SECTION I

TOXICITY DATA

PRINCIPLES FOR ASSESSING TOXIC EFFECTS IN ANIMALS

by
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The main objective of toxicity studies in animals is to determine the adverse effects on biological systems. The results provide the experimental data necessary for the prevention of harmful effects in humans exposed to tested chemical compounds.

In principle, all new chemicals require toxicity testing before their large-scale manufacture is undertaken. However, the growing number of chemicals introduced annually into industrial production and the limited resources available necessitate the establishment of priorities in the selection of chemicals for testing. Essential criteria for such testing are:

- indication or suspicion of hazard to human health and type and severity of potential health effects;
- probable extent of production and use;
- potential for persistence in the environment;
- potential for accumulation in the biota and the environment; and
- type and size of population likely to be exposed.

A priority chemical for testing would rate highly with respect to all or most of these criteria (1). The above-mentioned criteria should also be considered when the extent of toxicity testing required is being proposed. In general, a higher priority for testing is linked with the larger scope of toxicity testing.

Before beginning an experimental toxicity evaluation an approximate estimation of toxicity may be useful, based on the chemical structure, the physical and chemical properties of the substance and on the known correlation of these variables with biological activity (2-5). Based on the chemical structure and toxicity of chemically related compounds, the preliminary prediction of the nature and site of toxic action may be possible. Stability of the chemical in various ambient conditions, possibility of photodecomposition and knowledge of decomposition products are essential, both for designing the toxicological experiments and for avoiding loss of the test compound during storage and preparation for

exposure. The determination of oil organic solvents and water solubilities of the chemical, its partition coefficient between these liquids and the extent of its ionization in water are helpful because they influence the absorption and distribution of a compound in living organisms. Volatility of a chemical substance will indicate the likelihood of human exposure through inhalation. The chemical impurities of the test compound samples may highly influence toxic action; therefore, the chemical purity of the tested substance should be determined before the onset of experimentation.

The fact that evaluation of chemical safety cannot be standardized is generally accepted (1,6), as is the acceptance that the design, execution and interpretation of results of a toxicity testing programme should be left to experienced investigators. However, a certain scope of toxicity testing remains which should be performed for each new chemical. The results of this toxicity assessment should allow for the following: evaluation of hazard of acute intoxication; determination of signs of acute intoxication and systems or organs most sensitive to a given chemical; determination of irritation potential; evaluation of skin sensitization; and determination of hygienic measures necessary to prevent toxic effects in humans.

Minimal Scope of Toxicity Studies

At the Institute of Occupational Health, Lodz, the minimal scope of toxicological investigations (7) includes determination of lethal doses (Table 1), evaluation of morphological alterations of inner organs, assessment of eye and skin irritation (Table 2), and sensitization (Table 3).

The injury of inner organs after a single application of a chemical may persist for several hours or days. Knowledge of the reversibility of these effects is important for their proper assessment. In order to assess the reversibility of morphological injuries induced by chemicals, the animals are killed either 48 hours or 14 days after single oral application (or inhalation exposure) on at least two dose (concentration) levels. The first dose is in the range between 0.5 - 1 LD_{50} (LC_{50}); the second one is in the range of 0.1 - 0.2 LD_{50} (LC_{50}). The scope of additional tests depends on the results of the standard investigations and properties

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(Tables 4 and 5).

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Table 1. Propos
lethal doses

Determined dose		Procedure
1.	Approximate, lowest lethal dose after oral or intraperitoneal administration	- Animals: at least rats - Number of animals: 5-10, 1 animal per one dose - Doses: geometrically increasing by progression coefficient 1.5 - Observation period: 14 days
2.	Approximate, lowest lethal dose after dermal administration	- Animals: rats or rabbits, 6-10 - 24 hours closed dermal exposure - Doses and observations as under 1
3.	Median lethal dose after oral administration (LD_{50}) and/or lethal concentration (LC_{50})[a]	- Animals: at least rats, 5 animals per dose or concentration - At least 3 doses or concentrations causing mortality rate above 0% and below 100% - Duration of inhalation exposure: 4 hours - Observation period: 14 days

[a] Calculation of LD_{50} or LC_{50}, according to Litchfield and Wilcoxon method.

Table 2. Procedure for irritation testing (7)

Performed tests
Skin:
- Single open exposure (ears or flanks) - 4 rabbits
- Single closed 24-hour exposure on abraded and intact skin (flanks) - 6 rabbits
- Repeated open exposure through 10 days (ears) - 6 rabbits
Eye:
- Single injection into the conjunctival sac - 6 rabbits

Table 3. Testing for skin sensitization

Animals: guinea pigs: 10-20 (treated; 5-10, control treated identically except for application of the test compound)
Induction procedure
Stage I: Intradermal injection into the shoulder region with Freund's adjuvant, test compound or test compound and adjuvant
Stage II: 7 days later - 48 hours topical, closed exposure to the test compound
Challenge procedure (14 days later)
- 24 hours topical, closed exposure to the test compound on flanks of animals
- Readings of the skin reactions - 24, 48 and 72 hours after challenge exposure

Table 4. Recommended compleme
systemic toxicity (7

Test	Indications
LC_{50}	- Highly a - Liquids - Aerosols (a) hig (b) sli but
Determination of the approximate lowest dose after application on the rabbit skin	- Substanc
LD_{50} in rats or rabbits after skin application	- Substances with high dermal toxicity
Determination of dermal toxicity through the rat tail	- Liquids
Morphological examination of inner organs after skin application	- Substances absorbed through the skin
Determination of the rate of skin absorption	- Substances of high dermal toxicity
LD_{50} and LC_{50} in other animal species	- Substances highly toxic for rats - Possibility of body uptake of high doses of substances in industrial environment - Large human population at risk
Functional examinations of systemic effects	- Appearance of signs of injury of the nervous system and/or respiratory system
Estimation of threshold doses or concentrations	- High toxicity - Morphological changes at low doses - Functional changes

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Table 5. Recommended complementary tests for local effects (7)

Test	Indications
Estimation of a threshold of acute irritation of rabbit skin	- Substances with high irritating potential
Estimation of a threshold of irritation of rabbit skin after repeated exposure	- Substances with high irritating potential
Estimation of irritating effect after chronic exposure	- Chronic exposure in industry
Histopathological skin examination	- Substances with high irritating potential - Lack of microscopic signs of irritation
Histopathological examination of the skin of sensitized guinea pigs	- Substances with irritating and sensitizing potential

between the dose and the magnitude of graded effect, either in an individual or in a population. Dose-response curves demonstrate the relation between dose and the proportion of individuals responding with a quantal effect. The threshold for an adverse effect of a chemical is defined as the minimum exposure level or dose that gives rise to biological changes beyond the limits of homeostatic adaptation. Determination of a threshold in acute toxicity testing is especially recommended for a substance showing irritative, neurotoxic, hepatotoxic and nephrotoxic effects at a dose or concentration not lethal to laboratory animals.

Before discussing further steps of assessment of toxic effects in animals, certain considerations essential for proper experimental design should be pointed out.

Selection of Laboratory Animals

The species of animals selected for toxicity testing should be those closely resembling humans in sensitivity to the expected toxic action and biotransformation of the chemical. In many cases of subacute or chronic toxicity tests, a biotransformation of a chemical in humans and other animals is not known prior to their onset. The laboratory mammals generally available for toxicity testing of chemicals are rodents (rat, guinea pig, mouse, hamster and gerbil), lagomorphs (rabbits), carnivores (dogs and cats) and primates (monkeys). Similar toxicity in more than one species of laboratory animal increases the predictability of toxic effects in humans. In order to provide data on a sufficient number of animals for valid statistical analyses, small laboratory rodents (usually rats) are commonly used for large-scale toxicity tests. Another animal species frequently included in toxicity studies is the dog. An advantage of these animal species is the large amount of background and historical information available for comparative purposes. To ensure uniformity, animals used for toxicity evaluation should be healthy and derived from the same colony source.

However, experiments to evaluate toxicity in special animal models might be designed which would reflect potentially hypersusceptible segments of the human population (e.g. rats with spontaneous hypertension or hamsters with cardiomyopathy).

Routes of Exposure

The route of exposure in toxicity studies should be the same as the one through which humans are likely to be exposed. Inhalation exposure lasting five hours a day, five days a week is recommended for gases and volatile chemicals. Food additives, pesticides and other chemicals likely to come into contact with food or water should be administered orally in the food or drinking-water. The route of exposure may determine the likelihood and type of biotransformation before the chemical reaches the specific sites of action. The route of entry may also affect distribution of the compound in the body. Thus, the route of exposure may influence the apparent toxicity of a tested substance.

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Examinations in Toxicity Testing

At each stage of toxicity assessment, but especially in acute and subacute experiments, animals should be observed for signs of toxicity. Not only incidence but also time of appearance of such signs should be recorded for each animal. Physical examinations should be frequently performed according to standardized screening procedures and recorded in the prepared tables.

Periodic monitoring of body weight and food consumption should be done in all studies. The food efficiency, that is, a weight gain per unit of food consumed, should be calculated. Water consumption should be measured when appropriate to the chemical's effect or when the chemical is incorporated into the drinking-water.

In general, function tests may be divided into biochemical, physiological, haematological and immunological tests. In the liver, hepatotoxic substances may cause the following types of functional disturbance or signs of injury: metabolic impairment; changes in secretory or excretory efficiency; diminished ability for detoxication; and serum enzyme alterations.

To evaluate the metabolic impairment of liver, various tests may be used including determination of serum level of protein components, assessment of blood coagulation system levels of serum lipids, amino acids in plasma and urine and levels of vitamins in the liver or plasma. The secretory and excretory functions of the liver may be assessed by measuring the conjugated bilirubin content in serum or the rate of disappearance of bromosulfonphthalein in the blood after intravenous injection. The quantity of hippuric acid in urine after benzoate loading and the extent of sulfanilamide acetylation, glucuronide conjugation or hydroxylation of hexobarbital (evaluated by sleeping time) may serve to measure the detoxifying capacity of the liver.

Determination of enzyme activity in serum frequently indicates initial cell degeneration occurring in advance of overt liver injury. Thus, serum enzyme assays are used in early diagnosis and to detect hepatotoxic effects: they are most useful in detecting acute liver dysfunction. Organ enzyme specificity and serum enzyme behaviour, depending on the intensity of liver damage and

on a particular group of applied chemi
discussed by De Bruin (8).

Standard tests of the urinary system
urine specific gravity, level of prot
glucose, uric acid, amino acid urine,
creatinine in blood and urine and uri
examinations, may be applied to eluc
but they usually lack sufficient sen
subtle changes in kidney function.
method for detection of kidney injuries seems to be the
kidney histopathological examination (9). Functional
kidney tests may be divided into three assessment groups:

- rate of glomerular filtration assessed by renal clearance of creatinine or inulin (9,10);
- rate of renal plasma flow assessed by a clearance of p-aminohippurate (PAH) (11); and
- proximal tubular secretory function assessed by maximal secretory capacity for PAH or the phenolsulfonphthalein excretion test (9,12).

Water reabsorption capacity may be evaluated by water dilution or concentration tests (9). Two valuable methods for detection of kidney injury are the determination of certain enzyme activity in urine (13-16) and the determination of specific protein components in urine (17,18).

Haematological tests usually include erythrocyte and reticulocyte counts, haematocrit, haemoglobin concentration, total and differential leucocytes count, platelet count and prothrombin time.

When such effects are expected in more detailed studies, other tests, such as methemoglobin level determination, examinations of bone marrow and imprints of lymph nodes or spleen, evaluation of red cell fragility and coagulation tests, are also performed.

Physiological tests are derived from methods elaborated in animal physiology. In toxicity testing, they are mainly used to assess effects of chemicals on the nervous, respiratory and cardiovascular systems.

The site of action frequently involved in systemic toxicity is the central nervous system. Although many

compounds have prominent effects elsewhere, the central nervous system, particularly the brain, may be affected, as demonstrated by the use of appropriate and sensitive methods. Functional assessment of the effects induced in the nervous system is much more sensitive than are the pathomorphological studies. Behavioural as well as neurophysiological methods used in toxicity assessment in animals have been reviewed in several publications (19-22).

The functional test most commonly used for the respiratory system of small rodents is a measurement of respiration frequency - the regularity of breathing rhythm and the ratio of aspiration to expiration time (23,24). More sophisticated methods may yield information on pulmonary flow resistance, compliance and tidal and minute volume (25-30). Dalhamn (31) and Dalhamn & Rohdin (32) developed methods for measuring both the rate of mucus transport and the frequency of ciliary beats in the trachea of living animals under carefully controlled conditions of temperature and humidity. The measurement of pO_2, pH and pCO_2 in arterial blood may serve as indirect tests of the lung function (33).

The evaluation of respiratory functions is most useful in studies of irritant gases or vapours. Determination of threshold concentrations for their irritant effects is essential for establishing the highest permissible concentrations in the air of the industrial environment (34).

Electrocardiography and arterial blood pressure measurements are the main methods for functional evaluation of the cardiovascular system. The ECG of animals often depends, however, on their emotional state: handling the animals, attaching the electrodes and restraining their activities may affect both the heart rate and ECG pattern. The electrocardiograms of many animal species (rat, mouse and hamster) differ from the human type in that the isoelectric ST segment is absent. On the other hand, the guinea pig, rabbit, cat and dog have patterns similar to that of the human ECG (35). Methods measure cardiac output, stroke volume, intraventricular pressure and of other parameters are also available (36).

Immunological tests are relatively rarely used in toxicological investigations. Evaluation of the immune system should take into account cellular and humoral

immunity as in the case of mice prenatally exposed to methylmercury dicyanoliamide (37). The effects of chemicals on innate and acquired immunity in exposed animals may be assessed (38,39).

Pathomorphological Examination

Functional toxicity tests do not usually cover all organ and body systems, and sometimes their results are unclear and difficult to interpret. Thus, pathomorphological investigations reveal sites of action and the nature of alteration unknown from functional tests. Morphological studies should be performed not only at the end but also at different intervals during chronic exposure and after its termination.

Subacute Toxicity and Cumulative Effects Evaluation

The main goal at this stage of toxicity assessment is the evaluation of cumulative properties and the detection of organs and systems affected by toxic action of the chemical under conditions of repeated exposure to the compound over a period up to 90 days. The assessment of cumulative properties and types of effect induced by prolonged exposure are important for establishing permissible levels of exposure for humans and are helpful in the proper design of chronic toxicity studies.

The development of an analytical method for quantitative estimation of the chemical in samples of biological tissues is highly desirable. When such a method is available, the rate of absorption from various routes of administration, the organ distribution, the rate of excretion and the biological half-life of the compound can be determined. Dosage schedules can be designed to preclude excessive accumulation, and the toxic effects observed in the animals may be related to tissue concentrations of the compound. Material accumulation of the compound due to a given level of exposure to a chemical may be assessed. Certain amounts of the above information can be gathered from studies of absorption, distribution and excretion of the test compound labelled with a suitable isotope.

This approach offers the possibility of evaluating the material accumulation of the compound in the body in

relation to a defined exposure level. Functional cumulative properties lead to the appearance of toxic effects induced by many repeated daily doses whereas a single dose of the same magnitude does not induce this effect or cause an increase of the effect with a prolongation of exposure.

The methods used for assessing functional cumulation are based on a ratio of cumulative doses, administered daily in small fractions for several weeks or months, and a single dose. However, cumulative doses and a single dose have to induce the same magnitude of effect or response in animals (40,41). Hayes (42) introduced the method which compares the median lethal dose in a single administration with a dose level of the compound which leads to mortality of 50% of animals during 90 days of exposure. The simplest method is used by Kagan (40). It assumes daily oral administration to experimental animals of a certain fraction of LD_{50} of the test compound (usually in the range 1/5-1/20 of LD_{50}) for two to four months. Based on mortality data, the median cumulative lethal dose is calculated. The functional cumulation will also depend on the magnitude of the daily dose; therefore, at least three exposure levels should be used. The ratio of the median accumulative lethal dose and median single lethal dose gives the coefficient of functional cumulation of the test substance:

$$K_{cum} = \frac{cumLD_{50}}{LD_{50}}$$

The coefficient of functional cumulation may also be calculated as a ratio of a cumulative dose and a single dose causing the same effect in 50% of experimental animals or a 50% change in the magnitude of a given effect. The coefficient of cumulation may vary with the magnitude of the daily dose administered in subacute exposure. The smaller the coefficient value obtained in this way, the higher the cumulative properties of the substance.

To evaluate toxic effects induced by the test compound during this subacute exposure and to detect organs and systems affected by the chemical, the control and exposed animals should be examined prior and during the exposure period. For assessing the reversibility of the observed toxic effects, the animals should be examined at a

specified time after termination of exposure. The scope of investigations should include physical examinations, general measurements, organ function tests and pathomorphological examinations of inner organs. The selection of applied tests depends on the data obtained in acute toxicity testing and the characteristics of toxic effects induced by chemically related compounds.

Subacute inhalation toxicity testing should be performed for the volatile compounds or substances which may appear as aerosols during the technological process of production or application. Principles of toxicity tests are similar to those used in subacute oral toxicity studies; however, special emphasis should be given to airway and lung tissue evaluation.

Chronic Toxicity Studies

The aim of chronic toxicity studies is to establish the "maximal no-observed-adverse-effect level" as well as to determine the signs of chronic intoxications and the organ and body systems affected by chronic exposure. The determination of the time sequence of effects appearing during the exposure and their reversibility during certain periods after termination of exposure is reasonable. The establishment of the biological indices sensitive to the toxic action of the compound which may be used for early diagnosis of chronic intoxication in humans is essential. Based on detected sites of chronic action of the substance, the types of disease or health alterations in humans which will contraindicate employment in industry or agriculture with such exposure may be identified.

The results of chronic toxicity studies should allow permissible levels of exposure (highest permissible concentrations) to the substance in the air of the workplace to be set. In other branches of experimental toxicology, the assessment of toxic effects evoked by chronic exposure should permit the setting of acceptable daily intakes or safe limits for the chemical in food, water and air in the general environment.

The duration of chronic exposure usually varies from 4 to 12 months but may include an animal's entire lifespan. The toxic properties and size of human population to be exposed influence not only the duration of exposure but also the extent of testing. The selected scope of animal

examinations is based on effects observed in acute and subacute experiments and knowledge of the toxic effects induced in chronic exposure by chemically related compounds.

The range of examinations includes physical examination, general measurements, blood enzyme assays and blood and/or urine constituents, organ or system function tests and pathomorphological investigations of inner organs. Examinations should be performed prior to the exposures (usually only some of them), several times during the exposure and at certain intervals after termination of exposure. Most of the chronic toxicity studies are carried out on rats, but the use of nonrodent animal species is recommended. Levels of exposure are chosen according to dose-effect and/or dose-response relationships found in acute and subacute experiments, considering also the cumulative properties of the test compound. Three or more levels of exposure are recommended. The highest exposure levels should produce evident chronic intoxication of animals. The lowest is not expected to induce adverse effects. The other levels are in between. The control animals must be included in every subacute or chronic experiment.

The determination of organ distribution and the route and rate of excretion of the compound is desirable to relate the toxic effects observed in the animals to tissue, urine or faeces concentration. Biotransformation of the compound, including the proportion of its main excreted metabolites, should be determined for animals used in toxicity testing. This consideration may be important when extrapolating the obtained data to humans.

Studies of Specific Effects

Evaluation of toxic effects of a systemic and local nature in the course of acute, subacute and chronic exposure does not permit the assessment of all hazards related to exposure to the test substance. Additional studies must be made to reveal the possibilities of toxic effects of a different character.

Pollution of air and water contamination of food usually involves simultaneous exposure to more than one harmful chemical agent. The toxic action of one of them may sum up, potentiate or antagonize the toxicity of the

other(s). To elucidate the nature of interaction between two chemicals and the living organism, tests must be designed to provide such information. Other tests include embryotoxic and teratogenic tests, reproduction tests and mutagenic tests.

The objective of toxicological testing is not only to provide data for protection of the population to be directly exposed but also to protect future generations; therefore, the above tests are strongly recommended. Mutagenic activity of the substance is highly correlated with its carcinogenic properties. The need for information of this type depends upon the scale of production, size and type of population to be exposed and physicochemical properties of the compounds.

The necessity for performing carcinogenicity tests depends on the results of mutagenic tests and the chemical structure of the test compound. The establishment of the carcinogenic potential of the compound under study is desirable.

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