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Acute Toxicity Tests
LD50 (LC50) Determinations
and Alternatives

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FOREWORD

Over the past few years the European Chemical Industry Ecology and Toxicology Centre (ECETOC) has published a number of Monographs in which it has attempted to clarify, and express its views on, some of the more important problems in toxicology. This Monograph is a further addition to the series.

The results of animal studies are relied upon widely to ensure that chemicals can be safely produced and used. At the time of writing, a wide-ranging debate about the use of animals in such evaluations is going on in the public, scientific and regulatory domains. The chemical industry and its employees are part of the public domain and have a vital interest in the other two. One area in which the use of animals is of most concern is in assessing the acute toxicity of chemicals, in particular the determination of LD_{50} and LC_{50}. As an organisation of scientists in W. European chemical companies, ECETOC is, with this Monograph, contributing to the debate by surveying the possibilities for reducing the number of animals, or eliminating their use entirely, in testing for acute toxicity.

In introducing this Monograph to its readers I wish to make an important point which is not widely appreciated, i.e. that there has been a steady reduction in the number of animals used for many years now. This can be seen from the following table which gives the percentage reduction in the use of common animal species for all types of biomedical research, including toxicology, in 3 major industrialised countries :

	USA (DHSS, 1980) 1968 - 1978	W. Germany (BPI, 1982) 1977 - 1979	Switzerland (Anon, 1984) 1977 - 1983
Mice	-41	-17	-35
Rats and hamsters	-29	-14	-42
Rabbits	-13	-21	-44

There is no doubt that some part of this change results from the use of modified or new techniques made possible by advances in science. This is a trend which all concerned with both animal and human welfare will wish to see continued.

Dr. H.J. Heller
Chairman of ECETOC Board
Director of Ciba-Geigy

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A. INTRODUCTION

It is generally accepted that chemicals and their mixtures and formulations require an appraisal of their toxic potential so that they may be handled, transported and used as safely as possible. Acute toxicity forms an integral part of such appraisals and information on it is required by many of the present laws and regulations which are concerned with the control of chemicals.

Scientists involved in toxicology, the regulatory authorities and the general public, are becoming increasingly concerned about the numbers of animals being used in the evaluation of the safety of chemicals. This arises from a wider concern about the need to use animals for medical and biological research in general, together with a feeling that toxicity tests required by regulatory bodies may cause unnecessary suffering to animals and be wasteful of animal life.

Although a variety of important information can be derived from a well-designed acute toxicity study, an estimate of the lethal dose (i.e. an LD_{50} or an LC_{50} value) is often specifically required by regulations. Over a number of years the use of LD_{50} (LC_{50}) as the basis for classifying acute toxic hazard has led many to assume that these values are synonymous with acute toxicity. Thus the acute toxicity of a material is often described simply as a number, the LD_{50} (LC_{50}). This is often quoted as a point value with a confidence interval, i.e. the upper and lower limits which are likely to include the true value. Large numbers of animals may be required in order to derive a narrow confidence interval. This situation has been criticised on scientific and ethical grounds. Scientifically, LD_{50} and LC_{50} figures do not represent all of the important facets of acute toxicity and they ascribe a spurious accuracy to a number which is recognised to be of limited value. The determination of LD_{50} and LC_{50} values also results in a wasteful use of animals. As a consequence, various organisations and regulatory agencies have begun to reappraise their position regarding the necessity for LD_{50}(LC_{50})s and thence the recommended experimental protocols for determining them (Dutch Health Council, 1983; VCI, 1983; Bass et al., 1983; BTS, 1984; EPA, 1984; Dayan et al., 1984). In addition, there has been an increased emphasis on research into methods which may help to determine acute toxicity without the need to use live animals.

An ECETOC Task Force was therefore set up to examine these problems and was given the following Terms of Reference :

1. To describe the purpose of acute toxicity tests and the utility of LD_{50}(LC_{50}) values, a) in regulatory notifications, b) as a contribution to the scientific understanding of the toxicology of a chemical, and c) in providing information useful for protecting human health.

2. To assess with what effectiveness the OECD guidelines for these tests meet the above purposes.

3. To state, if possible, how the purposes could be met using fewer animals in the test.

4. To state, if possible, how the purposes would be met by alternative methods in which whole animals are not used.

5. To consider whether the results from alternative tests would need to be correlated with the historical data from the conventional methods and, if so, to recommend how this could be done.

The Task Force has defined some of the terms used in this report as follows.

Toxicity : the inherent property of a substance to cause an adverse biological effect.

Acute toxicity: adverse biological effect or effects, which occur within a short period of time after a short-term exposure, i.e. a single oral administration, or a dermal or inhalation exposure not exceeding 24 hours. Other routes of exposure may also be relevant, e.g. intraperitoneal (i.p.), subcutaneous (s.c.), intramuscular (i.m.) and intravenous (i.v.).

LD_{50} value (median lethal dose) : a statistically-derived dose which, when administered in an acute toxicity test, is expected to cause death in 50% of the treated animals in a given period.

LC_{50} value (median lethal concentration) : a statistically-derived concentration which over a defined period of exposure, is expected to cause death in 50% of the animals within a given period. This term is applicable to experiments in which the inhalation route of exposure is used and to aquatic toxicity testing.

Acute toxicity test : an experiment which provides information on acute toxicity over a range of dosage or concentration levels. This may include information on the lethal dose, the organs, tissues and functions affected and the time to onset, duration and severity of effects.

LD_{50} (LC_{50}) test : an experiment which aims at determining an LD_{50} (LC_{50}) value and in which only the mortality incidence is recorded.

Precision : the degree to which the individual observations cluster around the mean value. The closer the individual observations are to the mean value, the higher is the precision. The mean value is not necessarily the same as the absolute or true value. Precision is usually expressed by the 95% confidence limit.

Accuracy : the closeness of an observed or calculated quantity to the defined or true value.

Reproducibility : the closeness of agreement between the results obtained when a defined procedure is applied several times under prescribed conditions.

In this report all types of chemicals are considered for which acute toxicity data are required (i.e. industrial chemicals, pesticides, pharmaceuticals, food additives). When answering the questions raised by the Terms of Reference, different aspects of acute toxicity have been emphasised depending on the type of chemical and its use. This document is concerned mainly with mammalian toxicity but the arguments and proposals can be applied to other species, e.g. birds and fish, in which the acute toxicity of chemicals may also be assessed.

B. PURPOSES OF ACUTE TOXICITY TESTING

It is important to emphasise that $LD_{50}(LC_{50})$ and acute toxicity tests are not synonymous. Acute toxicity tests aim to determine all of the toxicological consequences of a short-term exposure. The $LD_{50}(LC_{50})$, which provides information only on the lethal dose of a chemical, is just one of the parameters which may be derived from a well-designed acute toxicity test. An LD_{50} (LC_{50}) is statistically derived from the mortality incidence in an individual experiment but it is not an absolute value (Schütz, 1969) since many factors influence its reproducibility (Zbinden and Flury-Roversi, 1981). Even when used as an index of the lethal dose, the LD_{50} (LC_{50}) value should not be considered in isolation since the shape of the dose-response curve is of equal importance. The fact remains that the LD_{50} (LC_{50}) is widely used as a numerical index of overall acute toxicity. The reasons for this are mainly historical and result from various attempts to categorise chemicals according to their toxic hazard. Such categorisations have been based on numerical data for simplicity and ease of transfer of information since in many instances they are intended for use by personnel who handle chemicals but may have a limited understanding of toxicology.

Acute toxicity testing provides information that may be used to meet the requirements of regulatory authorities, to further the toxicological knowledge of a chemical or to provide data relevant to the protection of human health. The various types of information which may be derived from such a test are listed in Table 1. The need to obtain certain data depends on the material (e.g. it may be a consumer product, a pesticide, a pharmaceutical or an industrial chemical) and/or the foreseeable hazard associated with its production, transport, application, specific use, etc. .

Most of the information listed in Table 1 is necessary to make a full assessment of the acute toxicity profile of a chemical and would be relevant to recommendations regarding human health protection (ECETOC, 1984). To ensure the most accurate prediction of a possible hazard to man, the design of the experiment should take into account the substance's physical and chemical properties, its intended use and the possibilities of accidental exposure. Acute toxicity tests with some materials (e.g. solid polymers) are irrelevant by virtue of their physical properties and testing should not be required in these cases.

TABLE 1

Information which may be derived from an acute toxicity study

- Signs of intoxication
- Time to onset, and duration, of toxic effects
- Reversibility of toxic effects
- Occurrence of delayed toxic effects
- Dose/toxic-response relationships
- Sex-specific effects
- Organs, tissues and functions affected
- Mode of toxic action
- Highest non-toxic, lowest toxic, and lowest lethal dose
- Median lethal dose/concentration with confidence limits, i.e. LD_{50} (LC_{50})

The route of exposure may influence the toxic response and thus its selection is of paramount importance in designing studies. Parenteral routes such as intravenous administration are directly relevant only for pharmaceuticals, whereas oral, inhalation and dermal routes are more relevant for industrial chemicals. It is often important to have acute toxicity information from several routes of exposure since absorption may change significantly with the route of administration of a substance.

An essential part of any well-designed acute toxicity test is the observation of signs of intoxication. An awareness of these signs, which may appear at sub-lethal doses, is often far more important than the knowledge of a lethal dose. In addition, it may be valuable to examine more than one animal species since there may be significant differences in absorption, excretion, metabolism and toxicity between species which would be very relevant to the assessment of the hazard to man.

In Table 2 are listed the purposes for which LD_{50} (LC_{50}) values are currently used in relation to regulations, general toxicological evaluation and health protection. The major application of LD_{50}(LC_{50})s is for the classification of substances according to their toxicity. There are a multitude of laws and regulations worldwide which are based to some degree on these numerical values and it must be realised that their substitution by approximate, semi-quantitative or qualitative terms would produce many administrative

problems. $LD_{50}(LC_{50})$s have applications in a limited number of other areas, e.g. calculating a therapeutic index, determining bioavailability or developing antidotes.

TABLE 2

Current Uses of LD_{50} (LC_{50})s

	By Regulatory Authorities	For Toxicological Evaluation	For Health Protection
1. Hazard evaluation; prediction of toxic effects in man and animals.	x	x	x
2. Registration and classification (for labelling, packaging, transport, etc. purposes).	x		x
3. Calculation of the lethal dose of mixtures for classification.	x		
4. Standardisation of a biological reagent or complex mixture which cannot be chemically analysed.			x
5. Comparison of structurally-related compounds.		x	x
6. Determination of a therapeutic index.		x	
7. Development of antidotes.		x	x
8. Determination of the hazard of combined exposure (e.g. synergism or antagonism).		x	x
9. Evaluation of toxic hazard for especially susceptible populations (e.g. according to sex, age, etc.).		x	x
10. Provision of information for the design of repeated-dose toxicity tests.		x	
11. Provision of information on bioavailability, e.g. from different routes of exposure.		x	x

Objections have been made to the use of $LD_{50}(LC_{50})$s for all of the purposes listed in Table 2 (Sperling, 1976; Zbinden and Flury-Roversi, 1981).The TF also disagreed with many of the current applications of the LD_{50} (LC_{50}) values and its opinion on when a knowledge of the lethal dose is needed, and to what precision, is given in the following section.

1. The Need for a Knowledge of the Lethal Dose or an LD_{50} (LC_{50})

1.1. Hazard evaluation. The complete toxicological evaluation of a substance must include an examination of its lethal dose or concentration as well as of all other aspects of its acute toxicity. A knowledge of toxic effects at dosage levels below the lethal dose is necessary for determining hazard to man. When a deliberate or adventitious over-exposure cannot be ruled out then it is important to know the probable lethal dose, the affected organs or tissues and associated signs of toxicity. However, for this purpose it is seldom necessary to have a precise LD_{50} (LC_{50}). In the

evaluation of hazard to man it is irrelevant to determine the LD_{50} or LC_{50} if they exceed 2000 mg/kgbw, or 5 mg/l for 4 hours, respectively, since such high exposures are unlikely to occur. However, any incidence of mortality at these upper limit dose levels may constitute useful information.

1.2. Classification. The use of LD_{50} (LC_{50})s for classification is based more on expediency and regulatory demands than on science. Other parameters of toxicity could be used as a basis for such classification (cf. Chapter D.2.2). It must be recognised that the LD_{50} (LC_{50}) value has become an integral part of most regulatory systems, and the difficulty and length of time required to achieve any change in this situation should not be underestimated. However, in the case of the registration of pharmaceuticals a number of regulatory authorities have recently relinquished their requirement for an LD_{50} (LC_{50}) value (EEC, 1984-c; UK/CSM, 1984).

1.3. Mixtures. Attempts have been made to minimise the experimental determination of the LD_{50}(LC_{50}) of a large number of mixtures made from relatively small numbers of components. These proposals allow mixtures of chemicals to be classified on the basis of a calculated value derived from the LD_{50}(LC_{50})s of the components of the final formulation or preparation (Martins et al.,1984; EEC, 1984-b). The calculation system on which these proposals are based would have to be changed if LD_{50}(LC_{50})s were not available in the future because the classification of the individual components would then be based on a less precise indication of a lethal dose, or on alternative toxic effects (cf. Chapter D.2.2).

1.4. Biological standardisation. The LD_{50} test was originally developed to compare the activity of complex medicinal preparations derived from biological materials with the activity of a pure standard (Trevan, 1927). This use is of decreasing importance and is rarely necessary because of progress in modern analytical techniques.

1.5. Comparison of structurally-related compounds. Acute toxicity may be used for comparison and grading of substances, especially within a given chemical class. For such a grading, numerical LD_{50}(LC_{50})s may be used but are not essential.

1.6. Therapeutic index. This is defined as the ratio of the median effect dose and the $LD_{50}(LC_{50})$ value (Ehrlich and Hatta,1910; Schneiderman et al.,1964). If this is required, then it is necessary to determine the $LD_{50}(LC_{50})$. The value of the therapeutic index has been questioned (Neubert, 1975; Zbinden, 1983).

1.7. Antidotes. The development of antidotes should be based on a knowledge of the mechanisms of toxic action or on typical signs of intoxication. The effects of antidotal treatment are usually evident at below the lethal dose range but the ultimate test of a successful treatment is prevention of death. The determination of an $LD_{50}(LC_{50})$ should seldom be necessary to confirm this.

1.8. Combined exposure. The assessment of the hazard from the combined exposure to more than one substance follows the same principles are applied to an exposure to a single substance. Unless the modes of toxic action of the substances, alone or in combination, are known, a knowledge of the lethal dose after combined exposure is usually necessary to determine the possible hazard .In most cases, this need not involve the determination of an $LD_{50}(LC_{50})$.

1.9. Especially susceptible populations. The acute toxicity of a substance may be influenced by factors such as sex and age. An experiment to identify major differences in susceptibility to the toxic effects of a substance due to such factors would seldom need a knowledge of the lethal dose or the determination of an LD_{50} (LC_{50}).

1.10 Dose setting. Acute toxicity data are of value in setting the dosage levels in repeated-dose experiments designed to investigate toxic effects other than death. The selection of dose levels is rarely a precise exercise and although an indication of the approximate lethal-dose range following acute exposure may be useful an $LD_{50}(LC_{50})$ is not required.

1.11 Bioavailability. The lethal dose of a chemical depends on its bioavailability. Information on the lethal dose with different routes of exposure may give some information about bioavailability but this is not the preferred approach. In very few cases, e.g. in the absence of suitable

analytical methods, there may be a justification for using the lethal dose to compare absorptions under different routes of administration.

It is concluded from the above discussion that many of the applications of $LD_{50}(LC_{50})$s could be satisfied without determining a lethal dose with a high degree of precision. The views of the Task Force on the toxicological necessity for data on mortality and LD_{50} (LC_{50}) are summarised in Table 3.

TABLE 3

Toxicological Necessity for Data on Lethal Dose* and $LD_{50}(LC_{50})$.

Purpose	Need for Mortality Data	Need for LD50(LC50)s
1. Hazard evaluation, and prediction of toxic effects in man and animals	Usually	Seldom
2. Registration & classification (for labelling, packaging, transport, etc. purposes)	Usually	Never
3. Calculation of the lethal dose of mixtures for classification	Usually	Never
4. Standardisation of a biological reagent or complex mixture which cannot be chemically analysed	Always	Always
5. Comparison of structurally-related compounds	Usually	Seldom
6. Determination of a therapeutic index	Always	Always
7. Development of antidotes	Usually	Seldom
8. Determination of the hazard of combined exposure (e.g. synergism or antagonism)	Usually	Seldom
9. Evaluation of toxic hazard for especially susceptible populations (sex, age, etc.)	Seldom	Seldom
10. Provision of information for the design of repeated-dose toxicity tests	Seldom	Never
11. Provision of information on bioavailability, e.g. from different routes of exposure	Seldom	Never

* Data on lethal dose refer to limit, etc. tests (see Chapter D).

2. Required Precision of LD_{50} (LC_{50}) Data .

The $LD_{50}(LC_{50})$ is by definition derived from a statistical analysis of the dose-related mortality incidence. Perhaps as a result of its widely accepted use and its statistical definition, the $LD_{50}(LC_{50})$ value is erroneously considered to be a fundamental characteristic of a chemical and to have the

status of a physical constant (Schütz, 1969). An $LD_{50}(LC_{50})$ is not an absolute value and therefore its accuracy cannot be quoted although the level of precision of an individual determination may be stated.

It is not intended, nor is it feasible, to advise on the precision required of an $LD_{50}(LC_{50})$ in an individual study. Various authors have shown that the $LD_{50}(LC_{50})$ itself depends on a host of environmental and experimental conditions (Griffith, 1964; Weil et al., 1966; Weil and Wright, 1967; Schütz, 1969; Hunter et al., 1979; Lingk, 1982). Indeed, even when experiments are repeated with the same compound in the same laboratory under highly standardised conditions there may be considerable variation in the resulting $LD_{50}(LC_{50})$s (Weil et al., 1966). A high precision, indicated by a low 95% confidence interval, relates only to that experiment from which the $LD_{50}(LC_{50})$ was derived, and it does not increase the probability that the $LD_{50}(LC_{50})$ from any other experiment will be identical or even similar. A highly precise $LD_{50}(LC_{50})$ does not infer that it is accurate (Hunter et al., 1979; Lingk, 1982).

The majority of existing regulations (OECD, 1981; EEC, 1984-a) require a statement of the 95% confidence limits of a determination, i.e. the range of values within which the $LD_{50}(LC_{50})$ would fall 95% of the time if it were re-estimated in an experiment repeated under identical conditions. It is important to recognise that the existence of a calculated 95% confidence limit does not necessarily indicate that the range of possible values around a mean value will be narrow; indeed the range may be very wide. The width of this range and therefore the precision of the $LD_{50}(LC_{50})$ is influenced by the number of dose levels and animals per group and by the method of statistical analysis (Chanter and Heywood, 1982).

The need to revise the current uses of $LD_{50}(LC_{50})$ was addressed in the previous section. However, until the present circumstances are altered $LD_{50}(LC_{50})$s will continue to be required especially for regulatory purposes. In these instances the precision to which the $LD_{50}(LC_{50})$ is measured should be based on a consideration of the following factors :

a) the uses to which the chemical will be put;
b) its known biological/toxicological activity;
c) its physical/chemical properties;
d) the likelihood of human exposure;

e) any information concerning past or future toxicological assessments.

For pharmaceuticals, a high degree of precision may be needed when the difference between therapeutic and toxic doses is narrow. This may be contrasted with some industrial chemicals where the likelihood of exposure to lethal amounts is limited (e.g. for intermediates in a closed system). The higher the biological/toxicological or chemical activity of the compound, the more thorough should be the toxicological characterisation. The more likely the exposure of humans to toxic quantities (depending on the physical properties of the chemical) the more precise the $LD_{50}(LC_{50})$ determination may need to be. Depending on the amount and nature of toxicological data already available, or to be generated by future studies, the $LD_{50}(LC_{50})$ may need to be correspondingly more or less precise.

In general, the use of large numbers of animals to determine an $LD_{50}(LC_{50})$ with high precision is rarely necessary but the use of other means to increase the precision of the $LD_{50}(LC_{50})$ is recommended e.g. the choice of the most appropriate experimental design and statistical treatment of data (Chanter and Heywood, 1982).

C. REQUIREMENTS AND EFFECTIVENESS OF OECD TEST GUIDELINES

1. Requirements

Before a chemical can be marketed and used it may have to be tested for its acute toxic potential to satisfy the requirements of various registration authorities. The OECD test guidelines (OECD, 1981; Appendix 1) represent the harmonisation of numerous previously-existing guidelines, and they give an outline of how acute toxicity assessments should be conducted (Alder et al., 1981; Hayes, 1982). Full acceptance of the OECD test guidelines by all countries is essential if unnecessary duplication or repetition of studies is to be avoided.

2. Effectiveness

The OECD guidelines (Appendix 1) are essentially a set of outline protocols and the purposes of an acute toxicity test as detailed in the previous chapter of this report would be effectively met if the guidelines were followed. Depending on the exact purpose of the test, some modifications to the basic OECD protocol may be required. As stated earlier, the outcome of

toxicity studies may be influenced by many factors (Zbinden and Fluri-Roversi, 1981) some of which are listed in Table 4. The OECD test guidelines are flexible with respect to a number of these factors.

TABLE 4

Factors Known to Influence the Outcome of Acute Toxicity Tests

Species	Diet
Strain	Season of year
Age	Vehicle
Sex	Vehicle volume
Weight	Formulation characteristics
Health	Time of dosing
Stress	Duration of dosing procedure
Husbandry (temperature, humidity, etc.)	Fasting

It has been suggested that a more precise definition of age, species and strain, vehicle, etc., may lead to an increased reproducibility of experimental data, but this does not take account of the true size of the problem. It is impossible to eliminate all factors which contribute to the variability between any two biological experiments, and overspecification in guidelines must be resisted as it may reinforce an unjustified reliance on the precision of the experimental data. It may also lead to the elimination of toxicological judgement and expertise which are essential to the design of all studies.

The need to avoid overspecification in guidelines can be illustrated by the following two examples. Firstly, species differences in the toxicity of chemicals are well documented and flexibility permitting the use of alternative species to those quoted in the guidelines is essential. The choice of species may be influenced by, for example, technical considerations, the availability of data suggesting that other species are more relevant to man, or the need to correlate results with those from other studies in the chosen species. The second example concerns a specific requirement of the OECD guidelines for the use of fasted animals in oral acute toxicity studies. This requirement is based on the belief that fasting reduces the volume of, and variation in, the stomach contents. Thus, fasting may result in an increased bioavailability of the test chemical and a more

uniform absorption (Paget, 1970; Brown, 1983). Consequently, fasted animals are usually more susceptible to the systemic toxic action and to the local effects of a chemical on the gastric mucosa. However, equal variations in the stomach contents, and similar toxicity, in fed and fasted rodents have been reported (Schütz, 1969; Kast and Nishikawa, 1981). In addition, acute toxicity information from fasted animals may be less easily related to longer-term studies on unfasted animals, and fasting does not reflect the most common condition of exposure in man. Overall, the Task Force considers that the OECD guidelines should be changed to become flexible on the issue of fasting animals prior to oral dosing.

Whether the OECD guidelines represent an adequate protocol for determining the acute toxic potential of a chemical while minimising the number of animals is discussed in Chapter D. The Task Force endorses the approach of the OECD expert group on short-term toxicology in retaining the level of flexibility expressed in OECD guidelines such that "... the examination of the toxicity of a chemical substance is conducted as a scientific exercise rather than as a set of stereotyped tests to be conducted in a routine". This implies an absolute requirement that the exact experimental details of all studies are fully and accurately recorded (OECD, 1981).

D. ALTERNATIVE PROTOCOLS WITH FEWER ANIMALS

The majority of regulatory guidelines, including those of the OECD, demand an $LD_{50}(LC_{50})$ and its 95% confidence limits in addition to the range of qualitative information (Table 2) necessary to fully describe the acute toxicity of a substance. Various publications refer to methods in which fewer animals than recommended by OECD guidelines are used to provide comprehensive acute toxicity data, but in which the lethal dose may not be precisely determined. In some cases the methods still permit the calculation of an $LD_{50}(LC_{50})$. These methods are discussed below in relation to rodents only and with an emphasis on economy of animal numbers in determining the lethal dose range. In certain guidelines there is already some flexibility for using fewer animals with less commonly used species, e.g. dogs (Japan/MHW, 1984).

1. Typical Regulatory Acute Toxicity Protocols

These protocols demand the use of groups of 10 animals (5 of each sex) which are administered pre-determined doses of a substance (cf. Appendices 1 and

2). A minimum of three dose levels (at least 30 animals in total) is generally required, but 50 animals are most commonly used in the determination of an $LD_{50}(LC_{50})$. This is usually calculated from the probit log plot of those doses producing greater than 0 and less than 100% mortality. These data are also used to calculate the slope of the dose-response curve and the confidence limit. If nothing is known about the lethal dose range of the test material it is normally necessary to perform a range-finding test first in order to choose doses that will result in group mortalities greater than 0 and less than 100%. For substances of low acute toxicity some guidelines permit an alternative to the calculation of an $LD_{50}(LC_{50})$ value, i.e. a limit test performed at a specified single dose (cf. Appendix 1 and 2.2 below).

2. Alternatives to Typical Regulatory Protocols (see Table 5)

2.1. The range finding test. A common approach to the determination of an approximate lethal dose (Smyth and Carpenter, 1944) is the use of 1 male and 1 female animal to which the test chemical is administered simultaneously at several standard dose intervals, e.g. 40, 200, 1000 and 5000 mg/kgbw in an oral dosing study. With such widely-spaced doses a common outcome is for both animals to die at one dose, e.g. 1000 mg/kgbw, and for all lower-dosed animals to survive. In this case the dose range used to determine the LD_{50} is 200-1000 mg/kgbw. This study would need about 8-10 animals and gives a clear indication of the order of magnitude of the lethal dose.

2.2. The limit test (fixed-dose procedure). This test is normally performed with 5 male and 5 female animals. All receive the same dose, e.g. 5000 mg/kgbw as recommended by the OECD for oral administration (cf. Appendix 1). If none of the animals dies the material can be regarded as having an LD_{50} of greater than 5000 mg/kgbw. Other limit doses are recommended for other routes of exposure. Limit doses which are specified in guidelines do not always correlate with doses which define a toxicity class (OECD, 1984).

Where there is no information concerning the lethal dose range of a substance it is inappropriate to proceed with an upper limit dose study without first of all conducting a range-finding test.

TABLE 5

Summary of Acute Toxicity Test Protocols

Protocol	No. of Animals	No. of Dose Groups	Dosing		LD_{50} (LC_{50})	
			Simultaneous	Sequential	Value	Range
OECD	30-50	3-5	+		+	
Limit Dose	10	1	+			+
Fixed-dose Procedure	10-20	1-2		+		+
Range-Finding	8-10	4-5	+			+
Modified LD_{50}(LC_{50}) Tests	12-30	3-6	depends on specificic protocol		+	
Up-and-Down	6-10*	6-10		+	+	

* single sex figures only

In practice the limit-dose approach can be used at any pre-determined dose if the purpose of the study is purely to identify a hazard category within a classification system. Depending on the outcome with the first dose-group, a subsequent dose-group, moved up or down one "category" or "class", could give further definition in the case of an equivocal outcome. This approach would normally require no more than 20 animals, and is one of the possibilities for a fixed-dose-procedure protocol (FRAME, 1977; VCI, 1983). Fixed-dose procedures which are based on toxicological endpoints other than mortality have also been proposed (BTS, 1984).

2.3. Modified $LD_{50}(LC_{50})$ test. Several modified $LD_{50}(LC_{50})$ procedures have been recommended (Schütz and Fuchs, 1982; Lorke, 1983; Depass et al.,1984), all based on the use of fewer animals per dose group. The authors indicate that the use of an increased number of dose groups but with fewer animals (1 or 2 per sex, per group) leads to an $LD_{50}(LC_{50})$ of similar precision to that obtained in a typical regulatory protocol (cf. Chapter D.1).

Weil (1983) has shown that the slope of the dose-response curve can also be derived from such data in a procedure which would probably require 16-20 animals. An alternative to probit analysis for calculating an LD_{50} (LC_{50}) is the moving averages method which is particularly applicable to this modified type of study as it also uses data from groups with 0 and 100% mortality (Thompson, 1947).

2.4. "Up and Down" procedure. A sequential dosing procedure described by Bruce (1984) requires a total of 6 to 10 animals of each sex. One animal is given a single dose in the region of the anticipated $LD_{50}(LC_{50})$ and is observed for 48 hours. If it survives, the next animal is given a higher dose, increased by a factor of 1.3 (a proportional reduction of the original dose is given if the first animal dies). Once a reversal of the initial outcome has been obtained the dose for each successive animal is adjusted up or down depending on the outcome for the previous animal. After the first reversal of the initial result Bruce recommends that 5 more animals of each sex are dosed to determine a precise $LD_{50}(LC_{50})$.

3. Comments on Alternative Protocols with Fewer Animals

In general, all of the above protocols or methods (Table 5) lead to a reduction in the number of animals used in comparison to typical regulatory

protocols but they still allow the examination of dose-related acute toxicity effects such as time to onset, duration and reversal of clinical signs. However, where a single dose or widely-spaced doses are used these effects may be less well characterised. These alternative protocols differ from regulatory protocols primarily in that they either fail to generate an $LD_{50}(LC_{50})$ at all, generate a figure of lower precision, or simply indicate the order of magnitude of the lethal dose.

3.1. Range-finding. Usually an approximate $LD_{50}(LC_{50})$ is obtained, but in certain cases the results may be adequate to enable the calculation of an $LD_{50}(LC_{50})$ with confidence intervals. The slope of the dose-response curve can also be obtained.

3.2. Limit or fixed dose-test. These provide information on the approximate lethal dose range or toxicity class. An $LD_{50}(LC_{50})$ cannot be estimated.

3.3. Modified $LD_{50}(LC_{50})$ test and "up and down" procedure. The precision of the estimated $LD_{50}(LC_{50})$ may be lower than that obtained with more animals although it can be argued that the difference is smaller than that produced by other factors, e.g. differences between laboratories, strains, species, etc. Schütz and Fuchs (1982) have stated that their protocol achieves additional economies in numbers of animals if the selection of further doses is based on the outcome of the initial dose, and the experiment is continued with sequential dose selection.

3.4. General comments. A number of authors (Schütz and Fuchs, 1982; Bruce, 1984; Tattersall, 1982; Depass et al.,1984) have stressed that differences in the $LD_{50}(LC_{50})$ between the sexes are few and, if real, are usually less than 20%. Therefore, a reduction in the number of animals can be achieved by testing in one sex only. In this case a small number of animals of the opposite sex could then be given an appropriate dose to determine whether there were any gross differences in sensitivity.

Protocols involving sequential dosing procedures are more time-consuming and complicated to perform and may present logistical problems in the laboratory. In some protocols it is recommended to choose the next dose rather soon after administration of the first. Doses chosen in this way might be incorrect if death is delayed beyond 2 or 3 days after dosing,

although this may be rare in practice. Nevertheless, there is a greater possibility of having to include additional dose levels or having to repeat the experiment. Furthermore, an increased number of animals may have to be used in the "up and down" procedure if the approximate $LD_{50}(LC_{50})$ cannot be estimated before the test.

E. ALTERNATIVES TO MAMMALIAN SPECIES

The expression of acute toxicity in an animal is the consequence of many complex interactions. Acute toxic effects are the result of the perturbation by a chemical of a complex and highly sophisticated biological organism, affecting specific molecules, cells, tissues and organs, and is influenced by the way the chemical is absorbed, metabolised and excreted by the body. These considerations illustrate the difficulty of replacing live animals by simpler systems, and it is unlikely that a single approach or model will provide a comprehensive alternative to in vivo procedures.

The types of systems or models that have been considered as alternatives have been reviewed recently (Smyth, 1978; Paganuzzi-Stammati et al., 1981; Brown, 1983). The following discussion is not intended to give an exhaustive survey but to illustrate some of the experimental directions taken and to summarise the progress that has been made. It is emphasised that progress, to date, has been made only in relation to the estimation of the lethal dose; no system has been investigated which can give information on overall acute toxicity symptomology.

1. Alternatives

1.1. In vitro mammalian cell and tissue cultures. The concept of correlating the lethal dose in animals and man with parameters of cell perturbation or cytotoxicity in vitro has been examined in a number of studies. In most of these, chemically-induced cytotoxicity was expressed as an effect on cell viability or morphology. Studies in which the non-specific or general toxicity of chemicals to cell systems have been assessed are listed in Appendix 3.

Primary cell cultures, fibroblasts, and epithelial and tumour cells have been used, together with a wide range of end-points - growth inhibition and growth defects; inhibition of DNA, RNA and protein synthesis; morphological

and mitotic abnormalities; dye inclusion/exclusion - signifying subtle or overt toxicity and reduced viability. Various compounds, e.g. pesticides, drugs and industrial products, have been examined, but not always in the same systems. In most studies only a small number of chemicals related by structure or mode of action has been assessed. The variety of experimental conditions employed prevents a direct comparison of results between studies.

It is stressed that in very few studies has there been an attempt to validate tests by comparing in vitro findings with known in vivo results. Where this has been done there was a degree of correlation but the broader the toxicological spectrum and range of chemicals the less this correlation was maintained. For example, in one of the few well-validated studies in which a large number of chemicals with a variety of modes of action or toxic effects were used there was some agreement between toxicity in in vitro cell cultures and lethal dose in animals and man, but this held for only approximately two-thirds of the chemicals tested (Ekwall, 1980-a,b).

1.2. Plants, bacteria and invertebrate systems. There are a few studies in which higher plants have been used with the aim of replacing, or reducing the number of, animals in toxicity tests (Kordan, 1981; Anon, 1982). The use of algae as an indicator of lethal concentration for other aquatic species has gained some application but the comparison has not been extended to mammals (Bringmann and Kühn, 1980).

Although bacteria, mould, fungi, yeasts and insects have been employed to predict mutagenicity, in only relatively few studies have these organisms been used to predict other toxic effects in higher animals (Harwig and Scott, 1971; Johnson and Finley, 1980; Kaiser, 1980; Metcalf et al.,1983; Slooff et al.,1983; de Zwart and Slooff, 1983). In general there is a marked difference in susceptibility to individual chemicals within sub-mammalian species which reduces the degree of correlation with toxicity in mammalian species.

1.3. Sub-mammalian vertebrate systems (amphibians, fish, birds). Toxicity tests on amphibians have been reported and acute toxicity tests on fish and birds are currently required by several authorities as part of the overall appraisal of the impact of chemicals on the environment. Some attempts have

been made to correlate the toxicity in these species with that in rodents (Schafer, 1972; EPA, 1982) but in general the greater the phylogenetic difference the poorer is the correlation. The presence of a developed nervous system in amphibians, fish and birds, and their consequent ability to experience pain, may limit their use as acceptable alternatives to mammalian species (Smyth, 1978).

The injection of chemicals into fertile eggs prior to incubation has been proposed as a screening method in the evaluation of the toxicity of food additives, chemicals and drugs. In addition, the egg may also be used to indicate teratogenic potential (McLaughlin et al.,1962, 1963). Although a toxicity (embryotoxicity) ranking similar to that based on rodent LD_{50} values was observed with mycotoxins (Vesely et al., 1982), this work was too limited to permit an assessment of the usefulness of this technique for estimating the lethal dose.

1.4. Quantitative structure-activity relationships (QSARs). The principle of QSARs is straightforward but the construction of an appropriate data base from which to make predictions of toxic potential against chemical structure is a complex task. In general, the predictive accuracy and capacity is inversely proportional to the number of assumptions that have to be made in establishing any QSAR model (Golberg, 1983). QSAR predictions can be based on chemical characteristics such as physico-chemical properties, e.g. by "Hansch" analysis, or on specific mechanisms of action, e.g. the probability of interaction of electrophilic centres with DNA, and consequent genotoxic potential (Tinker,1983).

Several systems have been proposed for predicting an $LD_{50}(LC_{50})$ value by attributing toxic activity to atoms or groups of atoms whose activity may be modified by their immediate molecular environment (Cramer and Ford, 1978; Dunn and Wold, 1980; Craig and Enslein, 1983). These approaches may also take into account physico-chemical or other data.
So far, these predictions have fallen short of being reliable. Overall, QSARs are most likely to be effective for series of chemicals which are closely-related in structure and where a single effect is being considered or where mechanisms of toxicity are well understood. This is not the case with novel chemicals or with parameters of toxicity such as death, as

measured by the $LD_{50}(LC_{50})$, which may result from a variety of different mechanisms.

2. Comments

It is concluded that at present there are no procedures, either alone or in combination, which constitute a reliable alternative to the whole animal for establishing the acute toxic profile of a chemical. Given the biological simplicity of mammalian cells in culture relative to intact animals, and the anatomical and morphological differences between these and lower species and embryonic systems, it is, perhaps, not surprising that the alternatives are inadequate.

Although there is no alternative approach which would result in a complete replacement of animals, it is possible to apply some of the available systems as screening tests, or adjuncts to whole-animal tests, in order to reduce the number of animals that would otherwise be used. The Task Force considers that this would be most appropriate where a strong correlation has been derived based on previous information, e.g. on a mode of action, a specific toxic effect, or on several closely-related chemical analogues. Prior information or screening may aid dose setting or the selection of chemicals for ultimate testing, both of which will result in the use of fewer animals. It must be noted that toxicologists regularly make assessments based on their experience of chemicals and acute toxicology which influence the design of experiments and minimise the numbers of animals used.

Clearly, it will be difficult to discover an alternative to the whole animal which provides a comprehensive indication of the toxic potential of a chemical. In addition, the predictive ability of any alternative system would need to be validated fully against animal data (and human data, if available) to gain scientific and regulatory approval. The validation procedure should be such as to ensure that reliable results were obtained with an extensive range of chemicals having a high degree of variation in their physico-chemical characteristics, the tissues, organs or systems they perturb, the type of biological process affected, the modes of action and pharmacokinetic characteristics. Finally, if an acceptable correlation were achieved, it would be essential to establish the reliability and reproducibility of the technique(s) in a co-ordinated blind trial between a number of independent laboratories.

F. SUMMARY AND CONCLUSIONS

1. The incorrect use of various terms and the failure to recognize the distinction between acute toxicity, lethality and $LD_{50}(LC_{50})$ often leads to confusion in the discussion of acute toxicity.

2. An investigation of acute toxicity is an essential part of the toxicological characterisation of a chemical. It is important that all the information available from an acute toxicity study is considered in assessing toxic potential. While it may be necessary to have a knowledge of the lethal dose, too much emphasis is placed on determining an $LD_{50}(LC_{50})$.

3. The $LD_{50}(LC_{50})$ is statistically derived from biological data and thus is an inherently variable value. It cannot be considered to be a constant and therefore its accuracy cannot be determined.

4. The Task Force considers that the determination of an $LD_{50}(LC_{50})$ is seldom necessary. However, it is demanded by regulatory authorities for classification, labelling, packaging and registration purposes.

5. Several protocols exist in which only a small number of animals is used for the estimation of the lethal dose, in some cases as an $LD_{50}(LC_{50})$, with sufficient precision for most purposes. In most cases a chemical could be assigned to a toxicity class based on the results of a fixed dose procedure rather than according to its $LD_{50}(LC_{50})$.

6. The determination of LD_{50}s at above 2000 mg/kgbw, or LC_{50}s at above 5 mg/l for 4 h, are toxicologically irrelevant.

7. When assessing the acute toxic hazard to man it is advantageous to have information on the acute toxicity (including a knowledge of the lethal dose when necessary) in more than one species by the relevant route of exposure.

8. Many techniques, including the use of lower vertebrates, invertebrates, cultured tissues and mathematical/structural approaches, have been suggested for replacing conventional mammalian acute toxicity tests. They may be useful adjuncts to, but at present cannot replace, conventional mammalian acute toxicity tests.

G. RECOMMENDATIONS

1. More emphasis should be placed on the wide range of information that can be derived from acute toxicity tests and which is essential in determining the overall toxic effects of a chemical. (LD_{50}(LC_{50}) values do not define the toxicity of a substance; they are merely an index of the lethal dose).

2. Where an LD_{50}(LC_{50}) is required by regulatory authorities, they should accept data from scientifically valid experiments but should not specify a minimum number of animals.

3. Classification systems based on the LD_{50}(LC_{50}) should be discouraged and the adoption of fixed-dose procedures for the selection of a toxicity class should be encouraged. Greater worldwide harmonisation of the criteria which define toxicity classes, and the mutual acceptance of data, would prevent the need to duplicate tests.

4. Regulatory authorities should acknowledge that acute toxicity data may be unnecessary for the protection of human health for some products (e.g. those with certain physico-chemical properties) and/or in certain circumstances. Standard arguments to cover these conditions should be developed and accepted internationally.

5. The use of predictions based on QSARs, or tests on lower animals, etc. should be encouraged as an aid to dose selection. For single-dose tests in particular this could prevent the unnecessary use of animals.

6. The distinction between LD_{50}(LC_{50}), lethality and acute toxicity should be maintained in current usage, and in the discussion of these topics.

H. APPENDICES

APPENDIX 1 : OECD Test Guidelines (1981), Acute Toxicity

Route of Exposure	Oral	Dermal	Inhalation
Animals			
Species/strain	Preferred rodent is rat (commonly used strains)	Adult rat, rabbit or guinea pig	Idem oral
Number/sex	At least 10 (5 males, 5 females) at each dose. Females should be nulliparous and non-pregnant	Idem oral	Idem oral
Caging	May be group-caged by sex. Number must allow clear observation of individuals. Biol. properties of test substrate, or its toxicity, may indicate that individual caging is required	Individually caged	Inhalation equipment: dynamic air flow, 12-15 air changes per h, 19% O_2
Environmental Conditions	Temp., animal room : 21°C ± 3° Rel. Humidity: 30-70% Lighting : 12h light/12h dark	Rodents : 22°C (± 3°) Rabbits : 20°C (± 3°) Others, idem oral	Idem oral
Diet	Conventional diets Unlimited supply of water	Idem oral	Idem oral
Test Conditions			
Dose levels	Sufficient (at least 3), appropriately spaced, with a range of toxic effects and mortality rates. Data should permit an acceptable determination of LD_{50} (95% conf. limits).	Idem oral	Idem oral. Data should permit an acceptable determination of LC_{50}. Care should be taken to avoid explosive concentrations.
Limit test	If at a dose of 5000 mg/kgbw there is no compound-related mortality, no full study is necessary.	Idem oral, but dose = 2000 mg/kgbw	Idem oral, but exposure concentration = 5mg/l for 4 h.

APPENDIX 1 (continued)

Route of Exposure	Oral	Dermal	Inhalation
Exposure time	Single dose administration, or smaller fractions over a period not exceeding 24 h.	24 h.	4 h.
Procedure	Administration of test substance by gavage to fasted animals. After administration, food may be withheld for a further 3-4 h.	Test substance applied on shaved, unabraded skin, not less than 10% of total body surface. No fasting procedure before, during and after exposure.	Oro-nasal, head only or whole body (individual chamber) exposure in a dynamic inhalation system. No fasting before and after exposure.
Observation period	Duration not rigidly fixed: at least 14 d, determined by toxic reactions, rate of onset and length of recovery period.	Idem oral	Idem oral
Study observations			
Clinical data			
-frequency of observation	Daily	Daily	Daily
-cageside observation	Changes in skin, fur, eyes and mucous membranes; respiratory, circulatory, autonomic, central nervous system, somatomotor activity and behaviour pattern.	Idem oral	Idem oral
-particular observations	Tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.	Idem oral	Idem oral
-time of death	To be recorded as precisely as possible.	Idem oral	Idem oral
-body weight	Measured before administr. of test substance, a week after, at death or at end of test before sacrificing.	Idem oral	Idem oral

APPENDIX 1 (continued)

Route of Exposure	Oral	Dermal	Inhalation
Pathology			
-gross anatomy	Full necropsy on all animals, as indicated by nature of toxic effects. All gross pathological changes to be recorded.	Idem oral	Idem oral but with particular reference to respiratory tract.
-organs	Microscopic examination of organs showing evidence of gross pathology in animals surviving 24h or more should be considered.	Idem oral	Idem oral
Presentation of Data	Table for each dose level: no. of animals at start; time of death of indiv. animals; no of animals displaying other signs of toxicity; description of toxic effects and necropsy findings.	Idem oral	Idem oral
	LD_{50}	LD_{50}	LC_{50}
	with 95% confidence intervals, dose-mortality curve and slope		

APPENDIX 2

Comparison of Some International Regulatory Requirements

	EEC[x] (1984-c)	UK/CSM[x] (1984)	Japan/MHW[x] (1984)	OECD (1981), EEC (1984-a) and UK/HSC (1982)	EPA/FIFRA (1982)
No. of species	2	2	Rodent : 2 Non-rodent : 1	Several may be used	Rodent (preferred) or non-rodents
Sex/No. of animals	Equal nos. m*/f*	Equal nos. m/f	Rodent : 5m/5f Non-rodent : 2m/2f	5m/5f	5m/5f
No. of dose levels	Sufficient for approximate determination of the lethal dose	Sufficient for approximate determination of the lethal dose	Rodent: sufficient to permit determination of LD_{50}(LC_{50}) to OECD requirements Non-rodent: sufficient for approximate determination of the lethal dose	The data should be sufficient to produce a dose-response curve and permit an acceptable determination of the LD50(LC_{50}) (except for Limit Test)	At least 3, as OECD
Route of dosing	2 routes including clinical route (for injections, i.v. route alone is acceptable)	As proposed for clinical use, and one route to ensure systemic absorption	Rodent : oral and parenteral routes including clinical route Non-rodent : clinical route	Oral (dermal/inhal.)	Oral

* m : males
f : females

[x] for pharmaceuticals only

APPENDIX 3

Some General Toxicity Studies on the Effects of Chemicals With Cell and Organ Cultures

Compound	Cell Type	Effects	Reference
PESTICIDES			
42 compounds (e.g. phenazine, puromycin, lindane, chlorodimeform, etc.)	Culex pipiens ovarian cell line; L-cells	Inhibition of growth, respiration, and nucleic acid and protein synthesis	Yoshida et al. (1979)
5 commercial pesticides: Raid, Malathion 50, Isotox, Sevin, Orthoklor	HeLa cells	Various effects on growth; increased DNA, RNA and protein contents	Blevins and Sholes (1978)
11 pesticides (e.g. DDT, aldrin, dieldrin, etc.)	HEL 299 cells	Cellular uptake of environmentally persistent chemicals greater than that of non-persistent chemicals; growth inhibition did not correlate with pesticide uptake.	Murakami and Fukami (1978)
Aldrin, parathion, paraoxon	BALB/c 3T3, WI-38, HeLa cells, rat lung fibroblasts, human foreskin fibroblasts	Inhibition of growth, and of RNA and protein synthesis	De Vore (1978)
Lindane, DDT, malathion, bromophos, trichlorophon, aprocarb, dioxacarb, benlate	Monkey kidney, fibroblasts	Morphological alterations and dose-dependent growth inhibition	Desi et al.(1977)
Tetrachloro-dibenzo-p-dioxin	HeLa, BALB-3T3, SV 101, FS, NC-37 cells	No effect on cell morphology and growth	Beatty et al.(1975)

Appendix 3 (continued)

Compound	Cell Type	Effects	Reference
Chlorphenamidine and some structurally-related compounds	HeLa cells	Compound-related growth inhibition; no significant inhibition of nucleic acid and protein synthesis.	Murakami and Fukami (1974)
DRUGS			
21 drugs (i.e. ouabain, methyldopa, phenobarbital, prometazine, alcohol)	HeLa cells	Growth inhibition dependant on drug and its concentration, substrate adhesion, and increase of culture-medium pH	Ekwall and Sandstrom(1978-a)
17 drugs tested individually and as 61 pairs (e.g. propanol, amitripcyline, Na fluoride, etc.)	HeLa cells	Growth inhibition dependant on drug and concentration, substrate adhesion, and increase of culture medium pH	Ekwall and Sandstrom (1978-b)
Gentian violet and crystal violet	CHO cells	Mitotic anomalies and chromosomal aberrations	Au et al.(1978)
Aliomycin, eurocidin, xantholycin B and flavofungil	HeLa and L-5178 Y cells	Growth inhibition: L-5178 Y cells more sensitive than HeLa cells	Fisher et al.(1978)
10 non-steroid anti-inflammatory drugs (e.g. acetylsalicylic acid, sodium salicylate, phenylbutazone etc.)	Human foreskin, fibroblasts and rat hepatoma cells	Reversible dose-dependent growth inhibition; inhibition of protein, RNA and DNA synthesis	Hial et al.(1977)
20 carcinogens (e.g. diethylnitrosamine, methylnitrosourea, etc.) and non-carcinogens	Newborn rat kidney fibroblasts	Growth inhibition	Hooson and Grasso (1977)
15 antibiotics (e.g. ampicillin, chloramphenicol, gentamycin, streptomycin, etc.)	Human diploid skin fibroblasts	Decrease in cell survival; no detectable chromosomal damage	Byarugaba et al. (1975)

Appendix 3 (continued)

Compound	Cell Type	Effects	Reference
52 drugs (e.g.procaine, strychnine, methadone, alprenolol, chlorpromazine, etc.)	HeLa cells	50% inhibitory concentration (IC) to HeLa cells of 39 drugs was similar to approximate human LD; for 47 drugs, good correspondence of mouse i.v.LD and HeLa IC 50.	Ekwall (1980-b)
INORGANIC CHEMICALS			
13 metal compounds (e.g. $CaCrO_4$, $CdSO_4$, $Pb(C_2H_3O_2)_2$, etc).	CHO cells	Inhibition of DNA, RNA, protein synthesis and ornithine-decarboxylase induction, depending on metal compound	Costa (1979)
NaF	LS and LS_{FR6} cells	Different effects on membrane permeability depending on concentration	Holland and Hongslo (1979)
Asbestos	CHO and K-22 cells	Growth inhibition	Neugut et al.(1978)
$PbCl_2$, $HgCl_2$, $CdCl_2$	L-A cells	Decrease in cell viability; growth inhibition	Fischer (1976)
$PbCl_2$	L-A cells	Cell death; morphological alterations; depression in mitotic rate; increase in lactate production; no effect on lactate dehydrogenase leakage	Fischer (1975)
INDUSTRIAL ORGANIC CHEMICALS			
CH_3HgCl	HeLa S3 cells	Partially reversible inhibition of RNA, DNA and protein synthesis	Gruenwedel and Cruikshank (1979)
		Decrease in cell viability; increase in mitotic index	Gruenwedel and Fordan (1978)

Appendix 3 (continued)

Compound	Cell Type	Effects	Reference
Coal leachates	L-cells (clone 929); WI-38; primary human (amnion) fibroblasts	Growth-inhibition linearly related to log concentration	Christian and Nelson (1978)
Water fractions from oil-refinery effluents	L-M strain	Seasonal variations of growth inhibition	Richardson et al.(1977)

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