxicity studies.

Histopathology in standard toxicity studies often includes examinations of brain structures that are related to specific types of behaviour. Examples of such structures include:

- the hippocampus, which is important for memory;

- components of the limbic system, which are responsible for emotion;

- the hypothalamus, which is associated with autonomic integration and control of the endocrine system.

Histopathological examination of all nervous tissue is impracticable, however representative samples of nervous tissues are considered adequate (Hirano and Llena, 1980; Thomas, 1980). Furthermore, since practically all tissues include some nervous tissue, routine microscopic sections in standard toxicity studies (e.g., skin, intestine and muscle) also contribute to the comprehensive examination of the nervous system. In addition, since the functional and structural integrity of effector organs may be altered by changes in normal control by the nervous system, histopathological examination of tissues such as muscle, exocrine glands, viscera, reproductive organs, and, in particular, sensory organs and endocrine glands may give indirect indications of neurotoxic effects.

SPECIFIC NEUROTOXICITY TEST METHODS

Primary methods for neurotoxicological evaluation are behaviour, electrophysiology, neurochemistry and neuropathology. Additional methods are available for developmental neurotoxicity tests as well as *in vitro* and other alternative test methodologies.

Behavioural Tests

Behavioural changes following exposure to a neurotoxic chemical can be sensitive indicators of disturbed function of the nervous system since they may be observed earlier and/or at doses lower than demonstrable clinical symptoms or structural lesions (Rice, 1980; Alder and Zbinden, 1977, 1983; Walsh and Chrobak, 1987; Broxup et al, 1989; Schulze and Boysen, 1991). On the other hand, there is the possibility that some structural loss associated with neurotoxicity may occur in the nervous system while the animal remains

functionally normal due to the functional reserve capacity of the nervous system (Mitchell and Tilson, 1982).

A significant limitation of behavioural tests is the lack of specificity of most these tests. Not all behavioural changes necessarily represent the specific action of a chemical on the nervous system. Many behavioural tests are affected by changes in non-neural organs (Gerber and O'Shaughnessy, 1986; Rice, 1990) as well as by dietary restriction (Albee et al, 1987), hormonal state (Robbins, 1977), fatigue (Bogo et al, 1981), motivation (Cooper, 1981) or age (Soffie and Bronchart, 1988). Some behaviours are affected by housing conditions and, thus, may not be apparent in a particular test. For example, one of the characteristic signs of trimethyltin neurotoxicity in rats is aggression which may be detected as sparring between cage-mates (Dyer and Howell, 1982); however, aggression is not seen when animals are singly housed, rather self-mutilation is observed instead (Bouldin et al, 1981).

The choice of behavioural test depends upon the purpose of the study; some tests may be simple to perform, but lack sensitivity, whereas others are much more sensitive, but are complex and time consuming. On the other hand, a complex test is not necessarily a sensitive one. For example, a comparison of the relative sensitivity of a functional observational battery (FOB), motor activity (MA) and schedule-controlled operant behaviour (SCOB) indicated that the FOB was as sensitive or more sensitive than MA or SCOB in detecting treatment-related effects (Moser and MacPhail, 1990).

There are several problems related to data analysis and interpretation of neurobehavioural screening data (Tilson and Moser, 1992). Most screening batteries consist of several tests that yield different types of data which are each analysed by different statistical methods. A significant problem is that each measure in the battery is viewed as a unique endpoint. Since there are multiple tests in the battery, some statistically significant changes might occur just by chance (Type I error). This situation is compounded by the very large amounts of data in most screening experiments. Scientific solutions to these problems are necessary if data from multitask screens are to be useful in hazard identification.

Observation of behaviour can be incorporated into standard test protocols. Typically, observation of the animal for signs such as lethargy or hyperactivity, piloerection, salivation, convulsions, ataxia, abnormal gait, and tremor is an

integral part of standard toxicity studies. Thus, any assessment of behavioural neurotoxicity should begin with simple and rapid tests to determine if behavioural effects are present and then proceed, if necessary, with tests of increasing complexity and duration.

Electrophysiological Techniques

Electrophysiological techniques measure the electrical potentials of impulse transmission in the nervous system and thus reflect the functioning of neurons. These methods offer many advantages to the neurotoxicologist. These advantages include: the ease with which most biopotentials can be measured in experimental animals; the relative ease with which the data can be analysed, quantified and standardised; and the large amount of electrophysiological data that can be collected quickly. Some techniques are also non-invasive and allow monitoring of progression and/or recovery of functional disturbance.

Electrophysiological techniques in humans are commonly used by neurophysiologists and clinical neurologists. The procedures provide a large body of information against which animal test data can be compared (Thompson and Patterson, 1974; Barber, 1980). In addition, the biological basis of electrophysiology allows these techniques to be readily applied across species, including man (Seppalainen, 1975; Rebert, 1983; Dyer, 1985; Arezzo et al, 1985; Mattsson and Albee, 1988; Mattsson et al, 1989). Most electrophysiological data are easily extrapolated to man since these data are familiar to the medical community (Mattsson and Albee, 1988). In fact, typically the degree of comparability is higher for electrophysiolgical than for most behavioural measures (Winneke, 1992).

Neurochemical Tests

Various neurochemical methods have been designed to assess mechanisms and effects of psychopharmacological agents and are used increasingly to investigate mechanisms of neurotoxicity. Unlike blood chemical parameters which are used to assess systemic organ damage, neurochemical measurements in general are performed on nerve tissue. The tissues can be parts of peripheral nerves, the entire brain of animals, distinct brain structures obtained by dissecting whole brains, slices of whole brain or of particular brain structures, neurons or glial cells cultured in vitro or isolated from brain.

Since neuronal lesions generally are limited to specific areas of the brain and often to specific types of neurons, the sensitivity of neurochemical measurements decreases with increasing volume of nerve tissue in a single assay. In contrast, the chance of missing an effect increases with decreasing total volume of tissue. In addition, the relationship between nervous system function and observations made in neural tissue extract in a test tube is somewhat tenuous because the concentration of many endogenous substances or the activity of enzymes may change rapidly after death. Further problems arise due to the tendency of the nervous system to compensate for neuronal loss, for example, by increasing turnover rate of transmitters or by up- or down-regulation of receptors (Cooper et al, 1986).

Based on the above considerations, neurochemical methods are unsuitable as routine screens for neurotoxic effects. Too many different parameters have to be measured to assess comprehensively the neurotoxic potential of a given compound. In cases where the neurotoxic mechanism of a compound is known, a few critical parameters related to its neurotoxic effect can be measured to screen rapidly structural analogues for that specific neurotoxic mechanism.

An interesting and promising approach to overcome some of the problems related to neurochemical assays is the combination of neurochemical determinations and histopathology (histochemical staining techniques). Such techniques are specific, sensitive and have the advantage of showing the topographic distribution of any findings (Krinke and Hess, 1981).

Neuropathological Methods

The morphological complexity of the nervous systems must be taken into account in the application of histopathological techniques for the assessment of the neurotoxic potential of chemicals (Altman, 1973, 1976; Palay and Chan-Palay, 1974; Gardner, 1975; Peters et al, 1976). The many factors affecting the ability to detect these responses have been reviewed (Spencer and Schaumburg, 1980 ; WHO, 1986; Krinke, 1989).

Many neurotoxicological entities in man can be readily reproduced in the rat (Spencer and Schaumburg, 1980). However, certain neuropathological effects, such as those associated with tri-o-cresyl phosphate intoxication or high doses

of lovastatin, are not always manifested in this species (Berry et al, 1988; Somkuti et al, 1988).

Age of the test animal may affect detection of a neurotoxic change, certain responses being most marked at a particular stage of development. For example, haemorrhagic encephalopathy is associated with administration of tunicamycins or corynetoxins in the immature rat (Berry and Vogel, 1982; Finnie and O'Shea, 1988). Interpretation of pathological changes may be confounded by spontaneous background lesions in subchronic and chronic studies (Eisenbrandt et al, 1990). One example is spinal radiculoneuropathy in the rat which increases in incidence and severity with age (Burek et al, 1976; Krinke, 1983); these spontaneous lesions may complicate the interpretation of neurotoxic peripheral neuropathy in this species.

The distribution, morphology and time course (spatio-temporal pattern) of a lesion are essential considerations for detection. Most lesions in nervous tissues are assessable by standard semi-quantitative pathological evaluation. Nevertheless, certain pathological changes, particularly those associated with neurodevelopmental toxicants or chemicals causing low grade peripheral neuropathy, may only be detected using morphometry (Rodier, 1979, 1990; Broxup et al, 1990) or special techniques to demonstrate subtle changes such as those found in neurites of rats exposed prenatally to lead or ethanol (Averill and Needleman, 1980; West and Hodges-Savola, 1983). Certain neuropathological effects, such as those reported with amoscanate, pyridoxine or tunicamycin, characteristically occur within a few days of exposure (Krinke et al, 1985, 1988; Finnie and O'Shea, 1988). Other compounds such as high-dosed lovastatin in dogs, clinical evidence of neurotoxicity may not develop for several weeks (Berry et al, 1988).

When chemicals initially are tested for toxicity, effective use of animals and resource involves the choice of techniques that allow thorough examination of the central and peripheral nervous system, but does not disrupt pathological examination of other organs. Thus, standard pathological methods should be incorporated in a first tier of neurotoxicity screening when there has been no prior indication of any neurotoxic effect. Several investigators have described Tier 1 methods in the rat based on immersion fixation of nervous tissue complemented with appropriate functional assessment. When available data suggest a chemical produces neurotoxicity, second tier pathological techniques, usually incorporating perfusion fixation, may be appropriate (O'Donoghue,

1989; Mattsson et al, 1990b) with supplementary specialised procedures to define particular effects and to avoid misinterpretation of artifacts.

Neuropathology should be integrated with functional studies (Tilson et al, 1979; Johnson and Richardson 1983; WHO, 1986; O'Donoghue, 1989; Mattsson et al, 1989, 1990b). While neuropathology provides clearly interpretable data and high resolution (including single neurons and axons), the methods are limited to static evaluation of discrete sections. On the other hand, functional tests evaluate dynamic system functions and populations of cells; nevertheless, these tests are somewhat limited in resolution and interpretability and are subject to masking or compensation. Adequate definition of an encephalopathy or neuropathy may be enhanced by an understanding of the clinical or functional disturbance as well as the morphologic effect (Spencer and Schaumburg, 1980; Dyck et al, 1986; Krinke 1989, Mattsson et al, 1989, 1990b).

Developmental Neurotoxicity

Many teratogens may affect the nervous system (Rodier, 1990). Therefore, examination of effects on the nervous system of developing animals is an important aspect of the assessment of developmental toxicity. Methods for the detection of developmental neurotoxicity have been described by Altman and Sudarshan (1975), Adams (1986) and WHO (1986).

Exposure of developing animals to a chemical may result in quantitatively and qualitatively different effects than exposure of adult animals. Examples include ethanol (Meyer et al, 1990; Rees et al, 1990) and the relative resistance of the weanling rat to hexane neuropathy (Howd et al, 1983).

The stage of development of the nervous system at birth varies with different species. For example, the neonatal rat is at a stage of development most similar to that of man at the beginning of the third trimester of pregnancy (Nikimura and Shiota, 1977). A number of developmental landmarks have been defined which reflect normal development (Alder and Zbinden, 1977). Some of these physical landmarks are closely connected to the development of the nervous system and their evaluation may give a first indication for an impaired nervous system development.

Parallel to physical development, functional development of animals also may indicate an impairment of neural function. Some of the functional landmarks for rats are: surface righting, negative geotaxis, disappearance of pivoting, olfactory orientation, hind-limb support, auditory startle and mid-air righting. These functional tests are easy to conduct and can be included in routine reproductive toxicity studies.

More specific measurements of behaviour, sensory and cognitive functions such as odour or taste aversion, active and passive avoidance or motor activity can be conducted to characterise particular effects. Contrary to the simple functional tests, these more specific tests in general have to be conducted with older animals and cannot easily be integrated into standard protocols.

Large test batteries have been developed for comprehensive examination of developing animals. Four major test batteries have been described (Adams, 1986):

- the Collaborative Behavioural Teratology Study Battery,
- the Cincinnati Psychoteratogenicity Screening test battery,
- the Barlow Sullivan Screening battery
- the Japanese Battery for Behavioural Teratology Screening.

Common to these test batteries is the preferential assessment of physical and functional landmarks. These landmarks seem to be more sensitive indicators than the more specific measures of behaviour, sensory and cognitive functions (Elsner et al, 1988; Elsner, 1991) and thus are valuable tools for the detection of potential developmental neurotoxicity.

Both maternal toxicity and systemic toxicity in the offspring are taken into account in the assessment of specific effects on the developing nervous system. Test substances which induce severe maternal toxicity might generate false positive results in the pups. Developmental effects, especially altered behaviour, may occur as a consequence of maternal toxicity during gestation and/or lactation rather than being a direct effect of the test substance on the offspring (Francis, 1992).

Alternative *In Vivo* (non-Mammalian) and In *Vitro Methods*

No alternative *in vivo* or *in vitro* test is widely accepted as a routine pre-screening test for neurotoxicity. The difficulty of developing any *in vitro* pre-screening test to reflect the variety of complex responses of the *in vivo* nervous system is widely recognized.

The use of alternative methods to screen for neurotoxicity is likely to be appropriate only in exceptional cases such as compounds of a chemical structure related to known neurotoxicants. *In vitro* methods can be used to obtain important additional information on the mode of action of a neurotoxicant. Consequently, such methods are unlikely to serve as a replacement for common *in vivo* testing, but are more appropriately considered as complementary to whole-animal tests.

PRACTICAL TESTING STRATEGY

ADEQUACY OF DATA

Studies that investigate the potential effects of chemicals on the nervous system should provide adequate data for risk assessment in order to protect human health in the work place and in the environment. These data should include the following:

- nature of neurotoxic effect - determine if the chemical affects the central, peripheral or autonomic nervous system and if the material causes morphological effects and/or functional changes;

- dose response - the dose response and NOEL should be established;

- transient versus persistent effects - the time course and persistence of neurological effects should be determined;

- direct/indirect effect - establish whether the neurotoxic effects are direct or secondary to some other toxic effect.

Additional information may, in some cases, be necessary for adequate hazard assessment. Examples include:

- species specificity - susceptibility of a second animal species may be investigated. Activity in a second species suggests that the chemical is more likely to be active in man;

- toxicokinetics - investigation of toxicokinetics of chemicals would further the understanding of toxic effects in animals and may provide an indication of the relevance of the animal data to man;

- mechanism of action - the mechanism for a neurotoxic effect in animals may provide a better understanding of the potential for neurotoxicity in man.

The adequacy of available toxicity data from animal studies as well as any information from human exposure should be evaluated in relation to the need for hazard assessment. The depth of an assessment of the potential risk to man depends on the use of a chemical and the risk of human exposure.

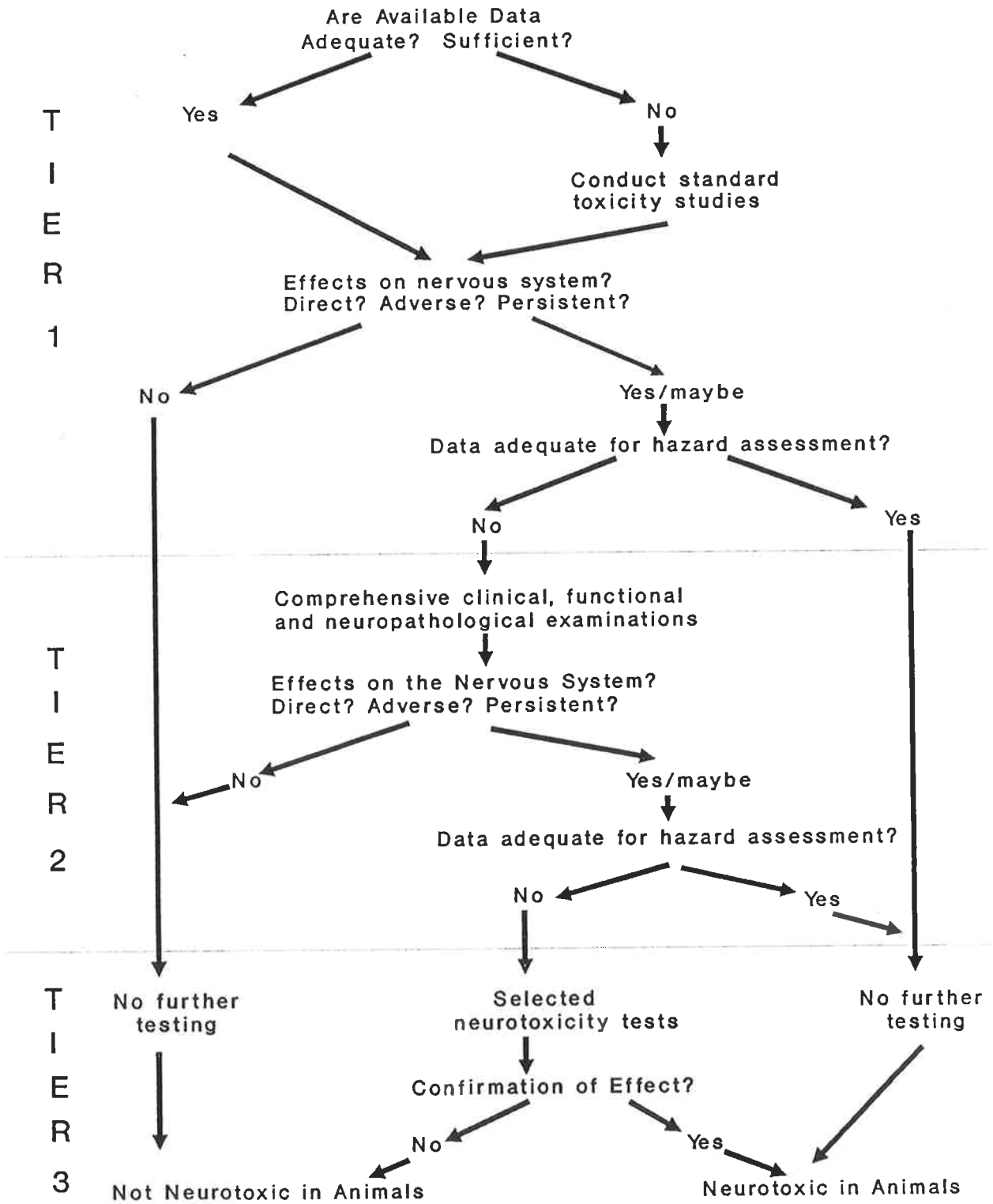
Inadequate data may need to be supplemented by additional studies in order to estimate the neurotoxic potential of a chemical and allow risk assessment.

Clinical observations and histological examination of nervous tissue are central to the identification of potential neurotoxicity and are performed in most standard toxicity studies. Enhancement of these studies by additional relevant parameters and careful evaluation of data from all of these studies would broaden the data basis for identification of a potential neurotoxic effect. The chance for detecting a potential neurotoxic effect is increased by not relying on a single species and route of administration. The evaluation of relevant data in a special report could eliminate the need for separate screening tests to identify potential neurotoxicity. Such an approach can be applied to new as well as existing chemicals and would reduce the amount of testing as well as expedite the assessment of the neurotoxic risk of chemicals.

TEST STRATEGY

A test strategy for the evaluation of the neurotoxic potential of chemicals should not be rigid, but should be determined with a case-by-case approach and depend on such factors as the structure and physical form of the chemical, systemic toxicity, and nature of any neurotoxic response. General guidance for a test strategy is schematically presented in Figure 1.

TEST STRATEGY FOR NEUROTOXICITY HAZARD IDENTIFICATION



Tier 1 - Screening Tests

Standard toxicity studies normally detect effects in the nervous system as well as effects in other body systems such as the digestive, urinary or cardiovascular systems. Standard toxicity studies are reliable Tier 1 "screening" tests for the detection of potential neurotoxicity and often are adequate for hazard assessment. Standard toxicity studies are appropriate for neurotoxicity screening when the available data do not suggest a potential for neurotoxicity.

Study design and requirements. Standard toxicity studies are conducted at relatively high dose levels with different durations and routes of administration and examine several species of animal. The number of animals required for standard studies are adequate for Tier 1 assessment of potential neurotoxic effects.

Carefully conducted and well documented clinical observations are important components of any toxicological evaluation and are essential for the assessment of potential neurotoxicity. Clinical signs should be recorded as they are observed and should include the time of onset, degree, and duration of effects. Observations should involve handling the animal and include observations inside and outside of the cage.

Parameters should include changes in skin, fur, eyes, mucous membranes, occurrence of secretions, changes in spontaneous behaviour pattern, activity, gait, muscular tone, sensory activity (e.g., response to visual, tactile or auditory stimulus), and autonomic activity (e.g., lacrimation, pupil size, piloerection, skin temperature, and unusual respiratory patterns).

A quantified measurement of grip strength or motor activity is not appropriate as a component of standard toxicity studies because of the confounding influence of systemic toxicity. Such investigations may be useful in more detailed studies on potential neurotoxicity (Tier 2) if conducted at dose levels that do not produce systemic toxicity.

Routine preservation of the brain, spinal cord, peripheral nerves and skeletal muscle in fixative should be in accordance with standard toxicity test guidelines. The brain should be included in the list of tissues to be weighed (ECETOC, 1991). Histopathological examination should include the brain and a peripheral

nerve; the spinal cord and skeletal muscle should be examined if other findings suggest a possible treatment related effect. Perfusion fixation of nervous tissue is not necessary for Tier 1 studies.

Evaluation. When there is no evidence of nervous system effects either in laboratory animals from standard toxicity tests or from experience of human exposure, and when the chemical structure of the substance suggests no concern for potential neurotoxicity, then the substance can be regarded as not neurotoxic. In this case, no further testing is warranted until any new cause of concern for the nervous system arises.

When standard toxicity studies, perhaps in conjunction with other data, provide sufficient evidence of direct, adverse, persistent effects on the nervous system, the chemical can be considered a probable human neurotoxicant. If the weight of evidence is sufficient and the data are adequate for hazard assessment, then no further testing is warranted.

When there is equivocal evidence of direct, adverse, persistent effects on the nervous system in standard toxicity studies and/or if there is a plausible structure relationship with known neurotoxicants, the chemical could be considered a possible human neurotoxicant. Tier 2 neurotoxicity studies should then be considered.

Tier 2 - Neurotoxicity Tests

Chemicals of concern are those that have structure/activity relationships to known neurotoxicants or those chemicals for which there is an indication of neurotoxic potential from standard toxicity studies or human exposure. These chemicals should be tested further if the available data are inadequate for risk assessment.

The goal of Tier 2 tests is to thoroughly evaluate the nervous system with broad, exploratory tests and to provide adequate data for risk assessment. Because of the multiplicity of possible effects in the nervous system, there is no single test method that can ensure the detection and identification of every possible change. Therefore, Tier 2 tests should evaluate major structures and functions of the nervous system. In certain circumstances Tier 2 test methods may be employed in conjunction with standard toxicity studies.

Study Design and Requirements. A subchronic study that incorporates specialised evaluations of the nervous system is recommended for Tier 2 testing since the study design allows for possible accumulation of a chemical within the body or the expression of delayed neurotoxic effects. If toxicokinetic parameters indicate rapid elimination, shorter studies may be appropriate.

The highest dose level for Tier 2 studies should challenge the nervous system, but should not be so high that there are complications from systemic toxicity.

Assessment of clinical signs should be as detailed as for the Tier 1 tests and may be supplemented by functional tests covering sensory, motor and autonomic nervous functions including testing of reflexes. These investigations normally include semi-quantitative estimation of animal locomotion. Measurement of motor activity with automated devices may provide additional but non-specific indications for potential neurotoxic effects.

Neuropathological evaluations should be performed on high-dose and control animals first and intermediate-dose animals only if necessary. Histological examination of central and peripheral nervous system should include as a minimum the following tissues: brain (several sections), spinal cord (cervical and lumbar enlargements, spinal roots, dorsal root ganglia), and peripheral nerves (e.g., sciatic and tibial nerves). Routine histopathology should include examinations of brain structures that are related to specific types of behaviour (e.g., the hippocampus, the hypothalamus, the cerebellum etc.). Additional tissues, special fixation (perfusion) and staining techniques may be necessary depending on preliminary findings. Neuropathological investigation may be supplemented by electron microscopic, morphometric or histochemical examinations of selected specimens to further clarify the diagnosis.

These neurotoxicological investigations can be incorporated into standard toxicity studies in order to provide evaluation of nervous system effects in relation to any other toxic effects of the chemical and minimise the use of additional animals. This also will help to establish whether the neurotoxic effect is direct or secondary to toxic effects in other organs.

Evaluation. Tier 2 tests will provide adequate data for risk assessment in most cases. When there is no evidence of a neurological effect in Tier 2 studies then the substance can be regarded as not neurotoxic. In this case no

further testing is warranted unless new evidence suggests concern for a potential neurological effect.

When direct, adverse, persistent effects on the nervous system are detected or confirmed with Tier 2 tests, the chemical may be considered a 'probable human neurotoxicant'. When the weight of evidence is sufficient and the data are adequate for risk assessment, no further testing is warranted.

Further specific neurotoxicity tests may be warranted in a Tier 3 characterisation phase if the effects on the nervous system are unclear or inconsistent.

Tier 3 - Characterisation of Neurotoxicity

A decision to conduct Tier 3 neurotoxicity studies depends upon factors such as the nature of the neurotoxicity, the potential of the chemical to accumulate in biological systems, the intended use of the chemical and the potential for human exposure. Neurotoxicity data from Tier 1 and Tier 2 studies should provide a majority of the information that is necessary for risk assessment. Tier 3 neurotoxicity tests may be appropriate for the advanced characterisation of known neurotoxicants in selected instances when Tier 1 and Tier 2 evaluations are inadequate for risk assessment. Investigation of the mechanism of action or toxicokinetics of the chemical may also form part of a Tier 3 evaluation.

Specific Neurotoxicity Methods. A case-by-case approach is necessary to determine the most appropriate methods for Tier 3 studies. The data from Tier 1 and Tier 2 studies should provide a basis for generation of a refined hypothesis for Tier 3 studies and guide selection of the most appropriate methodology. The following are categories of possible Tier 3 studies:

- cognitive function or other specialised behavioural methods;
- electrophysiological methods;
- neurochemical methods;
- specialised neuropathological techniques.

Evaluation. Specific hypotheses resulting from findings in Tier 1 or Tier 2 studies may not be confirmed in Tier 3 studies. If there is no additional evidence of neurotoxicity and the hypothesis is rejected, then the substance

can be regarded as not neurotoxic. In this case no further testing is warranted unless new evidence suggests concern for a potential neurological effect.

When direct, adverse, persistent effects on the nervous system are confirmed with Tier 3 tests, the chemical should be considered 'a probable human neurotoxicant'. When the weight of evidence is sufficient and the data are adequate for risk assessment, no further testing is warranted.

SPECIFIC COMMENTS ON ACUTE NEUROTOXICITY TESTS

Acute tests which typically are conducted at lethal or near-lethal dose levels are considered to be of limited value for the assessment of neurotoxicity. Substances which are not directly toxic to the nervous system can be associated with signs such as convulsions, tremors or ataxia at systemically toxic or lethal dose levels. Subacute or subchronic studies would provide more interpretable data on the nervous system because of the decreased severity of systemic toxicity as compared to acute (lethal or near-lethal) studies. We are not aware of any chemical that damages the nervous system after a single exposure that does not also have neurotoxic effects after repeated exposure although the neurotoxic effects are not necessarily the same (Yoshimura et al., 1992). Thus, emphasis on subacute or subchronic studies for the evaluation of the nervous system will provide adequate data for the protection of human health.

SPECIFIC COMMENTS ON DEVELOPMENTAL NEUROTOXICITY

Standard reproduction studies can detect disturbances in nervous system development. Thus, standard studies should incorporate an evaluation of physical and functional landmarks which assess sensory and motor function. These enhanced studies could then be considered as true screening tests for developmental neurotoxicants and in most cases would provide adequate data for hazard assessment.

When data are not adequate for hazard assessment, enhanced reproduction studies may be followed by more specific testing to further investigate and characterise the functional deficit. A case-by-case approach is necessary to determine the most appropriate methods for these special studies.

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