CANADA

TOXICITY DETERMINATIONS

AND THE

CHEMICAL WEAPONS CONVENTION

AUGUST 1990

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TABLE OF CONTENTS

LIST OF TABLES

PREFACE

This paper was first published in May 1990 for distribution to Canadian government agencies in order to promote discussion on issues related to the proposed Chemical Weapons Convention. The issue of toxicity testing in relation to the proposed Convention has not been discussed in detail since March 1982 and, as noted in the present rolling text (CD/961), there might be a need for these procedures to "be supplemented or modified and/or, if necessary, reviewed." This paper reviews more current procedures for toxicity determinations and compares them with those described in an annex to the rolling text. It concludes that it would be appropriate to carry out a careful review of the existing annex on toxicity determinations in view of the changes in the methodology of toxicity testing that have taken place in recent years in academia and in industry.

The paper was written by consultants from the Toxicology Research Centre in the University of Saskatchewan in conjunction with the Verification Research Unit of External Affairs and International Trade Canada. The paper and its recommendations are intended to promote discussion and do not necessarily reflect the views of the Canadian government.

EXECUTIVE SUMMARY

Article VI of the "Rolling Text" (CD/961) is concerned with Activities not Prohibited by the Convention, and a number of Annexes and Schedules describe toxic chemicals and the methods for determination of toxicity; the annex on toxicity determinations also suggests that the draft proposal for toxicity determinations might need to be modified or supplemented.

This report reviews various aspects of toxicity determinations, as set out in the "Rolling Text" and also methods generally used in industry and academia. After careful consideration of all aspects, it is concluded that it is advisable to initiate a very careful review of the existing annex on toxicity determinations.

A number of specific points are recommended as topics for review, in particular:

- establishment of categories of toxicity;
- selection of route of exposure, etc.;
- impact on industry, the public-at-large, and the animal welfare movement;
- critical evaluation of methods of determination of toxicity.

1.0 Introduction

Article VI of the "Rolling Text" (CD/961; 1989) describes Activities not Prohibited by the Convention and states that each State Party has the right, subject to provisions of the Chemicals Weapons Convention, to produce and use toxic chemicals and their precursors for purposes not prohibited by the Convention. However, facilities that produce, process or consume scheduled toxic chemicals or precursors are subject to various regimes for international monitoring.

A number of Annexes and Schedules describe toxic chemicals and the methods for determination of toxicity for the purpose of the Convention. A footnote to the annex on toxicity determinations in CD/961 indicates that "it was understood that these recommended standardized operating procedures (CD/CW/WP.30) for toxicity determinations might be supplemented or modified and/or, if necessary, reviewed." Item B of the annex indicates that modalities for revision of toxicity determination procedures have to be developed.

This paper addresses a number of issues concerning the toxicity determinations, and is a contribution to the discussion of further, potential modifications of the toxicity determination procedures.

2.0 Review of Procedures for Toxicity Determinations

2.1 Procedures as Described in the CWC Drafts

The current "rolling text" CD/961 refers to CD/CW/WP.30 as the source of recommendations for standardized procedures for toxicity testing. These recommendations resulted from consultations held in March 1982, and involved 32 experts from 25 countries. Three categories of agents were defined on the basis of their toxicity:

(i) super-toxic lethal chemicals;

(ii) other lethal chemicals;

(iii) other harmful chemicals.

Lethality limits in terms of LD_{50} for subcutaneous administration were established to separate three toxic categories at 0.5 mg/kg and 10 mg/kg, on the basis of CD/CW/CTC/7. Lethality limits in terms of LCt_{50} for inhalatory application were established to separate three toxic categories at 2,000 mg min/m^3 and 20,000 mg min/m^3, on the basis of CD/CW/CT/6. Many years of deliberations and discussions preceded CD/CW/WP.30, and while it is not possible to review here every single step of how the categories were arrived at, a few critical steps will be highlighted.

In their Working Papers (CCD/301; 1970, CCD/344; 1971), Japanese experts suggested that it would be desirable to establish a reporting system for CW agents. A lethal dose (LD_{50}) by hypodermic injection (s.c. = subcutaneous) was suggested as a criterion for limiting the scope of chemicals to be reported for this purpose. An LD_{50} (s.c.) of 0.5 mg/kg body weight (BW) was suggested because among the organophosporous compounds, none that were used for peaceful purposes, at that time, had LD_{50} values (s.c.) > 0.5 mg/kg. Such a toxicity threshold could then separate supertoxic substances from less toxic chemicals. It was further suggested that 0.5 mg/kg (s.c.) has the lethal equivalent to a dose of about 1.0 mg/kg (p.o. = oral application). Japan noted that more information was available for LD_{50} values by the s.c. route of administration for both chemicals and animal species than by i.p. (intraperitoneal), i.v. (intravenous) or p.o. (oral)

Italian experts (CCD/373; 1972) agreed that toxicity was an important criterion for classification but also suggested careful and correct appraisal of other factors (e.g., dissemination characteristics) could be important.

The Netherlands concurred that toxicity was a useful criterion (CCD/320; 1971), provided that the species of animals used in testing and the method of application were standardized. They further suggested that it is difficult to use the lethal

dose as a sole criterion for defining a range of agents that could be subject to unconditional prohibition. Japan agreed (CCD/344; 1971) with the Netherlands (CCD/320, CCD/383; 1972) that the general structural formula could be useful as a criterion for the classification of organophosphorous nerve agents. Other Working Papers (CCD/365, CCD/374, CCD/375, CCD/387, CCD/430, CCD/435, CCD/473) emphasized the importance of adapting standard experimental procedures for measuring toxicity if toxicity criteria were used to restrict or prohibit chemicals.

In 1972, Canada (CCD/387) recommended general procedures for lethality testing. It was felt that it is not possible to define rigid procedures, in detail, that should be followed in estimating the lethal potency of chemical substances with relevance to possible uses in warfare. Canada stated that the control of chemical substances cannot be based on lethal toxicity alone if the LD_{50} is greater than 1 mg/kg. Those agents with LD_{50} greater than 0.5 mg/kg but less than 1.0 mg/kg should be considered as potential lethal CW agents but it would also be necessary to assess their practicability as CW agents. Canada recommended that chemicals with an LD_{50} less than 0.5 mg/kg should be controlled and this should be the deciding criterion.

Japan (CCD/374; 1972) noted that a spectrum of LD_{50} values, consisting of measurements from tests carried out under

identical laboratory conditions, should be utilized since single LD_{50} values for chemicals can vary depending on experimental conditions (e.g., animal species, route of administration, etc.). The USA (CCD/435; 1974) also discussed the variability of LD_{50} values based on routes of exposure. The U.S. suggested that the military potential of supertoxic compounds is often closely related to toxicity by inhalation and so it seemed logical to establish a criterion based on the respiratory route of exposure as proposed by Canada (CCD/414; 1973). However, some chemicals (e.g., supertoxic carbamates), are not supertoxic by inhalation, but are extraordinarily toxic if carried into the body by a projectile which penetrates the skin. Thus, criteria based only on the respiratory route of exposure would not be sufficient.

The USA (CCD/435) agreed with Japan (CCD/374) that the proposed utilization of the s.c. or i.p. routes of exposure is less difficult and would be useful in supplementing the criterion based on the respiratory route. Since compounds, supertoxic by inhalation, would also be supertoxic by a parenteral route, these two routes of exposure were proposed. The s.c. route was particularly recommended because more data are available, for CW agents and also for mice than for any other animal species. The USA further suggested establishing an LD_{50} value of 0.5 mg/kg (s.c. mouse) as a limit to separate single purpose supertoxic agents from dual purpose chemicals (as suggested in CCD/301) .

In a Working Paper (CCD/372; 1972), Swedish experts indicated that nearly all CW agents classified as supertoxic were single purpose agents, i.e. they are only used for military purposes. However, it was noted that not all single purpose agents are supertoxic. Other chemicals may be considered dual purpose and be utilized as CW agents as well as having a civilian use and that future supertoxic CW agents may also be dual purpose.

A Japanese Paper (CCD/430) refers to a Canadian (CCD/414) and a Swedish (CD/427) working paper and considers three criteria:

- a toxicity level, e.g. a LD_{50} (i.p.) of 0.62 mg/kg or a LD_{50} of 0.50 mg/kg (s.c.), which can be considered as objective criteria;
- the chemical formula;
- determination whether or not the chemical has a peaceful use.

The suggestion was to use the lowest LD_{50} value, but the route of exposure was not stated. Additionally, in 1975, Japan provided (CCD/446) a listing of chemicals, but said little or nothing about methodology. Japan agreed with Canada (CCD/414) and Sweden (CCD/427) who suggested that upper and lower bound thresholds can be established for exposure by inhalation. The

lower bound threshold would be based on chlorine toxicity and have an LCt_{50} value of 20,000 mg min/m^3, whereas the upper bound threshold would be based on the toxicity of tabun and have an LCt_{50} of 500 mg min/m^3. Chlorine was selected because it is the least toxic CW agent, and tabun because it is the least toxic of the single purpose supertoxic lethal agents.

Canada followed this by a working paper on the "Use of Measurements of Lethality for Definition of Agents of Chemical Warfare" (CCD/473; 1975). The paper recommends the use of toxic reference materials (Table 1) and the adoption of separate standards of lethality for three groups of agents, according to their routes of entry into the human body, i.e.,

- inhaled gases or vapours
- percutaneously lethal materials, and
- supertoxic solids.

Table 1: Approximate lethal dosages of CW agents and other toxic materials (Canada, CCD/473; 1975)

Group I - Toxic Vapours and Gases

		Approximate Lethal Dose Inhaled	
	Name of Lethal Material	LCt_{50} mg min/m^3	LD_{50} mg/kg
	Carbon Monoxide	150,000	21
	Ammonia	70,000	10
	Sulfur Dioxide	40,000	5.6
	Chlorine	36,000	5.1
	Hydrogen sulfide	22,000	3.1
	Hydrogen cyanide	5,500	0.790
Proposed-> reference substance	Phosgene	3,000	0.43
	Ozone	2,000	0.28
	Non-persistent nerve gas	100	0.014

(Table continued next page)

Table 1 - continued

Group II - Percutaneously Toxic Liquids

	Approximate Lethal Doses Percutaneous		
Name of Lethal Material	Percutaneous mg/kg	Inhaled Vapour LCt_{50} mg min/m3	Injected mg/kg
Parathion	500		5
Diisopropyl flurorophosphate	100	5,000	4
Allyl alcohol	50	140,000	-
Proposed-> reference substance Nicotine (base)			
Mustard Gas	20(?)	2,000	10
Paraoxon	10		-
Persistent nerve gas	0.2	50	0.02

Group III - Supertoxic Solids

Name of Lethal Material	Approximate Lethal Dose Injected mg/kg (subcutaneous)
Strychnine	1.0
Physostigmine	0.5
Curarine	0.5
Proposed-> reference substance Neostigmine	0.4
Digitoxin	0.3
Carbachol	0.3
Snake Venoms	0.5 -> 50
Ricin	0.02
Carbamates	0.01
Bacterial Toxins	
- staphylococcus	0.00001
- tetanus	0.00000003
- botulin	0.00000002

Another submission from Japan (CCD/515; 1975), explained that chemicals have different LD_{50} values, depending on the route of application, and it is suggested that it would be advantageous to use a LD_{50} spectrum. It proposed listing the chemicals starting with the lowest LD_{50} values, regardless of the application route, but including data from animals other than rodents. 30 mg/kg (oral) is proposed as the spectrum's upper limit.

In 1977, Hungary (CCD/537/Rev 1; 1977), although noting previous efforts, proposed a more general approach. Using an LD_{50} of 200 mg/kg, would cover all toxic chemical warfare agents and also a significant number of irritants, etc. Using an LD_{50} of 30 mg/kg, would cover practically all lethal chemical warfare agents, but would exclude irritants, etc. An LD_{50} of 3 mg/kg, would cover supertoxic agents.

The following thresholds were suggested for inhalatory LCt_{50}:

- 35,000 mg min/m^3
- 3,000 mg min/m^3
- 2,350 mg min/m^3
- 500 mg min/m^3

The paper also suggested that it might be possible to convert the LCt_{50} to an LD_{50}. When the body-weight of the test animal and the amount of the air the animal breathes per minute are known, the value LCt_{50} can be converted to the LD_{50} by the following formula:

$$LD_{50} \text{ (inhaled)} = \frac{(LCt_{50} \text{ value}) \times (\text{inhaled air})}{\text{body weight}}$$

The USSR (CD/789, Annex 9; 1987), presented some views on standard methods for classifying supertoxic lethal chemicals. The paper proposed the determination of intravenous toxicity in rabbits, and described the details of the procedures; the chemicals are introduced in a water-acetone or water-alcohol solution, and diluted with distilled water (presumably at a 0.9% NaCl physiological level) so that the dose of the chemical to be tested is 0.05 ml of the solution.

Italy (CD/CW/WP.190; 1988) contributed important remarks on the toxicity index (LD_{50}) chosen as parameter for identification of chemicals not listed in Schedules (1), (2), (3) or (4)[1] of the then "Rolling Text." The paper also introduced a number of questions such as:

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[1]Schedule 4 is now incorporated as Schedule 2A

- What degree of reliability can be given to the LD_{50} toxicity index of 0.5 mg/kg?
- What impact on industry might result from having compounds of Schedule (4) subjected to measures contained in the Convention?

The paper argues that there is a very large number of compounds that have a Schedule 4 (2A) level of toxicity (i.e., 850 substances have an $LD_{50} \leq 0.5$ mg/kg, and 596 substances have an LD_{50} between 0.5 and 1.0 mg/kg). The paper also argues that toxicity values reported in the literature are the result of a variety of testing methods, and are not comparable when one considers the variety of species and different routes of exposure employed.

In a footnote to the annex "Procedures for Toxicity Determinations" in CD/961, it says that "it was understood that these recommended standardized operating procedures (CD/CW//WP.30) for toxicity determinations might be supplemented or modified and/or, if necessary, reviewed" and that "a view was expressed that appropriate methods for testing of non-lethal harmful chemicals need to be addressed at a later stage." This review paper takes a critical look at the proposed methods.

2.2 Conventional Methods for Toxicity Determination

2.2.1 Acute Toxicity and LD_{50}

The OECD (1983) defines acute oral toxicity as "the adverse effects occurring within a short time of oral administration of a single dose of a substance or multiple doses given with 24 hours". The objectives of acute toxicity testing are to define the intrinsic toxicity of the chemical, predict hazard to nontarget species or toxicity to target species, determine the most susceptible species, identify target organs, provide valuable information for clinicians to predict, diagnose, and prescribe treatment for acute overexposure to chemicals (Chan and Hayes, 1989). A carefully designed acute toxicity study can often provide important clues on the mechanism of toxicity and the structure-effect relationship for a particular class of chemicals (Chan and Hayes, 1989).

However, many acute toxicity studies have been conducted solely for the purpose of determining the LD_{50} of a chemical for regulatory purposes. It has to be understood here that acute toxicity testing is not equivalent to determination of the LD_{50}, because the LD_{50} is not an absolute biologic constant to be equated with such constants as pH, pKa, melting point, and

solubility. Rather, the LD_{50} is one of many indices used in defining acute toxicity (Chan and Hayes, 1989).

2.2.2 LD_{50} and Its Determination

Definition

The LD_{50}, in its simplest form, is the dose of a compound that would cause 50% mortality in a population of test animals under specified laboratory conditions. The OECD (1983) has defined the LD_{50} as the "statistically derived single dose of a substance that can be expected to cause death in 50% of the animals." Therefore, the LD_{50} value is simply a descriptive term designed to describe statistically the lethal response to a chemical in a particular population under a discrete set of experimental conditions (Chan and Hayes, 1989).

Origin and Significance of the LD_{50}

Many years ago, the use of plants or extracts of plant or animal tissues in medicine created a need to compare the therapeutic potency of different lots of materials (SOT, 1989). This led to the development of bioassay procedures which estimated the ED_{50} (effective dose/50% response or median effective dose) of similar materials so that comparisons could be made. If the effect measured was death, the ED_{50} became the LD_{50} (median lethal dose). Thus, the LD_{50} is one variant of

the more general term ED_{50}. Later, the LD_{50} became a measurement by which the relative toxicities of different substances could be compared (SOT, 1989).

In 1927, Trevan introduced the concept of a median lethal dose (LD_{50}) for the standardization of digitalis extracts, insulin and diphtheria toxin (Chan and Hayes, 1989). Trevan realized that the precision of the LD_{50} value was dependent on many factors such as seasonal variation and number of test animals. Since that time the number of factors identified as affecting the LD_{50} has increased to include sex, age, species, strain, diet, nutritional status, general health, animal husbandry, experimental procedures, route of administration, stress, dosage formulation (vehicle) and inter- and intralaboratory variations (Chan and Hayes, 1989). Thus the LD_{50} is an imprecise value.

Despite this imprecision, the numeric value of the LD_{50} has been used to classify and compare toxicity among chemicals. The numeric LD_{50} <u>per se</u> is not equivalent to acute toxicity. Lethality is just one of many reference points in defining acute toxicity. The slope of the dose-response curve, the time to death, pharmacotoxic signs, and pathologic findings are all vital or even more critical than the LD_{50} numerical value in the assessment of acute toxicity (Chan and Hayes, 1989). It is for these reasons that the LD_{50} is no longer considered as important as it once was.

2.2.3 Types of Acute Testing

It is generally agreed that a battery of tests under different conditions and exposure routes should be conducted to determine toxicity of a compound (Chan and Hayes, 1989). Lethality is just one important aspect of acute toxicity testing. It is equally important to study animals who survive for 7-14 days following the LD_{50} test dosing. The severity of symptoms, the duration of primary toxicity, the development of any secondary toxicity, the recovery from the toxic insult (or the lack of recovery) and at least a preliminary study of the mechanism(s) of actions should also be studied. Preferably, such tests should include oral, dermal, and inhalation toxicity determinations, and also skin and eye irritation studies. The number and kind of acute tests needed to establish the initial toxicity data base may not be the same for each chemical. However, the oral, dermal, inhalation, skin and eye irritation tests should be considered a must for initial investigations (Chan and Hayes, 1989).

Animals Tested

Species Differences/Effects

The responses elicited by a chemical often vary greatly among species, as Table 2 shows. Therefore, conducting acute

toxicity studies in a variety of animal species will indicate whether the signs and symptoms of acute toxicity of the chemical are consistent in all the species tested. If it is, then the extrapolation to man is less tenuous.

Table 2: Species Differences in the Oral LD_{50} Values for DDT

Species	mg/kg Body Weight
Frog	7.6
Rat	113.0
Mouse	135.0
Guinea Pig	150.0
Monkey	200.0
Rabbit	250.0

Source: Compiled from various sources.

There is no absolute criterion for selecting a particular animal species. However, priority should usually be given to animals with metabolism or other physiologic and biochemical parameters similar to man. Animal species should also be selected on the basis of convenience, economical factors, and the existing data base for the animal. For all these reasons, rats, mice, rabbits, and guinea pigs are most frequently chosen for acute toxicity studies.

Number and Sex

The precision of acute toxicity testing is dependent on the number of animals employed per dose level. Twenty rats (10 male and 10 female) per dose have been recommended in most regulatory guidelines, although there is now a tendency to reduce this number. The degree of precision needed and the number of animals per dose group needed depend on the purpose of the study. In screening tests or range finding tests, fewer animals per dose level or smaller number of dose levels may be considered. If a fairly precise LD_{50} is required, the number of dose levels (a minimum of 3 dose levels) and the number of animals per dose group have to be increased.

In 1986, the OECD updated their toxicity testing guidelines in a draft paper which recommended that acute toxicity studies be conducted using animals of one sex only and only five animals per dose (Auletta, 1988; Chan and Hayes, 1989). Administration of one dose to the opposite sex is recommended to confirm the absence of sex-related differences (Table 3). This approach halves the number of animals required in acute toxicity testing.

Table 3: Sex Differences Related to Oral LD_{50} Values for 2,3,7,8-TCDD

Species	Sex	mg/kg Body Weight
Rat	Male	0.022
	Female	0.045
Guinea Pig	Male	0.0006
	Female	0.0021

Source: Compiled from various sources.

Dose Levels

A sufficient number of dose levels should be used to allow for a clear demonstration of a dose-response relationship, and to permit an acceptable determination of the LD_{50}. Three dose levels are generally considered to be sufficient, although Japanese guidelines recommend five levels (Chan and Hayes, 1989). The selected levels should bracket the expected LD_{50} value with at least one dose level higher than the expected LD_{50} but not causing 100% mortality, and one dose level below the expected LD_{50} value but not causing 0% mortality, when the probit analysis method is applied to estimate the LD_{50} (Chan and Hayes, 1989). In any event, three or more dose levels with a wide range of toxic responses are recommended to establish the dose-

effect relationship and the slope of the line if no other toxicity data are available.

Routes of Exposure

Important information can be obtained from lethality studies comparing different routes of exposure (STC, 1985; and Annex I). A chemical is not usually equally toxic by all routes of exposure. Generally, a chemical is most toxic by the route which permits fastest and greatest entry into the body (Table 4).

Table 4: Effect of Route of Administration on LD_{50} Values for Parathion in Mice

Route	mg/kg Body Weight
Intraperitoneal	5.6
Oral	6.0
Intramuscular	7.2
Subcutaneous	11.5
Intravenous	17.4
Dermal	32.4

Source: Compiled from various sources.

Methods of application usually include oral (p.o.), dermal, subcutaneous (s.c.), intramuscular (i.m.), intraperitoneal (i.p.) and intravenous (i.v.). The employment of various routes of

application has not necessarily added to our understanding of toxicity. Two examples should illustrate this point (Schiefer, 1986). Does it really help our understanding of the toxicity of aflatoxin when the LD_{50} is established using intraperitoneal application, "because it was not possible to obtain a reliable LD_{50} after gavage, due to variation in the response of the animals?" What should we think of the observation that patulin is more toxic by s.c. and i.p. routes than i.v., but least toxic when given per os, the most likely route of consumption? Such reports give valuable insights into the mechanisms of action, but they should not be expected to serve as a guide for classification of a toxicant as being able to produce typical disease in one or the other organ, or for causing death, for that matter.

The standard rule, therefore, is that preference should be given to testing via the intended route of application. Data derived from such studies carry the greatest weight when it comes to evaluation of a new compound.

The more common types of exposure used in evaluation of inhalation toxicity are whole-body exposures, head-only or nose-only exposures (Kennedy, 1989). For inhalation studies where the entire animal is exposed (whole-body exposures), the exposure chamber is essentially a jar or box containing an access for placing and removing the test animals and for introducing and

removing the test chemical with provisions made to withdraw air chamber samples for chemical/physical analysis periodically throughout the test (Kennedy, 1989). This mode of operation reflects the situation usually encountered by humans, that is, the exposed individual may move about freely in an atmosphere containing the chemical such that absorption occurs mainly through the lung following inhalation. However, there is a possibility of uptake through the skin following contact with aerosols or vapours, and through the gastrointestinal tract after swallowing. Whole-body inhalation exposures will usually result in the chemical being taken up by the body, regardless of the physical form of the toxicant. Gases or vapours can dissolve in the mucous fluid lining the respiratory tract, and, via the mucociliary escalator, reach the pharynx where they are swallowed. Droplets or solid particles also reach the gastrointestinal tract via this mechanism. Further contributions to total absorbed dose can be seen following dermal absorption of the test agent (Kennedy, 1989).

Head-only exposure is regarded as being a more specific indicator of the inhalatory toxicity of a compound, and will typically yield different toxicity or lethality values compared to those obtained by whole-body exposure (Table 5). On the other hand, head-only or nose-only exposures are "unnatural."

Table 5: Effect of Inhalation Exposure Route on Toxicity of Anilines

Compound	LC_{50} in ppm		Ratio whole-body/ head-only
	Whole-body	Head-only	
Aniline	478	839	0.57
N-ethylaniline	263	424	0.62
N,N-diethylaniline	315	679	0.46

Source: Kennedy (1988).

The duration of exposure should be at least 4 hours, after equilibration of the chamber concentrations, and the observation period should be at least 14 days.

2.3 Other Approaches

2.3.1 Fixed Dose Procedure

In 1984, the British Toxicology Society (BTS) suggested a new approach to acute toxicity testing, based upon a fixed dose procedure (Anon, 1984). The procedure is designed to avoid the death of animals as an end-point and emphasizes instead observations of clear signs of toxicity that develop after application of a series of fixed dose levels. Test materials can then be classified on the basis of dose levels at which toxic

signs are observed. Since death does not necessarily determine classification under this system, any animal that is suffering unduly can be sacrificed without affecting the study results. The use of lower sub-lethal doses would tend to reduce the severity of toxic signs. For these reasons, such studies are more acceptable from the ethical and animal welfare standpoint and also reduce the number of animals required, possibly to as few as 10. Chemicals with high LD_{50} values which produce severe toxic effects at low doses (i.e., chemicals with shallow dose-response curves) would, in some cases, result in a higher toxicity classification than the LD_{50} value alone would have yielded. Also, it has been claimed that less inter-laboratory variation of classifications result from the use of the fixed dose method than with current procedures which rely on rather unreliable estimated LD_{50} values (Gilman, 1989).

In order to validate this fixed-dose procedure, studies have been carried out under the British Toxicology Society and the OECD, in accordance with the OECD Principles of Good Laboratory Practice (GLP). The Commission of the European Communities (CEC) sponsored part of these studies. Results were discussed at a meeting organized by the CEC on September 19-21, 1989; some of these results have been published earlier (Van den Heuvel et al., 1987).

A protocol for acute oral toxicity testing, utilizing the fixed-dose procedure, was developed previously (Van den Heuvel et al., 1987; and Annex 2). Briefly, the protocol suggests that the test substance be administered orally by gavage at a single dose level to a group of rats. The dose used is selected from a series of fixed, pre-set dose levels, and is the one judged most likely to produce evident toxicity, but no deaths. Evident toxicity is a general term describing clear signs of compound-related toxicity, but without very severe pain, distress or mortality. If no information is available on the likely toxicity of the chemical, a preliminary "sighting" study, using 3 to 6 animals, is carried out. A significant increase in the dose administered, i.e., to the next higher pre-set level, would be expected to result in severe toxic effects and probably mortality. Animals are closely observed following administration of the test chemical. Those in distress or pain are killed humanely. All animals which die during the test are necropsied, as are the remaining animals at the end of the test. If evident toxicity is not seen the chemical is retested at the next pre-set higher dose level. The dose level which produces evident toxicity, but no mortality, is used to allocate the substance to a toxicity class (Van den Heuvel et al., 1987). Table 6 summarizes the important features of the Fixed Dose procedure:

Table 6: Investigation of Acute Oral Toxicity Using a Fixed Dose Procedure: Criteria for Classification for Labelling Purposes

Test Dose (mg/kg body weight)	Result	Action
5	Less than 90% survival	Classify as very toxic
	90% or more survival; but evident toxicity	Classify as toxic
	90% or more survival; no evident toxicity	Retest at 50 mg/kg
50	Less than 90% survival	Classify as toxic Retest at 5 mg/kg if not already tested at that dosage
	90% or more survival; but evident toxicity	Classify as harmful
	90% or more survival; no evident toxicity	Retest at 500 mg/kg
500	Less than 90% survival	Classify as harmful Retest at 50 mg/kg if not already tested at that dose
	No evident toxicity	Unclassified

Source: Van den Heuvel et al., 1987

2.3.2 Range-Finding Studies

Range-finding studies, first proposed in 1945, may be useful for chemicals with unknown toxicity or those whose anticipated LD_{50} value may be below the upper limit for testing requirements (Auletta, 1988). A preliminary range-finding test is undertaken to provide guidance in selecting dose levels. At least four dose levels are administered to two animals (one male; one female) per dose. A wide range of doses (50 - 500 mg/kg) should be selected if no information is available. Animals are held for 7 days after dosing and observed for lethality.

On the basis of such range-finding studies, dose levels are then selected for the LD_{50} study. It is important to realize, however, that dose selection based on only two animals may not always be accurate. If an appropriate mortality range is not achieved initially, more tests may have to be added. If this is the case, it is essential that experimental conditions be maintained as closely as possible to those in the initial doses.

The advantage of this step-wise approach is that the total number of animals used is decreased when compared with traditional LD_{50} tests. The disadvantage is that it can sometimes lead to variable results and thus must be carefully administered (Auletta, 1988).

2.3.3 "Up - and - Down" Study

The "up - and - down" study method was introduced by Dixon and Mood in 1948 and revised by Bruce in 1985 (De Pass, 1989). Animals are dosed one at a time starting at an estimated LD_{50} dose. If the first animal survives, the next one receives a higher dose. If the first animal dies, the next one receives a lower dose. A constant multiplicative factor such as 1.3 is usually used to adjust the doses. Depending upon the fate of the previous animal, the dose is adjusted up or down for each successive animal. Comparison of the results with conventional probit-derived LD_{50} data using computerized simulations of this method, produced excellent agreement between the two methods. The up - and - down method generally requires only 6-9 animals compared with the 40-50 required by conventional methods (De Pass, 1989).

2.4 The LD_{50} and the Animal Welfare Movement

Concerns about the use of laboratory animals in biomedical research, particularly in toxicity testing, are not new. Antivivisection organizations were formed in the 18th Century, and a "Cruelty to Animals Act" was passed in Britain as early as 1876. This bill regulated painful research but did not abolish it and was therefore strongly opposed by antivivisection groups (see Rowan, 1984). From these beginnings,

people concerned with animal welfare have demanded that scientists reduce the number of animals used by refining their methodology, and replacing live animals by a variety of alternate experimental methods. Public pressure for such changes has grown over the years and activist groups have adopted a more radical stance on these issues.

One of the main targets of the animal welfare movement is the LD_{50} test (Rowan, 1984). The idea of deliberately administering a large enough dose of a substance to poison approximately 50% of a group of animals virtually guarantees protest. Also, an increasing number of government departments, industrial associations, and toxicologists are critical of the LD_{50} test. Zbinden (1973) has called the LD_{50} test a "ritual mass execution of animals" in which "scientific inventiveness and common sense have been replaced by a thoughtless completion of standard protocols" (Zbinden, 1976). Considering the shortcomings of the LD_{50} test, it can be expected that opposition to it will grow stronger in the future.

3.0 Critical Comparison of Current Rolling Text with Internationally Accepted LD_{50} or LCt_{50} Determinations

Proposal in CD/961, Appendix I, Pages 52 to 55	COMMENTS (on the basis of internationally accepted toxicological procedures)
A. Procedures for toxicity determinations[1,2]	
<u>Recommended standardized operating procedures for acute subcutaneous toxicity determinations</u>	
1. <u>Introduction</u>	
Three categories of agents were defined on the basis of their toxicity:	This classification is quite different from classifications used in toxicology generally. See section "Discussion" for details.
(i) super-toxic lethal chemicals;	
(ii) other lethal chemicals;	
(iii) other harmful chemicals.	It is incorrect to call the results of testing standard concentrations an LD_{50} (see "Discussion").
Lethality limits in terms of LD_{50} for subcutaneous administration were established to separate three toxic categories at 0.5 mg/kg and 10 mg/kg.	Subcutaneous administration is not always used routinely for toxicity testing (see "Discussion").

[1] It was understood that these recommended standardized operating procedures (CD/CW/WP.30) for toxicity determinations might be supplemented or modified and/or, if necessary, reviewed.

[2] A view was expressed that appropriate methods for testing of non-lethal harmful chemicals need to be addressed at a later stage.

2. Principles of the test method

The test substance is administered to a group of animals in doses corresponding exactly to the category limits (0.5 or 10 mg/kg respectively). If in an actual test the death rate was greater than 50 per cent, then the material would fall into the higher toxicity category; if it was lower than 50 per cent the material would fall into the lower toxicity category.

The category limits are somewhat similar to the fixed dose procedure which provides for 10-fold increments (5, 50, 500), whereas the CD/CW increment is 20-fold.

3. Description of the test procedure

3.1 **Experimental animal**

Healthy young adult male albino rats of Wistar strain weighing 200 ± 20 g should be used. The animals should be acclimatized to the laboratory conditions for at least five days prior to the test. The temperature of the animal room before and during the test should be 22 ± 3°C and the relative humidity should be 50-70 per cent. With artificial lighting, the sequence should be 12 hours light, 12 hours dark. Conventional laboratory diets may be used for feeding with an unlimited supply of drinking water. The animals should be group-caged but the number of animals per cage should not interfere with proper observation of each animal. Prior to the test, the animals are randomized and divided into groups; 20 animals in each group.

Why are only Wistar rats to be used? Which strain of Wistar rats is to be used? Many strains of rats are available (e.g., Sprague Dawley, F344, etc.). Numerous toxicity data exist, obtained using other strains. Will this requirement trigger an avalanche of new toxicity testing (and animal use)?

The allowable weight deviation (10%) may be too tight. (See other testing guidelines).

3.2 **Test Substance** Each test substance should be appropriately identified (chemical composition, origin, batch number, purity, solubility, stability, etc.) and stored under conditions ensuring its stability. The stability of the substance under the test conditions should also be known. A solution of the test substance should be prepared just before the test. Solutions with concentrations of 0.5 mg/ml and 10 mg/ml should be prepared. The preferable solvent is 0.85 per cent saline. Where the solubility of the test substance is a problem, a minimum amount of an organic solvent such as ethanol, propylene glycol or polyethylene glycol may be used to achive solution.

If dissolved in ethanol, propylene glycol or polyethylene glycol, would these substances contribute to toxicity? Would the toxicity of the solvent be tested in control animals?

What is the definition of a "minimum amount"? This could be a very important factor.

3.3 **Test method** Twenty animals receive in the back region 1 ml/kg of the solution containing 0.5 mg/ml of the test substance. The number of dead animals is determined within 48 hours and again after 7 days. If the death rate is lower than 10 animals, another group of 20 animals should be injected by the same way with 1 ml/kg of the solution containing 10 mg/ml of the test substance. The number of dead animals should be determined within 48 hours and again after 7 days. If the result is doubtful (e.g., death rate = 10), the test should be repeated.

Why 48 hrs and 7 days? According to Auletta (1988), the observation period is usually 14 days for dermal and oral LD_{50} determinations, except for US Department of Transport which recommends 48 hrs.

3.4 **Evaluation of the results** If the death rate in the first group of animals (receiving a solution containing 0.5 mg/ml) is equal to or higher than 50 per cent, the test substance will fall into the "super-toxic lethal chemical" category. If the death rate in the second group (receiving a solution containing 10 mg/ml) is equal to or higher than 50 per cent, the test substance will fall into the "other harmful chemical".

4. Data reporting

A test report should include the following information:

(i) test conditions: date and hour of the test, air temperature and humidity;

(ii) animal data: strain, weight and origin of the animals;

(iii) test substance characterization: chemical composition, origin, batch number and purity (or impurities) of the substance; date of receipt, quantities received and used in the test; conditions of storage, solvent used in the test;

It is also important to record the concentration of the solvent used.

(iv) results. the number of dead animals in each group, evaluation of results.

Recommended standardized operating procedures for acute inhalation toxicity criteria

1. In the assessment and evaluation of the toxic characteristics of chemicals in a vapour or aerosol state determination of acute inhalation toxicity is necessary. In every case, when it is possible, this test should be preceded by subcutaneous toxicity determination. Data from these studies constitute the initial steps in the estabishing of a dosage regimen in subchronic and other studies and may provide additional information on the mode of toxic action of a substance.

Explanation needed why subcutaneous toxicity is required "when possible"? In the case of a gas, or aerosol, such a requirement is not likely to be valid.

Three caterogies of agents were defined on the basis of their toxicity:

(i) super-toxic lethal chemicals;

(ii) other lethal chemicals;

(iii) other harmful chemicals;

Lethality limits in terms of LCt_{50} for inhalatory application were established to separate three toxic categories at 2,000 mg min/m^3 and 20,000 mg min/m^3.

2. **Principles of the test method**

A group of animals is exposed for a defined period to the test substance in concentration corresponding exactly to the category limits (2,000 mg min/m^3)

respectively. If in an actual test the death rate was greater than 50 per cent, then the material would fall into the higher toxicity category; if it was lower than 50 per cent, the material would fall into the lower toxicity category.

3. Description of the test procedure

Why Wistar rats?

3.1 Experimental animal

Healthy young adult male albino rats of Wistar strain weighing 200 ± 20 g should be used. The animals should be acclimatized to the laboratory conditions for at least five days prior to the test. The temperature of the animal room before and during the test should be 22 ± 3°C and the relative humidity should be 50-70 per cent. With artificial lighting, the sequence should be 12 hours light, 12 hours dark. Conventional laboratory diets may be used for feeding with an unlimited supply of drinking water. The animals should be group-caged but the number of animals per cage should not interfere with proper observation of each animal. Prior to the test the animals are randomized and divided into two groups; 20 animals in each group.

3.2 **Test substance** Each test substance should be appropriately identified (chemical composition, origin, batch number, purity, solubility, boiling point, flash point, vapour pressure, etc.) and stored under conditions ensuring its stability. The stability of

the substance under the test conditions should also be known.

3.3 **Equipment** A constant vapour concentration may be produced by one of several methods:

(i) by means of an automatic syringe which drops the material on to a suitable heating system (e.g., hot plate);

The temperature will be critical; glycerol dropped onto a hot hot plate is converted to acrolein, for example.

(ii) by sending airstream through a solution containing the material (e.g., bubbling chamber);

(iii) by diffusion of the agent through a suitable material (e.g., diffusion chamber).

A dynamic inhalation system with a suitable analytical concentration control system should be used. The rate of air flow should be adjusted to ensure that conditions throughout the equipment are essentially the same. Both a whole body individual chamber exposure or head only exposure may be used.

This is in agreement with OECD Guidelines.

There is quite a difference between LCt_{50} values obtained when whole-body exposure is used, as compared to head-only exposure. The values obtained from these two different exposure methods are generally not comparable.

3.4 **Physical measurements** Measurements or monitoring should be conducted of the following parameters:

(i) the rate of air flow (preferably continuously);

(ii) the actual concentration of the test substance during the exposed period;

(iii) temperature and humidity.

3.5 **Test method** Twenty animals are exposed for 10 minutes to the concentration of 200 mg/m^3 and then removed from the chamber. The number of dead animals is determined within 48 hours and again after 7 days. If the death rate is lower than 10 animals, another group of 20 animals should be exposed for 10 minutes to the concentration of 2,000 mg/m^3. The number of dead animals should be determined within 48 hours and agian after 7 days. If the result is doubtful (e.g., death rate = 10), the test should be repeated.

3.6 **Evaluation of results** If the death rate in the first group of animals (exposed to the concentration of 200 mg/m^3) is equal to or higher than 50 per cent, the test substance will fall into the "super-toxic lethal chemical" category. If the death rate in the second group (exposed to the concentration of 2,000 mg/m^3) is equal to or higher than 50 per cent, the test substance will fall into the "other lethal chemical" category; if it is lower than 50 per cent, the test substance will fall into the "other harmful chemical".

Kennedy (1989) indicates that 10 min exposures are used to determine upper respiratory tract irritation. Longer exposure should be used to determine LCt_{50}. For instance, exposures are usually conducted for a single 4 to 6 hr period to determine acute responses. The animals are to be observed for 14 days after treatment.

4. Data reporting

A test report should include the following:

(i) Test conditions: data and hour of the test, description of exposure chamber (type dimensions, source of air, system for generating the test substance, method of conditioning air, treatment of exhaust air etc.) and equipment for measuring temperature, humidity, air flow and concentration of the test substance;

(ii) Exposure data: air flow rate, temperature and humidity of air, nominal concentration (total amount of test substance fed into the equipment divided by volume of air), actual concentration in test breathing zone;

(iii) Animal data: strain, weight and origin of animals;

(iv) Test substance characterization: chemical composition, origin, batch number and purity (or impurities) of the substance; boiling point, flash poirt, vapour pressure; date of receipt, quantities received and used in the test; condition of storage, solvent used in the test;

(v) Results: Number of dead animals in each group, evaluation of results.

B. Modalities for revision of toxicity determination procedures.

(To be developed)

4.0 Discussion

4.1 Categories of Toxicity

The categories proposed in the rolling text are not consistent with the generally used classification or rating schemes of toxic compounds, as Table 7 shows. This is not to say that there is consistency of nomenclature or doses amongst the standard references listed in that table. However, understanding of the proposed CWC categories is not facilitated by introducing totally new and different categories, that have no resemblance to the generally used or accepted categories. The use of two dose levels (0.5 and 10 mg) leads to three categories, which can be described as following:

< 0.5 mg	> 0.5 mg < 10 mg	>10 mg
0.5 mg/kg	10 mg/kg	

The CD/961 proposal does not mention the "less than ..." or "more than ..." subcategories, which
are implied.

Further, it is incorrect to say that the lethality limits **in terms of LD_{50}** (emphasis added) can be established by administration of two standard concentrations. What the application of the standard concentration aims at, is the

determination of a Minimum Lethal Dose (MLD) or probable lethal dose, or a Fixed-Dose Toxicity Determination, not an LD_{50} determination. Given this completely different approach, it appears to be doubtful that existing LD_{50} data are easily used for the purposes of the CW Convention. That means, that either thousands of chemicals would have to be re-tested, and thousands more animals used, just to determine the toxicity limits for the purposes of the Convention, or that one would have to come to some agreement on how to translate existing data into the proposed CW Convention classification scheme.

Table 7: Comparison of Standard Oral Toxicity Ratings with Proposed (CD/CW) Toxicity Ratings Determinations

Toxicity Class or Rating	Probable Oral Lethal Dose (or equivalent) humans (70 kg)	dog (20 kg)	cow (450 kg)	CD/CW Proposal (s.c. dose; rat)
Supertoxic[a], or Extremely toxic[b], or Dangerously toxic[c], or Very toxic[d]	< 5 mg/kg (less than 7 drops) < 1 mg/kg < 1 mg/kg (a taste) 5 mg/kg (for lab animals)	(0.004 tsp)	(0.09 tsp)	Supertoxic-Lethal < 0.5 mg/kg
Extremely toxic[a], or Highly toxic[b], or Seriously toxic[c], or Toxic[d]	5-50 mg/kg (between 7 drops and 1 tsp) 1-50 mg/kg 1-50 mg/kg 50 mg/kg (for lab animals)	(0.2 tsp)	(4.5 tsp)	Other Lethal < 10 mg/kg
Very toxic[a], or Moderately toxic[b], or Highly toxic[c], or Harmful[d]	50-500 mg/kg (between 1 tsp and 1 oz) 50-500 mg/kg 50-500 mg/kg 500 mg/kg (for lab animals)	(2 tsp)	(1 cup)	Other harmful > 10 mg/kg
Moderately toxic[a], or Slightly toxic[b], or Moderately toxic[c]	0.5-5 g/kg (between 1 oz and 1 pt or 1 lb) 0.5-5 g/kg	(0.45 cup)	(2.5 quart)	(not applicable)

continued...

Table 7 - continued

Toxicity Class or Rating	Probable Oral Lethal Dose (or equivalent) humans (70 kg)	dog (20 kg)	cow (450 kg)	CD/CW Proposal (s.c. dose; rat)
Slightly toxic[a)], or	5-15 g/kg (between 1 pt and 1 qt)			
Practically non-toxic[b)], Slightly toxic[c)]	5-15 g/kg	(1.34 cup)	(2 gal)	(not applicable)
Practically non-toxic[a)], or	> 15 g/kg (more than 1 qt or 2.2 lb)			
Relatively harmless[b)], or	> 15 g/kg	(1.34 cup)	(> 2 gal)	(not applicable)
Extremely low toxicity[c)]	> 15 g/kg			

Footnotes:

a) Gosselin, R.E., R.E. Smith, H.C. Hodge and J.E. Braddock, page VI-3 in: Clinical Toxicology of Commercial Products. 5th Ed. Williams & Wilkins, Baltimore, MD, 1984; Klaassen, C.D., M.O. Amdur and J. Doull, page 13 in: Casarett and Doull's Toxicology. The Basic Science of Poisons, 3rd Ed. Macmillan, New York-Toronto-London, 1986.

b) Osweiler, G.D., T.L. Carson, W.B. Buck and G.A. van Gelder, page 5 in: Clinical and Diagnostic Veterinary Toxicology, 3rd Ed. Kendall Hunt, Dubuque, Iowa, 1985.

c) Sax, N.I. Page 1 in: Dangerous Properties of Industrial Materials, 6th Ed. Van Nostrand Reinhold, New York-Toronto-London, 1984.

d) Fixed dose Procedure; see Van den Heuvel et al., Human Toxicol. 6, 279-291, 1987.

4.2 Route of Exposure

The suggested method in the CW/CD drafts, i.e., subcutaneous injection, is a method which is not always employed and certainly not in standard toxicity testing as required for existing regulatory purposes. There are two further points. Firstly (Table 3) subcutaneous injection results in a somewhat reduced toxicity, as compared to oral application. Secondly, it is likely that there may be only limited data on subcutaneous effects, thus necessitating re-testing of a number of compounds.

4.3 Species and Strain of Animals

While there is nothing wrong with using rats as a species for testing, it appears that the CWC draft requirement to use Wistar rats exclusively is unnecessarily restrictive. On what grounds would data generated with other strains of rats be rejected? This could require that hundreds if not thousands of chemicals would have to be re-tested, and one should anticipate adverse reaction to what would be viewed as the unnecessary killing of laboratory animals.

4.4 Other Details

There are other minor details that have to be clarified or brought into line with other, already existing regulations. These include:

- Preferred or acceptable solvent(s) and its (their) effects
- Length of observation periods after application of test substance
- Rationale for selection of LC_{50} values
- Rationale for allowing both whole-body exposure and head-only exposure with respect to inhalation toxicity determinations.

4.5 Number of Compounds That Might Fall Under the Surveillance Clause

From the Italian document CD/CW/WP.190, it is apparent that 850 of the 80,000 chemicals in RTECS (Registry of Toxic Effects of Chemical Substances) have an LD_{50} value of $\leq$ 0.5 mg/kg, although neither the route nor the species is indicated. In addition, another 596 substances have an LD_{50} between 0.5 and 1 mg/kg. The Italians are correct in stating that a compilation of a schedule that lists all chemicals with LD_{50} < 0.5 mg/kg "may cause severe impairment to the chemical industries' research and development activities." This document also notes the report of the "US - National Research Council", that states that toxicity data are not available for 78% of chemical products marketed at the rate of 1 million lbs per year.

4.6 Limitations of LD_{50} Tests

The LD_{50} (median lethal dose test) was first proposed over 60 years ago to provide a measure of toxicity having a precise end-point (Chan and Hayes, 1989). The LD_{50} procedure requires large numbers of animals to be subjected to at least 3 dose levels, in order to calculate, statistically, the median lethal dose. The numerical values provided by the LD_{50} test have been widely used to classify hazardous chemicals. However, there are several limitations to the test itself according to Gilman (1989):

- The LD_{50} value is not a biological constant, but is highly influenced by both endogenous and exogenous factors.

- The LD_{50} test considers only mortality, but not morbidity.

- The predictive value for the lethal dose in man based on animal LD_{50} values may be low.

- Detecting special risks in human neonates is not reliable when based on a comparison of the LD_{50} in newborn and mature rodents.

- The LD_{50} does not provide a reliable basis for selecting doses for chronic toxicity studies.

- Comparing oral and parenteral LD_{50} values is a wasteful and unreliable method for assessing bioavailability.

- The use of LD_{50} values for classifying hazardous substances neglects many other effects worthy of consideration (e.g., subclinical, neurological, etc.). In addition, different countries use different classification criteria.

- Some animals used in the LD_{50} test are thereby subjected to extreme pain and distress.

Because of the shortcomings of the LD_{50} test, the fixed dose test procedure is now suggested by the Commission of the European Community as an alternative.

4.7 Fixed Dose Procedure

During the discussions which took place in the context of the Seminar "LD_{50} and Classifications Schemes - The Possibilities for Change" held in Brussels in 1989, under the auspices of the Commission of the European Community, it became evident that the existing differences in schemes for the

classification of dangerous substances and preparations within different countries, international organizations, and trading blocks constitute a definite barrier to moving away from traditional LD_{50} testing methods.

The Commission made the following statement:

"The Commission shares the opinion of the majority of the participants to the seminar that it is highly desirable to rationalize these classification schemes. It recognizes however that this is a medium-long term goal requiring a concerted effort in order to be met.

In order to make progress in this field, the Commission engages to take an initiative to evaluate the feasibility of such a rationalization. It will support financially the necessary preparatory work, and will formally invite all the OECD Member Countries as well as international organizations to become associated with this initiative.

Furthermore, recognizing that such an exercise may take several years to be completed, and considering that it is not justifiable to await the outcome of such an exercise before making progress towards the replacement for classification purposes of the traditional LD_{50} test by scientifically more valid and ethically more acceptable acute toxicity testing methods, the Commission:

- accepting that a fixed dose procedure which provides adequate toxicity data for classification labelling and risk assessment of dangerous chemicals and preparations is actually available,

- recognizing that this procedure uses less animals and causes less distress to them than the traditional LD_{50} test,

- considering that this has been adequately validated at an international level:

(a) undertakes to propose the introduction of the fixed dose test procedure into Annex V of Directive 79/831/EEC,

(b) undertakes to make the necessary modifications to Annexes VI and VII of this same Directive,

(c) undertakes that once these modifications are officially adopted and incorporated into the legislation, to accept the data derived from the use of the fixed dose procedure.

The Commission hopes that all the OECD Member Countries which are not Members of the European Communities will undertake similar action, in order to incorporate the fixed dose procedure into their legislative schemes. It is clear however that according to the principle of the Mutual Acceptance of Data it will continue to accept data submitted according to the agreed OECD protocols as well."

If this proposal is generally accepted, the fixed dose concept, which is expressed in the draft of the CW Convention, in principle, could be regarded as being in line with future internationally accepted procedures, leaving aside the matter of the subcutaneous route of administration, and the need for retesting of thousands of compounds.

4.8 Other Approaches for LD_{50} Determinations

Utilizing range-finding studies or the "up - and - down" method for toxicity assessment are useful in reducing the numbers of animals used for toxicity determinations, and could also be considered.

5.0 Conclusions

Following the ideas expressed in the first footnote to Appendix I (page 52) of CD/961, it is proposed that the Ad Hoc Committee on Chemical Weapons initiate a thorough review by experts of these proposed toxicity determinations and the applicable standardized operating procedures.

The review should address, inter alia, the following points:

- establishment of categories of toxicity, which should come as close as possible to generally or usually employed classification or rating schemes of toxic compounds;

- selection of route of exposure, species and strain of animals, use of solvents, method of inhalation exposure;

- impact of approval of whatever toxicity determinations and levels of toxicity are chosen, on the number of compounds that might fall under the surveillance clause;

- consideration of impact of need for additional requirements for animal testing on the public in

general, and members of the animal welfare movement in particular;

- critical evaluation of the benefits and drawbacks of using the traditional LD_{50} method, versus the Fixed-Dose Procedure, or any other approaches for LD_{50} determinations.

World-wide acceptance of Article VI of the "Rolling Text", and of a CW Convention, could be facilitated by the acceptance of methods or criteria (for the determination of toxicity of chemical compounds) that receive the maximally obtainable support both from industry and the public. While it may appear difficult to reconcile the views of industry, the animal welfare movements, and the global interest in a workable CWC, a careful re-examination and revision of the proposed toxicity determination methods could lead to a compromise that comes close to the ideals of each interest group.

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SOCIETY OF TOXICOLOGY OF CANADA

APPENDIX 1

POSITION PAPER ON THE LD50 *

Historical Background

In the early part of the 20th Century, many medicinal agents in use were available as impure mixtures or extracts of biologically derived materials ("biologicals") rather than as pure chemical forms. It was often difficult to prepare uniform products by such processes, since the amount of "active" ingredient varied considerably from product to product. For several of these agents, the active *therapeutic potency* of the mixture could be correlated with the *lethal potency* of the mixture or extract. If one could calculate with precision the lethal potency of the material, one could indirectly assess the therapeutic potency of the same material. Effective therapeutic "dosages" for biologicals were often expressed in "units of activity" rather than in units of weight. Thus, quantitative methods were devised to assess lethal potency with precision, as a means of establishing standardization of biologically derived medicinal agents.

For statistical reasons, the median lethal dosage (LD50, the dosage estimated to kill 50% of the universal population of the species under test) was found to be the most accurate means of quantifying lethal potency. Furthermore, the mathematical precision of the statistically estimated LD50 was found to be directly related to the number of animals that were subjected to each test dose and the number of dosage levels (yielding values between 10% and 90% mortality) utilized to derive the lethality dose-response data. Thus, the LD50 was introduced in pharmacology and toxicology because of an important need in the estimation of potency of certain classes of medicinal agents.

A more general application of the LD50 determination followed. The quantification of lethality became widespread. The LD50 became one of the first quantifiable experimental tools available to the toxicologist. With such a tool, toxicologists could classify and compare chemicals according to their quantitative lethal potencies. Extrapolations to the potential dangers to humans due to acute exposures to relatively large amounts of chemicals were made on the basis of LD50 data derived in animals. These determinations were carried out in a variety of species and by different routes of administration.

Present Situation

One cannot discuss the utility of the so-called "LD50 test" in isolation. The assessment of life-threatening qualities of chemicals is an absolutely essential component of the safety evaluation process employed for the toxicological evaluation of diverse chemical substances, such as medicinal agents, cosmetics, food additives, pesticides, chemicals encountered in the household or the occupational setting, chemicals encountered in recreation or hobbycrafts, and chemicals dispersed in the environment. The toxicologist determines the potentially adverse effects that such substances might cause when various living species are exposed to chemicals under a variety of conditions. The species of greatest interest is of course the human being, but it is important to realize that many other mammalian and non-mammalian species can be the biological target of concern.

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* Adopted at the STC Annual Meeting on December 3, 1985

The assessment of the lethal properties of chemicals is usually associated with the acute toxicity phase of the safety evaluation process. Both the dosages and the exposure conditions that lead to the lethal response must be established in properly performed toxicological assessments. If humans are likely to come in contact with a particular chemical (voluntarily or involuntarily, accidentally or by design), one must know where the lethal range exists, if these individuals are to be protected. The safe handling of potentially lethal chemicals depends on adequate knowledge of lethal dosages and exposure conditions. The design of treatment procedures or specific antidotes to be used in the case of chemical intoxications depends on adequate knowledge of the lethal process. Questions raised regarding the precision one needs when performing the "LD50 test" are legitimate questions. On the other hand, questions dealing with the necessity of lethality assessment must be rational and in keeping with the responsibility of protecting society.

Large amounts of LD50 data have been accumulated; their utility has been questioned by a number of toxicologists. Toxicologists have deplored the misuse of the LD50 value as a kind of "biological constant". Variability is the rule in biology. This is also true when the biological response is death. LD50 values exhibit both interspecies and intraspecies variation. Furthermore, factors such as age, nutritional state and environmental conditions are known to affect lethal potency. Thus, the LD50 value, regardless of its precision, can never be regarded as a constant.

Toxicologists also realize that a precisely determined (in a statistical sense) LD50 value (with its 95% fiducial limits) is still only an estimate of the situation that may prevail in the population of species under test. In view of the well known interspecies variation, is great precision really necessary? Toxicologists are questioning the need for precision in the determination of LD50 values.

Toxicologists can obtain significant information on lethal potency and the process leading to lethality without the calculation of a precise LD50 value (one with very small 95% fiducial limits). It is important that the animals given lethal or near-lethal dosages be observed closely to gain knowledge of the functional and pathological alterations manifested by the animals. Questions regarding lethal potency can be resolved by the use of less precise statistical estimates than the ones traditionally employed to calculate LD50 values. Methods that require fewer numbers of animals can certainly be used to estimate an LD50 value or to yield a reasonable estimate of the dosages that border the lethal range. It is doubtful that much meaningful knowledge is lost by the application of such techniques in the safety evaluation process. On the other hand, a more complete examination of the animals employed to estimate lethal potency is to be encouraged. More can be done to obtain more meaningful biological data from animals used in lethality studies.

Questions have been raised about the utility of determining LD50 values in a number of different animal species. It must be remembered that one of the goals of the safety evaluation process is to provide data where one can extrapolate the findings observed in laboratory animals to the potentially adverse effects that might be observed in humans, domestic and wild animals, or animals in captivity exposed to the same chemicals. If the lethal dosage of the chemical is found to be similar in several species, extrapolation of toxicity to humans is more secure. If similar toxicological effects are observed in several animal species, it is probable that a common mechanism of action is involved in these species and probably will occur in humans as well. Thus, extrapolation to humans should be more reliable. However, if the lethal dosage is found to very considerably in a number of different species, extrapolation to humans becomes tenuous. Such an observation indicates that the toxicity is species-related and that further investigations are needed to determine which species resembles the human. Thus, the determination of lethal potency in several species can have a marked influence on the confidence

with which extrapolation to the human exposure situation is carried out. Furthermore, such results can have an important influence on the kinds of additional toxicological or biological studies that might be required to resolve the issue. Thus, it would seem unwise to restrict *a priori* the number of species that should be tested in lethality studies.

Important information can also be obtained from lethality studies performed with different routes of administration. In the past, such observations have had an important bearing on conclusions regarding the relative bioavailability (amount absorbed) of various chemicals following exposure by different routes of administration. They have been essential for determining how chemicals can be handled safely. These data can also help to establish the exposure conditions that are relatively without risk when chemicals are to be used as articles of commerce. Thus, it would be unwise to limit *a priori* the routes of administration that should be employed in lethality studies.

Whether to employ a particular lethality test or not, or the precision one needs if the test is chosen, depends on the anticipated use that will be made of the data generated. This means that one must look at the toxicological questions that are being asked. Estimates of acute lethal potency are presently very important data for the classification of chemicals when these substances are transported as hazardous chemicals. In the case of accidental spills and derailments, for instance, the adverse effects of consequence to humans are those associated with the temporary acute exposure to high concentrations of the chemical. In the occupational setting, accidental discharges may occur, resulting in acute exposures to potentially unsafe amounts. Acquisition of sound LD50 data are essential in such situations.

It is important to point out that there are no known, validated alternatives to the use of animals for the assessment of lethal potency. Nor are such alternatives likely to appear in the near future. Attempts are being made to develop techniques that predict lethal properties of certain classes of chemicals on the basis of already known structure-activity relationships. Quantitative Structure-Activity Relationships (QSAR) and Quantitative Structure-Toxicity Relationships (QSTR) are examples of such approaches. The reliability of the QSAR approach depends on the availability of data reflecting (1) well-defined interactions between chemical substances (2) belonging to congeneric series of structures and (3) an already known active site in a biological system. The application of the QSAR approach is said to presuppose the presence of an active site coupled with unambiguousness (in terms of mechanism of action) of the observed biological effects. The present state of toxicological knowledge is far from providing the necessary data that could make use of the QSAR approach. Thus, while these efforts are to be encouraged, it is evident that they will not be reliable substitutes for experiments in laboratory animals.

There is an important political issue that also bears on the safety evaluation process. Toxicological assessments are used to protect the public from the potentially adverse effects of chemicals. Public perception is that individuals have the right to live in a so-called "safe" environment. The adversarial-litigation climate that reigns in North America reflects this public perception. This climate indirectly influences the practice of toxicology. What toxicologist or government regulator is likely to decide in favor of not performing a particular toxicological study, thought to be of limited value, when court litigation at some later date for this decision remains a possibility?

Conclusions

The position of the Society of Toxicology of Canada on the issue of the so-called "LD50 test" can be summarized as the following:

1. The assessment of the lethal properties of chemicals is an essential component of toxicological evaluations designed to protect the public and the environment.

2. Sound toxicological questions should determine the extent to which the evaluation of lethality should be pursued. The number of species tested, the range of dosages employed, and the number of routes investigated should be minimized but consistent with sound toxicological approaches.

3. In most instances, high statistical precision of the LD50 estimate does not appear to be essential. Consequently, procedures that permit the estimation of this parameter with a small number of animals should be the procedures of choice.

4. All efforts should be carried out for the worldwide dissemination and communication of such results to prevent the unnecessary repetition of such studies.

5. Toxicologists should contribute to the construction of biological data banks that may lead to the development of non-animal approaches to the estimation of lethal potency.

Appendix: Acute Oral Toxicity Project

Test protocol: BTS procedure*

Principle of the test method

The test substance is administered orally by gavage at a single dose level to a group of experimental animals. The dose used is selected from a series of fixed dose levels which are related to a classification and labelling system. The dose selected is that which is judged likely to produce evident toxicity, but no deaths. Where no information is available on which to predict the likely toxicity of the test substances, a preliminary 'sighting' study, using just 3 or 4 animals, should be carried out. Following administration, observations for effects are made. Animals showing distress or which die during the test are necropsied, and at the conclusion of the test the remaining animals are necropsied. Where evident toxicity is not seen at the chosen dose level, or where a severe toxic reaction requires, for animal welfare reasons, the removal of animals from the study, the substances should be retested at the next higher or lower dose level.

* For practical reasons, this protocol was drafted in the form of an OECD Guideline, but does not have this status.

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Description of the test procedure

Preparations
Healthy young adult rats are randomly selected and acclimatised to the laboratory conditions for at least 5 days prior to the test.

Where necessary, the test substance is dissolved or suspended in a suitable vehicle. It is recommended that wherever possible the use of an aqueous solution be considered first, followed by consideration of solution in oil (e.g. corn oil) and then by possible solution in other vehicles. For non-aqueous vehicles the toxic characteristics of the vehicle should be known, and if not known should be determined before the test. The maximum volume of liquid administered at one time should not exceed 1 ml/100 g body wt, except in the cases of aqueous solutions where 2 ml/100 g may be used. Variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels.

Experimental animals

Selection of species. The rat should be used. Commonly used laboratory strains should be employed. The weight variation in animals used in a test should not exceed ± 20% of the mean weight.

Number and sex. At least 10 animals (5 female and 5 male) should be used for each dose level which is investigated. The females should be nulliparous and non-pregnant.

Housing and feeding conditions. The temperature of the experimental animal room should be 22°C (± 3°) and the relative humidity 30–70%. Animals may be group-caged by sex, but the number of animals per cage must not interfere with clear observation of each animal. The biological properties of the test substance or toxic effects (e.g. morbidity, excitability) may indicate a need for individual caging. Where the lighting is artificial, the sequence should be 12 h light, 12 h dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

Test conditions

Dose level. The dose level to be used in the test should be selected from one of the three levels listed in the criteria for classification, (Appendix Table 1) namely, 5, 50 or 500 mg/kg body wt. The initial dose level chosen should be that which is judged likely to produce evident toxicity but no mortality. Where no information is available upon which to make such a judgement, an initial 'sighting' study should normally be carried out. Where evident toxicity does not result from administration of the chosen dose level, the substance should be retested at the next higher dose level. The animals, however, should continue to be kept under observation until the observation period is complete. Where a severe toxic reaction requires animals to be removed from the study, the substance should be retested at the next lower dose level. Again, animals that do not need to be removed from the study should be kept under observation for the full observation period.

Appendix Table 1 Investigation of acute oral toxicity using a fixed dose procedure criteria for classification for labelling purposes

Test dose (mg/kg)	*Result*	*Action*
5	Less than 90% survival	Classify as *very toxic*
	90% or more survival; but evident toxicity	Classify as *toxic*
	90% or more survival; no evident toxicity	Retest at 50 mg/kg
50	Less than 90% survival	Classify as *toxic* Retest at 5 mg/kg if not already tested at that dosage
	90% or more survival; but evident toxicity	Classify as *harmful*
	90% or more survival; no evident toxicity	Retest at 500 mg/kg
500	Less than 90% survival *or* evident toxicity and no deaths	Classify as *harmful* Retest at 50 mg/kg if not already tested at that dose
	No evident toxicity	*Unclassified*

Observation period. Except where a test is prematurely terminated for animal welfare reasons, the observation period should be at least 14 d. However, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions, rate of onset and length of recovery period, and may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear must be recorded. If deaths occur, or if animals are humanely killed, the time of death should be noted.

Procedure
Animals should be fasted prior to substance administration by withholding food overnight. Following the period of fasting, the animals should be weighed and then the test substance administered in a single dose to animals by gavage using a stomach tube or a suitable intubation cannula. If a single dose is not possible, the dose may be given in smaller fractions

over a period not exceeding 24 h. After the substance has been administered, food may be withheld for a further 3–4 h. Where a dose is administered in fractions over a period it may be necessary to provide the animals with food and water depending on the length of the period. Following administration, observations are made and recorded systematically with individual records being maintained for each animal. The investigation of a second or, in exceptional circumstances, a third dose level is dependent upon the results of the preceding dose level.

Clinical examinations

A careful clinical examination should be made at least once each day. Additional observations should be made during the first few days after dosing so that the test may be terminated if it becomes apparent that the initial dose level chosen was too high. Cage-side observations should include changes in the skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Individual weights of animals should be determined shortly before the test substance is administered, weekly thereafter and at death. Where possible, animals should be weighed daily during the first week. Weight changes should be calculated and recorded. At the end of the test surviving animals are weighed and then humanely killed.

Pathology

All test animals should be subjected to gross necropsy. All gross pathological changes should be recorded. Microscopic examination of organs showing evidence of gross pathology in animals surviving 24 h or more should also be considered.

Treatment of results

Data may be summarised in tabular form showing for each test group the number of animals at the start of the test; the number of animals displaying signs of toxicity; a description of the toxic effects and whether evident toxicity was observed; the time course of any toxic effects; and the necropsy findings. The report should include details of all dose levels investigated, and should provide information on the number of animals which died or were humanely killed.